Effect of Bosentan on Plasma Markers of Endothelial Cell Activity in Patients with Secondary Pulmonary Hypertension Related to Connective Tissue Diseases

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ABSTRACT. ***Objective.*** To evaluate plasma markers of endothelial cell activity in patients with pulmonary arterial hypertension (PAH) induced by connective tissue diseases (CTD) before and after 3-month administration of bosentan.

Methods. We quantified E, L and P-selectin (sE-S, sL-S, sP-S), thrombomodulin (TM), monocyte-chemotactic protein 1 (MCP-1), human soluble CD40 ligand (sCD40L), and nitric oxide (NO) in 18 patients and 18 controls. We evaluated right ventricular systolic pressure (RVSP) and the 6-minute walk test (6-MWT).

Results. All plasma markers but sL-S and TM at Time 0 were significantly higher in patients compared with controls. After 3 months of therapy, decreased levels were noted in NO (Time 0 24.05 ± 6.01 mmol/l, Time 1 13.92 ± 3.40 mmol/l; $p < 0.001$) and sCD40L (Time 0 1685.33 ± 866 pg/ml, Time 1 1055.11 ± 630.6 pg/ml; $p = 0.017$). In contrast, sP-S was significantly increased (Time 0 88.36 ± 47.76 ng/ml, Time 1 147.21 ± 94.43 ng/ml; $p = 0.021$). All patients remained stable in WHO class III, and in 9 patients we noted an improvement in 6-MWT. A correlation was found between Δ of RVSP and 6-MWT ($r^2 = 0.5355$, $p < 0.001$) as well as between Δ-sP-S and both Δ-6-MWT and Δ-RVSP. An increase sP-S level was found in 89% of nonresponder patients, whereas 55% of responders showed a stable or reduced sP-S level ($p = 0.016$ responder vs nonresponder).

Conclusion. Treatment with bosentan for 3 months induced a beneficial effect by restoring endothelial function through a decrease in the markers of endothelial cell activity, leading to stabilization or improvement of severe PAH. (J Rheumatol First Release Jan 15 2009; doi:10.3899/jrheum.080542)

Key Indexing Terms:
PULMONARY HYPERTENSION BOSENTAN SYSTEMIC SCLEROSIS NITRIC OXIDE

Pulmonary arterial hypertension (PAH) is a serious complication of connective tissue diseases (CTD) and a leading cause of death in such patients[1,2]. Under normal circumstances endothelium tends to favor vasodilation, inhibition of thrombosis, and leukocyte adhesion[3]. The endothelial cell can elaborate a variety of substances such as prostacyclin, nitric oxide (NO), endothelins, thrombomodulin (TM), selectins, and heparan sulfate, all regulating pulmonary blood flow and vascular resistance[4-6].

Altered endothelial cell and hemostatic markers like endothelin, CD40, selectins, von Willebrand factor, thrombin-antithrombin complex, fragments 1+2, ß-thromboglobulin, and platelet aggregates are present in patients with CTD and in patients with PAH[7-13].

Although currently there is no cure for PAH, many classes of drugs became available during the past decade improving survival and the relief of symptoms[14]. Among these drugs, bosentan, a nonpeptide orally active agent that blocks the actions of the endothelin receptors A and B (ET_A and ET_B), is very promising[15]. Endothelin 1 (ET-1) induces a variety of different effects through its receptors[16]. It binds ET_A and ET_B receptors on vascular smooth cells, inducing a potent vasoconstriction and mitogenic activity. In contrast, when ET-1 binds ET_B receptor on endothelial cells, it induces the production of NO and prostacyclin, and clearance of ET-1, under normal physiological conditions.

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Moreover, it stimulates the production of cytokines, growth factor, collagen, and aldosterone, thus inducing proinflammatory actions, and can induce cardiac hypertrophy[15,16].

Our aim was to evaluate and to correlate levels of plasma markers of endothelial cell activity with cardiopulmonary tests in patients with PAH induced by CTD before and after 3-month administration of bosentan.

MATERIALS AND METHODS

Study population. Subjects consisted of 18 patients with PAH associated with CTD (13 women, 5 men) followed in the rheumatology and cardiology units of the University of Padova. They were included in a prospective, noncontrolled study. Patient characteristics are summarized in Table 1 and concomitant treatments in Table 2.

The diagnosis was systemic sclerosis (SSc) in 13 cases, systemic lupus erythematosus (SLE) in 2, mixed CTD in 1, undifferentiated CTD in 1, and overlap syndrome (scleroderma and myositis) in 1.

At the start of therapy, the mean age of patients was 53.8 ± 13.1 years (range 34–75) and the mean duration of SSc was 11.8 ± 10.4 years (range 2–36). Patients fulfilled the American College of Rheumatology criteria for their disease[17]. Before entry to the study, 10 patients had received treatment with intravenous pulse iloprost, with minimal or no efficacy. At the beginning of the treatment all patients were in World Health Organization class III[18]. Patients with severe lung fibrosis were excluded from this study.

Bosentan (Tracleer; Actelion, Allscwil, Switzerland) was administered orally at a dose of 62.5 mg/bid during the first month and 125 mg/bid during the next 2 months. In a patient undergoing regular hemodialysis treatment due to a previous scleroderma-associated renal crisis, the dose remained 62.5 mg/bid over the entire year. Safety was assessed by monthly leukocyte and platelet counts as well as hemoglobin and aminotransferases levels in peripheral blood.

Cardiological assessment. Evaluation was performed at baseline and after 3 months of therapy. All patients underwent a transthoracic echocardiogram[19-22] and a 6-minute walk test (6-MWT)[23,24] during each assessment.

Although the diagnosis of PAH relies on invasive measurements of hemodynamics and echocardiogram alone, it is not sufficient to rule out postcapillary pulmonary hypertension; we used the value of right ventricular systolic pressure (RVSP) as ≥ 45 mm Hg by echocardiography as minimal diagnostic value since in patients with CTD the predictive accuracy of echocardiography compared to cardiac catheterization is 97% for values ≥ 45 mm Hg[19].

The determination of pulmonary artery systolic pressure (PASP) was performed by cardiac Doppler ultrasound examination, assuming 30 mm Hg of mean pulmonary artery pressure (mPAP) and 45 mm Hg of RVSP as minimal diagnostic values. Standard 2-dimensional, M-mode, and Doppler ultrasound examination was performed using a Sonos HP 5500 or Acuson Sequoia instrument (Siemens, Berlin, Germany), including parasternal long- and short-axis, 2-, 3-, 4-chamber and subcostal views, and Doppler recordings of valve flow velocities. RVSP was estimated by adding right atrial pressure to the transtricuspid gradient derived from maximal tricuspid regurgitant jet velocity in the continuous-wave Doppler flow profile obtained from an apical 4-chamber view. Right atrial pressure was assumed to be 5 mm Hg if tricuspid regurgitation was mild, 8–10 mm Hg if it was moderate with inferior vena cava inspiratory collapse > 50%, 12–15 mm Hg if it was severe with inferior vena cava collapse < 50%. RVSP was considered equal to PASP in the absence of pulmonary stenosis or right ventricle outflow obstruction. The mPAP was calculated by the right ventricle outflow tract (RVOT) acceleration time measured in the pulse-wave Doppler flow of RVOT visualized on parasternal short-axis view. The acceleration time was normalized by heart rate and mPAP estimated by Mahan's equation. For each variable, 3 representative beats were analyzed and the mean results calculated.

The 6-MWT was performed along a 45-meter corridor. Patients were requested to walk as far as possible for 6 minutes and the distance was measured. Baseline and peak exercise blood pressure, heart rate, and oxygen saturation by pulse oximetry were recorded.

The study was conducted according to the provisions of the Helsinki Declaration of 1975, as revised in 1983, and in adherence to local guidelines for good medical practice. The local ethics review committees approved the protocol, and written informed consent was obtained from all patients.

Plasma markers of endothelial cell activity. Venous blood samples were drawn, using a double-syringe butterfly technique, into 3.8% sodium citrate (9:1, v/v) at baseline. Blood samples were centrifuged at 3000 g for 20 min

Table 1. Features of 18 patients with severe pulmonary artery hypertension (PAH) related to connective tissue diseases.

Patient	Sex	Age, yrs	Smoker	WHO PAH Functional Class	Connective Tissue Diseases	Disease Duration, yrs	ANA Specificity
1	M	62	Ex smoker	III	SSc	13	ACA
2	M	41	No	III	SLE	7	Anti-nDNA
3	F	73	No	III	SSc	36	ACA
4	F	43	No	III	MCTD	18	Anti-nRNP
5	M	53	Ex smoker	III	SSc	11	ACA
6	F	60	No	III	SSc	13	ACA
7	F	61	7–8 cigarettes	III	UCTD	9	NA
8	F	52	No	III	SSc	10	Anti-topo I
9	F	55	No	III	SSc	26	Anti-topo I
10	F	32	No	III	SLE	13	Anti-nDNA
11	M	64	No	III	SSc	12	ACA
12	F	33	No	III	SM	11	Aspecific ANA
13	F	52	No	III	SSc	2	ACA
14	F	41	No	III	SSc	23	Anti-topo I
15	F	67	No	III	SSc	4	ACA
16	F	59	No	III	SSc	4	Anti-topo I
17	F	51	No	III	SSc	14	ACA
18	M	31	40 cigarettes/day	III	SSc	4	ACA

SSc: systemic sclerosis; SLE: systemic lupus erythematosus; MCTD: mixed connective tissue disease; UCTD: undifferentiated connective tissue disease; SM: overlap syndrome; ACA: anticentromere; Anti-topo I: anti-topoisomerase I.

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Table 2. Therapy of 18 patients with severe pulmonary artery hypertension related to connective tissue diseases.

Patient	Drugs								
	Calcium Blockers	Anticoagulants	Diuretics	ACE Inhibitors	Digoxin	Steroids	Antirheumatics and/or Immunosuppressors	E	PEX
1		•				•			
2		•	•	•	•	•			
3	•					•			
4	•		•			•	•		
5	•			•			•	•	•
6	•	•	•			•	•		
7	•					•			
8	•					•	•		
9	•								
10		•`	•	•		•	•		
11	•			•		•		•	
12	•					•	•		
13	•					•	•		
14	•						•		
15	•		•					•	
16	•								•
17	•	•	•	•		•			
18	•	•				•			

ACE: angiotensin-converting enzyme; E: epoprostenol; PEX: plasma exchange.

at 4°C. The plasma was divided in aliquots and kept frozen at –70°C until analysis.

Soluble adhesion molecules of the selectin class (sE-S, sP-S, and sL-S) with concentrations expressed as ng/ml and MCP-1/CCL2 and sCD40L expressed as pg/ml were determined using a commercial quantitative colorimetric sandwich ELISA (R&D Systems, Minneapolis, MN, USA). Nitrate + nitrite (NO_{-2}/NO_{-3}) in nmol/ml as a measure of NO metabolism was determined with a quantitative colorimetric assay (Cayman Chemical, Ann Arbor, MI, USA). Thrombomodulin antigen (ng/ml) was measured by commercial ELISA (Diagnostica Stago, Asnières sur Seine, France). All determinations were carefully performed according to the manufacturer's protocol.

Blood was drawn to evaluate the same biochemical plasma markers of endothelial cell dysfunction in a group of 18 healthy subjects matched for sex and age with our patients.

Statistical analysis. Results were expressed as mean ± standard deviation (SD). Analysis of the differences was by Student t test. The statistical significance level was set at $p < 0.05$. All probabilities reported were 2-tailed. Correlations were determined using Pearson linear correlation and the coefficient of determination r^2. Data analysis was carried out with Prism 4 for Macintosh, Version 4.0 (GraphPad Software).

RESULTS

Figure 1 summarizes the measurements of endothelial markers. No difference was found in the level of TM in controls compared to patients and between TM levels before and after treatment. NO levels were significantly increased in patients as compared with controls before treatment (controls 13.57 ± 2.8 nmol/ml, patients 24.05 ± 6.04 nmol/ml; $p < 0.001$). After treatment, NO levels had decreased significantly (Time 1 13.92 ± 3.40 nmol/ml; $p < 0.001$ compared to Time 0).

Soluble CD40 ligand (sCD40L) that was significantly higher in patients (1685.33 ± 866 pg/ml) compared to controls (245.74 ± 111.8 pg/ml; $p < 0.001$) showed a significant reduction after bosentan therapy (1055.11 ± 630.6 pg/ml; $p = 0.017$ compared to Time 0). MCP-1/CCL2 was also statistically significantly higher in patients (243.16 ± 140.2 pg/ml) compared to controls (141.5 ± 31.2 pg/ml; $p = 0.007$), but there was no significant difference after treatment.

sE-S and sP-S levels were significantly higher in patients (sE-S 112.51 ± 47.8 ng/ml; sP-S 88.32 ± 42.8 ng/ml) compared to controls (sE-S 68.98 ± 20.9 ng/ml, $p = 0.0019$; sP-S 45.18 ± 9.6 ng/ml, $p < 0.001$) before treatment. The level of sE-S remained unchanged after 3-month treatment, whereas sP-S increased significantly ($p = 0.021$ compared to Time 0).

sL-S level was significantly decreased in patients (1473 ± 688 ng/ml) compared with controls (1959 ± 470 ng/ml; $p = 0.038$) and it was not significantly changed after bosentan treatment.

At baseline, 15 patients had a severe RVSP (50–120 mm Hg), whereas 3 patients had a mild RVSP of 45 mm Hg. All patients remained stable in WHO functional class III during the 3-month followup. Bosentan in 14 patients induced a slight reduction in mean RVSP, and among them, 7 had reduction between 10 and 20 mm Hg.

Nine patients showed an improvement in the 6-MWT between 12 and 112 meters. In these responder patients, the RVSP was significantly reduced after bosentan treatment (–16.7% ± 5.13%) compared to patients who showed no significant improvement at the 6-MWT (–2.6% ± 3.3 %) ($p = 0.035$; data not shown).

Patients who received bosentan did not experience significant side effects.

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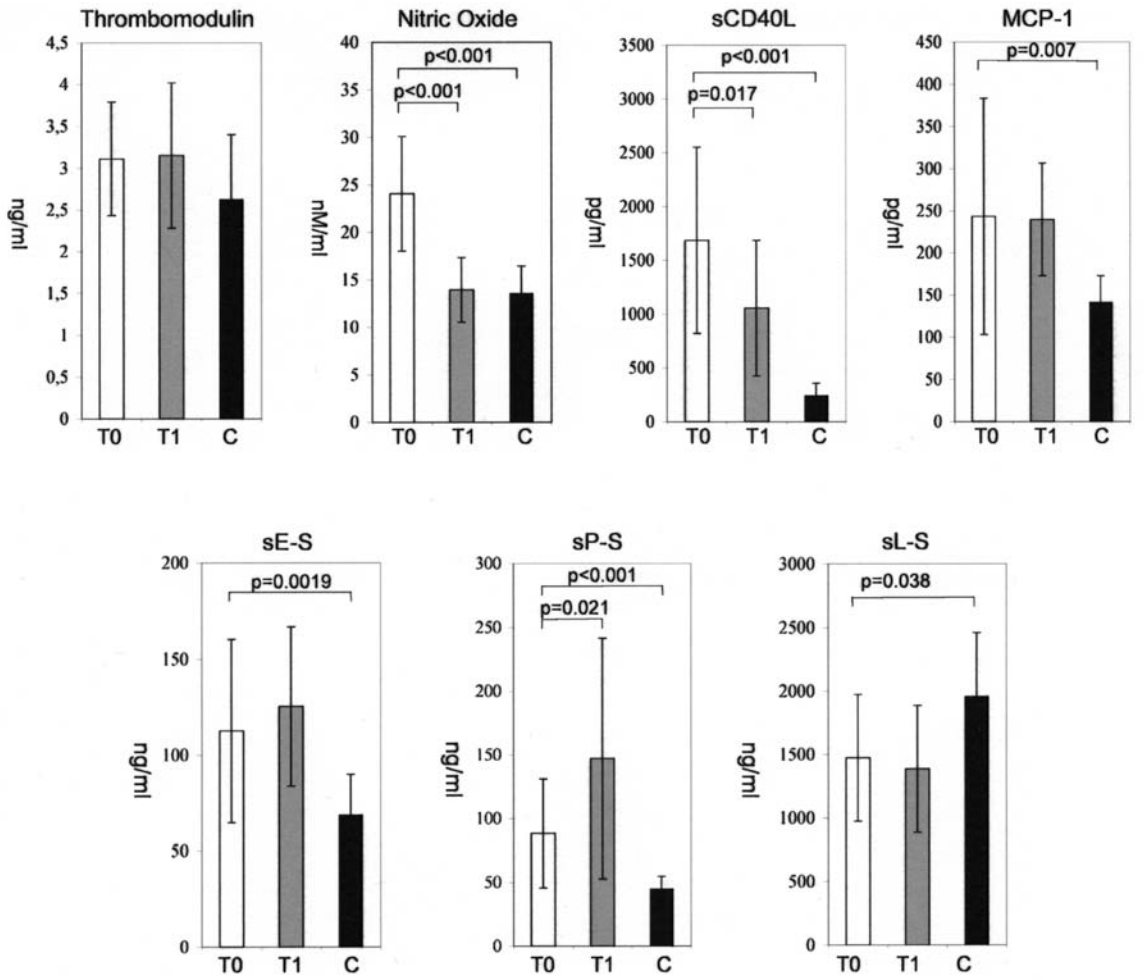

Figure 1. Plasma markers of endothelial cell activity before (Time 0: T0) and after (T1) 3 months of treatment with bosentan in patients with connective tissue diseases and in controls (C). MCP-1: monocyte-chemotactic protein 1.

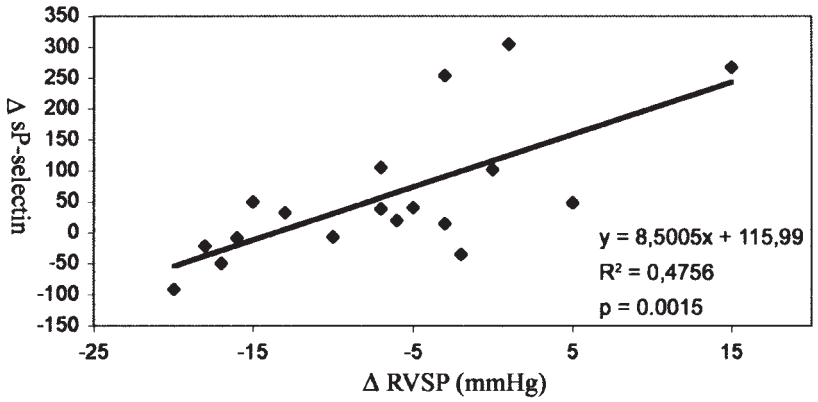

Figure 2. Correlation between Δ-sP-selectin and Δ-RVSP.

Correlations. We found a weak positive correlation between the levels of sP-S and the RVSP before and after bosentan (Δ-sP-S and Δ-RVSP; $r^2 = 0.4756$, $p = 0.0015$; Figure 2) and a weak negative correlation between the Δ-sP-S and the Δ-6-MWT ($r^2 = 0.3054$, $p = 0.017$; Figure 3). In contrast, the Δ-RVSP and Δ-6-MWT showed a good significant negative correlation ($r^2 = 0.5355$, $p = 0.0006$; Figure 4).

We then analyzed in more detail how sP-S correlated to

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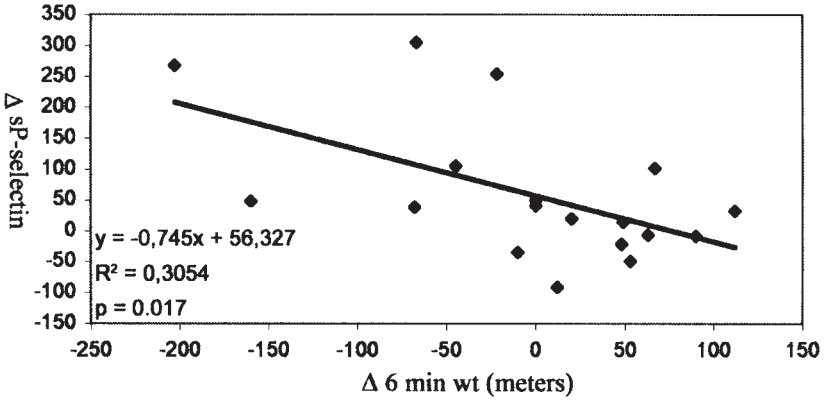

Figure 3. Correlation between Δ-sP-selectin and the distance walked on Δ-6-minute walk test.

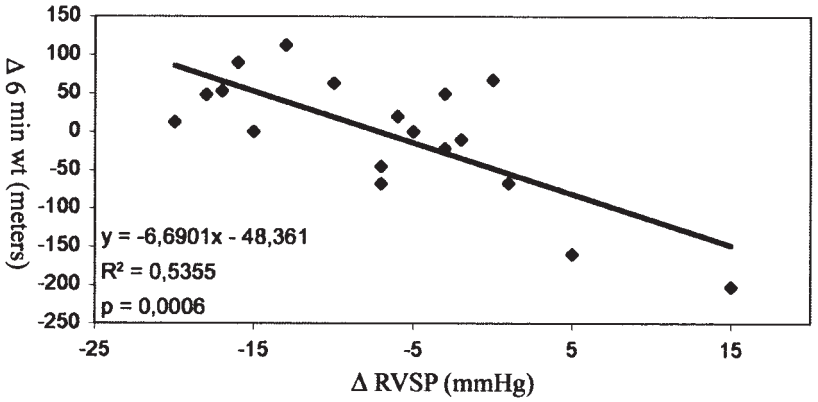

Figure 4. Correlation between Δ-RVSP and the distance walked on Δ-6-minute walk test.

response to bosentan treatment. As shown in Figure 5, 89% of patients who showed no improvement at the 6-MWT experienced an increase in sP-S levels. In contrast, the level of sP-S in responders was unchanged or reduced in 55% of cases (p = 0.016, responder vs nonresponder).

DISCUSSION

PAH, either idiopathic or related to CTD, is a severe clinical condition with a sustained elevation of pulmonary arterial pressure to > 25 mm Hg at rest or > 30 mm Hg with exercise[25]. ET-1 signaling has been found to play a role in the pathogenesis of PAH, and to be increased in CTD[14,25,26]. There is conflicting evidence regarding the effect of ET-1 on platelets, probably due to complex interactions between platelets and the 2 receptors ET_A and ET_B[27]. Recently, the orally active nonpeptide selective antagonist bosentan has been shown to significantly improve symptoms and increase survival of patients with PAH[15].

We evaluated endothelial cell dysfunction by measuring some plasma markers of endothelial activity in patients with PAH secondary to CTD, before and after 3 months of bosentan therapy. In our patient group we found significantly increased levels of sE-S and sP-S and reduction of sE-S compared with controls. Together with other adhesion molecules, selectins play a key role in the pathogenesis of inflammation, immunologic responses, thrombosis, and infection in the vasculature[28,29]. They are upregulated in a variety of disease states, and in some instances are released into the circulation, where they can be detected in soluble form. sP-S, stored in the α-granules of the platelets and in the Weibel-Palade bodies of endothelial cells, has been shown to be elevated in thrombogenic states such as myocardial infarction, deep venous thrombosis, thrombotic thrombocytopenic purpura, and heparin-induced thrombocytopenia[28,29]. sE-S is involved in the adherence of leukocytes to the endothelium that has been postulated to play an important role in atherosclerosis[28,29]. It is overexpressed in vascular endothelial cells by injury, ischemia, cytokines, tissue factor, and thrombin. The increased levels of sP-S and sE-S we observed could reflect endothelial dysfunction/inflammation or damage as well as platelet activation, as reported in both CTD and pulmonary hypertension.

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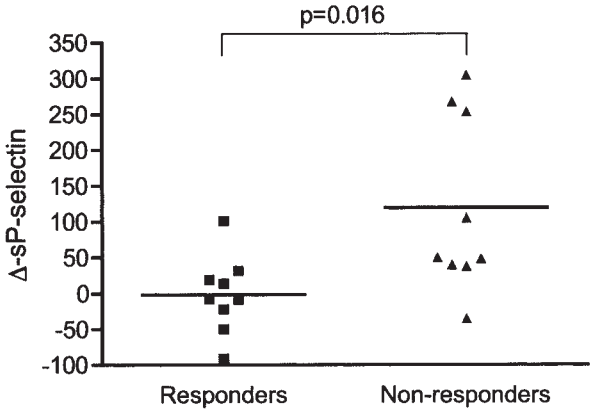

Figure 5. Δ-sP-selectin in patients according to response to Δ-6-minute walk test.

Bosentan did not significantly affect the level of sE-S, but interestingly, it differentially modulated sP-S levels. Indeed, sP-S was unchanged or slightly reduced in 55% of patients who showed an improvement on the 6-MWT, whereas sP-S levels rose significantly in almost 90% of nonresponders. This result may suggest that increasing levels of sP-S are predictive of treatment failure, whereas a beneficial functional effect of bosentan may be expected when the treatment is associated with a reduction in sP-S levels. How can bosentan-mediated sP-S changes correlate with response to treatment? P-selectin plays an important role in mediating interactions among neutrophils, platelets, and the vascular endothelium. This interaction is in part modulated by ET-1, which, through inhibition of P-selectin upregulation, prevents interactions between leukocytes and endothelium, particularly in the presence of vasoconstriction and inflammation[30]. As P-selectin levels in patients with SSc may also be related to extensive systemic inflammation[31,32], it is possible that the antagonist effect of bosentan on ET-1 receptor disrupts the compensatory mechanism, eventually leading to sP-S upregulation. This complex interaction may eventually translate into treatment failure. Recently, Iannone, *et al* found that response to 12-month bosentan therapy in patients with SSc and PAH was associated with dowregulation of sP-S as well as other serum endothelial markers[33]. Our results are in part consistent with this study, but they add further insight into the role of sP-S, as increasing plasma levels during bosentan therapy may identify patients who will fail to show any functional improvement.

sL-S was reduced in patients compared to controls, and no changes were seen after bosentan treatment. This was not unexpected, as it has been shown that sL-S is decreased in CTD[29], and most of the patients were treated with steroids, which are known to reduce the expression of L-selectin on granulocytes and lymphocyte surfaces, decreasing the level of the soluble form[34,35].

In patients with PAH of various origins, an exaggerated short-term pulmonary vasodilator response to L-arginine suggests a relative impairment of pulmonary vascular endothelial NO production that may contribute to increased pulmonary vascular tone, and thus be important in the pathophysiology of the disorder[36]. However, in our patients we found increased levels of nitrate + nitrite (NO_{-2}/NO_{-3}) as a measure of NO before treatment. Several groups have shown increased circulating NO plasma levels in CTD and specifically in SSc[36,37], and our results are consistent with these findings.

Although the protective role of NO is well known, since impaired basal NO production may contribute to development of vascular disease in CTD, the free radicals produced by inflammatory cells may react with the increased production of NO to form peroxynitrate, a powerful oxidant that mediates cellular and tissue injury, leading to endothelial apoptosis[38].

After treatment with bosentan, the levels of NO were significantly reduced and were similar to those of healthy controls. It is possible that by blocking ET_B on endothelial cells, bosentan, similarly to prostacyclin, curtails the release of NO[39]. This is also consistent with previous reports that ET-1 mediates the release of NO from endothelial cells[30]. Since NO and prostacyclin are powerful inhibitors of platelet activation, their reduction could be responsible for the increased circulating sP-S observed in some of our patients. Sfikakis, *et al* reported that bosentan improves endothelial dysfunction in patients with SSc as measured by the flow-mediated dilation (FMD) of brachial artery[40]. As FMD correlates with NO release, they suggest that the bosentan-mediated improvement in endothelial function is due to increased NO bioavailability. Our results are more consistent with a compensatory role of NO in the presence of vascular disease in CTD. Bosentan seems to stabilize endothelial dysfunction and to restore a normal NO endothelial release. Moreover, it must be taken into consideration that NO release from endothelium may differ significantly based on the extent of multi-organ endothelial involvement in patients with CTD.

CD40L is a transmembrane protein structurally related to the cytokine tumor necrosis factor-α. It can be cleaved from the cell surface, releasing a soluble form that is biologically active. It plays an important role in the induction of humoral and cellular immune responses, and it is a marker and mediator of inflammatory diseases such as atherosclerosis[41]. Komura, *et al* reported elevated circulating sCD40L concentration[9], and more recently similar results were obtained by Allanore, *et al* in patients with SSc associated with PAH and digital ulcers[42]. Our findings are in keeping with these authors' results. After bosentan therapy this inflammatory biomarker was significantly reduced, although it did not reach the levels seen in the controls. Since vasculopathy resulting from endothelial cell injury, recruitment of activated lymphocytes, and fibrinolytic and coagulation pathway abnormalities are well recognized features of CTD[7], the positive effect of bosentan on high levels of both NO and

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sCD40L could be important in reducing inflammation and cell injury in this condition.

It has been shown that CD40L on platelets can induce endothelial cells to express adhesion molecules[43]. In particular, sCD40L is positively correlated with circulating adhesion molecules and MCP-1 *in vivo*, and may influence the expression of soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, sE-selectin, and MCP-1 in endothelial cells *in vitro*[44]. Although we found increased levels of adhesion molecules in patients with CTD, the same markers showed no significant correlation with sCD40L. This is not completely unexpected, and is consistent with a recent report of improvement of vascular endothelial function in SSc patients after bosentan treatment, with no significant changes of adhesion molecule levels[40].

MCP-1 is a chemokine that activates mononuclear phagocytes, inducing migration to the sites of inflammation[41], and may play an important role in the development of pulmonary fibrosis in CTD[45]. As expected, we found elevated levels of it in our patients as compared to controls. However, bosentan treatment, in contrast with sCD40L, did not induce any significant variation.

High levels of circulating TM, a glycoprotein expressed on the surface of endothelial cells acting as a cell receptor for thrombin, have been reported in SSc and in other CTD. Circulating TM levels were not different in our patients compared to controls, and this may suggest a lack of reliability of this glycoprotein as a marker of endothelial injury, as recently suggested by Matucci Cerinic, *et al*[38].

Joglekar, *et al* found that bosentan was clinically beneficial in patients with PAH secondary to scleroderma, including patients with restrictive lung disease, since there was an improvement in the WHO class at 3, 6, and 9 months[46]. However, pulmonary hemodynamics and pulmonary function tests remained stable during treatment. Joglekar, *et al* suggested that during bosentan therapy the improvement in functional class might be due to the drug-related increase in oxygen delivery to the exercising muscle.

Recently, Denton, *et al*[47] analyzed the subgroup of patients with scleroderma in 2 controlled clinical trials[48,49]. The authors found a positive effect of bosentan based on the stabilization of the disease compared with a decline in functional ability seen in patients receiving placebo.

We previously found in a smaller group of 13 patients that 1-year treatment with bosentan was associated with a progressive improvement of exercise capacity in about half the patients with PAH related to CTD[50].

In the present study our patients remained stable in their WHO class III, and 7 out of 18 patients showed an improvement in the 6-MWT after 3 months of treatment, with an inverse correlation with the reduction of RVSP. Our data suggest that the increase in exercise capacity after bosentan treatment may be explained by the effects of the drug on a decrease of pulmonary vascular resistance, with reduction in RVSP, consistent with echocardiographic and invasive hemodynamic results in previous reports[48,51].

Among the markers studied, only Δ-sP-S showed a correlation with Δ-RVSP and Δ-6-MWT, and it may have a role in evaluation of the efficacy of bosentan.

Administration of bosentan for 3 months induced a beneficial effect by restoring endothelial function through a decrease in the markers of endothelial cell activity, leading to stabilization or improvement of severe pulmonary arterial hypertension.

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