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Putative Role of Serum Amyloid-A and Proinflammatory Cytokines as Biomarkers for Behcet's Disease

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Abstract: Behcet's disease (BD) is a multisystemic disorder of unknown etiology characterized by relapsing oral–genital ulcers, uveitis, and involvement of vascular, gastrointestinal, neurological, and musculoskeletal system. Although disease pathogenesis is still unclear, both innate and adaptive immunity have shown to play a pivotal role, and multiple proinflammatory cytokines seem to be involved in different pathogenic pathways that eventually lead to tissue damage.

The aims of our study were to evaluate serum cytokines levels of IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, CXCL9, and SAA levels in patients with BD, in comparison to healthy controls (HC), and to correlate their levels to disease activity.

We included 78 serum samples obtained from 58 BD patients and analyzed a set of proinflammatory cytokines including IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, and CXCL9 by multiplex bead analysis as well as SAA by enzyme-linked immunosorbent assay.

Compared to HC, BD patients showed elevated cytokine levels of IL-8, IL-18, IFN- α 2a, and IL-6, and low levels of CXCL11. BD patients with SAA serum levels >20 mg/L showed higher levels of proinflammatory markers than HC or group with SAA \leq 20 mg/L. IL-18, IFN- α 2a, and IL-6 were higher in BD group with SAA >20 mg/L than HC, while IL-8 and CXCL9 levels were higher than in patients with SAA \leq 20 mg/L and HC.

Active BD patients with SAA >20 mg/L exhibited elevated levels of inflammatory mediators, suggesting that may exist a relationship between SAA and proinflammatory cytokines in the intricate scenario of BD pathogenesis.

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Abbreviations: APC = antigen-presenting cell, BD = Behcet's disease, HC = healthy controls, PBMC = peripheral blood mononuclear cell, SAA = serum amyloid-A.

INTRODUCTION

Behcet's disease (BD) is a chronic systemic inflammatory syndrome at the crossroad between autoimmune and auto-inflammatory diseases.¹ Beside the classical symptoms represented by muco-cutaneous lesions and chronic relapsing bilateral uveitis, vascular, gastro-enteric, neurological, and joint involvement may also occur.² Although disease pathogenesis is still unclear, both innate and adaptive immunity have shown to play a pivotal role.^{3–5} The recent understandings on cellular and molecular biology seem to suggest that an imbalance of the Th1/Th2 polarization in favor of Th1 would be able to generate an inflammatory process leading to a CD4+ T lymphocytes clonal expansion, producing high concentrations of both proinflammatory cytokines (such as IFN- γ , IL-6, and IL-8) and cytotoxic CD8+ cells. Moreover, enhanced circulating Th17 lymphocytes as well as increased levels of IL-17A have also been reported in BD.⁶ In addition, high levels of the classical Th1 type cytokine IFN- γ have been found in patients with ocular involvement.⁷ Similarly, IFN- γ secretion is stimulated by natural killer cells through IL-18 action closely relating to BD disease activity and local inflammatory response.⁸ Also, IFN- γ leads to IFN- γ -inducible CXCR3 ligands production (including CXCL9, CXCL10, and CXCL11), known to be involved in various angiogenesis-related disease as well as immunological disorders by inducing the recruitment of activated Th1 cells.⁹ In this regard, few data are available on the role of these chemokines in BD pathogenesis with the exception of CXCL10 chemokine, known to be involved in inducing inflammation in nervous system of BD patients.^{10–16} Not least in importance is the action of antigen-presenting cells (APCs), leading to inflammatory T-cell response as well as neutrophils activation which through the secretion of IL-18, IL-1, IL-6, IL-8, and TNF- α .^{6,13,16,17}

To date, there are no specific laboratory clues to assess disease activity.¹⁸ In this regard, the acute phase protein serum amyloid-A (SAA), synthesized by the liver under the stimulus of proinflammatory cytokines such as IL-6, has been investigated as a potential laboratory marker for several rheumatologic disorders.¹⁹

Recently the role of SAA levels as a potential marker of disease activity in BD patients has been explored, and it has been reported that the occurrence of oral aphthosis, neurological and ocular disease are significantly associated with high serum levels of SAA.²⁰

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Therefore, the present study was aimed at investigating any potential correlation among cytokines profile, serum levels of SAA, and disease activity in BD patients.

MATERIALS AND METHODS

Patients

Seventy-eight serum samples were routinely collected from 58 BD patients (28 males, 30 females, mean age 44.7 ± 12.2 years) who met the International Study Group classification criteria for BD;²¹ the samples were collected every 3 to 4 months and in case of disease relapse.

Of these, 37 samples were obtained from patients with active disease and 41 from patients with inactive disease.

The primary aim of the study was to compare cytokines profile among patients with active BD, patients with inactive BD and healthy controls (HC); the secondary aims were to evaluate the levels of SAA according to BD activity; explore any potential correlation among levels of cytokines, SAA, and status of disease activity.

Patients were included in the active-BD group if they had at least 2 of the following BD-related clinical findings: uveitis, oral aphthosis, genital aphthosis, cutaneous disease, central nervous system involvement, vascular involvement, and gastrointestinal involvement. Samples were also obtained from 32 HC (6 males, 26 females, mean age 41 ± 8.3 years). Table 1 summarizes the clinical and demographic characteristics of BD patients.

The endpoints of the study were: to determine serum levels of IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, CXCL9, and SAA serum concentration every 3 to 4 months and in case of disease relapse; to correlate IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, CXCL9, and SAA serum concentration with the status of disease activity.

Written informed consent was obtained both from patients and HC. The study protocol was reviewed and approved by the Ethical Committee of the Medical University of Bari. Demographic and clinical information was obtained through structured interview, review of medical records, physical examination, and laboratory tests.

Multiplex Bead Analysis

Serum cytokine levels of IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, and CXCL9 were determined, using a Bio-Rad cytokine bead arrays according to the manufacturers' instructions. Data analysis was performed using the Bioplex manager software 6.0 and results displayed as mean \pm SD.

SAA Enzyme-Linked Immunosorbent Assay

SAA serum concentration was determined with a commercial solid phase sandwich enzyme-linked immunosorbent assay (Human SAA, BioSource Europe S.A., Belgium) according to the manufacturer's protocol. Although literature data suggest that SAA levels are elevated over the threshold of 10 mg/L,²² we referred in our analysis to a cut-off of 20 mg/L, since it represents the threshold of our laboratory.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Results were expressed as mean \pm standard deviation. Analysis of variance was used to analyze differences between groups. Two-tailed Mann-Whitney *U* test (for 2 nonparametric groups) and Student's *t*-test (for 2 parametric groups) were used for statistical comparisons between groups.

TABLE 1. Demographic, Laboratory, and General Clinical Characteristics of Patients Affected by Behcet's Disease Recruited in Our Study

	BD Patients (n = 58)	BD Serum Samples (n = 78)
Males n, %	28 (48)	35 (45)
Females n, %	30 (52)	43 (55)
Disease onset (mean \pm SD) in years	31.43 \pm 12.94	31.37 \pm 13.37
Disease duration (mean \pm SD) in years	12.74 \pm 9.31	13.81 \pm 9.94
HLA-B51 positivity, n, %	34 (59)	50 (64)
Serum amyloid-A levels mean \pm SD (range mg/L)	71.91 \pm 135.06 (793.40–4.78)	110.08 \pm 227.55 (1518.40–1.80)
Clinical features, %		
Uveitis	18/58 (31)	26/78 (33)
Oral aphthosis	23/58 (40)	34/78 (44)
Genital aphthosis	7/58 (12)	9/78 (12)
Cutaneous disease	17/58 (29)	20/78 (26)
Central nervous system involvement	6/58 (10)	8/78 (10)
Vascular involvement	3/58 (5)	4/78 (5)
Gastrointestinal involvement	14/58 (24)	17/78 (22)

BD = Behcet's disease, HLA = human leukocyte antigen, SD = standard deviation.

Significance was defined as $P < 0.05$. Correlations were calculated using Spearman correlation (2-tailed *P*-value) as well as Pearson correlation test when required.

RESULTS

The characteristics of 78 serum samples obtained from 58 BD patients are summarized in Table 1. Specifically, 37/78 (47%) were collected from patients with active disease; moreover, 69/78 were treated at the time of serum collection. Anti-TNF as monotherapy (13/78, 17%) or in combination with disease-modifying antirheumatic drugs (16/78, 21%) and disease-modifying antirheumatic drugs monotherapy (26/78, 33%) were the commonest treatments at the time of serum collection. Table 2 summarizes the treatment at the time of serum collection.

Cytokine levels of IL-8, IL-18, IFN- α 2a, IL-6, IFN- γ , CXCL10, CXCL11, and CXCL9 were analyzed in 78 serum samples from BD patients and 32 HC. In BD patients serum concentrations of IL-8 ($P = 0.0001$), IL-18 ($P = 0.0058$), IFN- α 2a ($P = 0.0181$), and IL-6 ($P = 0.0233$) were significantly higher than in HC. Conversely, CXCL11 resulted lower in BD than in HC ($P = 0.0369$), while no differences in CXCL10 and CXCL9 levels between BD and HC were found (Figure 1, Table 3). Detectable levels of IFN- γ were found in few samples both in HC and BD subjects, for this reason this cytokine was not

TABLE 2. BD Patients' Therapy at the Time of Serum Collection

Treatments	N, %
DMARDs	26/78 (33)
Anti-TNF	13/78 (17)
DMARDs + anti-TNF	16/78 (21)
Anakinra	7/78 (9)
Canakinumab	1/78 (1)
Tocilizumab	1/78 (1)
Chorticosteroid	5/78 (6)
No treatment	9/78 (12)

BD = Behcet's disease, DMARD = disease-modifying antirheumatic drug, TNF = tumor necrosis factor.

included in the analysis. In addition statistical analysis by Spearman rho test showed significant correlations between serum concentration of IL-8 and IL-18 ($r = 0.337, P = 0.002$), IL-6 ($r = 0.333, P = 0.004$) serum levels, and strong correlation was found between IL-8 and IFN- α 2a ($r = 0.661, P < 0.0001$). Moreover, also IL-6 serum levels positively correlated with IFN- α 2a ($r = 0.356, P = 0.0011$) serum concentrations. In order to understand whether disease activity may affect cytokine profiles, we compared serum cytokines levels between active-BD ($n = 37$), inactive-BD ($n = 41$), and HC ($n = 32$) subjects. IL-8 and IL-18 resulted higher in both active-BD ($P = 0.0001$ and 0.012 , respectively) and inactive-BD ($P = 0.0001$ and 0.0128 , respectively) than in HC. No differences were observed in serum levels of CXCL10 and CXCL9 between patients and HC. Interestingly IFN- α 2a ($P = 0.0141$) and IL-6 ($P = 0.0332$) serum levels were

significantly higher in active-BD than HC. Moreover, CXCL11 serum concentration was significantly lower in inactive-BD than HC ($P = 0.0154$) (Figure 2, Table 3).

When cytokines were evaluated in the active-BD group, serum IL-8 concentration positively correlated with IL-18 ($r = 0.381, P = 0.02$) and a strong correlation was found with IFN- α 2a ($r = 0.737, P < 0.0001$) serum levels. In addition, IFN- α 2a serum concentration also correlated positively with serum concentration of IL-6 ($r = 0.513, P = 0.007$).

When IL-8, IL-18, IFN- α 2a, and IL-6 serum levels were compare between BD patients with SAA serum levels ≤ 20 mg/L ($n = 20$), >20 mg/L ($n = 50$) and HC ($n = 32$), patients with SAA levels below 20 mg/L showed significantly increased IL-8 ($P = 0.002$), IFN- α 2a ($P = 0.0213$) and significantly decreased CXCL10 ($P = 0.0205$) and CXCL11 ($P = 0.0004$) serum levels compared to HC. Interestingly, no differences were observed in IL-18, IL-6, and CXCL9 serum levels between patients with SAA below 20 mg/L and HC. Patients with SAA >20 mg/L showed serum IL-8 levels significantly higher than patients with SAA ≤ 20 mg/L ($P = 0.0298$) and HC ($P = 0.0001$). Serum IL-18 ($P = 0.0044$), IFN- α 2a ($P = 0.0212$), and IL-6 ($P = 0.0042$) levels were significantly higher than in HC, but comparable to BD subjects with SAA ≤ 20 mg/L. In addition, CXCL9 resulted higher when SAA was higher than 20 mg/L compared to HC ($P = 0.032$) and patients with serum levels ≤ 20 mg/L ($P = 0.0242$). Furthermore, we observed that CXCL10 and CXCL11 serum levels showed to be significantly higher when SAA levels were >20 mg/L than in patients with lower SAA levels ($P = 0.0239$ and 0.0123 , respectively), but were comparable to HC group (Figure 3, Table 3).

Finally, we found SAA serum levels positively correlated with IL-8 ($r = 0.273, P = 0.022$), IL-6 ($r = 0.421, P = 0.0005$), CXCL10 ($r = 0.293, P = 0.014$), CXCL11 ($r = 0.318, P = 0.007$), and CXCL9 ($r = 0.336, P = 0.006$) serum levels.

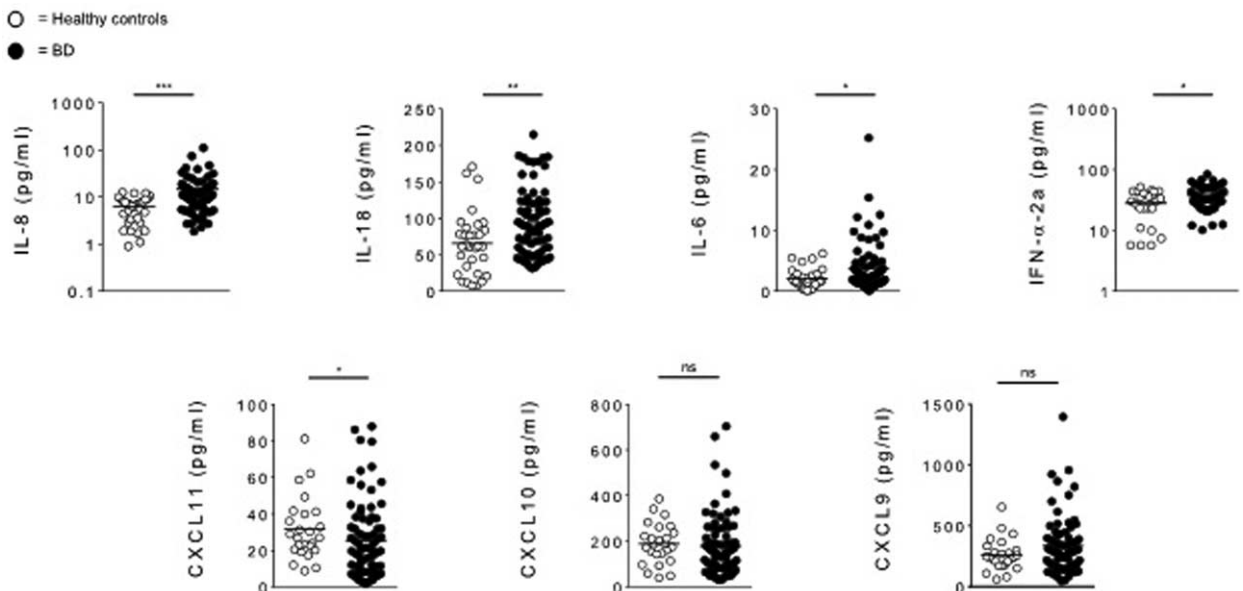


FIGURE 1. Serum cytokines levels in BD patients and HC. Circulating level of IL-8, IL-18, IL-6, IFN- α 2a, CXCL11, CXCL10, and CXCL9 were analyzed in serum samples obtained from BD patients ($n = 78$) and HC ($n = 32$). Data are expressed as mean \pm SD. Mann-Whitney U test and Student's *t*-test were carried out to check for statistical significance between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant). BD = Behcet's disease, HC = healthy controls, SD = standard deviation.

TABLE 3. Levels of Cytokines in HC and BD Patients

	HC	BD	Inactive-BD	Active-BD	Low-SAA	High-SAA
IL-8, pg/mL	6.18 ± 3.36	14.84 ± 15.67***	14.48 ± 17.47***	15.24 ± 13.64***	9.853 ± 4.74**	18.11 ± 18.56***,****,
IL-18, pg/mL	65.59 ± 42.91	92.97 ± 45.92**	94.65 ± 51.17**	91.1 ± 39.92**	90.19 ± 52.84	94.47 ± 43.88**
IFN-α-2a, pg/mL	28.33 ± 14.59	37.77 ± 16.35*	35.99 ± 16.44	39.56 ± 16.39*	40.46 ± 11.68*	38.72 ± 17.74*
IL-6, pg/mL	2.01 ± 1.69	3.77 ± 4.11*	3.32 ± 2.90	4.208 ± 5.01*	2.124 ± 1.24	4.499 ± 4.70**
CXCL10, pg/mL	188.85 ± 89.47	179.3 ± 133.6	166.3 ± 129.5	193.6 ± 138.4	143.6 ± 134.9*	201.2 ± 135.2****
CXCL11, pg/mL	31.52 ± 17.11	25.34 ± 21.07**	22.56 ± 19.25*	28.42 ± 22.78	15.67 ± 12.36***	30.44 ± 23.32****
CXCL9, pg/mL	263.52 ± 130.70	338.1 ± 238.2	320.8 ± 260.8	356.5 ± 213.8	240 ± 122	388 ± 261.9*,****

Data are expressed as mean ± SD. Mann–Whitney *U* test and Student’s *t*-test were carried out to check for statistical significance between groups. Significance between groups versus the HC group. BD = Behcet’s disease, HC = healthy controls, SAA = serum amyloid A. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.05 between low- versus high-SAA group.

DISCUSSION

Our study was aimed at exploring any potential correlation among cytokines profile, serum levels of SAA and disease activity in BD patients. First of all, we analyzed serum levels of a core set of cytokines (IL-8, IL-18, IFN-α2a, IL-6, IFN-γ, CXCL10, CXCL11, and CXCL9) and SAA serum concentration.

BD is a complex inflammatory disorder characterized by an abnormal innate and adaptive immune response leading to hyperactivation of proinflammatory mediators. In the present study, in line with previously reported data,^{11,23,24} we found elevated levels of several inflammatory markers in BD patients compared to HC. In particular, BD appears associated to high levels of IL-8, IL-18, IL-6, and IFN-α especially in patients with high disease activity and SAA serum levels higher than 20 mg/L.

More specifically, IL-8 is known to be involved in the neutrophils activation as well as in leukocytes migration. In addition, it promotes the differentiation of mononuclear to granulocytic infiltration and the adhesion of peripheral blood leukocytes to endothelial cells. Several studies reported elevated IL-8 levels in serum from BD patients compared to HC, but discordant results were obtained when a relation to disease activity was investigated. In particular, IL-8 significantly correlate to specific BD clinical characteristic such as oral ulcers, skin lesions, and ocular involvement due to its role in the inflammatory response and its capability to attract polymorphonuclear cells into the lesions.^{11–16} However, these findings are controversial.¹⁵

IL-18 is a proinflammatory cytokines over-expressed in BD.²⁴ It is principally produced by APCs and is involved in Th1

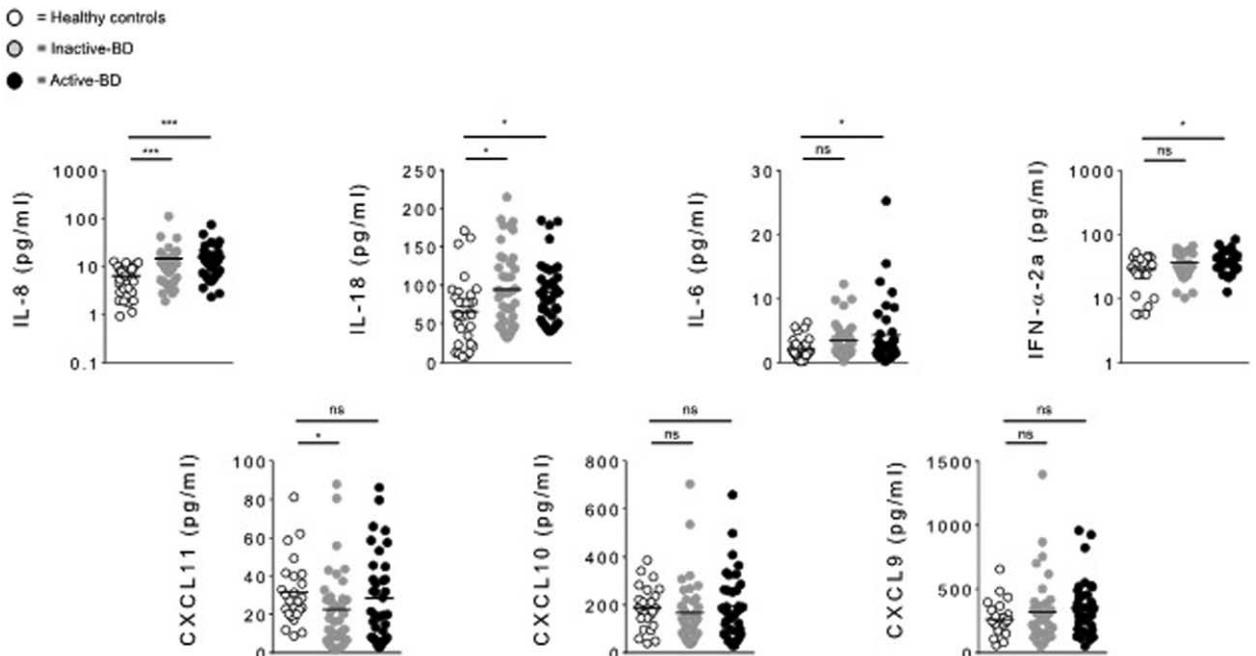


FIGURE 2. Serum cytokines levels in inactive-BD, active-BD, patients and HC. Circulating level of IL-8, IL-18, IL-6, IFN-α2a, CXCL11, CXCL10, and CXCL9 were analyzed in serum samples obtained from active-BD (n = 37), inactive-BD (n = 41), and HC (n = 32) subjects. Data are expressed as mean ± SD. Mann–Whitney *U* test and Student’s *t*-test were carried out to check for statistical significance between groups (**P* < 0.05, ***P* < 0.01, ****P* < 0.01, ns = not significant). BD = Behcet’s disease, HC = healthy controls, SD = standard deviation.

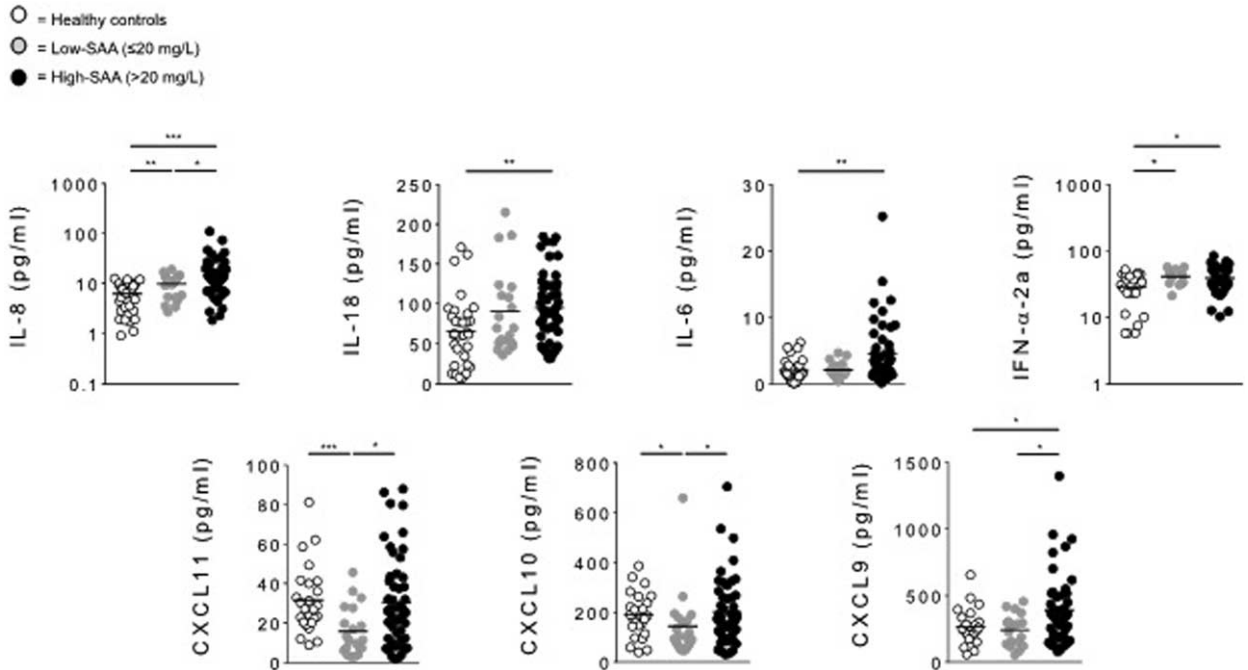


FIGURE 3. Serum cytokines levels in low-SAA BD, high-SAA BD patients, and HC. Circulating level of IL-8, IL-18, IL-6, IFN- α 2a, CXCL11, CXCL10, and CXCL9 were analyzed in serum samples obtained from BD patients with SAA serum levels ≤ 20 mg/L (low-SAA, $n = 20$), >20 mg/L (high-SAA, $n = 50$), and HC ($n = 32$). Data are expressed as mean \pm SD. Mann-Whitney U test and Student's t -test were carried out to check for statistical significance between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). BD = Behcet's disease, HC = healthy controls, SAA = serum amyloid-A.

polarized immune response as well as in IL-6, IFN- α , and IL-12 production.⁶ Moreover, IL-18 seems to enhance chemokine productions, especially CXCL9, and induce INF- γ release by NK cells, suggesting a role also in innate responses. Although serum IL-18 levels have been observed to be not significantly different between active and inactive BD patients,²⁵ we found that IL-18 correlates with disease activity. Notably, the detection of increased levels of IL-18 in patients with inactive disease might be explained by a smoldering subclinical inflammation during intercritical periods.

Interestingly, in our study serum IL-6 and IFN- α levels were significantly higher exclusively in patients with active disease compared to HC. IFN- α is a cytokine involved in BD pathogenesis presenting pleiotropic effects. Especially, IFN- α may significantly increase IFN- γ levels in memory CD4+ T cells and inhibit Th1 and Th17 cells.²⁶ Several studies suggest that IFN- α promotes a regulatory Th1 response causing anti-inflammatory effects mainly through an increase in soluble cytokine receptors, cytokine antagonists, and soluble adhesion molecules.²⁷⁻²⁹ Few data are available concerning IFN- α levels in BD. Kötter et al³⁰ observed increased IFN- α serum levels in a group of BD patients with ocular involvement. More recently, it has been suggested that high serum IFN- α levels in BD patients might be due to increased frequency and activation of IFN- α + plasmacytoid dendritic cells (pDC) resulting in Th1 type immune response.²⁷

With regard to IL-6, it is mainly produced by activated macrophages and monocytes, but is also released by other cell types such as APCs and lymphocytes. IL-6, known to be associated to monocyte differentiation and CD4+ T cells differentiation into Th17 cells, is linked to BD activity.^{6,31,32} In addition, it also seems to play a critical role in BD patients

presenting with central nervous system involvement,³³ albeit it might have a protective role on mucocutaneous BD features.³⁴

Since we recently demonstrated a correlation between increased SAA serum levels and various typical BD clinical manifestations,²⁰ in the present, study we also looked for possible different cytokine patterns in patients with low and high SAA serum levels.

Noteworthy, we observed that BD patients with SAA higher than 20 mg/L showed intriguing characteristics in terms of proinflammatory cytokines serum levels. In particular, IL-18, IFN- α 2a, and IL-6 were higher in patients with at least 20 mg/L SAA levels than in HC, while IL-8 and CXCL9 levels were higher in patients with SAA >20 mg/L than in patients with SAA ≤ 20 mg/L and in HC. Although SAA is mainly produced in the liver by proinflammatory stimuli such as IL-6, IL-1, and TNF α ,³⁵ also other cell types are known to produce SAA-including activated endothelial cells and monocytes/macrophages.^{36,37} Moreover, monocytes and monocytes derived macrophages have been reported to produce proinflammatory cytokines after SAA stimulation.³⁸ Indeed, it has been recently demonstrated that SAA may stimulate the release of mature IL-1 β from neutrophils, macrophages, and fibroblasts through inflammasome activation.³⁹⁻⁴¹ Interestingly, the release of IL-8 as well as TNF- α from SAA-stimulated neutrophils was also reported.⁴² In addition, it has been observed that SAA induces neutrophil transmigration, monocyte migration, and peripheral blood mononuclear cells adhesion in an IL-8 and monocyte chemoattractant protein-1 dependent manner.⁴³ The main limitations of our study are represented by the sample size that did not allow us to compare data according to specific organ involvement, and the absence of a disease control group, that could add more information regarding the disease specificity of

the alterations observed. Moreover, at the time of samples collection, all of the patients were already taking corticosteroids and immunosuppressive agents, which might have affected cytokines serum levels.

In conclusion, our findings corroborate that BD patients exhibit elevated levels of specific inflammatory mediators, especially during active disease periods and in patients with increased SAA serum levels. Notably the increased levels of proinflammatory cytokines found in our study show that the immune response in BD is skewed toward a Th1 pathway. However, no obvious differences in cytokine profile between active and inactive-BD patients were found. This lack of differences may be due to various grounds such as different subsets of patients, admission criteria, besides the treatment regimens. For the above reason it is difficult to attribute an actual pathogenic role for each cytokine as regards the cytokines profile between active and inactive patients. Together with all the results from studies aimed at exploring, the pathogenetic role of cytokines in BD, these findings, may certainly contribute to improve our knowledge regarding specific target for novel therapies or for a different and more specific use of biologic drug currently available.^{44–48} However, although our suggestion of a possible SAA role in the induction of BD inflammatory manifestations is intriguing, further studies are needed both to better understand this pathogenetic perspective and to investigate the possible role of SAA inhibition in such patients. In order to confirm the results of our proof of concept study on the role of SAA, cytokines profile, and disease activity, a validation study on a larger cohort is ongoing.

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