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Altered Expression of Unfolded Protein Response Genes in Macrophages from Behcet's Disease

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Endoplasmic reticulum (ER) stress triggers the unfolded protein response (UPR), which has been correlated with enhanced production of inflammatory cytokines. Given the important pathogenic roles of macrophages and inflammatory responses in the etiopathogenesis of Behcet's disease (BD), this study aimed to assess the mRNA expression pattern of genes involved in the UPR pathway in macrophages from smoker and non-smoker BD patients. This case-control study was conducted between 2015 and 2016 in Shariati Hospital, Tehran, Iran. Monocytes were enriched from obtained whole blood samples of 10 smoker and 10 non-smoker BD patients as well as 10 healthy individuals. Using macrophage-colony stimulating factor (M-CSF), separated monocytes were differentiated into macrophages. After total RNA purification and cDNA synthesis, quantification analysis of UPR genes, including *activating transcription factor* (*ATF) 4, ATF6*, X-box binding protein 1 (*XBP1*), *binding immunoglobulin protein (BIP)*, *C/EBP homologous protein* (*CHOP)*, *homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1* (*HERP), and growth arrest and DNA damage-inducible protein (GADD34)*, was performed using SYBR green master mix and real-time PCR. Among the measured genes, *HERP* mRNA was overexpressed in macrophages from BD patients in comparison with healthy macrophages. *HERP* and *GADD34* genes were upregulated in smoker BD patients compared with non-smoker BD patients as well as healthy subjects. Cigarette smoke can induce UPR gene expression in BD patients. The altered UPR gene expression in BD macrophages may contribute to BD pathogenesis.

Keywords: Behcet's syndrome, macrophages, unfolded protein response

Introduction

Behcet's disease (BD), traditionally known as Silk Road disease, is a chronic multisystemic autoimmune disease with relapsing/remitting episodes [1] that manifests most importantly as genital and oral ulcers as well as ocular involvement [2]. BD is generally considered a vasculopathy, occurring in genetically susceptible individuals, and triggered by many various environmental factors [3]. Prevalence ranges from the highest rate of 20-420 per 100,000 [4] in Turkey to 80 per 100,000 in Iran and to the lowest rate of 0.46 per 100,000 population in the UK [5]. Genetically speaking, HLA-B*51 has been mainly associated with disease susceptibility since the early 1970s [6] and reported in 60% of BD patients [7]. Concerning the immune system, it has demonstrated that innate inflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-12, IL-6, IL-15, IL-23, tumor necrosis factor-alpha (TNF-α), and adaptive cytokines from Th1 helper cells including interferon-gamma (IFN-γ), IL-2, and IL-17 and only IL-13 from Th2 lymphocytes have significant associations with BD pathogenesis. Any imbalance, especially in the expression of innate immunity cytokine profile [8] or the ratio of Th17 to Th1 cells, could be of great importance in the pathogenesis of the disease [9], which is still a controversial topic [6]. Regarding the role of pro-inflammatory cytokines in this disease, BD manifests as inflammation episodes in almost all organs and tissues such as joints, the gastrointestinal tract, and the nervous system, and it affects both arteries and veins [10]. Vasculitis is greatly dependent on macrophage functions in harmony with other immune cells, specifically Th1 cells,

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to form granuloma, present antigen, and to release cytokines, enzymes, and reactive oxygen species [11].

Under a continuing inflammatory state, the endoplasmic reticulum (ER) of macrophages undergoes prolonged stress. The normal process of protein folding is interrupted during ER stress and could ultimately lead to macrophage death [12]. ER stress induces unfolded protein response (UPR) signaling [13] and activates downstream proteins including PERK, eIF2α, ATF4, CHOP, GADD34, ATF6, XBP1, and a few others. Furthermore, it triggers the inflammatory responses of macrophages [14]. Increased amounts of some of these factors such as XBP1 [15] and BiP [16] have been reported in certain autoimmune diseases like rheumatoid arthritis (RA). Also, in osteoarthritis (OA), *XBP1, BIP, ATF6*, and *IREα* genes have increased expression levels [17], and the CHOP protein is directly associated with cartilage apoptosis and destruction [18, 19]. The latter is an important transcription factor activated through ER stress and induced cell death. Cultured cells with decreased CHOP activity have an increased survival rate [20].

There is enough evidence to support the association of UPR genes with other autoimmune diseases; however, the expression of UPR genes in the macrophages of BD patients has not been investigated, and the role of this pathway in the pathogeny of BD is still unclear. Because BD is an autoimmune vasculitis similar in some ways to other autoimmune diseases, and due to the role of macrophages in the inflammatory responses of BD and the role of ER stress in the induction of inflammation, the authors of this study intended to investigate possible changes in the expression level of UPR marker genes in macrophages from smoker and non-smoker BD patients.

Materials and Methods

Participants

Twenty Iranian BD patients and ten healthy controls were randomly included in this case-control study that was conducted between 2015 and 2016 in Shariati Hospital, Tehran, Iran. Patients were categorized into two subgroups, smokers and non-smokers, with each group comprising 10 BD patients (Table 1). Moreover, a normal age- and sex-matched control group comprising 10 individuals with no past or current medical history of autoimmune disease was added to the study. The inclusion criterion was defined as patients being formally diagnosed with BD according to the International Criteria for Behcet's Disease [21].

Mononuclear cells isolation

To isolate monocytes from peripheral blood mononuclear cells (PBMCs), first, 20 ml peripheral blood was drawn from each participant in tubes containing ethylenediaminetetraacetic acid (EDTA). Then, mononuclear cells were isolated through density gradient centrifugation using isotonic Ficoll-Hypaque (Inno-train, Germany). The buffy coat obtained after centrifugation was washed three times using phosphate-buffered saline (PBS; GIBCO Invitrogen) [22]. Harvested PBMCs were mixed with magnetic-activated cell sorting (MACS) buffer (4 °C) and loaded onto a MACS column (Miltenyi Biotec, Auburn, CA, USA) in the magnetic field of a separator to isolate monocytes according to the manufacture's guideline.

Confirmation of cell isolation

The purity of cells isolated from the column was evaluated through both cellular morphology (using an inverted microscope) and flow cytometry (using antibodies against CD14) after separation.

Macrophage differentiation

The collected monocytes were seeded in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin (Sigma), L-glutamine (2 mM, Biosera), and 100 µg/ml streptomycin (Sigma) in the presence of 50 ng/ml macrophage colony-stimulating factor (M-CSF; eBioscience) and cultured at 37 °C in a humidified incubator in an atmosphere with 85% humidity and 5% CO2 for 7 days.

Confirmation of differentiation

To evaluate cellular differentiation, after 7 days of cell treatment with M-CSF, the cellular morphology was evaluated by inverted microscope, and the cellular markers were confirmed by flow cytometry (targeting $CD163^+$ and $CD206^+$ markers).

RNA extraction and cDNA synthesis

After the differentiation of monocytes into macrophages, the mRNA expression level of genes involved in the UPR pathway such as *activating transcription factor (ATF)-4*, *ATF6*, *X-box binding protein 1 (XBP1), binding immunoglobulin protein (BIP)*, *C/EBP homologous protein* (*CHOP)*, *homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1* (*HERP)*, and *growth arrest and DNA damage-inducible protein (GADD34)* was evaluated using real-time PCR. First, total RNA was extracted from differentiated macrophages using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. For complementary DNA (cDNA) synthesis from the extracted total RNA, the Quantitect Reverse Transcription Kit (Takara, Korea) was used, and all steps were performed following the manufacturer's manual.

Real-time PCR

The primer sequences are presented in Table 2. The accuracy and specificity of the designed primers were checked by the BLAST tool of the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The level of selected gene expression was assessed by real-time PCR as a quantitative method using SYBR Green Master Mix and ABI Step-One-Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The expression data of the genes was normalized to the corresponding Ct values of *GAPDH* mRNA as the endogenous control. The comparative Ct method was applied to calculate the fold change in expressions between tested groups [23]. According to the melting results, a single pick with a specific temperature was detected for each gene.

Ethical considerations

The protocol of this study complied with the Declaration of Helsinki guidelines and was accepted by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.REC.1394.1941). All patients voluntarily signed a consent form before enrollment and after the aims and methods of the study were explained to them in detail.

Statistical Analysis

All collected data was analyzed using IBM SPSS version 23.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5 (La Jolla, CA, USA). The normality test was performed using the Kolmogorov–Smirnov test. To compare the level of gene expression of those with normal distribution and homogeneous variance data, one-way ANOVA and two independent *t*-tests were performed. For those with non-normal distribution or heterogeneous data, the Kruskal-Wallis H and Mann–Whitney U tests were used. Correlation analysis was done by Spearman's rank-order. Moreover, any possible relationship between the laboratory results and the clinical data was analyzed by χ^2 test and Spearman's rank correlation coefficient. A *p value* < 0.05 was considered statistically significant.

Results

Patients and controls

The demographics and laboratory findings of the BD patients and healthy controls are shown in Table 1.

Cell isolation and differentiation

After cell isolation, flow cytometry was performed to evaluate monocytes in the isolated suspension. The purity was 90%-94% for CD14 positive cells in the sample. As for macrophage differentiation, the morphology of the cells was evaluated and affirmed by inverted microscope. The purity of differentiated macrophages was also evaluated by flow cytometry, and the results showed that after 7 days of stimulation by M-CSF, the cells expressed macrophage markers CD206 and CD163, 91% and 95%, respectively [24].

Gene expression

As Table 3 shows, among the evaluated genes, *HERP* with a 1.55-fold change showed statistically significant overexpression in macrophages from BD patients compared with healthy controls (*p value*= 0.025); however, the difference was no longer significant after *p*-value adjustment.

Moreover, according to Table 4, *HERP* and *GADD34* gene expression levels were higher in patients with a positive history of smoking in comparison to the non-smoker groups with 1.57 and 1.87-fold changes, respectively (*p value*= 0.002 and 0.013, respectively). More details of gene expression in healthy controls, smoker, and non-smoker BD patients are provided in Table 4.

Relative fold changes comparing the expression levels of the mentioned genes between 1) patient and healthy groups, and 2) smoker and non-smoker patients are shown in Figures 1 and 2, respectively. As perceived from Figure 1, *HERP* was the only gene with statistically significant relative increased fold changes in patients in comparison with the healthy controls. As Figure 2 shows, in the smoker and non-smoker BD patients, *HERP* and *GADD34* were relatively overexpressed in smokers compared with non-smokers.

Table.5 demonstrates Spearman's correlation coefficients and the related *p* values between the expression levels of genes in BD patients. *ATF4* was significantly correlated with *ATF6*, *BIP, CHOP, HERP*, and *GADD34*. Moreover, the expression level of *ATF6* was significantly correlated with *XBP1* and *BIP,* and the correlation coefficient between *XBP1* and *HERP* expression was positively significant.

Table 1. Clinical characteristics of BD patients.

Characteristic	Non-smoker BD patients	Smoker BD patients	Healthy Controls
Male/Female	5 (50%)/ 5 (50%)	1 (10%)/ 9 (90%)	7 (70/ 3 (30%))
Age	38±11.23	36.1±8.30	33.3±6.92
HLA-B5	5 (50%)	2 (20%)	-
HLA-B51	2 (20%)	3 (30%)	-
Oral aphthous (Positive)	10 (100)	10 (100%)	-
Genital aphthous (Positive)	7 (70%)	6 (60%)	-
Uveitis (Positive)	7 (70%)	7 (70%)	-
Joint manifestation (Positive)	1 (10%)	5 (50%)	-
Pseudo folliculitis (Positive)	1 (10%)	3 (30%)	-
Erythema nodosum (Positive)	3 (30%)	2 (20%)	-
Monoarthritis (Positive)	1 (10%)	4 (40%)	-
Pathergy test (Positive)	2 (20%)	7 (70%)	-
Eshpilberg test score	47.5±11.81	51.33±13.77	-

Table 2. Primer sequences and product size of the studied genes.

Gene name	Sequence	Size (bp)
GAPDH	F: 5' GAGTCAACGGATTTGGTCGT 3′ R: 5' GACAAGCTTCCCGTTCTCAG 3′	185
CHOP	F: 5' TTGCCTTTCTCCTTCGGGAC 3′ R: 5' TGATTCTTCCTCTTCATTTCCAGG 3′	132
GADD34	F: 5' TAAAGGCCAGAAAGGTGCGCT 3′ R: 5' GGCTAAAGGTGGGTTCCTGAG 3′	167
XBP1	F: 5' GGCCATGAGTTTTCTCTCGT 3′ R: 5' CGAATGAGTGAGCTGGAACA 3′	97
ATF4	F: 5' CTCCGGGACAGATTGGATGTT 3′ R: 5' GGCTGCTTATTAGTCTCCTGGAC 3′	165
ATF6	F: 5' GCACCCACTAAAGGCCAGAC 3′ R: 5' ACTGGGCTATTCGCTGAAGG 3′	146
HERP	F: 5' GAGCCTGCTGGTTCTAATCG 3′ R: 5' GAAAGCTGAAGCCACCCATA 3′	168
BIP	F: 5' GAACGTCTGATTGGCGATGC 3′ R: 5' TCAACCACCTTGAACGGCAA 3′	146

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, *CHOP*: C/EBP homologous protein, *GADD34*: growth arrest and DNA damage-inducible protein, *XBP1*: X-Box binding protein 1, *ATF6*: Activating Transcription Factor 6, *ATF4*: Activating Transcription Factor 4, *HERP*: Homocysteine-induced endoplasmic reticulum protein, *BIP*: Binding immunoglobulin protein.

Table 3. Comparison of *HERP, GADD34, CHOP, BIP, XBP1, ATF4*, and *ATF6* mRNA expressions between BD patients and healthy controls.

Expression level	Patients (N=20)	Healthy Controls (N=10)	Fold Change	*P value*	Adjusted *P*
ATF4	1.16±0.48	1.05±0.33	1.10	0.53	0.74
ATF6	1.21±1.18	1.01±1.47	1.21	0.74	0.74
XBP1	2.05±1.98	1.10±1.17	1.86	0.17	0.39
BIP	1.35±0.74	1.17±0.79	1.15	0.50	0.74
CHOP	1.11±0.40	1.03±0.27	1.07	0.66	0.74
HERP	1.60±0.74	1.03±0.55	1.55	**0.025**	0.17
GADD34	1.56±0.87	1.05±0.33	1.49	0.083	0.29

Geometric mean± SD; FDR correction for multiple comparisons by Benjamini-Hochberg method; Relative expression for each sample was calculated using the following equation: $(2^{-\Delta Ct}) \times 10^3$

Table 4. Comparison of *HERP, GADD34, CHOP, BIP, XBP1, ATF4*, and *ATF6* mRNA expressions between smoker and non-smoker BD patients with healthy controls.

Expression level	Smoker Patients (N=10)	Non-smoker Patients (N=10)	Healthy Controls (N=10)	P-value	Adjusted *P*
ATF4	**1.37±0.58**	0.95±0.25	1.05±0.33	0.058	0.135
ATF6	**1.17±1.37**	1.29±1.01	1.07±1.47	0.721	0.841
XBP1	**2.06±1.72**	2.86±2.60	1.34±1.17	0.116	0.203
BIP	**1.12±0.52**	1.59±0.87	1.17±0.62	0.232	0.324
CHOP	**1.10±0.65**	1.16±0.31	1.03±0.27	0.844	0.841
HERP	**1.93±0.59**	1.23±0.54	1.03±0.25	**0.002**	**0.014**
GADD34	1.92±0.77	1.21±0.74	1.05±0.33	**0.013**	**0.045**

Geometric mean ± SD; FDR correction for multiple comparisons by Benjamini-Hochberg method; Relative expression for each sample was calculated using the following equation: $(2^{-\Delta Ct}) \times 10^3$.

Table 5. Matrix of Spearman's correlation coefficient between the mRNA expressions of genes in the patients.

	ATF4	*ATF6*	*XBP1*	*BIP*	*CHOP*	*HERP*	*GADD34*
ATF4	1	-0.397*	0.219	-0.441*	0.441*	0.50*	0.413*
ATF6	-	1	-0.463**	0.642***	-0.161	-0.110	-0.031
XBP1	-	-	1	-0.10	0.247*	0.398*	0.195
BIP	-	-	-	1	-0.18	-0.20	-0.06
CHOP	-	-	-	-	1	0.19	0.219
HERP	-	-	-	-	-	1	0.391*
GADD34	-	-	-	-	-	-	1

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

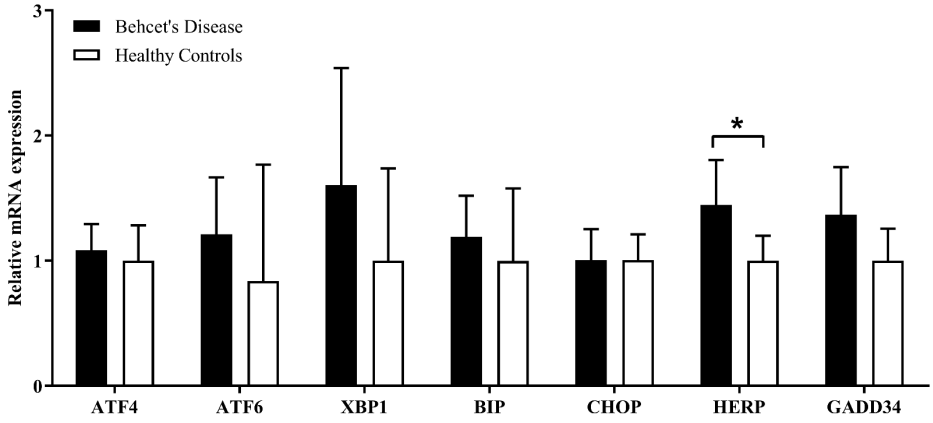

Figure 1. Comparison of relative fold changes in the expression level of *ATF4*, *ATF6, XBP1, BIP, CHOP*, *HERP*, and *GADD34* genes between Behcet's patients and healthy controls. The data are presented as the mean ± 95% CI (*$p \leq 0.05$).

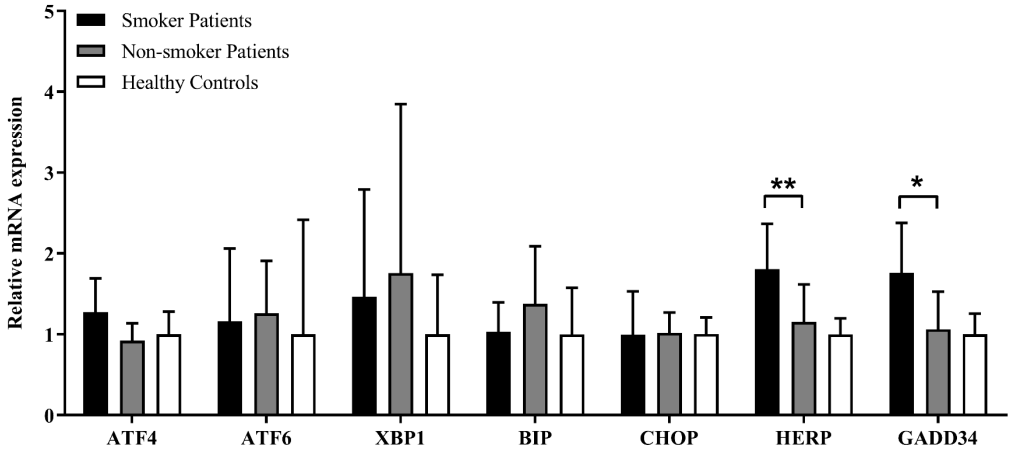

Figure 2. Comparison of relative fold changes in the expression level of *ATF4*, *ATF6, XBP1, BIP, CHOP*, *HERP*, and *GADD34* genes between smoker and non-smoker Behcet's patients with healthy controls. The data are presented as the mean ± 95% CI (*$p \leq 0.05$, **$p \leq 0.01$).

Discussion

BD is an autoimmune disease with an unknown etiology. It is a systemic disorder that involves various organs and burdens the patients predominantly with genital and oral ulcers as well as ocular complications. The ER organelle is mainly responsible for calcium hemostasis and lipid and protein synthesis. However, following oxidative stress and inflammation and upon increased cytokine production, ER triggers UPR to adjust the overload of accumulated misfolded/unfolded proteins. This is achieved through decreasing protein synthesis as well as activating signaling pathways that, in turn, lead to the regulation of genes that participate in the endoplasmic-reticulum-

associated protein degradation (ERAD) system, autophagy, and chaperons. ER stress and UPR can induce cytokine production in immune cells, especially macrophages [25]. Despite the role of the UPR pathway in macrophage functions, the expression of the UPR genes in the macrophages of BD patients has not yet been studied. As the current results showed, macrophages from BD patients expressed increased *HERP* gene compared with healthy controls. It was also found that macrophages from smoker BD patients expressed a higher level of *HERP* and *GADD34* genes.

UPR genes include *ATF4*, *ATF6*, *BIP*, *CHOP* [26], and *GADD34*. The latter is engaged in macrophage autophagy following ER stress [27]. *CHOP*, on the other hand, has been reported to induce apoptotic responses to pro-inflammatory cytokines in chondrocytes [28]. Although the role of UPR signaling in the pathogenesis of BD is still unclear, there is enough evidence to support the association of UPR genes with other autoimmune multisystemic diseases. Savic et al. studied ER stress response genes in the synovial fibroblast (SF) of active RA patients and patients in remission. They showed that toll-like receptor (TLR) induces the activation of *XBP1* and has a pivotal role in sustaining SF function in the active form of RA. They further demonstrated that *GADD34* and *CHOP* expression levels were higher in RA-remission patients than RA-active cases [15]. In another study on an arthritis model, Feng *et al.* found *BIP* to be significantly overexpressed in arthritis synovium where it was greatly observed in fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes (MLS). They also found that *CHOP/GADD153* expression was slightly raised [29] which is more consistent with the current results regarding *GADD34* expression level. In the present study, *GADD34* expression was more elevated in macrophages from BD patients than in healthy controls; however, the difference was not significant.

UPR genes including a spliced form of *XBP1* (*sXBP1*), *CHOP,* and *ATF6* have also been found to be highly expressed in the macrophages of OA patients [30]. A study on a fraction of synovial fluid mononuclear cells (SFMC) in spondyloarthritis (SpA) patients' joints validated the already observed upregulation of UPR genes, including *BIP* [31]. In inflammatory bowel disease (IBD), Kaser *et al.* indicated that *XBP1* abnormalities in epithelial cells of the intestine can relate ER stress responses to the inflammation occurring in IBD [32]. Although the current study detected a 2-fold increase of *XBP1* in BD macrophages in comparison with healthy ones, the difference was not statistically significant. *HERP* is an ER-localized protein that responds to cell stress and participates in ERAD and proper protein folding [33, 34]. No previous report regarding *HERP* function and expression in BD patients was found; however, the *HERP*-increased expression of macrophages from BD patients that was observed in this study may be due to its role in inflammatory responses.

Regarding the great difference in mRNA expression of *GADD34* and *HERP* between macrophages from smoker and non-smoker BD patients and healthy controls in the current research, many studies have established that cigarette smoke, as an *in vivo* inducer of chronic inflammation in airway ducts, can trigger the expression of UPR genes [35-37], including *CHOP* and *BIP* [38]. On the other hand, Kelsen *et al.* determined that reactive oxygen species (ROS) concentrated by cigarette smoke was an important risk factor of chronic obstructive pulmonary disease (COPD), partly because it interferes with protein folding and evokes UPRs including *ATF4*, an upstream regulator of *CHOP* [39]. It was also demonstrated that the expression of *ATF4* and subsequently *GADD34* increased in cigarette smoke-treated cells [36]. It is therefore plausible to propose that UPR genes are upregulated in smokers, which is consistent with the current results. Concerning the strong correlation found between the expressions of *ATF4* with *BIP*, there are well-documented facts stating that *ATF4* is an activator of *GRP78/BiP* promoter [40], while *ATF6* induces the mRNA expression of *XBP1* [41]. It has also been established that *ATF6* and *BIP* interact with each other, and *ATF6* activity is controlled by releasing from *BIP* [42]. Altogether, it is safe to say that the UPR signaling pathway may contribute to BD pathogenesis, as in other autoimmune chronic diseases, because there is evidence showing the association of involved genes in all these patients.

The current study is the first to consider the role of the UPR signaling pathway in the macrophages of BD patients. The expression of UPR genes associated with cigarette smoke was also analyzed in this research. However, the sample size in the current study was relatively small, and the protein expression of UPR pathway was not assessed. Therefore, to understand the exact pathogenic role of ER stress and UPR signaling in the BD pathogenesis, more studies on different immune cells with a larger sample size, considering the protein interactions of the pathway, are needed.

Conclusion

Autoimmune diseases with unknown etiologies, such as BD, require a lot of studies in order to identify predictive factors of both clinical and molecular natures. In the current study, aberrant expression patterns of UPR-related genes were observed in BD patients, especially in those who smoke. Given the role of UPR genes in ER stress and, therefore, inflammation induction, the authors hope that, upon further complementary studies, the diagnosis and treatment of BD patients would be facilitated.

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Conflict of interest

The authors declare they have no conflicts of interest.

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