
Anti-alpha-enolase antibodies in Behçet's disease: a marker of mucocutaneous and articular disease activity?

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ABSTRACT

Objective. To assess IgM anti-alpha-enolase antibodies (AAEA) in systemic Behçet's disease (BD) and its possible association with clinical manifestations and disease activity.

Methods. Ninety-seven consecutively selected BD patients were compared to 36 enteropathic spondyloarthritis (ESpA) [24 Crohn's disease (CD) and 12 ulcerative colitis (UC)] patients and 87 healthy controls. IgM AAEA was detected by immunoblotting. Disease activity was assessed by standardised indexes, Brazilian BD Current Activity Form (BR-BDCAF) for BD and Harvey-Bradshaw Index (HBI) for CD and UC patients. A second evaluation was performed in BD patients (n=56), regarding IgM AAEA presence, disease activity scores and C-reactive protein (CRP).

Results. Higher IgM AAEA prevalence was found in 97 BD (17.7%) compared to ESpA (2.8%) and healthy controls (2.3%), $p < 0.001$. IgM AAEA frequency was higher in active BD compared to inactive BD (30.2% vs. 7.4%, $p = 0.006$), a finding confirmed in the second cross-sectional evaluation of 56 of these BD patients (45.5% vs. 13.3%, $p = 0.02$). Mean BR-BDCAF scores were higher in IgM AAEA positive group on both evaluations (9.1 ± 5.4 vs. 4.9 ± 4.9 , $p = 0.002$; 5.0 ± 4.9 vs. 2.2 ± 2.9 , $p = 0.01$, respectively). BD patients with mucocutaneous and articular symptoms presented higher IgM AAEA positivity in the first and second evaluations (64.7% vs. 27.5%, $p = 0.005$; 36.4% vs. 7.1%, $p = 0.039$ respectively).

Conclusions. Our data support the notion that alpha-enolase is a target antigen in BD, particularly associated with disease activity, mucocutaneous and articular involvement. In addition, IgM AAEA may distinguish BD from ESpA, especially in patients with high disease activity.

Introduction

Behçet's disease (BD) is a multisystemic syndrome in which currently diagnostic criteria are established on clinical features, without specific serological markers, leading to misdiagnosis with other conditions, particularly inflammatory bowel diseases (1-3).

Anti-endothelial cell antibodies (AECA) are a heterogeneous group of antibodies against surface proteins in endothelial cells. AECA were described in several immune mediated diseases, as well as in BD patients' serum samples (4).

Alpha-enolase was described as a target antigen of IgM-type AECA in BD patients' sera (5), however clinical significance of this antigen is still undefined. IgM anti-alpha-enolase antibodies (AAEA) has moderate specificity for BD compared to other rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus and granulomatosis with polyangiitis) (6) and a higher prevalence of vascular lesions in IgM AAEA positive BD patients was described (7).

Conversely, Shin *et al.* detected IgM AAEA solely in intestinal BD with an association with a higher mean disease activity in this target organ. Furthermore, a weak correlation between Harvey Bradshaw-Index (HBI), commonly used for Crohn's disease (CD) activity assessment was observed (8). There is, however, no data regarding IgM AAEA presence related to established activity indexes used in BD, such as Behçet's disease current activity form (BDCAF) (9).

Therefore, the objective of this study is to assess IgM AAEA in BD, enteropathic spondyloarthritis (ESpA) and healthy controls. The possible association of this biomarker with clinical features and disease activity in systemic BD was also evaluated.

Methods

Subjects

This single-centre cross-section study was conducted at a spondyloarthritis and BD outpatient clinic in a rheumatology tertiary centre. One hundred and thirty-three consecutive subjects of both genders were consecutively selected, with 97 BD patients, fulfilling International Study Group for Behçet's Disease Criteria (10) and 36 ESpA patients, including 24 Crohn's disease patients and 12 Ulcerative colitis individuals, with both groups meeting the European Spondyloarthropathy Study Group (ESSG) classification criteria (11). Eighty-seven healthy individuals matched for age and sex with those with BD were selected to be the control group.

Study design

This study comprises: 1) a cross-sectional evaluation comparing 97 BD patients, 36 ESpA patients and 87 healthy controls age and sex matched to BD patients at baseline; 2) an additional follow-up evaluation of 56 BD patients from the cross-sectional group. All participants agreed to participate in the study and signed the informed consent, in accordance with the Declaration of Helsinki, previously approved by the local ethics committee (CAP-Pesq #0180/11).

Clinical evaluation

Data were obtained using a standardised electronic database protocol including demographic data, clinical, laboratorial findings, treatment and standardised and specific questionnaires to assess clinical disease activity were applied to all patients at two time-points. Brazilian Behçet's Disease Current Activity Form (BR-BD-CAF) (12) assessed patients with BD. Active BD was defined as BR-BDCAF scores >4 . Harvey-Bradshaw Index (HBI) (13) was used to assess Crohn's disease and ulcerative colitis subjects. Fifty-six BD patients were reassessed for BR-BDCAF index and IgM AAEA in a second visit after a minimum of two-years interval.

Laboratory evaluation

Serum samples were collected from all

Table I. Behçet's disease clinical features.

Variables	
Median age, \pm SD, years	44.0 \pm 11.3
Median disease duration, \pm SD, years	11.0 \pm 8.2
Female gender, % (n)	74.2% (72)
Active disease, % (n)	44.3% (43)
Median BR-BDCAF, \pm SD	4.0 \pm 5.3
Cumulative clinical history of BD manifestations	
Oral ulcers, % (n)	100% (97)
Genital ulcers, % (n)	86.6% (84)
Eye disease, % (n)	49.4% (48)
Cutaneous, % (n)	76.2% (74)
Articular, % (n)	45.3% (44)
Vascular, % (n)	28.8% (28)
Neurological, % (n)	24.7% (24)
Gastrointestinal, % (n)	12.3% (12)
Clinical symptoms at the first evaluation	
Cutaneous, % (n)	50.5% (49)
Articular, % (n)	58.8% (57)
Ocular, % (n)	7.2% (7)
Intestinal, % (n)	2.3% (2)
Neurological, % (n)	0.0% (0)
Vascular, % (n)	0.0% (0)

BR-BDCAF: Brazilian Behçet's disease current activity form; BD: Behçet's disease; n: number of patients.

subjects for the first and second evaluation.

IgM anti-alpha-enolase antibodies (AAEA) were detected by immunoblotting using a transinfected cell lysate with human alpha enolase (Santa Cruz Biotechnology, Inc., USA) as antigen and IgM and IgG AAEA (Sigma-Aldrich, USA) as secondary antibodies. C-reactive protein (CRP) was evaluated by nephelometry at two time-points in BD and ESpA patients. CRP levels were expressed in mg/L.

Statistical analyses

Statistical analysis was performed comparing disease groups and healthy controls. Continuous variables were expressed as median \pm SD and compared using the T test or Mann-Whitney test when comparing two groups and ANOVA/ANOVA on Ranks for more than two groups, as appropriated. Categorical variables were shown as percentage and evaluated through the Fisher Exact Test for two groups and Chi square in case of more than two groups, as appropriated. A *p* value less than 0.05 was considered to be statistically significant. Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of the biomarker were also

calculated. Statistical analyses were performed using SigmaStat version 3.1 (2005) and GraphPad Software.

Results

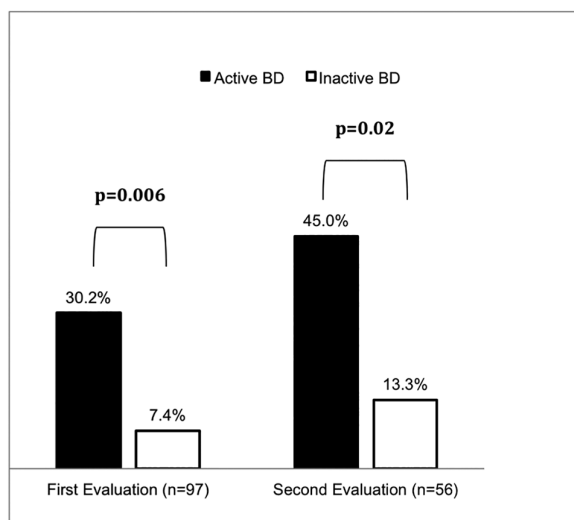
Demographic and clinical characteristics at first evaluation

BD patients, ESpA patients and control groups were similar regarding median age (44.0 \pm 11.3, 47.0 \pm 12.6 and 45.0 \pm 10.4 years; *p*=0.313, respectively) and female gender (74.2%, 77.7% and 78.1%; *p*=0.802). First evaluation of BD patients is summarised in Table I. Median disease duration was 11.0 \pm 8.2 years. Main BD clinical manifestations were oral ulcers (100%), genital ulcers (86.6%), cutaneous lesions (including pseudofolliculitis and erythema nodosum) (76.2%), ocular disease (49.4%), vascular involvement (28.8%), neurological involvement (24.7%) and intestinal disease (12.3%). Less than half (44.3%) of patients had active disease, with a median BR-BDCAF score of 4.0 \pm 5.3. Main current activity features at the first evaluation were cutaneous (50.5%), articular (58.8%), and ocular (7.2%) symptoms. Conversely, intestinal activity was found in only 2.3% of patients and none had vascular or neurological activity.

Table II. BD patients group according to IgM AAEA positivity and disease activity parameters at first and second evaluations.

IgM AAEA	First evaluation n=97			Second evaluation n=56		
	Positive n=17	Negative n=80	<i>p</i>	Positive n=11	Negative n=45	<i>p</i>
Disease Activity	76.5%	37.5%	0.006	45.5%	13.3%	0.02
Median BR-BDCAF, \pm SD	7.5 \pm 5.4	4.0 \pm 4.9	0.002	3.5 \pm 4.9	1.0 \pm 2.9	0.01
Median CRP, mg/L, \pm SD	3.6 \pm 26.3	2.1 \pm 7.2	0.07	5.8 \pm 10.5	2.4 \pm 4.7	0.13
Clinical Activity						
Mucocutaneous, % (n)	70.6% (12)	46.3% (37)	0.1	54.5% (6)	40% (18)	0.5
Articular, % (n)	88.2% (15)	52.5% (42)	0.006	54.5% (6)	15.6% (7)	0.01
Mucocutaneous and articular, % (n)	64.7% (11)	27.5% (22)	0.005	36.4% (4)	7.1% (4)	0.03
Ocular, % (n)	17.6% (3)	5% (4)	0.1	0% (0)	0% (0)	1.0

BR-BDCAF: Brazilian Behçet's disease current activity form; CRP: C-reactive protein.

**Fig. 1.** IgM AAEA frequency in active BD versus inactive BD.

frequency in active BD (45.5%) compared to inactive BD (13.3%), $p=0.02$ (Fig. 1). Moreover, median BR-BDCAF scores were higher in IgM AAEA positive group at first (7.5 ± 5.4 vs. 4.0 ± 4.9 , $p=0.002$) and at second evaluations (3.5 ± 4.9 vs. 1.0 ± 2.9 , $p=0.01$), compared to IgM AAEA negative group.

Regarding clinical manifestations, we confirmed the higher frequency of mucocutaneous and articular symptoms in IgM AAEA positive group (36.4% vs. 7.1%, $p=0.039$) compared to IgM AAEA negative group, as shown in Table II.

Comparing IgM AAEA positive and IgM AAEA negative groups, the higher median CRP levels at first (3.6 ± 26.3 vs. 2.1 ± 7.2 mg/L, $p=0.07$) and second evaluations (5.8 ± 10.5 vs. 2.4 ± 4.7 mg/L, $p=0.13$) in the former group did not reach statistical significance.

Notably, IgM AAEA presented high specificity for BD disease activity discrimination of 92.6% and 86.7% at first and second evaluations, respectively. Likewise, negative predictive value (NPV) were 62.5% at the first evaluation and reached 86.7% at the second evaluation. Sensitivity and positive predictive value (PPV) were 30.2% and 76.5% at first evaluation and 45.5% and 45.4% at second evaluation, respectively (Table III).

Discussion

The present study found a high prevalence of IgM AAEA in BD patients with systemic disease activity particularly in those with mucocutaneous and articular involvement.

Laboratorial baseline serum analysis

A higher IgM AAEA positivity was found in 17 BD patients (17.5%), compared to only 1 ESa patient with UC (2.8%), and 2 healthy controls (2.3%), $p<0.001$. BD patients group was evaluated according to BR-BDCAF score (active >4 and inactive ≤ 4). Notably, IgM AAEA frequency was higher in active BD (30.2%) compared to inactive BD (7.4%), $p=0.006$. Median CRP levels at baseline in BD and ESa patients were comparable (2.4 ± 12.6 vs. 6.3 ± 8.9 mg/L, $p=0.211$).

IgM AAEA positive and negative patients had comparable frequencies of previous history of oral ulcers (100% vs. 100%, $p=1.0$), genital ulcers (82.4% vs. 87.5%, $p=0.69$), erythema nodosum (58.8% vs. 52.5%, $p=0.79$), pseudofolliculitis (47.2% vs. 55%, $p=0.6$), ocular involvement (41.2% vs. 51.3%, $p=0.59$), articular involvement (41.2% vs. 46.3%, $p=0.79$), intestinal disease

(17.6% vs. 11.3%, $p=0.43$), vascular involvement (35.3% vs. 27.5%, $p=0.56$) and neurological disease (23.5% vs. 25%, $p=1.0$).

The evaluation of clinical features from four weeks prior to the laboratorial analysis revealed that BD IgM AAEA positive group presented a higher frequency of articular activity (88.2% vs. 52.5%, $p=0.006$) compared to BD IgM AAEA negative group. Moreover, selecting patients with mucocutaneous and articular activity at the same time revealed a higher IgM AAEA positivity (64.7% vs. 27.5%, $p=0.005$) (Table II).

Second evaluation in BD group

A second analysis of IgM AAEA frequency was performed on 56 BD patients reassessed from the original cross-sectional group, with a mean time interval of 26.6 ± 1.8 months between the two evaluations. This analysis also confirmed a higher IgM AAEA

Table III. IgM AAEA sensitivity, specificity, positive predictive value and negative predictive value for BD disease activity discrimination.

	First Evaluation		Second Evaluation	
	Value	95% CI	Value	95% CI
Sensitivity	30.2%	17.2% - 46.1%	45.4%	16.7% - 76.6%
Specificity	92.6%	82.1% - 97.9%	86.7%	73.2% - 94.9%
PPV	76.5%	50.1% - 93.2%	45.4%	16.7% - 76.6%
NPV	62.5%	50.9% - 73.1%	86.7%	73.2% - 94.9%

PPV: positive predictive value; NPV: negative predictive value; 95% CI: 95% confidence interval.

The simultaneous evaluation of IgM AAEA prevalence in BD, CD and UC performed herein confirmed previous observation from Eastern cohorts of a significantly higher frequency of this antibody in BD (7, 8) and emphasises the possible relevance of this biomarker to help to distinguish these conditions.

Moreover, we validated our finding for a multi-ethnic population with a lower prevalence of BD and lower frequency of intestinal involvement than Eastern reports and mainly consisting of sporadic cases, with infrequent familial history. Of note, the observed female predominance (72.4%) resembles the disease patterns of endemic areas, as previously described by our group (14), with similar frequency of visceral manifestations reported in greater series (15-17), such as ocular disease (49.4%), vascular involvement (28.8%), neurological involvement (24.7%) and intestinal disease (12.3%). Regarding clinical manifestations and IgM AAEA frequency, we demonstrated that previous major systemic manifestations did not influence antibody positivity. On the other hand, IgM AAEA seems to be a marker to BD flares, particularly associated with mucocutaneous and articular manifestations.

Concerning the possible pathogenic role of anti-alpha-enolase, previous evidences suggest that the target enzyme has not only innate glycolytic function but also a relevant role in several biological and pathophysiological processes. In fact, this enzyme may act as a plasminogen receptor in many cell types and seems to be a marker of pathological stress (18). Of note, alpha-enolase was described as a target antigen on many inflammatory diseases,

such as rheumatoid arthritis, systemic lupus erythematosus, vasculitides and systemic sclerosis (19). Accordingly, we observed a high prevalence of IgM AAEA in a subgroup of patients with clinical active BD.

This finding may help the differential diagnosis between BD and ESpA. Both of these chronic disorders are likely to affect patients at a younger age accompanied by fluctuating courses. Arthritis is a common feature of these conditions, together with oral ulcers, cutaneous lesions and intestinal manifestations. In BD, mucocutaneous symptoms are markers of disease activity and seem to be associated in up to 80% with articular involvement (20, 21). Our findings corroborate with this condition, since the group with mucocutaneous and articular involvement presented a higher frequency of IgM AAEA positivity. Therefore, IgM AAEA positivity may be helpful to determine BD flares.

Of note, using an established overall activity index for assessment of BD activity (BR-BDCAF) instead of HBI, commonly used for Crohn's disease, reinforces alpha-enolase role as a possible target antigen for systemic inflammation in BD and not restricted to the group with intestinal BD, as previously reported (8). In fact, BR-BDCAF scores are not influenced by intestinal activity and the low frequency of this involvement in our population may account for the lack of association of active intestinal involvement with AAEAs in the present study.

BR-BDCAF was validated for Brazilian Portuguese by Neves *et al.* (12) from the English version of BDCAF (9) and evaluates the past four weeks of symptoms. The Behçet's disease activity index (BD AI) (9), is simi-

lar to BR-BDCAF regarding activity symptoms evaluation, however the latter seems more suitable to evaluate cases in which mucocutaneous and articular symptoms intensity are relevant manifestations, scoring from zero to 4 points for each symptom, depending on its intensity (12). However, based on BR-BDCAF collected data, BDAI was applied in this study population and notably the association between BD disease activity and IgM AAEA positivity was sustained, with higher BDAI scores in BD IgM AAEA positive group (data not shown). Therefore, alternative activity scores may be used in future international studies regarding IgM AAEA ability to distinguish BD activity.

In conclusion, our data support the value of alpha-enolase as a target antigen in BD and suggest that IgM AAEA is a serological marker of flare in this disease. Further studies are necessary to confirm the relevance of this antibody to discriminate BD from ESpA, particularly in patients with high disease activity.

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