# Interaction between $PGI_2$ and ET-1 pathways in vascular smooth muscle from Group-III pulmonary hypertension patients

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**Abstract**

Pulmonary hypertension (PH) is characterized by an elevation of mean pulmonary artery pressure and it is classified into five groups. Among these groups, PH Group-III is defined as PH due to lung disease or hypoxia. Prostacyclin ($PGI_2$) analogues (iloprost, treprostinil) and endothelin-1 (ET-1) receptor antagonists (ERA) (used alone or in combination) are therapies used for treating PH. The mechanisms underlying the positive/negative effects of combination treatment are not well documented, and in this study, we tested the hypothesis that the combination of a $PGI_2$ analogue (iloprost, treprostinil) and an ERA may be more effective than either drug alone to treat vasculopathies observed in PH Group-III patients. Using Western blotting, $ET_A$ and $ET_B$ receptor expression were determined in human pulmonary artery (HPA) preparations derived from control and PH Group-III patients, and the physiologic impact of altered expression ratios was assessed by measuring ET-1 induced contraction of *ex vivo* HPA and human pulmonary veins (HPV) in an isolated organ bath system. In addition, the effects of single agent or combination treatments with a $PGI_2$ analogue and an ERA on ET-1 release and HPA smooth muscle cells (hPASMCs) proliferation were determined by ELISA and MTT techniques, respectively. Our results indicate that the increased $ET_A/ET_B$ receptor expression ratio in HPA derived from PH Group-III patients is primarily governed by a greatly depressed $ET_B$ receptor expression. However, contractions induced by ET-1 are not impacted in HPA and HPV derived from PH Group-III patients as compared to controls. Also, we found that the combination of an $ET_A$ receptor antagonist (BQ123) with iloprost provides greater inhibition of hPASMCs proliferation (-48±14% control; -32±06% PH) than either agent alone. Of note, while the $ET_B$ receptor antagonist (BQ788) increases ET-1 production from PH Group-III patients' preparations (HPA, parenchyma), even under these more proliferative conditions, iloprost and treprostinil are still effective to inhibit hPASMCs proliferation (-22/-24%). Our findings may provide new insights for the treatment of PH Group-III by combining a $PGI_2$ analogue and a selective $ET_A$ receptor antagonist.

**Key words:**

Human pulmonary artery, pulmonary hypertension, $PGI_2$, ET-1, endothelin receptor antagonist

## 1. Introduction

Pulmonary hypertension (PH) is a chronic and progressive disease defined by a mean pulmonary arterial pressure (mPAP) higher than 20 mmHg and is associated with a high mortality rate [1, 2]. Endothelial dysfunction is involved in the pathogenesis of PH and leads to increased production of vasoconstrictor/proliferative mediators [such as endothelin-1 (ET-1), thromboxane ($TxA_2$)] and reduced production of vasodilator/anti-proliferative mediators [such as prostacyclin ($PGI_2$), nitric oxide (NO)] [3-6]. Pharmacologic treatments for PH include $PGI_2$ analogues or mimetics (epoprostenol, iloprost, treprostinil, selexipag), ET-1 receptor antagonists (ERA: bosentan, ambrisentan, macitentan), phosphodiesterase inhibitors (tadalafil, sildenafil) or guanylate cyclase stimulators (riociguat) [7]. However, these treatments were primarily tested and found effective in PH Group-I (pulmonary arterial hypertension, PAH) patients according to classification established by the World Health Organization based upon etiology of disease [8]. On the other hand, PH Group-III is defined as PH due to lung disease (like chronic obstructive pulmonary disease, interstitial lung disease, or overlap syndromes) or conditions that cause hypoxemia (like obstructive sleep apnea, alveolar hypoventilation disorders) [8]. Although PH Group-III is the most common and lethal form of PH [9], treatment studies performed in these patients are limited, and none of the treatments described above for PH Group-I have been approved for use in PH Group-III [10].

Since PH is associated with enhanced plasma and arterial ET-1 levels, which are correlated with severity of the disease, the suppression of ET-1 activity is one of the therapeutic approaches for the treatment of PH [11-15]. Furthermore ERAs have been shown to improve 6-min walking distance (6MWD) and decrease pulmonary artery pressure and vascular resistance in PH Group-III patients [16-18]. However, the role of ET-1 in the pathogenesis of PH is complex, owing to the fact that it acts through two different receptor subtypes ($ET_A$ and $ET_B$). In humans, these receptors have different roles depending of the cells (endothelial or smooth muscle) and/or type of pulmonary artery (conductance, resistance) where they are expressed [19-22]. $ET_A$ receptors are expressed predominantly in pulmonary smooth muscle cells and induce vasoconstriction and smooth muscle cell proliferation, which contributes to the progression of PH [23]. On the other hand, $ET_B$ receptors are found on endothelial cells and to a much lesser extent on smooth muscle cells [24]. Activation of $ET_B$ receptors on endothelial cells releases vasodilator and anti-proliferative mediators (such as $PGI_2$ and NO) and mediates the clearance of ET-1, while $ET_B$ receptors on smooth muscle cells induce vasoconstriction [25, 26]. However, in human pulmonary artery (HPA), the major effect of $ET_B$ activation results in a vasoconstriction [21]. Despite the fact

that a non-selective ERA such as bosentan leads to clinical improvements of PH, inhibition of $ET_B$ receptors on endothelial cells is not desirable since inhibition of ET-1 clearance is unwanted in PH [27]. Therefore, the selective $ET_A$ receptor antagonist, ambrisentan might be considered the most appropriate ERA therapy for PH Group-I patients [16-18].

Another therapeutic approach for PH is administration of $PGI_2$ mimetics. $PGI_2$ is produced from pulmonary arteries/veins and acts via the IP receptor to cause vasodilation and inhibit smooth muscle cell proliferation [28-30]. The production of $PGI_2$ and expression of IP receptor are decreased in PH [5, 31, 32]; therefore, drugs targeting the $PGI_2$ pathway including synthetic $PGI_2$ (epoprostenol), $PGI_2$ analogues (iloprost, treprostinil, beraprost) and selective IP receptor agonists (selexipag) are treatment options for PH [29]. Although these treatments have not been recommended for PH Group-III patients by European Guidelines due to the lack of randomized controlled trials [33], several studies found that they are effective to significantly decrease pulmonary vascular resistance, mPAP, right heart dysfunction and/or to increase 6MWD Group-III patients, mostly with severe PH [34-38].

Despite the fact that approved PH drugs improve clinical and hemodynamic outcomes, morbidity and mortality remain high [39, 40]. The use of combinations of drugs with differential mechanisms of action is a strategy for PH treatment. In this study, we tested the hypothesis that the combining of a $PGI_2$ analogue (iloprost, treprostinil) with an ERA ($ET_A$ receptor antagonist: BQ123, $ET_B$ receptor antagonist: BQ788) may be more effective than either drug alone to decrease the elevated ET-1 levels, the elevated human pulmonary artery smooth muscle cells (hPASMCs) proliferation and the increased pulmonary vascular tone observed in PH Group-III patients.

## 2. Materials and Methods

### 2.1. Human pulmonary vascular preparations and lung parenchyma

After obtaining informed patient consent, the pulmonary preparations (pulmonary arteries, veins and lung parenchyma) were collected in the Department of Thoracic and Vascular Surgery at Bichat-Claude Bernard Hospital (Paris, France). The control pulmonary tissues were obtained from patients who underwent surgery for lung carcinoma (7 females, 6 males aged between 57-79 years old). HPA and human pulmonary veins (HPV) were carefully removed from macroscopically normal regions of the lungs. The PH pulmonary tissues have been obtained from patients who have undergone surgery for lung transplantation (explanted sick lung tissue). The category of PH patients is PH due to lung diseases and/or hypoxia (PH Group-III). The patient characteristics of PH Group-III patients are presented in Table S1. PH

lungs used in our study were from patients having catheter-measured mPAP ≥ 20 mmHg. The investigation conforms to the principles outlined in the Declaration of Helsinki. All research programs involving the use of human tissue are approved and supported by the INSERM Ethics Committee and the study (n° 11-045) was approved by the CEERB IRB00006477.

**2.2. Western blot analysis**

Samples of HPA were homogenized in liquid nitrogen, using a porcelain mortar. Homogenates were diluted (100 mg/ml) in Tris- HCl buffer with a protease inhibitor cocktail. Proteins were quantified by BCA protein assay kit and a 50 µg of protein sample loaded on a 12% polyacrylamide gel. Proteins were blotted onto nitrocellulose membranes. Membranes were blocked (TBS, 0.1% Tween 20, 5% non-fat dry milk) and incubated overnight at 4°C with a primary antibody specifically targeting $ET_A$ or $ET_B$ receptors (dilution ratio: 1/700, 1/350, respectively) in TBS/0.1% Tween- 20/1% non-fat dry milk. Subsequently, the membranes were incubated with an appropriate alkaline phosphatase- conjugated secondary antibody. Bands were visualized using the ECL prime luminescence system. For quantification, the film was scanned, and the integrated optical density of the bands was estimated with Scion Image (Scion Corporation, NIH, Frederick, MD, USA) and normalized to β-actin. For each sample, both $ET_A$ and $ET_B$ receptor expression were determined and calculated as a ratio of $ET_A$ to $ET_B$ receptor expression.

**2.3. Organ bath and isometric measurements**

Vascular preparations (pulmonary arteries and veins), cut as rings of 3 mm in width, were set up in 10 mL organ baths containing Tyrode's solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.5, gassed with 5% $CO_2$ and 95% $O_2$ at 37°C and pH 7.4. Each ring was initially stretched to an optimal load (~1.5 grams). Changes in force were recorded by isometric force displacement transducer (Narco F-60, Biosystems, Houston, TX, USA) and data acquisition system IOX (EMKA, Paris, France). Rings were then equilibrated for 90 min with bath fluid changes taking place every 10 min. After the equilibration period, the viability (contractility) of the vessel specimens was checked with norepinephrine (NE, 10 µM) stimulation and the preparations were washed until the initial resting tone was re-established. Thereafter, the vessels were contracted with increasing concentrations of ET-1 (0.001–0.3 µM) in a cumulative manner to establish the concentration–response curves.

**2.4. Ex- vivo tissue culture and ET-1 measurements**

Samples of HPA and lung parenchyma were dissected and cut into small pieces and placed into 12- well plates (100- 200 mg tissue/well) containing RPMI (pH 7.4) supplemented

with antibiotics (penicillin, 1000 IU/mL; streptomycin, 100 µg/mL) and an antimycotic agent (amphotericin, 0.25 µg/mL). Fresh pulmonary preparations were incubated in the presence or absence of one selective ET-1 receptor antagonist ($ET_A$ receptor antagonist: BQ123, $ET_B$ receptor antagonist: BQ788; 1 µM) or/and the $PGI_2$ analogue (treprostinil; 1 µM). The volume of the culture medium was adjusted to 1 mL for 70 mg of tissue. All tissue incubations were performed at 37°C in a humidified atmosphere of 5% $CO_2$ in air using a culture incubator for 12h. Subsequent to this exposure, ET- 1 concentrations were measured in culture media using an enzyme immunoassay kit.

**2.5. Culture of human pulmonary artery smooth muscle cells (hPASMCs)**

The culture of hPASMCs and all treatments were carried out in a biosafety level 2 laboratory in a vertical laminar flow hood. The hPASMCs were obtained from HPA samples from control or PH patients. These arteries were removed after dissection of lung lobes or whole lungs. First, proximal arteries were opened, cleaned of any connective tissue (parenchyma) and then rinsed with phosphate- buffered saline (PBS) containing 1/20 penicillin, streptomycin, amphotericin B (PSA). After rinsing, the artery media was isolated and cut with a scalpel into small 1- 2 mm pieces. This preparation was placed in a medium containing collagenase (Type 1) and elastase, and then incubated for 30 to 40 minutes at 37°C. After incubation and enzymatic digestion of the extracellular matrix, the preparation was filtered (40 microns filter) and centrifuged at 1000 rpm for 10 minutes at 20°C. After centrifugation, the supernatant was aspirated and the pellet was resuspended in a T25 flask containing Smooth Muscle Cell Growth Medium 2) supplemented with 20% fetal calf serum (FCS), PSA and growth factors [hEGF (epidermal growth factor), hbFGF (fibroblast growth factor), IGF (insulin- like growth factor)] to allow the proliferation of SMCs. .The cells were cultured in an incubator at 37°C in humid atmosphere containing 5 % $CO_2$. When confluence was reached, the hPASMCs were detached from the T25 flask using 1 ml of collagenase (0.3%) then 2 ml trypsin. In this first passage, the cells were diluted in 12 ml of culture medium and transferred to a larger flask (T75). Depending on the extend of cell confluence, passages were performed approximately every 2 weeks. The SMCs were confirmed morphologically; we obtained spindle shaped cells forming the "hill and valley" configuration which is typical of SMC.

**2.6. Pharmacological treatment of hPASMCs and MTT proliferation assay**

At passage 3- 4, the hPASMCs were washed twice with PBS (12 ml). After the wash, hPASMCs were detached as previously described and diluted in culture medium containing 20% FCS to a concentration of $3x10^6$ cells per 100 ml. Maintaining one cell culture derived from one individual, the cells in a homogeneous suspension were then seeded (200 µL /well) in

four 48 well plates. After proliferation of the cells in the 48- well plates (25- 50% confluence), the culture medium (with 20% FCS) was aspirated and replaced with a 0% FCS (200 µl) culture medium for 24 h in order to synchronize proliferation of the hPASMCs. After 24 hours of FCS deprivation, the medium was aspirated and replaced with 100 µl of culture medium (15% FCS) in each well in order to restart proliferation. 100 µl of a single pharmacological treatment (iloprost, treprostinil, BQ123 or BQ788) or combination (one analogue of $PGI_2$ + one ERA) were added in this medium to determine the effect of each treatment on the proliferation of hPASMCs. Within the same plate, each treatment was tested in triplicate and the control (no treatment, 100% proliferation) was tested in sextuplicate.

The MTT solution (5 mg/ml) was prepared in the specific culture medium for hPASMCs containing 0% FCS. The mixture was filtered (22 microns) and subsequently stored at 4°C protected from light. 3- 4 days after the pharmacological treatment, the medium in each well was removed. The hPASMCs were washed twice with RPMI-1640 (200 µl) and then incubated with the diluted MTT solution in RPMI 1/10 for 4 h at 37°C and 5% $CO_2$. After incubation (2h), the MTT solution was removed by gentle inversion of the plates. The formazan crystals (purple colored) obtained were visualized under a microscope, and after dissolution with DMSO, the violet coloration was measured using an OPTIMA spectrophotometer (Tokyo, Japan) at a wave length of 540 nm.

**2.7. Statistical analysis**

All results obtained from different patients (n) were expressed as mean ± standard error of the mean (SEM). The concentration–response curve induced by ET-1 was expressed as % of the $E_{max}$ of the NE (10 µM) control. Statistical analysis was performed by Student's t-test, Mann Whitney-U test or two-way ANOVA and Bonferroni's multiple comparison post hoc tests. The null hypothesis is that there is no difference between PH Group-III versus control patients or there is no difference between measurements with and without treatments in cell proliferation or ET-1 levels. The null hypothesis is rejected if the P value is less than 0.05 and indicates data significantly different. Statistical analyses were performed using SigmaStat version 3.5 (Systat Software, Point Richmond, CA, USA).

**2.8. Compounds and materials**

Protease inhibitor cocktail, NE, antibiotics, antimycotic, trypsin BQ123, BQ788, β-actin antibody and MTT colorimetric assay were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iloprost, ET-1 and ET-1 ELISA kits were obtained from Cayman Chemical (Ann Arbor, MI, USA). Treprostinil was a gift from United Therapeutics Corporation (Silver Spring, Maryland, ABD). RPMI, trypsin and collagenase were obtained from Gibco Invitrogen

(Paisley, UK). Elastase was purchased from Worthington (Lakewood, NJ, USA). BCA protein assay kit was from Thermo (Rockford, USA). Nitrocellulose membranes and ECL Plus® system were obtained from Amersham Biosciences (Buckinghamshire, UK). Antibodies against $ET_A$ and $ET_B$ were from Abcam (Cambridge, UK). Smooth Muscle Cell Growth Medium 2 was from PromoCell (Heidelberg, Germany).

## 3. Results

### 3.1. $ET_A$ and $ET_B$ receptor expression in human pulmonary arterial preparations from control and PH Group-III patients

The expression of $ET_A$ and $ET_B$ receptors was determined in HPA preparations derived from control and PH patients. There was no significant difference in $ET_A$ receptor expression between samples from control and PH patients (Figure 1A). However, the $ET_B$ receptor expression was significantly lower in PH Group-III patients as compared to control patients, resulting in a significantly higher ratio of $ET_A$ to $ET_B$ (Figures 1A, B).

### 3.2. Contraction induced by ET-1 in human pulmonary artery and vein derived from control and PH Group-III patients

ET-1 induced contraction in a concentration-dependent manner in HPA and HPV preparations, with no differences observed for control or PH Group-III patients (Figure 2). Of note, HPV preparations exhibited greater contractions induced by ET-1 versus HPA in both control and PH patients at concentrations above 10 nM (Figure 2).

### 3.3. The effect of ET-1 receptor antagonists and/or treprostinil on ET-1 levels in PH Group-III patients

HPA and lung parenchyma derived from PH patients were incubated in the presence or absence of a selective $ET_A$ receptor antagonist (BQ123, 1 μM) or $ET_B$ receptor antagonist (BQ788, 1 μM) or/and treprostinil (1 μM). Following 12 h of incubation, ET-1 concentrations were measured in the culture medium. Incubation with BQ788 or BQ123 statistically significantly increased ET-1 levels in HPA preparations. BQ788 also increased ET-1 levels in parenchyma from PH Group-III patients (Figures 3A, B). In HPA preparations derived from PH Group-III patients, co-incubation with treprostinil and BQ788 resulted in statistically significant higher concentrations of ET-1 as compared to control incubation or those incubated with treprostinil alone (Figure 3A).

### 3.4. The effect of $PGI_2$ analogues and/or ET-1 receptor antagonists on hPASMCs proliferations in control and PH patients

Globally, when considering each treatment presented in Figure 4, proliferation of hPASMCs derived from PH patients (-18±03%) were significantly less inhibited in comparison with those from control patients (-28±03%; P= 0.014).

In hPASMCs from control patients, single agent treatment with treprostinil (1 µM) or iloprost (1 nM / 1 µM) statistically significantly decreased proliferation to a similar degree (about -25%, Figure 4A). In hPASMCs preparations from PH Group-III patients, single agent treatment with 1 µM, but not 1 nM of treprosinil or iloprost statistically significantly decreased proliferation (-24±06% and -19±06%, respectively; Figure 4B).

In both control and PH patient hPASMCs, neither BQ123 (1 µM) nor BQ788 (1 µM) as single agent treatment caused a statistically significant inhibition of proliferation. In addition, combined treatments with treprostinil (1 µM) +BQ123 (1 µM) or +BQ788 (1 µM) did not increase the inhibition of hPASMCs proliferation observed with treprostinil alone. In contrast, combination treatments including iloprost (1 nM) +BQ123 (1 µM) showed an increased inhibition of proliferation in both control and PH patients (-48±14, -32±06, respectively, $P<0.1$) versus iloprost (1 nM) alone. These results suggest an additive effect of combining iloprost with the $ET_A$ receptor antagonist BQ123 (Figures 4).

## 4. Discussion

In the present study, first we characterized the ET-1 pathway for receptor expression and responsiveness in preparations from PH Group-III patients. Our results indicated an increased $ET_A/ET_B$ ratio in HPA preparations derived from PH Group-III patients, yet vascular contraction induced by ET-1 was not impacted (Figures 1, 2). We also showed that mostly incubation with an $ET_B$ receptor antagonist significantly increased ET-1 production in pulmonary arteries and parenchyma from PH Group-III patients, an effect that is magnified in pulmonary arteries when co-incubated with treprostinil (Figure 3). In the second part of our study, co-incubation of an $ET_A$ receptor antagonist with iloprost suggested additive inhibition of hPASMCs proliferation from both control and PH patients (Figure 4).

Under physiological conditions, the release of vasodilator and anti-proliferative mediators by $ET_B$ receptor activation balances the vasoconstrictor and proliferative effects of $ET_A$ receptor. However, this fine balance between $ET_A$ and $ET_B$ receptor-mediated effects is disrupted and transformed into detrimental effects in pathological conditions [41]. Increased $ET_A/ET_B$ receptor expression ratio results in enhanced proliferative effects mediated by ET-1

and may play a role in PH pathogenesis. Moreover, since $ET_B$ receptor is also responsible for ET-1 clearance in the circulation, disruption of functional $ET_B$ receptor promotes increased levels of ET-1 and results in enhanced adverse effects on vascular tone and proliferation [41]. In the present report, we demonstrated decreased $ET_B$ receptor expression and greater ratio of $ET_A/ET_B$ receptor expression in HPA derived from PH Group-III patients (Figure 1). A similar observation was recently published for lung tissue derived from patients with idiopathic pulmonary fibrosis who have a high prevalence of PH Group-III [42]. On the other hand, the studies performed on the other types of PH reported that there is an increase of $ET_B$ receptor expression or mRNA levels in the lungs, pulmonary artery or hPASMCs of patients with PH Group I and IV, while others demonstrated $ET_B$ mRNA levels was decreased in hPASMCs of PH Group-I [26, 43-46]. These contradictory results could be due to etiology, stage of disease, treatments, age of the patients and different experimental techniques.

Even though we have demonstrated a higher $ET_A/ET_B$ receptor expression ratio in PH Group-III patients, the contraction induced by ET-1 on HPA and HPV was not different between control and PH Group-III patients (Figure 2). In accordance with our findings, there was no change in ET-1 induced contraction in PH Group-I models of rats with decreased $ET_B$ receptor expression in pulmonary arteries [47]. Furthermore, the signalling mechanisms of the contractile responses to ET-1 of PH Group-I and control hPASMCs were very similar [48]. The decreased expression of the $ET_B$ receptor appears to not have an impact on vasoconstriction induced by ET-1 in isolated lung vasculature from PH Group-III patients. We also demonstrated that contractions induced by ET-1 were greater for HPV than for HPA, consistent with results previously described by our group in these human vessels [49, 50], by other research groups in many mammalian pulmonary vessels and also in human internal mammary arteries/veins [51, 52].

In the present study, treatments with ERAs significantly increased ET-1 concentrations in HPA preparations. In our results, the selective $ET_B$ receptor antagonist (BQ788) significantly increased ET-1 levels in both HPA and lung parenchyma preparations obtained from PH Group-III patients. On the other hand, the selective $ET_A$ receptor antagonist (BQ123) only statistically increased ET-1 levels in HPA, this unexpected result and a role in clearance for $ET_A$ should be confirmed (Figure 3). Our results may not only involve endothelial cells but also other cells present in the lung preparations, and a previous study suggested that ET-1 clearance occurs in both endothelial cells and hPASMCs [53]. Several studies have shown elevated levels of ET-1 in plasma and HPA derived from PH Group-III patients [13-15, 54]. Given our results, $ET_B$ receptor antagonism may not desirable in the context of PH Group-III patients who have already increased ET-1 levels. In fact, in accordance with our *in vitro* results, clinical studies in non-

PH Group-III patients demonstrated that treatment with a non-selective ERA (bosentan) increased plasma ET-1 levels, an effect not observed with a selective $ET_A$ receptor antagonist (sitaxentan) [55, 56]. Furthermore, a double-blind trial in PH Group-I patients indicated that sitaxentan therapy showed significant benefit over bosentan with respect to discontinuation of monotherapy, clinical worsening and survival rate [57]. However, the randomized control trials with ERA conducted in PH Group-III patients were limited and with results depending on severity of disease and duration of treatments. Twelve-weeks of treatment with bosentan did not improve the hemodynamic parameters [58], while longer-term treatment increased activity of daily living and 6MWD, overall survival, as well as decreasing pulmonary artery pressure and vascular resistance [16, 17].

Other treatments for PH include $PGI_2$ mimetics because of their vasodilator and anti-proliferative properties. Since both $PGI_2$ and ET-1 pathways are involved in the pathogenesis of PH Group-III, combination therapy targeting both pathways is indicated [59]. In our study the increased ET-1 level observed in lung preparations incubated with BQ788 was not significantly different from the co-incubation of BQ788 with treprostinil (Figure 3). However, in the presence of this increased ET-1 production in hPASMCs derived from PH patients, the $PGI_2$ analogues were still able to significantly reduce proliferations by 24% (Figure 4B).

In hPASMCs derived from control patients, single agent treatment with treprostinil (1 µM) or iloprost (1 nM or 1 µM) statistically significantly decreased proliferation (Figure 4A). In our assay, iloprost was slightly more effective than treprostinil when used at a low concentration (1 nM; Figure 4). However, a slightly greater anti-proliferative potency of treprostinil (1 nM) versus iloprost (1 nM) was previously demonstrated [60]. This discrepancy could be due to experimental variability or to the prior study's use of smaller diameter pulmonary vessels where a greater density of EP2 receptor or PPARγ expression might also contribute to treprostinil's anti-proliferative activity [32, 61]. In fact, it has been already shown that there is a clear difference in inhibition of hPASMCs proliferation by iloprost and cicaprost (a selective IP agonist) depending on whether distal or proximal pulmonary artery is used [62]. Single agent treatment of hPASMCs with ERAs did not statistically inhibit proliferation in our study. However, other published results with higher doses (10 µM) of cicaprost or BQ123 showed anti-proliferative effects on hPASMCs derived from control patients (about -30%)[63, 64]. Of note, hPASMCs are able to release ET-1 and this release could be stimulated by ET-1 from hPASMCs in a concentration-dependent fashion[63, 64]. In addition, the inhibitor of endothelin converting enzyme (phosphoramidon), which is responsible for ET-1 formation, reduced

the proliferation induced by FCS in hPASMCs and that is confirming an autocrine role for ET-1[63].

In hPASMCs derived from PH Group-III patients, iloprost and treprostinil behaved similarly when comparing their anti-proliferative effects, with only the 1 µM concentrations showing a statistically significant anti-proliferative effect (Figure 4). This finding is similar to the inhibitory effects observed in hPASMCs proliferations derived from children with idiopathic PAH [32]. The decreased anti-proliferative potency of iloprost in PH Group-III versus control patients could be due to a reduced IP receptor expression in the hPASMCs, as was observed in PH Group-I patients [32]. Interestingly, incubating hPASMCs derived from both groups of patients with iloprost (1 nM) and the $ET_A$ receptor antagonist BQ123 resulted in a strong tendency toward additive inhibition (Figure 4). In contrast the anti-proliferative effect of treprostinil in hPASMCs was not modified in the presence of either ERA.

In this study, we determined cell proliferation by MTT assay. Even though this technique is widely used, it has some disadvantages. Other cell proliferation techniques such as detection of proliferating nuclear antigen, cyclin D-E or cell counts could be performed to support MTT results. However, insufficient supply of human pulmonary artery is an important factor that limits performing these experiments. This is a limitation of our study and remains to be readdressed in further studies.

**Conclusion**

Despite the fact that PH Group-III is the most common and lethal form of PH [9], clinical studies on these patients have been limited and no conventional PH therapies are approved for use in this patient population [65]. In the present study, we used several tissues derived from PH Group-III patients including HPA, HPV, lung parenchyma and hPASMCs. The *in vitro* results presented support that the increased $ET_A/ET_B$ receptor expression ratio in HPA may be involved in the pathogenesis of PH Group-III, in ways not impacting *ex vivo* pulmonary vascular tone induced by ET-1. Using selective ERA antagonists, our results support mostly a role for $ET_B$ receptor regulation of ET-1 production in pulmonary tissue; a role in which $ET_B$ receptor antagonism or downregulation would lead to a detrimental increase in ET-1 concentrations. In addition, whereas both iloprost and treprostinil individually inhibited proliferation of hPASMCs from either patient group, the combination of the selective $ET_A$ receptor antagonist BQ123 and iloprost produced a greater tendency to inhibit hPASMCs proliferation derived from both groups of patients. This combination therapy is currently recommended for the treatment of PH Group-I in the guidelines of ESC/ERS [8], and our findings will hopefully provide

additional mechanistic information to consider when treating (severe) PH Group-III patients with a combination of a $PGI_2$ analogue and a selective $ET_A$ receptor antagonist.

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## Figure Legends

### Figure 1

**$ET_A$ and $ET_B$ receptor expression (Figure 1A and 1C) and $ET_A/ET_B$ receptor expression ratio (Figure 1B) in human pulmonary artery (HPA) derived from control and pulmonary hypertension (PH) Group-III patients**. Western blot analyses for endothelin receptors ($ET_A$ and $ET_B$) were normalized by β–actin in human preparations and then $ET_A/ET_B$ receptor expression ratios were calculated. Values are means ± SEM, n=4-7 patients. *Data significantly different between control and PH patient groups ($P<0.05$). A representative image of Western blot is presented in Figure 1C.

### Figure 2

**Contraction induced by endothelin-1 (ET-1) in human pulmonary arteries (HPA) and human pulmonary veins (HPV) derived from control and pulmonary hypertension (PH) Group-III patients**. Concentration-response curves for ET-1-induced contraction. Responses are expressed as a percentage of contraction induced by norepinephrine (NE, 10 µM). Values are means ± SEM, n=3-4 patients in each group. *Data significantly different from HPV for respective patient groups ($P<0.05$).

### Figure 3

**Endothelin-1 (ET-1) content in human pulmonary arteries (HPA, Figure 3A) and lung parenchyma (Figure 3B) preparations derived from pulmonary hypertension (PH) Group-III patients after different treatments.** Human preparations were incubated with $PGI_2$ analogue (TRP, treprostinil, 1 µM) and/or $ET_A$ receptor antagonist (BQ123, 1 µM), $ET_B$ receptor antagonist (BQ788, 1 µM). Black bars indicate human preparations without any treatment; white bars indicate human preparations with single treatment and lined bars indicate human preparations with combination treatment. The concentration of ET-1 in organ culture supernatant after 12h incubation was expressed as pg/mg of protein (A) or pg/mg of tissue (B). * indicates values significantly different ($P<0.05$). Values are means ± SEM, n=7-11 (HPA) or 3-4 (parenchyma) patients.

### Figure 4

**Proliferation of human pulmonary artery smooth muscle cells (hPASMCs) derived from control (Figure 4A) and pulmonary hypertension Group-III patients (PH, Figure 4B) after different treatments.** hPASMCs were incubated with $PGI_2$ analogues (TRP: treprostinil,

ILO: iloprost) and/or $ET_A$ receptor antagonist (BQ123), $ET_B$ receptor antagonist (BQ788). Black bars indicate human preparations without any treatment; white bars indicate human preparations with single treatment and lined bars indicate human preparations with combination treatment. The cell numbers are calculated as % of control (without any treatment). * indicates values significantly different versus control, # indicates values significantly different versus treprostinil (1 nM), † indicates values significantly different ($P<0.05$) versus BQ123 (1 µM), § indicates values significantly different versus BQ788 (1 µM) for respective group. Co-treatment with iloprost (1 nM) + BQ123 in Control group and with iloprost (1 nM) + BQ788 or + BQ123 in PH Group showed greater inhibition of proliferation versus respective iloprost (1 nM) incubation alone ($P<0.1$). Values are means ± SEM, n=6 Control and 5 PH patients.

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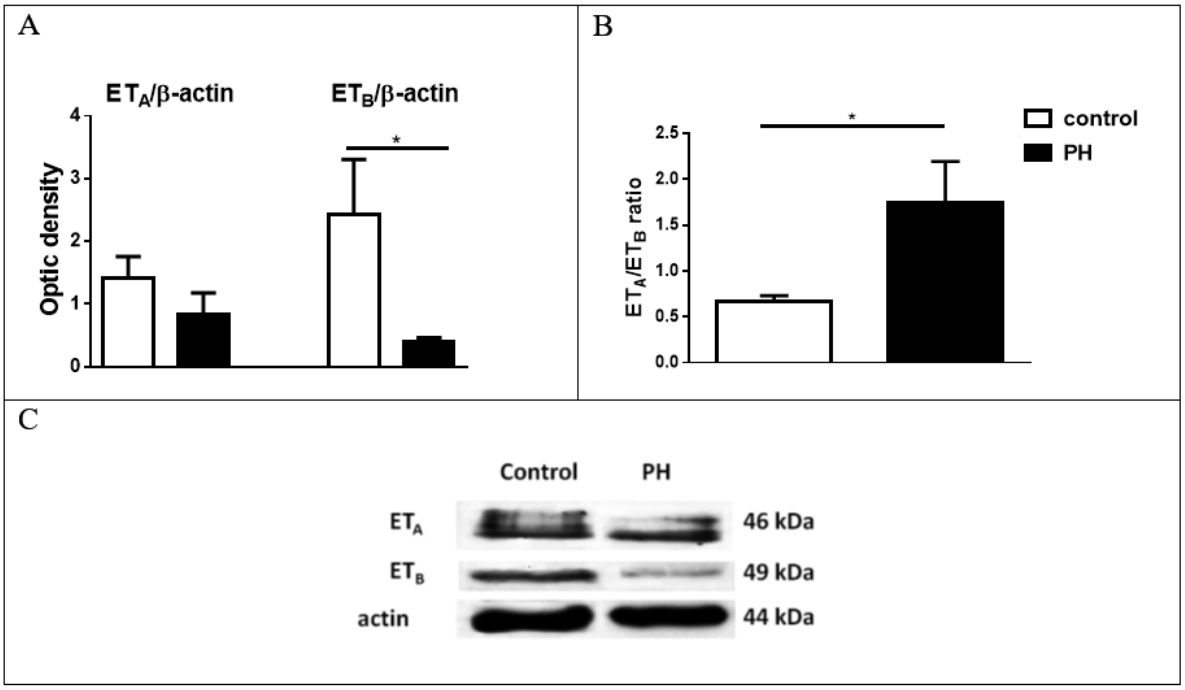
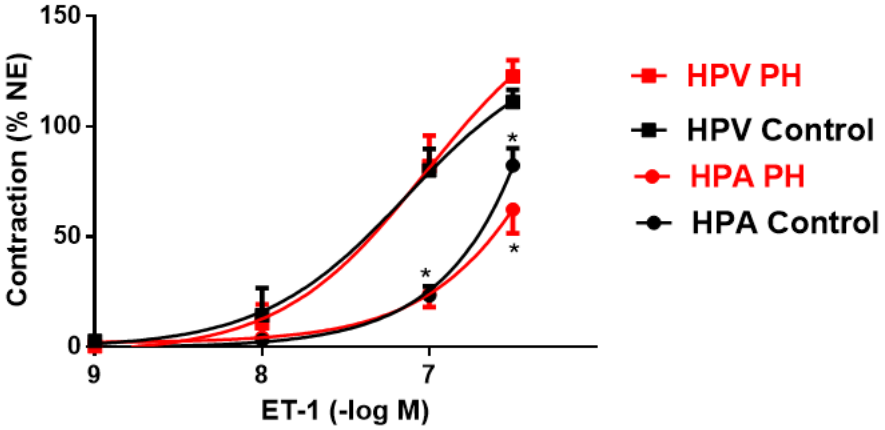
**Figure 1**



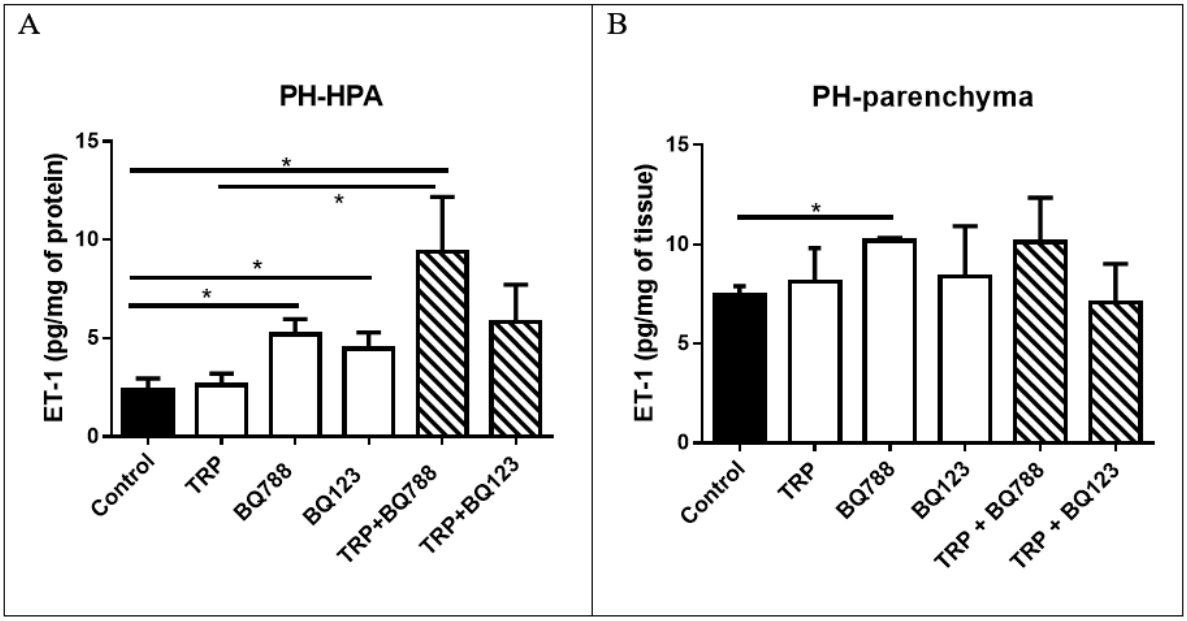

**$ET_A$ and $ET_B$ receptor expression (Figure 1A and 1C) and $ET_A/ET_B$ receptor expression ratio (Figure 1B) in human pulmonary artery (HPA) derived from control and pulmonary hypertension (PH) Group-III patients**. Western blot analyses for endothelin receptors ($ET_A$ and $ET_B$) were normalized by β–actin in human preparations and then $ET_A/ET_B$ receptor expression ratios were calculated. Values are means ± SEM, n=4-7 patients. *Data significantly different between control and PH patient groups ($P<0.05$). A representative image of Western blot is presented in Figure 1C.

**Figure 2**



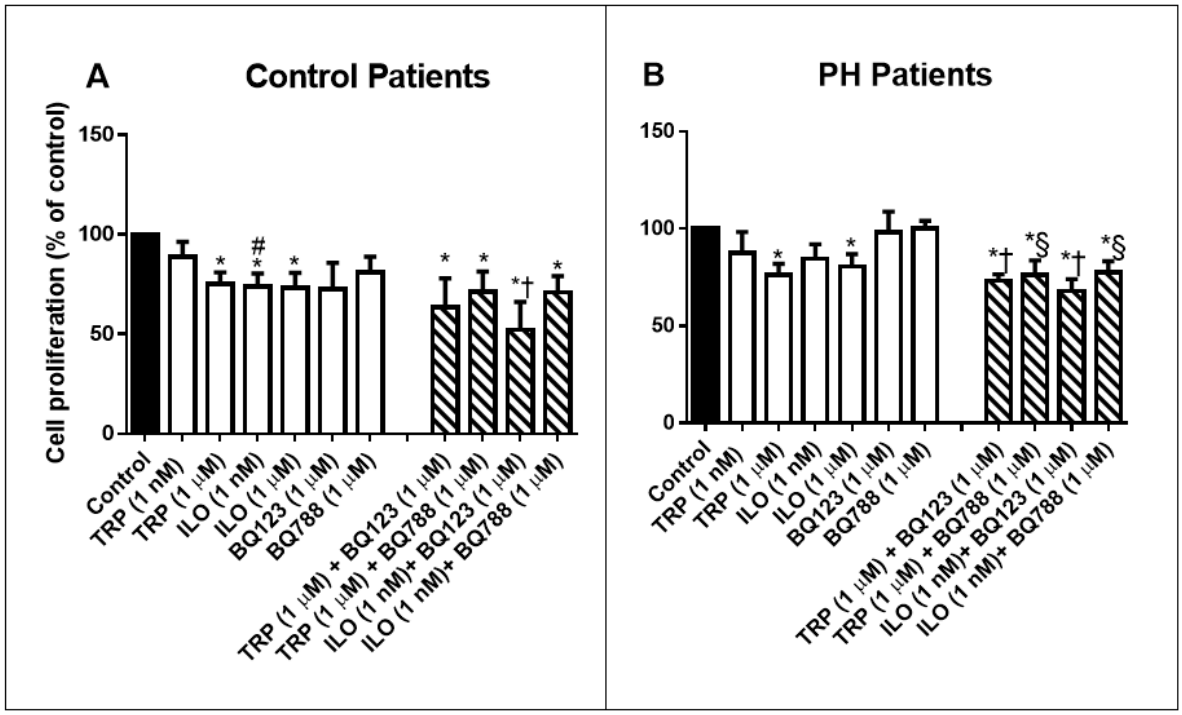

**Contraction induced by endothelin-1 (ET-1) in human pulmonary arteries (HPA) and human pulmonary veins (HPV) derived from control and pulmonary hypertension (PH) Group-III patients**. Concentration-response curves for ET-1-induced contraction. Responses are expressed as a percentage of contraction induced by norepinephrine (NE, 10 μM). Values are means ± SEM, n=3-4 patients in each group. *Data significantly different from HPV for respective patient groups ($P<0.05$).

**Figure 3**




**Endothelin-1 (ET-1) content in human pulmonary arteries (HPA, Figure 3A) and lung parenchyma (Figure 3B) preparations derived from pulmonary hypertension (PH) Group-III patients after different treatments.** Human preparations were incubated with $PGI_2$ analogue (TRP, treprostinil, 1 μM) and/or $ET_A$ receptor antagonist (BQ123, 1 μM), $ET_B$ receptor antagonist (BQ788, 1 μM). Black bars indicate human preparations without any treatment; white bars indicate human preparations with single treatment and lined bars indicate human preparations with combination treatment. The concentration of ET-1 in organ culture supernatant after 12h incubation was expressed as pg/mg of protein (A) or pg/mg of tissue (B). * indicates values significantly different ($P<0.05$). Values are means ± SEM, n=7-11 (HPA) or 3-4 (parenchyma) patients.

**Figure 4**




**Proliferation of human pulmonary artery smooth muscle cells (hPASMCs) derived from control (Figure 4A) and pulmonary hypertension Group-III patients (PH, Figure 4B) after different treatments.** hPASMCs were incubated with $PGI_2$ analogues (TRP: treprostinil, ILO: iloprost) and/or $ET_A$ receptor antagonist (BQ123), $ET_B$ receptor antagonist (BQ788). Black bars indicate human preparations without any treatment; white bars indicate human preparations with single treatment and lined bars indicate human preparations with combination treatment. The cell numbers are calculated as % of control (without any treatment). * indicates values significantly different versus control, # indicates values significantly different versus treprostinil (1 nM), † indicates values significantly different ($P<0.05$) versus BQ123 (1 μM), § indicates values significantly different versus BQ788 (1 μM) for respective group. Co-treatment with iloprost (1 nM) + BQ123 in Control group and with iloprost (1 nM) + BQ788 or + BQ123 in PH Group showed greater inhibition of proliferation versus respective iloprost (1 nM) incubation alone ($P<0.1$). Values are means ± SEM, n=6 Control and 5 PH patients.