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1. Title: **Procoagulant membrane microparticles correlate with the severity of pulmonary arterial hypertension.**

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Scientific knowledge on the subject: Procoagulant microparticles are circulating markers of thrombotic tendency in cardiovascular disorders. There is no information on their possible role in the pathophysiology of pulmonary arterial hypertension.

What this study Adds to the field: Procoagulant microparticles correlate with the severity of pulmonary arterial hypertension.

11. This article has an online data supplement, which is accessible from this issue's table of content online at [www.atsjournals.org](http://www.atsjournals.org)

## Abstract

**Rationale:** Procoagulant microparticles constitute valuable hallmarks of cell damage. Microparticles also behave as cellular effectors.

**Objectives:** We hypothesized that the extent of the vascular cell damage measured by circulating microparticles could be related to the severity of pulmonary arterial hypertension (PAH).

**Methods:** Circulating biomarkers of vascular damage and cell activation were measured in blood samples from 20 patients with PAH. Samples were withdrawn from occluded pulmonary artery and jugular vein. Peripheral venous blood samples were obtained in 23 control subjects. The microparticle procoagulant abilities were quantified by functional prothrombinase and tissue factor assays and their cellular origin were determined.

**Results:** Soluble vascular cellular adhesion molecule-1 and pro-inflammatory markers such as monocyte chemoattractant protein-1 and high-specific C-reactive protein were elevated in PAH patients compared to controls. Microparticles bearing active tissue factor and CD105 (endoglin) were also elevated in patients with PAH compared to controls ( $29 \pm 13$  fM versus  $16 \pm 6$  fM,  $p < 0.001$  and  $1.10 \pm 0.46$  nM PhtdSer Eq versus  $0.49 \pm 0.33$  nM PhtdSer Eq,  $p < 0.001$ , respectively). A further increase in endothelial-derived CD105 microparticles was observed in pulmonary arterial blood compared to venous blood in patients with PAH ( $1.73 \pm 0.77$ ,  $p = 0.038$ ). Microparticles bearing active tissue factor were at a higher level in patients in functional class III and IV and walking less than 380 meters at the 6-minute walk test.

**Conclusion:** Circulating markers of endothelium damage, pro-inflammatory markers and cell stimulation estimated with circulating microparticles appear valuable tools in determining the severity of PAH.

**Number of words:** 247

**Key words:** pulmonary hypertension, endothelium, tissue factor, VCAM-1, endoglin.

## Introduction

Pulmonary arterial hypertension (PAH) is a severe disease of the small pulmonary arteries characterized by vascular narrowing and raised pulmonary artery pressure leading to the development of right-sided heart failure and death (1). In PAH, vasoconstriction, remodeling of the pulmonary vessel wall, endothelial and vascular smooth muscle cell proliferation and dysfunction, and thrombosis contribute to increased pulmonary vascular resistance, right ventricle overload and stretch. Uncontrolled endothelial cell proliferation leading to the formation of plexiform lesions is frequently described. *In situ*, both hemostatic and fibrinolytic functions of the endothelium are altered, as suggested by the elevated plasma levels in von Willebrand factors (vWF), P-selectin, plasminogen activator inhibitor type-1 (PAI-1), and decreased thrombomodulin plasma concentrations (2). Thrombotic lesions and platelet dysfunction are common features in PAH. In the flowing blood, circulating endothelial cells exhibit CD36 and E-selectin two markers which correlate with pulmonary hemodynamic parameters (3).

The up-regulation of several cytokines and their elevated plasma levels has been demonstrated in severe PAH, emphasizing a possible influence on inflammatory mechanisms (4). The importance of such inflammatory mechanisms is underlined by the correlation observed between inflammatory markers and pulmonary vascular resistance (5). Interactions between activated platelets and the endothelium could also lead to thrombus formation and to the release of bioactive effectors of pulmonary vasoconstriction and remodeling. As an example, CD40L, a transmembrane protein found on lymphocytes and activated platelets, would thus contribute to inflammation by promoting the up-regulation and release of IL-8 and

MCP-1 chemokines leading to lung perivascular invasion by macrophages and lymphocytes. Furthermore, inflammatory cell infiltrates have been detected in plexiform lesions of pulmonary hypertension.

In the vasculature, procoagulant microparticles shed by apoptotic or stimulated cells upon membrane blebbing, constitute valuable hallmarks of cell damage (6-8).

Elevated levels of platelet, endothelial, monocyte-derived microparticles are a common feature of most thrombotic diseases including pulmonary embolism (9-12). Indeed, circulating microparticles provide an additional phospholipid surface for the assembly of clotting enzymes complexes promoting thrombin generation. Their catalytic property relies on a procoagulant anionic aminophospholipid, phosphatidylserine, translocated to the exoplasmic leaflet following membrane remodeling, and on the eventual presence of tissue factor, the main cellular initiator of blood coagulation. Circulating microparticles also behave as cellular effectors and may contribute to vascular inflammation and endothelial dysfunction. In the target cell, microparticles may promote cytokine release, cytoadhesins up-regulation, nitric oxide synthase inhibition, reduction of nitric oxide bioavailability, or redox balance impairment (7).

We hypothesized that microparticle generation, endothelial damage and neurohormonal stimulation could be related to the severity of PAH assessed by pulmonary hemodynamic parameters, the 6-minute walk test and the World Health Organisation (WHO) functional classification. Preliminary results of this study have been previously reported in the form of an abstract (13).

## Methods

### Patients and control subjects

We studied 20 patients with severe PAH (75% in WHO functional classes III and IV), 11 patients were recorded as idiopathic pulmonary arterial hypertension (IPAH), according to the criteria of the third world symposium on PAH (Venice, Italy, 2003). Other etiologies were PAH associated with connective tissue disease (n=5), HIV infection (n=2), and portal hypertension (n=2). Twenty-three patients referring to the cardiology department for atypical chest pain with normal cardiovascular profile were enrolled as control subjects. Infectious diseases, severe kidney failure and acute coronary syndrome within the past six months were criteria of exclusion. Individual predisposing factors for cardiovascular disease are listed in Table 1. In control subjects no acute cardiovascular event was recorded in the previous year. Written informed consent was obtained from all patients with approval of local Ethic Committee.

### Pulmonary hemodynamic studies

We used Swan-Ganz standard technique for right heart catheterization. During right-sided heart catheterization mean arterial pressure (mPAP) and pulmonary capillary wedge pressure (PCWP) were obtained. Cardiac output was determined by thermodilution. Pulmonary vascular resistance was calculated. The diagnosis of PAH was confirmed when mPAP was superior to 25 mmHg at rest with a normal PCWP (<15 mmHg).

## **6-minute Walk Test (6MWT)**

6MWT was conducted in accordance with the American Thoracic Society guidelines (14).

## **Blood sampling protocols**

During right heart catheterization, blood samples were collected from the occluded pulmonary artery and the jugular vein. In the control group, blood samples were withdrawn by venipuncture of a forearm vein. Platelet-poor plasma samples (PPP) were obtained by double centrifugation as previously described (15).

## **Isolation of circulating microparticles, determination of their procoagulant potential**

Procoagulant microparticles were captured from PPP onto insolubilized annexin-V and their phosphatidylserine content was measured by functional prothrombinase assay (15). Results were expressed as phosphatidylserine equivalent (PhtdSer Eq). We confirmed that the venous site, jugular vein versus forearm vein did not account for differences of microparticle levels (Table E1).

## **Search for the cellular origin of circulating microparticles**

Biotinylated monoclonal antibodies to various cell types: 1) anti-CD11a for leukocytes, 2) anti-CD31, a dual probe for apoptotic endothelial cells and platelet stimulation, 3) anti-E selectin (CD62E) for stimulated endothelial cells (16, 17), 4) anti-CD105 (endoglin) and anti-fractalkine for endothelial cells, and 5) anti-GPIIb for platelets, were insolubilized onto streptavidin-coated microtitration plates and incubated with PPP (15). Captured procoagulant microparticles were quantified by prothrombinase assay. In a given sample, no direct comparison between capture by

annexin-V and antibodies can be given because affinities for the respective ligands are different.

### **Assessment of tissue factor (TF) activity harbored by microparticles**

Microparticles bearing TF were isolated by capture onto insolubilized biotinylated specific antibody to human TF and quantified using a standardized tissue factor activity assay (Innovin, Dade Behring, Margburgh, Germany). Values are expressed as fentomolar of active TF.

### **Measurement of sP-selectin, sCD40L, sVCAM-1, MCP-1, IL-1 $\beta$ , IL-6**

Measurements were performed in PPP samples, as recommended by the manufacturer using the Flow Cytomix human cardiovascular multiplex fluorescent bead immunoassay (Bender MedSystems, Vienna, Austria).

### **Miscellaneous measurements**

RANTES was quantified in PPP samples by ELISA. Quantitative determination of plasma von Willebrand factor antigen (vWF:Ag) and high-specific C-reactive protein were realized by immunoturbidimetric assays. PAI-1 plasma concentrations were determined using a synthetic chromogenic substrate method. Circulating brain natriuretic factor (BNP) levels were determined by enzyme immunoassays.

### **Statistical analysis**

Statistical analysis was performed using SPSS 13.0 software. Results are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant.

Additional details of the methods used are provided in the online data supplement.

## Results

### Patients

Individual anthropometric data, treatments, right heart catheterization findings are given in Table 1. No significant difference in age, sex distribution, pulmonary hemodynamic and arterial blood gas parameters could be demonstrated between patients with idiopathic or associated PAH (data not shown). Control subjects were slightly younger but the difference did not reach the significance level. Dyslipidemia was significantly more frequent in control compared to PAH patients.

### Endothelium damage in pulmonary artery hypertension

Higher levels of soluble vascular cellular adhesion molecule-1 (sVCAM-1) were measured in the jugular vein of patients with PAH, compared to values detected in venous blood of control subjects ( $1977 \pm 805$  vs.  $1104 \pm 670$  ng/ml;  $p=0.001$ , respectively). However, no difference was observed between mean levels of sVCAM-1 in jugular vein compared with occluded pulmonary artery in PAH patients (Figure 1). Plasminogen activator inhibitor type-1 (PAI-1) concentrations were significantly higher in the plasma from patients presenting PAH compared to controls ( $19.8 \pm 7.7$  vs.  $11.5 \pm 6.7$  U/ml;  $p<0.01$ ) and were inversely correlated with the extent of fibrinolysis assessed by D-dimers in PAH patients ( $r=-0.805$ ;  $p=0.016$ ). No significant difference could be evidenced between plasma levels of Von Willebrand factor antigen (vWF:Ag) measured in PAH and control subset ( $1.66 \pm 0.81$  vs.  $1.45 \pm 0.38$  U/ml;  $p=0.7$ ).

## **Inflammatory status in pulmonary artery hypertension**

Higher concentrations of monocyte chemoattractant protein-1 (MCP-1) and RANTES pro-inflammatory chemokines, as well as high specific C-reactive protein ( $_{hs}CRP$ ), were measured in the jugular vein of PAH patients compared to values obtained in the venous blood of control subjects ( $413 \pm 237$  pg/ml vs.  $209 \pm 228$  mg/L;  $p < 0.01$  and  $1584 \pm 967$  vs.  $887 \pm 634$  mg/L;  $p = 0.01$ , respectively). Plasma levels of MCP-1 and RANTES measured in jugular vein and occluded pulmonary artery of PAH patients were comparable (Table 2). In occluded pulmonary artery blood, significant correlations between RANTES and CD 31- or CD11a-bearing microparticles were evidenced ( $r = 0.552$ ;  $p = 0.02$  and  $r = 0.524$ ;  $p = 0.03$ , respectively). MCP-1 levels were correlated with sVCAM-1 concentrations in both jugular vein and occluded pulmonary artery blood ( $r = 0.553$ ;  $p = 0.02$  and  $r = 0.572$ ;  $p = 0.02$ , respectively). No statistical difference could be observed in IL-1 $\beta$  and IL-6 circulating levels between controls and PAH patients (Table E2).

## **Procoagulant microparticles in pulmonary arterial hypertension**

### ***PAH patients compared to control subset***

Procoagulant microparticle levels measured by capture onto annexin-V were not significantly different in the jugular vein blood from PAH patients ( $6.9 \pm 4.4$  nM PhtdSer Eq) when compared to values measured in control subjects ( $5.4 \pm 2.1$  nM PhtdSer Eq;  $p = 0.37$ ) (Figure 2). Likewise, no difference in platelet-derived or leukocyte-derived microparticle levels could be evidenced (Tables 2 and E2). In patients with PAH, levels of endothelial-derived CD105-bearing microparticles (CD105 $^{+}$ -MPs) were significantly increased when measured in the jugular vein compared to controls ( $1.10 \pm 0.46$  vs.  $0.49 \pm 0.33$  nM PhtdSer Eq;  $p < 0.001$ ). Interestingly, a higher tissue factor (TF) activity was harbored by microparticles

(TF<sup>+</sup>-MPs) isolated from the jugular vein compared to control subsets ( $29.4 \pm 13$  vs.  $15.7 \pm 6$ ;  $p < 0.001$ ) (Table 2 and Figure 3).

### ***Comparison between the two puncture sites in PAH subset***

In the PAH subset, endothelial-derived CD105<sup>+</sup>-MPs were detected at higher levels in occluded pulmonary artery compared to jugular vein (Table 2 and Figure 4). Furthermore, in the jugular vein, levels of microparticles bearing active TF or exposing E-selectin, a marker of endothelial activation, were slightly correlated ( $r=0.489$ ;  $p=0.039$ ). Microparticle-borne TF activity in the pulmonary vascular bed was correlated to vWF:Ag concentration and BNP level assessed in the peripheral venous blood ( $r=0.530$ ;  $p=0.029$  and  $r=0.664$ ;  $p=0.01$ , respectively). Notably, levels of procoagulant microparticles captured on annexin-V or CD105 antibody were higher in occluded pulmonary artery than jugular vein (Figures 2 and 4). All other categories of microparticles and soluble markers were similar in both puncture sites (Figure 1, Figure 3, Table 2 and Table E2). The above difference suggests a gradient of circulating microparticles across the precapillary lung circulation. Values of the microparticle gradient detected by capture on annexin-V were related to those obtained by capture on CD105 ( $r=0.749$ ;  $p=0.008$ ).

### **Relationships between clinical or physiological parameters and circulating biomarkers**

The pulmonary vascular resistance (PVR), a physiological surrogate of distal pulmonary vascular remodeling, was significantly correlated with brain natriuretic peptide (BNP), a biomarker of right heart failure in patients with PAH ( $r=0.73$ ;  $p=0.002$ ). BNP was correlated in one hand with sVCAM-1 ( $r=0.59$ ;  $p=0.03$ ), a biomarker of the endothelium damage, and in the other hand with cardiac output ( $r=-0.77$ ;  $p < 0.01$ ). We also observed significant correlations between PVR and

sVCAM-1 in jugular vein and between PVR and sVCAM-1 measured in occluded pulmonary artery blood. In addition, mean pulmonary arterial pressure (mPAP), cardiac output, right atrial pressure, on one hand, and sVCAM-1 level in occluded pulmonary artery, on the other hand, were correlated (Table 3). Stepwise multiple linear regression analysis with PVR as a dependant variable demonstrated that BNP was the strongest independent predictor.

It must also be mentioned that distance at the 6-minute walk test (6MWD), the most frequent used primary end point in randomized trial dedicated to PAH, was negatively correlated with microparticles bearing active TF, in jugular vein and occluded pulmonary artery blood ( $r=-0.52$ ;  $p=0.046$  and  $r=-0.62$ ;  $p=0.017$ , respectively). This exercise test was also negatively correlated with BNP levels ( $r=-0.71$ ;  $p=0.003$ ).

Microparticles with tissue factor activity were detected at higher level in the subset of PAH patients in WHO functional class  $\geq 3$  and with walking performances less than 380 meters (Figure 5). Stepwise multiple linear regression analysis with 6MWD as a dependant variable demonstrated that sVCAM-1 measured in the jugular vein was an independent predictor.

Values of the microparticle gradient observed across the lung precapillary circulation between the occluded pulmonary artery and the jugular vein was correlated with mPAP ( $r=0.631$ ;  $p=0.01$ ) (Figure 6).

## Discussion

In this study, we demonstrated that elevated levels of microparticles bearing active TF and endothelial-derived microparticles bearing CD105 (CD105<sup>+</sup>-MPs) circulate in the plasma from patients with PAH. In addition, we found that higher amounts of procoagulant microparticles measured by capture on annexin-V, are present in the occluded pulmonary artery blood compared to jugular vein blood.

Other indicators of inflammation and vascular damage such as high specific C-reactive protein, RANTES, MCP-1, and sVCAM-1 were found significantly elevated in patients with PAH compared to a control subset with normal cardiovascular background. Endothelial damage, neurohormonal activation and circulating procoagulant microparticles, appeared indicative of the severity of the pulmonary arterial hypertension assessed by hemodynamic or functional parameters.

### Inflammatory status in PAH

Previous reports emphasize a role for inflammation in PAH. Enhanced MCP-1 expression was reported in humans (18, 19) and anti-MCP-1 therapy inhibits the development of PAH in rats. Furthermore, an enhanced expression of RANTES, mainly originating from endothelial cells, was demonstrated in lung samples from patients with severe pulmonary hypertension (20). Such tissue expression was associated with inflammatory cell infiltration. In the present study, significant correlations between MCP-1 and sVCAM-1, a reliable probe of endothelial stimulation, were observed regardless of the puncture site, thereby testifying to persisting vascular damage. In the pulmonary vascular bed, RANTES, was associated with procoagulant microparticles of leukocyte (CD11a<sup>+</sup>-MPs), endothelial

and platelet origin (CD31<sup>+</sup>-MPs), possibly reflecting multiple pathways in which microparticles contribute to inflammation and thrombus formation.

### **Neurohormonal activation**

Previous studies in patients with PAH have demonstrated the role for BNP as a marker of increased right cardiac pressure and volume overload. BNP has been shown to correlate with hemodynamic parameters, functional status, and prognosis of PAH patients (21-23). Our present study is in accordance with these reports. BNP was an independent predictor of pulmonary vascular resistance and was correlated negatively with cardiac output and 6MWD.

### **Endothelium damage in PAH**

In PAH alterations of the pulmonary vascular circulation include smooth muscle cell proliferation and arteriolar muscularization, fibrosis, endothelial cells proliferation, intimal thickening and *in situ* thrombosis. Endothelial cells may respond to injury in various ways affecting the process of vascular remodeling, including proliferation, apoptosis, release of vasoactive agents and growth factors (24). Following inflammatory, hypoxic, or shear stress, endothelial cells display proadhesive, proinflammatory and prothrombotic phenotypes and release sVCAM-1, vWF, PAI-1 and endothelial-derived microparticles. Our data strongly highlight the link between the severity of PAH and endothelial stimulation. An argument in support of this hypothesis is that sVCAM-1 concentrations were correlated to mPAP, PVR, CO and to 6MWD. However, we could not establish whether the elevation in plasma endothelial markers is the result of a severe raise in intravascular pressures or rather constitutes a deleterious cell signal possibly tuning the severity of the disease through leukocyte or platelet recruitment, altered fibrinolysis or tissue proteolysis. In

the present study, endothelial microparticle phenotypes may reflect a particular cellular response under the characteristic vascular settings of PAH (see below).

### **Procoagulant microparticles in PAH: noxious effectors?**

The combined alteration of coagulation and fibrinolysis was previously demonstrated in PAH, using a variety of circulating markers including tissue plasminogen activator, PAI-1, D-dimers, vWF, P-selectin, thrombomodulin (2, 25-28). Altogether, an hemostatic imbalance resulting from increased thrombotic factors and diminished fibrinolytic activity is believed to favor thrombosis (29, 30).

Membrane microparticles constitute a reservoir of potent actors in the adjustment of the hemostatic balance. Because of their dual procoagulant abilities, endothelial-derived microparticles have been reported possible effectors in cardiovascular and thrombotic disorders (16, 31). Shed from the plasma membrane of damaged endothelial cells, they exhibit phosphatidylserine, eventually combined to TF and can be endowed with proadhesive abilities (32, 33).

### **Active tissue factor borne by microparticles and PAH pathogenesis**

Recently, a circulating reservoir of TF, mainly conveyed by microparticles, of various cell origins, has been termed “blood-borne tissue factor” (34, 35). Multiple fusions and exchanges between monocytes, endothelial cells, platelets and derived microparticles would favor phosphatidylserine location at TF vicinity and promote the so-called de-encryption of its active form (35-37). The role generally assigned to vessel-bound TF in the initiation of blood coagulation is now believed to require additional blood-borne TF in the swift growth of the thrombus (38, 39).

TF has been poorly investigated in PAH. In a rodent model of severe pulmonary hypertension, specific TF staining of plexiform-like lesions has been shown (40).

Thrombin, as a potent inducer of TF expression by pulmonary artery smooth muscle cells, would amplify TF-driven coagulation responses (41-43). In the present study, the elevated microparticle TF procoagulant activity detected in patients with PAH is indicative of vascular cell damage or stimulation. Furthermore, the highest TF<sup>+</sup>-MP levels were measured in PAH patients with the lesser functional capacity (6MWD<380m and WHO functional class  $\geq 3$ ). These results are in agreement with the hypothesis of a noxious prothrombotic effect of TF<sup>+</sup>-MPs in PAH.

### **Endoglin, a possible vascular modulator at endothelial microparticle surface**

In the present study, levels of procoagulant microparticles captured onto annexin-V did not reach statistical significance in the jugular vein. However, values of procoagulant microparticles measured in the occluded pulmonary artery from PAH patients reached the high levels evidenced during acute cardiovascular diseases such as unstable angina (44), myocardial infarction in non diabetes mellitus patients (45), or acute cardiac rejection (unpublished personal data). Endothelial cells appeared a key contributor to procoagulant microparticle shedding since significant variations in sVCAM-1 as well as CD105-bearing microparticles could be demonstrated between PAH and control subsets. However, endothelial-derived microparticles expressing other phenotypes such as CD31, E-selectin or fractalkine were not significantly elevated in the pulmonary microvasculature. These observations probably reflect the specific contribution of endoglin (CD105) in PAH and confirm the lower threshold of the other endothelial markers investigated, as observed by our laboratory in other pathological settings. In PAH, the specific elevation in CD105-bearing microparticles emphasizes the importance of endoglin-mediated signalling in the pulmonary vasculature. Endoglin is an accessory receptor for transforming growth factor- $\beta$  (TGF- $\beta$ ) involved in endothelial cells proliferation. *In*

*vivo*, endoglin is predominantly expressed by angiogenic vessels and by *in vitro* proliferating endothelial cells. Recently, it was demonstrated that the ectopic expression of endoglin promotes endothelial cell proliferation (46). In the present study, an eventual microparticle mediated endoglin transfer to neighbouring endothelial cells may occur and promote cell proliferation. Such intercellular transfer has been described for monocyte microparticles able to deliver CCR5 to endothelial cells or Tissue factor to activated platelets (37, 47).

### **A gradient of endothelial circulating microparticles across the precapillary lung circulation**

Interestingly, the characteristic procoagulant microparticle gradient measured between occluded pulmonary artery and jugular vein (by capture on annexin-V) was also evidenced for CD105-bearing microparticles. This observation is suggestive of 1) an increased production at the vicinity of lung microvasculature, 2) a possible trapping in cell aggregates, as reported in coronary diseases,(48) or 3) an eventual lung sequestration. Although the study does not provide data regarding an eventual direct effect of procoagulant microparticles on pulmonary vascular remodeling, the possible sequestration of microparticles supported by endothelium damage and inflammation is in accordance with an eventual thrombus formation and the altered pulmonary vasomotion observed in PAH.

The clinical data in the present study strengthen the hypothesis of noxious pathways involving procoagulant microparticles in the pathogenesis of severe PAH: the gradient of procoagulant microparticles is linked to mean pulmonary arterial pressure and circulating amounts of microparticles bearing active TF are inversely correlated to the functional status (6MWD).

Regardless of their procoagulant properties, it is tempting to speculate that microparticles circulating in the pulmonary vascular bed, could also contribute to lung injury through multiple and intricate pathways including impaired perfusion, vascular remodeling, inflammatory response and leukocyte recruitment. A variety of modulators of cell-cell cross talk are borne by microparticles and could favour leukocyte infiltration and angiogenesis (49, 50). Furthermore, platelet-derived microparticles could promote the proliferation of smooth muscle cells and target vascular narrowing (51). Microparticles could also alter vasomotion through (i) the delivery of thromboxane A2, a potent regulator of the vascular tone, (52) (ii) the inhibition of endothelial nitric oxide synthase expression (53) (iii) the induction of oxidative stress (54) (iv) the reduction of nitric oxide bioavailability (55).

### **Study limitations**

The control subset refers to patients with atypical chest pain and normal cardiovascular background and should be considered as a reference for comparison purposes. No comparison can be done between microparticle measurements obtained by capture onto annexin-V and antibodies because the affinity for both ligands are not similar. Annexin-V is a highly specific probe for Ptdser with a Kd of  $10^{-14}$  M, whereas the best antibodies allow interactions with a Kd of about  $10^{-9}$  M. This is why studies on microparticle phenotypes should always compare clinical subsets to determine the relevance of the observed microparticle level variations. No significant elevation in endothelial-derived microparticles expressing CD31, E-Selectin or fractalkine could be evidenced. This observation may indicate detection limits in the measurement of these endothelial markers in PAH which depend on 1) the level of endothelial markers born by microparticles, 2) the antibody affinity, 3) the

availability of the antigen (steric hindrance or limited proteolysis leading to unrecognized epitope).

In conclusion, endothelium damage, neurohormonal activation and procoagulant microparticles are related to the severity of PAH. Higher levels of microparticles bearing the active form of Tissue Factor were measured in PAH patients with lesser functional capacity, suggesting a possible prothrombotic role. The specific elevation in circulating amounts of procoagulant endothelial-derived microparticles expressing endoglin, a transmembrane receptor involved in endothelial cell proliferation, and the observation of a microparticle gradient across the precapillary lung circulation, point at the endothelium as a key actor in the pulmonary microvasculature. Whether procoagulant microparticles play a major role in thrombus formation in PAH and subsequently contribute to the progression of the disease remains to be established.

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## Figures legends

### **Figure 1: Circulating levels of vascular cellular adhesion molecule-1 (sVCAM-1)**

sVCAM-1 was measured, using a fluorescent bead immunoassay: 1) in the peripheral venous blood from control patients (CTL), 2) in the jugular vein (PAH<sub>JV</sub>) and in the occluded pulmonary artery blood (PAH<sub>PA</sub>) from patients with pulmonary arterial hypertension (PAH). Values are expressed as ng/ml. ns: not significant.

### **Figure 2: Circulating procoagulant microparticles (MPs)**

MPs were isolated: 1) from the peripheral venous blood of control patients (CTL), 2) from the jugular vein (PAH<sub>JV</sub>) and the occluded pulmonary artery blood (PAH<sub>PA</sub>) from patients with pulmonary arterial hypertension (PAH). MPs were captured onto annexin-V and measured using functional prothrombinase assay. Values are expressed as nanomolar of phosphatidylserine equivalents (nM PhtdSer Eq.). ns: not significant.

### **Figure 3: Circulating microparticles bearing active tissue factor (TF<sup>+</sup>-MPs)**

Microparticles (MPs) were measured: 1) in the peripheral venous blood from control patients (CTL), 2) in the jugular vein (PAH<sub>JV</sub>) and in the occluded pulmonary artery blood (PAH<sub>PA</sub>) from patients with pulmonary arterial hypertension (PAH). Procoagulant MPs were captured onto insolubilized biotinylated specific antibody to human TF and TF-related procoagulant activity was measured as described in methods. Values are expressed as fentomolar of active tissue factor. TF: tissue factor; MPs: microparticles. ns: not significant.

**Figure 4: Circulating procoagulant endothelial-derived microparticles bearing endoglin (CD105<sup>+</sup>-MPs)**

Microparticles (MPs) were isolated 1) from the peripheral venous blood from control patients (CTL), 2) from the jugular vein (PAH<sub>JV</sub>) and the occluded pulmonary artery blood (PAH<sub>PA</sub>) of patients with pulmonary arterial hypertension (PAH). MPs were captured onto anti-CD105 antibody and measured using functional prothrombinase assay. Values are expressed as nanomolar of phosphatidylserine equivalents (nM PhtdSer Eq.).

**Figure 5: Relationships between circulating microparticles bearing active tissue factor (TF<sup>+</sup>-MPs) in the occluded pulmonary arterial blood (PAH<sub>PA</sub>) and the functional status assessed by 6-minute walk test (A) and World Health Organisation (WHO) functional classification (B).** Patients with pulmonary arterial hypertension (PAH) were dichotomized in two subsets according to the mean 6-minute walk distance (380 m) or WHO functional class (< 3). Procoagulant microparticles were captured onto insolubilized biotinylated specific antibody to human TF and TF-related activity was measured as described in methods. Values are expressed as femtomolar of TF. TF: tissue factor; MPs: microparticles.

**Figure 6: Correlations between the procoagulant microparticle gradient across the precapillary lung circulation and the mean pulmonary artery pressure (mPAP).** Each microparticle gradient was calculated by subtracting the jugular venous blood level from the pulmonary artery level of microparticles captured onto annexin-V.

**Table 1: Characteristics, treatments of patients and controls and right heart catheterization, and arterial blood gas findings**

	Number (%) or mean $\pm$ SD		<i>P</i>
	PAH subset (n = 20)	Control subset (n = 23)	
<b>Age (years)</b>	54 $\pm$ 17	44 $\pm$ 17	0.061
<b>Sex (M/F)</b>	8/12	16/7	0.118
<b>Cardiovascular risk factors</b>			
Hypertension	8 (40)	4 (17.4)	0.180
History of smoking	4 (20)	9 (39)	0.756
Dyslipidemia	3 (15)	11 (48)	<b>0.048</b>
Diabetes mellitus	4 (20)	3 (13)	0.682
<b>Body mass index (kg/m<sup>2</sup>)</b>	26.5 $\pm$ 8	24.9 $\pm$ 5	0.990
<b>PAH categories</b>		-	-
Idiopathic	11 (55)		
Associated with collagen vascular disease	5 (25)		
Associated with portal Hypertension	2 (10)		
Associated with VIH infection	2 (10)		
<b>World Health Organisation functional Class (%)</b>		-	-
I-II	25		
III-IV	75		
<b>Six-minute walk distance (m)</b>	380 $\pm$ 144	-	-
<b>Specific PAH treatments</b>		-	-
Calcium-channel blockers	2 (10)		
Endothelin receptor antagonist	6 (30)		
Prostacyclin analogues	9 (45)		
Sildenafil	3 (15)		
<b>Cardiovascular treatments</b>			
Diuretics (Furosemide / Spirolactone)	12 (60) / 6 (30)	1 (4) / 0 (0)	<b>&lt;0.01</b>
Fluindione	13 (65)	0 (0)	<b>&lt;0.001</b>
Low molecular weight heparin	1 (5)	1 (4)	1.000
Aspirin	2 (10)	9 (39)	0.075
Clopidogrel	1 (5)	3 (13)	0.613
Angiotensin converting enzyme inhibitors	3 (15)	3 (13)	0.644
Angiotensin II receptors blockers	1 (5)	0 (0)	0.452
$\beta$ blockers	0 (0)	3 (13)	0.239
Calcium-channel blockers	2 (10)	3 (13)	1.000
Statins	1 (5)	11 (48)	<b>&lt;0.01</b>
<b>Right heart catheterization findings</b>		-	-
Heart rate (beats/min)	86 $\pm$ 17		
mPAP (mm Hg)	46 $\pm$ 9		
RAP (mm Hg)	8 $\pm$ 4.5		
PCWP (mm Hg)	9 $\pm$ 5.9		
Cardiac output (l/min)	5.7 $\pm$ 2.3		
PVR (dynes.s.cm <sup>-5</sup> )	625 $\pm$ 407		
<b>PaO<sub>2</sub> (mm Hg)</b>	65 $\pm$ 15	-	-
<b>PaCO<sub>2</sub> (mm Hg)</b>	34.5 $\pm$ 5.5	-	-

PAH: pulmonary arterial hypertension; mPAP: mean pulmonary artery pressure; RAP: right atrial pressure; PCWP: pulmonary capillary wedge pressure; PVR: pulmonary vascular resistance. PaO<sub>2</sub>: arterial oxygen partial pressure; PaCO<sub>2</sub>: arterial carbon dioxide partial pressure

**Table 2: Biological characteristics in PAH and control subsets**

	PAH subset (n = 20)		Control subset (n = 23)	<i>P</i>	<i>P</i>
	PAH <sub>JV</sub>	PAH <sub>PA</sub>		PAH <sub>JV</sub> vs. CTL	PAH <sub>PA</sub> vs. PAH <sub>JV</sub>
hsCRP (mg/l)	8.8 ± 6.8	-	4.0 ± 0.5	<b>0.032</b>	-
BNP (ng/ml)	228 ± 236	-	-	-	-
PAI-1 (U/ml)	19.8 ± 7.7	-	11.5 ± 6.7	<b>&lt;0.01</b>	-
<b>Microparticles</b>					
AV <sup>+</sup> -MPs (nM PhtdSer Eq.)	6.9 ± 4.4	12.1 ± 8.2	5.4 ± 2.1	0.365	<b>0.002</b>
GPIb <sup>+</sup> -MPs (Platelets)	3.3 ± 1.1	3.7 ± 1.5	3.9 ± 1.8	0.344	0.155
CD11a <sup>+</sup> -MPs (Leukocyte)	2.1 ± 1	2.2 ± 1.1	1.7 ± 0.7	0.221	0.214
E-Sel <sup>+</sup> -MPs (Endothelium)	0.22 ± 0.43	0.35 ± 0.10	0.13 ± 0.34	0.444	0.655
CD31 <sup>+</sup> -MPs (Endothelium)	0.89 ± 0.10	0.11 ± 0.13	0.10 ± 0.25	0.480	0.414
CD105 <sup>+</sup> -MPs (Endothelium)	1.10 ± 0.46	1.73 ± 0.77	0.49 ± 0.33	<b>&lt; 0.001</b>	<b>0.038</b>
TF <sup>+</sup> -MPs (fM)	29 ± 13	24 ± 13	16 ± 6	<b>&lt;0.001</b>	0.346
<b>Chemokines – Selectins – Adhesion molecules</b>					
sP-selectin (ng/ml)	15 ± 34	22 ± 38	31 ± 65	0.626	0.144
sVCAM (ng/ml)	1977 ± 805	2136 ± 700	1104 ± 670	<b>0.001</b>	0.423
MCP-1 (pg/ml)	413 ± 237	439 ± 255	209 ± 228	<b>&lt;0.01</b>	0.569
RANTES (pg/ml)	1584 ± 967	1208 ± 969	887 ± 634	<b>0.01</b>	0.088

Biological characteristics in patients with PAH. Values measured in PAH and control subsets. Puncture sites in PAH patients: PAH<sub>JV</sub>: Jugular vein; PAH<sub>PA</sub>: Occluded pulmonary artery. BNP and hsCRP levels were performed on peripheral venous blood samples withdrawn from a forearm vein. hsCRP: High specific C-reactive protein. BNP: Brain natriuretic peptide. Procoagulant microparticles (MPs) levels are expressed as nanomole “phosphatidylserine equivalent” (nM PhtdSer Eq.). AV<sup>+</sup>-MPs: levels of MPs captured onto annexin-V. Cellular origin of circulating MPs was determined by capture onto specific antibodies; E-Sel: E-selectin. FT<sup>+</sup>-MPs: Levels of MPs bearing active tissue factor expressed as fentomolar of tissue factor. PAI-1: Plasminogen activator inhibitor type-1. sP-selectin: soluble P-selectin. sVCAM-1: soluble vascular cellular adhesion molecule-1. MCP-1: Monocyte chemoattractant protein-1. CTL: control subset.

**Table 3: Correlations between sVCAM-1 in jugular venous blood (PAH<sub>JV</sub>) and in occluded pulmonary artery blood (PAH<sub>PA</sub>), pulmonary hemodynamic findings and BNP level**

		mPAP	RAP	PCWP	CO	PVR	BNP
<b>sVCAM-1 (JV)</b>	<i>r</i>	0.09	0.40	0.14	-0.68	0.64	0.587
	<i>P</i>	0.72	0.11	0.61	<b>&lt; 0.01</b>	<b>&lt;0.01</b>	<b>0.03</b>
<b>sVCAM-1 (PA)</b>	<i>r</i>	0.51	0.74	0.29	-0.49	0.52	0.658
	<i>P</i>	<b>0.03</b>	<b>&lt; 0.01</b>	0.25	<b>0.04</b>	<b>0.03</b>	<b>0.008</b>

sVCAM-1: soluble vascular cellular adhesion molecule-1; mPAP: mean pulmonary artery pressure; RAP: mean right atrial pressure; PCWP: mean pulmonary capillary wedge pressure; CO: cardiac output; PVR: pulmonary vascular resistance; BNP: brain natriuretic peptide

Figure 1

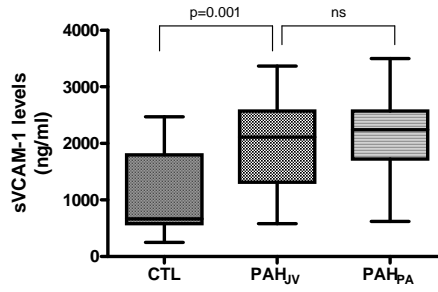


Figure 2

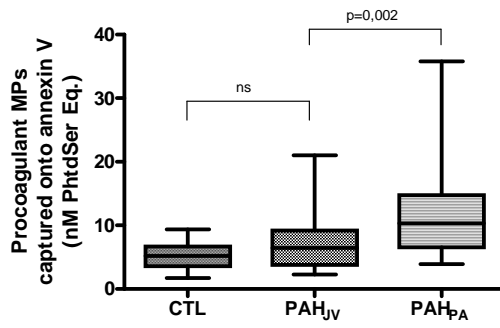


Figure 3

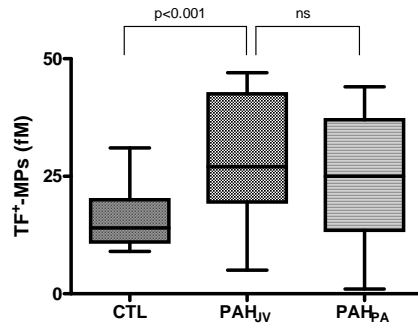


Figure 4

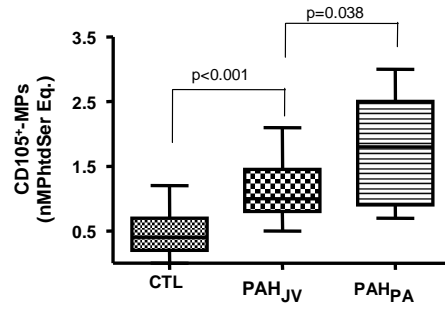


Figure 5

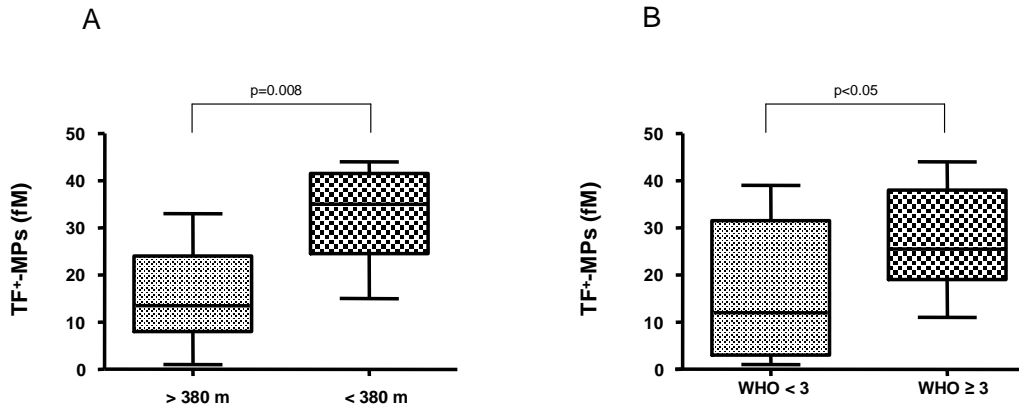
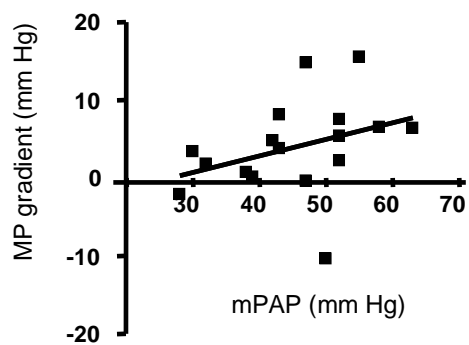


Figure 6

$r=0.631$ ;  $P=0.01$



## **ONLINE DATA SUPPLEMENT FOR MANUSCRIPT:**

**Procoagulant membrane microparticles correlate with the severity of pulmonary arterial hypertension.**

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## Detailed Methods and materials

### Patients and control subjects

We studied 20 patients with severe PAH (75% in World Health Organisation functional classes III and IV), 11 patients were recorded as idiopathic pulmonary arterial hypertension (IPAH), according to the criteria of the third world symposium on PAH (Venice, Italy, 2003). Other etiologies were PAH associated with connective tissue disease (n=5), HIV infection (n=2), and portal hypertension (n=2). Twenty-three patients referring to the cardiology department for atypical chest pain with normal cardiovascular profile by troponins, echocardiography, effort stress, were enrolled as control subjects. To avoid any clinical background known to interfere with the level of microparticles, infectious diseases, severe kidney failure and acute coronary syndrome within the past six months were defined as criteria of exclusion. In control subjects no acute cardiovascular event (acute coronary syndrome, typical angina pectoris or stroke) was recorded in the previous year. Written informed consent was obtained from all patients with approval of local Ethic Committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Strasbourg, France).

### Pulmonary hemodynamic studies

We used Swan-Ganz standard technique for right heart catheterization. During right-sided heart catheterization, systolic (sPAP), diastolic (dPAP), mean pulmonary arterial (mPAP) and pulmonary capillary wedge pressure (PWCP) were obtained at the end of expiration. Cardiac output (CO) was determined by thermodilution. Pulmonary vascular resistance was calculated by the formula  $PVR = (mPAP -$

PWCP)/CO. The diagnosis of PAH was confirmed when mPAP was superior to 25 mmHg at rest with a normal PCWP (< 15 mmHg).

### **6-minute Walk Test**

Patients performed the 6-minute walk test (6MWT), and the distance (6MWD) was recorded using a standardized protocol in accordance with the American Thoracic Society 2002 guidelines (E1).

### **Blood sampling protocols**

During right heart catheterization, samples were collected from the occluded pulmonary artery and from the peripheral venous blood in jugular vein into pyrogen free, tubes with 12.9 mM tri-sodium citrate anticoagulant. Blood was taken through a venous sheath in the jugular vein and from in-dwelling catheters in occluded pulmonary artery. In the control group, blood samples were withdrawn by venipuncture of a forearm vein. Patients were punctured using 18 G needles. For each withdrawn sample, the first 5ml blood tube was systematically discarded. Samples were kept at room temperature to avoid any thermic shock possibly, resulting in the artefactual shedding of platelet-derived microparticles. Platelet-poor plasma (PPP) samples were obtained by double centrifugation, performed within 90 min as detailed elsewhere (E2). PPP were immediately frozen at -80° and thawed only once.

### **Materials**

Monoclonal antibody (mAb) to human platelet glycoprotein GPIb and to human CD105 (RD systems, USA) was previously characterized and biotinylated as described elsewhere (E3). Monoclonal antibody to CD31 was from CALTAG Laboratories (Burlingame, CA, USA), mAb to CD11a and biotinylated

immunoglobulins were from Leinco Technologies (Ballwin, MO, USA). Biotinylated mAb to CD62E (E-selectin) were from Ancell, (Bayport, USA), biotinylated mAb to human fractalkine were from RD systems (USA), tissue factor were from American Diagnostica (Stamford, CT, USA). Human prothrombin (FII) was from Hyphen BioMed (Andresy, France), activated factor X (FXa) from Biogenic S.A. (Mauguio, France), and factor Va from American Diagnostica (Stamford, CT, USA). Biotinylated recombinant human annexin-V was prepared and characterized as previously described (E3). Innovin was from (Dade Behring, Margburgh, Germany). High binding capacity streptavidin-coated microtitration plates and Chromozym TH were from Roche Diagnostics (Mannheim, Germany). CD40 ligand, soluble P-selectin, MCP-1, VCAM-1, IL-1 $\beta$ , and IL6 quantification assay and RANTES ELISA kits were from Bender MedSystems (Vienna, Austria).

### **Isolation of circulating microparticles and determination of their procoagulant potential**

Procoagulant microparticles were captured from PPP onto insolubilized annexin-V and their phosphatidylserine content was measured by functional prothrombinase assay using a microplate reader equipped with kinetics software. In this assay, blood clotting factors (FXa, FVa, FII) and calcium concentrations were determined to ensure that phosphatidylserine is the rate-limiting parameter in the generation of soluble thrombin from prothrombin. FVa was in excess with respect to FXa in order to exclude any contribution of FVa, possibly associated with microparticles. Results were expressed as phosphatidylserine equivalent (PhtdSer Eq) by reference to a standard curve constructed with liposomes of known phosphatidylserine concentrations (E2). This purified system does not allow the capture of lipoproteins, (E4) and the eventual presence of tissue factor on captured microparticles does not

alter values corresponding to phosphatidylserine content, as it is based on a true prothrombinase assay. Each value was obtained from at least two independent measurements.

### **Search for the cellular origin of circulating microparticles**

Biotinylated monoclonal antibodies to various cell types (anti-CD11a for leukocytes, anti-CD31 a dual probe for apoptotic endothelial cells and platelet stimulation, anti-E selectin (CD62E) for stimulated endothelial cells (E5, E6), anti-CD105 (endoglin) and anti-fractalkine for endothelial cells, anti-GPIb for platelets), were insolubilized onto streptavidin-coated microtitration plates and incubated with PPP (E2). After washing, captured procoagulant microparticles were quantified by prothrombinase assay as described above. Background values obtained with corresponding irrelevant immunoglobulins were subtracted. In the same sample, no direct comparison between capture by annexin-V and/or antibodies can be afforded because affinities for the respective ligands are different. It has to be indicated that truly soluble forms of membrane antigens do not generate prothrombinase activity (E2). The method enables specific measurement of procoagulant phospholipids attached to each microparticle phenotype in different samples. Each value was obtained from at least two independent measurements. In our hands, interassay variations are below 5 % of the recorded value as measured in PPP pooled control samples over the last 5 years.

### **Assessment of tissue factor (TF) activity harbored by microparticles**

Microparticles bearing TF were isolated by capture onto insolubilized biotinylated specific antibody to human TF and quantified using a standardized tissue factor activity assay. Briefly, TF activity was measured in a purified system by enzymatic

assay detecting Factor Xa produced from factor X (150 nM) in the presence of factor VIIa (5 nM) and calcium chloride (5 mM). Values are expressed as fM of active Tissue Factor by reference to a calibration curve constructed using a standardized preparation combining purified recombinant human TF and synthetic phospholipids (Innovin). Each value was obtained from at least two independent measurements. Values are expressed as fentomolar of active Tissue Factor.

### **Measurement of sP-selectin, sCD40L, VCAM-1, MCP-1, IL-1 $\beta$ , IL-6 by fluorescent bead immunoassay**

Measurements were performed in PPP samples, as recommended by the manufacturer using the Flow Cytomix human cardiovascular multiplex fluorescent bead immunoassay (Bender MedSystems, Vienna, Austria). Acquisitions were realized using a FacsScan flow cytometer (Becton Dickinson, USA). 10000 events were recorded for each sample.

### **Miscellaneous measurements**

RANTES was quantified by ELISA performed on PPP samples as recommended by the manufacturer (Instant ELISA, Bender MedSystems, Vienna, Austria). PAI-1 concentrations were determined on PPP using a synthetic chromogenic substrate method (Stachrom PAI, Stago, Asnières, France) and measured as arbitrary units following the manufacturer's recommendations. Quantitative determination of plasma von Willebrand factor antigen (vWF:Ag) was realized by immunoturbidimetric assay (STA-Liastest vWF kit, Stago, Asnières, France). High specific C-reactive protein (hsCRP) was measured by an immunoturbidimetric method (Roche Diagnostics). Circulating BNP level was determined by enzyme immunoassays (Shionoria BNP,

CIS Diagnostics, Gif-sur-Yvette, France). BNP, <sub>hs</sub>CRP and vWF:Ag levels were performed on peripheral venous blood samples from a forearm vein.

### **Statistical analysis**

Results are expressed as mean  $\pm$  SD. Statistical analysis was carried out using Mann-Whitney test for continuous variables and Fisher's exact test for qualitative variables. Comparisons of variables measured in the jugular vein and in occluded pulmonary were performed by the Wilcoxon matched-pairs test. Correlations were calculated by the Spearman rank coefficient test for univariate analysis. Covariates found to be significant ( $p < 0.05$ ) according to the correlation analysis were evaluated in a multiple analysis using a stepwise multiple linear regression. Probability values (2 sided) were considered significant at a value of  $P < 0.05$ . All analyses were performed using SPSS 13.0 software.

## References for online data supplement:

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- E2. Hugel B, Zobairi F, Freyssinet JM. Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2004;2:1846-7.
- E3. Aupeix K, Hugel B, Martin T, Bischoff P, Lill H, Pasquali JL, Freyssinet JM. The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection. *J Clin Invest* 1997;99:1546-54.
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- E5. Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G. Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2006;26:112-6.
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**Table E1: Procoagulant microparticle (MP) levels in simultaneously withdrawn samples from jugular and forearm veins of 10 patients with PAH.**

<b>PAH patients</b>	<b>PAH<sub>JV</sub></b>	<b>PAH<sub>PV</sub></b>
Number 1	7.6	8.3
Number 2	11.3	7.3
Number 3	9.1	12.6
Number 4	12	11.2
Number 5	10.1	7
Number 6	9.8	7.7
Number 7	4.6	4.1
Number 8	4.1	4.1
Number 9	5.1	4.2
Number 10	7.3	5.6
<b>Mean values (nM PhtdSer Eq.)</b>	<b>8.1 ± 2.82</b>	<b>7.2 ± 2.9</b>

MPs were isolated from the peripheral venous blood (PAH<sub>PV</sub>), the jugular vein (PAH<sub>JV</sub>) and the occluded pulmonary artery blood of 10 PAH patients, captured onto annexin-V and measured using functional prothrombinase assay. Values are expressed as nanomolar of phosphatidylserine equivalents (nM PhtdSer Eq.). Absence of statistical difference between MP levels in jugular vein and peripheral venous blood (p= 0.14) by Wilcoxon matched-paired test. Mean MP levels in pulmonary artery (14.3 ± 4 nM PhtdSer Eq) vs. peripheral or jugular veins were still statistically different in this particular subset by Wilcoxon matched-paired test (p= 0.018).

**Table E2: Biological characteristics in PAH and control subsets**

	PAH subset (n = 20)		Control subset (n = 23)	P	
	PAH <sub>JV</sub>	PAH <sub>PA</sub>		PAH <sub>JV</sub> vs. CTL	PAH <sub>PA</sub> vs. PAH <sub>JV</sub>
hsCRP (mg/l)*	8.8 ± 6.8	-	4.0 ± 0.5	<b>0.032</b>	-
BNP (ng/ml)*	228 ± 236	-	-	-	-
vWF:Ag (U/ml)*	1.66 ± 0.81	-	1.45 ± 0.38	0.726	-
PAI-1 (U/ml)	19.8 ± 7.7	-	11.5 ± 6.7	<b>&lt;0.01</b>	-
AV <sup>+</sup> -MPs (nM PhtdSer Eq.)	6.9 ± 4.4	12.1 ± 8.2	5.4 ± 2.1	0.365	<b>0.002</b>
GPIb <sup>+</sup> -MPs (Platelets)	3.3 ± 1.1	3.7 ± 1.5	3.9 ± 1.8	0.344	0.155
CD11a <sup>+</sup> -MPs (Leukocyte)	2.1 ± 1	2.2 ± 1.1	1.7 ± 0.7	0.221	0.214
CD95 <sup>+</sup> -MPs (Leukocyte)	0.15 ± 0.26	0.10 ± 0.16	0.65 ± 0.12	0.197	0.739
E-Sel <sup>+</sup> -MPs (Endothelium)	0.22 ± 0.43	0.35 ± 0.10	0.13 ± 0.34	0.444	0.655
CD31 <sup>+</sup> -MPs (Endothelium)	0.89 ± 0.10	0.11 ± 0.13	0.10 ± 0.25	0.480	0.414
FKN <sup>+</sup> -MPs (Endothelium)	0.10 ± 0.47	0.18 ± 0.39	0.17 ± 0.39	0.298	0.564
CD105 <sup>+</sup> -MPs (Endothelium)	1.10 ± 0.46	1.73 ± 0.77	0.49 ± 0.33	<b>&lt; 0.001</b>	<b>0.038</b>
TF <sup>+</sup> -MPs (fM)	29 ± 13	24 ± 13	16 ± 6	<b>&lt;0.001</b>	0.346
sP-selectin (ng/ml)	15 ± 34	22 ± 38	31 ± 65	0.626	0.144
sVCAM (ng/ml)	1977 ± 805	2136 ± 700	1104 ± 670	<b>0.001</b>	0.423
MCP-1 (pg/ml)	413 ± 237	439 ± 255	209 ± 228	<b>&lt;0.01</b>	0.569
RANTES (pg/ml)	1584 ± 967	1208 ± 969	887 ± 634	<b>0.01</b>	0.088
CD40 L (pg/ml)	267 ± 341	214 ± 302	380 ± 521	0.645	0.689
IL-1β (pg/ml)	23.5 ± 15.5	21.4 ± 9.1	22.3 ± 12.8	0.818	1.000
IL-6 (pg/ml)	21.5 ± 4	20.6 ± 2.4	21.2 ± 3.1	0.350	0.204

Biological characteristics in patients with PAH. Values measured in PAH and control subsets. Puncture sites in PAH patients: PAH<sub>JV</sub>: Jugular vein; PAH<sub>PA</sub>: Occluded pulmonary artery. BNP, hsCRP and vWF:Ag levels were performed on peripheral venous blood samples withdrawn from a forearm vein (\*). hsCRP: High specific C-reactive protein. BNP: Brain natriuretic peptide. Procoagulant microparticles (MPs) levels are expressed as nanomole “phosphatidylserine equivalent” (nM PhtdSer Eq.). AV<sup>+</sup>-MPs: levels of MPs captured onto annexin-V. Cellular origin of circulating MPs was determined by capture onto specific antibodies; FKN: Fracktalkine, E-Sel: E-selectin. FT<sup>+</sup>-MPs: Levels of MPs bearing active tissue factor expressed as fentomolar of tissue factor. vWF Ag: Von Willebrand factor antigen. PAI-1: Plasminogen activator inhibitor type-1. sVCAM-1: soluble vascular cellular adhesion molecule-1. MCP-1: Monocyte chemoattractant protein-1. CD40L: CD 40 ligand. IL-1β: interleukin 1 beta. IL-6: interleukin 6. CTL: control subset.