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ORIGINAL ARTICLE

Environmental, inflammatory, and anti-inflammatory squad in acne vulgaris pathogenesis: AhR, IL-36, and IL-38

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Abstract

Background: Acne vulgaris (AV) is an extraordinarily common skin condition. The high prevalence of AV is linked to the exposure factors, as environmental pollutants and climatic factors, occupational, psychosocial, and lifestyle factors. The AhR plays a critical part in environmental toxic action. The AhR expression and imbalance in the IL-36 & 38 expression may have a role in inflammation and AV pathogenesis.

Aims: To detect possible links between environmental, inflammatory, and anti-inflammatory factors in AV pathogenesis through measuring AhR, IL-36, and IL-38 mRNA gene expression levels.

Patients and Methods: Total of 100 subjects (70 AV patients and 30 apparently healthy control subjects) were tested for AhR, IL-36γ, and IL-38 mRNA levels by quantitative real-time PCR.

Results: The median levels of AhR and IL-36 mRNA gene expression were considerably greater, while that of IL-38 was essentially lower in AV than healthy subjects ($p < 0.001$, 0.021 and 0.002, respectively). The AhR and IL-36 mRNA gene expression levels increased, while IL-38 decreased significantly with higher grades of severity ($p < 0.001$, 0.001, and <0.001, respectively). ROC curve showed that AhR mRNA gene expression level had the best AUC for diagnosis of AV, with better sensitivity and specificity than IL-36 and IL-38.

Conclusions: Higher levels of AhR, IL-36, and lower levels of IL-38 gene expression were significantly associated with AV patients and higher grades of severity. AhR had better diagnostic ability than IL-38 and IL-36.

KEYWORDS
acne vulgaris, aryl hydrocarbon receptor (AhR), IL-36, IL-38, quantitative real-time PCR

1 | INTRODUCTION

Acne vulgaris (AV) is a pilosebaceous chronic inflammatory condition, in which many components have been embroiled, including hormonal, follicular hyperkeratinization, Cutibacterium acnes multiplication, genetic, inflammatory, and environmental factors.[1] The prevalence of AV is about 28.9 to 91.3% among adolescents.[2] Familial preponderance clearly indicates a genetic basis for acne vulgaris, but solid genetic associations were lacking.[3]

The aryl hydrocarbon receptor (AhR) is a transcription factor and cytoplasmic receptor. The AhR gene is located on the 7p15 chromosome.[4] Environmental compounds, diet, and microbiome can influence AhR activity.[5] The AhR is a vital player in integrity of the skin, homeostasis, and immunity.[6] Molecular mechanisms of AhR sense

environmental stimuli as pollution to control sebocytes innate immunity and give an understanding into their link in AV pathogenesis.[7] Nuclear factor kappa beta (NF-κB) induced levels of tumor necrosis factor-α (TNF-α) and ILs (1, 8, and 10) in AV pathogeneses was through AhR interaction with the toll-like receptors (TLRs) and NF-κB signaling pathway.[8,9]

Interleukin-36 (IL-36) is a pro-inflammatory IL-1 family cytokine, including four members: IL-36Ra (receptor antagonist), IL-36 (α, β, and γ) (receptor agonists).[10] On chromosome 2q13, the IL-36 genes are mapped to the IL-1 locus.[11] The induction of keratinocytes IL-36 occurs by IL-36 itself, IL-17, IL-22, and TNF-α.[12] The IL-36 has a significant impact on defense mechanisms and maintaining homeostasis.[13] Inflammation could be a main component within AV pathogenesis, an increment in pro-inflammatory cytokine activity; IL-1 is found before hyperproliferation around follicles and is thought to activate keratinocyte proliferation.[14] The IL-1 stimulates pilosebaceous unit remodeling and promotes comedogenesis.[15]

Interleukin-38 (IL-38) is the 10th member of the same family (IL-1). The gene of IL-38 is mapped on chromosome 2p13 alongside IL-1 receptor antagonist (IL-1Ra) and IL-36Ra genes, within the IL-1 gene cluster.[16] The IL-38 is a well-known antagonist of the IL-36 receptor, it decreases inflammation by blocking agonist receptor ligands binding to IL-36R, a special IL-38 receptor.[17] The main biological role of IL-38 is to block the binding of IL-36 cytokines to IL-36R. So, IL-38 has its anti-inflammatory effect due to its activity as a receptor antagonist.[18] IL-38 binds to IL-1R6 also and so, has anti-inflammatory function.[17]

Thus, to investigate the potential influence of environmental factors in AV pathogenesis and evaluate the interplay between environmental factors, inflammatory and anti-inflammatory cytokines in AV, AhR, IL-36, and IL-38 mRNA gene expression levels were investigated.

2 | AIM OF THE STUDY

The aim was to assess the mRNA levels of AhR, IL-36γ, and IL-38 in AV patients, to assess the effect of disease severity on their mRNA levels and whether the severity could be determined accordingly.

3 | SUBJECTS AND METHODS

From those attending Dermatology, Venereology, and Andrology Department outpatient clinic in cooperation with the Molecular Biology and Biotechnology Unit, this case-control work was performed. Written informed consent was filled by subjects before being included in this study. The work has been agreed by the Scientific Ethics Committee of Faculty of Medicine, according to Helsinki Declaration principles.

Diagnosis of AV was clinically settled by two expert dermatologists, severity was scored by Global Acne Grading System (GAGS).[19] The AV subjects included were either never treated or stopped treatment for at least 8 weeks. Control subjects included were age and sex matched. Patient less than 15 and above 35 years old, lactating, or pregnant women, having systemic inflammatory diseases and with acute or chronic infection were not included in this work.

3.1 | IL-36, IL-38, and AhR gene expression by quantitative real-time PCR

3.1.1 | Sampling

A venous blood sample (2 ml) was taken from each subject in the study, placed in a sterile vacutainer tube having EDTA, mixed thoroughly and aliquoted into 2 Eppendorff tubes, which maintained at −80°C until mRNA extraction.

3.1.2 | Steps

A. Extraction of RNA.

It was done with 100 μl EDTA whole blood via Total RNA Purification Kit with elimination of remaining DNA by gDNA removal kit (Jena Bioscience, Germany) in accordance with the manufacturer's guidelines.

B. Quantitation of extracted RNA.

By Nanodrop 2000 Spectrophotometer ultraviolet spectrophotometric quantification of RNA was done (Thermo Fisher Scientific, Wilmington, USA). The optical density (OD) ratio of pure RNA preparations was 1.9–2.3 at 260/280 nm.[20]

3.1.3 | Two-step relative quantification of each mRNA gene

1. In Veriti™ Thermal Cycler (Applied Biosystems), reverse transcription (RT) of RNA into complementary DNA (cDNA) is performed, by HiSenScript™ RH (-) RT PreMix Kit (*Intron*). To each RT tube supplied; 5 μl RNA template and 15 μl nuclease-free water were added. Thermal states were set at 42°C for 1 h then RTase inactivation at 85°C for 10 min.
2. Relative quantitation of gene expression using *Hera Sybr Green qPCR kit* (*Willowfort*, *UK*). Endogenous housekeeping gene was human β-actin. The primers for IL-36γ and IL-38 were FP: 5′-GGTCGTGTGCTTGGAGGA-3′, RP: 5′-GGTACCATTCCCAATGCTGA-3′ and FP: 5′-AAGGTCC CCATTTTCCTGGG-3′, RP: 5′-CTCAATGTTCACATCCTCCAGC-3′, respectively,[21] AhR were FP: 5′CAAATCCTTCCAAGCGGCATA3′, RP:5′CGCTGAGCCTAAGAACTGAAG-3′,β-actin were FP:5'-AGACGCAGGATGGCATGGG-3′ and RP:5′-GAGACCTTCAACA

CCCCAGCC-3′.[22] Singleplex reactions were done, each reaction mix contained 10 μl Hera Sybr master mix (2X), 1 μl FP, 1 μl RP, 4 μl cDNA, and up to 20 μl nuclease-free water. Amplification took place in *Stepone Real-Time Cycler* (*Applied Biosystem*, *Singapore*). After a 2-min holding stage at 95°C, 45 cycles of denaturation at 95°C for 10 s and annealing/extension at 59°C for 30 s were conducted. Each run was subjected to a melting curve analysis to validate the assay's specificity.C. Data analysis:

According to Stepone software v2.2.2, data presented as sigmoid-shaped amplification curves, with the cycles number plotted versus normalized reporter fluorescence (Rn). IL-36γ, IL-38, and AhR gene expression levels in the control group were set to 1. Relative quantitation of target gene expression was normalized to that of human β actin. The fold changes in gene expression were estimated by $2^{-\Delta\Delta CT}$ equation.[23] ΔCt values were determined by subtracting the threshold cycle (Ct) value of β-actin from Ct value of target gene (IL-36γ, Il-38, and AhR), ΔΔCt was evaluated by subtracting the ΔCt of controls from ΔCt of cases.

3.2 | Statistical analysis

The Statistical Program for Social Science (IBM Corp., 2017) was used to analyze data. Version 25.0 of IBM SPSS (IBM Corp., Armonk, NY). For statistical difference of a non-parametric variable Mann-Whitney Test was used, and between more than two study categories carried out by Kruskal-Wallis test. The receiver operating characteristic (ROC) curve evaluated the sensitivity and specificity for quantitative diagnostic measures. Linear regression analysis was used for risk factors prediction. If *p* value is less than 0.05 at the 95% confidence interval, it is considered significant.

4 | RESULTS

The study included 70 AV patients (29 males and 41 females) with a mean age of 22.14 ± 4.60 years, in addition to 30 apparently healthy subjects as controls (13 males and 17 females). The mean age of the control subjects was 23.15 ± 5.31years. The mean age of onset of AV was 17.47 years, the mean disease duration was 4.63 years, 48.6% of cases has intermittent course and 51.4% of them has a progressive course. 62.9% of patients have family history of AV.

The median of AhR and IL-36 mRNA gene expression levels was significantly higher, while median IL-38 mRNA gene expression was significantly lower in AV patients than that in controls ($p < 0.001$, 0.021 and 0.002, respectively) (Table 1 and Figure 1A). The AhR and IL-36 mRNA gene expression levels were increased, while IL-38 was decreased significantly with higher grades of AV severity ($p < 0.001$, 0.001 and <0.001, respectively) (Table 2 and Figure 1B).

A statistically significant positive correlation between IL-36 and AhR mRNA gene expression levels, significant negative correlations between IL-38 and AhR as well as between IL-38 and IL-36 in AV group were found (Figure 2A–2C, respectively).

The ROC curve was done discriminating AV cases and control groups. Best cutoff values and performance characteristics are showed in (Table 3, Figure 2D). It is noticed that AhR mRNA gene expression level had the best AUC for diagnosis of AV cases, with better sensitivity and specificity than IL-38 and IL-36 mRNA levels.

Linear regression analysis was utilized for AV severity prediction, by covariates as age, gender, family history, onset, course, duration, AhR, IL-36, and IL-38 gene expression levels. Higher AhR, IL-36, and lower IL-38 gene expression levels were suggested to be independent predictors for more severe AV cases in uni- and multivariable analyses (Table 4).

5 | DISCUSSION

Acne vulgaris is a very common inflammatory condition with complicated pathophysiology.[24] Crosstalk between environment, innate and adaptive immunity, and cells, such as sebocyte, keratinocytes and fibroblasts, underpins AV pathogenesis.[25] The adaptive immune reaction toward Cutibacterium acnes antigens present to CD4+ T cells, via TLR2-4, cause activation of Th17 axis and cytokines (IL-1, 6, 8, 17, and TNF) activate pathogenic steps in AV.[26]

The AhR included in sebocytes homeostasis in both stable and inflammatory conditions.[27] The interaction between sebaceous glands and AhR is unique, because AhR and cytochrome p450 1A1 (CYP1A1) are emphatically communicated in sebocytes.[28,29] The AhR agonist dioxin increase TNF-α and IL-8 production in pretreated sebocytes with peptidoglycan (PGN).[30]

TABLE 1 Comparison between the two studied groups according to AhR, IL-36, and IL-38 gene expression levels

Variables	Control group (*n* = 30) Median (range)	Acne vulgaris group (*n* = 70) Median (range)	*p*
AhR gene expression (RQ)	0.84 (0.56–1.78)	2.20 (1.39–3.91)	<0.001**
IL–36 gene expression (RQ)	0.89 (0.51–1.95)	1.28 (0.65–2.28)	0.021*
IL–38 gene expression (RQ)	1.84 (0.51–2.31)	1.09 (0.47–1.80)	0.002**

Abbreviation: RQ, relative quantitation.

*Significant, **High significant.

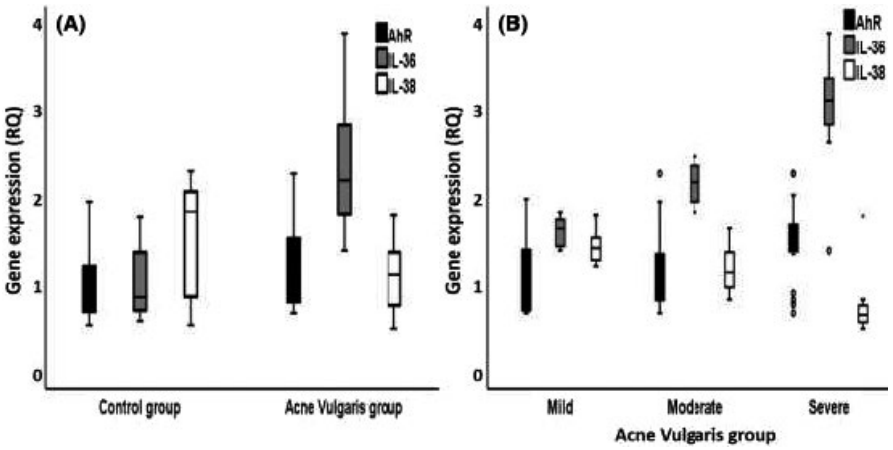

FIGURE 1 Gene expression levels of AhR, IL-36, and IL-38: (A) in acne vulgaris cases versus controls and (B) in cases categorized by severity

TABLE 2 Relation between the degree of disease severity and AhR, IL-36, and IL-38 gene expression levels in the patient group

Variables	Acne Vulgaris Severity			*p*
	Mild ($n = 11$)	Moderate ($n = 38$)	Severe ($n = 21$)	
	Median (range)	Median (range)	Median (range)	
AhR gene expression (RQ)	1.65 (1.39–1.83)	2.18 (1.83–2.48)	3.12 (1.39–3.91)	<0.001**
IL–36 gene expression (RQ)	0.74 (0.65–1.98)	0.98 (0.65–2.28)	1.50 (0.65–2.28)	0.001**
IL–38 gene expression (RQ)	1.42 (1.19–1.80)	1.12 (0.81–1.65)	0.63 (0.47–1.79)	<0.001**

Abbreviation: RQ, relative quantitation.

**High significant.

The uneven expression of IL-36/IL-38 levels may be the cause of dysregulated inflammatory condition initiated by IL-36.[21] Agonist and antagonist functions imbalance in IL-1 family could play a critical part in skin inflammation.[31] IL-38 may be closely related to inflammatory conditions caused by IL-36. Thus far, the IL-1 receptor antagonist has been affirmed as IL-1-targeting agents for the treatment of inflammatory diseases.[32]

The current study revealed that gene expression levels of AhR, IL-36 mRNA were significantly higher, while that of IL-38 was significantly lower in AV patients ($p < 0.001$, 0.021 and 0.002, respectively), the AhR, IL-36 mRNA gene expression levels increased, while IL-38 decreased significantly with higher severity ($p < 0.001$, 0.001 and <0.001, respectively).

This was in line with Fabbrocini et al.[33] who reported that stimulation of AhR leads to increased CYP1A1 expression in skin which is crucial biomarker for AhR stimulation and that might initiate comedogenesis. Also, Furue et al.[34] found that hyperactivation of AhR is included in the AV pathogenesis, although the precise way is not understood well. Exceedingly lipophilic dioxins show up to accumulate in and to be excreted by means of sebum and sebaceous glands.

A cross-relation between AhR which reacts to environmental factors and TLRs has been reported. Activation of TLR-2 and consequent inflammatory reaction lead to AV lesion formation. The AhR can balance PGN-stimulated TNF-α and IL-8 expression in human sebocytes including the myeloid differentiation factor 88 (MyD88) and phospho-p38MAP kinase (p-p38MAPK) signaling pathway which likely show a brand-new pathway in TLR-2-mediated AV.[28]

Krutmann et al.[7] said that there is a tie between AV and contact with environmental toxins through hazy pathways. However, Wei et al.[35] revealed that AhR stimulation appears to be vital for immunological reactions and decreasing inflammation through upregulated IL-22 and downregulated Th17 response. Knocking down of AhR for the most part downregulates the innate immunity genes expression.

The IL-36 cytokines are most active cutaneous barrier.[36] In normal cutaneous tissue, low amounts of IL-36 cytokines are expressed constitutively. Upon cutaneous injury, RNAs from harmed cells stimulate TLR3 and IFN-β, which enhance the generation of IL-36γ, which then enhance regenerating family member 3 alpha (REG3A), which directs keratinocyte proliferation and differentiation.[37] Nevertheless, as these cytokines are upregulated and overexpressed illnesses, they may lose homeostatic adjust, causing abnormal pro-inflammatory milieu.[38]

Interleukin-36 cytokines are basically expressed in monocytes/macrophages and keratinocytes, it plays a critical part within the balance of Th1 and Th17 immune reactions.[39] IL-36-mediated induction of inhibitor of NF-κB was necessary for release of downstream genes included in inflammation signaling pathway, neutrophil recruitment, and leukocyte stimulation.[40] Pro-inflammatory IL-36 subfamily members are enhanced in cutaneous AV lesions, highlighting the possibility of their commitment in its pathogenesis.[41]

Interleukin-36 cytokines bind IL-36R recruiting intracellular signaling molecules; MyD88, IL-1R-associated kinase (IRAK), and TNF receptor-associated factor 6 (TRAF6) to potentiate NF-kB and

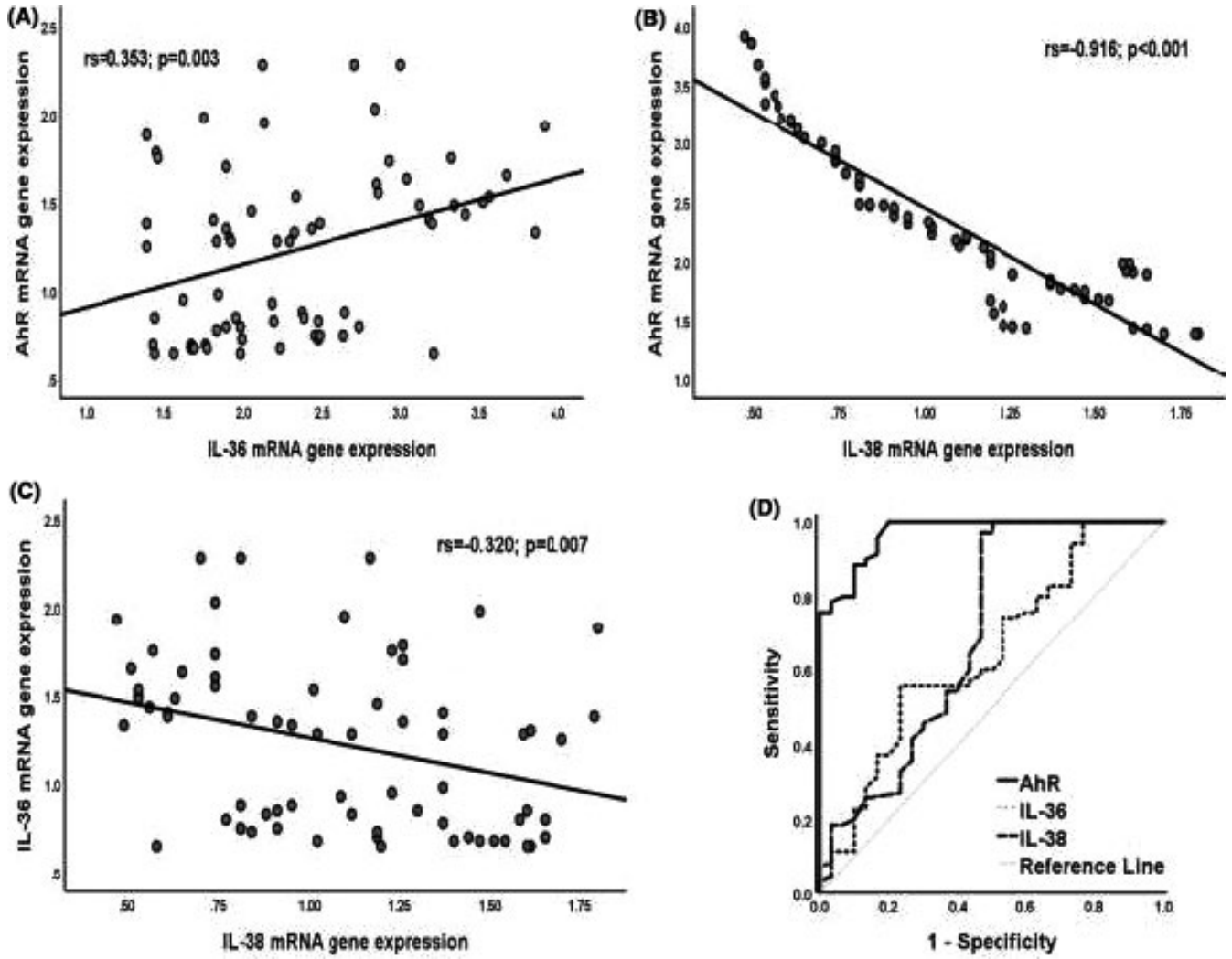

FIGURE 2 Correlations between AhR, IL-36, and IL-38 among cases (A, B, and C) and ROC curve analysis (D) for discrimination between cases and controls. Abbreviation: r_s, Spearman correlation coefficient

TABLE 3 Validity of AhR, IL-36, and IL-38 gene expression levels for discrimination between acne vulgaris cases and controls

	AhR gene expression	IL−36 gene expression	IL−38 gene expression
AUC	0.971	0.646	0.697
95% CI	0.942–0.999	0.526–0.766	0.567–0.826
Cutoff (RQ)	1.38	1.22	<1.725
Sensitivity (%)	88.6	55.7	97.1
Specificity (%)	90	76.7	53.3
PPV (%)	95.4	84.8	97.1
NPV (%)	77.2	42.6	53.3
Accuracy (%)	89	62	84

Abbreviations: AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; RQ, relative quantitation.

MAPK.[42] Stimulation of MAPKs, such as c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERK1/2), leads to a quick enhanced phosphorylation of inhibitor-kappa-B-alpha (IkB-α), a strong inhibitor of NF-kB. Phosphorylated IkB-α dissociates and frees NF-kB activating NF-kB and/or MAPK-related pro-inflammatory cascades.[43] This activation then enhances the generation of other cytokines, chemokines, and anti-microbial peptides that magnify the pro-inflammatory reaction.[44] IL-36 expression is substantially inducible by various inner and outer components and signaling pathways. IL-36 cytokines are strongly upregulated responding to microbial stimuli.[45] Also, cellular damage brings about IL-36γ upregulation.[46]

Interleukin-38 has anti-inflammatory property by antagonizing IL-36 pathways by direct binding to its receptor.[47] Blocking the IL-1, 18 and 36 receptors pathways is how IL-38 and Th17 cells work.[17] So, the impact of IL-38 on Th17 cells was comparable to inhibiting IL-1 and 36 receptors pathways that suppressed IL-17 & 22 production.[48] Mononuclear cells activated by IL-36γ with the presence of IL-38 revealed decreased secretion of IL-8, supporting the idea that IL-38 appears to stop IL-36γ-induced IL-8.[17] Knockdown of IL-38 in blood mononuclear cells appears that generation of

TABLE 4 Regression analysis for prediction of severity (assessed by GAGS)

Variables	Univariate analysis		Multivariate analysis	
	β	*p*	*β*	*p*
Age (years)	0.406	0.129		
Gender	0.300	0.874		
Positive family history	3.088	0.199		
Onset	0.208	0.441		
Course	1.182	0.276		
Duration (years)	0.527	0.147		
AhR gene expression	2.767	<0.001**	1.092	<0.001**
IL−36 gene expression	2.110	0.002**	1.038	0.042*
IL−38 gene expression	−3.892	<0.001**	−2.598	0.035*

Abbreviations: GAGS, global acne grading system; *β*, regression coefficient.
*Significant, **High significant.

IL-6, CCL-2 were expanded in reaction to TLR ligands, so IL-38 acted like antagonist in this case.[49] Because higher quantities of IL-38 modestly boosted IL-22 production, low concentrations of IL-38 were more efficient in lowering IL-17 and 22 secretion than higher amounts.[17] Moreover, IL-38 mRNA exhibited lower levels in the skin, epidermis, keratinocytes of mice with imiquimod-induced inflammation versus the control mice.[50] In addition, the highly expressed IL-38 in murine demonstrate of arthritis ameliorated the diseases. IL-38 may have anti-inflammatory properties in rheumatoid arthritis, which might enable it to be used as a line in a therapeutic strategy.[51]

However, Mora et al.[52] reported that IL-38 could have both agonist and antagonist actions depending on processing and concentration. In contrast, IL-38 mRNA levels were higher in inflamed colonic biopsies from Crohn's disease patients than in non-inflamed biopsies from the same individuals and were link to IL-1β, IL-17A, and IL-6 production.[51] Systemic lupus erythematosus patients had increased concentrations of IL-38 compared to controls and patients with active disease had higher serum IL-38 levels than those with inactive disease.[49]

Furthermore, our results found that there was significant positive relation between IL-36 and AhR mRNA gene expression levels and significant negative correlations between IL-38 and AhR as well as between IL-38 and IL-36 in AV group. Thus far, our data indicate that environmental factors influence inflammation. Supported by Barker et al.,[53] who found that dysregulated AhR led to aberrant inflammation and enhanced stem cell proliferation. Crosstalk between environmental factors and inflammation, supported by Karl Walter Bock,[54] who reported that AhR may quicken or weaken inflammation and consequent resolution. It modulates inflammation by a mixture of genomic and non-genomic signaling pathways. Also, the presence of a diverse adjust in the IL-36/IL-38 expression among diverse inflammatory conditions. There was a noteworthy negative relation between fold gene expression of IL-38 and IL-36 proposing that IL-36/IL-38 ratio could impact the expression of this pro-inflammatory cytokine.[55]

It is noticed that AhR mRNA gene expression level had the best AUC for diagnosis of AV cases, with better sensitivity and specificity than IL-38 and IL-36 mRNA levels. We suggest that AhR is maestro players regulating inflammatory and anti-inflammatory pathways.

6 | STUDY LIMITATIONS

As with most studies, design of the current study is subject to small sample size limitation.

7 | CONCLUSIONS

The uneven expression of IL-36/IL-38 may be responsible for the inflammatory processes in AV pathogenesis which may be triggered by environmental-induced elevation of AV pathogenesis. Higher AhR, IL-36, and lower IL-38 gene expression levels were suggested to be independent predictors for more severe AV.

Higher AhR, IL-36, and lower IL-38 gene expression levels were significantly associated with patients with AV. In addition, AhR, IL-36 levels increased, while IL-38 level decreased significantly with higher grades of severity. AhR had better diagnostic ability than IL-38 and IL-36.

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CONFLICT OF INTEREST

The authors have declared that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Fatma Mohamed El Esawy and Doaa M. Elhabak: Desinated the research. Shuzan Ali Mohammed: Performed the Biochemical work. Ebtesam Nasar Zargon Nasar, Sara Hemdan Mostafa and Doaa M.

Elhabak: Collected the cases and performed the work. Doaa M. Elhabak and Shuzan Ali Mohammed: Wrote the paper.

This research was conducted in Dermatology, Venereology, & Andrology Department and in Medical Biochemistry & Molecular Biology Department. Faculty of Medicine, Benha University, Egypt.

ETHICAL APPROVAL

The work has been agreed by the Scientific Ethics Committee of Faculty of Medicine, according to Helsinki Declaration principles. Written informed consent was filled by subjects before being included in this study.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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