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ICAM-1 promotes the abnormal endothelial cell phenotype in chronic thromboembolic pulmonary hypertension

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KEYWORDS:

Chronic thromboembolic hypertension;
Endothelial dysfunction;
ICAM-1;
Adhesion molecules;
Survival signalling pathway

BACKGROUND: Pulmonary endothelial cells play a key role in the pathogenesis of Chronic Thromboembolic Pulmonary Hypertension (CTEPH). Increased synthesis and/or the release of intercellular adhesion molecule-1 (ICAM-1) by pulmonary endothelial cells of patients with CTEPH has been recently reported, suggesting a potential role for ICAM-1 in CTEPH.

METHODS: We studied pulmonary endarterectomy specimens from 172 patients with CTEPH and pulmonary artery specimens from 97 controls undergoing lobectomy for low-stage cancer without metastasis.

RESULTS: ICAM-1 was overexpressed in vitro in isolated and cultured endothelial cells from endarterectomy specimens. Endothelial cell growth and apoptosis resistance were significantly higher in CTEPH specimens than in the controls ($p < 0.001$). Both abnormalities were abolished by pharmacological inhibition of ICAM-1 synthesis or activity. The overexpression of ICAM-1 contributed to the acquisition and maintenance of abnormal EC growth and apoptosis resistance via the phosphorylation of SRC, p38 and ERK1/2 and the overproduction of survivin. Regarding the ICAM-1 E469K polymorphism, the KE heterozygote genotype was significantly more frequent in CTEPH than in the controls, but it was not associated with disease severity among patients with CTEPH.

CONCLUSIONS: ICAM-1 contributes to maintaining the abnormal endothelial cell phenotype in CTEPH.

Chronic thromboembolic pulmonary hypertension (CTEPH) is a subtype of pulmonary hypertension (PH) resulting from the obstruction of the pulmonary vascular bed by non-resolving thromboemboli.^{1–3} The prevalence of symptomatic CTEPH following acute pulmonary embolism varies from 0.6% to 4.6%.^{3–5}

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The failure to resolve pulmonary emboli is a complex, multifactorial process in which endothelial cells play a central role.^{6–10} We previously reported the abnormal phenotype of pulmonary endothelial cells (PEC) directly in contact with the thrombus and demonstrated its role in the persistence and remodeling of the thrombus.⁹ In a targeted screen, we analyzed the levels of markers reported to be increased in CTEPH or to be involved in the pathogenesis of pulmonary arterial hypertension in PEC media, including growth factors, inflammatory cytokines, and cell adhesion molecules.^{11–14} Interestingly, soluble ICAM-1 levels in PEC culture supernatants exhibited the highest increase in CTEPH patients compared with the controls.⁹

The ICAM-1 protein belongs to the immunoglobulin superfamily synthesized by endothelial cells.¹⁵ It promotes the adhesion and transmigration of circulating leukocytes by binding to and activating surface leukocyte function-associated antigen 1 (LFA-1),^{16,17} an $\alpha\beta$ heterodimeric transmembrane glycoprotein consisting of an α L subunit (CD11a) and a β 2 subunit (CD18) that physically link extracellular ligands to the cytoskeleton. The interaction between LFA-1 and ICAM-1 is important for several cellular events, including the regulation of leukocyte migration from the blood into the tissues and intercellular communication during the specific immune response. The ability of ICAM-1 to induce PEC proliferation is directly related to the level of gene expression, which is controlled by environmental and genetic factors. Inflammation is a strong inducer of ICAM-1 expression. The gene expression levels are also determined genetically via a single nucleotide polymorphism in ICAM-1 exon 6 (E469K).¹⁸ The relationship between E469K and heart disease has been documented in atherosclerosis, coronary artery disease, and preeclampsia,^{19–22} and E469K may play a very early role in the thrombotic process.

In this study, we hypothesized that pulmonary endothelial ICAM-1 contributes to the dysfunctional PEC phenotype, contributing to CTEPH pathophysiology. To test this, we investigated (1) *in situ* ICAM-1 protein expression in CTEPH pulmonary endarterectomy specimens and control pulmonary arteries; (2) ICAM-1 mRNA and protein levels in cultured endothelial cells from CTEPH and control patients *in vitro*; (3) the consequences of ICAM-1 inhibition on endothelial cell function; and (4) the frequency and clinical correlates of the ICAM-1 KE polymorphism in CTEPH patients.

Methods

Ethics statement

The Marie Lannelongue Hospital Institutional Review Board and the local ethics committee (CPP Ile-de-France, Le Kremlin-Bicêtre: C0-09-015; Biobanking declaration: DC-2009-1032) approved this study that was conducted in accordance with the amended Declaration of Helsinki. All patients gave written informed consent.

Study population and samples collected

This study included 172 operable CTEPH patients undergoing pulmonary endarterectomy at Marie Lannelongue Hospital (France)

and 97 controls (Figure 1). The control subjects underwent lobectomy for stage 1 lung cancer without metastasis or PH on pre-operative echocardiography (i.e., right ventricular systolic pressure < 35 mm Hg estimated from the tricuspid regurgitation maximal velocity). Endarterectomy specimens were collected from CTEPH patients; for controls, pulmonary artery rings were collected as far as possible from the tumor. Pre-operative peripheral blood samples were collected from both groups.

Pulmonary endothelial cells isolation and culture

PEC isolated by enzymatic digestion of endarterectomy or pulmonary arterial specimens from a randomly generated subset of 20 CTEPH and 20 control patients

Pharmacological inhibition of ICAM-1

We assessed the effects of the ICAM-1-induced activation of downstream effector proteins, particularly on endothelial cell growth and apoptosis resistance. The PECs were seeded in 96-well plates for the evaluation of proliferation or 6-well plates for apoptosis and Western blot analyses; the wells were pre-coated with 0.1% gelatin and the cells seeded at different densities, depending on the experiment. The PECs were allowed to grow for 24 hours and then serum-starved (1% serum) in MCDB131 medium for 24 hours. Subsequently, they were incubated with 50 or 500 nM of each of two ICAM-1 inhibitors, namely A205804 (I1, inhibits ICAM-1 synthesis)^{23–25} and A286982 (I2, inhibits ICAM-1 activity as an LFA-1 antagonist)²⁶ (Tocris, Bio-technie, France) for 24 hours in MCDB131 medium. Doses of the compounds were chosen according to the literature and the IC₅₀.^{23–26} As a positive control, the cells were treated with 5% fetal calf serum (FCS) for 24 hours.

Functional analyses

Proliferation and apoptosis were assessed in the PECs from both groups ($n = 7$ in each group; each condition was analyzed in triplicate). The cells were seeded at a density of 5×10^3 cells per well. After 24 hours of starvation (1% serum), the cells were treated for 24 hours with two doses (50 and 500 nM) of I1 or I2 or 5% FCS. Cell growth was measured using the DELFIA Cell Proliferation Kit (PerkinElmer, Courtaboeuf, France) and a time-resolved fluorometer EnVision™ multilabel reader (PerkinElmer). The same protocol was also performed, but the PECs were maintained for 72 hours and then counted by a cell counter with trypan blue dye exclusion.

To assess apoptosis resistance, the PECs were seeded at a density of 5×10^4 cells per well. After 24 hours of starvation (1% serum), the cells were incubated with I1 or I2 (50 and 500 nM) for 24 hours. Floating cells were collected and combined with adherent cells harvested by trypsin/EDTA treatment and stained with annexin V-FITC (BD Biosciences, Pont de Claix, France), and then analyzed by flow cytometry using a MACSQuant (Miltenyi Biotec). At least, 20,000 cells in each sample were counted by FACS analysis; additionally, floating cells were collected and combined with adherent cells harvested by trypsin/EDTA treatment, stained with Hoechst, and then analyzed by flow cytometry using a MACSQuant (Miltenyi Biotec).

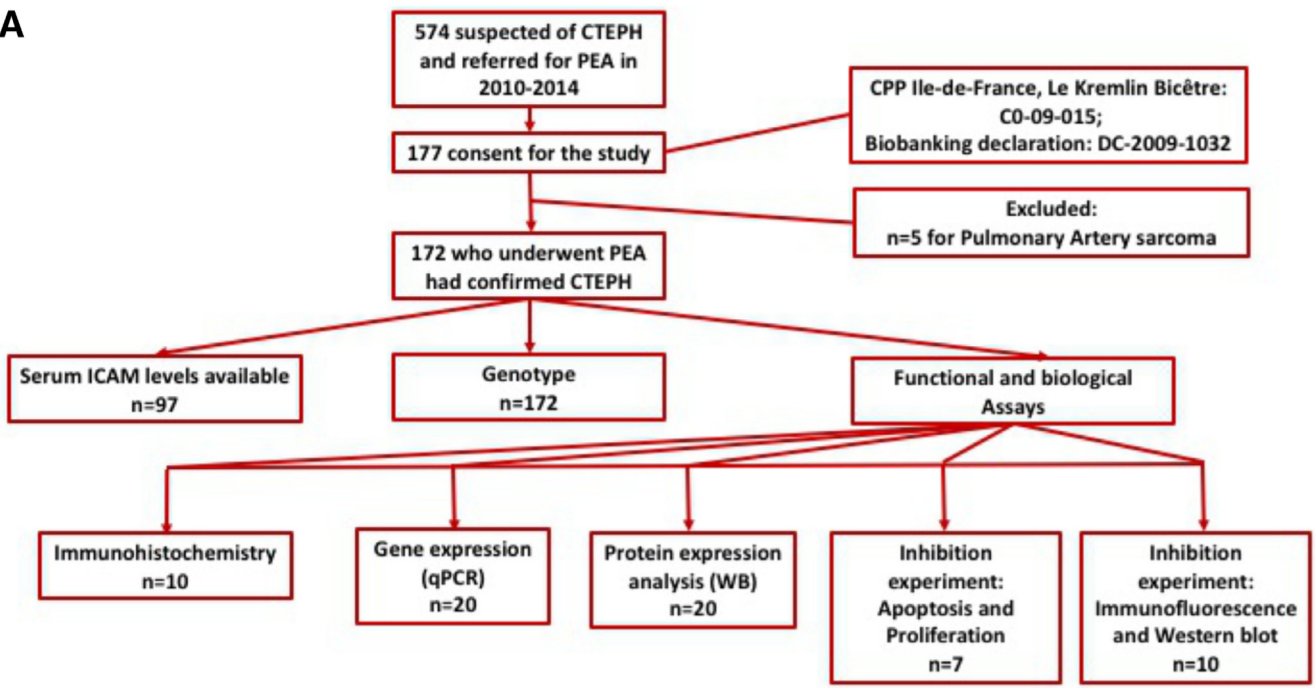
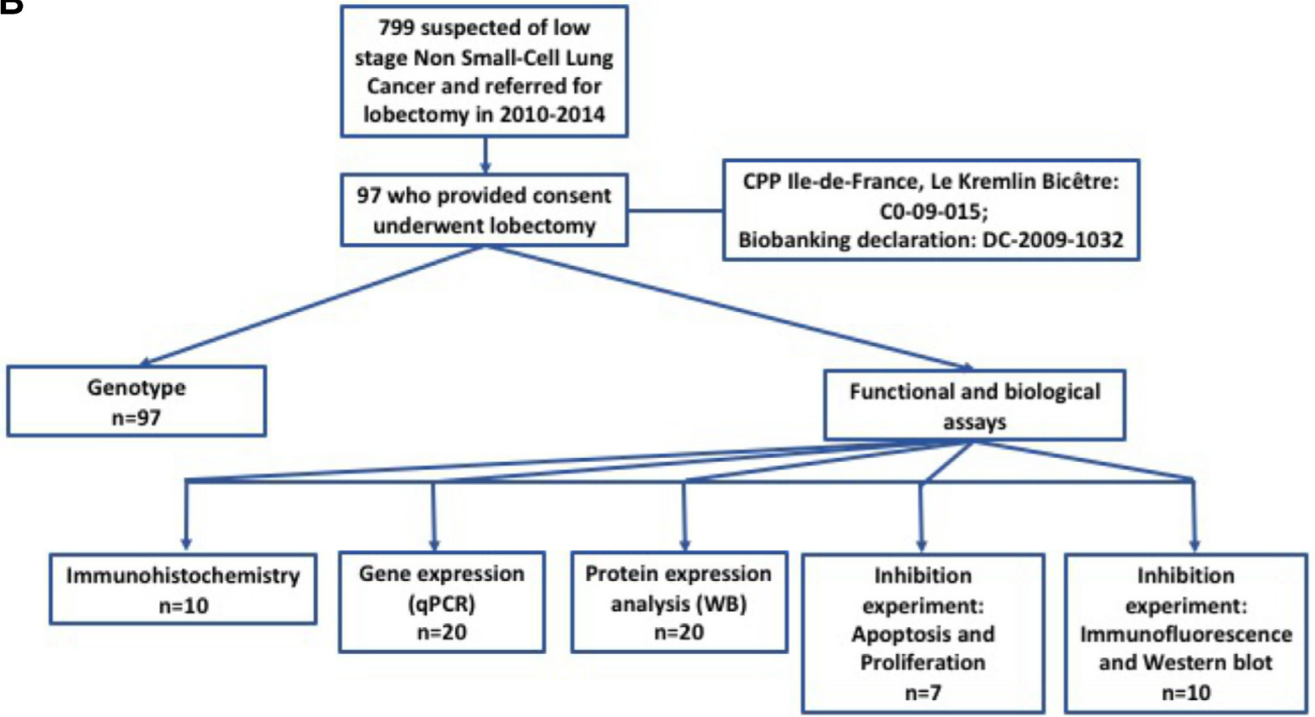
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Figure 1 Flowchart representing the study design, divided into (A) patients with CTEPH and (B) control subjects. CTEPH, Chronic Thromboembolic Pulmonary Hypertension; ICAM, Intercellular adhesion molecule; PEA, Pulmonary endarterectomy; WB, Western blotting.

To explore the effects of endothelial cell-derived ICAM-1, human peripheral blood mononuclear cells were isolated from the whole blood of healthy donors (n=5) using Ficoll density gradient centrifugation followed by Percoll density gradient centrifugation to isolate monocytes. Briefly, monocytes (1×10^4 per well) were seeded in the upper chamber and endothelial cells from controls or CTEPH patients (5×10^4

per well) in the lower chamber (n=5 in each group). Endothelial cells were incubated in the presence of I1, I2, or vehicle during 24 hours. The migratory cells that have moved through the pores toward the bottom wells were stained and quantified. The mean number from 10 randomly chosen high-power ($\times 400$) fields on the undersurface of the filter was computed.

Gene and protein expression analyses

The levels of ICAM-1 and LFA-1 mRNA in the PECs were measured by real-time quantitative PCR ($n=20$ samples per group). Total RNA was extracted using phenol-chloroform, and its concentrations were determined by spectrophotometry. Reverse transcription was performed using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). TaqMan primers for ICAM-1 and LFA-1 (Applied Biosystems, Foster City, CA) were used for qRT-PCR. Relative quantification was calculated by normalizing the Ct (threshold cycle) of the gene of interest to the Ct of 18S rRNA in the same sample, using the comparative $\Delta\Delta Ct$ method.

The quantification of ICAM-1 and LFA-1 was performed by Western blotting in PECs from 20 CTEPH and 20 controls. The effects of ICAM-1-induced activation of downstream effectors were assessed. Steady-state levels of SRC, p38, and ERK phosphorylation, as well as survivin levels, were estimated by immunoblotting. PECs were seeded at a density of 1×10^5 cells per well and allowed to grow in the same medium for 24 hours. After 24 hours of starvation (1% serum), cells were treated with I1 or I2 (50 or 500 nM) for 24 hours, washed twice with ice-cold phosphate-buffered saline (PBS), scraped off the wells, pelleted, lysed, and sonicated in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail. Then, 20 μg of total protein was used to detect ICAM-1, CD11a, CD18 (Santa Cruz Biotechnology, Santa Cruz, CA), SRC, p-SRC, ERK, p-ERK, p38, phospho-p38, survivin (Cell signaling), and β -actin (Sigma-Aldrich, St Louis, MO).

Immunostaining

Paraffin sections (5 μm) were cut from pulmonary endarterectomy specimens and fixed with 4% paraformaldehyde, dewaxed in 100% toluene, and rehydrated by immersion in decreasing ethanol concentrations (100%, 95%, and 70%) followed by distilled water.

In each group, the PECs from 10 individuals were seeded at a density of 1×10^5 cells per well in 6-well plates pre-coated with 0.1% gelatin, covered with glass cover slides, and allowed to grow in the same medium for 24 hours. Following starvation (1% serum) in MCDB131 medium for the next 24 hours, the PECs were treated or not with increasing I1 or I2 concentrations, washed twice with ice-cold PBS, and fixed with methanol. Cells were not permeabilized to avoid staining the intracellular adhesion molecules. After blocking with PBS containing 1% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20, the sections or glass cover slides from both groups were incubated at 4°C overnight with anti-human ICAM-1, CD11a, CD18 monoclonal antibodies (1/200, Santa Cruz Biotechnology), anti-human platelet and endothelial cell adhesion molecule 1 PECAM-1/CD31 monoclonal antibody (1/40, Dako, Agilent, Santa Clara, CA), anti-human α -actin (1/200, Santa Cruz Biotechnology), SRC, ERK, p38, and survivin (Cell Signaling). The slides were then incubated with anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 597 secondary antibodies (Thermo Fisher Scientific, Waltham, MA) for 1 hour. The nuclei were counterstained using 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) according to the manufacturer's recommendations. The slides were imaged using epifluorescence microscopy (Nikon, Minato, Tokyo, Japan) and an LSM700 confocal microscope (Zeiss, Marly Le Roi, France). The mean fluorescence intensity was quantified using Zen software (Zeiss) or ImageJ (National Institutes of Health, Bethesda, MD).

ICAM-1 genotype analysis

The ICAM-1 genotype was determined by PCR using genomic DNA²² from tissue specimens for the CTEPH group and peripheral blood for the control group (owing to limited tissue sample size).

Pre- and peri-operative characteristics

Pre-operative demographics, CTEPH risk factors, PH-specific therapy, functional status (New York Heart Association class, six-minute walk test distance), hemodynamics using catheterization, and pulmonary functional tests were collected. The thrombus type and location were classified according to Jamieson's classification²⁷ and the recently proposed Madani's classification^{28,29} are presented in the Supplementary Methods online. Post-operative intrahospital outcomes (including all-cause death or lung transplantation) were also collected.

Statistical analysis

Continuous variables are described as the means \pm standard deviation or median and interquartile ranges, according to the distribution (normality was tested using the Kolmogorov-Smirnov test) and categorical variables as the number (%). Continuous variables were compared using the Student's t-test or a two-way analysis of variance if normally distributed, or the Mann-Whitney or Kruskal–Wallis test if not (with Bonferroni post-hoc correction). Categorical variables were compared using the chi-square test. Statistical analyses were performed using GraphPad Software (La Jolla, CA) or SPSS software V.24 (IBM, Chicago, IL). p values <0.05 were considered statistically significant.

Results

Endothelial localization of ICAM-1 in the CTEPH specimens and control

The cell surface expression of ICAM-1 in pulmonary artery specimens from 10 CTEPH and 10 controls were identified by confocal microscopy. In both groups, ICAM-1 colocalized with CD31 but not smooth muscle alpha-actin (Figure 2A and B), demonstrating the presence of ICAM-1 in endothelial cells but not in smooth muscle cells. The fluorescence staining of ICAM-1 was higher in paraffin-embedded specimens of CTEPH patients than in control endothelial cells (Figure 2C). The endothelial ICAM-1 staining was also stronger in pulmonary arteries from CTEPH patients (Figure 2C and D) and in neovessels observed in specimens from these patients |

I

Expression of ICAM-1 and LFA-1 in cultured PECs

The levels of ICAM-1 mRNA in PECs of CTEPH patients were 3-fold higher than in control PECs ($p < 0.01$), while the LFA-1 mRNA levels did not differ (Figure 3A). Regarding protein expression, both immunofluorescence (Figure 3B) and Western blot (Figure 3C) analyses showed higher ICAM-1 levels in the PECs from CTEPH than those

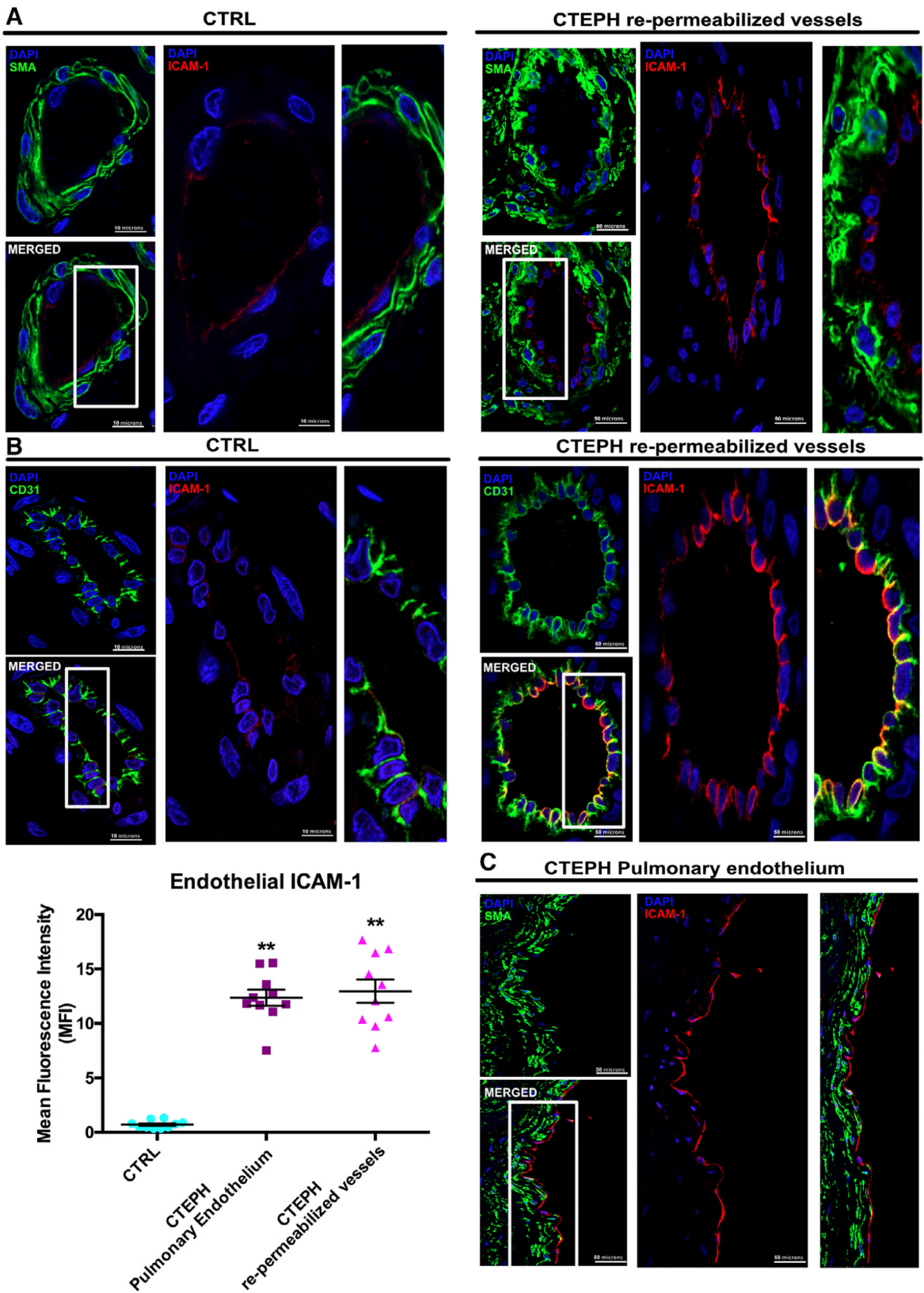


Figure 2 (A–B) Localization of ICAM-1 (red) in controls (left panels), re-permeabilized vessels (right panels), or (C) pulmonary endothelium from endarterectomy specimens from the main right pulmonary artery of patients with CTEPH, double-labeled with ICAM-1 and either α -actin or CD31 (green). Fluorescence intensity quantification of endothelial ICAM-1 in CD31-positive cells of 10 fields per subject ($n = 10$ per group). ICAM-1 immunoreactivity was strong in endothelial cells in both pulmonary endothelia and new vessel formation in the thrombi of CTEPH patients, and absent from smooth muscle cells. The bar graph shows the means \pm SEM. * $p < 0.05$; ** $p < 0.01$. DAPI = 4',6'-diamidino-2-phenylindole. No immunoreactivity was seen in the section incubated with the secondary antibody but no primary antibody. Scale bar = 50 μ m. CTEPH, Chronic Thromboembolic Pulmonary Hypertension; CTRL, control.

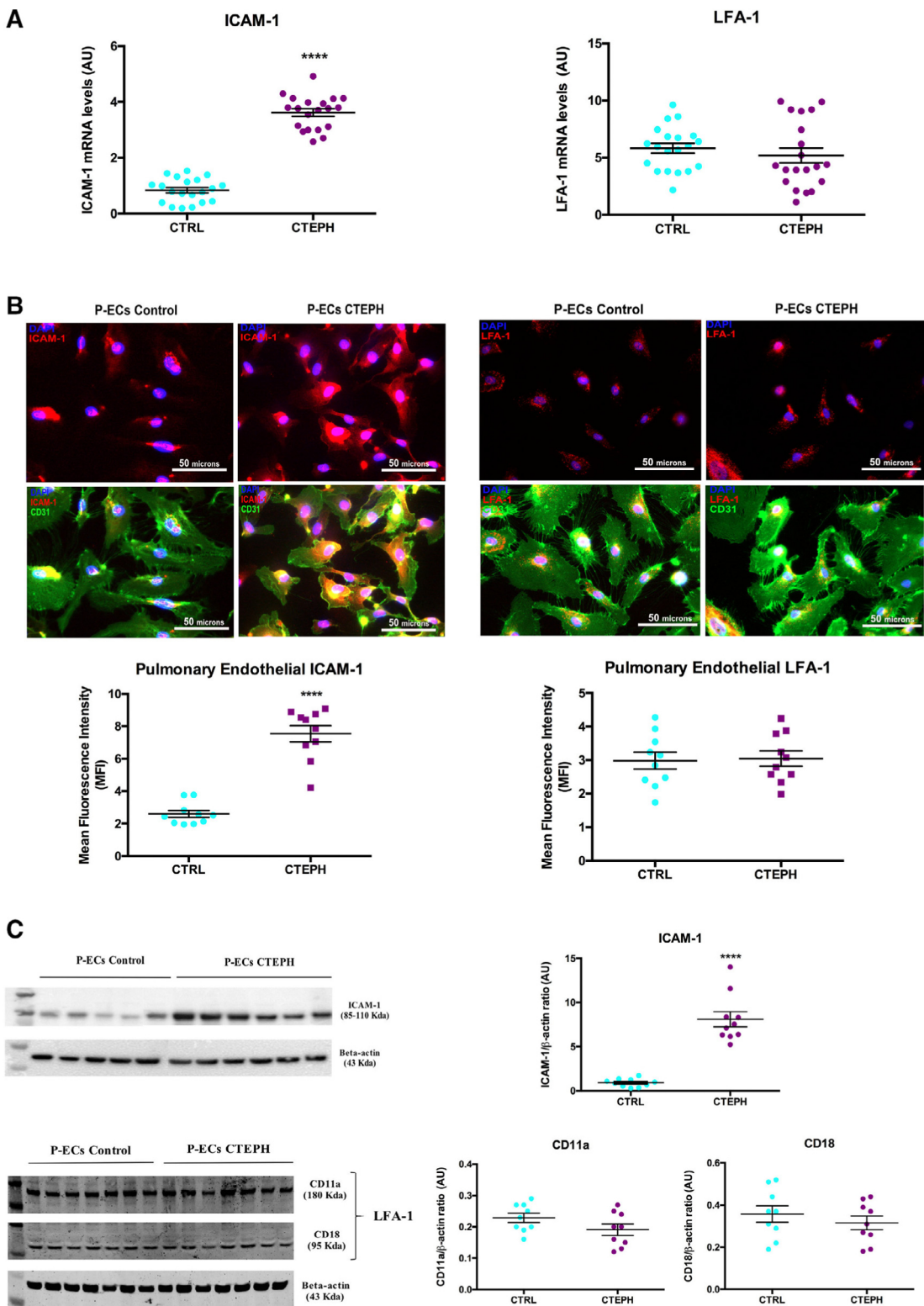


Figure 3 (A) Real-time quantitative PCR assessing the mRNA levels of ICAM-1 and its receptor LFA-1 in PECs from CTEPH or control patients. Values are presented as the means \pm SEM of values from 20 CTEPH and 20 control patients. $**p < 0.01$ for patients versus controls (using the Mann-Whitney test). (B) Expression of ICAM-1 (red) and its receptor LFA-1 (red) in cultured PECs from CTEPH or control patients by immunostaining and quantification. For immunostaining, LFA-1 was targeted using an anti-CD18 antibody. No immunoreactivity was seen in cells incubated with the secondary antibody but no primary antibody. Each bar represents the mean \pm SEM (arbitrary units) signal intensity in cells from 10 CTEPH and 10 control patients. $**p < 0.01$ compared versus controls (using the Mann-Whitney test). Scale bar = 100 μ m. (C) Immunoreactivity for ICAM-1 and the two subunits of its receptor LFA-1 (CD11a and CD18) in PECs from CTEPH and control patients and quantification of the signal in each. CTEPH, Chronic Thromboembolic Pulmonary Hypertension; CTRL, control; ICAM, Intercellular adhesion molecule; LFA-1, leukocyte function-associated antigen 1; PEC, pulmonary endothelial cells.

from control patients but similar levels of LFA-1 and its 2 subunits. Pulmonary endothelial cells from both groups exhibited similar CD31 signal intensity, suggesting that this phenomenon was specific to ICAM-1 and did not affect other endothelial cell adhesion molecules like PECAM-1/CD31.

ICAM-1 inhibition diminished growth and migration and increased PECs apoptosis

After PEC incubation with low concentrations of FCS (serum deprivation), apoptotic cell numbers were lower in untreated CTEPH samples than in the controls. Inhibiting ICAM-1 synthesis or activity dose-dependently increased the number of apoptotic cells in CTEPH to a greater extent than in the controls ($p < 0.001$) (Figure 4A and B).

Serum-deprived PECs presented a more marked increase in 5-bromo-2'-deoxyuridine (BrdU) incorporation in CTEPH than in the controls (Figure 4C and D). Pre-treating the PECs with increasing concentrations of I1 or I2 inhibited cell proliferation. When treated with the inhibitors, PECs from CTEPH patients had reduced DNA synthesis compared with the basal condition and also to the controls. These results were confirmed by cell counting. However, BrdU incorporation did not significantly differ according to the dose of ICAM-1 inhibitors, suggesting the absence of dose-dependent inhibition.

The presence of PECs in the Transwell lower chamber induced the migration of monocytes (Figure 4E). Migration was more marked in the presence of PECs from patients with CTEPH as compared with control endothelial cells (45 ± 6 vs 27 ± 4 monocytes per fields, respectively, $p < 0.01$). This effect was partially reduced in the presence of pharmacological inhibitors of ICAM-1.

Differential effects of ICAM-1 on survival signaling pathways in PECs

To assess the role of ICAM-1 in the phosphorylation and expression of survival effector proteins, we evaluated the expression of SRC, p38, ERK, and survivin in growing PECs from both groups following inhibitor treatment (Figures 5 and 6). The phosphorylation and/or expression levels of these proteins were higher in CTEPH than control PECs. Immunofluorescence for SRC, p38, ERK, and survivin decreased significantly after PEC exposure to I1 or I2 (Figures 5A and 6A). Similarly, immunoblotting (Figures 5B and 6B) showed higher protein levels in the PECs from CTEPH than from controls. Incubating PECs with either inhibitor decreased SRC, survivin, p38, and ERK phosphorylation and/or expression levels. However, there was no difference in the phosphorylated over total protein ratios, suggesting the apparent decrease in phospho-SRC, -p38, and -ERK1/2 levels was attributable to reduced protein expression rather than reduced activation.

ICAM-1 E469K polymorphism

The control group followed the Hardy-Weinberg equilibrium. The theoretical numbers of patients with the KK, KE, and EE genotypes were 55.1, 51.7, and 12.1, respectively, while the observed numbers were 58, 51, and 16, respectively ($p = 0.23$). The frequency of allele K was 0.68 (0.32 for allele E). The genotype frequency distribution (KK, KE, and EE) differed significantly in the 172 CTEPH patients compared with the controls ($p < 0.001$) (Figure 7B). The proportion of KK homozygotes was lower in CTEPH than in the controls, while the proportion of KE heterozygotes was higher in CTEPH than in the controls.

When comparing the 172 CTEPH patients according to the 3 genotypes, there was no significant difference in demographics, functional status, hemodynamics, perioperative characteristics, or post-operative outcomes (Table 1), except for longer 6-minute test distances in the KK patients among the subgroup with available data. Moreover, neither serum ICAM-1 content nor ICAM-1 endothelial cell mRNA levels differed between patients with KK vs KE or EE (respectively).

Serum ICAM-1 levels and clinical phenotypes

When dividing CTEPH patients according to pre-operative tertiles of serum ICAM-1 levels ($n = 97$), no significant difference was found in demographics, functional status, hemodynamics, therapy, or perioperative characteristics, except for a lower body mass index in the lower ICAM-1 tertile.

Discussion

We investigated the contribution of ICAM-1 to endothelial cell dysfunction in CTEPH. Expression of ICAM-1 in endothelial cells from pulmonary arteries and neovessels within CTEPH thrombi were similar, suggesting a similar dysfunctional phenotype in the 2 cell types. We further demonstrated the crucial role of ICAM-1 overexpression in PEC proliferation and apoptosis resistance in CTEPH through several downstream effectors (including survivin, SRC, p38, and ERK), as inhibition of ICAM-1 synthesis or activity decreased PEC proliferation and increased apoptosis. The overproduction of ICAM-1 acts on downstream effectors of cell survival, suggesting that ICAM-1 acts in an autocrine manner to regulate endothelial cell growth and viability. Finally, the high prevalence of the ICAM-1 E469K polymorphism in the CTEPH patients did not translate into differences in clinical severity at the time of surgery.

The pathophysiology of CTEPH has been characterized as the presence of unresolved thrombi within pulmonary arteries, leading to fibrotic organization of the thrombi and to pulmonary arterial endothelium remodeling.^{6,30,31} Pulmonary endothelial dysfunction is believed to be crucial for CTEPH development and progression. Animal and in vitro

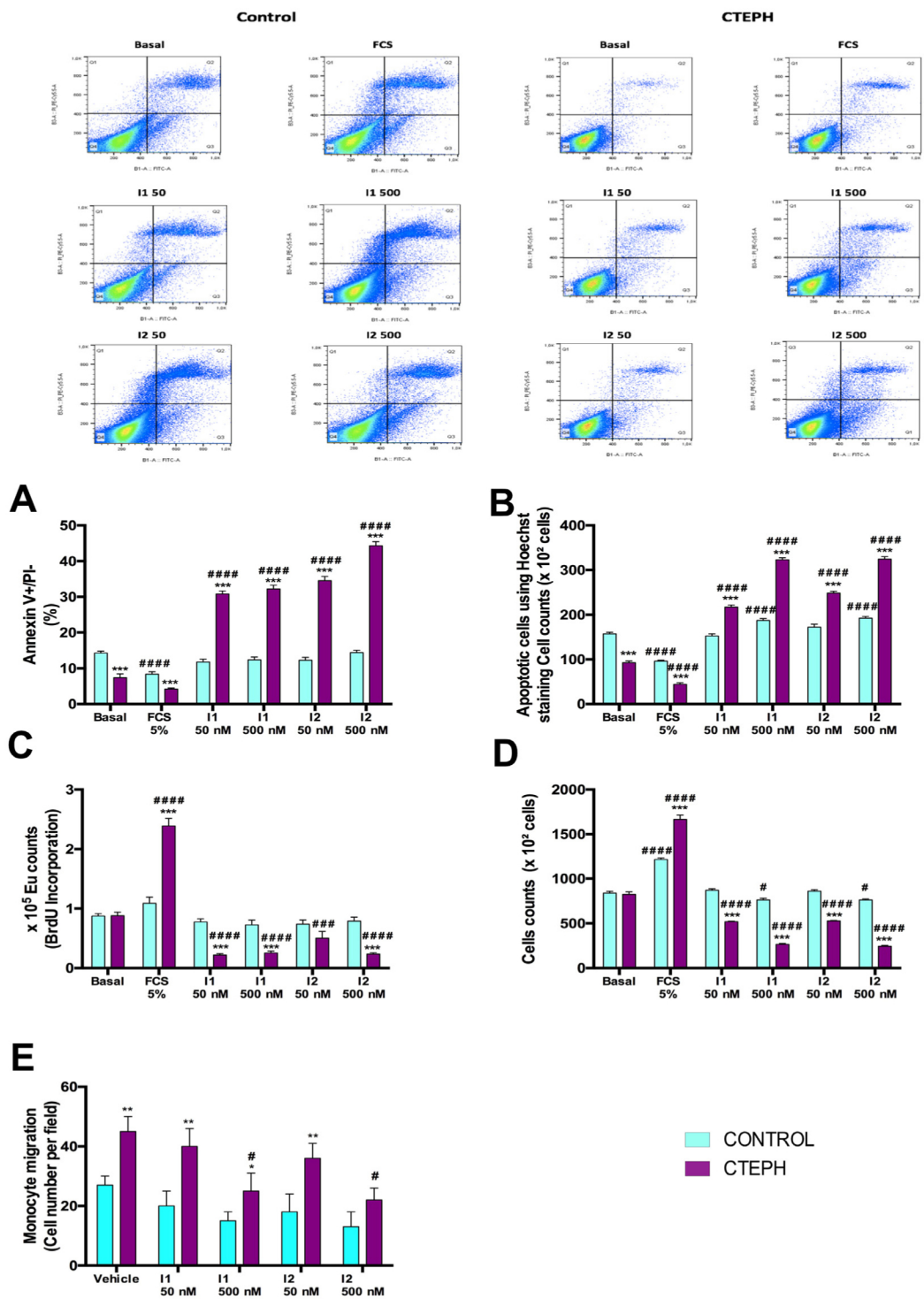
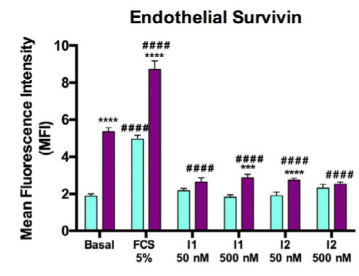
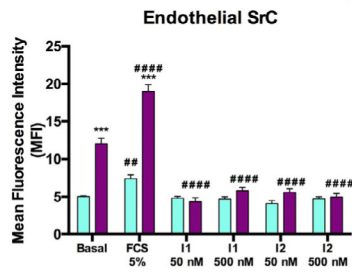
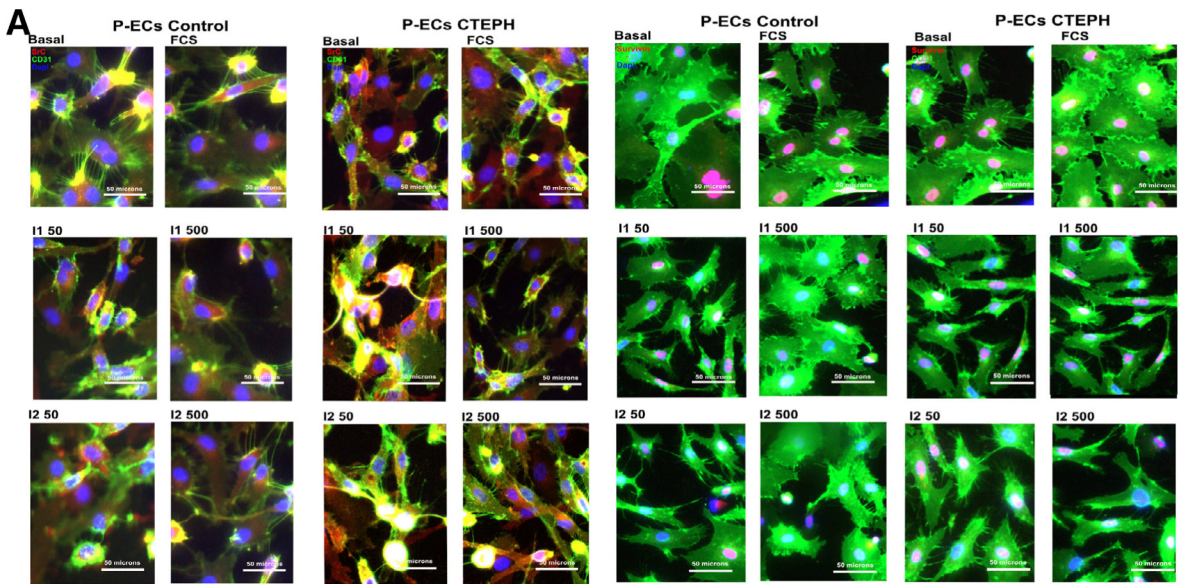


Figure 4 In vitro proliferation, apoptosis and migration of PECs from CTEPH and control patients. After 24 hours of serum deprivation (1% serum), PEC growth and apoptosis were assessed under basal conditions or in the presence of increasing concentrations of two ICAM-1 pharmacological inhibitors: I1 (inhibitor of ICAM-1 synthesis) and I2 (inhibitor of ICAM-1 activity) for 24 hours in MCDB131 medium, at 2 concentrations: 50 and 500 nM. Apoptosis was estimated under basal conditions or with treatment using Annexin V-PI. (A) Annexin V (FITC-X axis) and PI (PerCP-Y axis) and (B) Hoechst staining. Staining is also shown in histograms. Proliferation of PECs was assessed by (C) 5-bromo-2'- deoxyuridine (BrdU) incorporation and (D) cell counts under basal conditions or with treatments. (E) Consequence of endothelial cell-derived ICAM-1 on monocyte migration. Monocyte migration in the presence of endothelial cells from controls and CTEPH incubated with increasing concentrations of I1 or I2 or vehicle using Transwell. * $p < 0.05$, ** $p < 0.01$ in comparison between the chemoattractant effect of EC from CTEPH and from controls. # $p < 0.05$ in comparison with vehicle in the same group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between PECs from patients with CTEPH and controls subjected to the same treatment. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with PECs under basal conditions (n = 7 in each group and each condition in triplicate; two-way ANOVA). Each experiment has been repeated 3 times for reproducibility. CTEPH, Chronic Thromboembolic Pulmonary Hypertension.



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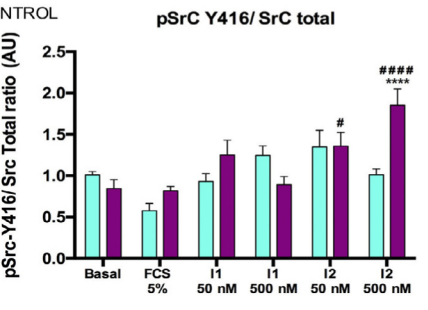
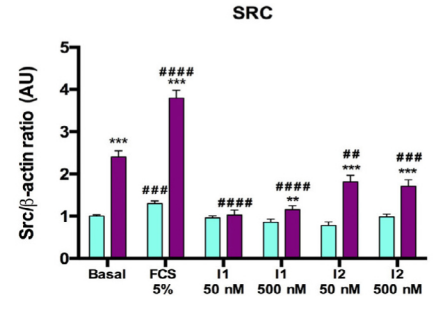
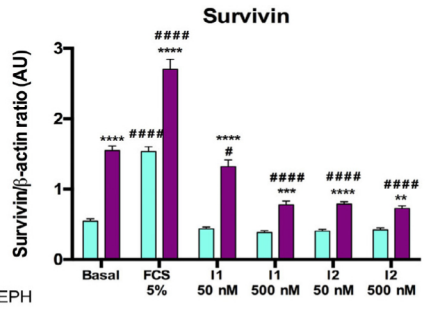
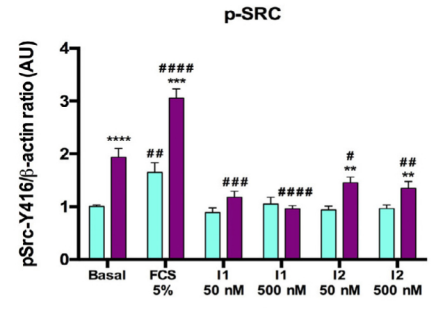
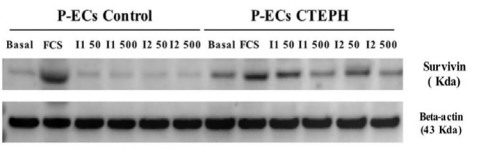
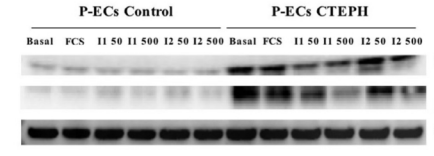
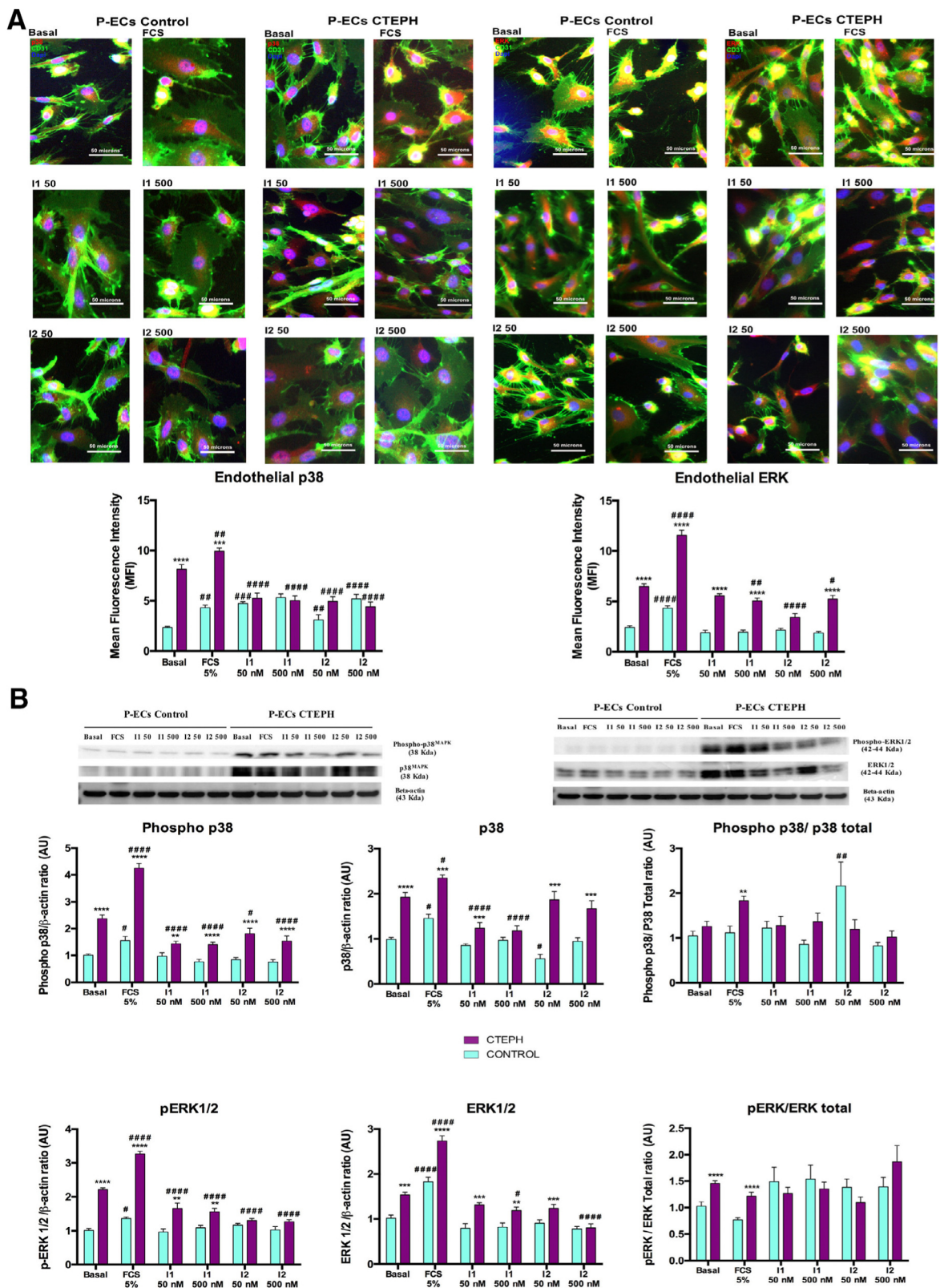


Figure 5 (A) Immunostaining and quantification, and (B) immunoblot analyses of the expression and activation of survival pathway effectors Src (left panel) and survivin (right panel). Phosphorylated and total Src and survivin protein levels were estimated in PECs from patients with CTEPH and controls incubated in the presence of two ICAM-1 pharmacological inhibitors: I1 (inhibitor of ICAM-1 synthesis) and I2 (inhibitor of ICAM-1 activity) (Tocris, Bio-technie, France) for 24 hours in MCDB131 medium, at 2 concentrations: 50 nM and 500 nM. Each bar is the mean \pm SEM protein level in extracts from 10 patients with CTEPH and 10 controls. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between PECs from patients with CTEPH and controls subjected to the same treatment. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with control PECs under basal conditions (using a two-way ANOVA). CTEPH, Chronic Thromboembolic Pulmonary Hypertension.



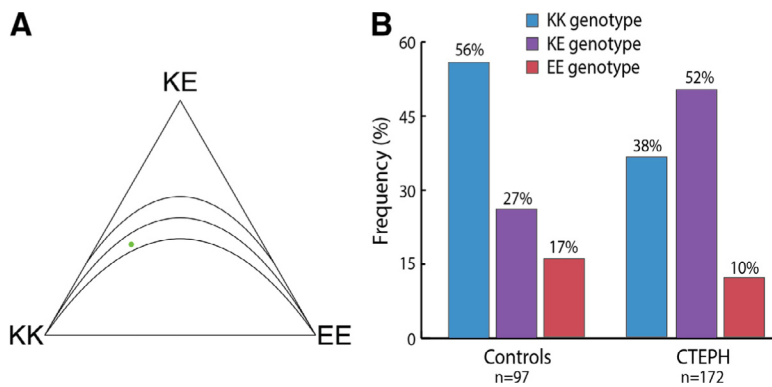


Figure 7 ICAM-1 E469K polymorphism frequency analysis. (A) Hardy-Weinberg equilibrium in the control group. (B) Histogram displaying the different frequencies of the KK, KE, and EE polymorphisms in the 2 groups (controls and chronic thromboembolic pulmonary hypertension CTEPH), $p < 0.0001$ using the Chi-square test. CTEPH, Chronic Thromboembolic Pulmonary Hypertension.

studies have shown the loss of the normal balance between PEC proliferation and apoptosis in CTEPH,^{13,32} but the mechanisms underlying this imbalance are unknown. Apoptosis is a multistage and genetically controlled process ensuring normal cell turnover and can be influenced by physiological and pathological regulatory processes mediated by growth factors and cytokines.^{11,32–34} We have previously reported the abundance of growth factors involved in acquiring and maintaining PEC.⁹

This study quantitatively demonstrates the overexpression of ICAM-1 in the PECs from patients with CTEPH as compared with controls without PH. While the pulmonary endothelial cell expression of ICAM-1 in patients with pulmonary arterial hypertension has been previously demonstrated,^{35,36} data in CTEPH remains scarce and derive from discrepant qualitative fluorescence report from 2 publications by the same group. In 2012, Wynants et al have reported the absence/low baseline expression of ICAM-1 by PECs from both 26 patients with CTEPH and 15 controls with PH,³⁷ contrasting with our results and previous literature on the high expression of ICAM-1 by PECs from patients with pulmonary arterial hypertension. In a second study published the following year, it can be observed that pulmonary endothelial cells from patients with CTEPH express ICAM-1 at baseline, although no quantitative comparative data is provided. Our study further elucidates the question by providing quantitative data in comparison with controls without PH, confirming the higher expression of ICAM-1 at baseline in patients with CTEPH. Overall, the emerging role of ICAM-1 in CTEPH requires further studies to better elucidate its importance in the disease.

Receptor activation by ICAM-1 activates several intracellular signaling pathways, including SRC and ERK1/2 that are involved in cell migration, proliferation, and apoptosis resistance.^{7,16,17,33,38} This activation may facilitate transmigration by triggering endothelial cell contraction and weakening junctional adhesion bonds. While the contribution of ICAM-1 in the interaction between circulating leukocytes and pulmonary endothelial cells has been documented, promoting leukocyte migration through the vascular endothelium,³⁷ this study documents the additional role

of ICAM-1 in promoting PEC proliferation and apoptosis resistance within organized thrombi in CTEPH. In our study, ICAM-1 receptor activation increased the SRC expression and phosphorylation, thereby increasing ERK expression. Cell proliferation, differentiation, and survival involve the MAPK signaling pathway, comprising four sub-pathways: ERK1/2, c-Jun N-terminal kinase, p38, and ERK5,³⁹ and it cannot be excluded that ICAM-1 receptor activation may also activate these pathways.

The above responses were more marked in the CTEPH group than in controls and were completely abrogated by the inhibition of ICAM-1 synthesis or activity. This suggests that overactivity of the ICAM-1 autocrine loop contributed to the hyperproliferative and apoptosis-resistant PEC phenotype seen in CTEPH, although roles for other growth and angiogenic factors cannot be excluded.^{40,41} We further assessed whether ICAM-1 contributed to the endothelial production of survivin, a protein that promotes angiogenesis by reducing apoptosis and stimulating endothelial cell proliferation and migration. Survivin is overexpressed in most human cancers, where it is thought to provide a major growth advantage. In our study, the PECs from CTEPH patients overexpressed survivin, and ICAM-1 inhibition induced a marked drop in survivin protein levels, indicating that survivin overexpression is mediated by ICAM-1.

In our study, monocyte migration was dramatically reduced in the presence of high concentration of ICAM-1 inhibitors, suggesting the involvement of ICAM-1 in the chemotaxis of monocytes. Those results are consistent with the previously reported role of ICAM-1 in facilitating leukocyte transmigration by endothelial cells, junctional adhesion molecules weakening and contraction via inducing intracellular Ca^{2+} levels leading to the activation of p38 and Rho. We did not find any demographic, functional, hemodynamic, or perioperative difference according to serum levels in our CTEPH cohort. Additionally, we found no association between the ICAM-1 E469K polymorphism and ICAM-1 mRNA or protein levels. The T>C substitution in exon 6 of ICAM-1 results in the glutamate (E) being substituted by lysine (K) in the 469th amino acid in the ICAM-1 Ig domain 5 sequence. This polymorphism may

Table 1 Comparative Pre- and Perioperative Characteristics of the Total Chronic Thromboembolic Pulmonary Hypertension (CTEPH) Cohort (n = 172) According to the ICAM Genotype.

	KK n = 47	KE n = 97	EE n = 28	p value ^a
Age (years)	63.0 [49.0-71.0]	63.0 [51.2-71.2]	66.0 [54.0-76.5]	0.69
Female gender (%)	21 (44.7)	46 (47.4)	15 (53.6)	0.63
Body mass index (kg/m ²)	26.2 [23.8-30.4]	25.8 [22.9-29.4]	24.6 [221.6-27.6]	0.26
Body surface area (m ²)	1.82 [1.72-1.91]	1.85 [1.68-1.98]	1.83 [1.68-1.86]	0.72
History of deep venous thrombosis or acute pulmonary embolism (%)	35 (74.5)	81 (83.5)	22 (78.6)	0.24
Inferior vena cava filter (%)	2 (4.3)	6 (6.2)	1 (3.6)	0.72
Presence of endovascular device (%)	1 (2.1)	3 (3.1)	1 (3.6)	0.71
History of splenectomy (%)	1 (2.1)	3 (3.1)	1 (3.6)	0.71
Thrombophilia or blood disorder (%)	4 (8.5)	18 (18.6)	5 (17.9)	0.11
Active or recent (<3 years) smoker (%)	11 (23.4)	37 (38.1)	8 (28.6)	0.12
Time since first symptom (years)	2.1 [1.0-8.7]	3.5 [1.3-7.8]	2.3 [0.7-7.7]	0.77
NYHA functional class				
II	9 (19.1)	18 (18.6)	6 (21.4)	
III	35 (74.5)	70 (72.2)	18 (64.3)	0.71
IV	3 (6.4)	9 (9.3)	4 (14.3)	
Six-minute walk test distance (m)	(n = 24) 368.5 [250.5-413.8]	(n = 52) 417.5 [338.8-489.3] ^b	(n = 12) 405.0 [270.5-459.8]	0.03
Hemodynamics				
Right atrial pressure (mm Hg)	8.0 [5.0-10.0]	7.0 [5.0-9.0]	7.0 [4.0-9.0]	0.17
Mean PAP (mm Hg)	48.0 [37.0-55.0]	44.0 [37.5-51.5]	45.5 [38.6-54.8]	0.59
PAWP (mm Hg)	(n = 19) 7.0 [5.0-9.0]	(n = 49) 7.0 [6.0-10.0]	(n = 5) 6.0 [3.0-11.0]	0.90
Cardiac Index (L/min/m ²)	2.3 [2.1-2.7]	2.5 [2.1-2.9]	2.5 [2.3-2.8]	0.28
Total pulmonary resistance (WU)	10.7 [8.7-12.5]	9.6 [7.0-12.5]	9.7 [7.2-12.1]	0.20
Total Pulmonary Resistance index (WU/m ²)	19.9 [15.7-21.8]	17.9 [13.2-22.3]	16.4 [13.2-21.8]	0.26
Pulmonary vascular resistance (WU)	(n = 19) 8.6 [5.6-10.2]	(n = 49) 7.8 [5.6-9.7]	(n = 5) 10.3 [9.4-12.1]	0.62
Pulmonary vascular resistance index (WU/m ²)	(n = 19) 16.5 [10.8-20.0]	(n = 49) 13.9 [11.2-17.3]	(n = 5) 18.8 [15.6-22.4]	0.64
Pulmonary functional tests	(n = 44)	(n = 90)	(n = 25)	
FEV1 (% of predicted value)	85.0 [74.0-97.3]	84.0 [68.9-97.0]	89.0 [68.0-99.5]	0.85
DLCO (%)	73.0 [57.0-83.0]	68.0 [54.0-78.4]	62.9 [49.0-70.0] ^b	0.08
Resting paO ₂ (mm Hg)	64.8 [61.3-68.0]	65.9 [58.8-74.0]	63.2 [57.2-71.8]	0.15
Pre-operative PH-specific therapies				
Treatment naïve (%)	41 (87.2)	73 (75.3)	26 (92.9)	0.23
Double therapy (%)	3 (6.4)	10 (10.3)	0 (0)	0.72
Prostanoid therapy (%)	0	0	0	-
Phosphodiesterase inhibitors (%)	3 (6.4)	14 (14.4)	1 (3.6)	0.28
Endothelin receptor blockers (%)	6 (12.8)	20 (20.6)	1 (3.6)	0.52
Perioperative characteristics				
Jamieson classification (%)				
Group 1 (%)	6 (12.8)	8 (8.3)	4 (14.3)	0.26
Group 2 (%)	32 (68.1)	64 (66.0)	16 (57.1)	
Group 3 (%)	6 (12.8)	22 (22.7)	8 (28.6)	
Group 4 (%)	3 (6.4)	3 (3.1)	0	
Madani classification (%)				
Level I (%)	6 (12.8)	8 (8.3)	4 (14.3)	0.82
Level II (%)	32 (68.1)	64 (66.0)	16 (57.1)	0.72
Level III (%)	5 (10.6)	16 (16.5)	6 (21.4)	
Level IV (%)	4 (8.5)	9 (9.3)	2 (7.1)	
Associated procedure (%)	2 (4.3)	5 (5.2)	2 (7.1)	
Cardiopulmonary bypass duration (min)	212.0 [195.0-237.0]	226.0 [199.0-251.0] ^b	222.0 [200.8-240.0]	0.04
Aortic clamping duration (min)	98.0 [81.0-106.0]	98.0 [86.0-113.5]	92.0 [84.5-114.0]	0.43
Circulatory arrest duration (min)	29.0 [21.0-35.0]	28.0 [22.0-36.5]	31.5 [26.0-36.8]	0.63

(continued)

Table 1 (Continued)

	KK n = 47	KE n = 97	EE n = 28	p value ^a
Need of extracorporeal support after surgery (%)	5 (10.6)	6 (6.2)	2 (7.1)	0.35
Post-surgical embolization of bronchial artery (%)	1 (2.1)	3 (3.1)	1 (3.6)	0.71
Mechanical ventilation (days)	1.0 [1.0-2.0]	1.0 [1.0-6.0]	1.5 [1.0-3.0]	0.12
Intensive care unit stay (days)	9.0 [5.0-17.0]	9.0 [5.0-21.0]	9.0 [5.0-15.0]	0.84
Postop residual PH	4 (8.5)	13 (13.4)	3 (10.7)	0.68
In-hospital death (%)	1 (2.1)	1 (1.0)	1 (3.6)	0.82
Circulating cytokines	n = 27	n = 53	n = 17	
ICAM-1 (ng/ml)	166.8 [111.7-202.3]	166.8 [137.9-298.5]	163.2 [116.9-227.4]	0.22
VCAM (ng/ml)	416.0 [378.5-464.9]	400.0 [344.1-470.1]	405.3 [334.5-509.7]	0.68
IL-1 (ng/ml)	2.9 [1.1-6.8]	1.2 [0.6-2.9]	1.1 [0.2-4.7]	0.03
IL-6 (ng/ml)	12.0 [3.0-50.0]	14.6 [2.5-56.3]	22.3 [2.7-54.1]	0.72
MCP-1 (ng/ml)	327.0 [245.3-456.2]	455.8 [303.4-765.8]	395.8 [237.7-623.2]	0.08
Ang 1 (ng/ml)	16450.4 [11886.8-26337.4]	15382.9 [12298.1-19775.0]	12015.9 [8390.7-18456.6] ^b	0.23
Ang 2 (ng/ml)	3195.6 [2376.2-6452.9]	3921.6 [2472.3-5558.6]	3734.3 [1991.8-8602.0]	0.69
FGF2 (ng/ml)	12.8 [8.6-23.1]	12.5 [6.3-22.5]	13.6 [5.8-26.5]	0.76
PDGF (ng/ml)	1262.3 [892.3-1931.2]	1262.3 [888.2-1866.6]	945.4 [638.9-1600.1]	0.42
VEGF (ng/ml)	130.0 [75.3-280.4]	99.9 [20.8-262.7]	53.7 [5.9-224.6] ^b	0.16

DLCO, carbon monoxide diffusing capacity; MCP-1, monocyte chemoattractant protein-1; NYHA, New York Heart Association; PAWP, pulmonary wedge pressure; PDGF, platelet-derived growth factor; PH, pulmonary hypertension; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

^bp-value <0.05 for comparison with KK genotype group.

^aComparison between KE + EE genotypes and KK genotype. Presence of endovascular device includes implantable port, pacemakers, ventricular-arterial derivation. Associated procedures refer to coronary arterial bypass graft and aortic valvular replacement.

influence ICAM-1 serum levels and activity and has been associated with cardiovascular diseases.^{21,22,39,42}

The genotype distribution of our control population with lung cancer was similar to that previously reported for healthy cohorts.^{21,22} Our study demonstrated a significantly higher frequency of the KE genotype in CTEPH than in control patients referred for lobectomy. Overall, these results suggest that the E469K polymorphism may be involved in CTEPH pathogenesis by interfering with ICAM-1 stability and/or ICAM-1 affinity for the LFA-1 receptor. In our CTEPH group, we found no association between genotype and disease severity, although this was likely limited by the statistical power. Taken together, our results suggest that, while ICAM-1 may be involved in endothelial cell function and CTEPH pathophysiology, it does not seem to correlate with disease severity in patients with established CTEPH referred for endarterectomy, and further studies are needed to better understand the timeline of the ICAM-1 contribution to CTEPH pathophysiology.

Study limitations

Our study had several limitations. First, it was monocentric by design. However, Marie Lannelongue Hospital performs all the endarterectomies in France.⁴³ Second, owing to ethical considerations, we used pulmonary arterial samples from patients undergoing lobectomy for malignancy instead of healthy people as controls. Controls were selected from patients with low-stage lung cancer and no feature suggesting metastasis, and we previously demonstrated their

similarity to 3 commercial human pulmonary artery endothelial cell lines.⁹ As cancer is a known inflammatory state, using controls with lung cancer may have increased the possibility of finding no differences between CTEPH and the controls. With regards to the ICAM-1 genotype, the frequency in our control population was similar to that previously published for healthy cohorts, limiting the risk of bias. Third, the small number of specimens may have limited our ability to show differences in mRNA expression, apoptosis, or proliferation associated with the E469K polymorphism. Regarding the question of “recanalizing vessels”, we are not able to provide any information as regards of the origin of “recanalizing vessels”.

In conclusion, our study highlights the role of ICAM-1 overexpression by pulmonary endothelial cells in endothelial cell phenotype alteration in CTEPH, characterized by hyperproliferation and resistance to apoptosis. The ICAM-1 signaling pathway may therefore constitute a promising target for therapy aimed at preventing CTEPH development after acute pulmonary embolism.

Disclosure Statement

The authors have nothing to disclose.

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