The relationship between NETosis findings and disease activity in Behçet's disease: an exploratory study

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Abstract

Objective

This study aimed to investigate the relationship between the neutrophil extracellular traps (NET) and NETosis findings and disease activity in Behçet disease (BD).

Methods

The study group consisted of 30 active BD patients and 10 healthy individuals as controls. Serum and saliva samples were collected from the patients during their active and remission periods. A subset of them were followed longitudinally. Serum and saliva cf-DNA, NE, MPO, and cit-H3 levels were measured as indirect NETosis findings, subsequently the results were adjusted based on the peripheral blood neutrophil counts. Unadjusted and adjusted levels of the NETosis findings were evaluated.

Results

In active BD, unadjusted serum cf-DNA and NE levels were significantly higher than in controls, whereas adjusted serum MPO and cit-H3 levels were found to be lower. In inactive BD, unadjusted serum NE levels remained elevated compared to controls, while unadjusted serum MPO, adjusted serum MPO, and adjusted serum cit-H3 levels were lower. No significant differences were observed in salivary NETosis findings between the patients and controls. Longitudinal follow-up revealed a decrease in both unadjusted serum cf-DNA and saliva cf-DNA levels in parallel with reduced clinical activity. Saliva and unadjusted serum cf-DNA showed a positive correlation with inflammatory markers, whereas adjusted serum MPO and cit-H3 correlated negatively.

Conclusion

Indirect NETosis findings varied in relation to the systemic and/or local activity of BD patients. The changes after adjustment suggest that serum NETosis markers can be influenced by increased neutrophil turnover during the active phase of the disease.

Key words

neutrophil extracellular traps, NETosis, neutrophil, Behçet's disease, saliva, oral aphthous ulcer

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Introduction

Behçet disease (BD) is a multisystem inflammatory disorder of unknown aetiology and classified as a variable vessel vasculitis and a member of neutrophilic dermatoses (1, 2). Endothelial dysfunction and tissue damage resulting from the activation of the immune system with environmental triggers in individuals genetically predisposed to BD, particularly those carrying HLA-B*51, are thought to be the principal mechanism in the pathogenesis of BD (3-5). Neutrophil activation plays a prominent role in the pathogenesis of BD, contributing to tissue damage. It has been documented that the levels of neutrophil-associated cytokines and chemokines increase, as well as the neutrophil-mediated oxidative stress, in patients with BD. In addition, inflammation characterised by neutrophil infiltration of the vascular wall and endothelial damage contribute to vascular involvement in BD (4). Despite the long-standing hypothesis regarding the role of neutrophils in the pathogenesis of the disease, the precise mechanisms underlying the development of manifestations remain elusive.

A number of studies have demonstrated the potential role of neutrophil extracellular trap (NET)-induced mechanisms in the pathogenesis of BD (6-8). However, further studies are warranted to clarify the mechanism and to elucidate its impact on the pathogenesis of the disease. NETs are defined as a web-like structure consisting of granular and nuclear content derived from neutrophils, which can kill bacteria outside the cell (9). The phenomenon that develops at the end of the NET formation and the occurrence of cell death are also defined as NETosis (10). NETosis is regarded as a pivotal mechanism in innate host defence, tissue damage, thrombosis, regulation of sterile inflammation, as well as in autoimmunity and oncoimmunity (11). NETosis is a dynamic process that can occur in two distinct ways, classical (suicidal) NETosis and vital NETosis. The cellular pathway commences with the release of neutrophil granule contents, including neutrophil elastase (NE) and myeloperoxidase (MPO), into the cytoplasm by various mechanisms, histone modification-mediated chromatin decondensation, and ends with chromatin swelling, nuclear lobulation, plasma membrane disintegration and the release of DNA and other nuclear fragments such as cell-free DNA (cf-DNA) or citrullinated histone H3 (cit-H3), and their complexes with neutrophil contents out of the cell (12, 13). NETosis analyses remain a developing field, and there is currently no gold-standard assay to document suicidal or vital NET formation in systemic circulation, and all of the methods have certain limitations (14, 15). In this exploratory study, we aimed to investigate the utility of a set of NETosis-related markers in Behçet disease, assessing their association with oral and systemic disease activity to evaluate their potential for use in future studies using serum and saliva samples.

Material and method

Patients and study design

This study was designed as a cross-sectional and longitudinal experimental analysis in a single centre, and it was conducted between September 2021 and November 2022. Adult BD patients with oral and/or systemic active disease findings were included in the study group and a group of healthy individuals were included as a control group. International Study Group (ISG) 1990 criteria set was used for classification (16). Patients with additional inflammatory conditions and using biologic agents or >5 mg prednisolone were excluded. A subset of the subjects was also followed longitudinally, and serum and saliva samples were collected from the patients during both their active and remission periods. Patients with oral aphthous ulcers at the time of sample collection were defined as "orally active disease", while patients with other mucocutaneous manifestations (genital ulcer, erythema nodosum-like lesion, etc.) with elevated inflammatory markers or systemic manifestations were defined as 'systemically active disease'. Moreover, 'inactive disease' was defined as patients with no manifestation and normal acute phase reactants for at least one month after appropriate treatment

modifications. Patients' demographic characteristics, cumulative clinical data, characteristics of oral (number, type, duration, and recurrence frequency of aphthous ulcers) and/or systemic manifestations, previous and current treatments, laboratory parameters including complete blood count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) on the day of sample collection were obtained from patient's medical records and noted on a standardised form.

This study protocol was approved by Istanbul Faculty of Medicine, Clinical Research Ethics Committee (2021-231298), and all subjects provided written informed consent.

Sample collection and storage

Venous blood samples were collected from subjects during both active and inactive disease periods, as well as from healthy individuals. Serum samples were obtained by centrifugation at 3000 rpm for 10 minutes and subsequently aliquoted into cryotubes and stored at -80°C until analysis.

A total of 3–5 ml of unstimulated whole saliva samples were collected from subjects during orally active and inactive disease periods and from healthy individuals in 15 ml falcon tubes in 5-10 minutes. All saliva samples were collected between 08:00 and 12:00 hours. Study participants interrupted eating and drinking at least 1.5 hours before sampling and did not smoke on or before the day of collection, and they gently rinsed the inside of their mouths with sterile water. Supernatants were obtained by centrifugation of saliva samples at 3500 rpm for 15 minutes. Subsequently, the samples were immediately aliquoted into cryotubes and stored at -80°C until analysis.

Analyses of indirect NETosis markers

We used cf-DNA, cit-H3, MPO, and NE measurements, to be able to assess their utility in following changes in association with systemic and local inflammatory activity. The level of cf-DNA in serum and saliva was measured by using Invitrogen Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. Briefly, the standards were prepared based on the high-range standard curve. 100 µl of Quant-iT PicoGreen dsDNA reagent was added to each sample and standard, and samples were incubated for 5 minutes at room temperature. Fluorescence signals were measured using an Invitrogen Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, USA) with the standard wavelength settings, for excitation at 480 nm, and for emission at 520 nm.

The levels of NE, MPO, and cit-H3 in serum and saliva were measured by using Human NE/ELA2 (Neutrophil Elastase/Elastase-2) ELISA Kit (Elab Science, E-EL-H1946), Human MPO ELISA Kit (Hycult Biotech, HK324) and Human Citrullinated Histone H3 ELISA Kit (MyBiosource, MBS3804611) following the manufacturer's instructions, respectively.

The levels of all markers were given as ng/ml. Adjustment of these measurements was made by the calculation of the amount of each serum marker per 1 million neutrophils, by using the absolute neutrophil counts in the complete blood counts that were already collected on the day of sample collection.

Statistical analysis

The quantitative variables were described as the mean ± standard deviation (SD) or median [interquartile range (IQR)], while the categorical variables were presented as a number and percentage. Continuous variables were compared using either the Wilcoxon signed-rank test or the Mann-Whitney U-test, depending on the non-parametric nature of the variables. Correlation analyses were conducted using Spearman's rho, given that the relevant variables were not normally distributed in at least one group. The diagnostic decision-making properties of the markers were determined by calculating the area under the curve (AUC) from receiver operating characteristic (ROC) curves. All statistical analyses were

Table I. Characteristics of patients whose serum samples were taken in the active and inactive state.

	All BD patients (n=30)	Longitudinally followed BD patients (n=18)	
	Active period	Active period	Inactive period
Sex, male, n (%)	21 (70)	11 (61.1)	
Age, year, mean ± SD	35.3 ± 10.7	36.5 ± 9.1	
Disease duration, m, med (IQR)	54 (10-150)	48 (10-70)	
Family history of BD, n (%)	6 (20)	4 (23.5)	
HLA-B51, n (%)	10[¥] (76.9)	9[ǂ] (75)	
Smoking, n (%)	8 (26.7)	7 (38.9)	
Pathergy positivity, n (%)	19[§] (73.1)	12[ɛ] (75)	
Cumulative involvement, n (%)			
Mucocutaneous	30 (100)	18 (100)	
Arthritis	12 (40)	8 (44.4)	
Uveitis	8 (26.7)	2 (11.1)	
Neurologic	2 (6.7)	1 (5.6)	
GIS	1 (3.3)	0 (0)	
Vascular	7 (23.3)	6 (33.3)	
Other	1 (3.3)	1 (5.6)	
Treatments, n (%)			
Naive	7 (23.3)	4 (22.2)	0 (0)
Colchicine	23 (76.7)	14 (77.7)	18 (100)
Azathioprine	7 (23.3)	3 (16.6)	10 (55.6)
Corticosteroid	3 (10)	2 (11.1)	9 (50)
TNFi	0 (0)	0 (0)	1 (5.6)
Laboratory values			
Neutrophil, 10^3/µl, med (IQR)	6.3 (4.7-7.7)	6.6 (5.1-8.5)	4.7 (3.4-6.6)
CRP, mg/l, med (IQR)	7.5 (3.16-26.5)	10.7 (4.03-50)	2.44 (0.6-4.46)
ESR, mm/h, med (IQR)	10.5 (7-23.25)	13.5 (7.7-27)	9 (3-15)

BD: Behçet's disease; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; GIS: gastrointestinal system; IQR: interquartile range; m: month; med: median; n: number of patients; SD: standard deviation; TNFi: tumour necrosis factor inhibitor.
([¥] n=13, [ǂ] n=12, [§] n=26, [ɛ] n=16).

Table II. Characteristics of oral aphthae in patients whose saliva samples were obtained in the orally active and inactive state (during the active period).

	All orally active patients (n=15)	Longitudinally followed patients (n=7)
Number of OA, med (IQR)	1 (1-3)	2 (1-4)
Type of OA, n (%)		
Minor	11 (73.3)	5 (71.4)
Major	4 (26.7)	2 (28.6)
Herpetiform	0 (0)	0 (0)
Day of sampling, day, med (IQR)	4 (2-7)	4 (3-7)

IQR: interquartile range; med: median; n: number of patients; OA: oral aphthae.

performed by using SPSS software v. 26.0 (IBM Corp., Armonk, USA), and two-tailed p-value <0.05 was considered statistically significant.

Results

Patients and control group characteristics

A total of 30 consecutive adult BD patients with oral and/or systemic active disease findings were included in the study group. All but one patient fulfilled the ISG-1990 diagnostic criteria, and the exceptional patient was diagnosed with BD based on recurrent oral aphthae, pathergy positivity, and typical vascular involvement.

Ten individuals were included in the study as the healthy control (HC) group, which consisted of 5 (50%) males, and their median age was 30 (26.75–47.5) years. The number of smokers in the control group was 3 (30%). There was no statistically significant difference between the control group and the patients in terms of sex, age, or smoking status. Twenty-two patients had systemic activation, while eight patients had only oral activation. Fifteen patients were classified as orally active. Follow-up serum samples were obtained from 18 of 30 active patients during their inactive period, while follow-up saliva samples were obtained from 7 of 15 orally active patients. All active patients had significantly higher inflammatory markers than the HC group [neutrophil counts 4 (3.2–4.6) 10^3/µl, CRP 1.35 (0.55–2.32) mg/l (normal range: 0–5 mg/l), and ESR 4 (4-6) mm/hour; p=0.002, p<0.001 and p=0.002, respectively], whereas there was no statistically significant difference between the inactive patients and HCs. Neutrophil counts, CRP and ESR levels were significantly elevated during the active disease phase compared to the inactive disease phase among longitudinally followed patients (p=0.019, p<0.001, and p=0.004, respectively). The demographics, clinical characteristics, and laboratory findings of the patients whose serum and saliva samples were collected during both active and inactive disease periods are given in Table I, and the characteristics of oral aphthae in all orally active patients and longitudinally followed patients (at the time of activation) are shown in Table II. The demographic, clinical, and laboratory features of systemic and isolated oral active patients are shown in Supplementary Table S1.

Indirect NETosis markers in the study groups

The unadjusted serum cf-DNA and NE levels were significantly higher in active patients (aBD) than the levels in HC [3980 (3540–4337) *vs*. 3115 (2780–3202) ng/ml, p<0.001 and 5.06 (3.39–5.97) *vs*. 2.95 (1.52–3.81) ng/ml, p=0.012, respectively], whereas unadjusted serum MPO and cit-H3 levels were lower without statistical significance. After the adjustment of these measurements according to the absolute neutrophil counts, there was no difference between serum cf-DNA and NE levels of aBD patients and HC. However, adjusted serum MPO and cit-H3 levels were found to be significantly lower in aBD patients compared to the levels in HC [3.66 (2.4–5.24) *vs*. 6.91 (5.94–8.09) ng/ml, p<0.001 and 0.95 (0.36–1.72) *vs*. 3.07 (1.38–3.69) ng/ml, p=0.005, respectively].

Unadjusted serum NE levels were found to be significantly higher in inactive BD (iBD) than in HC [4.56 (3.30–5.80) *vs*. 2.95 (1.52–3.81) ng/ml, p=0.027], in contrast to cf-DNA. Unadjusted serum MPO levels were found to be significantly lower in iBD than in HC [23.80 (16.18–27.04) *vs*. 28.83 (23.66–30.30) ng/ml, p=0.049], and unadjusted serum cit-H3 levels were also found to be lower, but the difference did not reach statistical significance. The difference in adjusted serum MPO and cit-H3 levels between iBD patients and HC groups became more prominent [4.56 (3.30–5.39) *vs*. 6.91 (5.94–8.09) ng/ml, p=0.001 and 1.63 (0.89–2.06) *vs*. 3.07 (1.38–3.69) ng/ml, p=0.035, respectively], while the statistical significance had disappeared for the adjusted serum NE levels.

Only the adjusted NE levels among all serum NETosis markers were significantly different between systemically active and isolated orally active patients, interestingly it was higher in orally active ones [0.65 (0.34–1.02) *vs*. 1.19 (0.91–1.80) ng/ml, p=0.022] (Supplementary Fig. S1). In the patients with vascular involvement (V-BD), the difference between the level of NETosis markers of HC and the patient groups was quite similar to the general cohort, particularly unadjusted serum cf-DNA level was found to be significantly higher, whereas adjusted serum MPO and cit-H3 levels were significantly lower in active V-BD patients (Suppl. Fig. S2A-H).

In the analyses of the saliva, no significant difference was observed in indirect NETosis markers between aBD and HC, as well as between iBD and HC samples. The levels of cf-DNA and NE were numerically higher in aBD than in HC, although these differences did not reach statistically significant level [462 (288–1240) *vs*. 226 (185–818) ng/ml, p=0.257 and 4.34 (0.88–6.63) *vs*. 2.98 (1.25–5.56) ng/ml, p=0.594, respectively]. The levels of serum and saliva NETosis markers in HC, aBD, and iBD are shown in Figure 1.

Longitudinal changes in indirect NETosis markers

The unadjusted serum cf-DNA levels of aBD patients decreased significantly when they became inactive during the longitudinal follow-up [from 3910

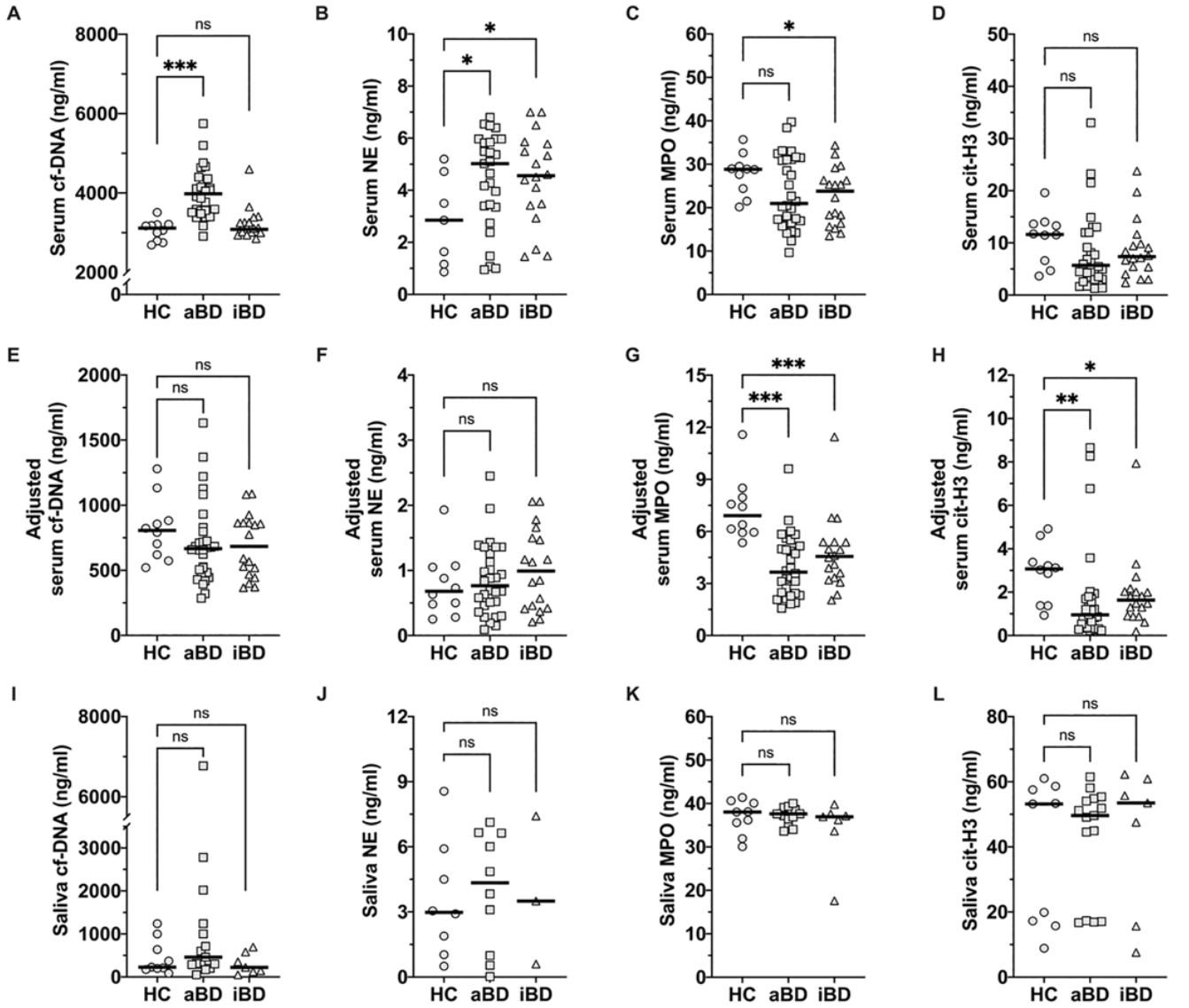

Fig. 1. Unadjusted serum NETosis markers (**A**, **B**, **C**, and **D**), adjusted serum NETosis markers (**E**, **F**, **G**, and **H**), and saliva NETosis markers (**I**, **J**, **K**, and **L**) in HC, aBD, and iBD. Mann-Whitney U-test; ns=non-significant.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.
aBD: active Behçet disease; iBD: inactive Behçet disease; HC: healthy control; cf-DNA: cell-free DNA; NE: neutrophil elastase; MPO: myeloperoxidase; cit-H3: citrullinated histon-3.

(3460–4195) to 3085 (3007–3292) ng/ml, p=0.002], whereas no significant change was detected in unadjusted serum NE, MPO, and cit-H3 measurements. There was no significant change in adjusted serum cf-DNA during the longitudinal follow-up. The changes in the level of other NETosis markers during follow-up were also quite similar to the trend observed in the general cohort, particularly adjusted serum cit-H3 level significantly increased during the follow-up with the decreased disease activity (Suppl. Fig. S2I-P).

In the saliva, the level of cf-DNA significantly decreased during the follow-up, like unadjusted serum cf-DNA levels [from 1240 (600–2780) *vs*. 222 (122–581) ng/ml, p=0.018]. Saliva MPO levels measured during active disease period were decreased in the inactive disease period [from 37.60 (36.88–39.33) to 36.94 (33.65–37.61) ng/ml, p=0.028], while there was no significant change in the levels of NE and cit-H3. The alterations of serum and saliva NETosis markers during the longitudinal follow-up are shown in Figure 2.

Correlation analysis of indirect NETosis markers

In correlation analysis between inflammatory markers and NETosis markers, the unadjusted serum cf-DNA levels were positively correlated with the neutrophil count, CRP, and ESR (r=0.300, p=0.039; r=0.558, p<0.001; r=0.343, p=0.017, respectively). Unadjusted serum MPO levels were also positively correlated with the neutrophil count (r=0.313, p=0.030). After the adjustment of values with the absolute neutrophil count, serum MPO and cit-H3 levels were negatively correlated with CRP (r= -0.380, p=0.008 and r= -0.344, p=0.017, respectively), as well as ESR (r= -0.307, p=0.034 and r= -0.314, p=0.030, respectively). In saliva, a pos-

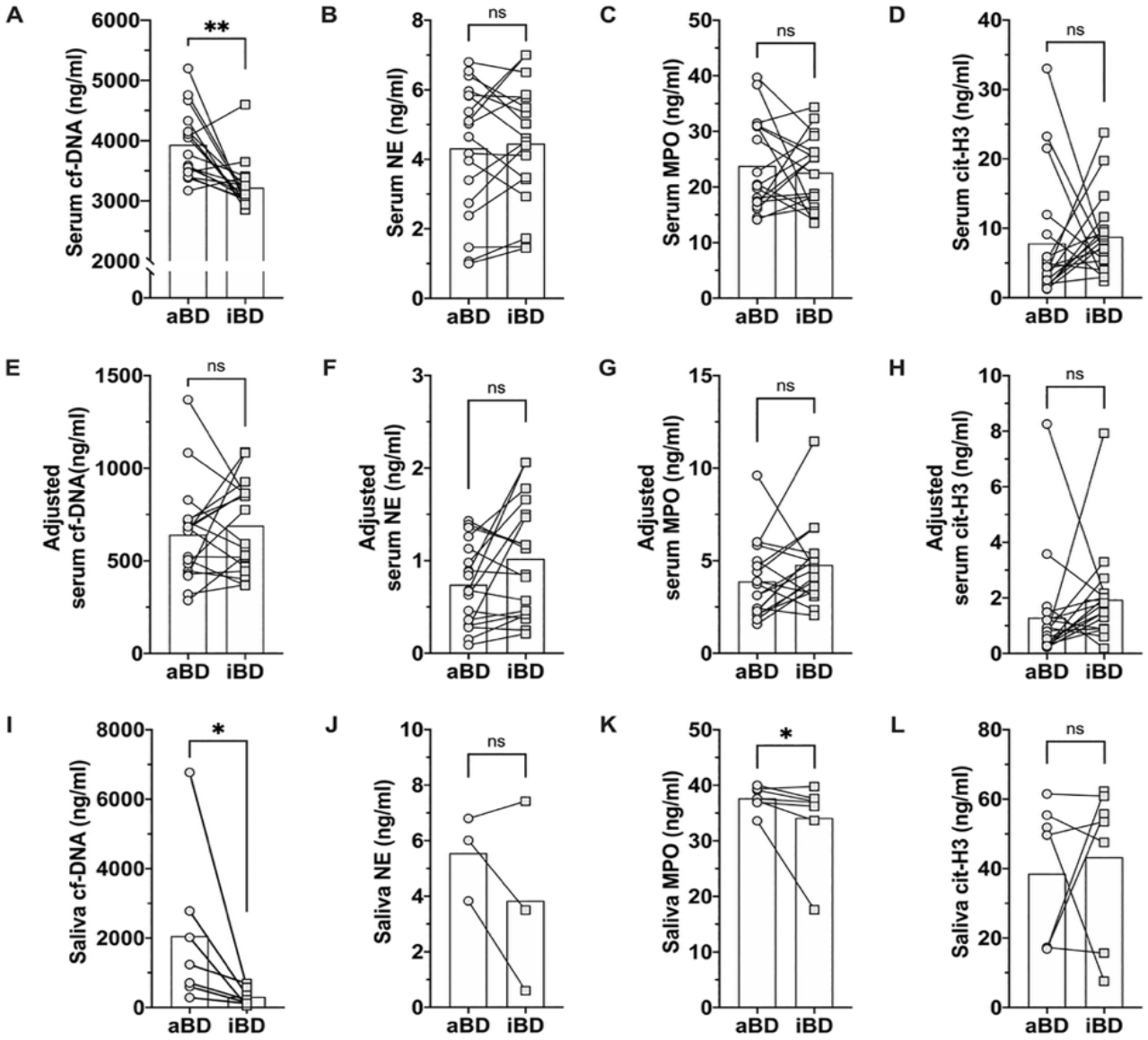

Fig. 2. Alterations in unadjusted serum NETosis markers (**A**, **B**, **C**, and **D**), adjusted serum NETosis markers (**E**, **F**, **G**, and **H**), and saliva NETosis markers (**I**, **J**, **K**, and **L**) during the longitudinal follow-up. Wilcoxon signed-rank test; ns=non-significant.
* $p<0.05$, ** $p<0.01$.
aBD: active Behçet disease; iBD: inactive Behçet disease; cf-DNA: cell-free DNA; NE: neutrophil elastase; MPO: myeloperoxidase; cit-H3: citrullinated histon-3.

itive correlation was observed between cf-DNA levels and inflammatory markers, particularly neutrophil count and CRP (r=0.427, p=0.047 and r=0.552, p=0.008, respectively) (Suppl. Table S2).

Correlation analysis between the NETosis markers revealed that only unadjusted serum MPO and cit-H3 levels positively correlated with each other among all unadjusted serum NETosis markers (r=0.457, p=0.001). However, the levels of all adjusted serum NETosis markers positively correlated with each other (adjusted cf-DNA and NE, r=0.711, $p<0.001$; adjusted cf-DNA and MPO, r=0.572, $p<0.001$; adjusted cf-DNA and cit-H3, r=0.367, p=0.010; adjusted NE and MPO, r=0.626, $p<0.001$; adjusted NE and cit-H3, r=0.366, p=0.010; adjusted MPO and cit-H3, r=0.550, $p<0.001$) (Fig. 3).

No significant correlation was found between the levels of NETosis markers in saliva. Furthermore, saliva NETosis markers did not correlate with both unadjusted and adjusted serum NETosis markers (Suppl. Table S3).

Indirect NETosis markers as diagnostic and activity indicators

ROC curves were calculated for the discrimination of the aBD from HC using the NETosis markers that showed a significant difference. AUC for unadjusted serum cf-DNA level was 0.947 (0.879–1.000) with a sensitivity of 93% and specificity of 90% for the cut-off value of 3295 ng/ml, and AUC for unadjusted serum NE was 0.767 (0.619-0.914) with a sensitivity of 73% and specificity of 70% for the cut-off value of 3.43 ng/ml ($p<0.001$, and p=0.012, respectively). AUC for adjusted serum MPO level was 0.930 (0.851-1.000) with a sensitivity of 87% and specificity of 90% for the cut-off value of 5.84 ng/ml, and AUC for adjusted serum cit-

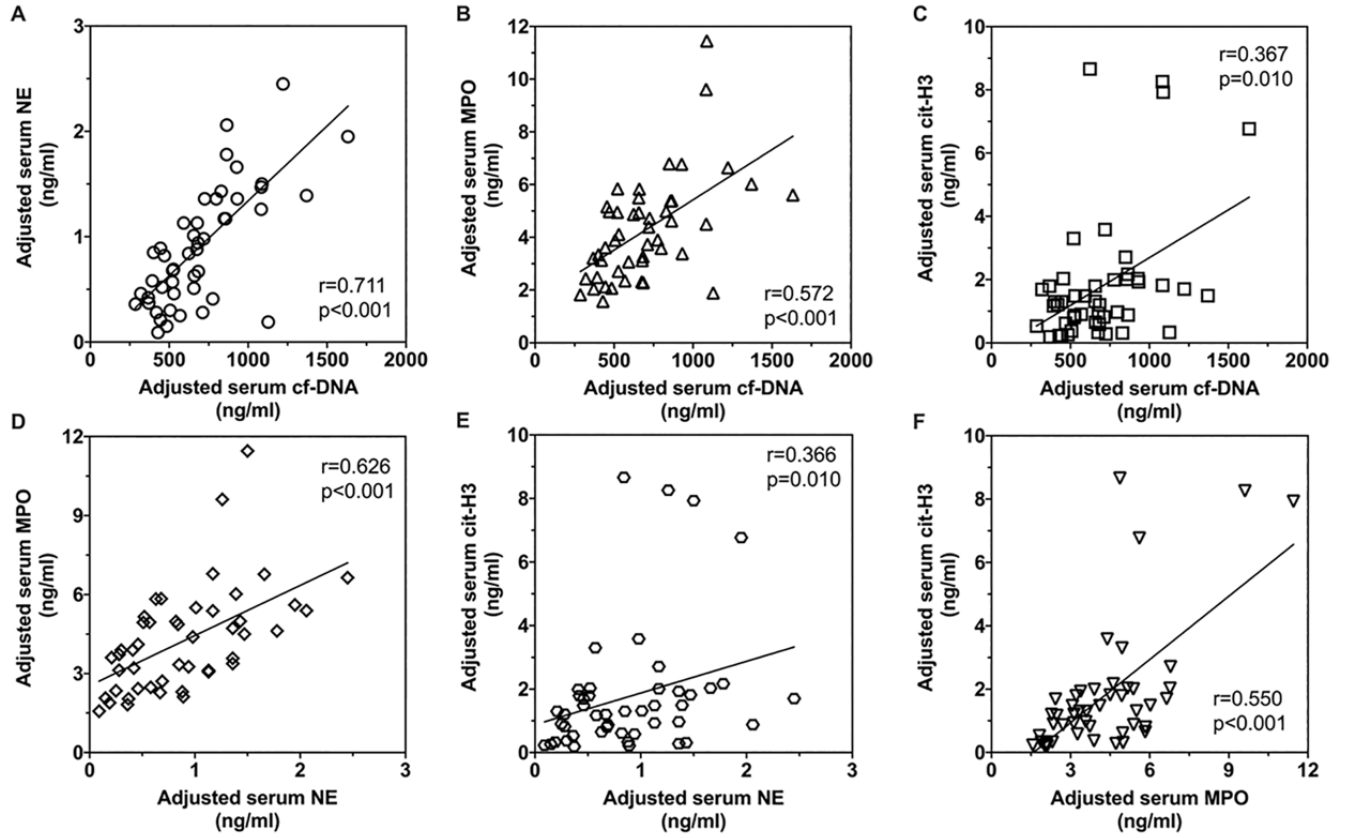

Fig. 3. The correlation analysis between levels of adjusted serum NETosis markers in the patients with BD, including adjusted serum cf-DNA and NE (**A**), adjusted serum cf-DNA and MPO (**B**), adjusted serum MPO and cit-H3 (**C**), adjusted serum NE and MPO (**D**), adjusted serum cit-H3 and NE (**E**), and adjusted serum MPO and cit-H3 (**F**). Spearman's *rho*.
R: correlation coefficient; cf-DNA: cell-free DNA; NE: neutrophil elastase; MPO: myeloperoxidase; cit-H3: citrullinated histon-3.

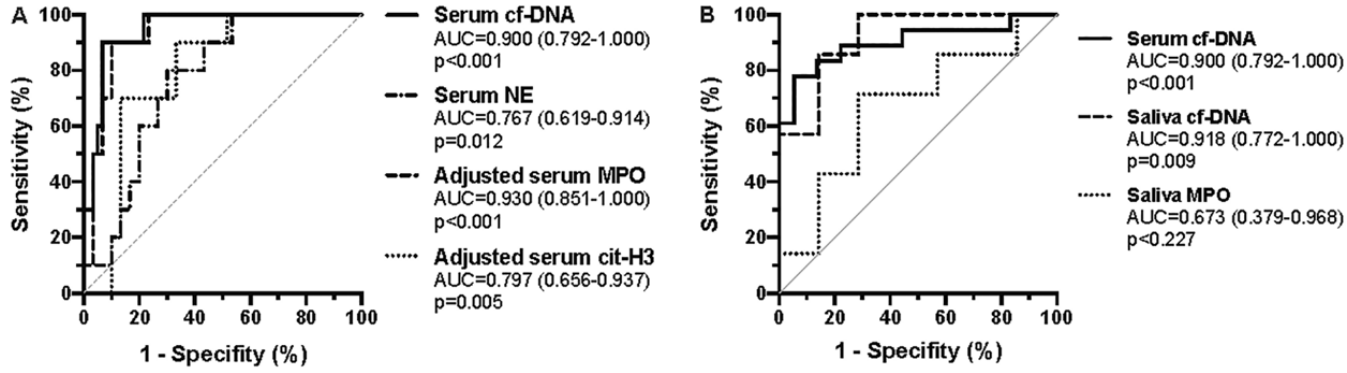

Fig. 4. ROC curves of NETosis markers for differentiation of aBD from HC (**A**). ROC curves of NETosis markers for differentiation of aBD from iBD (**B**).
cf-DNA: cell-free DNA; NE: neutrophil elastase; MPO: myeloperoxidase; cit-H3: citrullinated histon-3.

H3 value was 0.797 (0.656–0.937) with a sensitivity of 67% and specificity of 90% for the cut-off value of 1.34 ng/ml (p<0.001, p=0.005, respectively) (Fig. 4A).

ROC curves were calculated for the discrimination of aBD patients from iBD using the NETosis markers that showed a significant difference. AUC for unadjusted serum cf-DNA level was 0.900 (0.792–1.000) with a sensitivity of 78% and specificity of 89% for the cut-off value of 3445 ng/ml, and, AUC for saliva cf-DNA was 0.918 (0.772-1.000) with a sensitivity of 86% and specificity of 86% for the cut-off value of 590 ng/ml (p<0.001, p=0.009, respectively) (Fig. 4B).

Additionally, ROC curves of neutrophil count [AUC=0.835 (0.708-0.962) for differentiation of aBD from HC, p=0.002 and AUC=0.674 (0.497-0.852) for differentiation of aBD from iBD, p=0.074)] and CRP [AUC=0.893 (0.795-0.991) for differentiation of aBD from HC, p<0.001 and AUC=0.819 (0.683-0.956) for differentiation of aBD from iBD, p=0.001)] were calculated. Unadjusted serum cf-DNA and adjusted serum MPO levels provided better discriminatory power than inflammatory markers in differentiating aBD from HC, while unadjusted serum

cf-DNA and saliva cf-DNA levels provided better discriminatory power than inflammatory markers in differentiating aBD from iBD (Fig. S3).

Discussion

The activation of neutrophils, which encompasses enhanced chemotaxis, phagocytic activity, and superoxide production, plays a substantial role in the pathogenesis of BD. The contribution of neutrophils to tissue damage is particularly evident in vascular involvement in BD (4). In this context, investigations focused on the relationship between NETosis and the predisposition to inflammatory thrombosis in BD. The role of NETosis in the pathogenesis of BD has been the subject of several studies, yet the mechanism remains poorly understood (8). The objective of the current study was the measurement of some of the indirect NETosis markers in serum in order to evaluate the systemic effects of NETosis, and also in saliva, in order to assess their utility in the local disease activity. In addition, given the potential influence of absolute neutrophil counts on the neutrophil-associated markers, we repeated the analyses after the adjustment of NETosis markers by calculating their amounts per 1 million neutrophils. To our knowledge, previous studies analysing NETosis markers in systemic circulation have not routinely adjusted their results for the absolute neutrophil count. We expect that this adjustment will provide critical insights into the interpretation of the findings.

In active BD, serum cf-DNA and NE levels were significantly higher than in controls, whereas adjusted serum MPO and cit-H3 levels were found to be lower. On the other hand, no significant differences were observed in salivary NETosis findings between the patients and controls. This finding may be associated with the technical issues associated with the measurements in saliva or the size of the study group, since the results of the longitudinal follow-up analyses revealed a consistent decrease in both serum and saliva cf-DNA levels in parallel with reduced clinical activity.

A previous study in BD also showed an increase in serum cfDNA levels that have not been adjusted for neutrophil count (7). In an *in vitro* study in which PMA-stimulated and unstimulated neutrophils were examined, it was found that the cf-DNA level was higher in the BD group (17). The findings of the current study indicate that although there is an apparent increase in serum cf-DNA levels, the significance of this difference disappears following adjustment with the absolute neutrophil count, which suggests that the level of cf-DNA in serum is unlikely to be significant in the absence of overt systemic activation in BD. Furthermore, increased saliva cf-DNA levels did not reach statistically significant levels in orally active patients, but a significant decrease of saliva cf-DNA levels during follow-up supports the contribution of NETosis to the development of oral aphthae.

cf-DNA is a double-stranded DNA fragment of approximately 200 base pairs that is found in whole blood, plasma, serum, and other body fluids (18). cf-DNA levels are elevated in inflammatory diseases, such as systemic lupus erythematosus and rheumatoid arthritis (19). Our results provide evidence that serum and saliva cf-DNA had a strong correlation with inflammatory markers, and it has good discriminatory power in distinguishing active from inactive patients or active patients from healthy controls, with a potential utility as a non-specific marker of inflammation.

NE and MPO are enzymes of neutrophilic azurophilic granules, and they are released from neutrophils along with DNA and histones during NET formation. These enzymes and their complexes with DNA are used to screen NETosis frequently, with a better sensitivity for the latter ones despite all limitations (14, 15). In this study, an increase was observed in NE levels in the BD patients compared to healthy controls. However, it is notable that the mean neutrophil count of the patient group was significantly higher than that of the control group (20). In another study, it was reported that the amount of NET-related elastase was elevated in both active and inactive BD patients (6). Saliva NE levels were reported to be high in BD, and abnormal NE levels may stimulate the production of cytokines and chemokines, as well as epithelial damage (21, 22). In accordance with previous studies, our findings indicated that serum and saliva NE levels were elevated in the patient group, although this increase was not statistically significant for saliva. However, serum NE levels of the patients became similar to the levels in healthy controls following the adjustment, which might indicate that the observed changes may be related to high neutrophil turnover rather than to pathogenesis-related NETosis. Additionally, the significant difference between the adjusted NE of systemically active and isolated orally active patients, with higher in orally active ones, suggests that the treatments may also affect these results, which may be more potent in patients with systemic activity.

Some studies have reported increased plasma MPO activity or level in patients with BD, which are mainly attributed to neutrophils and, to a lesser extent, monocytes. However, in one of these studies, neutrophil MPO activity was found to be similar to that of the control group (23-25). In a study, serum MPO levels were found to be not significantly different between the patient and healthy control groups (26). Another study showed that the saliva MPO level was elevated in individuals with oral inflammatory conditions, whereas no difference was found between recurrent aphthous stomatitis and control group (27, 28). In this recent study, the serum MPO levels in patients with BD were found to be significantly lower than those observed in the control group, particularly when adjusted for neutrophil counts. The results of our investigation indicate that saliva MPO does not accurately reflect the oral manifestation of the disease. In this context, the investigation of MPO-DNA complexes would be useful in follow-up studies to distinguish the differential effects of free MPO enzyme versus its complexed forms on the observed results.

The presence of increased citrullinated histone at the tissue and cellular level, and increased mRNA expression of PAD4, an enzyme that is responsible for citrullination, were reported in BD (7, 29). Additionally, it was reported

that cit-H3 might result in endothelial barrier damage (30). In considering the aforementioned circumstances and our findings, it may be postulated that low serum cit-H3 in patients with active vascular involvement may be due to its accumulation in damaged tissues, which may contribute to endothelial damage. Moreover, we showed that saliva cit-H3 levels were not a reliable indicator of oral disease activity, as MPO. The observation, that serum MPO and cit-H3 levels were lower, particularly during the active disease period, and their normalisation with the decreased disease activity, warrants further studies to explore their contribution to the pathogenesis of the disease. It can be hypothesised that endogenous inhibitors exist as "counter-regulatory systems" that suppress the enzymatic activity and control inflammation during the active phase of the disease. We can speculate that a reduction in MPO and cit-H3 levels may be attributed to an increase in endogenous inhibitors as a regulatory mechanism or their deposition into the damaged tissues. Interestingly, reactive oxygen species (ROS) and phagocytic activity, which may be MPO-related, were shown to be decreased in BD (31). An alternative hypothesis for these results is that, despite the identical methodology employed across all groups, the observed effects might have been attributable to precipitation with the coagulum, given that the samples were serum rather than plasma. Lastly, the half-lives of MPO and NE enzymes, as well as their complexes with DNA or inhibitors, may vary depending on the state of disease activity and the activation of compensatory mechanisms. These differences could contribute to the variations observed in this study. Further research is warranted to elucidate the impact of these epiphenomena on the pathogenesis of the disease.

The limitations of this study include its single-centre design, relatively low number of patients, lack of adjustment of saliva NETosis markers for saliva total protein amount, using serum but not plasma samples in the analyses, and the use of only the ELISA method for the measurement of NE, MPO, and cit-H3 levels, and absence of analyses for MPO-DNA and NE-DNA complexes. The impact of confounding factors, such as gingivitis and periodontitis, on saliva sample collection, the duration and severity of oral aphthae, and the presence of patients on immunosuppressive therapy, such as azathioprine and colchicine, were other limitations, as well. This exploratory work provided a framework for the design of future studies aiming to assess the indirect findings of NET formation in Behçet disease.

In conclusion, the indirect NETosis findings showed changes in association with systemic and/or local activity of BD patients in relation to the disease manifestations. In particular, cf-DNA levels were potentially indicative of local and systemic disease activity, while NE levels were elevated in both active and inactive periods. Changes of the NETosis findings following the adjustment of the results according to the peripheral blood neutrophil counts may indicate that the observed NETosis findings in serum could be related to high neutrophil turnover in the active phase of the disease, and all future studies are advised to include similar adjustments. The results of this exploratory study should be validated in larger and more homogeneous patient populations along with disease controls, which are potentially associated with neutrophil activation. Further studies are required to clarify the mechanism underlying the low adjusted serum MPO and cit-H3 levels, particularly in active patients, and to assess the biomarker potential of NETosis findings in BD.

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