# Dual endothelin receptor antagonists contrast the effects induced by endothelin-1 on cultured human microvascular endothelial cells

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## Abstract

### Objective

*To evaluate the ability of dual endothelin (ET) receptor antagonists ($ET_A$/$ET_B$ -$ET_{A/B}$RAs) to contrast the ET-1-induced effects on cultured human microvascular endothelial cells (HMVECs).*

### Methods

*Some cultured HMVECs were untreated, or treated with ET-1 (100nM) or transforming growth factor β1 (TGFβ1, 10 ng/mL) alone for 6 days, in order to induce the endothelial-to-mesenchymal transition (EndoMT). Other cultured HMVECs were pre-treated for 1hr with $ET_{A/B}$RAs bosentan (10 μM) or macitentan (1μM, 10 μM) before the stimulation with ET-1 for 6 days. At the end of treatments, a mechanical injury was induced to cultured HMVECs (by scratching the cell monolayer with a sterile tip), and then the cell ability to re-fill the damaged area was determined after 24hrs. EndoMT phenotype markers and monocyte chemoattractant protein-1 (MCP-1) were evaluated by qRT-PCR and Western blotting. Statistical analysis was performed using Mann-Whitney-U non-parametric test.*

### Results

*Both ET-1 and TGFβ1 induced EndoMT and the MCP-1 over-expression in cultured HMVECs, as well as reduced the process of endothelial cell damage repair. Pre-treatment with $ET_{A/B}$RAs let cultured HMVECs to significantly restore the in vitro damage of the cell monolayer and antagonised the EndoMT process as well as the MCP-1 over-expression (range $p<0.05$ – $p<0.001$). Conversely, untreated or TGFβ1-treated HMVECs were found unaffected by the $ET_{A/B}$RAs treatments.*

### Conclusion

*The treatment with dual $ET_{A/B}$RAs seems to partially restore the altered cell function induced by ET-1 in cultured endothelial cells, and might justify their therapeutic efficiency in clinical conditions characterised by increased concentrations of ET-1.*

**Key words**

endothelial cells, endothelin-1, TGFβ1, endothelial-to-mesenchymal transition, dual endothelin receptor antagonists, fibrotic process

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## Introduction

Endothelial cell dysfunction contributes to the microvascular damage, which is considered an early event in the pathogenesis of fibrotic diseases, including systemic sclerosis (SSc) (1, 2).

In SSc, chronic microvascular endothelial cell activation and progressive damage contrast vascular recovery, by inducing a reduction in peripheral capillary density and an impaired angiogenesis (3, 4). These events, linked with the activation of local immune-inflammatory reaction, precede the activation of fibroblasts into pro-fibrotic myofibroblasts (5, 6).

Endothelial cells may contribute to the development of tissue fibrosis through several mechanisms, including the production and secretion of cytokines and pro-fibrotic molecules, such as transforming growth factor-β1 (TGFβ1), connective tissue growth factor (CTGF) and endothelin-1 (ET-1) (7, 8). The release of these mediators allows the recruitment and the activation of myofibroblasts to over-produce extracellular matrix (ECM) proteins, primarily type I and III collagens (COL-1 and COL-3), as well as fibronectin (FN) both *in vivo* and *in vitro* (9-11).

Among the mechanisms and the cell types involved in the fibrotic process, vascular endothelial cells have been reported to acquire matrix-producing myofibroblast features and to induce ECM deposition through the endothelial-to-mesenchymal transition (EndoMT) process, contributing to dermal fibrogenesis (12, 13).

EndoMT is characterised by the ability of endothelial cells to over-express myofibroblast phenotype markers, such as α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (S100A4), and COL-1 and to down-regulate endothelial phenotype markers, such as platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and vascular endothelial (VE)-cadherin (13, 14). Recently, TGFβ1 has been shown to induce the EndoMT process in human dermal microvascular endothelial cells (HMVECs) (14).

ET-1 is a vasoconstrictor and pro-fibrotic molecule primarily secreted by endothelial cells and it contributes to the activation of myofibroblasts and the over-production of ECM proteins (15, 16). Moreover, activated fibroblasts have been shown to over-produce and release ET-1, contributing to the persistence of fibrosis (17). In SSc, higher serum levels of ET-1 have been detected in patients with skin and lung fibrosis, correlating with the severity of the fibrotic phenotype (18). ET-1 exerts its effects through the binding to its receptors ($ET_A$ and $ET_B$), which are expressed on several cell types, including fibroblasts, macrophages, and endothelial cells (7, 19, 20). Based on these observations, the blockage of the interaction between ET-1 and its receptors through the ET receptor antagonists (ETRAs) was considered a possible strategy to prevent the ET-1 effects. ETRAs may either contrast the binding between ET-1 and both receptors ($ET_A$ and $ET_B$), as with bosentan and macitentan, or antagonise its interaction with one specific receptor selectively, as with ambrisentan, sitaxentan or BQ123 (20, 21).

The aim of this study was mainly to investigate the effects of the dual ET receptor antagonists ($ET_{A/B}$RAs, bosentan and macitentan) in contrasting the endothelial cell damage and the EndoMT processes induced by ET-1 on cultured HMVECs.

## Materials and methods

### *Cell cultures and treatment*

HMVECs were purchased from Lonza Clonetics (Lonza Sales, Basel, Switzerland) and cultured in EGM-2MV medium (Lonza) at 5% of fetal bovine serum (FBS). The cells have been isolated from female healthy donors and characterised as CD31 positive, von Willebrand Factor VIII positive and α-SMA negative cells, as certified by Lonza Clonetics, which also excludes the presence of other cell types (*i.e.* fibroblasts and epithelial cells). The cells were used between the 3rd and 5th culture passages. Some cultured HMVECs were treated with ET-1 (100 nM; Enzo Life Sciences, UK) or TGFβ1 (10ng/ml; PreproTech, London, UK) alone for 6 days, in order to induce the EndoMT process, in accordance with recent studies (14, 22, 23). Other cultured HMVECs were treated for 1hr with bosen-

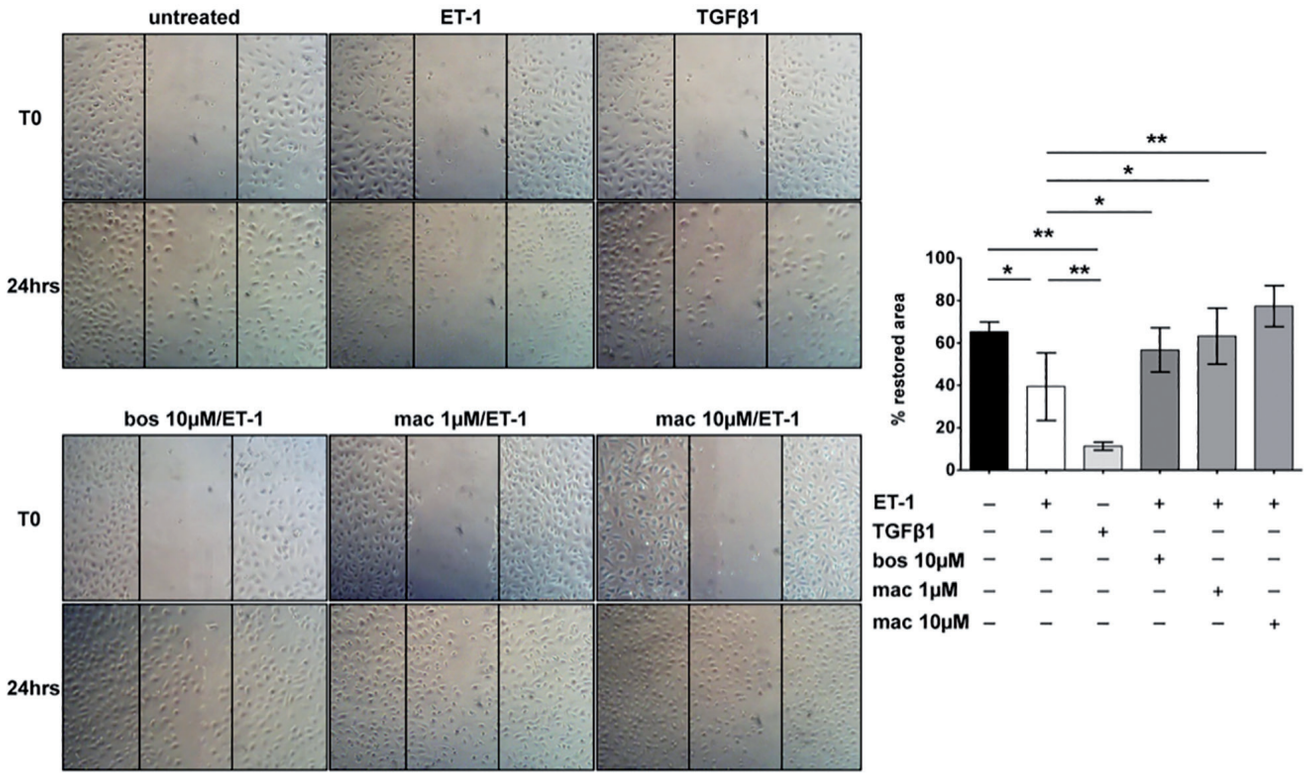


**Fig. 1.** *In vitro* endothelial cell damage repair process in cultured HMVECs stimulated for 6 days to undergo endothelial-to-mesenchymal transition (EndoMT) process.
Evaluation of the ability to restore the *in vitro* damaged area and cell monolayer 24hrs after the induction of a mechanical injury in cultured human dermal microvascular endothelial cells (HMVECs) stimulated for 6 days to undergo endothelial-to-mesenchymal (EndoMT) process. Some cultured cells were maintained in growth medium without any treatment (untreated cells) or stimulated with endothelin-1 (ET-1, 100nM) and transforming growth factor-β1 (TGFβ1, 10ng/ml) alone for 6 days. Other cultured cells were treated for 1hr with bosentan (bos) 10 μM, macitentan (mac) 1μM and 10 μM and then stimulated with ET-1 for 6 days.
For each experimental condition, the ability of cultured HMVECs to repair the damaged area and restore cell monolayer was determined by the difference in the cell-free area between the moment of injury in the endothelial cell monolayer (T0) and 24hrs later. *$p$<0.05; **$p$<0.01.

tan (10μM) or macitentan (1μM and 10μM) (Actelion Pharmaceutics, Basel, Switzerland) and then stimulated with ET-1 for 6 days. Cultured HMVECs maintained in EGM-2MV medium at 5% of FBS for 6 days were used as untreated cells. The EGM-2MV medium and the stimulation with ET-1, TGFβ1, and $ET_{A/B}$RAs were renewed every 48hrs. The concentrations of bosentan and macitentan used for the *in vitro* experiments were in accordance with several studies (16, 17, 22, 24). Six independent *in vitro* experiments were performed using four different consecutive lots of HMVECs from Lonza Clonetics (Lot numbers: from 0000432056 to 0000432059). Results obtained from all *in vitro* experiments were expressed as mean±standard deviation (±SD).

*In vitro endothelial cell damage assay*
HMVECs were cultured in 12-well tissue culture plates ($3x10^3$ cells/$cm^2$) and treated as described in the "Cell cultures and treatment" paragraph. At the end of treatments, an *in vitro* endothelial damage was obtained by scratching the HMVECs monolayer with a sterile 200μL tip, in accordance with several *in vitro* studies (25, 26). Cultured HMVECs were washed with Dulbecco's phosphate buffer solution (DPBS), in order to remove the floating cells and then maintained in EGM-2MV medium at 5% of FBS for additional 24hrs. Endothelial cell injury was evaluated immediately after scratching (T0) and after 24hrs.
For each experimental condition, the ability of cultured HMVECs to repair the damaged area and to restore the cell monolayer integrity was determined by the difference in the cell-free area between the two investigated time points (T0 and 24hrs), using Leica Q500MC Image Analysis System (Leica, Cambridge, UK). Such a difference was indicated as percentage (%) of restored area. The ability of cultured HMVECs to repair the damaged area was related to their migration to the recovery space (25, 26). Every experimental condition was tested in duplicate in each independent *in vitro* experiment.

*Quantitative real time polymerase chain reaction (qRT-PCR)*
Total RNA was extracted with NucleoSpin RNA/protein (Macherey-Nagel, Duren, Germany) and quantified with nanodrop (Thermo Scientific, Wilmington, USA), which was also used to evaluate the RNA integrity. For each experimental condition, 1μg of total RNA was used to synthesise first-strand cDNA by the QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy). The qRT-PCR was performed on an Eppendorf Realplex 4 Mastercycler using Real MasterMix SYBR Green detection system (Eppendorf, Milan, Italy).
Primers for monocyte chemoattract-

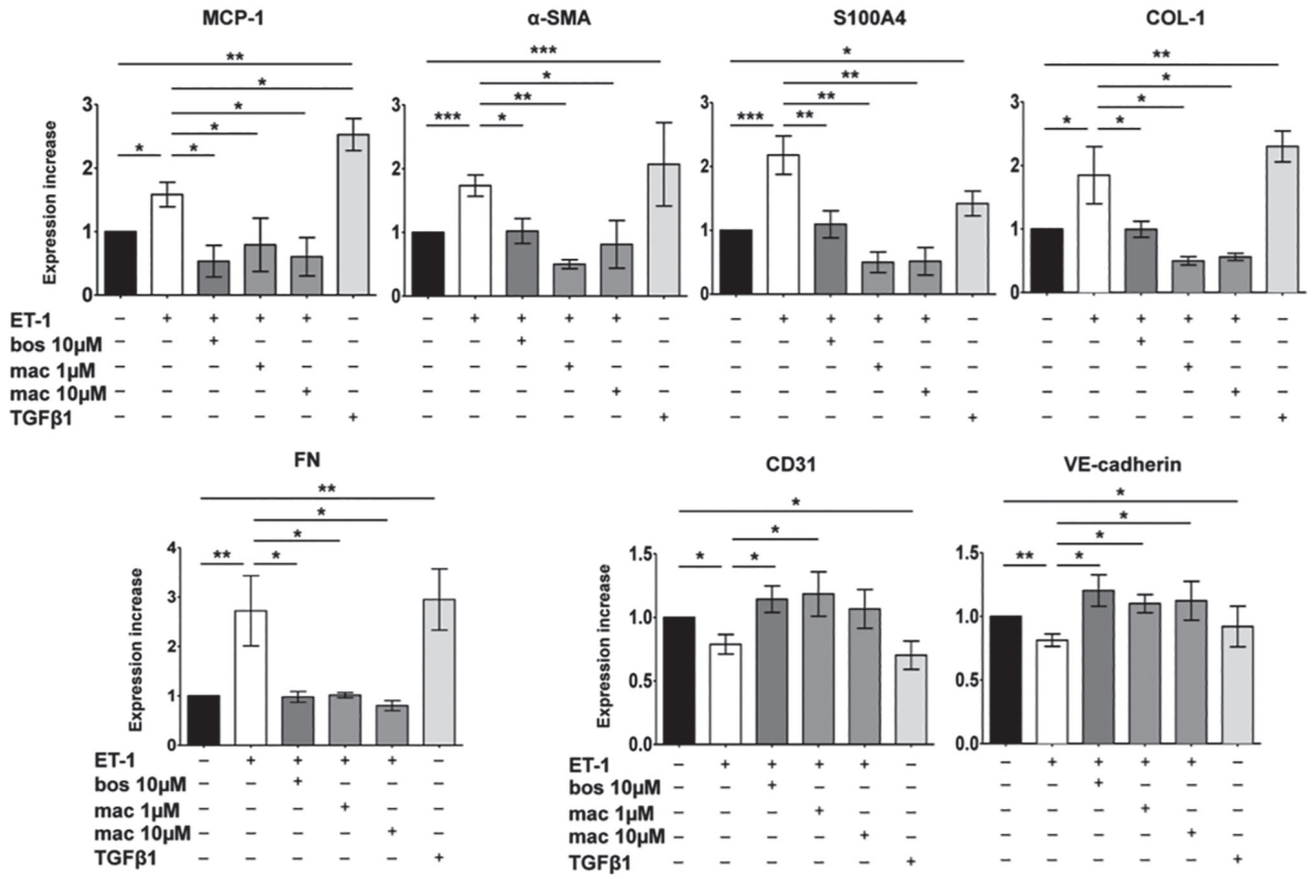


**Fig. 2.** Gene expression levels of MCP-1, myofibroblast and endothelial phenotype markers in cultured HMVECs.
Quantitative real time polymerase chain reaction (qRT-PCR) of gene expression levels of monocyte chemoattractant protein-1 (MCP-1), α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (S100A4), type I collagen (COL-1), fibronectin (FN), platelet endothelial cell adhesion molecules (PECAM-1 or CD31), and vascular endothelial cadherin (VE-cadherin) in cultured human dermal microvascular endothelial cells (HMVECs).
Some cultured cells were maintained in growth medium without any treatment (untreated cells) or stimulated with endothelin-1 (ET-1, 100nM) alone for 6 days. Other cultured cells were treated for 1hr with bosentan (bos) 10 μM, macitentan (mac) 1μM and 10μM and then stimulated with ET-1 for 6 days. Finally, some other cultured HMVECs were treated with transforming growth factor-β1 (TGFβ1, 10 ng/ml) alone for 6 days (as positive control of EndoMT process). For each experimental condition, gene expression values corresponded to a fold expression (expression level or fold increase) of the target gene compared to that of the untreated cells, taken as unit value.
The data of gene expressions of MCP-1, α-SMA, S100A4, COL-1, FN, CD31 and VE-cadherin were indicated as mean±SD and showed as gene expression level. Results of qRT-PCR were obtained by six independent *in vitro* experiments. **p*<0.05; ***p*<0.01; ****p*<0.001.**p*<0.05; ***p*<0.01; ****p*<0.001.

ant protein-1 (MCP-1; NM_002982), α-SMA (NM_001613), S100A4 (NM_002961), COL-1 (NM_000088), FN(NM_002026),CD31(NM_000442), VE-cadherin (NM_001795), and β-actin (NM_001101, housekeeping gene) were supplied by Primer design (Primer design, UK).

For each experimental condition, the gene expression values were calculated using the comparative ΔΔCT method and corresponded to the expression level increase (fold expression) of the target gene compared to untreated cells, taken as unit value by definition (27). The melting curve confirmed the specificity of the SYBR green assay, in accordance with the manufacturer's protocol (Eppendorf).

*Western blotting*

Cells were lysed with NucleoSpin RNA/protein (Macherey-Nagel) and the protein amount quantified by Bradford method.

For each experimental condition, 30μg of protein were separated by electrophoresis on a 4-12% SDS-PAGE gel (Thermo Scientific) to detect the protein synthesis of α-SMA, S100A4, COL-1, FN, CD31, and VE-cadherin and then transferred onto Hybond-C-nitrocellulose membranes (Life Technologies, Carlsbad, CA, USA).

After 1hr in blocking solution (PBS1x, 0.1% triton-X and 5% of bovine serum albumin), membranes were incubated overnight at 4°C with primary antibodies anti-human α-SMA (dilution 1:500; Cell Signaling Technology), S100A4 (dilution 1:200; Santa-Cruz Biotechnology), COL-1 (dilution 1:600, Proteintech, Manchester, UK), FN (dilution 1:1,000; Sigma-Aldrich), CD31 (dilution 1:1,000; Santa-Cruz Biotechnology), and VE-cadherin (dilution 1: 1,000; Cell Signaling Technology). Membranes were subsequently incubated with secondary antibodies (dilution 1:2,000) anti-mouse IgG for FN, anti-rabbit IgG for α-SMA, S100A4, COL-1, VE-cadherin (Cell Signaling Technology), and anti-goat IgG for CD31 (Santa-Cruz Biotechnology). Membranes were also incubated with HRP-conjugated antibody to human glyceraldehyde 3-phosphate dehydrogenase (GAPHD, dilution 1:3,000; Cell

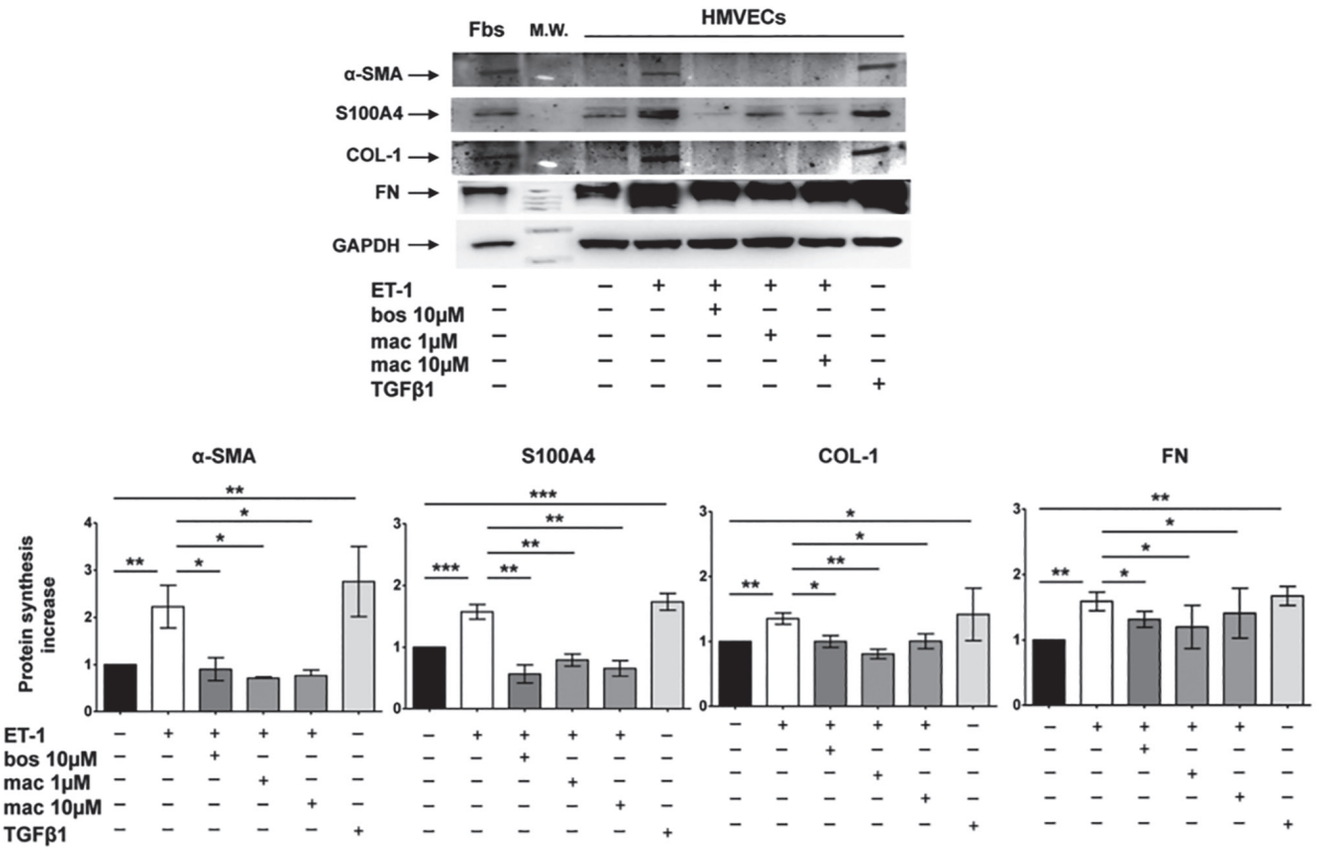


**Fig. 3.** Evaluation of protein synthesis of myofibroblast phenotype markers in cultured HMVECs.
Western blotting and related densitometric analysis of protein synthesis of α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (S100A4), type I collagen (COL-1) and fibronectin (FN), in cultured human dermal microvascular endothelial cells (HMVECs).
Some cultured cells were maintained in growth medium without any treatment (untreated cells) or stimulated with endothelin-1 (ET-1, 100nM) alone for 6 days. Other cultured cells were treated for 1hr with bosentan (bos) 10 μM, macitentan (mac) 1μM and 10 μM and then stimulated with ET-1 for 6 days. Finally, some other cultured HMVECs were treated with transforming growth factor-β1 (TGFβ1, 10 ng/ml) alone for 6 days (as positive control of EndoMT process). Activated skin myofibroblasts (Fbs) were used as positive controls for the synthesis of α-SMA, S100A4, COL-1, and FN (not evaluated by densitometric analysis). For each experimental condition, the values of the investigated proteins were normalised to those of the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The values of protein synthesis obtained for each treatment were normalized to that of untreated cells taken as unit value.
The data of α-SMA, S100A4, COL-1 and FN synthesis were indicated as mean±SD and showed as level of protein synthesis. Results were obtained by six independent *in vitro* experiments. *$p$<0.05; **$p$<0.01; ***$p$<0.001.

Signalling Technology), in order to confirm similar loading of gel and efficiency in the electrophoretic transfer.
The densitometric analysis was performed by UVITEC Alliance analysis software (UVItec Limited, Cambridge, UK). For each experimental condition, the values of the investigated proteins were normalised to those of the corresponding GAPDH. The resulting values of each treatment were normalised to that of the untreated cells (taken as unit value by definition) in order to obtain the increased level of protein synthesis.

*Immunocytochemistry*
Cells were fixed in methanol and incubated with primary antibodies to human α-SMA (dilution 1:100, Dako Citomation, Denmark), S100A4 (dilution 1:100; Santa Cruz Biotechnology), COL-1 (dilution 1:50, Proteintech), and FN (dilution 1:100; Sigma-Aldrich).
Human MACH1 universal horseradish-peroxidase-streptavidin polymer kit (Biocare Medical, CA, USA) was used to detect the expression of the investigated proteins.
For each experimental condition, the protein expression of the investigated myofibroblast phenotype markers as well as the morphological changes in cultured HMVECs were analysed by light microscopy (magnification 40x) (Leica, Cambridge, UK), evaluating the same number of cells.

*Statistical analysis*
The statistical analysis was carried out by GraphPad Prism 5 software, using the Mann-Whitney-U non-parametric test. Significance was set at $p$<0.05. Reports on endothelial cell damage assay, qRT-PCR and Western blotting represented the mean of the results obtained from all *in vitro* independent experiments and they were expressed as mean ± SD.

## Results

*Effects of ET-1, $ET_{A/B}$RAs and TGFβ1 on the endothelial cell damage repair process in cultured HMVECs treated for 6 days to undergo EndoMT process*
Twenty-four hours after the induction of the endothelial cell damage, cultured HMVECs maintained for 6 days in endothelial growth medium (untreated cells) showed the ability to restore the 65% of the scratched area (Fig. 1). On the contrary, the same capability result-

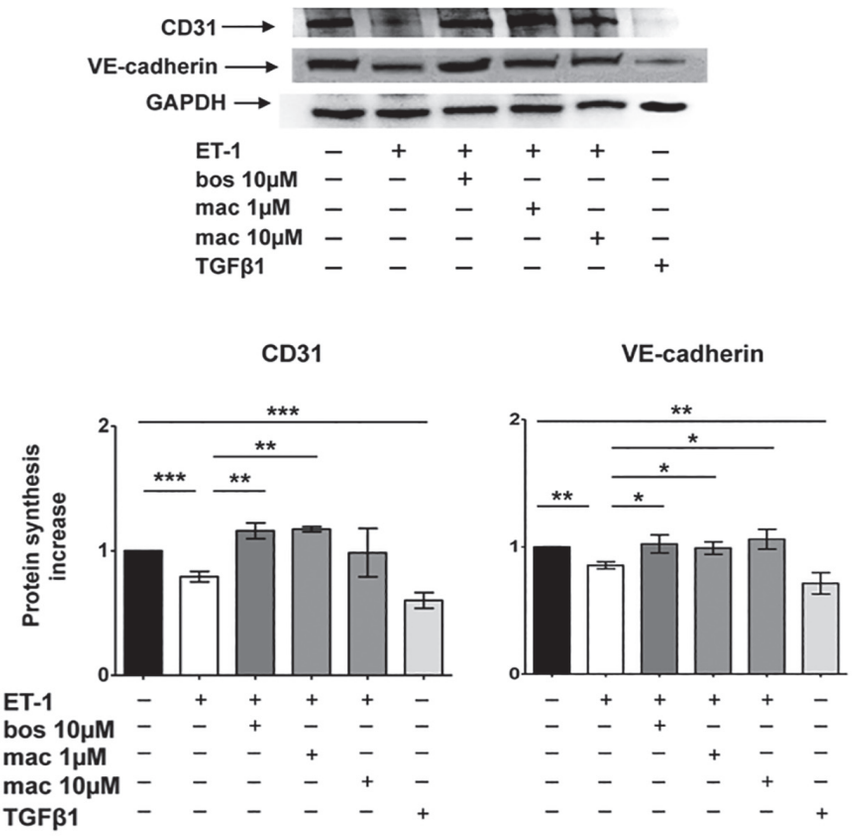


**Fig. 4.** Evaluation of protein synthesis of endothelial phenotype markers in cultured HMVECs.
Western blotting and related densitometric analysis of protein synthesis of platelet endothelial cell adhesion molecules (PECAM-1 or CD31) and vascular endothelial cadherin (VE-cadherin) in cultured human dermal microvascular endothelial cells (HMVECs).
Some cultured cells were maintained in growth medium without any treatment (untreated cells) or stimulated with endothelin-1 (ET-1, 100nM) alone for 6 days. Other cultured cells were treated for 1hr with bosentan (bos) 10μM, macitentan (mac) 1μM and 10 μM and then stimulated with ET-1 for 6 days. Finally, some other cultured HMVECs were treated with transforming growth factor-β1 (TGFβ1, 10 ng/ml) alone for 6 days (as positive control of EndoMT process). For each experimental condition, the values of the investigated proteins were normalized to those of the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The values of protein synthesis obtained for each treatment were normalised to that of untreated cells taken as unit value.
The data of CD31 and VE-cadherin synthesis were indicated as mean±SD and showed as level of protein synthesis. Results were obtained by six independent *in vitro* experiments.
**p*<0.05; ***p*<0.01; ****p*<0.001.

ed significantly decreased in cultured HMVECs stimulated with ET-1 alone for 6 days when compared to untreated cells (39.4±15.9% *vs*. 65.3±4.6%, respectively, p<0.05) (Fig. 1).
Of note, bosentan 10μM and macitentan at both concentrations (1μM, 10μM) significantly allowed cultured HMVECs to maintain their ability to replace the scratched area with a new monolayer of cells, contrasting the ET-1-induced effects on the *in vitro* endothelial cell damage (56.8±10.4% for bosentan 10μM, *p*<0.05; 63.3±13.1% for macitentan 1μM, *p*<0.05; 77.4±9.7% for macitentan 10μM, *p*<0.01 *vs*. 39.4±15.9% for ET-1) (Fig. 1). No significant differences were observed between bosentan 10μM and the two concentrations of macitentan in antagonising these effects (Fig. 1).
Cultured HMVECs stimulated for 6 days with TGFβ1 alone lost their ability to restore the scratched area compared to untreated cells (11.3±1.9 *vs*. 65.3±4.6%, *p*<0.01), even showing a significantly stronger effect than that induced by ET-1 (11.3±1.9 *vs*. 39.4±15.9%, *p*<0.01) (Fig. 1). Bosentan 10*μ*M and macitentan (1μM and 10μM) did not allow cultured HMVECs stimulated with TGFβ1 to maintain the ability to restore the scratched area with a new cell monolayer (data not shown).

*Effects of ET-1, $ET_{A/B}$RAs and TGFβ1 on MCP-1 gene expression in cultured HMVECs treated for 6 days*
In cultured HMVECs, ET-1 induced a significant up-regulation of MCP-1 gene expression compared to untreated cells (*p*<0.05) (Fig. 2). This up-regulation was significantly antagonised by the action of bosentan 10μM, macitentan 1μM and 10μM compared to cultured HMVECs treated with ET-1 alone (*p*<0.05 for all $ET_{A/B}$RAs) (Fig. 2).
TGFβ1 significantly increased the gene expression of MCP-1 compared to untreated cells (*p*<0.01), once again showing a significantly stronger effect than that induced by ET-1 (*p*<0.05 *vs*. ET-1-treated cells) (Fig. 2).

*Effects of ET-1, $ET_{A/B}$RAs and TGFβ1 on the gene expression of specific myofibroblast and endothelial phenotype markers in cultured HMVECs treated for 6 days*
In cultured HMVECs, ET-1 significantly induced the gene expression of α-SMA, S100A4 and COL-1 compared to untreated cells (*p*<0.001; *p*<0.001; *p*<0.05) (Fig. 2). Moreover, ET-1 significantly up-regulated the FN gene expression (*p*<0.01 *vs*. untreated cells). At the same time, ET-1 determined a significant down-regulation in the expression of CD31 and VE-cadherin genes compared to untreated cells (*p*<0.05; *p*<0.01) (Fig. 2).
In cultured HMVECs, the ET-1-induced effects were significantly antagonised by $ET_{A/B}$RAs (Fig. 2). Bosentan (10μM) and macitentan (1μM, 10μM) significantly antagonised the increase in the gene expression of α-SMA (*p*<0.05; *p*<0.01; *p*<0.05), S100A4 (*p*<0.01 for all $ET_{A/B}$RAs), COL-1 and FN (*p*<0.05 for both genes, for all $ET_{A/B}$RAs) compared to cultured HMVECs treated with ET-1 alone (Fig. 2).
Of note, bosentan 10μM and macitentan 1μM significantly antagonised the down-regulation of CD31 and VE-cadherin gene expressions induced by ET-1 (*p*<0.05, for both genes *vs*. ET-1 treated cells) (Fig. 2). Macitentan 10μM contrasted the down-regulation of CD31 (no significantly), whereas significantly antagonised the down-regulation of VE-cadherin mediated by ET-1

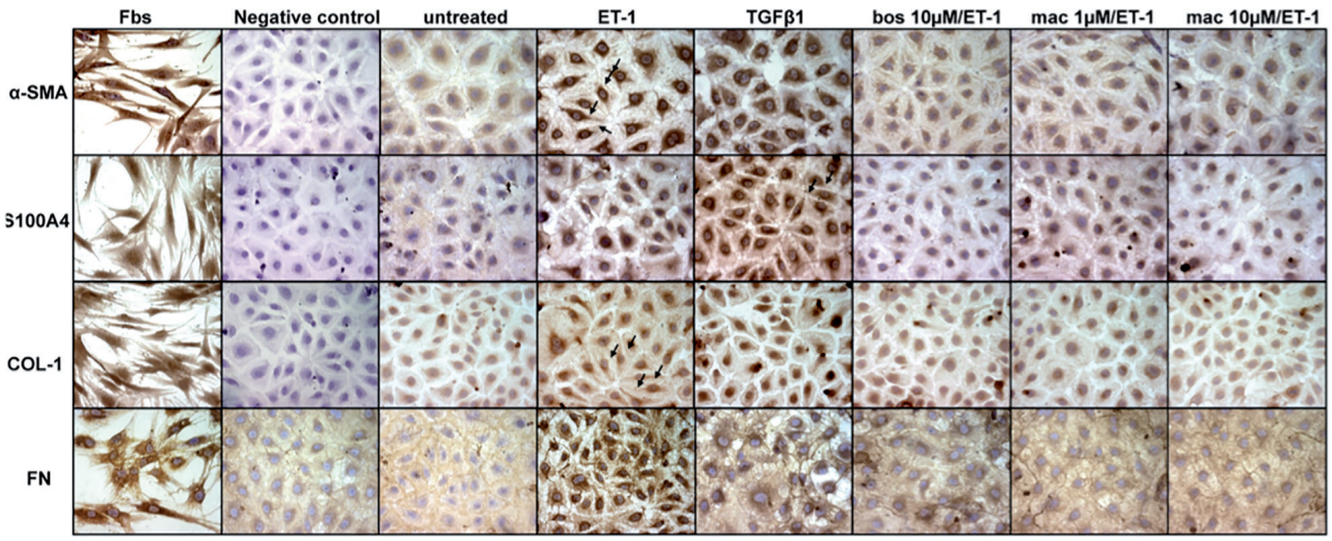


**Fig. 5.** Evaluation of morphological changes in cultured HMVECs.
Immunocytochemistry of α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (S100A4), type I collagen (COL-1) and fibronectin (FN) protein synthesis in cultured human dermal microvascular endothelial cells (HMVECs). Some cultured cells were maintained in growth medium without any treatment (untreated cells) or stimulated with endothelin-1 (ET-1, 100nM) and transforming growth factor-β1 (TGFβ1, 10ng/ml) alone for 6 days. Other cultured cells were treated for 1hr with bosentan (bos) 10μM, macitentan (mac) 1μM and 10μM and then stimulated with ET-1 for 6 days. Results of immunocytochemistry were obtained by six independent *in vitro* experiments. The images of immunocytochemistry (magnification 40x) are representative of the results. The black arrows indicate the cells showing a myofibroblast-like morphology.

($p$<0.05 *vs*. ET-1 treated cells) (Fig. 2).
In cultured HMVECs, TGFβ1 significantly induced the gene expression of myofibroblast markers and up-regulated the expression of FN compared to untreated cells ($p$<0.001 for α-SMA; $p$<0.05 for S100A4; $p$<0.01 for COL-1 and FN) (Fig. 2). At the same time, TGFβ1 significantly down-regulated the gene expression of CD31 and VE-cadherin compared to untreated cells ($p$<0.05, for both endothelial phenotype markers) (Fig. 2).
The results obtained by the treatment with ET-1, macitentan 1μM and TGFβ1 were found in accordance with recent *in vitro* studies (13, 14, 22).

*Effects of ET-1, $ET_{A/B}$RAs and TGFβ1 on the protein synthesis of myofibroblast and endothelial phenotype markers in cultured HMVECs treated for 6 days*

The ability of ET-1, $ET_{A/B}$RAs and TGFβ1 to modulate the expression of the EndoMT markers was also investigated at protein level by Western blotting.
In cultured HMVECs, ET-1 significantly induced the synthesis of α-SMA, S100A4, and COL-1 and increased the FN production compared to untreated cells ($p$<0.01; $p$<0.001; $p$<0.01; $p$<0.01, respectively) (Fig. 3).
At the same time, ET-1 induced a significant decrease in CD31 and VE-cadherin protein synthesis ($p$<0.001; $p$<0.01 *vs*. untreated cells) (Fig. 4).
The ET-1 effects were significantly contrasted by $ET_{A/B}$RAs (Fig. 3-4). Bosentan (10μM) and macitentan (1μM and 10 μM) significantly antagonised the ET-1-induced increase in myofibroblast phenotype marker and FN synthesis compared to cultured HMVECs treated with ET-1 alone (α-SMA: $p$<0.05 for all $ET_{A/B}$RAs; S100A4: $p$<0.01 for all $ET_{A/B}$RAs; COL-1: $p$<0.05 for bosentan 10μM and macitentan 10 μM, $p$<0.01 for macitentan 1μM; FN: $p$<0.05 for all $ET_{A/B}$RAs) (Fig. 3).
These results on myofibroblast phenotype marker protein expression were further confirmed by immunocytochemistry (Fig. 5).
Bosentan 10 μM and macitentan 1μM also significantly antagonised the ET-1-induced decrease in protein expression of CD31 and VE-cadherin ($p$<0.01 for CD31; $p$<0.05 for VE-cadherin, for both $ET_{A/B}$RAs) (Fig. 4). Macitentan 10μM antagonised the ET-1-mediated decrease in the synthesis of CD31 and significantly contrasted that of VE-cadherin compared to cultured HMVECs treated with ET-1 alone ($p$<0.05) (Fig. 4).
In cultured HMVECs, TGFβ1 induced a significant increase in the protein synthesis of myofibroblast markers and FN compared to untreated cells ($p$<0.01 for α-SMA; $p$<0.001 for S100A4; $p$<0.05 for COL-1; $p$<0.01 for FN) and significantly decreased CD31 and VE-cadherin protein expression ($p$<0.001 and $p$<0.01 *vs*. untreated cells) (Fig. 3-4). The data on protein synthesis were in accordance with those observed at gene expression levels.

*Effects of ET-1, $ET_{A/B}$RA and TGFβ1 on the cell morphology of cultured HMVECs treated for 6 days*

In addition to the induction of specific myofibroblast phenotype markers and the down-regulation of endothelial cell phenotype markers, ET-1 determined a morphological change into a spindle-shaped/myofibroblast-like appearance in some cultured HMVECs compared to untreated cells after 6 days of treatment (Fig. 5). This initial morphological change mediated by ET-1 was similar to that induced by TGFβ1 (Fig. 5). The treatment with bosentan 10μM, macitentan 1μM and 10μM contrasted the ET-1-mediated transition of cultured HMVECs into a myofibroblast appearance (Fig. 5).

## Discussion

The results of this study showed that ET-1, together with the induction of the EndoMT process in cultured endothelial cells, further reduced their ability to restore the integrity of the cell monolayer previously mechanically damaged. This result might suggest a possible new action of ET-1 on endothelial cells interfering with the endothelial cell damage repair process and contributing to the vascular dysfunction.

Endothelial cell injury is believed to be a possible trigger that precedes the development of fibrosis in several diseases, including SSc (8, 28). As known, injured endothelial cells may contribute to vascular dysfunction, which is considered one of the earliest features in SSc (29-31). However, the initial events responsible for the injury of endothelial cells and their subsequent activation are still not clearly known.

The ET-1 effects were found contrasted by $ET_{A/B}$RAs (bosentan and macitentan), whose treatment let cultured HMVECs to be able to restore the integrity of their cell monolayer. Therefore, the blockage of the interaction between ET-1 and its cell receptors through $ET_{A/B}$RAs may contribute to improve the endothelial cell damage repair process.

Together with microvascular endothelial cell damage, the presence of inflammatory infiltrate represents an early feature in the pathogenesis of the fibrotic process, as in SSc (1, 2). Inflammatory infiltrate is dominated by macrophages and T cells, which are located primarily in perivascular areas (32, 33). The migration of immune inflammatory cells is orchestrated by pro-inflammatory and pro-fibrotic chemokines, such as MCP-1, which is highly expressed in fibrotic diseases (34). MCP-1 can be released by several cell types, including mononuclear, epithelial, and endothelial cells. This chemokine is over-expressed in the skin and blood of SSc patients and it might therefore drive the infiltration of monocytes in the skin of patients characterised by an early and diffuse disease (35, 36). The up-regulation of MCP-1 induced by ET-1 in cultured HMVECs might provide these cells with the ability to attract monocytes.

Of note, bosentan and macitentan efficiently contrasted the up-regulation of MCP-1 induced by ET-1 in cultured HMVECs. Moreover, the comparison between both $ET_{A/B}$RAs in the efficiency to contrast the ET-1-mediated effects on the endothelial cell damage repair process and the MCP-1 up-regulation, showed that a 10-fold lower concentration of macitentan (1μM) was already effective.

These results might define a possible further important involvement of ET-1 in the early phases of the fibrotic process, which are characterised by an alteration of the endothelial cell function and the subsequent up-regulation of the chemokines involved in the activation of immune inflammatory cell migration. These important effects of ET-1 are mediated by the interaction with its receptors as demonstrated by the action of bosentan and macitentan, that efficiently contrasted the pro-fibrotic properties of ET-1 in cultured HMVECs.

As known, the production and release of pro-fibrotic molecules by injured/activated endothelial cells may directly stimulate inflammatory cells, vascular smooth muscle cells and fibroblasts (1, 8,16, 37, 38).

The present study further confirmed the ability of ET-1 to directly induce the gene expression and protein synthesis of EndoMT phenotype markers in endothelial cells, promoting a morphological change of some cultured HMVECs into a spindle-shaped/myofibroblast-like cell appearance. This morphological change was similar to that induced by TGFβ1. Interestingly, the evidence that only a low percentage of cultured cells treated with ET-1 and TGFβ1 acquired a spindle-shaped morphology is consistent with recent studies indicating that the transition from endothelial cells into myofibroblasts may not proceed through a complete trans-differentiation (39, 40).

In these studies, it was observed that only a partial transition of endothelial cells (as well as epithelial cells) into myofibroblasts may be sufficient for the initiation or progression of a pathological fibrogenesis (39, 40). The lower percentage of endothelial cells trans-differentiated into myofibroblasts was confirmed by another study showing that, in patients with SSc-associated pulmonary arterial hypertension, endothelial cells characterised by a double positivity for von Willebrand factor and α-SMA were detected in up to 5% of pulmonary vessels (41, 42).

Moreover, in the liver of patients affected by idiopathic portal hypertension (some of whom also affected by SSc) it was observed that only a small fraction of endothelial cells became α-$SMA^+$/$CD31^+$ and $S100A4^+$/$CD31^+$cells and began a change into myofibroblast-like phenotype during the fibrotic process (14).

In a model of bleomycin-induced pulmonary fibrosis, endothelial cells over-expressed S100A4 and FN, contributing to a pro-fibrotic environment (8). Moreover, S100A4 was shown to be strongly over-expressed in both fibrotic skin and activated SSc fibroblasts, where this molecule is essentially required to mediate the fibrotic process through the induction of α-SMA and COL-1 synthesis (9). Thanks to its role as a mediator of fibrosis, S100A4 was recently indicated as a possible candidate for novel anti-fibrotic therapies (9).

FN is considered one of the most abundant ECM molecules produced by myofibroblasts contributing to cardiac, pulmonary and skin fibrosis as well as synovitis (14, 16, 43-46). FN has been shown to be highly produced by endothelial cells in the early phase of the fibrotic process, preceding the development of pulmonary fibrosis (1, 2, 8).

Of note, the results of our study showed that ET-1, as already known for TGFβ1, up-regulated gene and protein expression of S100A4 and FN in cultured endothelial cells. These results might support recent observations, which demonstrated that, after injury, endothelial cells induced a local change in the pro-fibrotic milieu and directly contribute to fibrosis through the up-regulation and over-expression of fibrotic proteins, including S100A4, FN and osteopontin (8)

Therefore, in SSc, the EndoMT process may not only be responsible for the mere increase in the number of pro-fibrotic myofibroblasts, but also favour the loss of microvascular endothelial cells, contributing to capillary rarefac-

tion (17, 26). In SSc, capillary rarefaction is a clinical feature determined by the microvascular endothelial cell injury and in part due to an impaired or not evident angiogenesis (47-50).

Although the cultured HMVECs used in this *in vitro* study derived from female healthy subjects, a limitation of this research is that the effects of ET-1 on endothelial cell damage and in inducing monocyte attraction was not investigated in cultured HMVECs isolated from SSc patients. Therefore, the antagonistic effects of $ET_{A/B}$RAs (bosentan and macitentan) should be carried out also on SSc HMVECs. In addition, this study only evaluated the effects exerted by ETRAs acting on both $ET_A$ and $ET_B$ receptors and not singularly. However, it was recently demonstrated that under pathological conditions characterised by vascular alteration, the dual $ET_A$ and $ET_B$ receptor antagonism can provide superior vascular effects compared to $ET_A$-selective receptor blockade, such as ambrisentan (51, 52).

In conclusion, the antagonism of both $ET_A$ and $ET_B$ receptors by the dual ETRAs seem to interfere with the synthesis of several molecules involved in the fibrotic process as well as it might partially restore the altered cell function induced by ET-1 on cultured microvascular endothelial cells, and justify their therapeutic efficiency in clinical conditions characterised by increased concentrations of ET-1.

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