

A pilot study into bosentan (Tracleer®) as an immunomodulating agent in patients with Behçet's disease

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Abstract

Background: Behçet's disease (BD) is an auto-inflammatory vasculitis characterized by aphthous oro-genital ulcers, inflammatory skin changes and uveitis. Treatment is mainly immunosuppressive. Interestingly, elevated endothelin-1 (ET-1) levels suggest a possible beneficial effect of treatment with an ET-1 receptor antagonist.

Objectives: The aim of our study was to investigate the possible beneficial effect of the ET-1 inhibitor bosentan.

Methods: We performed a prospective double-blind placebo controlled pilot study into the effect and safety of bosentan in BD patients. Disease activity was measured using the Behçet Disease Current Activity Form. The primary objective of the study was to determine whether bosentan is therapeutically effective in patients with BD. Secondary endpoints were safety, tapering of medication and the effect of bosentan on possible disease activity markers such as ET-1, circulating endothelial cells (CECs), soluble interleukin-2 receptor (sIL2R) and cytokine levels.

Results: Ten patients were randomized to either bosentan or placebo. Overall, no effect on disease activity was observed, although one patient responded clinically and continued treatment after the study period. Despite one SAE, bosentan seems safe to use. No effect on tapering of medication, CECs, sIL2R and cytokine levels was found. In the bosentan group, ET-1 levels were elevated during the treatment period, with no correlation with disease activity.

Conclusions: Although this is a small pilot study, bosentan appears to be safe in BD patients. One patient had a durable and significant clinical response. Our observations should be confirmed and extended in a larger patient cohort to be of significant impact in the treatment options for BD.

Key words: Behçet's disease, bosentan, BDCAF, treatment, trial

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Abbreviations:

- BD Behçet Disease
- BDCAF Behçet Disease Current Activity Form
- CEC Circulating Endothelial Cells
- ET-1 Endothelin-1
- sIL2R soluble Interleukin-2 Receptor
- IFN- γ Interferon- γ
- TNF- α Tumor Necrosis Factor- α
- IL-1 Interleukin-1

Introduction

Behçet's disease (BD) is an auto-inflammatory vasculitis of unknown etiology.¹ It originates in countries alongside the Silk Route and is most common in Turkey.^{1,2}

Patients present with aphthous oro-genital ulcers, inflammatory skin changes and uveitis, but may also demonstrate arthritis, thrombosis, neurological symptoms or colitis. BD can be diagnosed by fulfilling the criteria outlined by the International Study Group for BD.³ The disease-specific pathology test,

in which a sterile skin puncture yields a sterile pustule, is one of these criteria. In light of this, BD may worsen after traumatic (skin) events provoking a Th-1 response in which the (pro) inflammatory cytokines interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) are mainly involved.² The affected sites may illustrate vaso-occlusive vasculitis with infiltration of predominantly activated T-cells and neutrophilic granulocytes. Therefore, therapy is mainly directed against T-cells, granulocytes or TNF- α . Toxicity and therapy failure limit the effect of immunosuppressive therapy.^{1,2}

Endothelin-1 is mainly secreted by endothelial cells and is primarily known for its vasoconstricting effect. In addition, studies show a pro-inflammatory effect via the activation of transcription factors such as NF κ B and the production of pro-inflammatory cytokines such as IL-1, TNF- α and IL-6.⁴

The endothelin-1 (ET-1) receptor antagonist bosentan effectuates vasodilation and inhibits endothelial cell proliferation, inflammation and subsequent fibrosis in patients with pulmonary hypertension (PHT).^{5,6} This beneficial effect might also be relevant for patients with BD, since high levels of ET-1 have been shown in serum and bronchoalveolar lavage fluid samples of BD patients with active pulmonary manifestations at a level that is comparable with PHT patients.^{7,8} Furthermore, decreased or extremely elevated nitric oxide (NO) levels in serum and affected tissues in BD patients might reflect a dysfunctional balance with ET-1 and endothelial stress.⁹⁻¹¹ In addition, endothelial progenitor cells are reported in active BD patients.¹² The presence of endothelial progenitor cells, of which circulating endothelial cells (CECs) are a subgroup, is also suggestive of endothelial stress. To investigate the possible beneficial effect of ET-1 inhibition on BD, we performed a prospective double-blind placebo controlled pilot study into the safety and efficacy of bosentan on disease activity in BD patients.

Methods

Study design and participants

In this pilot study, a prospective double-blind placebo designed controlled trial, we enrolled 10 BD patients, classified according to the criteria of the International Study Group for BD.³ All participants provided written informed consent. The study and protocol were reviewed and approved by the ethics committee of the Erasmus MC (METC number: MEC-2008-042).

Disease activity was measured using the Behçet Disease Current Activity Form (BDCAF) and by the presence of oral ulcerations.¹³ Oral ulcerations were counted as 1 point for every week that there was at least one present in the past four weeks, thereby scoring a minimum of 0 and a maximum of 4 points.

The primary objective of the study was to determine whether bosentan is an effective treatment in patients with BD, which was determined as a decrease in BDCAF of ≥ 2 in patients with a BDCAF score of ≥ 4 , after 24 weeks of treatment. In addition to this, disease activity was measured by the presence of oral ulcerations. A decrease in oral ulcerations of $\geq 25\%$ was regarded as effective treatment.

Secondary endpoints were safety, tapering of medication and the effect of bosentan on possible disease activity markers

such as ET-1, CECs, soluble interleukin-2 receptor (sIL2R) and cytokine levels.

Patients with active mucocutaneous disease, defined as BDCAF ≥ 4 , but without organ- or life-threatening disease, were included and randomized into two groups to receive either bosentan 125 mg twice daily or placebo.

Inclusion criteria were BD diagnosed according to international criteria,³ with a disease activity ≥ 4 according to BDCAF, despite therapy. Contraceptive measures in female patients of childbearing age were required during and for 6 weeks after the study period.

Screening laboratory test should meet the following criteria: Hemoglobin ≥ 6.5 mmol/L, WBC $\geq 3.0 \times 10^9/L$, neutrophils $\geq 1.5 \times 10^9/L$, and platelets $\geq 100 \times 10^9/L$. Liver enzyme levels should be within 3 times the upper limit of the normal range for the laboratory conducting the test.

Exclusion criteria were age below 18 years, eye- or life-threatening disease activity, pregnancy or planning a pregnancy within 38 weeks after enrollment, and hypotension defined as systolic blood pressure less than 85 mmHg. Medication use of glybenclamide, calcineurin inhibitors (e.g. cyclosporine A, and tacrolimus) or fluconazole was not allowed because of potential interactions.

Patients were randomized 1:1 to receive bosentan 125 mg or placebo for 24 weeks, with a maximum follow-up of 32 weeks.

Clinical and laboratory assessments were performed at baseline and every four weeks. BDCAF, oral ulcerations, adverse events (AE), liver enzymes and infection parameters were monitored.

Determination of ET-1 levels

ET-1 levels were measured according to a previously reported protocol.¹⁴

Enumeration of CECs

CECs were enumerated according to our previously reported flow cytometric approach¹⁵ which demonstrated excellent reproducibility of the assay between duplicate CEC samples. The following directly conjugated monoclonal antibodies were used to identify CECs – CD34-FITC (clone 8G12; BD Biosciences, San Jose, CA, USA), CD146-APC (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD45-PerCP (clone 2D1; BD Biosciences). DRAQ5 (Bioss Ltd, Shepshed, UK) was used as a cell permeable nuclear dye to exclude platelets and microparticles. CECs were defined as CD34+, CD146+, CD45- and DRAQ5+.

Samples were acquired on a FACS Canto II or Fortessa flow cytometer (BD Biosciences) and analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA).

Determination of sIL2R and cytokine levels

Venous blood samples were centrifuged at 3000 rpm immediately after collection for a duration of 10 minutes. Plasma was stored at -80°C and thawed to room temperature for sIL2R analysis. sIL2R plasma levels were quantified using an enzyme-linked immunosorbent assay (Human sCD25/sIL2R ELISA kit, Besancon, Cedex, France) in accordance with the manufacturer's instructions. sIL-2R levels were expressed in

picograms per milliliter (pg/ml), and levels > 2500pg/ml were considered elevated.

Cytokine levels of GM-CSF, IFN- γ , IL-10, IL-12(p70), IL-13, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 and TNF- α were measured using a standard 13-plex Luminex kit (Millipore, Bedford, MA, USA). Levels above 10 pg/ml were considered elevated.

Statistical analysis

Because of the low number of patients included, our analysis is only descriptive and exploratory.

Results

Patients

A total of 10 patients were included and randomized to either bosentan or placebo. Baseline characteristics were comparable, except for the lower mean age and higher prevalence of prior uveitis in the placebo group (**Table 1**). Patients in the bosentan group were slightly older with a mean age of 50 years (range 39–63), placebo 41 years (range 30–46). Mean disease activity was higher in the placebo group 6 (range 5–7) compared to the bosentan group 4.25 (range 3–5).

All patients expect one in the placebo group who was treated with interferon were treated with colchicine. In the bosentan group, one patient received thalidomide; in both groups, one patient was treated with hydroxychloroquine. One patient in both groups used NSAIDs.

Eight of the ten patients completed the study period. One patient in the placebo group terminated the study due to the development of a vitritis of his left eye, for which treatment with prednisolone was started. The other patient in the

Table 1. Patient details

	Bosentan	Placebo
Patients	5	5
% females	20%	40%
Age (mean, years)	50	41
Oral ulceration	100%	100%
Genital ulceration	100%	80%
Skin lesions	100%	100%
Positive pathology	60%	40%
Prior uveitis	20%	80%
HLA-B51 positive	20%	40%
Disease duration (mean, years)	8	9
Disease activity, BDCAF (mean, range)	4.25 (3-5)	6 (5-7)

bosentan group discontinued the trial because of depressive complaints that were not related to therapy and did not resolve after stopping the study medication.

Effect of bosentan on disease activity

After treatment with bosentan, no decrease in disease activity in the bosentan group was found (**Figure 1A**). In comparison with the placebo group, no beneficial effect of bosentan in the bosentan group was found. However, one patient in the bosentan group (patient 7) demonstrated a minor clinical response. **Table 2** shows detailed BDCAF score of all patients before and after treatment (week 24).

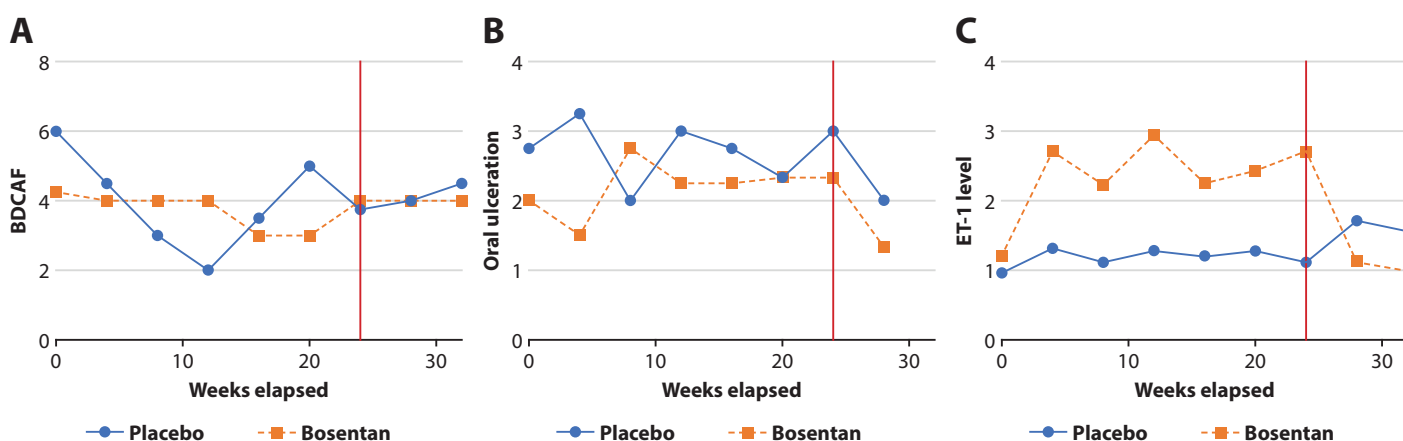


Figure 1. Disease activity and ET-1 levels before (BT) and at the end (AT) of the study period

A. Median disease activity as measured with BDCAF

Dotted line represents the placebo group; the solid line represents the bosentan group. The red line represents the last use of medication, week 24.

B. Median disease activity as measured by oral ulcerations. Oral ulcerations were counted as 1 point for every week there was at least one present (range 0-4). Dotted line represents the placebo group; the solid line represents the bosentan group. The red line represents the last use of medication, week 24.

C. Median levels of ET-1 (pg/ml).

Dotted line represents the placebo group; the solid line represents the bosentan group. The red line represents the last use of medication, week 24.

Table 2. BDCAF before and after treatment

	Headache	Oral ulceration	Genital ulceration	Erythema nodosum	Pustules	Arthralgia	Arthritis	N/V/AP	Diarrhoea	Toal BT	Total AT
Placebo											
Patient 1 BT	1	1	0	1	1	1	1	0	0	6	
Patient 1 AT	0	1	0	0	0	0	0	0	0		1
Patient 2 BT	1	1	1	0	1	1	0	1	0	6	
Patient 2 AT	0	1	1	0	1	1	0	0	0		4
Patient 3 BT	1	1	0	0	1	1	0	1	0	5	
Patient 3 AT	1	1	0	0	1	1	0	1	0		5
Patient 4 BT	1	1	1	1	1	0	1	1	0	7	
Patient 4 AT	1	1	1	0	1	0	0	1	0		5
									Mean	6	3.75
Bosentan											
Patient 5 BT	1	1	0	0	1	1	1	0	0	5	
Patient 5 AT	1	1	0	0	1	1	0	0	0		4
Patient 6 BT	1	1	0	0	0	1	1	1	0	5	
Patient 6 AT	1	1	1	0	0	1	0	1	0		5
Patient 7 BT	1	1	0	0	1	1	0	0	0	4	
Patient 7 AT	1	1	1	0	0	0	0	0	0		3
Patient 8 BT	1	1	0	0	1	1	0	0	0	3	
Patient 8 AT	1	1	0	0	1	1	0	0	0		4
									Mean	4.25	4

BDCAF scores of all patients, upper half displays the placebo group, lower half displays the bosentan group. BT - before treatment, AT - after treatment, N - nausea, V - vomiting, AP - abdominal pain. On the right, total scores are displayed, together with mean score before and after the treatment period.

Treatment with bosentan was not effective on the presence of oral ulcerations, as shown in **Figure 1B**. In addition, concomitant immunosuppressive medication could not be tapered off during the study period in any patient.

Safety of bosentan in BD patients

Bosentan was well tolerated. Liver enzymes in both groups remained stable and within normal limits. The only SAE reported, in both the placebo and bosentan groups, included hospitalization of a patient in the bosentan group four months after enrolment. This patient was suspected of a sinusitis which required treatment with antibiotics i.v., with rapid recovery. Upon resolution of the symptoms, the patient restarted bosentan treatment. In the follow-up of his sinusitis, p-ANCA was detected and the patient was regarded as having ANCA-associated vasculitis with orbital involvement urging to start systemic steroids. Both ANCAs and the initiation of steroids occurred 1 month after the follow-up period of this study. ANCAs had not been tested before in this patient.

Effects of bosentan on CECs, sIL2R, cytokine profiles or immune cell populations

CECs were measured at 0, 12, 24, 28 and 32 weeks, without showing significant differences or trends (**Figure 2A**). The same applies to sIL2R, in which no correlation with disease activity was found (**Figure 2B**).

Cytokine profiles during our study were determined to differentiate between a Th1 or Th2 immune reaction and to gain more insight into inflammation markers. No skewing towards either a Th1 or Th2 response was seen in either group. In addition, no correlation between disease activity or relation to treatment was found after analyzing the pro-inflammatory cytokines (data not shown).

The different immune cell populations (regulatory T cells, B-, CD4+-, CD8+ and NK cells) were comparable in all patients and did not change during the treatment period (data not shown).

Endothelin-1 rises during treatment with bosentan and falls after discontinuing treatment

ET-1 levels at the start of this study were comparable between the two patient groups and were within normal limits.

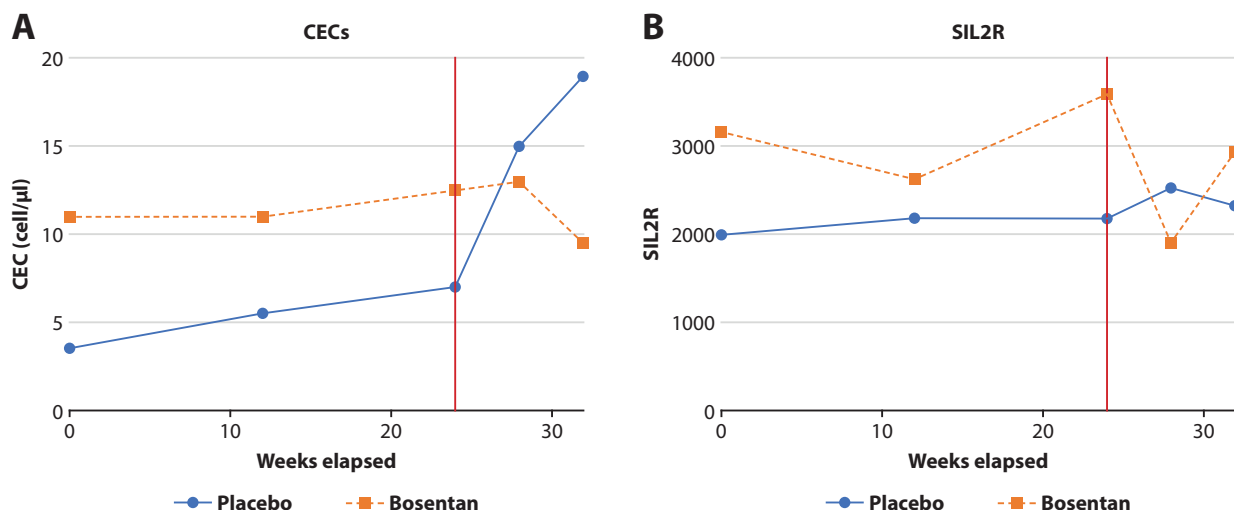


Figure 2. SILR2 and CEC levels during study period

A. Median levels of CECs (cell/µl).

Dotted line represents the placebo group; the solid line represents the bosentan group. The red line represents the last use of medication, week 24.

B. Median levels of SIL2R (pg/ml).

Dotted line represents the placebo group; the solid line represents the bosentan group. The red line represents the last use of medication, week 24.

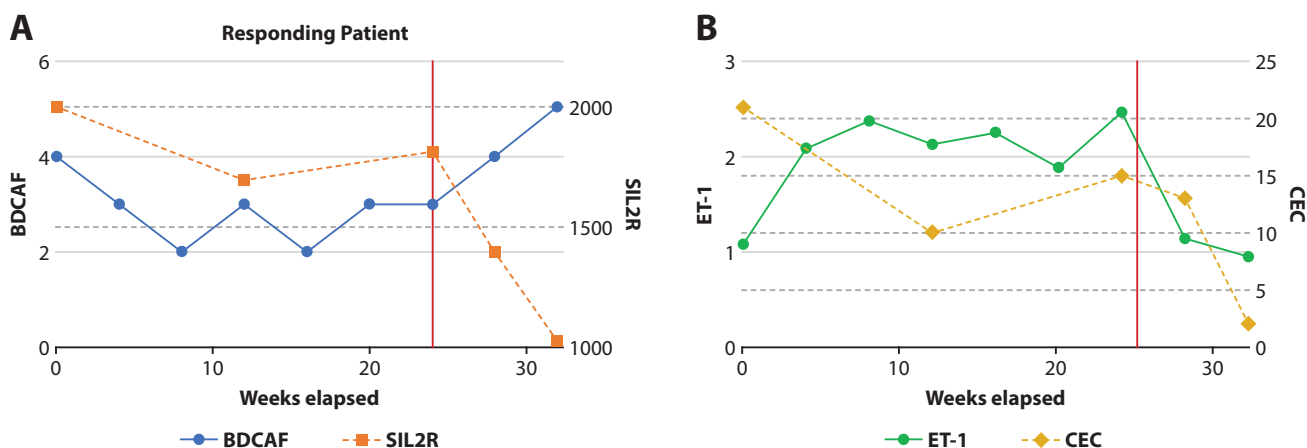


Figure 3. Successful treatment with bosentan

Reduced disease activity (BDCAF) during successful treatment with bosentan with CECs (cells/µl), SIL2R and ET-1 levels (both pg/ml) in one patient.

The red line represents the last use of medication, week 24.

Interestingly, a different profile of ET-1 was shown in the bosentan group compared to the placebo group (Figure 1C). ET-1 levels rose immediately after starting the treatment and levels fell again after the treatment period was over. In the placebo group, ET-1 levels remained stable during the course of the study. There was no correlation between disease activity and ET-1 levels.

Extended treatment in one patient

One patient (#7, a 63 years-old male) had a clinical response to treatment with bosentan; therefore, bosentan was continued after the study period, and showed a decrease in BDCAF from 4 to 3 during treatment with bosentan. After discontinuing bosentan, BDCAF increased to 5. Therefore, in January

2011, bosentan was restarted at a dose of 125 mg twice daily with a similar therapeutic efficacy.

This patient experienced positive effects of bosentan for a further 12 months with BDCAF decreasing to 3. Hereafter, immunosuppressive treatment was intensified with adalimumab because of a flare (BDCAF 6).

Because of headaches, which were possibly caused by the combination of adalimumab and bosentan, the latter was stopped in March 2013, with no significant effect.

As shown in Figure 3, successful treatment in this patient was reflected by a decreasing trend in CECs and SIL2R levels. Cytokine levels, percentage of FoxP3-positive cells, ET-1 levels and cell populations showed similar patterns to the other bosentan-treated patients.

Discussion

In this prospective double-blind placebo controlled pilot study we could not demonstrate the therapeutic efficacy of bosentan on disease activity in a small cohort of 10 patients with Behçet's disease. However, one patient clinically responded to bosentan.

Bosentan appears to be safe in patients with BD. However, the lack of a significant clinical effect of bosentan in the present study might restrict its clinical use in BD. One of the reasons for the apparent lack of response might be the small numbers of patients included. A substantial increase in numbers is required to potentially demonstrate a significant clinical effect. A sample size with a power of 80% and an alpha of 0.05 requires two cohorts of at least 46 patients to find a significant result of treatment with bosentan taking into account that 20% of the patients might respond as suggested in the present study. Although small, both groups are heterogeneous in age and gender and representative for typical BD patients that usually demonstrate a variable disease activity including typical "spontaneous" remission and relapse patterns.¹

Within the bosentan group, one patient demonstrated a sustainable decrease of disease activity. Therefore, the treatment period was extended, but bosentan failed to suppress disease activity after one-year, and adalimumab was added, which remained effective for two years. Apparently, the BD in this patient constitutes a severe therapy refractory sub-type, signifying the positive clinical therapeutic activity of bosentan in this case. In particular, the rise and fall in disease activity after stopping and restarting bosentan is illustrative for a potential beneficial clinical effect. Although blocking the pro-inflammatory effects of ET-1 could theoretically be beneficial in the treatment of vasculitis, no cases have been described in literature to date. Case reports describe a beneficial effect in digital ulcers caused by vasculitis (SLE and PAN), although it remains unclear whether the improvement is caused by the anti-inflammatory or vasolidating effect of bosentan.^{16,17}

ET-1 is a protein that causes vasoconstriction by smooth muscle contraction, thereby contributing to endothelial dysfunction. ET-1 levels are increased in BD and have been reported to be correlated with disease activity in some studies.^{18,19,20} In the present study, we could not show such elevated baseline levels of ET-1 or correlation with disease activity. Interestingly, we did show an increase in ET-1 levels during treatment with bosentan. Both a decrease and increase of ET-1 levels during bosentan treatment have been reported, with and without a correlation with the efficacy of treatment; our study adds to these observations.²¹⁻²³

CECs are associated with the degree of endothelial damage, are elevated in vascular disorders and are associated with disease activity in BD.^{24,25} Interestingly, in the responsive patient, CECs (and SIL2R) decreased according to the clinical response. This association of decreased CECs and the therapeutic efficacy of bosentan might indicate a clinical response to bosentan in BD.

IL-2 induces T cell proliferation and levels of its soluble receptor have been found to be correlated with T-cell mediated diseases and are considered a marker for T cell activation.²⁶ In Behçet's disease, no data for SIL2R and disease activity are available. In our study, we saw no correlation between SIL2R

levels and disease activity, except in the responsive patient. Studies on the value of SIL-2R as a disease activity marker in our BD cohort are ongoing.

Unfortunately, two patients dropped out during the study period. Indicatively for the heterogeneous and fluctuating presentation of BD, one patient in the placebo group developed vitritis. This was sustainably and effectively treated with steroids and infliximab. In the bosentan group, one patient was excluded because of compliance difficulties caused by depressive complaints. To the best of our knowledge, no relation between depression and bosentan is known. Interestingly, one study reveals a possible antidepressant-like effect of bosentan in mice, caused by an increase in IL-6.²⁷ In our patient however, IL-6 levels remained stable. Remarkably, one patient in the bosentan group developed an ANCA associated vasculitis (AAV). We cannot rule out an association with bosentan, however, it has not been described before and there is no reasonable mechanistic relation between ET-1 and ANCA. Although BD in combination with AAV is rare,²⁸ we do not believe our patient has been misdiagnosed with BD, because of a long history of recurrent oral aphthosis, genital ulcerations, pustular skin lesions and one episode of uveitis.

In conclusion, bosentan appears to be safe in BD patients. Although our cohort is too small to demonstrate an effect on disease activity, one patient out of five had a response showing decreased disease activation markers. Our observations should be confirmed and extended in a larger patient cohort to have a significant impact on the treatment options for BD.

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Disclosures and conflicts of interest

Actelion has partly financed this study and provided bosentan and placebo.

The authors declare no other conflicts of interest regarding the publication of this paper.

Key messages

- Bosentan appears to be safe in Behçet's disease patients.
- We show one patient who clinically responded to bosentan treatment, which should be confirmed and extended in a larger patient cohort.

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