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The significance of α-defensins 1-3 in Behcet's disease: a case-control study among Egyptian patients

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Abstract

Background: Behcet's disease (BD) is associated with uveitis, oral ulcers, genital ulcers and arthritis. Enhanced activity of innate immunity components, such as neutrophils, natural killer (NK) cells and endothelial cells, is a prominent feature of BD. The aim of this study was to clarify the significance of α-defensins 1-3 in BD and detect their correlation with disease activity, severity and oral ulcer activity.

Results: Significant differences were found between Behcet's patients and controls as regards serum α-defensins 1-3 (median 10.1, IQR 4–45 μg/ml versus median 7.4, IQR 0.6–13.2 μg/ml, $p = 0.001$) and salivary α-defensins 1-3 (median 17.4, IQR 5.7–44 μg/ml versus median 8.6, IQR 3.3–11 μg/ml, $p < 0.001$). Receiver operating characteristic (ROC) showed a fair area under curve (AUC) (AUC = 0.743), with sensitivity of 40% and specificity 100% at cutoff value of 17.3 μg/ml for serum α-defensins 1-3, while salivary α-defensins 1-3 showed excellent AUC (AUC = 0.936), with sensitivity of 93.3% and specificity of 86.7% at cutoff value of 9.8 μg/ml. The levels of serum and salivary α-defensins 1-3 > cutoff value were significantly higher in active severe Behcet's patients with active oral ulcers ($p < 0.001$).

Conclusion: α-Defensins 1-3 may be involved in the pathogenesis of BD and could be valuable markers in the determination of disease activity, severity and oral ulcer activity.

Keywords: Behcet's disease, Salivary α-defensins 1-3, Serum α-defensins 1-3, Innate immunity, Oral ulcers, IL6

Key points

- α-Defensins 1-3 are involved in the pathogenesis of BD.
- α-Defensins 1-3 are valuable markers of Behcet's disease activity.

Background

Behcet's disease (BD) is a systemic chronic disease characterised by oral ulcers, vasculitis, arthritis, genital ulcers and uveitis. It is more common in Turkey, China, Korea and Japan [1].

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The innate immune system is suggested to participate in the pathogenesis of BD, as manifested by the expression of toll-like receptor expression in affected cells, increased mucocutaneous symptoms and neutrophil hyper-reactivity to streptococci or heat shock proteins [2]. It was found that BD neutrophils are more active and express more adhesion molecules than in healthy controls [3].

Defensins include three subfamilies α-, β- and γ-defensins. Neutrophils are generous in α-defensins that are liberated extracellularly during inflammation, leading to cell adhesion stimulation, chemokine liberation, reactive oxygen species overexpression and T cell chemotaxis [4].

Initially, human alpha defensin peptides were found in neutrophils and are named human neutrophil peptides [5].

Human neutrophilic peptides 1-3 are encoded by two genes DEFA1 and DEFA3 found at chromosome 8, site

8p23.1. DEFA1 and DEFA3 encode similar peptides the difference in the first amino acid from alanine in HNP-1 to aspartic acid in HNP-3; HNP-2 is an N-terminally truncated iso-form without the first amino acid [6].

Defensins initially attracted interest due to their potent antimicrobial functions and highly conserved structure. Consequently, several immunomodulatory functions like chemotactic effects and the ability to induce the production of cytokines have been identified, suggesting that these peptides connect innate and adaptive immune responses [7]. Their importance for autoimmune disorders is supported by the recent finding of modulated defensin expression in patients with systemic lupus erythematosus [8].

Interestingly, high α-defensin levels have been reported in the bronchoalveolar lavage fluid and plasma of one Wegener's granulomatosis (WG) patient previously [9] and furthermore in other vasculitic diseases, like microscopic polyangiitis [10] and cutaneous vasculitis [11].

Defensins have a great role in the control of inflammatory and immunologic processes like complement activation, cytotoxicity, chemotaxis of immature dendritic cell, CD4+/CD45RA+ naïve T cell, CD8+ T cells and monocytes, cytokine induction and enhancement of humoral and cellular response [12]. Several studies found that one of the genes of chromosome 8 perhaps could be involved in the pathogenesis of BD [13].

Alpha defensins might be among the main effector molecules in BD lesions. Neutrophils and NK cells that invade the site of inflammation and secrete defensins in harmful amounts in response to stress signals may be involved in the pathogenesis of the disease. The examined skin biopsies from the forearm especially in the corium after pathergy reactions reveal an increase in neutrophils rich in HNP 1-3 [14].

BD is a disorder with oral tissue inflammation leading to ulcer development; the first line of defences with neutrophils can be expected to be enhanced in patients with BD. Various functions of neutrophils such as chemotaxis, phagocytosis and oxidative burst responses are increased in patients with BD, and over-reactive neutrophils have been suggested to participate to tissue damage [15].

As there is no universally recognised pathognomonic laboratory marker of BD [16], this study aimed to clarify the significance of serum as well as salivary α-defensins 1-3 in BD and their relation to disease activity and severity among Egyptian patients.

Methods
Study participants
This is a case-control study in a cohort of Egyptian patients and included 60 patients with BD with age ranging from 30 to 45 years who were diagnosed according to the international diagnostic criteria of BD [17]. Patients were recruited from the outpatient clinics of Rehabilitation, Rheumatology, and Internal Medicine, Mansoura University Hospital. They were compared to 60 age- and sex-matched healthy controls. A written informed consent was taken from all participants before the beginning of the study. The study was approved by the Institutional Research Board Mansoura University Faculty of Medicine code (R/16.03.118).

Exclusion criteria

– Oral mucosal disorders not caused by BD: ex. Pemphigus vulgaris, mucous membrane pemphigoid, lichen plans and erythema multiform
– Autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and spondyloarthropathies
– Cancer
– Infection

Data collection and clinical examination
Demographic and clinical data were taken from all patients including age, sex, disease duration, systemic clinical signs and symptoms, full musculoskeletal examination and intraoral examination.

Evaluation of BD
Disease activity was assessed by Behcet's Syndrome Activity Score (BSAS) [18]. *BSAS* has 10 questions, which consists of visual analogue scales for patient's level of discomfort with regard to oral ulcers, genital ulcers, skin lesions and current disease activity along with the number of oral ulcers, genital ulcers and skin lesions present, and records symptoms attributable to the gastrointestinal, vascular or eye involvement. The VAS questions are all scored 0–10, and the remaining are scored categorically, 0, 5 or 10 depending on the response, for a total score of 0–100; patients complete the BSAS at the time of the visit to the treating doctor, with no input from the physician.

Disease severity was estimated following Krause et al. study [19].

Severity score will be calculated as the sum of 1 point for each mild manifestation, 2 points for each moderate manifestation and 3 points for each severe manifestation.

Mild
Oral aphthosis, genital ulcer, typical skin lesions, arthralgia, recurrent headache, mild gastrointestinal symptoms, epididymitis, pleuritic pain, superficial vein thrombosis

Moderate
Arthritis, deep venous thrombosis of the legs, anterior uveitis, gastrointestinal bleeding

Severe

Posterior/panuveitis, retinal vasculitis, arterial thrombosis or major vein (vena cava, hepatic) thrombosis, aneurysms, neuro-Behçet, bowel perforation

Oral ulcer activity was estimated by Mumcu composite index [20]. Mumcu et al. proposed a composite index to monitor the clinical manifestations associated with oral ulceration in BD patients.

This index evaluates the activity of the oral ulcer, ulcer pain and functional disability.

➢ Oral ulceration activity is recorded as the number of ulcers in the past 30 days. This is scored 0 point if there were no ulcers and scored 1 point for the presence of oral ulcers in the previous month.
➢ Pain was evaluated by 100-mm visual analogue scale (VAS; 0, no pain, to 100, severe pain) by patients. Then, the VAS score was categorised to calculate the score as follows: ≤ 10: 0; 11–20: 1; 21–40: 2; 41–60: 3; 61–80: 4; and 81 and over: 5 points.
➢ Functional status (the effect of oral ulcer on tasting, talking, eating, chewing and swallowing) was evaluated by a 5-point Likert-type scale:
- None of the time (0 points)
- Little of the time (1 point)
- Some of the time (2 points)
- Most of the time (3 points)
- All of the time (4 points)

Laboratory assessment

Serum and salivary samples were obtained from both patients and controls. Samples of unstimulated whole saliva were obtained during 15 min in the morning due to the circadian rhythm of saliva secretion. Salivary and serum α-defensins 1-3 were assayed using α-defensins 1-3 ELISA kit (PELOBIOTECH GmbH-Am Klopferspitz 19-82152 Planegg, Germany). IL-6 was assayed using ELISA Kit (Elabscience, Bethesda, MD, USA). Erythrocyte sedimentation rate (ESR), neutrophil count and C-reactive protein were measured.

Statistical analysis

The collected data were revised, coded, tabulated and introduced to a PC using Statistical Package for Social Science (SPSS 20 for windows; SPSS Inc, Chicago, IL, 2011). The Student T test was taken to assess the statistical significance of the difference between two study groups means. The Mann-Whitney test (U test) was taken to assess the statistical significance of the difference of a non-parametric variable between two study groups. Differences were significant at the level $p \leq 0.05$.

Results

The present study comprised 60 patients with Behcet's disease. They were 38 males and 22 females and their mean age was 36.16 ± 8.05 compared to 60 healthy controls, who were 32 males and 28 females and have a mean age of 34.46 ± 6.18. No statistically significant difference was found between both groups as regards age and sex (p = 0.09, 0.35).

In Behcet's patients, oral ulcers were the most common clinical manifestation (70%) followed by arthralgia (60%), posterior uveitis (40%), genital ulcers (26.7%), deep venous thrombosis (DVT) (20%), anterior and panuveitis (16.7%), arthritis and neurological manifestations (6.7%). The severity score was median 14.5, IQR 6–25; the activity score was median 27.5, IQR 12–55); and the oral ulcer activity score was median 4. 5, IQR 0–9.

There were significant differences between Behcet's patients and the control group as regards ESR 1st h (median 28, IQR 4–76 mm/h versus median 10, IQR 5–30 mm/h, p < 0.001), ESR 2nd h (median 45, IQR 4–112 mm/h versus median 10, IQR 5–30 mm/h, p < 0.001), CRP (median 6, IQR 2–48 mg/L versus median 1, IQR 1–5 mg/L, p < 0.001) and blood neutrophilic count (median 4.5, IQR 1.6–14.6 × 10^3/mm^3 versus median 3.4, IQR 1.4–5 × 10^3/mm^3, p < 0.001).

Significant differences were found between Behcet's patients and controls as regards serum α-defensins 1-3 (median 10.1, IQR 4–45 μg/ml versus median 7.4, IQR 0.6–13.2 μg/ml, p = 0.001), salivary α-defensins 1-3 (median 17.4, IQR 5.7–44 μg/ml versus median 8.6, IQR 3.3–11 μg/ml, p < 0.001) and IL6 (median 16.4, IQR 2.9–40 pg/ml versus median 10.81, IQR 5.4–16.2 pg/ml, p < 0.001).

ROC curve for salivary and serum α-defensins 1-3 was performed to discriminate between patients and controls. Performance characteristics and best cutoff values are shown in Fig. 1.

Table 1 shows that serum α-defensins 1-3 more than the cutoff value was not significantly associated with age (p < 0.082) but was higher in the male gender (p < 0.001). As regards to clinical manifestations, there were only significant associations between α-defensins 1-3 > cutoff value and oral ulcers (p < 0.001), arthritis (p < 0.022) and panuveitis (p = 0.029). Higher Behcet's disease severity, activity and oral ulcer activity scores were found to be associated with serum α-defensins 1-3 > cutoff value (p < 0.001).

Table 2 shows significant associations between salivary α-defensins 1-3 > cutoff value and oral ulcers (p = 0.006). Salivary α-defensins 1-3 > cutoff value was significantly associated with Behcet's disease severity (p < 0.001), activity (p < 0.001) and oral activity scores (p = 0.006). However, salivary α-defensins 1-3 more than the
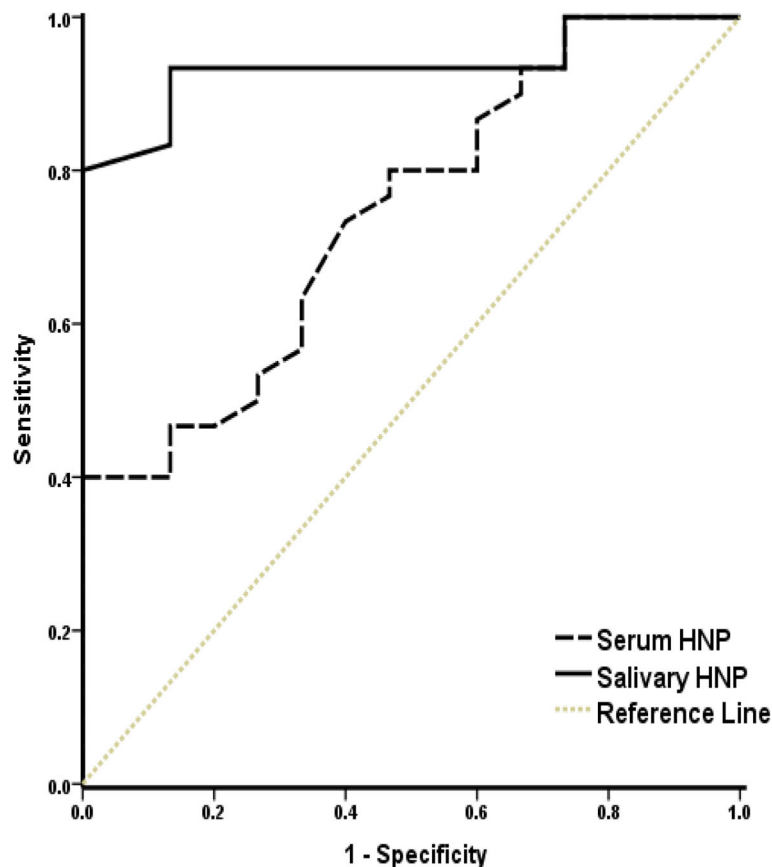
Fig. 1 Receiver operating Characterestic (ROC) analysis on utility of serum and salivary α defensins 1-3 for discrimination between cases and controls. Serum α-defensins 1-3 showed a fair area under the curve (AUC) = 0.743, 95% confidence interval (CI) for the AUC (0.657–0.830) with sensitivity of 40% and specificity 100% at cutoff value of 17.3 μg/ml, while salivary α-defensins 1-3 showed excellent AUC = 0.936, 95% CI for the AUC (0.887–0.984) with sensitivity of 93.3% and specificity of 86.7% at cutoff value of 9.8 μg/ml

cutoff value showed a statistically significant higher level of serum CRP ($p = 0.017$) and serum α-defensins 1-3 ($p = 0.001$).

Discussion

In the present study, the α-defensins 1-3 serum level in BD patients was significantly higher than that in healthy controls. α-Defensins 1-3 have a crucial role in the regulation of immunologic and inflammatory processes such as immature dendritic cell chemotaxis, cytotoxicity, complement activation, induction of cytokines and stimulation of cellular and humeral response [12]. The present study detected higher α-defensins 1-3 salivary level in Behcet's patients as compared to controls. This can be explained by neutrophil migration via junctional epithelium surrounding the teeth to the oral cavity. In BD, there are inflamed oral mucosa, increased oral ulceration and neutrophil overactivity which secrete a pathological amount of α-defensins 1-3 [14].

Our results detected a significant correlation between α-defensins 1-3 serum level and arthritis, panuveitis, BD severity and activity.

α-Defensins induce the release of several cytokines such as IL-1, IL-8 and TNF-α; IL-8 and other inflammatory cytokines such as TNF-α were found to increase in BD [12]. The essential role of IL-8 is neutrophil chemotaxis which is the main origin of α-defensins 1-3, and this makes a positive feedback circuit between α-defensins and inflammatory cytokines, leading to a vicious cycle of inflammation in BD [21]. Several studies detected α-defensin accumulation in synovial cavity of rheumatoid arthritis patients, and the defensin level in synovial fluid was correlated significantly with joint destruction and erosion [22]. Thus, it is assumed that α-defensins may have a crucial role in aggravating or developing arthritis in BD.

Joong et al. [23] detected that high copy numbers of the *DEFA1* gene and the associated increase in α-defensin-1 expression might initiate or increase the inflammatory response in the intestine of BD patients.

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Table 1 Comparison of demographic data, clinical symptoms, clinical scores and laboratory investigations according to serum α-defensins 1-3 cutoff value

	α-Defensins 1-3		α-Defensins 1-3		p
	< cutoff value, N = 36		> cutoff value, N = 24		
Demographic data					
Age (years) (mean ± SD)	37.22 ± 6.21		34.5 ± 8.78		< 0.082
Males, *n* (%)	14	38.9%	24	100%	< 0.001
Females, *n* (%)	22	61.1%	0	0%	
Clinical data					
Genital ulcers, *n* (%)	8	22.2%	8	33.3%	0.340
Oral ulcers, *n* (%)	18	50%	24	100%	< 0.001
DVT, *n* (%)	6	16.7%	6	25%	0.517
Arthritis, *n* (%)	0	0%	4	16.7%	0.022
Arthralgia, *n* (%)	20	55.6%	16	66.7%	0.389
CNS, *n* (%)	2	5.6%	2	8.3%	0.630
All uveitis, *n* (%)	26	72.2%	18	75%	0.812
Anterior uveitis, *n* (%)	4	11.1%	6	25%	0.178
Posterior uveitis, *n* (%)	16	44.4%	8	33.3%	0.431
Panuveitis, *n* (%)	6	16.7%	4	16.7%	0.029
Assessment scores					
BD severity (median, IQR)	13	9–13	20.5	18–22	< 0.001
BD activity (median, IQR)	23	16–25.5	38.5	34–41.25	< 0.001
Oral ulcer score (median, IQR)	3	0–4	8	7–8.75	< 0.001
Laboratory data					
ESR 1st h (mm/h)	28	13–35	29	15.25–48.75	0.565
ESR 2nd h (mm/h)	40.5	30–70	46.5	19–84.5	0.809
CRP (mg/dl)	6	5–7	7.5	5–20.25	0.273
Blood neutrophils (× 10^9/L)	3.8	2.8–5.9	5	3.275–6.925	0.103
Salivary α-defensins 1-3 (μg/ml)	14.5	10.8–15.4	32.2	25.225–34.275	< 0.001
IL6 (pg/ml)	16.7	16.4–17	15.05	10.3–23.75	0.415
Drugs					
Corticosteroid, *n* (%)	26	72.2	22	91.7%	0.100
Azathioprine, *n* (%)	32	88.88%	17	70.83%	0.097
Colchicinee, *n* (%)	18	50%	12	50%	1
Cyclosporine, *n* (%)	6	16.66%	8	33.33%	0.212
Infliximab, *n* (%)	8	22.22%	8	33.33%	0.558
Adalimumab, *n* (%)	8	22.22%	4	16.66%	1
Mycophenolate, *n* (%)	6	16.66%	2	8.3%	0.457

DVT deep vein thrombosis, *CNS* central nervous system, *BD* Behcet disease, *ESR 1st h, ESR 2nd h* erythrocyte sedimentation rate at the first and second hour, *CRP* C-reactive protein, *IL-6* interleukin 6

Accordingly, high *DEFA1* gene copy numbers may be involved in the increased susceptibility to intestinal pathology in patients with BD.

Our results showed a positive significant correlation between α-defensins 1-3 serum level with a number of oral ulcers, oral ulcer activity score and α-defensins 1-3 salivary level. Saliva is considered as an ultra-filtrate of the blood, and elevated neutrophil count in the blood may lead to elevated α-defensins 1-3 salivary level [24]. The locally expressed or systemically secreted α-defensins 1-3 act as chemical barriers in the protection of oral mucosa with innate immune response [25].

The release of pathological amount of α-defensins 1-3 in saliva of BD patients from infiltrating neutrophils and

Table 2 Comparison of demographic data, clinical symptoms, clinical scores and laboratory investigations according to salivary α-defensins 1-3 cutoff value

	Salivary α-defensins 1-3		Salivary α-defensins 1-3		*p*
	< cutoff value, *N* = 4		> cutoff value, *N* = 56		
Demographic data					
Age (years) (mean ± SD)	40 ± 6.271		35.89 ± 8.138		< 0.164
Males, *n* (%)	4	100	34	60.7	0.286
Females, *n* (%)	0	0	22	39.3	
Clinical data					
Genital ulcers, *n* (%)	2	50	14	25	0.287
Oral ulcers, *n* (%)	0	0	42	75	0.006
DVT, *n* (%)	0	0	12	21.4	0.574
Arthritis, *n* (%)	0	0	4	7.1	0.580
Arthralgia, *n* (%)	1	25%	22	39.28	0.57
CNS, *n* (%)	0	0	4	7.1	0.573
All uveitis, *n* (%)	4	100	40	71.4	0.565
Anterior uveitis, *n* (%)	2	50	8	14.3	0.125
Posterior uveitis, *n* (%)	2	50	22	39.3	0.626
Panuveitis, *n* (%)	0	0	10	17.9	1
Assessment scores					
BD severity (median, IQR)	6.5	6–7	15	13–19.5	0.001
BD activity (median, IQR)	12.5	12–13	28.75	23–37.25	0.001
Oral ulcer activity score (median, IQR)	0	0–0	5	3–7.75	0.006
Laboratory data					
ESR 1st h (mm/h)	20.5	11–30	28	13.5–48.75	0.312
ESR 2nd h (mm/h)	41.5	24–59	45	28.5–85	0.593
CRP (mg/dl)	18	12–24	18	6–48	0.784
Blood neutrophils (× 10^9/L)	4.7	3.5–5.9	4.35	2.825–6.325	0.589
Serum α-defensins 1-3 (µg/ml)	4	4–4	10.5	7.9–28.75	0.001
IL6 (pg/ml)	16.7	16.4–17	15.05	10.3–23.75	.812
Drugs					
Corticosteroids, *n* (%)	2	50%	46	82.1%	0.175
Azathioprine, *n* (%)	0	0%	26	46.4%	0.126
Colchicinee, *n* (%)	2	50%	28	50%	1
Cyclosporine, *n* (%)	0	0%	12	21.4%	0.574
Infliximab, *n* (%)	2	50%	14	25%	0.287
Adalimumab, *n* (%)	2	50%	7	12.5%	0.103
Mycophenolate, *n* (%)	2	50%	6	10.71%	0.082

DVT deep vein thrombosis, *CNS* central nervous system, *BD* Behcet disease, *ESR 1st h*, *ESR 2nd h* erythrocyte sedimentation rate at first and second hour, *CRP* C-reactive protein, *IL-6* interleukin 6

those hyperactive and excessive neutrophils are blamable for increased oral ulcer activity and increased tissue damage [15].

We expected in our study that an increase in blood neutrophilic count and significant correlation between it and serum α-defensins 1-3 could be present, but we found no statistically significant difference in blood neutrophilic count between patients and controls and also there was no significant correlation between α-defensins 1-3 serum level and blood neutrophilic count. It seems that an increased serum level of α-defensins 1-3 is due to increased α-defensins 1-3 load in primary granules of neutrophils and increased neutrophil degranulation not due to increased number of neutrophils [26].

Elif Ugurel et al. [27] revealed that innate immunity is primarily involved in acute attacks of neuro-Behcet's disease (NBD) and treatment regimens attacking neutrophils might prove efficacy in the treatment of NBD and perhaps replace steroids that are already used for this disease.

In our results, serum and salivary α-defensins 1-3 levels were not significantly correlated with ESR and CRP; this goes with results of Katsantonis et al. [28], who found that ESR and CRP are not specific indicators for the process of inflammation and IL-8 is the most reliable indicator of disease activity and inflammation in Behcet's disease.

Serum and salivary α-defensins 1-3 were not significantly correlated with IL6; this could be explained by the study of Brook et al. [29] who found that α-defensins 1-3 act as molecular brake on macrophage-driven inflammation and inhibit the release of multiple cytokines (TNF-α, IL6, IL8 and IL1B) and are responsible for pathogen clearance and resolution of inflammation with minimal tissue damage; they prevent an excessive proinflammatory response that would create its own collateral damage while still acting as powerful antimicrobial peptides. This opens the way for developing similar peptide-based therapeutics that would act as effective combined antiinflammatory and antimicrobial agents.

This is the first study to measure both salivary and serum levels of α-defensins 1-3 and to construct ROC curve for both to discriminate between cases and controls, and we found that salivary α-defensins 1-3 were more sensitive and had excellent AUC. Future studies are needed to clarify the role of alpha defensins in bridging between innate and cellular immunity by measuring different cytokines such as IL8, TNF and IL1B.

Conclusion

- Alpha defensins are highly involved in the pathogenesis of Behcet's disease since it is correlated with activity and severity of the disease and with oral ulcer activity. It could be used as a biological marker for the assessment of treatment response. Future studies are suggested to examine if it could be used as a future target for therapy in Behcet's disease.
- Salivary defensins are more sensitive and specific markers for the disease than serum defensins.

Abbreviations
AUC: Area under the curve; BD: Behcet's disease; BSAS: Behcet's Syndrome Activity Score; ESR: Erythrocyte sedimentation rate; IQR: Interquartile range; IL: Interleukins; ROC: Receiver operating characteristic; TNF: Tumour necrosis factor; WG: Wegener's granulomatosis

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