# Biomarkers in Behçet's disease: diagnosis and disease activity

Behçet's disease (BD) is a multisystem inflammatory disorder currently classified as a vasculitis. Its etiopathogenesis is unclear, but environmental, genetic and autoimmune factors have been considered. There are ongoing efforts to elucidate the etiology of BD and numerous attempts have been made to identify biomarkers predictive of the course and severity of the disease. Much of this – mostly cross-sectional – work has yielded some valuable results, but more longitudinal studies are needed. This review provides an overview of some of the biomarkers which have been investigated in BD so far, and aims to evaluate their usefulness in the diagnosis and estimation of disease activity. This is exemplified by certain clinical situations. Clinicians, clinical investigators and basic researchers alike are given a concise database of biomarkers studied in association with BD, as well as a reflection of methods for estimation of disease activity.

**KEYWORDS: BDCAF ■ Behçet's disease ■ biomarkers ■ disease activity ■ HLA ■ immunomarkers ■ neutrophiles ■ Th17 pathway**

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Behçet's disease (BD) is a multisystem inflammatory disorder, currently classified as a vasculitis. Its etiopathogenesis is unclear, but environmental, genetic and autoimmune factors have been considered.

The disease is most frequent in population groups along the 'Old Silk Road', however, it seems to have a worldwide distribution with different clinical features, severity and male:female ratios in different geographical regions. Caucasians are affected less frequently and often have mild disease, with the most severe cases occuring in the Middle East. Blacks are rarely affected. BD is well known in the Far East, and it is one of the leading causes of blindness in Japan.

The multiple manifestations, their unpredictable occurence in the course of the disease and its syndromatic character make BD disease difficult to diagnose, evaluate and treat. This is especially true in light of the lack of seromarkers specific for this disorder or predictive of its severity and course.

Over the past decades, there have been ongoing efforts to elucidate the etiology of BD. Many studies have investigated genetic aspects and immunologic features of the disorder in states of active disease or remission, in comparison with healthy individuals or other, often autoimmune-mediated, diseases. A large number of proteins have been implicated as possible auto-antigens and studies of cytokine profiles have yielded conflicting results. Many of these – mostly cross-sectional – studies have provided some valuable information and shed light on the complexity and intricacy of this disorder. However, there certainly is a need for more prospective, controlled and longitudinal studies, not an easy endeavor considering the rarity and heterogeneity of BD.

It is the aim of this review to give an overview of some of the biomarkers that have been investigated in BD so far and to try to evaluate their usefulness in the diagnosis, differential diagnosis and estimation of disease activity of BD, as well as in certain clinical situations.

We hope to stimulate further research and provide clinical investigators and basic researchers alike with a concise database of biomarkers that have already been studied to some extent in association with BD (Table 1).

## General considerations regarding biomarkers in BD

*"Not all that counts is countable, and not all that's countable counts"*
– Albert Einstein.

A biomarker is defined as 'a characteristic that can be objectively measured and evaluated as an indicator of normal biological process, pathogenic processes or pharmacologic responses to therapeutic intervention' by the NIH Biomarkers Definitions Working Group [1]. This definition implies the possible use of characteristics

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**Table 1. Biomarkers in Behcet's disease.**

| Disease manifestation | Genetic markers | | Markers associated with molecular mimicry | Immunomarkers | | | Coagulation and endothelium | Markers related to neutrophiles |
|---|---|---|---|---|---|---|---|---|
| | *HLA* | *Other* | | *Cytokines* | *Cells and cell activation markers* | *AB/compl.* | | |
| Overall disease activity/ association/ susceptibility/ predisposition | B51* [80*,85‡,86‡,87‡,88‡]; B52 [86]‡; B57‡; B5101 [89]‡; B5108 [89]‡; B5701 [90]‡; B15 [80]*; MICA [91,92]‡; TNF-1031C allele [90]‡ | Polymorphisms of genes encoding: eNOS [93,94]‡; VEGF alleles I and -634C [81]‡; TNF-α-1031C allele [83]‡; ICAM-1 241 [82]‡; IL-18 promoter (early symptom onset) [84]*; CYP450 (CYP2C19–2) [95]‡; MEFV mutations [25]‡; Chromosome regions: 6p22–24, 12p12–13 [96]‡ | Anti-*Mycobacterium tuberculosis* 65 KDa HSP-overlapping synthetic peptides 111–25, 154–72 and 311–26 IgA and IgG [75]‡; Anti-*Streptococcus sanguis* AB [97]‡; Anti-*Streptococcus pyogenes* HSP60 AB [98]‡; Antiretinal HSP60 AB [98]‡ | MBL (l) [99]‡§ ADA [61,41,42]*<br><br>TH1 group: IFN-γ [34]*; IL-6* [34*,39‡,38*] IL-8* [32*,33*,39‡,38*,100*]; IL-12 [34–36]*; IL-18 [28,34]*; IL-18 (BA)*<br><br>TH2 group: IL-4 [34,47]‡; IL-4 (OU) [7]‡; IL-10* [34*,36‡,47‡]; IL-13 [47]‡<br><br>Other: TNF-α* [39‡,38*]; sIL-2R [38]*; IL-1 [39]‡; IL-17 [34]*; sTNFR-75 [36]* | CD80B cells [101]‡;<br><br>TH1 activity: TH1 cells [35,102]*<br><br>TH2 activity: CD30 (S) [50]* γ-δ T cells * [103*,104‡,105‡]; CD28 [22]*; CD8+cytotoxic T cells reactive to MICA [106]* | AECA [26,107]*; anti-CTLA-4 AB [108]‡; anti-KIR2DL AB [109]‡; anti-oxLDL AB [110]‡; ASCA [9,111]‡; anti-kinectin AB [112]‡; ACA-IgG, IgM [19]‡; | vWF Ag [107]‡; NO [65,66]*; ADMA & NO(l) [113]‡; Homocysteine* [11‡,67*,114‡]; IgM AECA to α-enolase [115]‡; VGEF [67]*; Leptin [68]*; Prothrombin-fragment [116]‡; Plasmin/ α2-antiplasmin complexes [116]‡; Thrombomodulin (l) [116,117]‡; APC (l) [117]‡ | PMN leukocyte elastase [58]*; MPO [57]*; AOPP [57]*; Thiol (l) [57]*; ICAM-1 [59]*; E-selectin [60]*; L-selectin [60]*; P-selectin [60,61]*; Actin (NE) [118]‡; ADA [62,41,42]*; TBARS [62,38]*; G-CSF [63]*; CD14 [119]‡ |

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*Markers which are not specifically designated by (l) are elevated with statistical significance in comparison to those in a control group.*
**Markers proposed to be related to increased disease activity and/or severity.*
*‡Markers that have been associated with Behcet's disease.*
*§Innate immunity.*
*AB: Antibody; ACA: Anticardiolipin antibody; ADA: Adenosine deaminase; ADMA: Asymmetric dimethylarginine; AECA: Anti-endothelial cell antibodies; AH: Aqueous humor; Anti-CTLA-4: Anticytotoxic T-lymphocyte antigen 4; Anti-KIR2DL: Antikiller immunoglobulin-like receptor 2DL; Anti-oxLDL: Oxidatively modified low-density lipoprotein; AOPP: Advanced oxidation protein products; APC: Activated protein C; ASCA: Anti-*Saccharomyces cerevisiae *antibodies; BA: BAL fluid; CCR5: Chemokine receptor 5 (Th1-related); CIC: Circulating immune complexes; compl.: Complement; CSF: Cerebrospinal fluid; CYP: Cytochrome P; eNOS: Endothelial nitric oxide synthase; GI: Gastrointestinal lesions; HSP: Heat shock protein; ICAM: Intracellular adhesion molecule-1; l: Low levels; MBL: Mannose-binding lectin; MC: Mucocutaneous lesions (aphthae +/- skin); MCP-1: Monocyte chemoattractant protein 1; MEFV: Mediterranean fever; NE: Neutrophiles; NKT: Natural killer T cells; NO: Nitrate; OA: Osteoarthritis; OU: Oral ulcers; PMN: Polymorph nuclear; S: Serum; SBP: Selenium-binding protein; sIL: Serum interleukin; SK: Skin lesions; sTNFR: Soluble TNF receptor; SY: Synovial fluid; TBARS: Thiobarbituric acid-reactive substances; tPA: Tissue-type plasminogen activator; Txk: Tec family tyrosine kinase specific to Th1 cells; vWF: von Willebrand factor.*

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| Mucocutaneous | B51 [80]‡ | TNF-α-1031C allele polymorphism [83]‡ | – | IL-4 (OU) [7]‡; IL-12 (OU )[7]‡; INF-γ (OU) [7]‡; TNF-α (OU) [7]‡; IL-8 (MC) [8]‡; IL-10 (MC) [8]‡; IL-12 (MC) [120]‡; IL-12 (SK) [120]‡; IL-18 (SK) [120]‡; INF-γ (MC) [8]‡; MCP-1 (MC) [8]‡ | γ-δ T cells (OA) [104]‡; γ-δ T cells (S) [105,121]‡ | CIC [122]‡ | VEGF (OU) [69]*; CD34 (OU) [69]* | L-selectin [60]* |
| Arthritis | B51 [80]‡ | ICAM-1 G/R 241 polymorphism [82]‡ | – | IL-1β (SY) [15]‡ | – | AECA [103]‡ | NO (nitrite levels in SY) [65] | – |
| Ocular | B51 [80,85]‡; Co-expression of the TNFB-2 allele with HLA-B51 [85]* | VEGF allele plus 936T polymorphism [81]‡; *MEFV* mutations (less ocular disease) [25]‡ | Anti-mycobacterium 65 Kda HSP-peptides 111–25, 311–26 and anti-human 136–50, 336–51 IgA, IgG [75]*; Anti-*Streptococcus sanguis* AB [97]‡; Anti-strep HSP60 AB [98]‡; Anti-retinal HSP60 AB [98]‡ | INF-β [114]*; INF-γ (AH) [12]‡; IL-6 [123]‡; IL-8 [123]‡; TNF-α [123]‡; TNF-α (AH) [12]‡; IL-15 (AH) [12]‡ | γ-δ T cells (S, AH) [13]*; $CD8^+$ ,$CD56^+$ NKT cells (S, AH) [13]*; S-antigen responsive T-lymphocytes [112]* | IgA,G [124]‡; C3,4 [124]‡; AECA [26]‡; anti-CTLA-4 AB (less uveitis) [108]‡; Anti-Tropomysin AB [14]‡; Anti-SBP AB [125]*; CIC [122]‡ | NO (nitrite levels in AH) [126]‡; VGEF [67]‡; FVIII (macular edema) [70]* | AOPP [57]*; E-selectin [123]*; ICAM-1 [123]* |
| CNS | B51 [81]‡ | – | anti-αB-crystallin IgG (S, CSF) [127]‡ | – | – | CIC [122]‡ | – | – |

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| Thrombosis | – | MEFV mutation [25]‡ | – | – | – | AECA [26]‡; ACA-IgG [19]‡ | Homocystein [128]‡; APC (l) [117]‡; tPA (l) [27]‡; Lp (a) [119]‡ | – |
| Vascular | – | – | – | – | CD28 [22]* | – | ADMA & NO (l) [113]‡ | – |
| GIT | – | – | HSP60 (GI) [18]‡ | IL-12 (GI) [119]‡; IL-18 (GI) [119]‡; CCR5 (GI) [18]‡ mRNA expression: INF-γ (GI) [18]‡; TNF-α (GI) [18]‡; IL-12 (GI) [18]‡; Txk (GI) [18]‡ | – | ASCA [129]* | – | – |

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not necessarily related to laboratory investigation, such as the number of oral ulcers in a BD patient, as a predictor of disease outcome or specific clinical events. However, most biomarkers are searched for in body fluids, most often in peripheral blood. This constitutes one of the main problems in identifying biomarkers since many of these potential 'seromarkers' are proteins of varying size, making the detection of rare and small molecules in proteomic scans difficult. By contrast, improved laboratory techniques create large numbers of false positives [2]. Also, it might be argued that biomarkers in BD should be looked for (or better looked for) at sites of actual disease involvement, including oral or genital ulcers as well as skin and so on.

## Quality & validity of studies on biomarkers in BD

To date, the overwhelming majority of putative biomarkers in BD have been evaluated in cross-sectional studies. This very much limits the validity and usefulness of their results. In a heterogeneous disorder with an unpredictable clinical course, these one-point-in-time observations often yield conflicting results. Many investigators define patient groups as either being 'active' or 'in remission', and propose one or several potential biomarkers, which are measured at a certain point of time, and – if statistical significance between the two groups is observed – publish those results. Often, another control group of age- and sex-matched healthy individuals is included and sometimes uses patients with other autoimmune diseases.

There are a number of drawbacks and limitations to this approach: first, the lack of a longitudinal, prospective study design. Observations in BD need to be made over time, and markers need to be measured in the same individuals over that period of time against a well-defined control group. Patients in one group may be in different stages of their disease evolution than others. This can create numerous problems considering the pleiotrophic biological effects of many markers, such as some cytokines, which may vary over time. IL-10, for instance, acts as an anti-inflammatory cytokine through its inhibitory effect on antigen-presenting cells early in the immune-response, however, it enhances cytotoxic T cells later on. TNF-α and VGEF are other, similar examples.

Second, a disorder with a syndrome-like character, such as BD, is best broken down into its various clinical subentities, such as uveitis, arthritis, oral ulcers and so on, for which a possible biomarker should then be sought. Individuals in the same group of active patients may have very different disease manifestations, up to extremes such as isolated uveitis in some and and pustular rash in others. Even definitions such as ocular involvement or mucocutaneous lesions may be too general. Neither anterior uveitis, retinal vasculitis nor pyoderma gangrenosum and oral ulcers are interchangeable clinical and pathological entities, to give only a few examples.

Problems with defining disease activity are one of the major current obstacles in the comparability of study results and will be discussed later in this article.

Last but not least, statistical significance does not mean clinical relevance. Only markers with high predictive values, accessiblility and cost–effectiveness will find their way into everyday clinical practice.

What are biomarkers in BD good for then? As in many other diseases, biomarkers in BD might help researchers to get a better grasp of pathophysiological mechanisms. They might be of use in making a correct diagnosis of BD in uncertain cases or may indicate disease activity and/or severity. They may assist in defining a certain subset of patients, and – more importantly – predict specific clinical events, such as the development of retinal vasculitis or deep venous thrombosis.

Defining response to therapy is another, very important and intensely sought aspect of the possible future utility of biomarkers in BD.

## Diagnosis, differential diagnosis & biomarkers in BD

There is no single serologic test for the diagnosis of BD. Repeated studies evaluating the prevalence of standard rheumatologic laboratory tests, such as antinuclear antibody, antineutrophil cytoplasmic antibody (ANCA), and so on have failed to show an increased prevalence of those markers in BD.

To date, BD remains a clinical diagnosis, based on its disease manifestations, most of which have a long list of possible differential diagnoses. Perhaps the most specific finding is pathergy, although it also is a feature of the other neutrophilic dermatoses, such as Sweet's syndrome and pyoderma gangrenosum, and has recently been reported to be positive in 18% of patients with recurrent aphthous stomatitis (RAS) [3,4].

The International Study Group for Behçet's disease has established diagnostic criteria for BD, in which recurrent oral ulcers are a mandatory

finding, along with two of four of the following: recurrent genital ulcers, eye involvement, skin lesions and pathergy [5].

Those diagnostic criteria may serve as a first example of the use of biological markers in BD, since some of them fit the above mentioned definition of a biological marker (i.e., oral and genital ulcers or pathergy). Despite their easy accessibility and low cost of evaluation, powerful studies of their predictive values are scarce. Some of them, such as pathergy, are of limited use in certain ethnic groups, owing to the low incidence of positive findings [6].

The mere existence of diagnostic criteria for BD is an indicator of the fact that diagnostic difficulty and uncertainty are frequently encountered when evaluating patients for this disorder. This is particularly true when disease manifestations develop at different points in time, or when constellations of signs and symptoms fulfill the criteria for the diagnosis of BD, although, in fact, the patient has a different disease. For example, an inflammatory bowel disease (IBD)-patient may have recurrent oral ulcers, erythema nodosum and uveitis and therefore be easily misdiagnosed as a BD patient with gastrointestinal (GI) involvement.

The following discusses some clinical situation in which biomarkers could be of use.

## Patient with recurrent oral ulcers, but insufficient diagnostic criteria for BD: BD versus RAS

Those individuals need to be evaluated for spondyloarthropathy, reactive arthritis, systemic lupus erythematosus (SLE), celiac disease, IBD and so on. After exclusion of these disorders, the main differential diagnosis to BD remains RAS.

The TH1-associated cytokines IL-12, IFN-γ, TNF-α, as well as the TH2-cytokine IL-4, have all been found in elevated amounts in oral ulcers of patients with BD [7]. They were also elevated in RAS, with the exception of IL-4, which was found exclusively in the ulcers of BD patients compared with those in RAS in one study by Dalghous *et al.* [7]. However, Ben Ahmed *et al.* did not find IL-4 in mucocutaneous lesions of patients with BD [8]. IgG-anti-*Saccharomyces cerevisiae* antibodies (ASCA) were significantly increased in BD versus RAS in a study by Krause *et al.*, although this was not found for IgA-ASCA [9]. A later study did not find any significant differences for ASCA or perinuclear antineutrophil cytoplasmatic auto-antibody levels in patients with BD and GI symptoms, compared with RAS patients [10].

Ozkan *et al.* reported significantly increased levels of homocysteine in the serum of BD patients versus those with RAS [11].

## Patient with acute uveitis

Uveitis may be the initial presenting sign of BD and a large number of other autoimmune or infectious disorders. Ahn *et al.* studied the cytokine environment in aqueous humor and peripheral blood of patients with BD uveitis and those with other causes of uveitis, as well as in healthy controls. An extreme TH1 polarization in the aqueous humor of BD patients was characteristic of BD, and IL-15 was exclusively found in the aqueous humor of uveitis patients who had BD [12]. Yu *et al.* found predominance of $CD8^+CD56^+$ natural killer T cells in the aqueous humor and serum of uveitis patients with BD compared with those with uveitis owing to other causes, including idiopathic and Voght–Koyani–Harada syndrome-associated uveitis in which $CD4^+$ cells were predominant [13]. Mahesh *et al.* evaluated peripheral blood mononuclear cells (PBMCs) from BD patients with posterior uveitis, patients with other forms of noninfectious uveitis and healthy controls for their antigen-specific lymphoproliferative responses to α-tropomysin and anti-α-tropomysin antibodies. Only PBMCs from patients with BD-associated uveitis showed increased proliferative responses and antibodies to α-tropomysin [14].

## Patient with arthritis

These patients pose a special diagnostic challenge, especially when arthritis occurs in conjunction with oral ulcers and/or ocular disease, a constellation shared with diseases such as reactive arthritis and spondyloarthropathy (SpA).

Similar to BD, neither the spondyloarthropathies nor reactive arthritis have diagnostic serological markers if IBD-related SpA with associated ASCA is excluded.

To the best of our knowledge, no trials have been conducted with the ultimate aim of finding markers specific for arthritic involvement in BD using a control group of patients with reactive arthritis or SpA. However, Pay *et al.* compared IL-18, IL-1β, TNF-α and metalloproteinase-3 levels in the synovial fluid of BD patients with those of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) joint effusions. They found significantly higher levels of IL-18, TNF-α and metalloproteinase-3 in RA than in BD and OA. Cytokine levels did not differ between BD and OA, apart from IL-1β,

which was found to be elevated in BD compared with OA, but without a significant difference in RA. Joint effusions in BD were differed by a low overall cytokine content and significantly lower levels of metalloproteinases from those in RA [15].

## Patient with gastrointestinal complaints

Gastrointestinal symptoms and signs, including bloody diarrhea, can be part of the clinical spectrum of BD. Diarrhea or other GI complaints in the setting of skin lesions, such as erythema nodosum, pyoderma gangrenosum, joint symptoms, as well as oral ulcers or ocular disease, may signify the presentation of IBD, as well as of BD.

An increasing number of studies have tried to examine the prevalence of ASCA in BD. These antibodies, usually found in 50–60% of patients with Crohn's disease, were postulated to be a new seromarker for BD by Krause *et al.* (see also 'Patient with oral ulcers' section) [9]. Oshitani *et al.* showed differences in the IgG-subclasses of ASCA in GI BD patients versus IBD [16]. A study by Fresko *et al.* demonstrated ASCA levels in BD patients to be similar to those in healthy controls, ulcerative colitis and ankylosing spondylitis patients. As expected, Crohn's patients had high levels, but, interestingly, there was a significant trend for BD patients with GI involvement and these patients had elevated ASCA levels when compared with BD patients without GI involvement [17].

The quality of all future studies investigating ASCA in BD will depend on their ability to reliably exclude the possibility of Crohn's disease in BD study group patients, and vice versa.

Imamura *et al.* found heat shock protein (HSP) 60 mRNA expression in the GI lesions of BD and Crohns's disease, however, these were absent in patients with ulcerative colitis and in normal controls [18]. In the same study, which investigated intestinal lesions and peripheral blood lymphocytes, no difference was found for TH1-specific mRNA expression in BD and Crohn's disease alike; however, ulcerative colitis patients did not have TH1-specific mRNAs, but they expressed CCR4, a TH2-specific chemokine receptor.

## Patient with intracardiac thrombus or peripheral arterial thrombosis

Here, a correct and fast diagnosis is essential, since it implies different forms of treatment and diagnostic error can have detrimental consequences for the patient. The main differential diagnosis is between BD and antiphospholipid syndrome (APS). Homocystinuria is another, very rare possibility. First diagnostic clues to APS can be a prolonged partial thromboplastin time and evidence of lupus anticoagulant, anticardiolipin antibodies and anti-b2-glycoprotein I antibodies, however, these have also been described in BD with varying prevalence [19,20,21]. Considerable confusion can initially be caused by a SLE patient with antiphospholipid antibodies who presents, for example, with arthritis and oral ulcers. However, a positive antinuclear antibody will soon put an end to diagnostic uncertainty in most of these cases. A greater challenge is posed for patients with arterial thrombosis who do not have any additional features of BD at the time of presentation, but have some positive serology for antiphospholipid antibodies. If BD is diagnosed for these patients, anti-inflammatory treatment is needed more than anticoagulation, and there is no time for a wait-and-see approach.

Several studies have investigated the prevalence of antiphospholipid antibodies in BD with contradicting results. Musabak *et al.* found a higher than normal prevalence in Turkish BD patients, especially in those with vascular involvement [19]. In a study of 36 Romanian BD patients by Tanasenu *et al.*, 55% had antiphospholipid antibodies – a prevalence as high as in SLE [20], whereas Toky *et al.* could not confirm those observations in a study including 128 BD patients [21]. Only 7% of his patients with BD – no significant difference in comparison to healthy controls – were found to be positive for anticardiolipin IgG and IgM.

There is no conclusive explanation for these contradictory results, although regional differences have been proposed [21]. It seems, however, that antiphospholipid antibodies do not play a significant role in the thrombovascular pathology of BD. This assumption can be made from the observation of poor treatment outcome of BD patients with arterial thrombosis who have been treated with anticoagulation instead of anti-inflammatory drugs.

The identification of a marker highly predictive of arterial thrombosis would be a great step forward towards a better overall risk stratification of BD patients, as well as in the management of the small subset of patients affected by this life-threatening disease manifestation.

## Patient with large vessel vasculitis

Large vessel vasculitis in BD may affect the pulmonary arteries, the aorta and other major peripheral vessels. Often, the clinical picture with characteristic aneurysma formation hints

to the diagnosis, and is augmented by imaging techniques such as angio-computed tomography (CT), positron emission tomography–CT or angiography. The main differential diagnoses are Takayasu's arteritis and Giant cell arteritis, however, the combination of aneurysma formation in the main pulmonary arteries, together with an intracardiac thrombus, is virtually diagnostic of the disease.

There is considerable confusion in the current literature regarding the term 'vascular involvement'. This is not always clearly defined, and some authors seem to include manifestations, ranging from venous thrombosis over arterial thrombembolism to major large vessel vasculitis, under this category. One study by Hamzaoui *et al.* showed an increase in serum levels of soluble CD28 in BD patients and in those with RA, compared with normal controls. CD28 levels were especially high in patients with active BD and in those with BD and vasculitis [22].

We are not aware of any studies that have evaluated markers in BD in comparison with those in Takayasu's or Giant cell arteritis.

There is an increasing interest in ANCA-associated large vessel vasculitis in the recent literature [23,24]. Some of the features of the few cases described, such as prominent perivasculitis and frequent dissection, are reminiscent of BD-associated vasculitis and pose a contrast to the stenotic nature of Takayasu's lesions. However, there is currently not sufficient evidence to draw a line between ANCA-positivity and vasculitis associated with BD.

### Patient with venous thrombosis and features suggestive of an inflammatory disorder

Venous thrombosis occurs with increased frequency in BD, as well as in other inflammatory disorders, and may be the presenting manifestation in a BD patient. Similar to arterial thrombosis, the role of anticoagulation is only of a supplementary nature, and immunosuppression is first-line treatment in these patients.

Rabinovich *et al.* made the interesting observation of an increased incidence of the mutated *MEFV* gene in Israeli BD patients [25]. BD patients carrying the gene were significantly more likely to have thrombosis than noncarriers. There was no significant difference in gender, cutaneous lesions, joint disease or disease severity. Uveitis in carriers was less frequent than in noncarriers.

Another study by Aydintug *et al.* showed an increased incidence of thrombosis in BD patients who tested positive for anti-endothelial cell antibodies in comparison with those who did not [26]. There was no correlation with other clinical features, and anticardiolipin antibodies were negative in this series.

Yardukul *et al.* showed significantly lower tissue-type plasminogen activator levels in patients with deep venous thrombosis caused by BD in contrast with those with deep venous thrombosis due to other causes [27].

## Biomarkers in the evaluation of disease activity in BD patients

### Estimation of disease activity in BD

Estimation of disease activity in patients with BD remains a challenge. While certain patients display symptoms and signs, such as oral aphthosis or panniculitis, virtually all the time – intermingled with bouts of other manifestations – most patients tend to have unpredictable disease flairs and no or few symptoms in between. Neither the nature nor the frequency and duration of these flairs are foreseeable. However, it is quite certain that the overall disease activity slowly diminishes with increasing age.

The lack of a reliable, standardized tool for the evaluation of disease activity in BD is a major obstacle in trying to find biomarkers indicative of such activity and limits the comparability of study results. There are, however, some promising attempts to develop tools for the estimation of disease activity in everyday clinical practice and research. The best-studied, and probably most widely used tool, is the BD Current Activity Form (BDCAF), sometimes referred to as the Leeds Activity Inventory [28]. This questionnaire is an easy to use tool, which can provide a reasonable estimate of the overall disease activity in most BD patients within a comparably short amount of time. Its main characteristic is the approach of recording symptoms and signs over a predefined time interval (28 days), relating the level of activity to the duration of these signs and symptoms. No record is made of the number of oral, genital or skin lesions, nor the degree of severity of a certain disease manifestation at a given time. Good interobserver reliability for general disease activity was reported in one study by Bhakta *et al.* [29]. Most physicians in this study agreed on the degree of activity of oral, genital and joint manifestations, whereas great interobserver variability was found in the evaluation of GI symptoms. Neurological manifestations were accurately identified if they appeared as new symptoms, however, there was considerable disagreement on the specific site of the CNS lesion.

In our experience, the BDCAF is a valid, reliable, time- and cost-effective tool for most aspects of disease activity evaluation in BD patients. However, we did not find it helpful for the evaluation of patients with large vessel involvement who clearly depended on imaging studies (CT angiography or positron emission tomography) for obtaining reasonably precise information on disease activity and progression. The answers given in the questionnaire were rarely useful in making a decision regarding the indication for those imaging studies. Another problem is the time-length approach. Disease activity is not necessarily synonymous with disease duration. We feel that a BD patient presenting with aseptic meningitis or acute uveitis for 3 days may be as active, or even more active, than someone with oral ulcers, arthritis and erythema nodosum for the 4 consecutive weeks.

Furthermore, the BDCAF in itself does not propose an overall disease activity score, which could theoretically be calculated from the answers to the various items on the form. This very much limits the comparability of disease activity between individual patients, patient-groups and countries. An important attempt to identify those clinical features that can be summoned to form an overall index of disease activity, which would be appropriate for research studies internationally, has been made by Lawton *et al.* [30]. His work comprised 542 BDCAFs who had been filled out over a period of 7 years in five different countries (UK, Turkey, Iraq, Korea and China). Following some adjustments (rescoring of the 4-graded time scale into a dichotomous scale of 'symptom present or absent within the past 4 weeks'), as well as the exclusion of inferior data from China and Iraq, data from the remaining three countries – UK, Turkey and Korea – was pooled. After removal of two items from the BDCAF ('disease activity as seen by the patient' and 'disease activity as seen by the physician'), the summation of the remaining 13 items were a good fit with the Rasch statistical model and enabled comparisons between the three countries.

Few, if any, studies investigating markers of disease activity have used the BDCAF in a statistically relevant mode. Most authors define the disease as active when symptoms and/or signs appear in at least two to three organ systems. Others have used the BDCAF, however, they do not describe how they defined their score of activity, whereas some authors give hardly any description of what they consider to be active disease at all. The further implementation of the BDCAF, taking into consideration the results and conclusions of the important work by Lawton *et al.*, remains to be realized when clinically evaluating BD for disease activity. This especially applies to designing and carrying out studies that relate to aspects of disease activity in these patients.

## Role of erythrocyte sedimentation rate & C-reactive protein in the evaluation of disease activity

The erythrocyte sedimentation rate and C-reactive protein have long been recognized as inaccurate markers of disease activity in BD [31]. However, it is widely agreed that a significant elevation of these parameters or a marked change form the baseline of an individual patient should prompt further investigation.

## Immunomarkers & disease activity

Most of the studies dealing with the exploration of biomarkers possibly indicative of disease activity in BD have involved immunomarkers, such as cytokines, various immune cells, their activation markers, as well as antibodies and complement. Initially, the primary objective of many of those studies was to elucidate the pathoimmunology of the disease, however, more recently, the identification of markers of disease activity has become a primary concern.

The wide-spread concept of a TH1-cell predominance in BD has been challenged in numerous recent studies, as TH2 cytokines also seem to play a role in the pathogenesis of BD. It is probable that phenotypic changes of both subgroups of TH cells occur in the disorder. A newly recognized group of TH cells – IL-23 dependent TH17 cells – has drawn the attention of many investigators to their possible role in the pathogenesis of many autoimmune and rheumatologic disorders, including BD.

Correlation of overall disease activity with the serum levels of the TH1 cytokines IL-6, IL-8, IL-12, IL-18 and INF-γ has been demonstrated in a number of studies [32–38]. Other studies have found increased levels of these cytokines in BD, regardless of disease activity [39].

Adenosine deaminase (AD), an enzyme required for lymphocyte proliferation and differentiation, has been the focus of at least four studies dealing with disease activity markers in BD [40–43]. The enzyme – which is typically increased in disorders dependent on T-lymphocyte activation – was found to correlate well with disease activity in BD by all of the four above mentioned authors. Calis'

*et al.* findings were consistent with those of the other groups [41]. He also found AD levels to be independent of colchicine therapy in BD.

The TH2 cytokines IL-4, IL-10 and IL-13 have been found in increased levels in BD patients [7,34,36,37]. Hamazaoui *et al.* showed a significant increase of IL-10 in active BD patients compared with those in remission [34]. However, in the same study, he also demonstrated an increase of IL-6 and a striking increase in IL-17, IL-18 and INF-γ levels, concluding that a shift towards TH1 activity may occur in active BD, compared with patients in remission.

In light of a number of studies with contradicting results, the role of TH2-phenotype lymphocytes in BD remains unresolved. Decreased levels of $CD8^+$ lymphocytes, as well as IL-4 and IL-10 have been found by some investigators [44–46], whereas many others have demonstrated an increase in CD8:CD4 ratios, IL-4, IL-6, IL10 and IL-13 [34,36,47–49].

Apart from increased serum IL-10 levels, Turan *et al.* found a correlation between IL-12 and sTNFR and disease activity in a study consisting of 66 patients with BD [36].

Another clue to increased TH2 activation was provided by the findings of a study by Duzgun *et al.,* which revealed increased levels of soluble CD30 in BD [50]. CD30 is released from $CD4^+$ TH2 type T cells and is considered to be a marker for TH2 activity. In his study, levels of sCD30 were significantly higher in patients with active BD compared with those with BD in remission, RA patients and healthy controls. CD28 also was shown to be related to increased disease activity and to vascular complications by Hamzaoui *et al.* [22].

As mentioned previously, TH17 cells have recently attracted much attention amongst researchers in immunology [51–55]. New insights leading to a better understanding of the IL-23/TH17 axis or IL-23/IL-17 pathway have challenged the traditional concept of TH1/TH2 segregation in understanding TH-cell activation and function. It now seems clear that IL-23 induces the differentiation of $CD4^+$ T cells into TH17 cells, which secrete IL-17, IL-6, IL-8 and TNF-α. By contrast with TH1-cell populations – classically thought to be major players in autoimmunity – these cells do not produce INF-γ. Another characteristic of the IL-23/TH17 pathway is the activation of neutrophiles and monocytes – a feature likely to be of relevance in BD. It is now evident that the IL-23/TH17 pathway plays a major role in the autoimmunity of experimental allergic encephalomyelitis, IBD and collagen-induced arthritis, and there is increasing evidence for its role in RA and psoriatic arthritis [54,55].

This paradigm shift cannot be ignored by researchers studying the pathoimmunlogy of BD. Currently, we are aware of one published study by Chi *et al.,* who found increased IL-23 and IL-17 levels in the sera and PBMCs in Chinese BD patients with active uveitis [56].

## Markers related to neutrophiles

Neutrophiles have long been thought to play a central role in the pathogenesis of BD. This has even led to the classification of BD under the group of neutorphilic dermatoses by some authors, along with Sweet's syndrome and pyoderma gangrenosum, with which the disorder undoubtedly shares a number of clinical characteristics, the most prominent being pathergy.

Yazici *et al.* conducted a study exploring neutrophil activation in relation to disease activity in BD. His group measured myeloperoxidase (MPO) as a marker of neutrophil activation and found significantly increased plasma MPO activity in patients with active BD, as compared with inactive BD patients [57]. Two biomarkers of oxidative stress, advanced oxidation protein products (AOPP) and thiol, were also quantified. Increased levels of AOPP correlated well with increased disease activity, whereas thiol levels were low in active BD patients. Additionally, there was a significant correlation between uveitis and AOPP, however, not with MPO or thiol.

An earlier study by Deger *et al.* showed good correlation of polymorphonuclear leukocyte elastase with disease activity [58].

Other markers that have been studied are circulating intracellular adhesion molecule-1 [59], E-, L- and P-selectin [60,61] – all of which have been correlated with disease activity in BD. In addition, serum L-selectin was found to be significantly elevated in BD patients with erythema nodosum, and colchicine therapy was associated with lower selectin levels [60].

An interesting thought was introduced by Erkilic *et al.,* who investigated the relation of AD, thiobarbituric acid-reactive substances (TBARS) and antioxidant enzymes with disease activity in BD [40]. He found a significant, positive correlation between AD and TBARS, whereas there was a negative correlation of AD and TBARS with antioxidant enzymes, suggesting a link between T-cell activation and neutrophil hyperfunction.

G-CSF is elevated in Sweet's syndrome, a neutrophilic dermatosis showing some clinical similarity to BD. Exogenous G-CSF administration has previously been reported to cause Sweet's syndrome [62]. Kawakami *et al.* found increased levels of G-CSF in BD patients – although less than in Sweet's syndrome – in a study including patients with both disorders, also reporting a significant association of G-CSF levels with activity in both diseases [63].

## Markers associated with coagulation & endothelium

The main markers that have been found to have some correlation with disease activity are nitric oxide (NO), homocysteine, VGEF and leptin [64–68]. Positive VGEF staining of oral aphthous lesions was associated with a persistence of those lesions for more than 6 months in one study by Yalcin *et al.* [69]. Probst *et al.* showed increased factor VIII levels to be associated with an increase in the risk for macular edema in ophthalmologic BD patients [70].

## Molecular mimicry & activity markers

The thought that cross-reactivity of human antigens with bacterial or other foreign antigens may be the cause of BD has been favored by many investigators up to today. Even though this mechanism is unlikely to be the only one responsible for the disease, there is some evidence that strongly suggests molecular mimicry plays at least a partial role. This evidence is derived from various observations, such as increased levels of circulating antibodies to streptococcal HSPs, hypersensitivity reactions and systemic exacerbations of BD after streptococcal skin injections and a more favorable outcome of mucocutaneous lesions after combined treatment with penicillin plus colchicine over colchicine alone [71–73].

Heat shock proteins are the best-studied cross-reactive self antigens in BD. Apart from streptococcal HSP, mycobacterial HSP seem to play a role as shown by Stanford *et al.,* who induced anterior uveitis in Lewis rats following the injection of mycobacterial and homologous human heat-shock protein T-cell peptide epitopes specific for T lymphocytes in BD into their footpads [74].

A later study from the same group showed increasing IgA and IgG antibody titers to one or three peptides derived from the sequence of the Mycobacterium TB 65 kDa HSP and the homologous human mitochondrial 60 kDa HSP following their mapping in sera from BD patients in sequential antibody studies, thus suggesting a possible increase during exacerbations of ocular disease [75].

## Genetic markers & disease severity

Behçet's disease is associated with a number of HLAs, especially HLA-B51. The largest prevalence of *HLA-B51* carriers amongst BD patients is found in countries along the Old Silk Road, and familial cases of BD have higher HLA-B51 prevalences than sporadic cases. An increased risk of developing BD has also been observed amongst *HLA-B52* carriers in Israel and *HLA-B57* carriers in the UK.

It has been proposed that severe forms of BD may be partially linked to HLA positivity. Most of the studies in support of this assumption have found an association of HLA-B51 with particularly debilitating disease manifestations, such as posterior uveitis and CNS involvement [76–79]. This concept has been challenged by a number of authors. Choukri *et al.*, for example, did not find particularly severe disease or a certain subset of clinical manifestations to be associated with HLA-B51 or B15 in 86 Moroccan patients [80]. However, he observed a different disease course in HLA-B51 or B15 BD patients, characterized by an increase of symptoms over time out of proportion to the symptom increase in non-*HLA-B51/15* carriers, followed by complete remissions, thus concluding that earlier treatment might be justified in HLA-B51/15-positive individuals with BD.

A larger study from Turkey, which included 148 individuals with BD, did find increased frequencies of genital ulcerations, skin lesions, positive pathergy and eye disease in HLA-B51-positive patients. However, no significant association between HLA-51 positivity and a more severe disease course was found. HLA-B51 homozygosity was not related to increased severity of BD either.

Overall, it remains controversial whether HLA-B51 or another human leukocyte antigen is predictive of increased disease severity.

Non-*HLA* genes have also been associated with susceptibility to BD. Some examples are polymorphisms of the *VGEF* gene, the *ICAM-1* gene and *TNF* genes [81–83]. Not increased disease severity, but an earlier symptom onset was associated with polymorphisms of the *IL-18* promoter gene in a study from Korea [84]. The results of another interesting study by Verity *et al.,* which included 102 BD patients of Middle Eastern descent in the UK, suggest that co-expression of the *TNFB-2* allele with HLA-B51 might contribute to the severity of ocular disease [85].

Regardless of the role of genetic markers in terms of estimation of disease severity, it is important to state here that, male gender and young age at disease onset currently remain the most reliable known predictors of severe BD.

## Conclusion

Behçet's disease remains a clinical diagnosis. Notwithstanding the sheer multitude of proposed biomarkers with possible value in the diagnosis and estimation of disease activity, not a single one of them has emerged to the status of a feasible, easy to use, reliable and valid tool in everyday clinical practice or research so far.

This does not depreciate the many attempts that have been made in the search for these markers. Keeping in mind the pleotrophic effects of many seromarkers, the wide range of intrapatient variation over time and the frequently encountered uncertainty regarding disease activity, appreciation of the difficulty involved in carrying out these studies is warranted, as opposed to euphoria about a published statistically significant correlation. Conflicting results are the rule in this complex area of research, especially in this rare disorder, with its obscure pathogenesis, genetic, inter-regional and clinical heterogeneity.

There definitely is a need for longitudinal study design, appropriate control groups, well-characterized patient groups, outcome definitions and accurate clinical assessment of disease activity.

Some real progress with the estimation of disease activity has been made through the introduction of the Behçet's Disease Current Activity Form. The further implementation of this practical tool in clinical practice and research should be encouraged.

## Future perspective

Some future developments are desirable and worth considering:

- Improvement of study designs of clinical studies trying to identify biomarkes;
- Increased international cooperation and pooling of clinical data of patients from different countries;
- Standardization and further development of reliable clinical tools for disease activity evaluation;

### Executive summary

***General considerations regarding biomarkes in Behçet's disease***

- The lack of longitudinal, prospective study design limits the usefulness of many study results.
- The heterogeneity and rarity of the disease make the design of methodologically valuable studies a challenge.
- The syndromatic character of Behçet's disease (BD) may require separate investigations of different subgroups of disease manifestations or well-defined 'clusters' of signs and symptoms.

***Diagnosis, differential diagnosis & biomarkers in Behçet's disease***

- No serological marker is diagnostic of the disease.
- Putative markers might be especially helpful when evaluating patients for BD presenting with isolated disease manifestations, such as oral ulcers, uveitis or arthritis.
- Pathergy is a characteristic feature of BD in certain ethnic groups, but it is not pathognomonic for the disease as it may also occur in the neutrophilic dermatoses Sweet's syndrome and pyoderma gangrenosum.
- Markers indicative of BD versus antiphospholipid antibody syndrome in antiphospholipid antibody-positive BD patients presenting with intracardiac thrombi and/or peripheral arterial thrombosis may pave the way to faster diagnosis and timely initiation of life-saving therapy in the future.
- BD and inflammatory bowel disease are frequently confused entities and future studies investigating biomarkers in BD must be able to reliably exclude patients with inflammatory bowel disease.
- Currently, the best approach to diagnosing BD patients with large vessel involvement remains the use of imaging studies, such as angio-computed tomography, positron emission tomography-computed tomography or angiography.

***Biomarkers in the evaluation of disease activity in Behçet's disease patients***

- Estimation of disease activity in BD is difficult.
- The Behcet's disease current activity form (BDCAF) currently constitutes the best and easiest-to-use tool for disease activity estimation with an acceptable interobserver reliability for general disease activity.
- Some modifications of the BDCAF and its scoring scale may enable researchers to compare results indicative of degrees of disease activity between patients form different parts of the world.
- Erythrocyte sedimentation rate and C-reactive protein are unreliable indicators of disease activity in BD.
- Immunomarkers are widely researched but often problematic owing to their pleiotrophic effects.
- Neutrophile markers are an important and promising research target.
- Molecular mimicry remains a meaningful concept in BD, and associated markers – in particular various heat shock proteins – are in the focus of important research.
- Genetic markers, especially HLA-B51, may be weaker indicators of disease severity than previously assumed.

- Increased use of proteomic scans and critical interpretation of the data obtained from these scans;
- Reinforcement of a target-lesion-focused approach in trying to identify biomarkers;
- Focus on biomarkers that could be of help with diagnoistic dilemmas, such as BD versus IBD, APS or large vessel vasculitides;
- Careful (re)consideraton of the clinical relevance of a given biomarker. Statistical significance is not enough;
- A larger number of studies aiming to elucidate the role of the IL-23/TH17 pathway in BD.

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*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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