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Role of Thrombospondin-1 in pulmonary hypertension : a proof of concept

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geb. am 05.12.1977 in Luxemburg

This work is dedicated to my parents,

Armand and Marie-Thérèse Frantz-Neu.

Without their encouragement and financial support

I would not have achieved my medical studies and residency training.

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2 Abstract

Objective: One of the main treatment principles of pulmonary hypertension is vasorelaxation of pulmonary resistance vessels by a NO mediated accumulation of cGMP in the vascular smooth muscle cells. The NO pathway can be counteracted by the binding of TSP-1 to its receptors CD36 and CD47. Thrombospondin-1 is synthesized from both smooth muscle cells and endothelial cells under conditions of pathological shear stress or hypoxia as found in pulmonary hypertension. We examined the clinical relevance of TSP-1 in patients with PH as a new pathophysiological concept and biomarker of shear stress.

Methods: After informed consent, 73 patients were tested for pulmonary hypertension. Blood samples were obtained and stabilization of thrombocytes was performed by using CTAD tubes. We processed and measured levels of TSP-1, Big-ET, PDGF-ββ and PF-4 by ELISA according to vendor instructions.

Results: Compared to the control group, patients with pulmonary hypertension (N=56) showed a significant elevation of TSP-1 (82±16 vs 460±101 ng/ml, $p < 0.05$), Big-ET (0.77+-0.25 pg/ml, $p < 0.05$), PDGF-ββ (139±17 vs 754±151 pg/ml, $p < 0.05$). In the subgroup analysis, a significant difference of TSP-1 and Big-ET concentrations was confirmed in each pulmonary hypertension group (PAH, lung disease, CTEPH). PGDF-ββ was significantly increased in PAH and lung disease but not in CTEPH. With a cut-off at 95 ng/ml, TSP-1 yielded a sensitivity of 69.09% and a specificity of 72.22 % for the diagnosis of pulmonary hypertension and the number needed to diagnose was 2.4. Furthermore, TSP-1 levels correlated with PVR ($r = 0.41$, $p = 0.002$) as well as CO ($r = - 0.34$, $p = 0.01$).

Conclusion: While previous biomarkers focus on endothelial function or myocardial strain, we examined for the first time TSP-1 as a biomarker of endothelial shear stress in pulmonary hypertension. Our data suggest that pathological blood flows lead to an induction of TSP-1 in pulmonary hypertension independently of the underlying cause of disease. Usability of TSP-1 in a clinical setting seems valuable: 1. TSP-1 levels could give a predictive insight of treatment response especially in medications using the NO-pathway; 2. TSP-1 levels could be used for treatment efficiency monitoring; 3. Blockage of TSP-1 by biologicals as a new treatment approach in pulmonary hypertension should be subject of future investigations.

3 Introduction

3.1 Pulmonary Hypertension

3.1.1 Definition of pulmonary hypertension

Pulmonary hypertension (PH) is a hemodynamic and pathophysiological condition defined as an increase in the mean pulmonary artery pressure (PAP_m) above 25 mmHg measured at rest by right heart catheterization (Badesch, Champion et al. 2009). In healthy subjects, the average PAP_m during rest is 14.0 mmHg with an upper limit of normal of 20.8 mmHg in supine position. These values are independent of gender and ethnicity and only slightly influenced by posture and age. PAP_m during exercise on the other hand are dependent on exercise level and age, thus no normal exercise value has been defined so far (Kovacs, Berghold et al. 2009).

3.1.2 Symptoms of pulmonary hypertension

Initial symptoms of pulmonary hypertension are shortness of breath during exercise, fatigue or chest pain. Syncope or signs of right heart insufficiency can occur in advanced stages of the condition. Symptoms range in severity and a given patient may not have all of the symptoms. During auscultation of the heart a holosystolic murmur of pulmonary arterial origin or a splitting of the second heart sound might be found, while the examination of the lungs is usually normal (Perloff 1967; Rich, Dantzker et al. 1987).

3.1.3 Classification and epidemiology of pulmonary hypertension

The first classification of PH was proposed by the World Health Organization in 1973. It has been modified during the second (Evian, 1998), the third (Venice, 2003) and fourth World Conferences (Dana Point, 2008). Clinical pathologies with pulmonary hypertension can be classified into 5 distinct groups according to their underlying mechanism as well as their pathological, pathophysiological and therapeutic characteristics (table 1).

TABLE 1	Updated Clinical Classification Of Pulmonary Hypertension
Group 1	**Pulmonary Arterial Hypertension (PAH)** 1.1 Idiopathic PAH 1.2 Heritable PAH 1.2.1 Bone morphogenetic protein receptor type 2 mutations 1.2.2 Activin receptor-like kinase 1 mutations 1.3 Drugs and toxins induced 1.4 PAH associated with (APAH) 1.4.1 Connective tissue disease 1.4.2 HIV-Infection 1.4.3 Portal hypertension 1.4.4 Congenital heart disease 1.4.5 Schistosomiasis 1.4.6 Chronic hemolytic anemia 1.5 Persistent pulmonary hypertension of the newborn
Group 1'	**Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis**
Group 2	**Pulmonary hypertension due to left heart disease** 2.1 Systolic dysfunction 2.2 Diastolic dysfunction 2.3 Valvular disease
Group 3	**Pulmonary hypertension due to lung diseases and/or hypoxia** 3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary disease with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental abnormalities
Group 4	**Chronic thromboembolic pulmonary hypertension**
Group 5	**PH with unclear and/or multifactorial mechanisms** 5.1 Hematological disorders (myeloproliferative disorders, splenectomie) 5.2 Systemic disorders: Sarcoidosis, Langerhans cell histiocytosis, lymphangioleiomyomatosis. 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders. 5.4 Other: Tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Table 1 : Updated clinical classification of pulmonary hypertension.
Adapted from (Simonneau, Robbins et al. 2009)

Currently only scant epidemiological data on the prevalence of the different groups of PH are available. In an Australian monocentric study the prevalence of PH, evaluated by echocardiography and defined as a systolic pulmonary arterial pressure (PAP_{sys}) over 40

mmHg was 10.5% in an unselected population. The distribution of pulmonary hypertension was as follows (Gabbay 2007):

Group 2 (Left heart disease)	78.7 %
Group 3 (Lung disease/Hypoxia)	9.7 %
Group 1 (PAH)	4.2 %
Group 4 (Chronic thromboembolic pulmonary hypertension)	0.6 %
PH not classifiable	6.8 %

3.1.4 Pathophysiology of pulmonary hypertension

The pathophysiology of pulmonary hypertension is complex and multifactorial. Different pathobiological features characterize the diverse clinical PH groups (Galie, Hoeper et al. 2009).

In group 1, PAH is a consequence of an increased pulmonary microvascular resistance (figure 1). Vasoconstriction, proliferative and obstructive remodeling of the pulmonary vessel wall, inflammation and thrombosis are the most important factors responsible for this increase. Severe abnormalities of several vascular cell types including endothelial cells, vascular smooth muscle cells (VSMC) and fibroblasts have been discovered. Furthermore, platelets may play an important role in PAH, as prothrombotic abnormalities with local thrombus formation in the vascular bed have been demonstrated (Humbert, Morrell et al. 2004; Humbert 2010). Finally the release of cytokines and chemokines from activated endothelial cells mediate the influx of inflammatory cells such as monocytes, T or B lymphocytes which contribute to pulmonary artery smooth muscle cell proliferation (Hassoun, Mouthon et al. 2009).

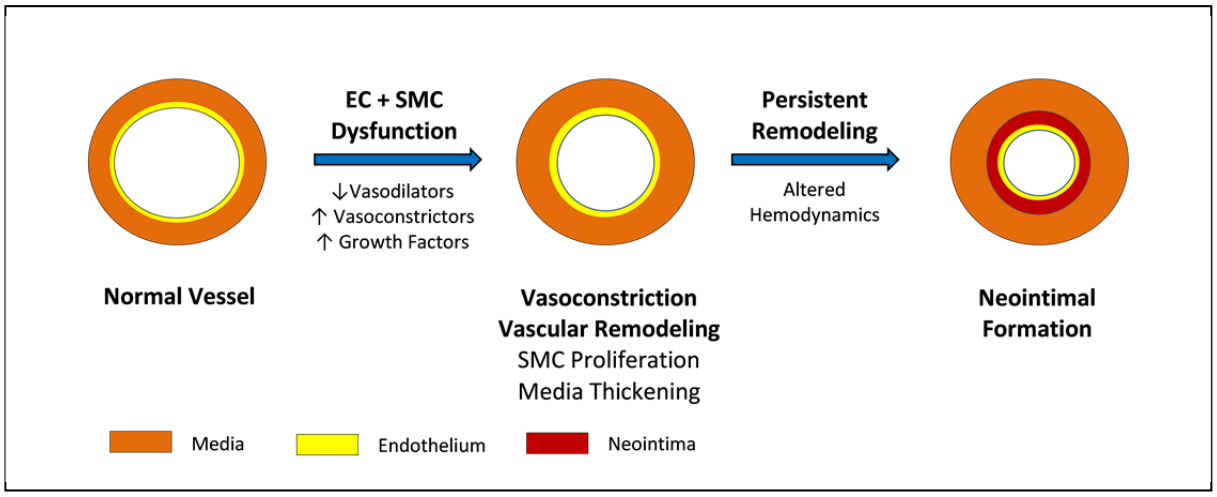

Figure 1 : **Pulmonary vascular remodeling in PAH.**
Adapted from (Botney 1999). EC : endothelial cells, SMC : smooth muscle cells

In group 2, backward transmission of the left pressure elevation due to chronic heart failure induces a reactive pulmonary hypertension. Chronic heart failure may be due to systolic or diastolic cardiac dysfunction or due to valvular diseases (Delgado, Conde et al. 2005).

Group 3 is characterized by multiple mechanisms. Hypoxic vasoconstriction, mechanical stress of hyperinflated lungs, loss of capillaries, inflammation as well as toxic effects of cigarette smoke are involved (Presberg and Dincer 2003).

In group 4, chronic thromboembolic pulmonary hypertension (CTEPH) is induced by failure to resolve acute embolic masses which later undergo fibrosis leading to a mechanical obstruction of the pulmonary arteries. Abnormalities in platelets or in the clotting cascade may cause or aggravate the initial thrombosis and provoke a chronic obstruction of the vascular bed (Humbert 2010). In a subset of patients a pulmonary arteriopathy without pulmonary embolism is discussed (Egermayer and Peacock 2000).

Group 5 consists of several forms of PH with an unclear or multifactorial etiology as well as heterogenic pathophysiology. Various pathologies such as hematological disorders (myeloproliferative disorders, splenectomy), systemic disorders (sarcoidosis, pulmonary Langerhans cell histiocytosis, lymphangioleiomyomatosis), metabolic disorders (glycogen

storage disease, Gaucher disease, thyroid disorders) or patients with chronic renal failure on dialysis are grouped in this category (Simonneau, Robbins et al. 2009).

3.1.5 Assessment of pulmonary hypertension

The non-specificity of PH symptoms makes the initial diagnosis difficult. PH should be considered in patients with exertional dyspnea, progressive limitation of exercise capacity, syncope or angina, especially when apparent cardiovascular or respiratory risk factors are missing. Furthermore, a history of lung embolism, familial cases of PH or a history of specific drug use such as anorectic stimulants (Aminorex) or amphetamines can lead to the assumption of the diagnosis.

Echocardiography is the most important non-invasive technique for screening and initial assessment of PH. A tricuspid regurgitation velocity over 3.4 m/s and a PAP_{sys} over 50 mmHg is highly suggestive of PH while increased right ventricular dimensions, an increased right ventricular wall thickness, an abnormal shape or function of the interventricular septum reinforce the suspicion (table 2). Echocardiography may also guide the etiological assessment of PH toward left heart pathology.

TABLE 2	Echocardiographic Assessment Of Pulmonary Hypertension
PH unlikely	Tricuspid Regurgitation velocity < 2.8 m/s PAP_{sys} < 36 mmHg No additional echocardiographic variables suggestive of PH
PH possible	Tricuspid Regurgitation velocity < 2.8 m/s PAP_{sys} < 36 mmHg Presence of additional echocardiographic variables suggestive of PH
	Tricuspid Regurgitation velocity 2.9-3.4 m/s PAP_{sys} 37-50 mmHg With/without additional echocardiographic variables suggestive of PH
PH likely	Tricuspid Regurgitation velocity > 3.4 m/s PAP_{sys} > 50 mmHg With or without additional echocardiographic variables suggestive of PH

Table 2 : Assessment of pulmonary hypertension by echocardiography.
Adapted from (Simonneau, Robbins et al. 2009)

If left heart disease was ruled out, the next diagnostic step is the assessment of pulmonary diseases by chest radiography, pulmonary function tests and a high resolution CT of the chest.

Chronic thromboembolic pulmonary hypertension must always be ruled out by a ventilation/perfusion scan of the lungs, even in patients without a history of venous thrombosis or lung embolism. If perfusion defects are found, a thoracic CT scan as well as a pulmonary angiography must be performed in patients who might be candidates for a pulmonary endarterectomy.

Finally, if no other cause was found, pulmonary arterial hypertension is probable. A right heart catheterization is required to confirm the diagnosis of PAH, to evaluate its severity and to guide the treatment options.

During right heart catheterization, the systolic, diastolic and mean pulmonary arterial pressure, the right atrial and ventricular pressure, the pulmonary arterial wedge pressure (PAWP) and the cardiac output (CO) must be measured and the pulmonary vascular resistance (PVR) calculated. Pulmonary hypertension is confirmed if the PAP_m at rest is above 25 mmHg. Precapillary PH is defined by a PAWP lower than 15 mmHg while a PAWP above 15 mmHg proves a postcapillary PH (Badesch, Champion et al. 2009).

For patients with PAH, a vasoreactivity testing with inhaled nitric oxide (NO) or ilomedin should be performed in order to identify those patients who may benefit from long term therapy with calcium channel blockers. In vasoreactivity testing, the patient inhales NO while the pulmonary vascular response is monitored. The test is considered positive if a reduction of the $PAP_m \geq 10$ mmHg with a reduction ≤ 40 mmHg is obtained. Furthermore cardiac output must be either increased or unchanged.

3.1.6 Severity of pulmonary hypertension

WHO Functional Class

The severity of symptoms and the degree of physical limitation in patients with PH should be assessed by World Health Organization functional class (table 3). Such assessment provides information on the current level of physical function, correlates to prognosis and can help guide decisions on therapy. There are four WHO functional classes, with class I being the least severe and class IV being the most advanced (Grünig, Barner et al. 2010). Functional classification is strongly predictive of mortality and is an important factor in the choice of PAH therapy (Badesch, Champion et al. 2009).

TABLE 3	WHO Functional Class
Class I	Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause dyspnea or fatigue, chest pain or near syncope.
Class II	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

Table 3 : **WHO Functional Class.** Adapted from (Rubin 2004)

Six minutes walking test

Another useful test for assessing the patient's exercise capacity is the six minutes walking test, which measures the maximal distance a patient is able to walk within a period of six minutes. The six minutes walking test is a submaximal exercise test, but it correlates well with the maximal cardiopulmonary exercise test. This test is also widely used for measuring the response to therapeutic interventions (ATS Statement,2002) and for providing prognostic information (Badesch, Champion et al. 2009).

3.2 Treatment of pulmonary hypertension

3.2.1.1 Treatment of pulmonary arterial hypertension (PAH)

Even though pulmonary hypertension was first described in 1891 (Romberg 1891), the first specific PAH medication did not appear until 1984 (Higenbottam, Wheeldon et al. 1984). Significant advances in the treatment of PH have been achieved recently. Besides the nonspecific treatment of PH, which includes oral anticoagulation, diuretics, oxygen, digoxin and calcium channel blockers, up to nine specific PAH drugs involving three pathways are available today.

Favorable clinical and prognostic results of long term administration of high doses of oral calcium channel blockers have been proven in patients with positive acute vasoreactivity testing results during the initial diagnostic right heart catheterization (< 10 % of all PAH-patients). Treatment with specific PAH therapies should be considered if the vasoreactivity testing was negative or if WHO Functional class I or II cannot be obtained despite calcium channel blocker treatment (Barst, Gibbs et al. 2009).

Prostanoids

The first drug used for PAH was epoprostenol, a synthetic prostacyclin (Watkins, Peterson et al. 1980). Its obligatory intravenous administration made the treatment prone to multiple complications such as systemic adverse effects (arterial hypotension, headaches) and catheter-related infections. Previously intravenous epoprostenol was the only medication with documented survival benefit. The development of more pharmacologically stable prostacyclin agonists, also called prostanoids, made an inhalative administration possible thus significantly reducing complications.

Through the prostaglandin I_2 (PGI_2) mediated increase of intracellular cAMP in the VSMC, prostanoids induce a vasodilation of pulmonary vessels (figure 2). Furthermore, prostanoids have an antiproliferative effect on the vascular endothelium and smooth muscle vasculature which results in less obliteration of the lumen and improvement in hemodynamics. Clinically, IV and inhaled prostanoid therapy lead to an improvement of respiratory symptoms, exercise capacity and clinical events related to PAH (Barst, Gibbs et al. 2009).

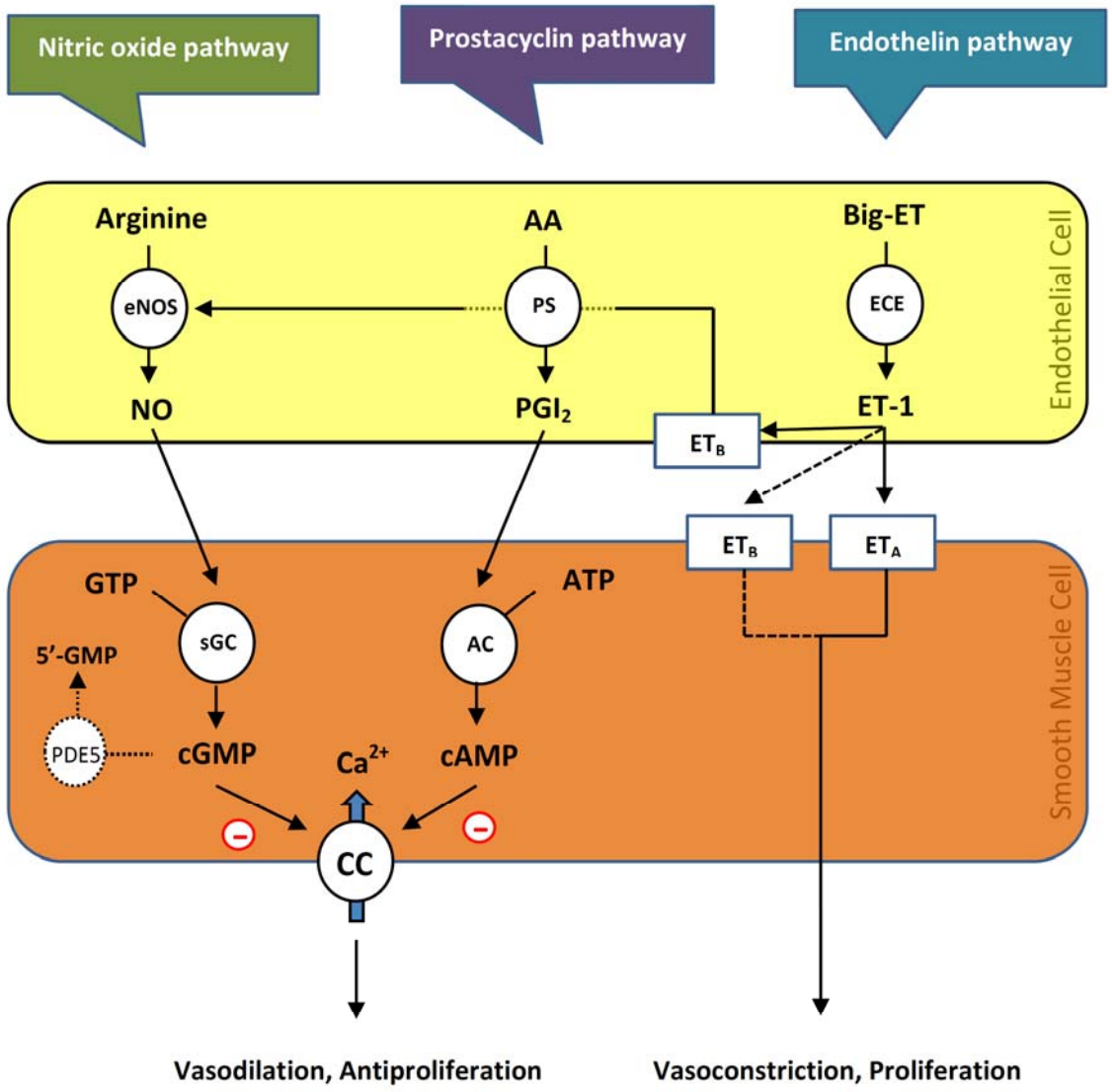

Figure 2 : **Treatment Pathways in pulmonary arterial hypertension**

Modified from (McGoon and Kane 2009)

AA : arachnidonic acid, eNOS : endothelial nitric oxide synthase, PS : prostacyclin synthase, ECE : endothelin-converting enzyme, PGI2 : prostaglandin I2, ET-1 : Endothelin-1, ETA : Endothelin receptor A, ETB : Endothelin receptor B, GTP : guanylate triphosphate, sGC : soluble guanylate cyclase, cGMP : cyclic guanylate monophosphate, PDE5 : phosphodiesterase 5, 5'-GMP : 5'-guanylate monophosphate, ATP : adenosine triphosphate, AC : adenylate cyclase, cAMP : cyclic adenosine monophosphate CC : calcium channel.

Endothelin receptor antagonist (ERA)

Another important pathway involved in the treatment of PAH is the endothelin pathway (figure 2). Inhibition of endothelin receptors located on the vascular smooth muscle cells causes a vasodilative and antiproliferative effect. In addition to non-selective ERAs which block both endothelial receptor A and B, selective ERAs acting only on the ET_A receptors have been developed. Overall ERAs improve exercise capacity, functional class, hemodynamic status, echocardiographic morphological and doppler variables as well as the time to clinical worsening (Barst, Gibbs et al. 2009).

PDE-5 Inhibitors

The NO pathway plays an important role in the treatment of PAH and will be discussed in more detail on page 15. Inhibition of phosphodiesterase type 5 (PDE-5) causes an accumulation of cyclic guanosine monophosphate (cGMP) in the smooth vascular cell resulting in vasodilation and improvement of hemodynamics. Clinically, PDE-5 inhibitors improve exercise capacity and the hemodynamic status of patients with PAH (Barst, Galie et al. 2006).

3.2.1.2 Treatment of Non-PAH pulmonary hypertension

Treatment of PH related to left heart disease

Optimal correction of the underlying pathology is necessary in the management of PH related to left heart disease. Patients with end-stage heart failure may be candidates for heart or heart-lung transplantation. The use of PAH specific drugs is not recommended for the majority of patients with left heart disease (Hoeper, Barbera et al. 2009; Rosenkranz, Bonderman et al. 2010).

Treatment of PH in chronic lung disease

Treatment of the underlying lung disease (e.g chronic obstructive pulmonary disease or lung fibrosis) is essential. Furthermore, the use of long term oxygen therapy in patients with chronic hypoxemia is necessary to reduce the pulmonary arterial pressure. There is insufficient evidence that drugs currently used for PAH are safe and effective in patients

with PH associated with chronic lung disease (Hoeper, Barbera et al. 2009; Hoeper, Andreas et al. 2010).

Treatment of patients with CTEPH

Lifelong oral anticoagulation therapy with a target international normalized ratio (INR) of 2.0-3.0 is essential in treating CTEPH. The therapy of choice is surgical pulmonary endarterectomy (PEA), since it is the only curative treatment option. After diagnosis of CTEPH, the case must be reviewed by an experienced surgeon in a PEA center for assessment of operability. A PEA can be performed if the embolic lesions are accessible at the segmental and subsegmental level of pulmonary arteries. Medical treatment with drugs used in PAH may be considered for inoperable disease or when PH is still persistent or recurrent after PEA (Wilkens, Lang et al. 2010; Fedullo, Kerr et al. 2011; Madani, Wittine et al. 2011).

3.3 The nitric oxide pathway

Nitric oxide (NO) is a bioactive gas produced through the conversion of L-Arginine to L-Citrulline by three nitric oxide synthases (NOS): neuronal NOS, inducible NOS, endothelial NOS. Endothelial NO-synthase (eNOS) is the predominant isoform expressed in endothelial cells and platelets (Kam and Govender 1994; Andrew and Mayer 1999).

When NO is formed in the vascular endothelium, it rapidly diffuses into the vascular lumen where it binds to hemoglobin. More importantly, it also diffuses into the adjacent vascular smooth muscle cells where it stimulates the soluble guanylyl cyclase (sGC). This interaction allows sGC to convert 5'-guanylate triphosphate (GTP) into cyclic guanylate monophosphate (cGMP).

cGMP acts on several downstream targets and many cellular effects are mediated through the activation of the cGMP dependent kinase (cGK). cGMP is degraded to 5’-GMP by proteins known as phosphodiesterases. The conversion of cGMP to 5’-GMP effectively blocks further nitric oxide signaling.

Activation of the cGK leads to an activation of potassium channels and inhibition of calcium channels in VSMC. This in turn leads to a decrease of intracellular calcium concentration and as a consequence induces the dephosphorylation of myosin light chains 2 (MLC2) by myosin light chain phosphatase. The cGK also induces a dephosphorylation of MLC2 through the intermediary of telokin and Rho-kinases. The ultimate consequence is the relaxation of endothelial VSMC and thus vasodilation (Sauzeau, Le Jeune et al. 2000; Friebe and Koesling 2003; Yao and Huang 2003; Isenberg, Frazier et al. 2008).

Other known vascular actions of the NO pathway include inhibition of platelet adhesion to the vascular endothelium and inhibition of smooth muscle hyperplasia. As a consequence, impairment of the NO system leads to thrombosis due to platelet aggregation and adhesion to vascular endothelium as well as vascular hypertrophy and stenosis (Michelakis 2003).

3.4 Thrombospondin-1 (TSP-1)

3.4.1 Overview

Thrombospondin-1 is a glycoprotein first discovered in the early 1970s belonging to the thrombospondin gene family which includes TSP-1, TSP-2, TSP-3, TSP-4 and COMP/TSP-5. TSP-1 is a homotrimer and each polypeptide subunit consists of 1152 amino acid residues.

TSP-1 was first isolated in platelets where it comprises 3 % of total platelet protein and 25 % of total platelet secreted protein (Baenziger, Brodie et al. 1971). TSP-1 is also produced in smooth muscle cells (Mumby, Abbott-Brown et al. 1984), endothelial cells (McPherson, Sage et al. 1981; Mosher, Doyle et al. 1982), monocytes (Jaffe, Ruggiero et al. 1985) and fibroblasts (Jaffe, Ruggiero et al. 1983). Plasma levels in healthy patients are low (Booth and Berndt 1987; Kehrel, Flicker et al. 1996).

TSP-1 falls into the category of “immediate early” response proteins and is up regulated in many stress conditions such as heat shock or hypoxia (Adams 1997). TSP-1 mRNA and protein levels in fibroblasts is up regulated by numerous polypeptide growth factors including platelet derived growth factor-ββ (PDGF-ββ), transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) (Dameron, Volpert et al. 1994). Furthermore, TSP-1 protein levels are down-regulated by many pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α (TNF-α) without change of the mRNA levels suggesting a post-translational processing alteration (Mettouchi, Cabon et al. 1994; Adams 1997).

TSP-1 disrupts the activity of NO and thus prevents the dephosphorylation of MLC2 and vasodilation of VSMC. Two known mechanisms of TSP-1 interfering with the NO pathway are inhibition of NO signaling through receptor CD47 and inhibition through receptor CD36 (see figure 3).

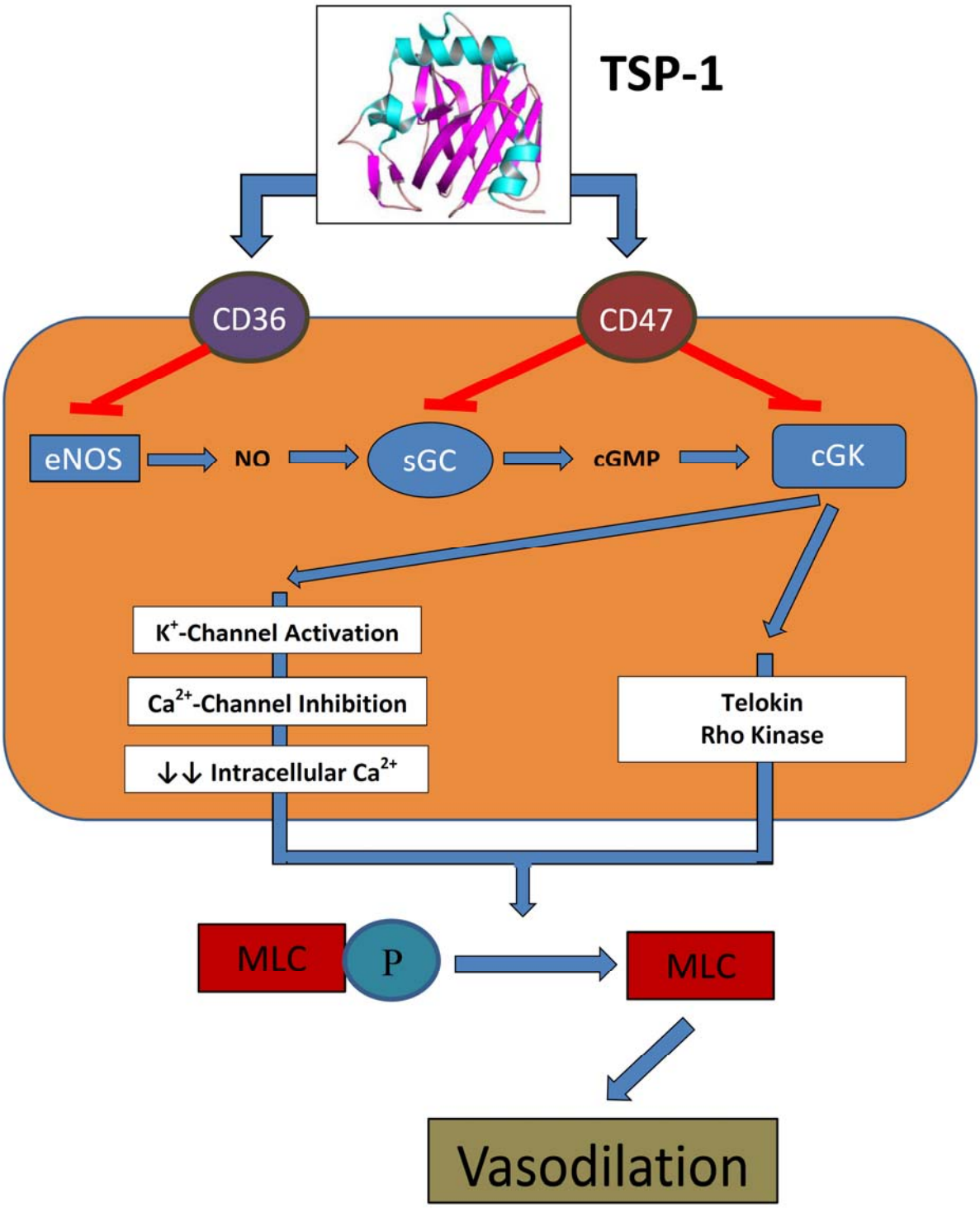

Figure 3 : **Mechanism of action of TSP-1 on the NO pathway**

eNOS : endothelial nitric oxide synthase, sGC : soluble guanylate cyclase, cGMP : cyclic guanylate monophosphate, cGK : cyclic guanylate monophosphate dependent kinase, MLC : Myosin light Chain.

3.4.2 Inhibition of the NO pathway via receptor CD47

CD47, a known receptor of thrombospondin family members, which is expressed by all vascular cells, has been ascribed roles in regulating integrin–matrix protein interactions, self-recognition and immunity.

The major role of CD47 in the NO pathway was recently described. The interaction of TSP-1 with its receptor CD47 is necessary and sufficient to inhibit NO-driven responses in both endothelial and VSMCs (Isenberg, Ridnour et al. 2006). This activation triggers two responses. First, small concentrations of TSP-1 are sufficient to completely block NO stimulation of sGC through receptor CD47, thus provoking an inability to accumulate cGMP in endothelial cells (Miller, Isenberg et al. 2010; Yao, Roberts et al. 2011). Second, phosphorylation of both cGMP kinase-I and vasodilator-stimulated phosphoprotein (VASP) are suppressed by the activation of CD47, causing the downstream blocking of the NO-pathway by TSP-1 (Isenberg, Frazier et al. 2008; Isenberg, Romeo et al. 2008).

As a consequence, TSP-1 by its action on receptor CD47 provokes severe impairment of vasodilation, thus leading to vascular modifications as seen in pulmonary hypertension.

3.4.3 Inhibition of the NO pathway via receptor CD36

CD36, a member of the scavenger receptor B family, is a TSP-1 receptor which is selectively expressed in microvascular endothelium. It acts as a fatty acid translocase activator for numerous proteins but predominantly for myristic acid (Kerkhoff, Sorg et al. 2001). Myristic acid plays an important role in the NO pathway as it activates eNOS in a CD36-dependent way thus contributing to an enhancement of vasodilation (Pollock, Klinghofer et al. 1992).

It has been recently proven that TSP-1 inhibits upstream NO signaling via receptor CD36 by inhibiting cellular myristic acid uptake and thus reducing the activation of eNOS. The resulting vasoconstriction may play an essential role in the pathogenesis of pulmonary hypertension (Isenberg, Jia et al. 2007).

3.4.4 TSP-1 regulation by shear stress

Studies of TSP-1 regulation by shear stress remain scarce. However, two studies independently showed an increase of extra-cellular TSP-1 levels by shear stress.

In the first study, exposure of human umbilical vein endothelial cells (HUVECs) to elevated continuous shear stress enhanced secretion of TSP-1 into extracellular matrix. TSP-1 levels remained unchanged by application of normal flow conditions (Gomes, Legrand et al. 2005). Those findings were confirmed by a second study analyzing TSP-1 expression in whole muscle homogenates (Bongrazio, Da Silva-Azevedo et al. 2006) and by an unpublished thesis (Rupp 2008). Furthermore, TSP-1 expression seems to depend on the type of shear stress applied. Indeed it has been shown that disturbed flow raises TSP-1 mRNA expression more than steady laminar flow in human aortic endothelial cells (Kaplan and Owen 1981).

On the contrary, the loss of flow-induced shear stress on HUVECs resulted in an increased secretion of TSP-1 in the extracellular matrix, supporting the hypothesis that a certain amount of hemodynamic forces are necessary for TSP-1 homeostasis (Freyberg, Kaiser et al. 2000).

As a consequence, TSP-1 expression seems to be modified by hemodynamic forces although the minimum and maximum thresholds as well as the nature of the shear stress (laminar or turbulent, pulse or continuous) leading to these changes remain to be elucidated.

3.4.5 TSP-1 regulation by hypoxia

The exposure of endothelial cells to hypoxic environments regulates the expression of a number of genes whose products that are vasoactive or mitogenic for vascular tissue.

It has been shown that TSP-1 mRNA expression is upregulated gradually and reversibly by low oxygen tensions. TSP-1 protein levels remained elevated for at least 72 hours of continuous hypoxic exposure but regained normal levels after 7 days of exposure (Phelan, Forman et al. 1998; Kwapiszewska, Wilhelm et al. 2005).

Moreover TSP-$1^{-/-}$ knockout mice had diminished pulmonary vasoconstriction response and were less responsive to hypoxia induced pulmonary hypertension than their wild type counterparts (Ochoa, Yu et al. 2010).

Both cellular and mouse model observations suggest that TSP-1 could play an active role in the pathogenesis of pulmonary hypertension.

3.4.6 TSP-1 and angiogenesis

The role of TSP-1 on angiogenesis has been extensively studied in cancer research. TSP-1 plays a major role in vascular remodeling as it stimulates endothelial cell apoptosis and downregulates angiogenesis (Lawler 2002).

The induction of apoptosis by TSP-1 in endothelial cells requires the sequential activation of CD36, the Src-family tyrosine kinase, Caspase-3-like proteases and p38 mitogen-activated protein kinase (MAPK). Finally, stimulation of activator complex-1 (c-Jun and c-Fos) leads to apoptosis (Jimenez, Volpert et al. 2000; Jimenez, Volpert et al. 2001).

On a cellular level the antiproliferative effect of TSP-1 has been confirmed in VSMC (Crawford, Stellmach et al. 1998; Ochoa, Baker et al. 2008) and endothelial cells (Hugo, Pichler et al. 1999; Iruela-Arispe, Lombardo et al. 1999).

However, the role of TSP-1 in vascular remodeling in the setting of pulmonary hypertension remains to be elucidated.

3.4.7 TSP-1 in idiopathic pulmonary fibrosis

Clinical data of TSP-1 levels in disease remains scarce. The sole clinical study published to date was conducted in idiopathic interstitial pneumonia. Serum TSP-1 levels were found to be significantly higher in patients with usual interstitial pneumonia and nonspecific interstitial pneumonia compared to either sarcoidosis or healthy controls. Moreover serum

TSP-1 correlated inversely with pulmonary vital capacity (Ide, Ishii et al. 2008). Unfortunately patients with idiopathic interstitial pneumonia were not screened for pulmonary hypertension. since pulmonary hypertension is present in about 30-80% of patients with advanced pulmonary fibrosis (Lettieri, Nathan et al. 2006), the TSP-1 elevation might have been attributed more to this condition than to the disease itself.

3.5 Big-Endothelin-1 (Big-ET)

Endothelin-1, a polypeptide of 21 amino acids discovered in 1988, is considered one of the most potent human vasoconstrictors (Yanagisawa, Kurihara et al. 1988). The ET-1 gene is translated to a 203 amino acid precursor, which is then cleaved to form Big-ET. Big-ET is subsequently cleaved by the ET-converting enzyme into functional ET-1. Endothelin-1 is produced in numerous tissues including the respiratory endothelium, the endocardium and the kidney (Hemsen and Lundberg 1991). Endothelin-1 acts on the vascular smooth muscle cells by binding to two different ET-1 receptors, ET_A and ET_B. This interaction triggers a calcium release from the sarcoplasmic reticulum through inositol triphosphate (IP3)-coupled signal transduction. This then causes smooth muscle contraction (Kasuya, Takuwa et al. 1989). ET_B receptors are also found on the endothelium. When ET-1 binds to endothelial ET_B receptors, the formation of nitric oxide is stimulated (Benigni and Remuzzi 1999). In patients with CTEPH, a selective upregulation of ET_B receptor mRNA transcripts has been found (Bauer, Wilkens et al. 2002).

Endothelin-1 plays an important role in the pathogenesis of pulmonary arterial hypertension. Indeed, patients with pulmonary hypertension have substantial alterations in plasma endothelin-1 levels, which reflect either changes in net release or clearance of ET-1 by the lung (Stewart, Levy et al. 1991; MacLean 1998). It has been proven that the blockade of endothelial receptors constitutes a major benefit in the treatment of pulmonary arterial hypertension and CTEPH (Rubin, Badesch et al. 2002; Hoeper, Kramm et al. 2005; Olschewski 2009; Vassallo, Kodric et al. 2009; Wilkens, Lang et al. 2010).

3.6 Platelet Derived Growth Factor – ββ (PDGF-ββ)

Platelet derived growth factor-ββ is produced by endothelial cells, smooth muscle cells (SMC), platelets and monocytes (Ross, Raines et al. 1986). It acts as a strong chemoattractant for SMC migration and is involved in the abnormal proliferation of SMCs (Perros, Montani et al. 2008).

Hypoxemia, as found in pulmonary hypertension, decreases expression of numerous protein tyrosine kinases (PTPs) resulting in reduced dephosphorylation of PDGF-ββ receptors. This in turn leads to enhanced receptor activation and proliferation of human pulmonary arterial smooth muscle cells (hPASMC) (ten Freyhaus, Dagnell et al. 2011) and consequently in the proliferative vascular remodeling found in pulmonary arterial hypertension (Balasubramaniam, Le Cras et al. 2003). Furthermore, hypoxic conditions significantly increased PDGF-ββ mRNA in cultured human umbilical vein endothelial cells by enhancing the transcription rate of its gene (Kourembanas, Hannan et al. 1990).

Effect of shear stress on PDGF-ββ has been tested in several studies. Progressive continuous laminar flow induced a proportional elevation of PDGF-ββ levels in the extracellular matrix of aortic smooth vessel cells. This elevation was still present 24 hours after flow cessation (Sterpetti, Cucina et al. 1994). Moreover, it was shown that high but not low cyclic strain increased PDGF-ββ chain transcription and protein synthesis in bovine aortic endothelial cells (Sumpio, Du et al. 1998).

The relationship between PDGF-ββ and TSP-1 remains unclear. It has been shown that TSP-1 mRNA in rat vascular smooth cells is induced by PDGF-ββ (Majack, Mildbrandt et al. 1987). However, the treatment of human umbilical vein endothelial cells (HUVECs) with recombinant PDGF-ββ or hypoxic-endothelial cell conditioned medium does not result in the induction of TSP-1 gene expression. Moreover the induction of PDGF-ββ in endothelial cells by hypoxemia is temporally delayed relative to the induction of TSP-1 transcripts (Phelan, Forman et al. 1998; Faller 1999).

3.7 Platelet Factor 4

Platelet factor 4 (PF-4), also known as CXC-chemokine Ligand 4 (CXCL4), is a small cytokine present abundantly in α-granules of platelet cells. PF-4 is released upon platelet activation and promotes blood coagulation. PF-4 levels are elevated in patients with cancer, atopic dermatitis and psoriasis (Abbasciano, Bianchi et al. 1995; Tamagawa-Mineoka, Katoh et al. 2008). Moreover, PF-4 is released upon heparin therapy (Cella, Scattolo et al. 1985).

In clinical research, PF-4 levels are currently the most frequently applied method to differentiate in vivo platelet activation from an in vitro artifact during blood processing (Kaplan and Owen 1981).

4 Aim of the study

Thrombospondin-1 has been largely studied in vitro for the last two decades. However, little is known about thrombospondin-1 in vivo. The aim of this thesis was to confirm older data on TSP-1 levels in healthy subjects. Furthermore, TSP-1 levels in different groups of pulmonary hypertension patients (PAH, PH related to lung diseases and CTEPH) were to be established and the diagnostic value of TSP-1 in pulmonary hypertension to be investigated. The relationship between TSP-1 and hemodynamic parameters as well as the association of TSP-1 with known biomarkers of pulmonary hypertension was also subject of this current study. Using those results, a pathophysiological model was to be formulated.

5 Materials and methods

5.1 Study population

During the period from 01.06.2008 to 31.12.2010, a total of 73 patients who underwent a consultation for diagnosis, follow up or exclusion of pulmonary hypertension were enrolled. Subjects over 18 years of age were included while the exclusion criteria were left heart disease, organ failure, infections, neoplasm, hematological and rheumatologic disorders, chronic alcohol intoxication and drug abuse. The control group included healthy partners of our patients, subjects screened for PH with a negative test result or co-workers and members of the research team. In eight out of seventeen controls a right heart catheterization was performed while in nine persons without any medical history of chronic disease or respiratory symptoms, PH was considered absent. In all patients with PH a right heart catheterization was performed. The study was approved by the Ethics Committee of the Medical Council of Saarland and all patients provided written informed consent.

5.2 Study Design

During either a diagnostic or follow-up right heart catheterization, 12.2 ml of venous blood (7.5 ml with citrate theophylline, adenosine, dipyridamole (CTAD) and 4.7 ml serum) was drawn from the pulmonary artery through an Arrow® HANDS-OFF® Thermodilution catheter (7.5 FR, 4 lumen, 110 cm). Particular attention was paid to avoid hemolysis. Vasoreactivity testing with inhaled iloprost was performed in 24 patients with suspicion of PAH. 15 minutes after inhalation of the test medication, another 12.2 ml sample of venous blood (7.5 ml CTAD and 4.7 ml serum) were taken. In order to prevent ex vivo platelet activation, the blood was immediately cooled on ice until centrifugation.

During the right heart catheterization, the following parameters were monitored: PAP_{sys}, PAP_{dia}, PAP_{m}, PAWP, CO, PVR, central-venous oxygen saturation ($S_{cv}O2$), Heart rate (HR), systolic and diastolic systemic blood pressure (BP_{sys}, BP_{dia}). Normal values for the hemodynamic parameters are listed in the table 4 below.

Table 4	Normal Hemodynamic Values At Rest		
Value		Average	Range
PAP_{sys}	[mmHg]	25	15-30
PAP_{dia}	[mmHg]	9	4-12
PAP_{m}	[mmHg]	15	9-19
PAWP	[mmHg]	9	4-12
CO	[l/min]	4.9	4.0-8.0
PVR	[dyn·s·cm^{-5}]	70	20-130
TPG	[mmHg]	-	≤ 12
$S_{cv}O2$	[%]	-	≥ 70
HR	[bpm]	-	60-80
BP_{sys}	[mmHg]	130	90-140
BP_{dia}	[mmHg]	70	60-90

Table 4 : **Normal hemodynamic values at rest.**
Adapted from (Braunwald 2007)

Pulmonary hypertension was considered present if PAP_m exceeded 25 mmHg at rest (Badesch, Champion et al. 2009). After catheterization, the pulmonary hypertension was classified into its specific Dana Point group. In patients with PAP_m below 21 mmHg, PH was considered absent.

5.3 Preanalytical platelet stabilization

Anticoagulation of blood samples is necessary to avoid platelet activation and clotting. Previous studies clearly showed that ethylene-diamine-tetraacetic acid (EDTA) should be avoided while CTAD (citrate, theophylline, adenosine, dipyridamole) is the anticoagulant of choice for TSP-1 measurement (Bergseth, Lappegard et al. 2000). As a matter of fact, theophylline and dipyridamole inhibit cAMP phosphodiesterase activity while adenosine stimulates membrane adenylyl cyclase. The consequence is an increase in the platelet's intracellular cAMP concentration and the inhibition of Ca^{2+}-mediated response leading to a reduction in platelet activation (Macey, Azam et al. 2002).

Test tubes (Saarstedt S-Monovette, 7.5 ml, No Ref: 01.01728.001) were filled with 1 ml of CTAD at the following concentrations: citrate 109 mmol/l, theophylline 15 mmol/l, adenosine 3.7 mmol/l, dipyridamole 0.198 mmol/l (Contant, Gouault-Heilmann et al. 1983). The withdrawn blood sample was cooled immediately (0-4°C) and centrifugation was performed within 10 minutes.

5.4 Platelet-poor plasma

In order to further reduce errors due to ex vivo activation of platelets, all plasma levels of TSP-1, PDGF-ββ and Big-ET were analyzed in platelet poor plasma (PPP). PPP was obtained by centrifugation of the CTAD blood samples at a temperature of 2°C at 1800g (Hettich Rotixa/P) for 20 minutes. The plasmatic platelet poor supernatant was transferred to polypropylene cryotubes (Nunc cryotube Vials, 1.8 ml No Ref: 347627) and frozen at -20°C until analysis.

5.5 Enzyme-Linked Immunosorbent Assays

Enzyme-linked immunosorbent assay (ELISA) is a very sensitive and specific biochemical technique used mainly to detect the presence of low concentration of antibodies or antigens (proteins, hormones, drugs) in a solution (serum, urine and culture cell supernatant). Serum containing the antigen (AG) to be measured is added to the ELISA wells containing the matching antibodies (AB). AG-AB binding is detected by a second antibody which is labeled by an enzyme. This enzyme proportionally converts a colorless substrate (chromogen) according to the quantity of AG-AB binding present. Thus the initial amount of the antigen present can be deduced by the intensity of color change (figure 4).

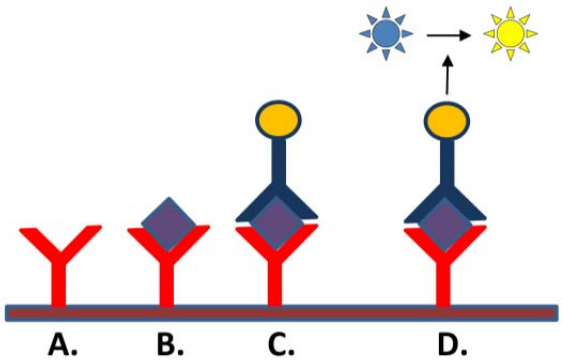

Figure 4 : Principles of Sandwich ELISA

A. ELISA plate is coated with a capture antibody
B. Sample is added, and the respective antigen present binds to capture antibody
C. Conjugated secondary detection antibody is added, and binds to the antigen captured by the first antibody
D. Conjugated antibody catalyzes a color change proportional to the amount of antigen present in the sample

5.5.1 TSP-1 and PDGF-ββ ELISA

TSP-1 platelet poor plasma levels were analyzed using the Quantikine® Human Thrombospondin-1 Immunoassay (R&D Systems, Minneapolis, USA, Catalog Number DTSP10). PGDF-ββ platelet poor plasma levels were measured by the Quantikine® Human PDGF-ββ Immunoassay (R&D Systems, Minneapolis, USA, Catalog Number DBB00).

All reagents and samples were processed as indicated in the User's Manual. The stored samples were slowly thawed in a warm water bath. Platelet-poor plasma samples were diluted two-fold by adding 100 µl of calibrator diluent RD5-33 to 100 µl of the sample. 100 µl of assay diluent RD1-56 were added to each well, followed by 50 µl (TSP-1) or 100 µl (PDGF-ββ) of standard, control, or sample. The plate was then covered with an adhesive strip and incubated for two hours at room temperature on a horizontal orbital microplate shaker at 500 ± 50 rpm. After the first incubation each well was washed four times with 200 µl of wash buffer. Particular attention was paid to the complete removal of liquid at each step. After the last wash, residual wash buffer was removed by aspiration. Finally the plate was inverted and blotted on clean paper towels. Then 200 µl of TSP-1 or PGDF-ββ conjugate were added to each well. Again, the plate was covered with a new adhesive strip followed by incubation for two more hours at room temperature on a shaker. The wells were then washed four times. Afterwards 200 µl of substrate solution were added to each well and incubate for 30 minutes at room temperature on the benchtop.

Finally 50 µl of stop solution were added to each well and the optical density of each well was measured within 10 minutes, using a Tecan Spectra III reader set to 450 nm against 620 nm reference wave length.

TSP-1 Assay Quality

The TSP-1 assay had a minimal limit of detection of 0.355 ng/ml. The intra-assay reproducibility coefficient of variation was 5.08%. Inter-assay reproducibility was calculated as 11.28%.

5.5.2 Big-Endothelin ELISA

The Big-Endothelin ELISA kit was obtained from the Biomedica Group, Vienna, Austria (Catalog Number BI-20082). The reagents and sample preparation as well as analysis were done according to the manufacturer's manual.

All samples were brought to room temperature by a water bath. 50 µl of standard, sample or control were pipetted into all wells except blank and then 200 µl of conjugate was added into all wells except blank. The plate was covered tightly and incubated for 4 hours at room temperature in the dark. After incubation, the wells were aspirated and washed with 300 µl of wash buffer for a total of five times. The residual buffer was removed by blotting the plate on paper towel. 200 µl of substrate were added into each well and the plate was again incubated for 30 minutes at room temperature in the dark.

Finally, 50 µl of stop solution were added into each well and the optical density was measured with a Tecan Spectra III reader set at 450 nm against 620 nm as reference.

5.5.3 PF-4 ELISA

The PF-4 ELISA kit was obtained from Ray Biotech, Inc. (Catalog Number ELH-PF4-001). The preparation of all reagents and samples was performed as described in the User's Manual. The stored samples were slowly thawed in a warm water bath.

100 µl of each standard as well as the samples were added into the appropriate wells. The plate was covered and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and the wells were washed four times with 300 µl of wash buffer. Particular attention was paid to complete removal of liquid at each step. After the last wash, any residual wash buffer was removed by aspirating or decanting. 100 µl of the included biotinylated antibody were pipetted into to each well and the plate was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and the wells were washed four times with 300 µl of wash buffer as described above. 100 µl of the included Streptavidin solution were added to each well and the plate was incubated for 45

minutes at room temperature with gentle shaking. Again, the plate was washed four times with wash buffer before 100 µl of TMB One-Step Substrate Reagent were added to each well. The incubation was performed for 30 minutes at room temperature in the dark with gentle shaking.

Finally, 50 µl of stop solution ended the reaction and optical density was measured with a Tecan Spectra III reader set at 450 nm against 620 nm as reference.

5.6 Statistical Analysis

All normally distributed values are expressed as the median ± standard deviation. Parameters were tested for normal distribution with the Shapiro-Wilk test. In case of normal distribution, homogeneity of variance between the different groups was assessed by the Levene Test. In case of given homogeneity of variance, the means were calculated and differences between groups tested by an ANOVA test, while a Gabriel's test for post hoc analysis was used. In case of non-homogeneity, the Dunnett T3 correction was applied. If the variable was not normally distributed, levels are expressed as median [range] and a non-parametric comparison with Mann-Whitney-U test was performed.

The receiver operator characteristics curve was calculated for pulmonary hypertension. The cut-off point for calculation of sensitivity, specificity, positive and negative predictive value was optimized by the ROC curve. Results were controlled by stepwise increase of TSP-1 cut-off and calculation of Matthew correlation coefficients.

Univariate correlations of TSP-1 with biomarkers and hemodynamic variables were calculated as Spearmans ranked order correlation coefficients, as most of the variables were not normally distributed.

A p-value below 0.05 denoted a statistically significant difference.

Data was stored in a Microsoft Excel 2007 file and analyzed with the statistical analysis software SPSS, Version 17.

6 Results

6.1 Study population

A total of 73 patients were enrolled from 01.06.2008 to 31.12.2010. Basic characteristics are shown in table 1. In 17 patients pulmonary hypertension was ruled out (controls) while 34 patients had proven PAH (Dana Point Group 1), 14 had a PH related to a lung disease (Dana Point Group 3) while 8 patients suffered from CTEPH.

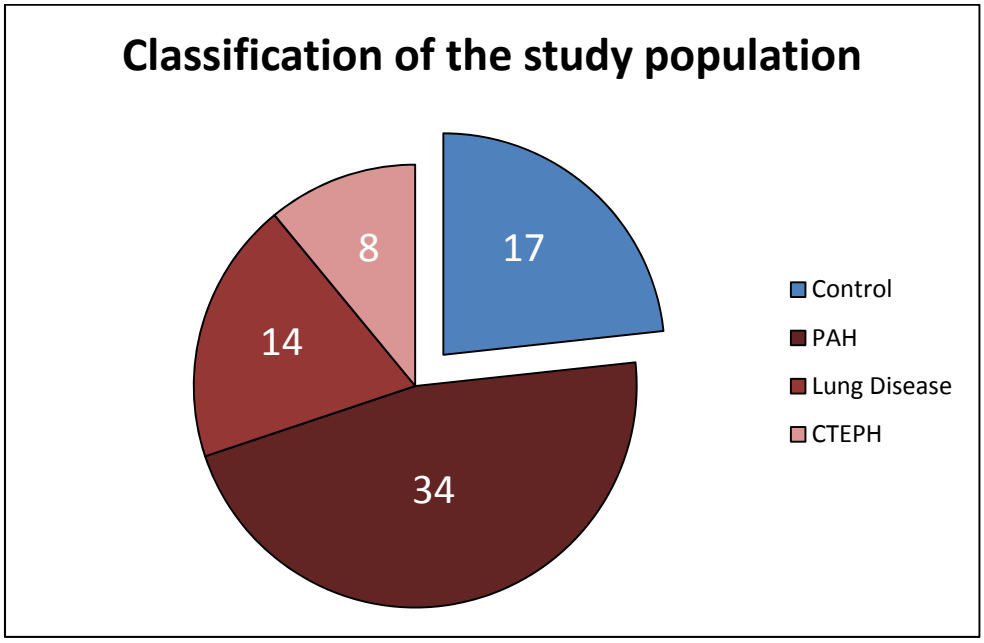

Figure 5 : Classification of study population

6.1.1 Basic characteristics

TABLE 5		Basic Characteristics Of The Study Population			
		Control	PAH	Lung Disease	CTEPH
Numbers	[n]	17	34	14	8
Age	[years]	45.4±14.2 (Range: 27-77)	57.8±17.7 [a b] (Range : 18-88)	68.6±12.2 [a c] (Range : 44-80)	68.9±6.8 [a] (Range : 54-75)
Gender [d]	[Male/Female]	6/11	10/24	12/2	1/7

a: p<0.05 vs control; b: p<0.05 vs Lung disease; c: p<0.05 vs PAH; d: p<0.001 (χ^2 = 17.1656)

Table 5 : Basic characteristics of the study population

The lung disease group included 9 patients with idiopathic pulmonary fibrosis (IPF), 2 patients with chronic obstructive lung disease (COPD), 1 with combined pulmonary fibrosis and emphysema (CPFE), 1 with hypoxemia due to obesity hypoventilation syndrome and 1 with extrinsic allergic alveolitis (EAA).

Table 6	Types Of Lung Diseases Included
Disease	**Number**
Idiopathic pulmonary Fibrosis	9
COPD	2
CPFE	1
Obesity hypoventilation	1
Extrinsic allergic alveolitis	1

Table 6 : Types of lung diseases included

Age

The mean age of the whole study population was 58.2±17.1 years with a range from 18 to 88 years. There was a significant difference between the age of the control group (45.4±14.2 years) and the age of the patients with PAH (57.8±17.7 years), PH related to lung disease (68.6±12.2 years) as well as the CTEPH group (68.9±6.8 years). Furthermore, a significant difference in the age of patients with PAH compared to the lung disease group was found.

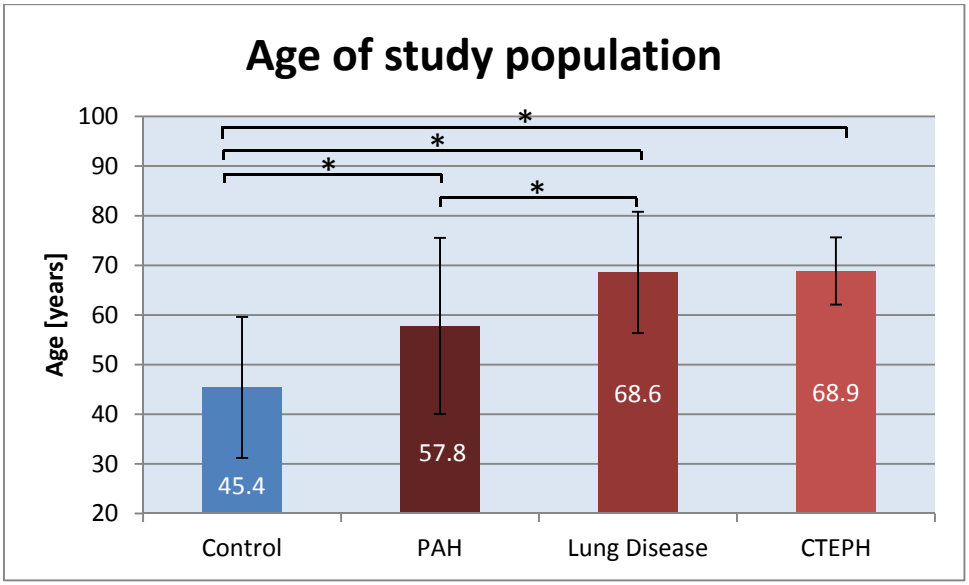

Figure 6 : Age of study population (* $p < 0.05$)

Gender Ratio

In all groups but in the lung disease group, a female predominance (Control: 64 %, PAH: 70 %, Lung disease: 14 %, CTEPH: 87 %) was found ($p<0.001$, $X^2 = 17.1656$).

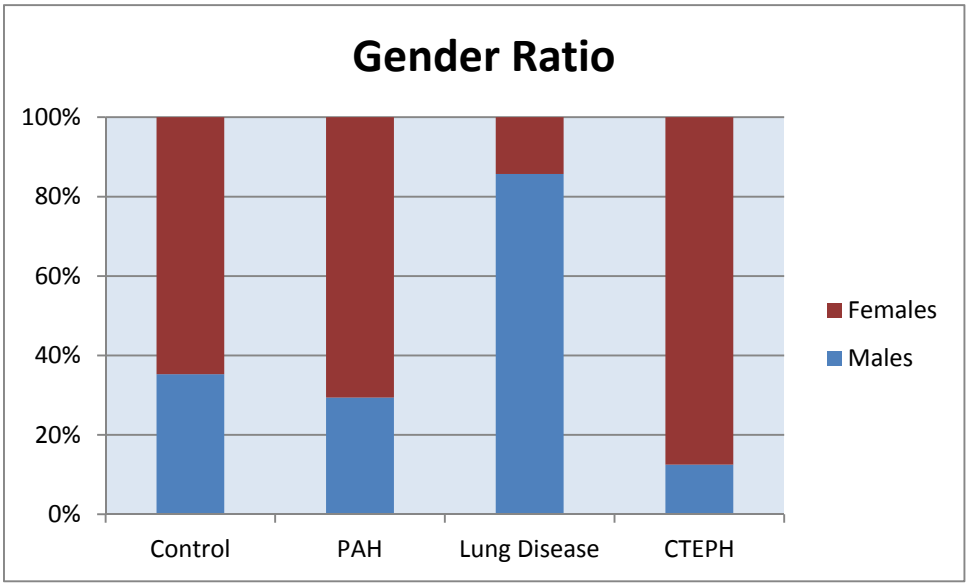

Figure 7 : Gender Ratio

6.1.2 Hemodynamic measurements

Pulmonary Arterial Pressures (PAP_{sys}, PAP_{dia}, PAP_m)

Table 7		Pulmonary Arterial Pressures							
		Control		PAH		Lung Disease		CTEPH	
Parameter		Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
PAP_{sys}	[mmHg]	23.1±4.4	19-30	76.2±19.2 [a b]	37-117	50.2±13.3 [a c]	27-74	68.0±22.3 [a]	37-89
PAP_{dia}	[mmHg]	10.1±2.0	7-13	31.6±9.0 [a b d]	15-66	24.4±6.0 [a c]	15-37	24.3±6.8 [a c]	18-36
PAP_m	[mmHg]	15.0±1.7	13-18	47.4±10.6 [a b]	27-73	37.2±9.9 [a c]	19-52	41.0±10.9 [a]	27-56
a: p< 0.05 vs control; b: p<0.05 vs Lung disease; c : p< 0.05 vs PAH d : p < 0.05 vs CTEPH									

Table 7 : Pulmonary arterial pressures of the study population

All pulmonary arterial pressures in control patients were normal at rest and significantly lower than those in the 3 groups with PH-patients.

By definition, PAP_m was higher than 25 mmHg in all PH-groups while the PAP_m levels in the controls were normal. As expected, the PAP_m was significantly higher in the pulmonary hypertension groups than in the control group ($p<0.05$) (see table 7).

The mean PAP_{sys} was also significantly higher in patients with PH compared to the controls ($p<0.05$). The same significant pattern was observed for the PAP_{dia} ($p<0.05$) (see table 7).

Significantly higher PAP_{sys}, PAP_{dia} and PAP_m were found in patients with PAH when compared to patients with lung disease while PAP_{dia} was also significantly different in PAH when compared to the lung disease or CTEPH group. One patient with COPD had normal PAP_m, PAP_{sys}, PAP_{dia} at rest. This patient was nevertheless included in the pulmonary hypertension group as there was an abnormal vascular reaction during light effort with a pathological increase of all pulmonary artery pressures.

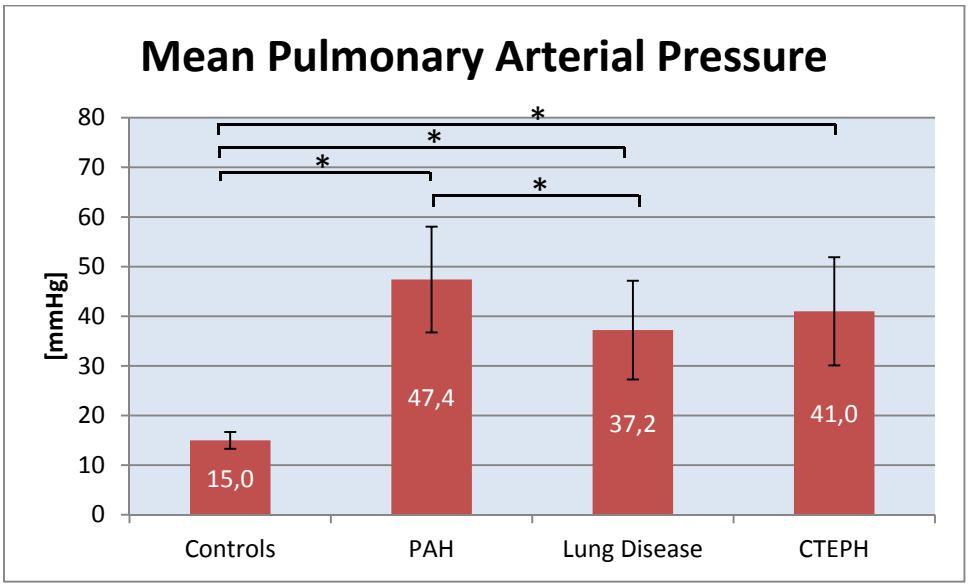

Figure 8 : **Mean pulmonary arterial pressure** (* $p < 0.05$)

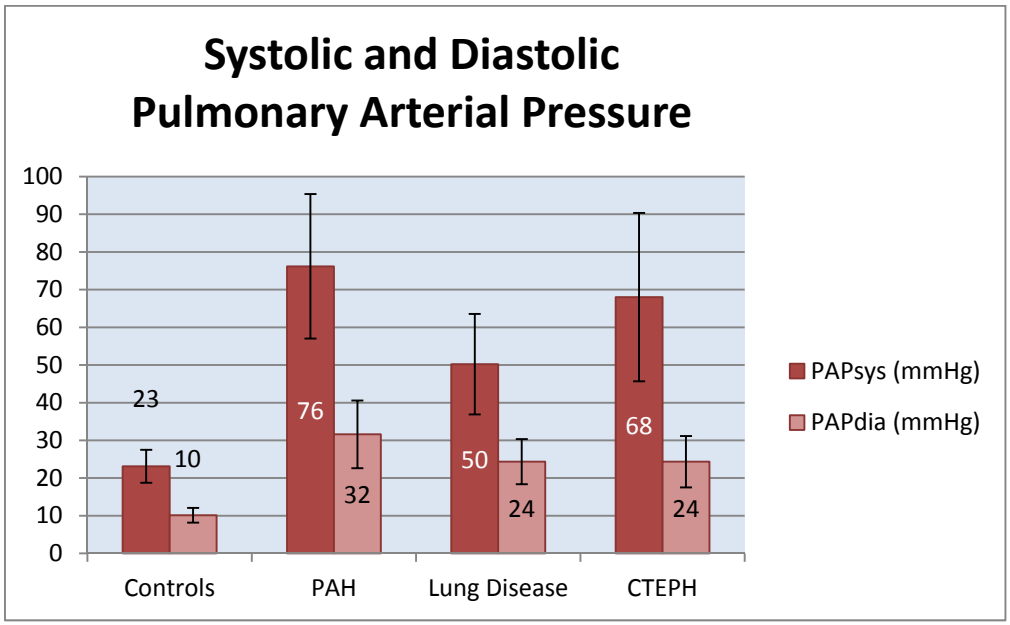

Figure 9 : **Systolic and diastolic pulmonary arterial pressure**

Cardiac output (CO) and index (CI)

Table 8	Cardiac Output and Index							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
CO [l/min]	4.81±0.93	3.27-5.90	4.81±1.53	2.00-8.67	5.32±1.39	2.62-8.00	4.56±2.18	2.72-8.46
CI [l/min/m^2]	2.8±0.56	2.21-3.39	2.55±0.74	1.50-4.78	2.62±0.43	1.77-3.28	2.57±1.23	1.47-4.80
No significant differences found								

Table 8 : Cardiac output and index

The mean cardiac output at rest was normal in all groups. No significant differences between the various groups were found (table 8).

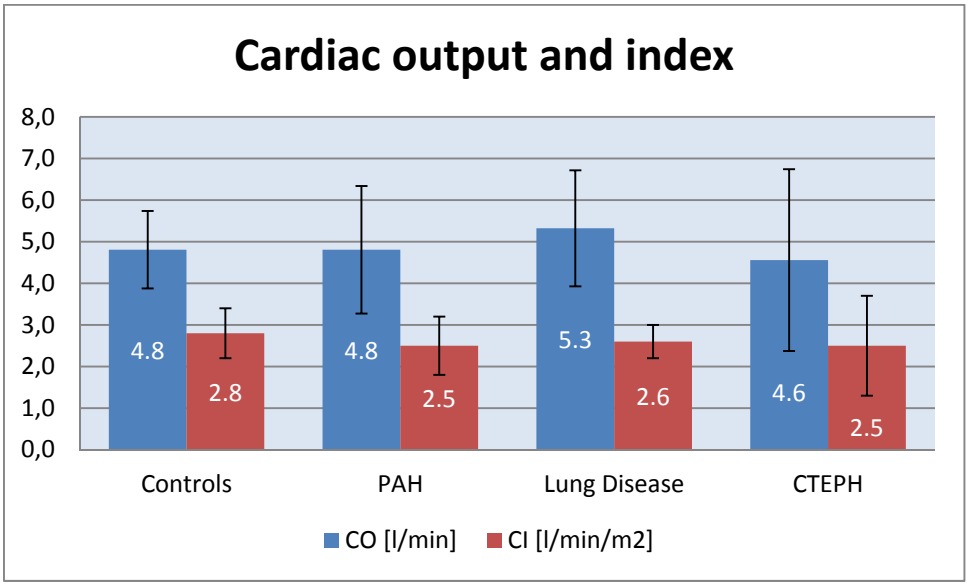

Figure 10 : Cardiac output and index

Pulmonary vascular resistance (PVR)

Table 9	Pulmonary Vascular Resistance							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
PVR [dyn·s·cm^{-5}]	123±39	81-174	711±351 [a,b]	240-1712	451±269 [a,c]	170-1099	656±373 [a]	132-1088
a: p< 0.05 vs control; b: p<0.05 vs Lung disease; c : p< 0.05 vs PAH								

Table 9 : Pulmonary Vascular Resistence

The mean pulmonary vascular resistance was normal in the control population. Two control subjects with a normal PAP_m had a discretely elevated PVR (171 and 174 dyn·s·cm^{-5}) but no clinical hemodynamic pathology was evident.

All PH patients had an elevated PVR. The mean PVR of the PAH, lung disease and CTEPH group was significantly higher than the control group ($p<0.05$). There was also a significant difference between the PAH and lung disease group, but not between other pulmonary hypertension groups (see table 9).

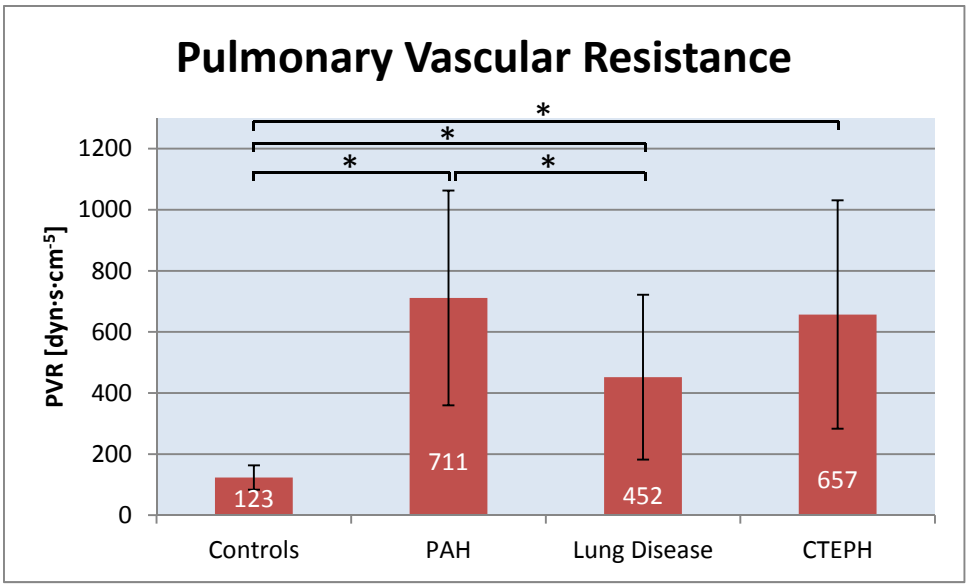

Figure 11 : Pulmonary vascular resistance (* p < 0.05)

Pulmonary Arterial Wedge Pressure (PAWP)

Table 10		Pulmonary Arterial Wedge Pressure							
		Control		PAH		Lung Disease		CTEPH	
Parameter		Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
PAWP	[mmHg]	7.6±2.4	3-11	9.7±2.6	6-15	10.4±1.8[a]	8-14	11.5±3.8	8-18
a: p< 0.05 vs control									

Table 10 : **Pulmonary Arterial Wedge Pressure**

The mean pulmonary arterial wedge pressure at rest was normal in all groups. A significantly higher, but still normal mean PAWP was found in patients with lung diseases compared to the control group. One patient with proven CTEPH had a PAWP over 15 mmHg suggesting a coexisting left heart disease (table 10).

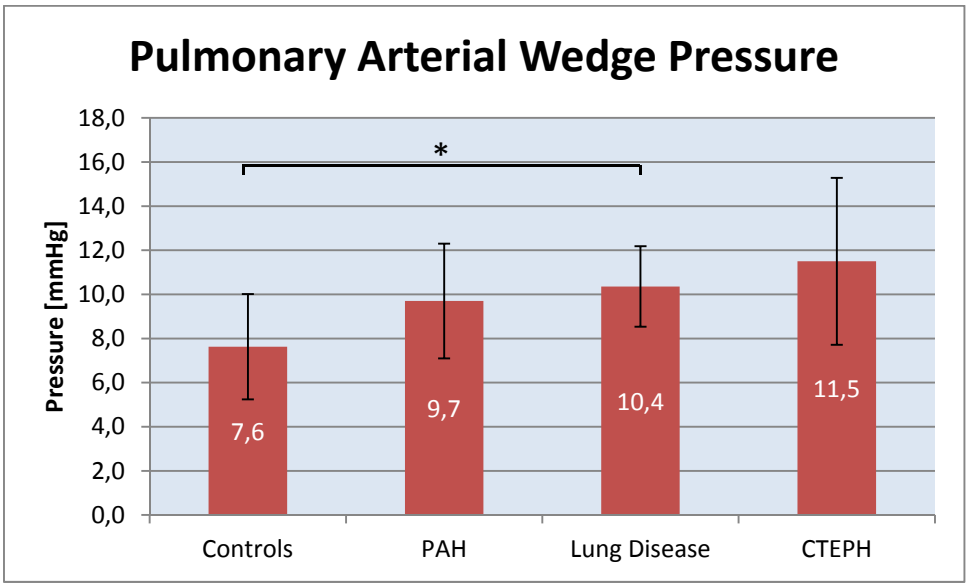

Figure 12 : **Pulmonary Arterial Wedge Pressure** (* p < 0.05)

Transpulmonary gradient (TPG)

Table 11	Transpulmonary Gradient							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
TPG [mmHg]	7.4±2.0	6-11	37.7±11.2[a b]	19-61	26.9±10.3[a c]	10-42	29.5±10.5[a]	14-39
a: p< 0.05 vs control; b: p<0.05 vs Lung disease; c : p< 0.05 vs PAH								

Table 11 : Transpulmonary Gradient

As expected, the TPG was significantly higher in the pulmonary hypertension groups (p<0.05) (see table 11). Furthermore, there was a significant difference between the PAH and lung disease group.

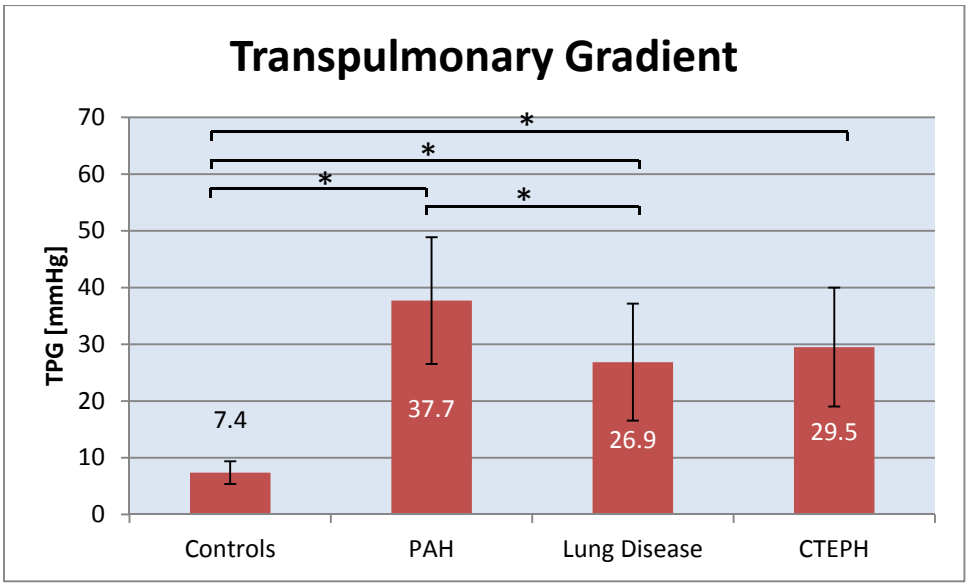

Figure 13 : Transpulmonary gradient (* p < 0.05)

Central venous oxygen saturation ($S_{cv}O_2$)

Table 12	Central Venous Saturation							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
$S_{cv}O_2$ (%)	74.3±2.7	70-77	64.0±6.4[a]	53-75	66.2±7.4	56-78	63.2±8.0[a]	52-74
a: p< 0.05 vs control								

Table 12 : Central Venous Saturation

Central venous oxygen saturation at rest was normal in all control patients. A significant difference in the mean $S_{cv}O_2$ was found in the PAH and CTEPH group compared to the controls ($p<0.05$) (see table 12). No differences between the different lung diseases and controls and in between PH groups were noted.

Heart rate

Table 13	Heart Rate							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
HR [bpm]	71.1±13.2	54-97	74.2±10.6	53-102	81.2±17.3	57-110	69.7±11.1	55-85
No significant differences between groups								

Table 13 : Heart rate

All groups had a normal mean heart rate at rest and no significant differences between the groups were found (table 13).

Blood Pressure

Table 14	Blood Pressure							
	Control		PAH		Lung Disease		CTEPH	
Parameter	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
BP_{sys} [mmHg]	119.1±12.2	107-136	122.7±16.3	100-160	136.4±17.7	107-174	136.2±13.9	113-153
BP_{dia} [mmHg]	72.7±7.3	63-80	69.1±9.3	42-87	78.8±10.3	62-103	75.8±15.6	52-93
No significant differences between groups								

Table 14 : Blood pressure

Blood pressure was normal in all patients. No significant difference between the various groups was found (table 14).

6.2 Concentration of biomarkers in healthy persons and in PH

TABLE 15		Concentrations In Controls And Pulmonary Hypertension			
		Controls		Pulmonary Hypertension	
		Mean ± SEM	Range	Mean ± SEM	Range
TSP-1	[ng/ml]	**82±16**	8-285	**460±101** [a]	33-3382
bigET	[pg/ml]	**0.77±0.25**	0.18-2.60	**2.50±0.36** [a]	0.18-9.60
PDGF-ββ	[pg/ml]	**139±17**	0-209	**754±151** [a]	0-4870
PF-4	[pg/ml]	**2169±265**	772-4629	**2998±277** [a]	928-9845
a: p< 0.05 vs control					

Table 15 : Concentrations of biomarker in controls and pulmonary hypertension

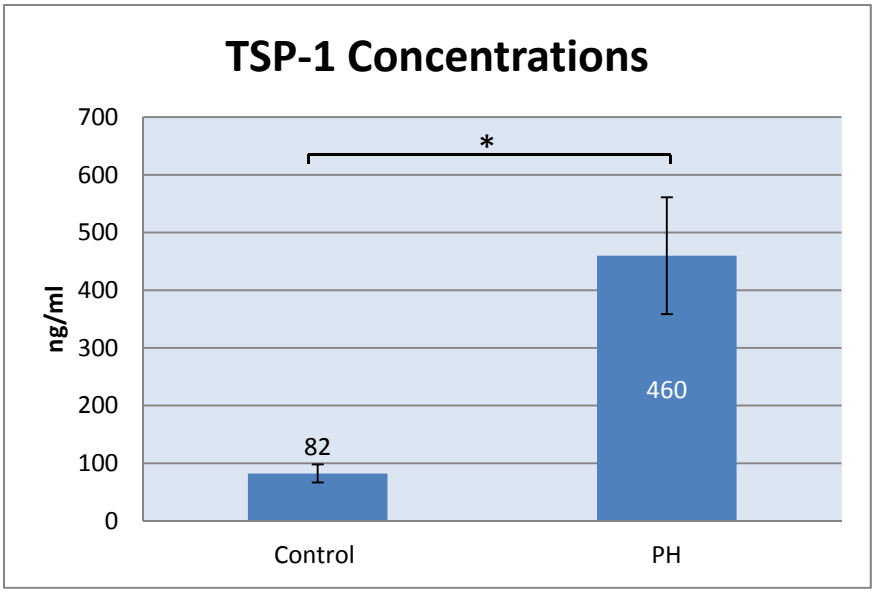

Figure 14 : Thrombospondin-1 concentrations (* p < 0.05)

We found a 5.6-fold elevation of plasma TSP-1 in patients with PH compared to the healthy group ($p < 0.05$) (figure 14). No significant gender difference in TSP-1 concentrations could be demonstrated in either healthy (males: 69±18 ng/ml, females: 89±22 ng/ml) or sick subjects (males: 342±107 ng/ml, females: 545±156 ng/ml). No association with age could be proven by Spearman rank ordered correlation.

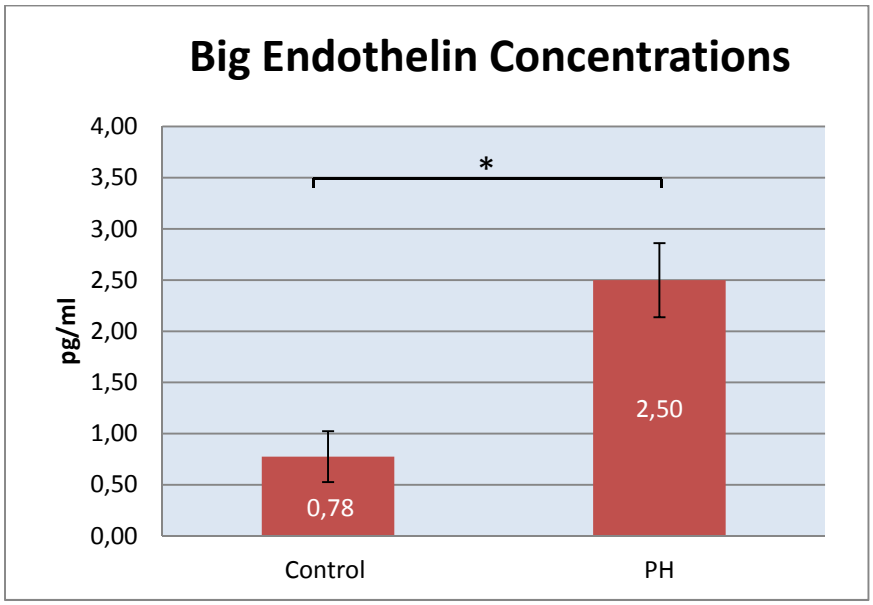

Figure 15 : **Big-ET concentrations** (* $p < 0.05$)

As expected, a significant elevation of Big-ET levels was found in the PH-group when compared to the control group (0.77±0.25 vs. 2.50±0.36 pg/l, $p< 0.05$) (figure 15).

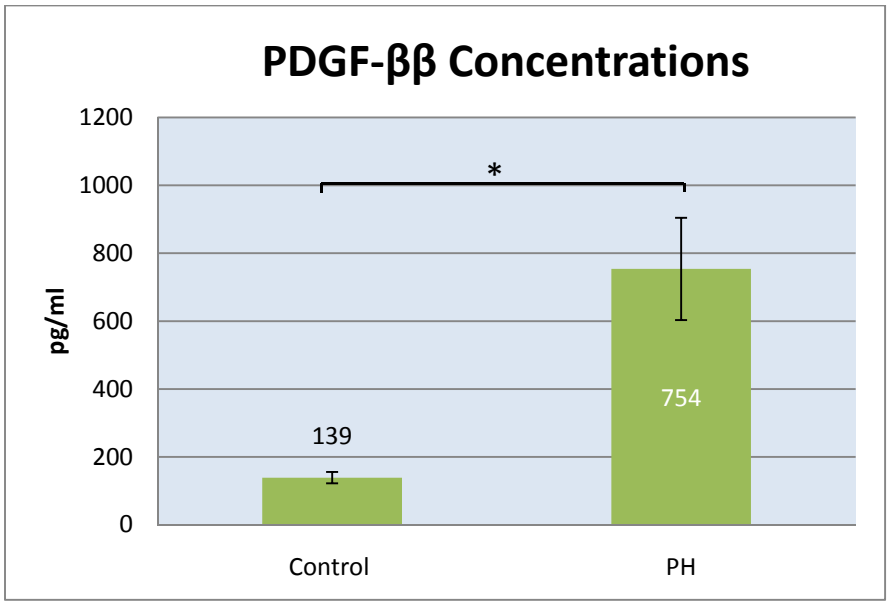

Figure 16 : **PDGF-ββ concentrations** (* $p < 0.05$)

PDGF-ββ was significantly increased in patients with PH compared to the control group (139±17 vs 754±151 pg/ml, $p< 0.05$) (figure 16).

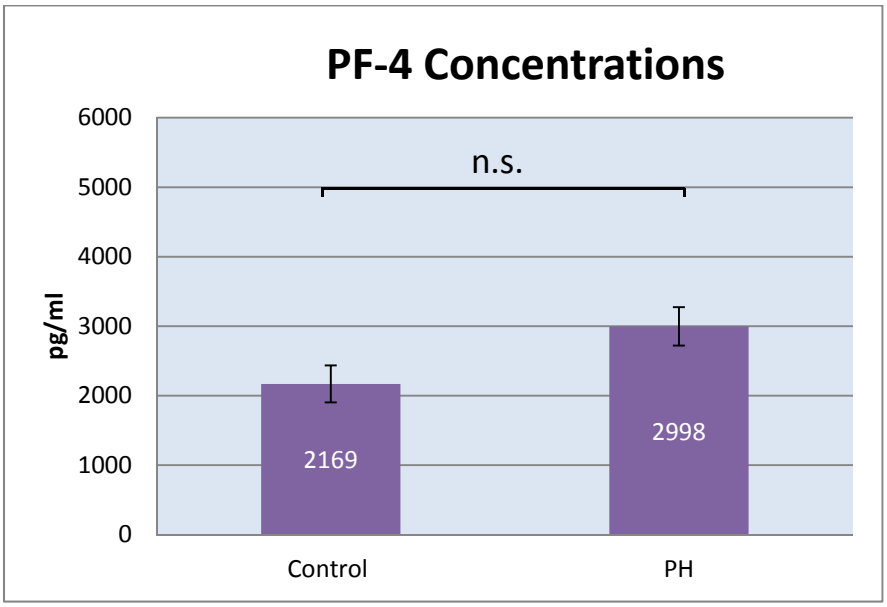

Figure 17 : **Platelet Factor 4 concentrations** (n.s. : not significant)

No significant difference in PF-4 concentrations was found in the PH-population and healthy subjects (figure 17).

6.3 Subgroup analysis of concentrations according to the DANA point classification

In a subgroup analysis, we investigated the concentrations of TSP-1, Big-ET, PDGF-ββ and PF-4 according to the DANA Point classification of pulmonary hypertension (see table 16).

TABLE 16		Subgroup Analysis of Biomarker Concentrations							
		Controls		PAH		Lung Disease		CTEPH	
		Mean ±SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
TSP-1	**[ng/ml]**	**82±16**	8-285	**498±134** [a]	38-3382	**238±91** [a c]	33-1292	**719±428** [a b]	54-3241
Big-ET	**[pg/ml]**	**0.77±0.25**	0.18-2.60	**2.62±0.48** [a]	0.18-2.60	**2.06±0.49** [a]	0.18-9.60	**3.45±2.72** [a]	0.72-6.17
PDGF-ββ	**[pg/ml]**	**139±17**	0-209	**905±253** [a]	82-4870	**614±139** [a]	133-1278	**420±180**	0-957
PF-4	**[pg/ml]**	**2169±265**	772-4629	**2811±298**	928-7520	**3441±687**	1088-9845	**2800±682**	1142-5282
a: $p < 0.05$ vs control; b: $p < 0.05$ vs Lung disease; c: $p < 0.05$ vs CTEPH									

Table 16 : Subgroup analysis of biomarker concentrations

We found a significant increase of TSP-1 in all analyzed PH groups compared to the control population. Furthermore, we found a difference between plasma concentration of TSP-1 in lung disease and CTEPH ($p < 0.05$) while no difference between PAH and CTEPH or PAH and lung disease could be found (figure 18).

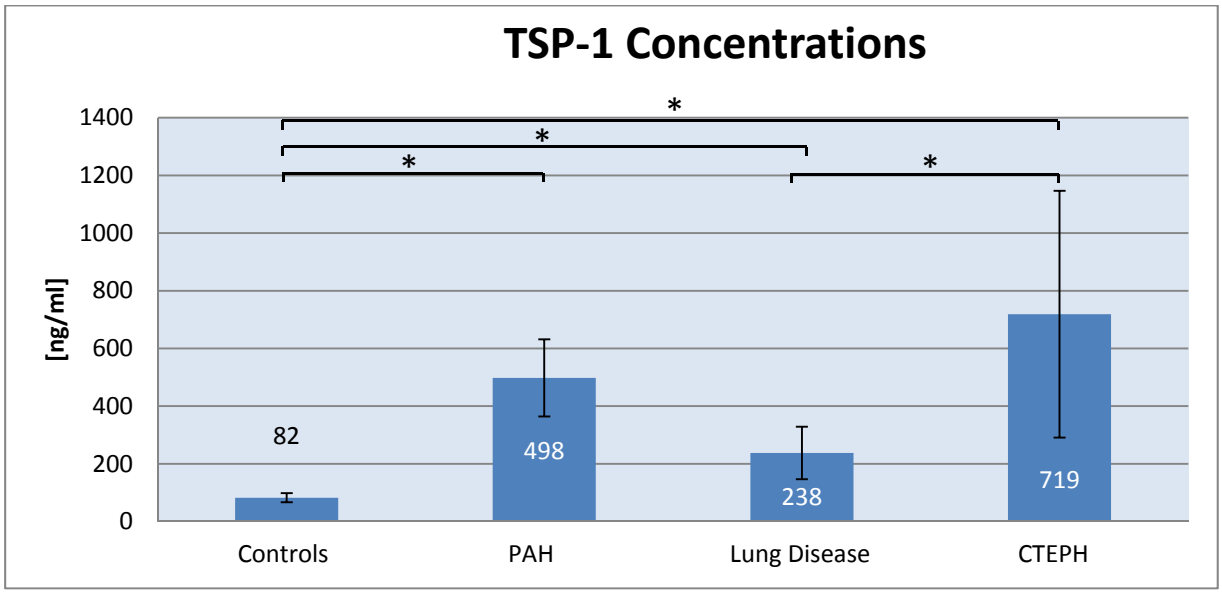

Figure 18 : **Subgroup analysis of TSP-1 concentrations** (* $p < 0.05$)

Big-ET was significantly elevated in all PH groups compared to the control population ($p < 0.05$). No difference between the various PH groups could be found (figure 19).

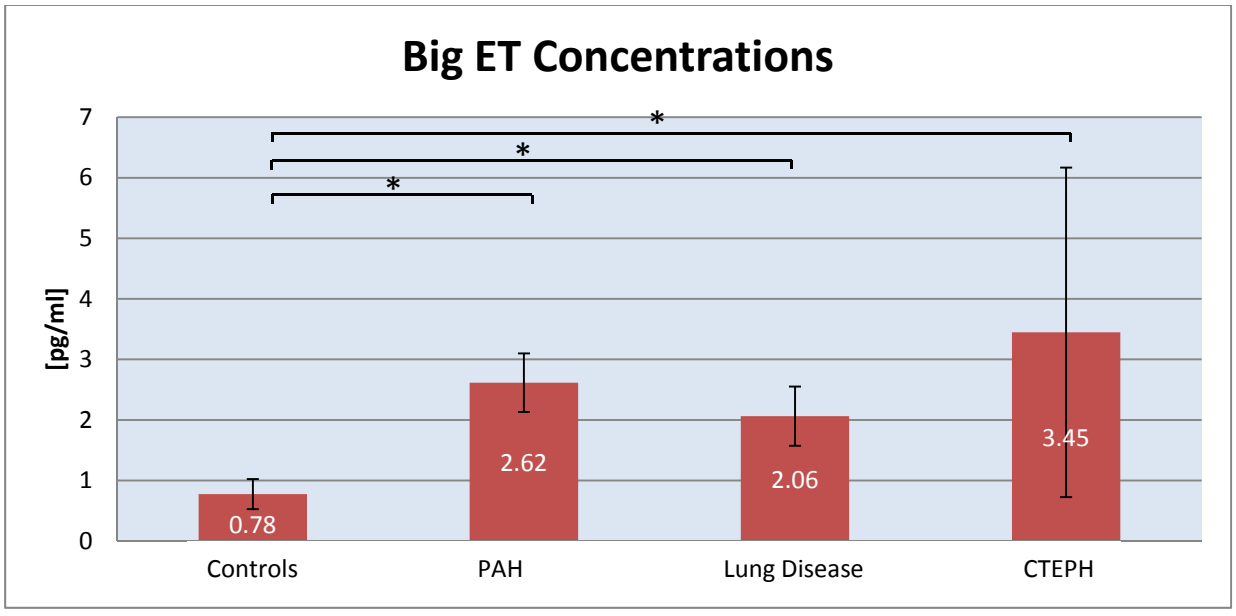

Figure 19 : **Subgroup analysis of Big-ET concentrations** (* $p < 0.05$)

There was a significant elevation of serum PDGF-ββ concentrations in PAH and lung disease compared to the controls. No significant difference between controls and CTEPH as well as between the PH groups was found (figure 20).

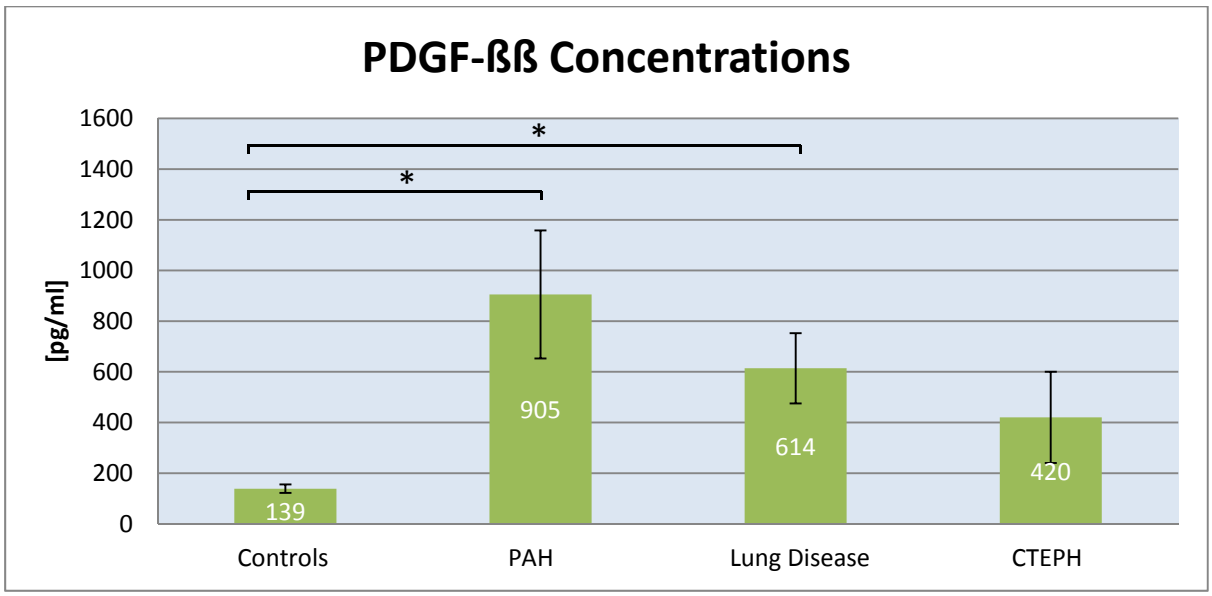

Figure 20 : **Subgroup analysis of PDGF-ββ concentrations** (* $p < 0.05$)

No significant difference in PF-4 serum concentrations was found in the different PH groups when compared to the controls. In addition no difference between PAH, lung disease or CTEPH was found (figure 21).

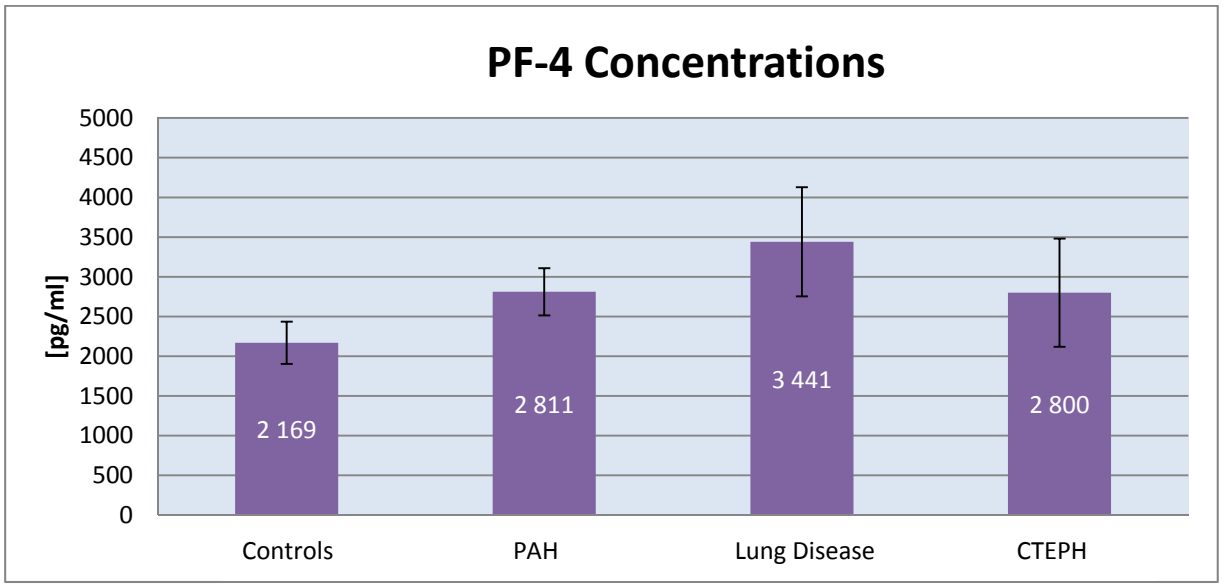

Figure 21 : **Subgroup analysis of PF-4 concentrations**

6.4 *Receiver Operating Characteristic Curve of TSP-1 for the diagnosis of Pulmonary Hypertension*

Based on the receiver operator characteristics curve the TSP-1 cut-off for prediction of pulmonary hypertension (defined as mean pulmonary artery pressure of more than 25 mmHg) was optimized.

The predictive values, sensitivity, specificity, accuracy and Matthews correlation coefficient (MCC) were determined stepwise for a range between 80 ng/ml and 140 ng/ml. The results are described in table 17. Additionally, X^2 and p-value of the corresponding contingency table are listed.

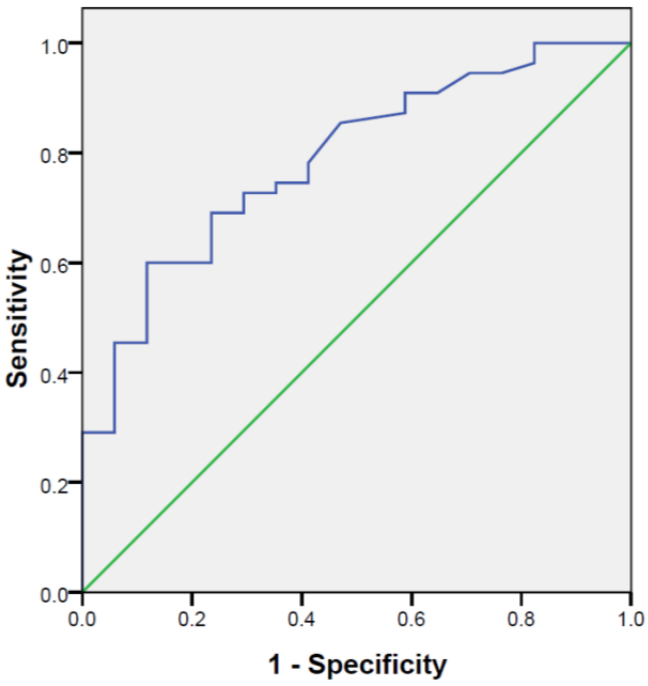

Figure 22 : ROC curve of TSP-1 for the diagnosis of pulmonary hypertension

Table 17	Area Under Curve	
Area under Curve	Standard error	Significance
0.788	0.06	< 0.05

Table 17 : AUC of TSP-1 for the diagnosis of pulmonary hypertension

TABLE 18	Diagnostic Value Of TSP-1 At Different Cut-offs							
Cut-off	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**ACC**	**MCC**	X^2	**p**
80ng/ml	74.54	55.55	83.67	41.66	0.6986	0.2762	5.56817	0.018
95ng/ml	69.09	72.22	88.37	43.33	0.6986	0.3619	9.56191	0.002
110ng/ml	61.81	77.77	89.47	40.00	0.6438	0.2941	6.6349	0.012
125ng/ml	60.00	77.77	89.18	38.88	0.6438	0.3257	7.74331	0.005
140ng/ml	47.27	83.33	89.65	34.09	0.5616	0.2696	5.30545	0.021

Table 18 : Diagnostic value of TSP-1 at different cut-offs

According to the table above, the optimum for a TSP-1 cut-off at 95 ng/ml was confirmed by maximization of the parameters as stated above and the maximum of Matthew's correlation coefficient. The two-way contingency table for this cut-off is shown in table 19.

TABLE 19	Contingency Table For PH, TSP-1 cut-off 95ng/ml		
	Controls	**Pulmonary Hypertension**	
TSP-1 < 95ng/ml (neg.)	13	17	*30*
TSP-1 > 95ng/ml (pos.)	5	38	*43*
	18	*55*	**N=73**
X^2 = 9.56191, p = 0.002			

Table 19 : Contingency table for PH at TSP-1 cut-off 95 ng/ml

The cut-off yielded a sensitivity of 69.09% (55-80) and a specificity of 72.22% (46-90). Positive and negative predictive values were determined as 88.37% (74-96) and 43.33% (25-62) respectively. Accuracy of TSP-1 at a cut-off 95ng/ml for pulmonary hypertension was 69.86%. The Matthew's correlation coefficient (MCC) reached a maximum of 0.36191 compared to the other tested cut-off points.

Number needed to diagnose

The number needed to diagnose at a TSP-1 cutoff of 95 ng/ml is 2.4 (Range: 1.6- 8.9).

6.5 TSP-1 Correlations

The association of TSP-1 concentration and both hemodynamic parameters and circulating markers was examined and is summarized in table 20. As most of the variables were not normally distributed, Spearman´s ranked order correlation was used.

TABLE X	**TSP-1 Correlations**		
	r_s (tie corrected)	**p**	
PAP_{sys}	0.2354	0.075	n.s.
PAP_{dia}	0.2411	0.068	n.s
PAP_m	0.2356	0.075	n.s
PAWP	-0.1180	0.378	n.s.
TPG	0.2498	0.059	n.s.
CO	-0.3344	0.012	*
PVR	0.3075	0.020	*
RR_{sys}	0.0701	<0.5	n.s.
RR_{dia}	0.1552	0.253	n.s.
ScvO2	-0.0120	<0.5	n.s.
Big-ET	0.4989	<0.001	*
PDGF-ββ	0.4668	<0.001	*
PF-4	-0.1356	0.297	n.s.

Table 20 : **TSP-1 Correlations** (* $p < 0.05$, n.s. : not significant)

A moderate negative correlation of TSP-1 concentration with cardiac output was found with r_s of -0.3344 ($p = 0.012$). Additionally, PVR demonstrated a slightly weaker correlation of r_s = 0.3075 ($p = 0.020$). Pulmonary artery pressures showed lower correlation coefficients with p-values slightly above statistical significance. Both Big-ET and PDGF-ββ demonstrated significant associations of r_s = 0.4989 and r_s = 0.4668 respectively ($p < 0.001$). There was no correlation of TSP-1 with PF-4 as a marker of platelet activation and degranulation.

6.6 Hemodynamic Correlations

There was an increase in plasma TSP-1 concentration when correlated to the pulmonary vascular resistance (r = 0.41, p = 0.002) (figure 23).

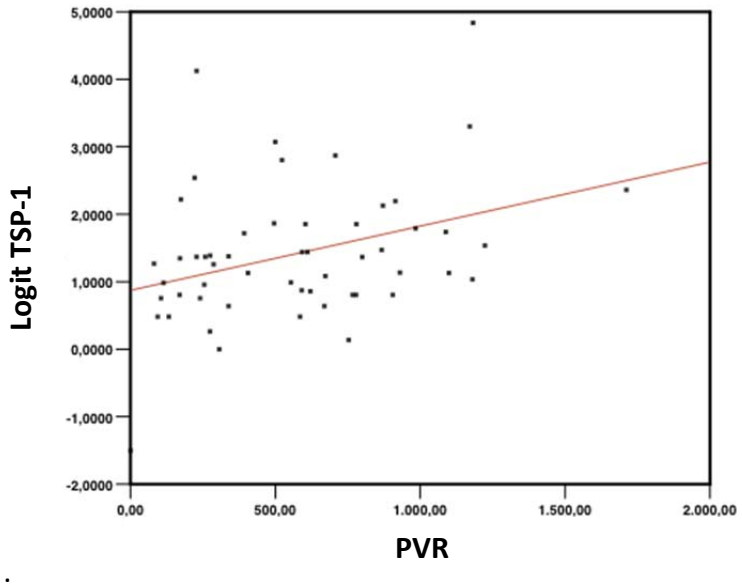

Figure 23 : Correlation between TSP-1 and PVR

A weak but significant negative correlation between logarithmically normalized TSP-1 concentrations and the cardiac output could be found (r = - 0.34, p = 0.01) (figure 24).

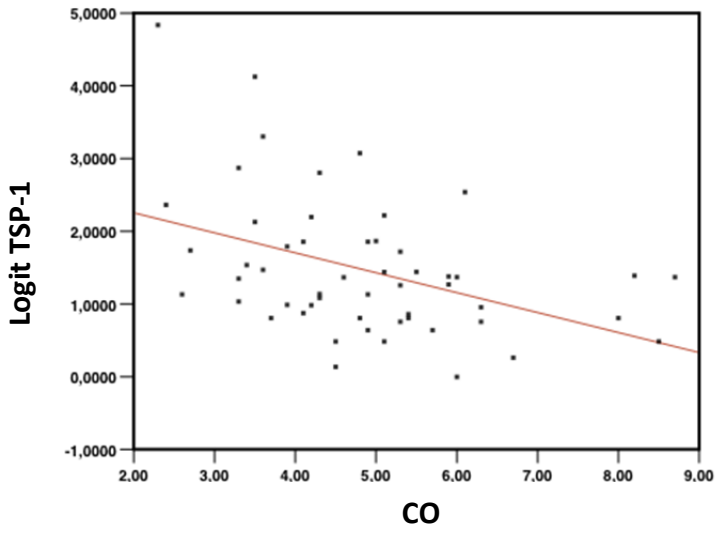

Figure 24 : Correlation between TSP-1 and CO

7 Discussion

TSP-1 in healthy subjects

We found a mean serum concentration of 82±16 ng/ml in the healthy controls. No significant difference between males and females (69±18 vs 89±22 ng/ml) was found and there was no correlation between age and TSP-1 concentrations.

Our TSP-1 concentrations in healthy subjects were consistent with previous studies where levels ranged from 60.6 ± 10.7 ng/ml (Switalska, Niewiarowski et al. 1985) (n=4) to 105.0 ± 31.0 ng/ml (Dawes, Clemetson et al. 1983) (n=4). One study (n= 66) showed slightly lower values (40±21 ng/ml) (Kehrel, Flicker et al. 1996) while another study (n= 20) showed higher values 175 ng/ml) (Saglio and Slayter 1982). This last study did not find a correlation with age or a difference between genders either.

The different concentrations found between those studies can be explained by the varying anticoagulants used. Indeed, Switalska et al. showed that TSP-1 concentrations were 60.0±10.7 ng/ml with acid-citrate-dextrose, 97.2±31,0 ng/ml with EDTA + theophylline + prostaglandin E1 and 145.9±56.4 ng/ml with EDTA alone. Serum levels topped 10101±6800 ng/ml (Switalska, Niewiarowski et al. 1985).

TSP-1 in pulmonary hypertension

In our patients with pulmonary hypertension, TSP-1 concentrations were significantly increased by a factor of 5.6 when compared to healthy subjects, a finding which has not yet been shown. This elevation was present in any of the groups of pulmonary hypertension. Thus the different pathophysiological entities may share a common mechanism leading to TSP-1 elevation.

Between the pulmonary hypertension groups, a significant difference between patients with lung disease and CTEPH was found. Even though each hemodynamic parameter did not differ significantly between the two groups, the combination of both higher PAP_m and higher PVR together may have led to an increased peak flow in CTEPH patients and by that means to increased shear stress at an endothelial level. However, this hypothesis cannot be

proven as no objective measurement of shear stress in vivo exists to date. Another explanation may be the small size of the CTEPH group with a wide range.

Recent studies suggested the regulation of TSP-1 in age-related left heart failure by miRNA-18 and miRNA-19 (van Almen, Verhesen et al. 2011). Hence, as age may influence TSP-1 levels in pulmonary hypertension, correlation between age and TSP-1 levels were analyzed in our patients. However, such correlations were not being found in this study either. In addition no difference between genders was found in the pulmonary hypertension groups.

PF-4

It was important to monitor platelet activation and degranulation as TSP-1 is present abundantly in the platelet's alpha-granules. In vitro activation of platelets may lead to an artificial rise of measured TSP-1 levels. We chose the commonly used platelet activation marker PF-4 for monitoring platelet activation in vitro and in vivo (Kaplan and Owen 1981).

Only one study analyzed PF-4 in an animal model of CTEPH (Guarneri, Molinari et al. 1988). No changes in PF-4 levels in minipigs before and after induction of pulmonary microembolism was noted. As a consequence, it is very likely that in non-thromboembolic origins of pulmonary hypertension (PAH, lung disease), PF-4 levels would have a similar pattern and would not be altered despite in situ thrombosis playing an important role in PAH. Thus an elevation of PF-4 would be the mere product of platelet activation.

However, no significant difference of PF-4 concentrations was found between groups. Those findings strongly eliminated artificial in vitro activation as the mechanism of TSP-1 elevation. Furthermore, no correlation between PF-4 concentrations and TSP-1 levels was found, indicating that platelets are very unlikely to be a source of TSP-1 in the background of pulmonary hypertension. Finally, as we did not find any association of PF-4 with either biomarkers or hemodynamic variables, the role of platelet activation in stable pulmonary hypertension seems limited, as already suggested by Guarneri et al. in the context of CTEPH (Guarneri, Molinari et al. 1988).

Basic characteristics

Our study has the inherent limitations of any small case series. Due to the low prevalence of pulmonary hypertension, we had a limited number of patients to enroll. The size of the subgroups varies significantly, limiting possibilities for statistical calculations. Furthermore, the study was designed as an observational cross-sectional study. Neither timelines for outcome nor reactions to medications were monitored.

As patients were included on the basis of hospital visits, we were not able to match the different groups in regards to age and gender. This led to a statistically significant difference between the patients and the control group. The higher female proportion in the lung disease group might be attributed to the small sample size. Though there was a considerable heterogeneity of the different diseases in this group, the majority had idiopathic pulmonary fibrosis, which affects more male than female patients in the observed age range (Raghu, Weycker et al. 2006).

However, the differences noted above should not influence the results of the main read-out as it has already been shown by Kehrel and co-workers that there is no correlation between TSP-1 concentration and age in healthy individuals. Additionally, no gender differences were found in the same study (Kehrel, Flicker et al. 1996). As age may still influence TSP-1 levels in pathologies (van Almen, Verhesen et al. 2011), corresponding calculations were nonetheless performed. Neither gender differences nor any association between age and TSP-1 levels could be demonstrated in healthy or disease states.

Hemodynamics

All pulmonary pressures (PAP_m, PAP_{sys}, PAP_{dia}) as well as the PVR and TPG were significantly higher in the PH groups compared to the controls. There was a significant difference between PAP_m, PAP_{sys}, PVR and TPG in patients with PAH and lung disease. These findings are consistent with the current literature. In general, the degree of PH in patients with lung disease tends to be low or moderate in magnitude when compared to PAH (Nathan, Shlobin et al. 2007; Hoeper, Barbera et al. 2009).

No difference in cardiac output or cardiac index was found between the different patient groups. However, the range of cardiac output in pulmonary hypertension was broader than in the control group with the lowest cardiac output values being in the disease group. It is also noteworthy to emphasize that right heart catheterization is performed during rest and that measurements during effort are not performed routinely. Thus cardiac output at rest does not reflect the actual cardiac effort capacity which may still be pathological in the pulmonary hypertension group.

Usability of TSP-1 as a biomarker

Usability of TSP-1 as a biomarker for PH seems limited in this first study for several reasons. First, great care was given to sampling conditions by stabilization of platelets using manufactured sampling tubes and quick cooling of the samples, which cannot be guaranteed in a clinical setting. Second, no standard anticoagulant for TSP-1 sampling has been defined. However, previous publications as well as our own examinations describe different normal values and considerable variations between anticoagulants (Switalska, Niewiarowski et al. 1985). Third, an increase of TSP-1 in other pathologies involving endothelium and platelets is to be expected. Indeed, unpublished data suggested an increase in peripheral arterial disease and post myocardial infarction followed by percutaneous coronary intervention. Finally, non-normality of distribution is expected with a broad range especially in advanced stages of the disease.

TSP-1 and hemodynamics

The univariate correlations showed a significant association of circulating TSP-1 and pulmonary vascular resistance ($r = 0.41$, $p = 0.002$) and cardiac output ($r = - 0.34$, $p = 0.01$). This finding supports the initial hypothesis leading to this study.

The increase of pulmonary resistance should lead to increased shear stress resulting in a release of TSP-1 from pulmonary endothelial cells. However, the endothelial origin of TSP-1 release cannot be proven beyond absolute certainty in this study. Peripheral arterial blood samples were not part of our standard right heart catheterization procedure, leading to an inability to monitor TSP-1 level gradients. For a variety of biomarkers such pulmonary

gradients have been shown as a result of increased production or reduced clearance of the marker (Dupuis, Cernacek et al. 1998; Wilkens, Bauer et al. 2003). In our case, the peripheral vascular bed might serve as a source of TSP-1 due to altered hemodynamics or due to an elevation of circulating peptide hormones activating endothelial cells.

Furthermore, the effects of elevated TSP-1 on peripheral vascular reactivity have not been examined in pulmonary hypertension. Flow mediated dilation as a NO-mediated effect has been described to be decreased in idiopathic pulmonary hypertension (Wolff, Lodziewski et al. 2007). Associations of TSP-1 concentration with peripheral vascular tone or reactivity were not investigated yet and are currently subject to further investigations of our research group.

TSP-1 and in vivo shear stress

While previous biomarkers focus on neuroadrenergic activation (ANP, BNP) and myocardial damage or stretch (hs-Troponin, ANP, BNP), TSP-1 has both properties of a biomarker at the site of the damage (endothelium) and the potential as a biomarker for disease activity and usability of specific medications.

As proven in vitro, increased shear stress forms the basis for the endothelial upregulation and release of TSP-1. However, hemodynamic parameters to determine shear stress in humans remain scarce. The extent of shear stress in a specific vessel is determined by flow velocity, vessel diameter and even by acceleration of flow in pulsatile flow. Therefore shear stress is clinically influenced by both right ventricular contractility and properties of the vascular bed of the lung. Both sets of variables are difficult to obtain in vivo. PVR remains the best surrogate variable of standard right heart catheterization for estimation of shear stress. Nevertheless, individuals with similar PVR and different velocities as determined by CO and TPG may have different shear stress (see figure 25).

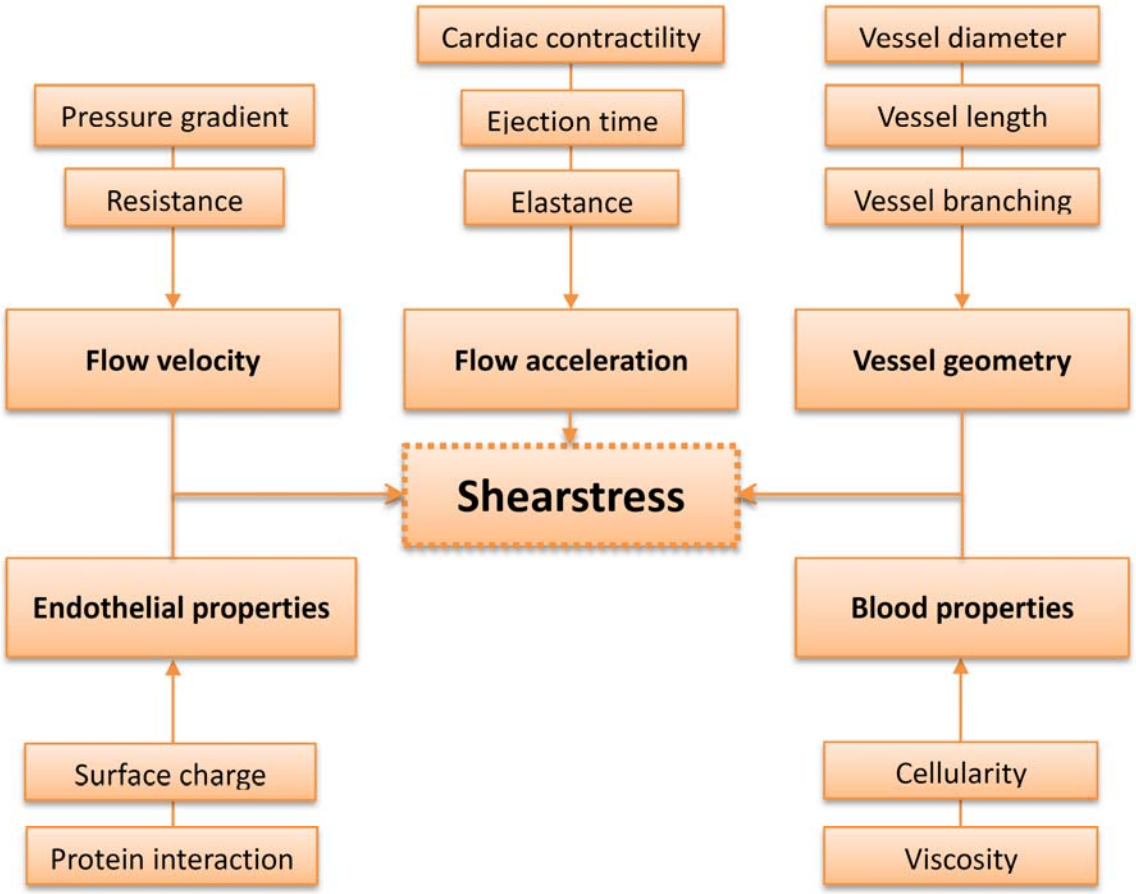

Figure 25 : Parameters influencing vascular shear stress

Furthermore, shear stress varies by location and the extent of TSP-1 net release depends on the amount of endothelial surface area suffering from pathological shear stress which is unknown in the clinical setting (figure 26). The specific threshold of a patient's endothelium for release of pathological levels of TSP-1 is not known, nor are alternative mechanisms of stimulation (platelet properties or levels of growth hormones). Considering the fact, that pathologically decreased shear stress also results in TSP-1 release leads to the assumption that at optimal shear stress a minimum of TSP-1 is produced (Rupp 2008). Thus the dependency of TSP-1 release and shear stress is neither linear nor monotonically increasing.

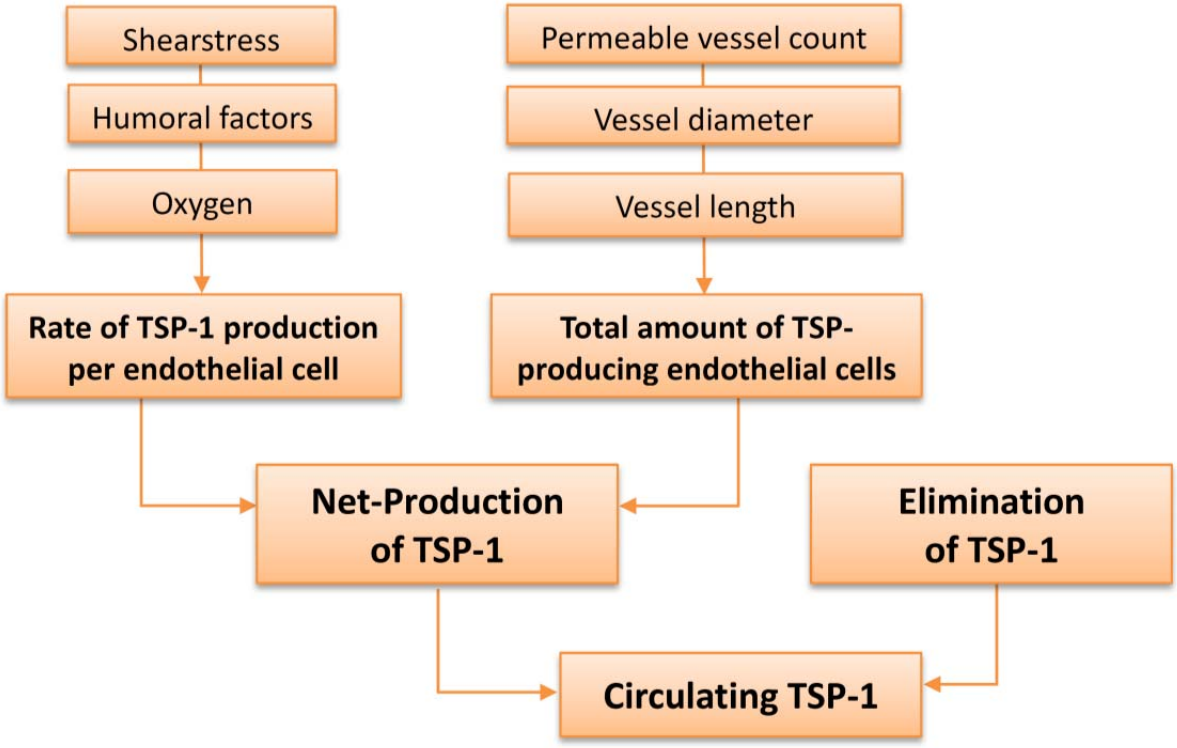

Figure 26 : Factors influencing circulating TSP-1 concentrations

During the course of disease, PVR is increasing and endothelial surface area is lost due to in situ thrombosis and occlusion of vessels. Provided that cardiac contractility and output is maintained, an increase of vascular resistance and reduction of vascular cross sectional area will lead to increased shear stress and TSP-1 production. On the other hand, the loss of total endothelial surface area will result in a reduced net production of TSP-1.

Furthermore, depending on the extent of pulmonary vascular resistance and the time course of increase, right ventricular dilation and insufficiency develops. When cardiac output and flow velocity decrease, intra-vascular shear stress will be reduced again (figure 27).

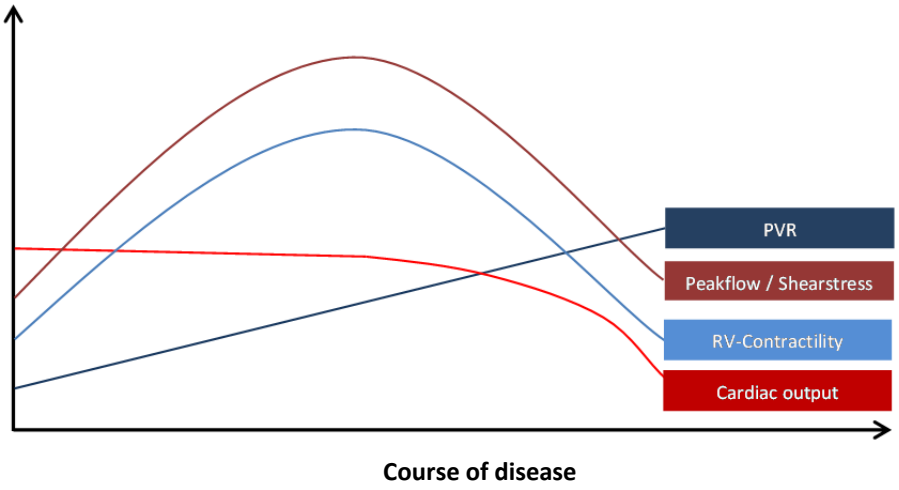

Figure 27 : Hemodynamics in the time course of pulmonary hypertension

Considering the complexity of hemodynamics and shear stress in the course of pulmonary hypertension with a nonlinear evolution of cardiac output, RV-contractility and peak flow, a similar complexity and nonlinearity of TSP-1 concentrations is to be expected. As our study was designed as an observational cross-sectional study, we included patients in different stages of the disease. While some had newly diagnosed PH, others were in the end stage of their illness. Thus a non-matched comparison of TSP-1 levels between patients and groups seems a serious limitation in this study.

Big-Endothelin

We analyzed the well-established pulmonary hypertension biomarker Big-ET in our study population and confirmed a significant elevation of Big-ET levels in patients with pulmonary hypertension. Previous studies suggested a correlation between Big-ET levels and severity of disease, mortality and time to clinical worsening with increased Big-ET levels (Rubens, Ewert et al. 2001).

In this current study, we found slightly lower Big-ET levels than previously described. This difference may be explained by the different sites of blood sampling. While older studies

focused on peripheral blood samples, we used blood drawn from the pulmonary artery during right heart catheterization. On one hand, the pulmonary vascular bed removes a considerable amount of Big-ET from the circulation with every passage in healthy subjects ($Big\text{-}ET_{Art}/Big\text{-}ET_{PA} < 1$). On the other hand, our research group previously demonstrated a dysregulation of this function in pulmonary hypertension resulting in an increase of $Big\text{-}ET_{Art}/Big\text{-}ET_{PA} > 1$ (Dupuis, Goresky et al. 1996; Dupuis, Cernacek et al. 1998; Wilkens, Bauer et al. 2003). Thus an accurate comparison of Big-ET levels from different sample sites cannot be performed.

A negative correlation between cardiac output and Big-ET level was found ($r_s = -0.4940$, $p = 0.002$). This findings confirm the relationship between severity of pulmonary hypertension and Big-ET levels as described in a previous study (Rubens, Ewert et al. 2001).

Finally, a significant correlation between Big-ET and TSP-1 levels was found in our study ($r_s = 0.4989$, $p<0.001$). Current literature does not provide possible mechanisms of an interaction between Big-ET and TSP-1. On one hand, a common denominator of hemodynamic alteration might be responsible for this statistical result. On the other hand, both Big-ET and TSP-1 levels are elevated in pulmonary hypertension based on endothelial damage or stimulation. Therefore this finding both supports the hypothesis of TSP-1 being released from pulmonary endothelium and the association of TSP-1 with disease severity (Kwapiszewska, Wilhelm et al. 2005). Nevertheless, TSP-1 effects on Big-ET expression and release should be subject to further investigation.

PDGF-ββ

Expression of PDGF-ββ in context of pulmonary hypertension was first described in animal models of hypoxia induced PH (Arcot, Lipke et al. 1993; Katayose, Ohe et al. 1993). The endothelial over-expression in these models formed the basis for measurements of circulating PDGF-ββ.

In this study, PDGF-ββ levels in the control group as well as in the pulmonary hypertension group were similar to the concentrations described in previous studies (Selimovic, Bergh et

al. 2009). In this study, we found a 5.4-fold elevation of PDGF levels in PH patients when compared to healthy subjects.

The ability of PDGF-ββ to stimulate vascular fibrosis and fibroblast growth rose the question of alternative treatment options by PDGF-inhibition (Bishop, Butt et al. 1998). Attempts have been made to treat pulmonary arterial hypertension with unspecific tyrosine kinase inhibitors. One of the first substances proving efficacy was imatinib, which binds predominantly to the cytosolic ATPase-domain of PDGF-receptors. Treatment with imatinib resulted in decreased circulating PDGF-concentrations, though the exact mechanism remains to be elucidated. Despite improved hemodynamics and clinical parameters, no correlations to PDGF-levels were found in the previous study (Ghofrani, Morrell et al. 2010). Such correlations were not be found in this study either.

We found a positive correlation between TSP-1 and PDGF-ββ (r_s = 0.4668, p<0.001). A possible induction of TSP-1 by PDGF-ββ could explain this finding, especially as this interaction was described in recent studies (Hogg, Hotchkiss et al. 1997; Breitkopf, Sawitza et al. 2005). However, this induction seems to be of minor importance as TSP-1 but not PDGF-ββ correlates with hemodynamic parameters such as CO or PVR. Moreover, older studies already showed a delay in induction of TSP-1 and PDGF-ββ transcripts, at least in hypoxemic conditions (Phelan, Forman et al. 1998; Faller 1999).

A possible explanation of PDGF-ββ elevation might be an upregulation by hypoxia (Kwapiszewska, Wilhelm et al. 2005). Arterial blood samples were not part of our study protocol and no association of either PDGF-ββ or TSP-1 with central venous oxygen saturation was found. Inflammation is another plausible reason for PDGF-elevation (Mannaioni, Di Bello et al. 1997). Markers of inflammation were not determined in this study and association of PDGF with inflammatory activity in the setting of pulmonary hypertension remains the subject of further investigation.

Aside from the arguments discussed above, the role of PDGF-ββ in pulmonary hypertension remains unclear. In spite of the correlation of PDGF-ββ with TSP-1, no causal connection

appears obvious. Both parameters might be affected by the total amount of pulmonary endothelium at risk, but might also respond to different triggers. These interactions go beyond the scope of this investigation.

8 Perspectives

In this observational cross-sectional study, we showed that TSP-1 levels are significantly elevated in pulmonary hypertension. Even though usability of TSP-1 as a biomarker for the diagnosis of pulmonary hypertension seems limited, TSP-1 levels nonetheless hold the opportunity to gain insight into the complex hemodynamic mechanisms of this disease. While current biomarkers focus on the repercussions of pulmonary hypertension such as myocardial damage, TSP-1 locally monitors endothelial injury and activation at the very source of the pathology. Therefore longitudinal studies might provide insight into the ability of TSP-1 to predict disease activity and progression by means of time to clinical worsening and survival. TSP-1 might also serve as a marker of treatment efficacy with TSP-1 levels falling as a result of improved endothelial hemodynamics.

Local endothelial shear stress needs further investigation in patients with pulmonary hypertension and correlations to TSP-1 levels should be made. Promising techniques for capillary flow and endothelial surface measurements are Magnetic Resonance Imaging and the experimental micro-electro-mechanical systems (Soundararajan, Hsiai et al. 2004).

Moreover, as TSP-1 interacts significantly with the NO pathway at least in experimental data, several perspectives seem promising. Observational studies should evaluate TSP-1 as a marker for possible non-responders before initiation of therapy with medication dependent on intracellular cGMP and cGK-activity such as PDE-5-inhibitors or the novel sGC-stimulator Riociguat. Moreover, TSP-1 blocking by biologicals might be a new experimental therapeutic approach especially as experimental data on TSP-$1^{-/-}$ knockout mice showed a diminished pulmonary vasoconstriction response and were less responsive to hypoxia induced pulmonary hypertension.

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10 Posters and Presentations about Thrombospondin-1

2010 Thrombospondin-1 as a Biomarker in Pulmonary Hypertension
R. Kaiser, **C. Frantz**, R. Bals, M. Böhm, H. Wilkens, Chest 2010,138:893A

Thrombospondin-1 als neuer Shear stress-Biomarker bei pulmonaler Hypertonie
C. Frantz, R. Kaiser, K. Rentz, G.W. Sybrecht, H. Wilkens
Vortrag V320 Deutsche Gesellschaft für Pneumologie

Thrombospondin-1 in pulmonary hypertension: A potential indicator of shear stress
C. Frantz, R. Kaiser, K. Franzen, G. W. Sybrecht, H. Wilkens
Poster P 1141 European Respiratory Society Congress

2011 Endotheliale TSP-1 Ausschüttung durch Scherstress bei pulmonaler Hypertonie
C. Frantz, R. Kaiser, C. Lensch, R. Bals, H. Wilkens
Poster P367 Deutsche Gesellschaft für Pneumologie

TSP-1 als Biomarker für endotheliale Scherkräfte bei pulmonaler Hypertonie
C**. Frantz**, R.Kaiser, R. Bals, M. Böhm, H. Wilkens
Vortrag YIA7, Endrundenteilnahme DGIM Young Investigator Award

11 Curriculum vitae

Personal information

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13 Appendix

13.1 Abbreviations

ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
AUC	Area under the curve
bFGF	Basic fibroblast growth factor
Big-ET	Big Endothelin
BNP	Brain natriuretic peptide
BP_{dia}	Diastolic blood pressure
BP_{sys}	Systolic blood pressure
cAMP	Cyclic adenosine monophosphate
cGK	Cyclic guanosine monophosphate dependent kinase
cGMP	Cyclic guanosine monophosphate
CI	Cardiac index
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
CPFE	Combined pulmonary fibrosis and emphysema
CTAD	Citrate, Theophylline, Adenosine, Dipyridamole
CTEPH	Chronic thromboembolic pulmonary hypertension
CXCL4	CXC-chemokine Ligand 4
EAA	Extrinsic allergic alveolitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERA	Endothelin receptor antagonist
ET-1	Endothelin-1
GTP	5'-guanylate triphosphate
hPAMSC	Human pulmonary arterial smooth muscle cells
HR	Heart rate
hs-Troponin	High sensitivity troponin
HUVEC	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
IPF	Idiopathic pulmonary fibrosis
MAPK	Mitogen-activated protein kinase
MCC	Matthews´s correlation coefficient
miRNA	Micro ribonucleic acid
MLC	Myosin light Chain
mRNA	Messenger ribonucleic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PAH	Pulmonary Arterial Hypertension
PAP_{dia}	Diastolic pulmonary arterial pressure
PAP_{m}	Mean pulmonary arterial pressure
PAP_{sys}	Systolic pulmonary arterial pressure
PAWP	Pulmonary arterial wedge pressure
PDE-5	Phosphodiesterase-5
PDGF-ββ	Platelet derived growth factor-ββ
PEA	Pulmonary endarterectomy
PF-4	Platelet Factor 4
PH	Pulmonary Hypertension
PPP	Platelet poor plasma
PTP	Protein tyrosine kinase
PVR	Pulmonary vascular resistance
ROC	Receiver operating characteristic
ScvO2	Central venous oxygen saturation
sGC	Soluble guanylyl cyclase
SMC	Smooth muscle cells
TGF-β	Transforming growth factor-β
TPG	Transpulmonary gradient
TSP-1	Thrombospondin-1
VASP	Vasodilator-stimulated phosphoprotein
VSMC	Vascular smooth muscle cells

13.2 Table of figures

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