Extracellular vesicles in chronic pulmonary vascular diseases: novel promising biomarkers?

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INTRODUCTION

1. Introduction

1.1 Pulmonary hypertension

1.1.1 Definition and epidemiology

Pulmonary hypertension (PH) is a progressive, fatal disease characterized by pulmonary vessel obstruction, remodeling, and an increase in pulmonary vascular resistance (PVR) resulting in right-heart dysfunction/hypertrophy, failure, and death. PH is defined as a chronic elevation in the mean pulmonary arterial pressure (mPAP) measured during right heart catheterization above or equal to 25 mmHg at rest and appears as a consequence of vasoconstriction and remodeling of the pulmonary vasculature (1, 2). Even though the pathogenesis of PH is not fully understood to date, it can be associated with many disease-predisposing factors and contributors such as inflammation, endothelial cell dysfunction, vascular wall cell proliferation, metabolic signaling, elastases, proteases, and mutations in the gene coding for bone morphogenetic protein receptor 2 (Bmpr2) (3, 4).

Considering all clinical forms, the worldwide prevalence of PH is around 100 million in total (3), and approximately 1% of the world population might be suffering from this disease (5). However, the prevalence may be above 10% in the age group over 65 years (6). According to the literature, the prevalence of schistosomiasis-induced PH is the most common cause of PH worldwide, constituting 1% of the chronic schistosomiasis infections (7, 8). In Germany, the incidence and prevalence of PH in adults in 2014 was 3.9 and 25.9 per 1 million, respectively (9). The incidence of CTEPH (10) in Germany was 4 per 1 million in 2014 (6). The prevalence of patients with left heart disease reported in Germany is 1,3 million (5). In Germany, 50 000 patients were diagnosed with severe PH due to left heart disease, which is approximately half of the patients with left heart disease and 10% of them with both pre and postcapillary PH (5, 6). In Germany, the prevalence of the second largest group of PH due to chronic lung diseases is similar to PH due to left heart disease (6). Pulmonary arterial hypertension (PAH) has been reported in a group of patients with an average age of 65 years, which was earlier believed to be a disease of young women (9, 11).

1.1.2 Classification of pulmonary hypertension

According to old classification, PH was categorized in two groups: 1) primary PH, and 2) secondary PH depending on different risk factors or causes (12). In the second world

symposium on PH (WSPH) in 1998 (13), the classification was revised and categorized into the following 5 groups: 1) PAH 2) LHD-PH 3) PH due to chronic lung diseases and or hypoxia 4) CTEPH, and 5) PH due to unclear multifactorial mechanisms. In the fifth world symposium on PH in Nice, 2013 (Graphic 1), where the updated clinical classification of PH has been re-discussed, some specific pediatric PH was added (14). PH can be caused due to chronic pulmonary diseases like chronic obstructive pulmonary disease (COPD), chronic sleep apnea, lung fibrosis, and/or cardiac diseases like valvular heart disease, left to right shunt, or unknown pathogenesis like in idiopathic PAH (15).

1.1.2.1 **Pulmonary arterial hypertension (PAH)**

PAH is observed during right heart catheterization (RHC) in patients with pre-capillary PH with mPAP of ≥25 mmHg and a normal end-expiratory pulmonary artery wedge pressure (PAWP) of ≤15 mmHg and elevated PVR >240 dyn.s.cm^{-5} or >3 Wood units (WU) (1, 5). Further, PAH has been classified in different subgroups: 1) idiopathic pulmonary arterial hypertension (iPAH); 2) heritable pulmonary arterial hypertension (hPAH); 3) drug and toxin-induced PAH; 4) associated PAH; 5) pulmonary veno-occlusive disease and or pulmonary capillary hemangiomatosis (1') and persistent PH of the newborn (1'') (14). In 80% of familial PAH mutations of BMPR2, which is a member of the tumor growth factor (TGF) beta family, has been implicated (14, 16). Other rare mutations mentioned in the literature are activin like receptor kinase (ALK1) (17), endoglin (ENG) (18), and Smad 9, which also belongs to the TGFβ family (19). Furthermore, two new gene mutations caveolin-1 (CAV1) (20) and potassium channel subfamily K member-3, KCKN3 are involved in the pathogenesis of PH (21). It has been mentioned that the development of PAH can be caused by various drugs and toxins such as aminorex, fenfluramine, dexfenfluramine, benfluorex, selective serotonin reuptake inhibitor (SSRIs), amphetamines, L-tryptophan, methamphetamines, and dasatinib depending on their mechanism and strength (14). PAH has been shown in 2% - 6% portal hypertension patients, which is named as portopulmonary hypertension (POPH) (22, 23). It has been reported that there is no connection between the risk of developing POPH and the degree of liver damage, but the prognosis depends on liver cirrhosis and cardiac function (24). The prevalence of PAH in adults with congenital heart disease (CHD) is around 10% and directly affects their life standard (25-27). Schistosomiasis-PAH (Sch-PAH) has been described as the most prevalent cause of PAH worldwide (14), affecting 200 million patients with 1/10th of them being diagnosed with hepatosplenic schistosomiasis (28). The symptoms and hemodynamics similarity between Sch-PAH and POPH have also be mentioned in the literature (29).

1.1.2.2 **Pulmonary hypertension due to left heart disease (LHD-PH)**

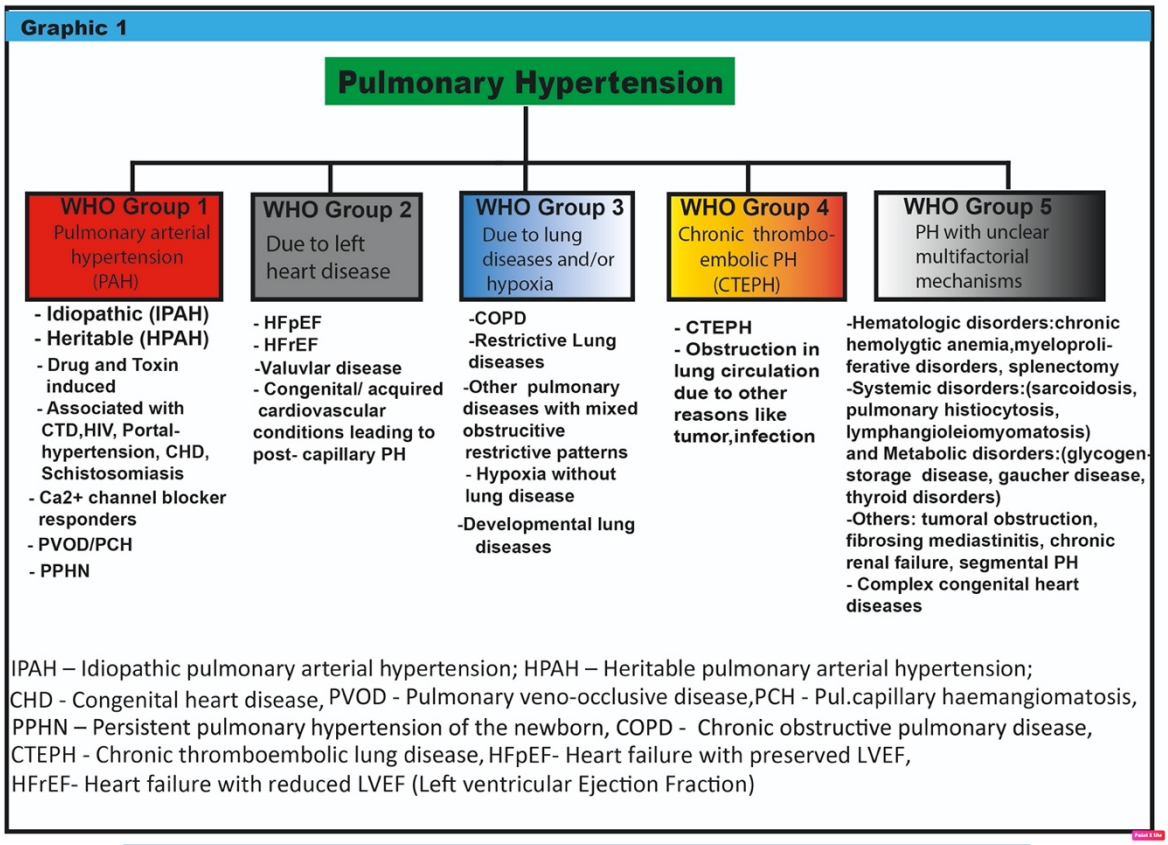
PH has been indicated as a widely known complication in left heart disease. It has been mentioned as the most common complication in patients diagnosed with left heart diseases as well as heart failure with preserved/reduced ejection fraction (HFpEF/ HFrEF). (30-32) It has also been reported that patients with LHD-PH are older and in addition, females with more comorbidity like systemic hypertension and metabolic syndrome (33, 34). LHD-PH includes the following parameters like mean mPAP ≥25 mm Hg, PAWP>15mmHg, and a normal or reduced cardiac output (CO) (35, 36). There are difficulties in diagnosing pre-and post-capillary PH in patients with HFpEF (35). “Reactive” and “passive” PH can be defined according to the transpulmonary pressure gradient (TPG > mPAP – PAWP), which is >12 mm Hg in reactive form and <12 mm Hg in passive form (35).

1.1.2.3 **Pulmonary hypertension due to chronic lung diseases and/or hypoxia**

PH in chronic lung diseases with poor prognosis includes COPD, hypoxia (e.g., exposure to high altitude), diffuse parenchymal lung disease (DPLD), idiopathic pulmonary fibrosis (IPF), and sarcoidosis (37). Most of the COPD patients belonging to the GOLD D (Global initiative for chronic obstructive lung disease) group are observed to have elevated mPAP, ranging between 20-40 mmHg (37-39). Compelling literature suggests that, there is a correlation between vascular lesions in COPD patients and the severity of PH, similar to IPAH severe condition(s) (40). The mortality of COPD patients with the presence of PH (COPD-PH) is very high, showing no correlation between mPAP, PVR and survival (38, 41, 42). Elevated mPAP has also been noticed in most of the patients with advanced and end-stage lung fibrosis (Fibrosis-PH) (37, 43, 44).

1.1.2.4 **Chronic thromboembolic pulmonary hypertension (CTEPH)**

CTEPH is an interventional or surgically treatable form of PH (45). The ventilation/perfusion (V/Q) ratio has been suggested for the screening of CTEPH (10, 36, 46, 47). It has been recommended that CTEPH patients who were treated with pulmonary endarterectomy (48) have better prognosis compared to those treated with pharmacological therapy. The lifelong anticoagulation therapy has also been suggested for CTEPH patients (45).



Graphic 1: Updated clinical classification of PH. Adapted from (5, 14, 49).

1.1.3 **Physiology of pulmonary circulation and pathophysiology of pulmonary hypertension**

1.1.3.1 **Physiological mechanisms in pulmonary circulation**

The anatomical branching of the respiratory bronchi into bronchioles, alveolar duct/sac, and alveoli simultaneously comes along with pulmonary arteries, which terminate into capillaries. These are important morphological characters of the pulmonary circulation. Deoxygenated blood from the body is pumped via the right ventricle to the lung through the pulmonary arteries. Gas exchange takes place in the pulmonary capillaries, and the saturated blood flows back to the left atrium through the pulmonary veins. The gas exchange occurs due to oxygen (O_2) and carbon dioxide (CO_2) partial pressure difference in the capillaries with an area of approximately 100 m^2. The oxygenated blood then passes through the pulmonary venules, veins, and left heart to the systemic circulation (50). The left ventricle forwards the oxygen-rich blood to the systemic circulation in the body.

Histologically vessels are made up of the following three layers: *tunica adventitia, tunica media, and tunica intima*. Endothelial cells (EC) build a monolayer that acts as a non-thrombogenic boundary between circulating blood and smooth muscle cells (SMC). EC take part in different biological functions like vascular tone, cell proliferation, apoptosis, inflammation, and thrombosis by secreting certain molecules/mediators (51). *Tunica media* is the thickest layer which is composed of SMC, connective tissue and elastic fibers. This layer is responsible for vasoconstriction and vasodilatation which further maintains the lumen diameter and thus, vascular resistance (Hagen-Poiseuille equation) (52). The partial muscularization of the smooth muscle cell layer of the pulmonary vasculature allows less resistance in the laminar flow due to more lumen diameter in comparison to the non-pulmonary vasculature, which has a thicker SMC layer, and leads to lumen narrowing and increased resistance. According to the Hagen-Poiseuille law, flow resistance is inversely proportional to the 4^{th} power of the radius. Therefore, healthy non-or partial-muscularized pulmonary resistance vessels have low flow resistance. The outer surrounding layer, *tunica adventitia*, consists of connective tissue with elastic and collagen fibers, fibroblasts, inflammatory cells, and vasa vasorum (52).

Different mechanisms/factors like the arterial baro-reflex or catecholamines are responsible for the regulation of the vascular tone in the systemic circulation, whereas the pulmonary circulation has its own autonomic regulatory mechanisms (e.g., hypoxic pulmonary vasoconstriction). Vascular tone regulation in the pulmonary circulation is not only influenced by the concentration of cyclic nucleotides like cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), catecholamine, nitric oxide (NO), prostacyclin and endothelin, but also by the cytoplasmic calcium concentration in pulmonary vessels. It has been noticed that the interaction between factors like vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor, angiopoietins, and tyrosine kinase receptors play a key role in the formation of blood vessels. The effect of a range of factors like hypoxia-inducible transcription factors on VEGF and vasoactive peptides play major roles in endothelin and NO production and thus in pulmonary vascular development. (53)

1.1.3.2 **Pathophysiology of pulmonary arterial hypertension (PAH)**

PAH is a multifactorial disease with complex pathophysiology that is still not sufficiently understood. The elevated pulmonary artery pressure (PAP) and PVR reported in PAH can be explained with vascular remodeling and in-situ thrombosis of pulmonary arteries and arterioles with small and medium-size and sustained vasoconstriction (54).

Pulmonary vasoconstriction

Vasoconstriction is the narrowing of the blood vessel lumen, altering the PVR and PAP (55). The complex pathophysiology of PAH can be partly explained by an imbalance of vasoactive and vasoconstrictive mediators. During this process, it was elegantly shown that vasodilatory mediators like prostaglandin I_2, NO, and cGMP are reduced (3, 56, 57), while vasoconstrictive mediators like thromboxane, endothelin-1 and 5-hydroxytryptamine (5-HT) (serotonin) are increased (58, 59).

In-situ thrombosis

In-situ thrombosis of pulmonary arterioles is a meaningful histo-morphological finding in PH (60). The pro-coagulant activities in the pulmonary vascular bed due to endothelial dysfunction, platelet activation, and clotting cascade dysfunction are pathological players in the pathogenesis of PH (54, 55). It has been described in the pathogenesis of iPAH patients, that, there are increases in fibrinopeptide1, and von Willebrand factor (vWF) in the blood plasma (61). Also, the vasoactive mediators play an important role in platelet aggregation and increased thrombosis in pulmonary vasculature by increasing pro-aggregatory mediators like thromboxane A2 and decreasing anti-aggregatory mediators like prostacyclin and NO (3).

Pulmonary vascular remodeling

The term “pulmonary vascular sclerosis” was first mentioned by the German physician Ernst von Romberg in 1881 (62). The understanding of the pathophysiology of PAH has made remarkable progress in the last decade but is still not well understood because of various signaling pathways and the additional impact of cardiopulmonary and systemic diseases (51). Pulmonary vascular remodeling (Figure 1) represents a structural and functional redesign of the pulmonary artery (PA) wall with increased muscularization and extracellular matrix deposition. Thus, the cross-sectional area of the PA wall increases, resulting in a decrease of lumen diameter. Therefore, the structural remodeling results in functional alterations such as an increase in PVR and PAP, following the Hagen-Poiseuille law. Small pulmonary arteries, which are normally non-muscularized, become muscularized (*neo-muscularization*) during the remodeling process (63, 64). It has been shown in animal models with hypoxic PH that there is increased vessel collagen production in large PA, which leads to the wall hypertrophy and narrowing of vessels with loss of compliance (65). Endothelial injury causes a serum leak, including elastases in the intima resulting in matrix degradation and release of growth factors, which then leads

to SMC proliferation and migration (53). Histopathology of PAH has been reported to have features like neo-muscularization, neointima and plexiform lesions, adventitial proliferation and in-situ thrombosis (63, 64, 66).

Intimal changes

Intimal lesions are another cause of decrease in the luminal area of small pulmonary arteries, and therefore, have a large impact on the PVR regulation. Eccentric intima thickening and concentric and fibrotic plexiform lesions are the characteristics of these lesions (67).

Intima thickening: It may have different forms like concentric laminar, eccentric, or concentric non-laminar, consisting of cells with characteristics of fibroblasts, myofibroblasts, and SMC and also a fibrotic pattern with extracellular matrix deposition (66, 67).

Plexiform lesions are a hallmark of PAH with complex vascular formations derived from remodeled pulmonary arteries (68). Plexiform lesions are multiplex pulmonary arterial proliferative lesions, including a network of channels, which is lined by endothelial cells and consists of matrix proteins and myofibroblasts in the stroma (54, 69). The formation of plexiform lesions in PAH are complex vascular changes in redesigned PA which seems like neoplastic disorders and glomeruloid like lesions in glioblastomas (68). The plexiform lesions in PAH may be explained with the upregulation of VEGF and vascular endothelial growth factor receptor 2 (VEGFR-2) and furthermore, the dysregulation of vascular endothelial proliferation (3). About the importance of plexiform lesions in the pathogenesis of PH is still elusive. Thus, the changes in intima with the unique plexiform formation and endothelial cell proliferation lead to the obstruction of the PA and a decrease in compliance (3).

Neointima is defined as vascular remodeling of small and large arteries, characterized by cells and extracellular matrix between the endothelium and the internal elastic lamina (66). The exact pathogenesis of neointima and the origin of cells involved in this process is still unknown.

Medial hypertrophy

Hypertrophy and hyperplasia of SMCs cause medial thickening in muscularized vessels (70). *Neo-muscularization* in the distal pulmonary arteries due to enhanced SMC proliferation results in the thickening of the media layer (63, 64, 66). In addition,

increased proliferation is accompanied by decreased apoptosis of SMCs (67). *De novo* formation of muscular media is noticed in pre-capillary vessels, which are usually non-muscularized. SMCs design the new media, which are mentioned to be derived from either differentiation of intermediate cells of vessels or from migration and differentiation of (myo)fibroblasts from the adventitia (70, 71).

Adventitial proliferation

It has been observed in animal models exposed to chronic hypoxia that the structural changes begin in the adventitia of pulmonary arteries, which continue in the medial and intimal layers (72). The heterogeneous population of pulmonary artery adventitial fibroblasts (PAFBs) is able to proliferate and secrete different chemokines to attract inflammatory cells and differentiate into myofibroblasts in response to stress (71). Collagen and other extracellular matrix proteins have been reported to be produced by myofibroblasts, and the movement and accumulation of myofibroblast to the media or intima layer further support the neointima formation (69).

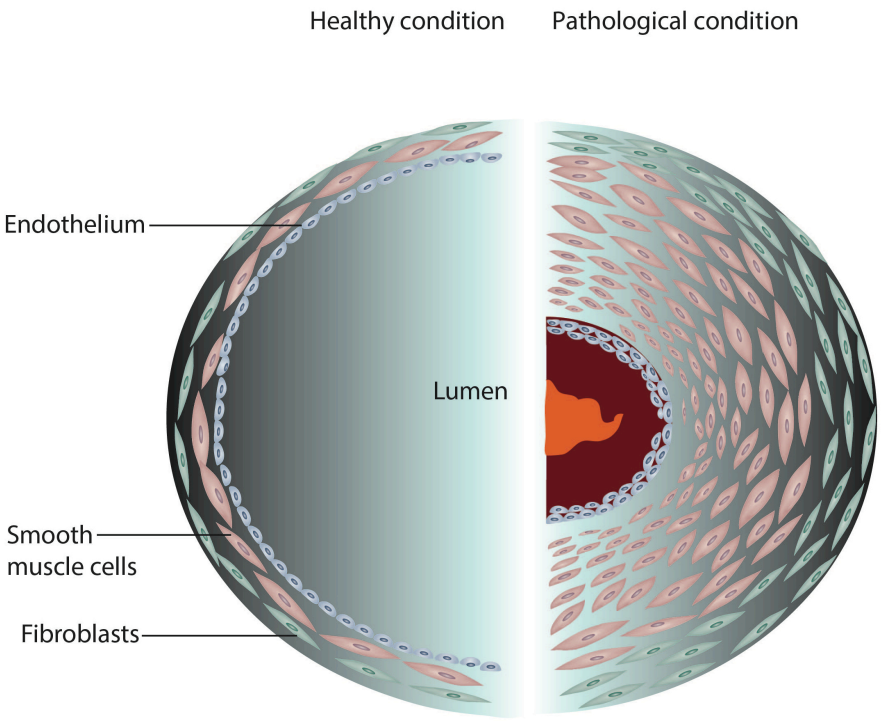


Figure 1 a. Remodeling of pulmonary vasculature in pulmonary arterial hypertension. Modified from (3).

Right heart hypertrophy

Increased pulmonary vasoconstriction and vascular remodeling of all three layers leads to the loss of the cross-sectional area and thus, elevation of PAP and right ventricular afterload (3). The pathogenesis of PH consists of several phases: vasoconstriction (hypoxia-induced: Euler-Liljestrand mechanism) followed by structural remodeling with intimal and endothelial hyperplasia, which leads to an increase in PAP, right ventricular afterload and right-heart decompensation with cardiac arrhythmia (15). Dilation or hypertrophy of the right ventricular wall with the increment of afterload in pulmonary arterial hypertension may result in *cor pulmonale* (73).

1.1.1.3 **Inflammation in pulmonary arterial hypertension (PAH)**

Inflammation is a programmed and sophisticated process that precedes tissue repair and /or apoptosis. It is either categorized as acute or chronic, depending on its severity and duration. Multiple cellular processes have been described during the inflammatory process, and molecular candidates such as cytokines and chemokines are compounding contributing factors. It was elegantly demonstrated that during inflammation, elevated levels of some of these soluble proteins are recorded in various diseases (74). Inflammation widely occurs in many cardiopulmonary diseases in response to injuries from biological and exogenous agents (75). For example, several facets of evidence elucidate inflammation to be one of the underlying contributors to PAH (76). It is, however, noteworthy that inflammation in PAH is usually initiated by an array of disease conditions spanning from scleroderma, systemic lupus erythematosus (77-79) to infections such as human immunodeficiency virus (HIV), Schistosomiasis, human herpes virus (Kaposi sarcoma (HHV-8), Epstein-Barr virus und Cytomegalie virus. Conversely, the inflammatory mechanisms that trigger the pathogenesis of PAH in the great vasculature remain elusive (3, 74, 76).

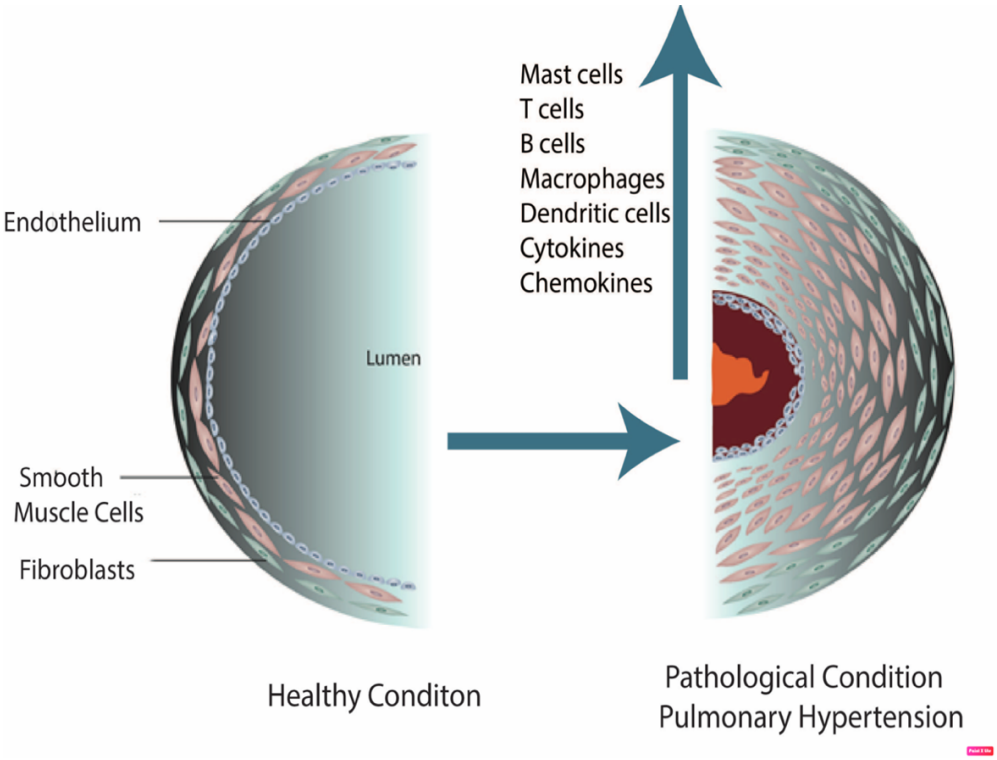


Figure 1b. Inflammatory mechanisms via various pathogenic drivers in the pathogenesis of pulmonary arterial hypertension. Modified from (3).

The migration of immune cells such as T cells, macrophages, and B cells to inflammatory sites has explained their roles as pathogenic drivers of PAH. Additionally, in human iPAH (except Eisenberger PAH), lymphoid follicles located at pulmonary arteries inhabit B lymphocytes, T lymphocytes, and dendritic cells (DC) (80). These immune cells are known to be connected to vessels undergoing remodeling through stromal networks (81). Collective data have shown that mast cells are present at the perivascular areas in iPAH and CHD-PAH (82-84). Also, some populations of DC have been indicated in the *tunica adventitia* and *media* of muscularized pulmonary arteries exclusively in human iPAH (85). These findings, therefore, support evidence of increased amounts of cytokines like IL-1-β, IL-8 (86, 87), and chemokines such as chemokine (C-C motif) ligand (CCL2 /monocyte chemotactic protein (MCP)-1 (88), and CCL5 in iPAH patients (80).

Role of immune cells in PAH

As already mentioned, immune cells are important candidates in PAH pathogenesis. Therefore, their functional roles are duly expounded in the following section.

Innate Immune Cells:

Mast Cells:

Mast cells originate from the bone marrow and consist of histamine and heparin, which have large granules. They play a vital role not only in wound healing and immunity against pathogens but also act in hypersensitivity reactions. Increased levels of mast cells are shown in different subgroups of PAH, but the pathophysiology regarding their contribution in PAH is unclear. It was however, suggested to produce vasoactive substances that further activate remodeling via the production of matrix metalloproteinases. (80, 82, 84, 89)

Monocytes/ Macrophages:

Another group of cells implicated in PAH is macrophages. They differentiate from monocytes and function in phagocytosis of damaged cells and foreign materials, among others. In PAH, it was mentioned that increased levels of these cells are located near pulmonary vessels undergoing remodeling (85, 90). Both, DC and macrophages possess antigens on their surface, which can bind to major histocompatibility complex class 2. Due to this, they are easily sensed by the T cells. (80)

Dendritic cells (DCs):

DCs are known as the antigen-presenting cells, which play a vital role in the activation of T Cells (74). As illustrated by Perros *et al*. (85), DCs are involved in infiltrating vascular lesions in iPAH patients. DCs also present antibodies to endothelial cells, fibroblasts, and nuclear antigens. The authors further demonstrated the accumulation of immature dendritic cells in redesigned pulmonary vessels (85, 91-93).

Adaptive Immune System:

T Cells:

T cells function in the adaptive immune response. They are classified into T helper cells (cluster of differentiation CD 4^+), T regulatory cells (T reg cells ($CD4^+$ CD25hi$FoxP3^+$CD 127 low)) and cytotoxic T cells (Tc cells ($CD8^+$)). The CD4+ T cells are further classified into Th1, Th2, and Th17. In general, Th cells are involved in B Cell differentiation and macrophage activation. The Tc cells help in fighting infections by killing infected cells. They do this by binding to major histocompatibility complex class 1 molecules. (80, 94)

The T reg cells control the Th1 and Th2 responses (94, 95). Besides this role, studies are ongoing to investigate other functions of T reg cells (80).

B Cells:

B cells are associated with PAH because increased amounts of antinuclear antibodies (produced by B cells) were revealed in PAH patients. Furthermore, it has been reported that the autoantibodies, which act against EC and fibroblasts, are increased in PH, contributing to the complex pathogenesis of pulmonary remodeling. (91, 92, 96, 97).

Cytokines and Chemokines in PAH

Cytokines are small proteins released by cells, which have a specific effect on the interactions and communications between cells. They are critical players in cell signaling. Chemokines are a subgroup of cytokines (called chemotactic cytokines), which play significant roles in the enrollment of leucocytes (98). Inflammatory cells of the innate immune system and vascular wall or adventitia are mainly responsible for the production of these two mediators (99). It is worth noting that cytokines and chemokines are elevated in the serum and tissue of PAH patients (86, 87).

1.1.4 Clinical symptoms

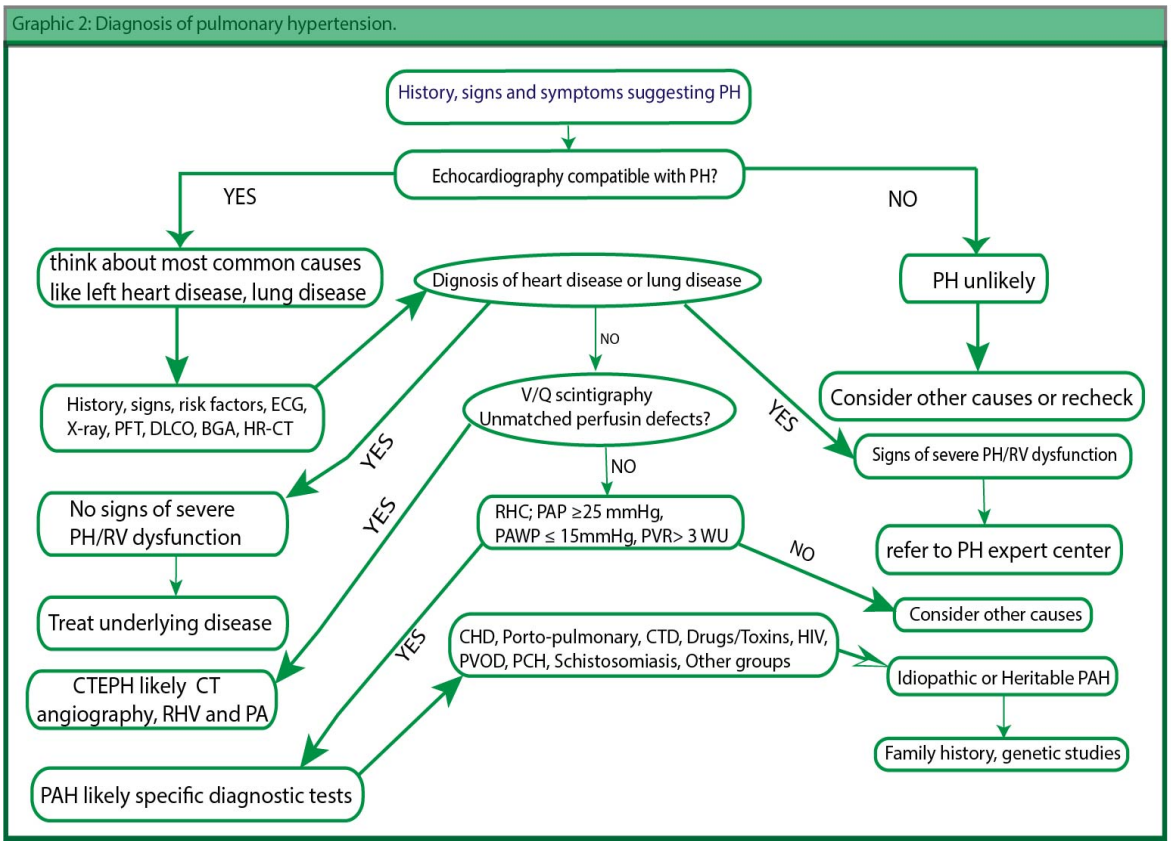
In the early stage of disease, patients may show no symptoms or unspecific symptoms like dyspnea after continuous exercise and lethargy, which is the reason why there is delay of many months or years between the early symptoms and diagnosis (5). Patients may develop symptoms like cyanosis, weakness, dizziness, exercise induced dyspnea and/or syncope on exertion, dyspnea on bending down (bendopnea), fatigue, chest pain, sinus tachycardia and paleness in the later stage of disease progression. With a higher degree of decompensated right-heart failure, the risk of serious cardiac arrhythmia leading to a bad prognosis is high (15, 73). Cardiac decompensation leads to frequent syncope even after light activities and also portray the triad of cervical venous congestion, ascites and edema with increase in right heart filling pressure, which is also a sign of worse prognosis (5).

1.1.5 Diagnosis of pulmonary hypertension

There are different methods to diagnose PH: Doppler echocardiography, right-heart catheterization, auscultation, chest X-ray, and electrocardiography (ECG) (Graphic 2).

Right heart catheterization is a gold standard for diagnosis of PH, which measures mPAP and PAWP in postcapillary arterioles (15, 73). Patients with a positive history and symptoms indicating PH, get along with ECG and laboratory reports, including parameters like Brain natriuretic peptide (BNP) and N-terminal pro B-type natriuretic peptide (NT-pro BNP), echocardiography to measure the PAP and the right heart index (1, 5). Normal ECG and laboratory reports suggest that there are lesser chances of PH. In patients with pathological ECG and, echocardiography report, further possible causes of PH for example due to left heart disease and/or lung diseases should be cleared with the help of X-ray, pulmonary function testing (PFT) including diffusion capacity of the lung for carbon monoxide (DLCO), blood gas analysis (BGA), high-resolution CT scan (HR-CT) and risk factors (1). After confirmation of heart disease or lung disease, therapy options can be different according to the signs of the severity of PH and right ventricle (RV) dysfunction (1).

Patients with signs of RV dysfunction and higher severity of PH are referred to PH centers (1). Ventilation/perfusion (V/Q) scintigraphy is done for patients with a negative diagnosis of heart or lung disease. With a positive V/Q scintigraphy report, there is a high possibility of CTEPH, which can be confirmed with computer tomography angiography (CT-Angio), RHC ,and pulmonary angiography at pulmonary endarterectomy (48) centers (1). With the help of right RHC, mPAP, PAWP, and PVR are measured to diagnose PAH in the case of a negative V/Q scintigraphy report. Further causes of PAH due to CTD, CHD, drugs/toxins, HIV, POPH, Schistosomiasis, and others should be examined (1). Finally, if no cause of PAH is found, those patients are categorized in idiopathic or inheritable PAH group. It has been suggested that right heart catheterization is an undisputable confirmation method in the case of PAH and CTEPH, whereas not necessary for PH due to left heart disease and chronic lung diseases (5). It has also been suggested that the dual energy CT scanners might not only provide the conventional images but also regional lung perfusion without the use of irradiation or contrast medium (5).



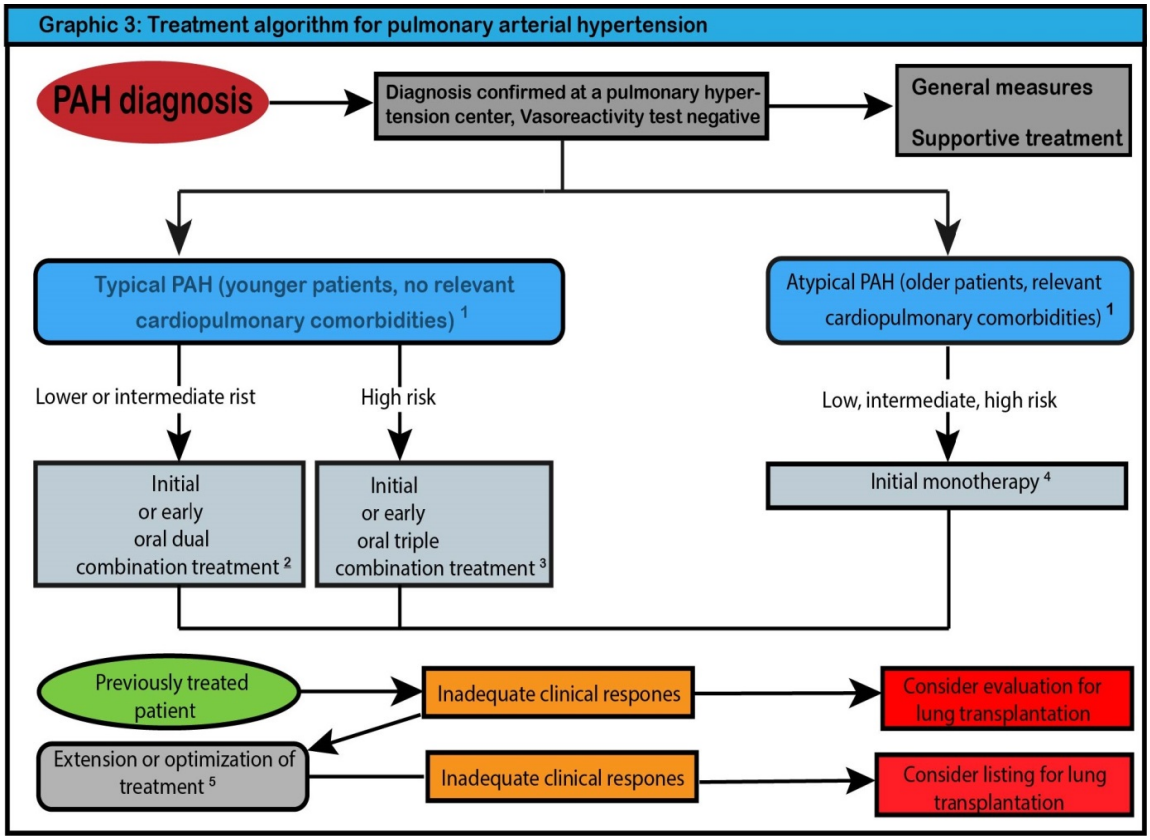
Graphic 2: Diagnosis of pulmonary hypertension. Based on (1).

PVR is usually given by the unit dyn.s.cm^{-5}. However, it was suggested to use the wood units (mmHg/l*min), which can be calculated directly from PAP (100) and CO measurements (1). The specificity of widely used PAWP ≤15 mmHg by diagnostic centers in pre-capillary PH is very high, which is a potential parameter for PAH clinical trials (1). Patients with mPAP between 21–24 mmHg so-called “borderline PH” (101) with CTD and family members having iPAH and/or hPAH are reported to be candidates with high risk for developing PAH (3).

It has been explained by the current guidelines that PAWP (left ventricular end-diastolic pressure, LVEDP) ≤15 mmHg is observed in pre-capillary PH, and ≥15 mmHg is seen in left heart disease PH (1). Fluid challenge technique can be useful to distinguish PAH from occult HFpEF. It has been observed that there is an increase in PAWP above 15 mmHg after patients are administered 500 ml saline in 5–10 minutes compared to healthy individuals, where values do not exceed 11 mmHg (1).

1.1.6 **Current treatment options**

There are three main current therapy options for PAH (Graphic 3): 1. General measures and calcium channel blockers as chronical therapy with supportive therapies; 2. PAH drugs which have already been approved; 3. Combination therapy, balloon atrial septostomy, and as ultima ratio lung transplantation (102).



Graphic 3: Treatment of PAH; Modified from (5); [1]Cardiopulmonary diseases like hypertonia, coronary heart disease, diabetes mellitus, obesity and other risk factors; [2]Initiation immediately or within 3 months of diagnosis with endothelin receptor antagonists and phosphodiesterase-5 inhibitors or stimulators of soluble guanylate cyclase; [3]Initiation immediately or within 3 months of diagnosis with endothelin receptor antagonists and phosphodiesterase-5 inhibitors or stimulators of soluble guanylate cyclase plus a prostacyclin derivative; [4]elderly patients with cardiac or pulmonary comorbidity, monotherapy is suggested; [5]individual strategies with typical PAH treatment modification like treatment escalation with prostacyclin derivatives, SC/IV prostacyclin, shifting from phosphodiesterase-5 inhibitor to sGC stimulator and in atypical PAH improving supportive treatments and rehabilitation measures.

There are also general measures which include disease management possibilities in pregnancy, rehabilitation, and exercise training (102). There is a trend to increase in healthy newborn babies from PH patients. Therefore, PAH in pregnancy can be treated to decrease complications (102). Rehabilitation and exercise training support to achieve better physical activity, less fatigue, better 6-min walk distance (6MWD),

cardiorespiratory function thus, collectively, helping to increase the living standard of the patients (103, 104). Further supportive therapies can be long term oxygen therapy, anticoagulants, diuretics, and digitalis. The expert centers are referred to as providing all the possible diagnostic and therapeutic options. It has been suggested that patients with idiopathic, hereditary, or drug-induced PAH after they are identified as responders with the help of right heart catheterization followed by vasoreactivity testing can get benefit from long-term high doses calcium-channel blocker therapy (5, 105, 106).

The activated endothelin pathway has been shown to play a key role in the pathobiology of PAH, but if it is cause or consequence of PAH remains unknown (107-109). Endothelin receptor–A and –A/B antagonists are used for the treatment of PAH (102). The approved PAH medication ambrisentan is a selective endothelin receptor-A antagonist (ERA), which seems to be beneficial for PAH patients of certain categories having side effects like worsening of liver function and peripheral edema (102). Another approved PAH medication is bosentan, a dual endothelin A and B receptor antagonist, which helps PAH patients to improve the symptoms but also has liver toxicity as a side effect (102). Therefore, patients on this medication must have a regular liver checkup. Phosphodiesterase-5 inhibitors like sildenafil, tadalafil, vardenafil inhibit cGMP degradation, which can keep the vasodilator nitric oxide (NO) longer in play, and has also been used as erectile dysfunction therapy (102). Meanwhile, soluble guanylate cyclase (sGC) stimulators increase the NO production. Thus, both PDE-5i and sGC stimulators have been shown to possess anti-proliferative and anti-remodeling effects (102). Riociguat shows a combined effect by working together with endogenous NO and by activating sGC. The tyrosine kinase inhibitor, imatinib, with anti-proliferative nature works as a selective antagonist of platelet derived growth factor receptor which has been used for chronic myeloid leukemia, is reported to be beneficial for PAH patients (110). Prostacyclin formed by endothelial cells is a vasodilator that inhibits platelet aggregation. Prostanoids like beraprost, epoprostenol, iloprost, and treprostinil also improve the symptoms. Prostacyclin IP-receptor agonist, selexipag, is also on trial for PAH treatment (102).

Different parameters like World health organization functional class (WHO-FC), exercise capacity, cardiac index, right atrial pressure, NT-pro BNP, and echocardiographic parameters are considered after 3-6 months of treatment to find out the clinical responses of initial therapy, so that the escalation with combined therapy option can be recommended. Interventional procedures like balloon atrial septostomy can be performed by constructing a right-left shunt to achieve more pre-load in the left ventricle and cardiac index (35).

Therefore, medicines like 1. PDE-5i: sildenafil, tadalafil 2. Endothelin receptor antagonists: bosentan, ambrisentan, and 3. Long-acting prostacyclin analogs: iloprost for inhalation, are included to reduce the PAP as well as heart-dysfunction treatment (111). In addition, cumarine therapy can be applied as an anticoagulating drug. As ultima ratio, heart/lung transplantation can be done (15).
In combination with other pharmacological therapies, patients suffering from PH and/or heart disease are being treated with long term oxygen inhalation (minimum 16 hours daily) to minimize the hypoxia-induced vasoconstriction (Euler-Liljestrand-mechanism) (15).

1.1.7 **Prognosis**

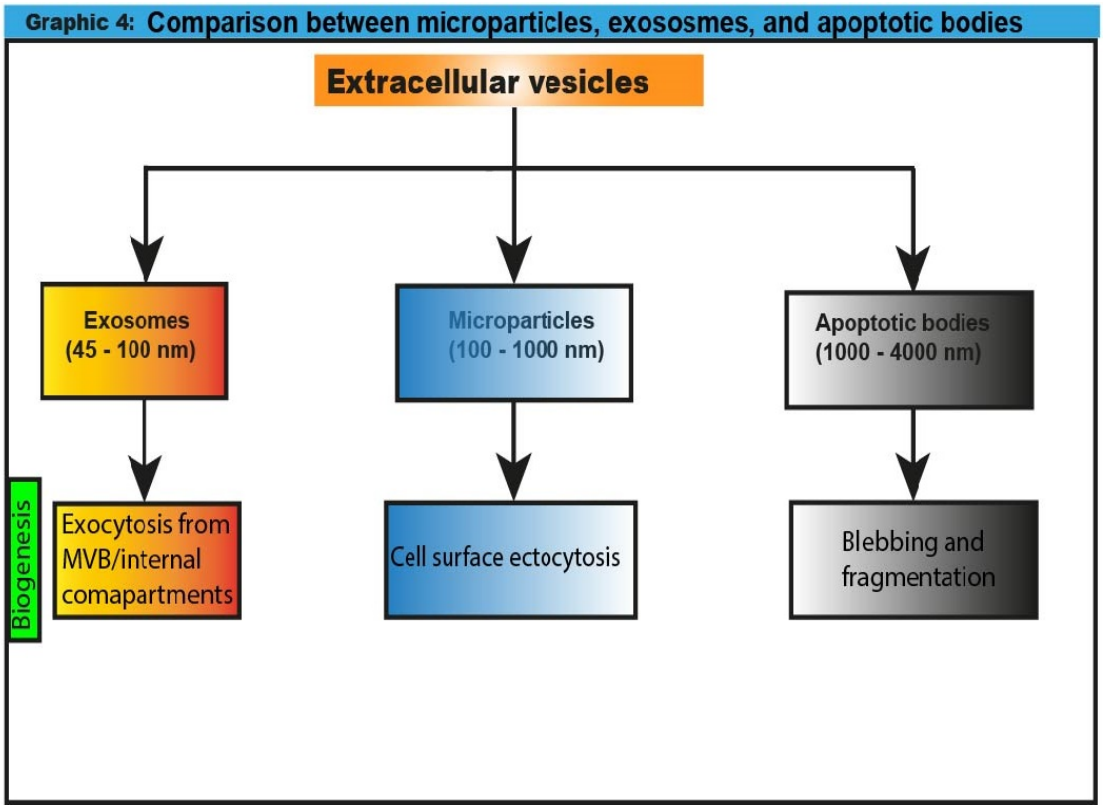
From the viewpoint of diagnosis, an average of three years of life expectancy for pulmonary arterial hypertension has been observed to increase from 40 to 70-80% in the last three decades (11). It has also been noticed that there is an increase in the survival rate of CTEPH patients (5). PH due to left heart disease and chronic lung diseases have been explained to be associated with more symptoms and poor prognosis (6).

1.2 **Extracellular vesicles (EVs) and Microparticles (MPs)**

1.2.1 **Formation and release of EVs/ MPs**

One of the types of EVs, such as MPs (21) where first mentioned as “platelet dust” in 1967 by Wolf et al. (112). They are shed membrane vesicle fragments with 0,1–1 μm in size, and can be released during different activation of various cells and/or apoptosis (113, 114).

Literature suggests that increase in the number of circulating MPs is associated with several cardiovascular diseases, inflammation and renal failure (4, 115, 116). MPs are reported as markers of endothelial injury and vascular remodeling (115, 116). There is a correlation between the increase in the number of MPs exposed and the severity of different cardiovascular diseases (116, 117).



Graphic 4. Comparison between microparticles, exosomes and apoptotic bodies. Adapted from (113).

EVs are membrane-derived structures, such as exosomes (40–100 nm), apoptotic bodies (1000–4000 nm), and MPs (100-1000 nm). They can be differentiated based on their size and properties (Graphic 4) (113). With regard to apoptotic bodies, it has been described that during the process called “budding”, plasma membrane blebbing followed by karyorrhexis and separation of cell fragments into apoptotic bodies occurs (118). Different types of membrane-bound vesicles like microvesicles, exosomes, and apoptotic bodies are formed and released by normal and healthy cells, functioning as intracellular communicators and signal transductors (119). It has been reported that, the manipulation of phagocyte clearance of apoptotic cells may be therapeutically useful in the treatment of inflammatory and autoimmune diseases and also for vaccine strategies against cancer and infections (120). Exosomes are believed to be formed by inward budding into the endosomal lumen of small intra-luminal bodies, which form multivesicular bodies (MVB) after aggregation (113). Exosomes have been mentioned to be enriched with CD63, and major histocompatibility complex (MHC) I and II molecules, but with less phosphatidylserine (PS) and pro-coagulation factors on the surface (121-124). The outer surface of MPs and apoptotic bodies consists of the anionic

phospholipid, phosphatidylserine, which is involved in the activation of coagulation and complement cascade and supports the activity of secreted phospholipase (114).

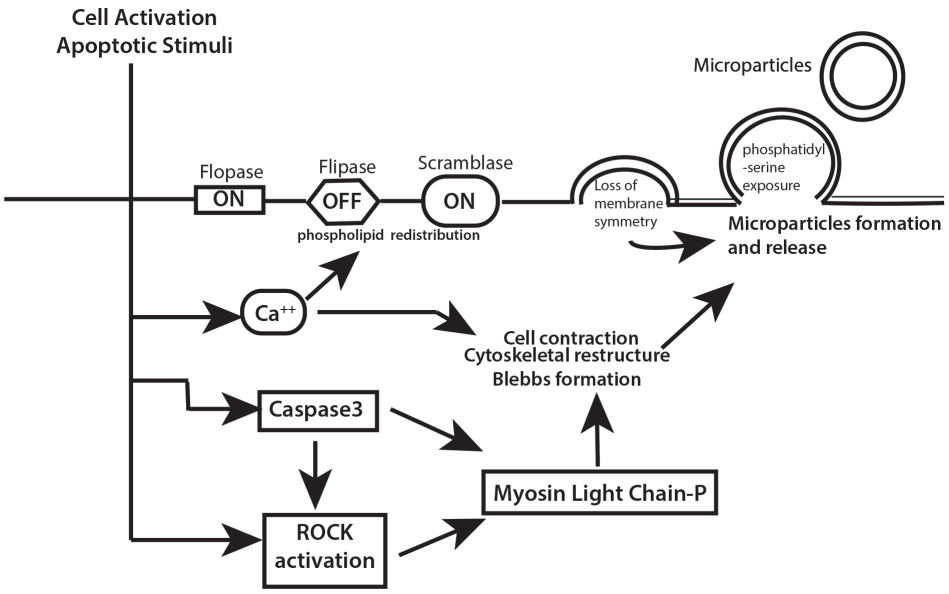


Figure 2. Formation and release of microparticles. Adapted from (114)

There are different mechanisms for the formation and release of MPs (Figure 2), which are considered to be possible prognostic markers of endothelial dysfunction in cardiovascular diseases. With an increase in the cytosolic calcium concentration, there is a reorganization of the cytoskeleton by the activation of flopase and scramblase, leading to blebs formation and thus, MP formation and release. Another mechanism by which MPs can be formed is through the activity of activated caspase 3 leading to the activation of Rho-associated protein kinase (ROCK), which in turn phosphorylates myosin light chain, thus resulting in MP formation. (114)

The characteristics of MPs depend on their lipid, protein, nucleic acid, cytokines contents, and surface receptors, which vary according to their cellular origin (4). MPs are formed from different cells so that they can avoid the apoptotic process or phagocytosis by removing signaling molecules like phosphatidylserine (114). Platelet derived MPs depend on the cytosolic cysteine proteasome Calpain, whose activity varies with calcium concentration (114).

1.2.2 **Microparticles in other diseases**

MPs play a major role in the pathogenesis of different diseases such as end-stage renal failure, myocardial infarction and inflammation of airway epithelial cells and among

others. The studies in MPs in patients with end-stage renal failure showed remarkable relation between endothelial-derived MPs and endothelial and arterial dysfunction. MPs derived from endothelial cells lead to reduced endothelial nitric oxide levels, which is highly correlated with endothelial and arterial pathology in end-stage renal failure (ESRF). (116)

Patients with ESRF have cardiovascular risk because of microparticle-induced endothelial pathology and arterial rigidity (116). It has been shown that the altered MPs derived from different cells influence the arterial dysfunction in ESRF (116). Increased level of Annexin V+ MPs derived from endothelial cells, erythrocytes, and platelets were investigated in ESRF, where endothelial cell-derived MPs are associated with enhanced common carotid artery augmentation index, aortic pulse wave velocity and flow-mediated dilatation (116). Investigation on MPs of monocytes/macrophages origin has been shown to be associated with the formation and release of more pro-inflammatory mediators by human airway epithelial cells, contributing to the pathogenesis of inflammatory lung disease (125).

1.2.3 **Microparticles in pulmonary hypertension**

MPs may represent not only potential biomarkers for PH, but also active players in the complex pathology of the disease. Patients with PH presented significantly higher levels of circulating E-selectin- and VE-cadherin-MPs, which are endothelial cell derived-MPs. VE-cadherin-MP levels are correlated with increased mPAP values (115). The increased number of CD62E, which are endothelial cell-derived MPs, has been described to correlate with clinical complications and a poor prognosis in PH patients (126). Elevated platelet and endothelial cell-derived MPs levels are involved in vascular coagulation in PH patients. Thus, it has been suggested that patients with PH benefit from platelet-derived microparticles (PMP) reduction (127). It has been described that the hypoxic MPs are responsible for endothelial dysfunction in an animal model by downregulating endothelial Nitric oxide synthase (eNOS) activity and elevated reactive oxygen species (ROS) production (2).

1.2.4 **MiRNA: diagnostic and therapeutic markers in various diseases**

MiRNA are small, non-coding RNA molecules consisting of 22 nucleotides which control post-translational gene regulation by binding to messenger RNAs (128). More than 1000 different types of miRNAs control one-third of the human protein-encoding genes and thus protein expression (129). MiRNAs can be very useful as diagnostic markers in

cardiovascular diseases since they are present in cardiac tissue and circulating blood (130).

The presence of RNase, which destroys RNA in the plasma, is known, but there are questions arising about the presence and protection of miRNA in plasma. Diehl *et al*. illustrated that the MPs work as transport vehicles to carry and protect the miRNAs in the plasma (129). Literature suggests that MPs also interfere in the protein expression of their target cells (131). Interestingly, miRNA and EVs-mediated communication between endothelial cells and SMC was elegantly shown by Hergenreider *et al*. (132).

1.3 Hypothesis and aims of the study

Hypothesis

Based on the literature discussed, MPs can be considered as carriers of pathological information via a variety of miRNAs and potentially, other “danger” signals during the pathogenesis of PH, thus, presents them as possible biomarkers and active players in the complex pathological symphony of the disease (Figure 3). Circulating endothelial cell-derived MPs may be involved in the pathogenesis of PH, and even more, some of the MPs might help to understand the severity of disease (115, 133). Although the current knowledge suggests the possible predictive nature of endothelial cell-derived MPs, very little is known about inflammatory cell-derived MPs. Literature indicates that inflammatory cell-derived MPs might be involved in the pathogenesis of other inflammatory diseases (125). Augmented inflammation is indeed an important phenomenon found in PH. Therefore, we hypothesize that different inflammatory cell-derived MPs may play a role in patients with various clinical forms of PH (Figure 3).

In general, there was a visible research effort with regard to the involvement of MPs in the pathology of PH. However, many aspects remain unknown and mostly limited to the platelet and endothelial cell-derived MPs. Even though, altered inflammation is an important characteristic of PH pathology, there is almost no existing knowledge about the role of inflammatory cell-derived MPs in this severe pulmonary vascular disease. In addition, the exact profile of endothelial cell-derived MPs in various clinical forms of PH are yet to be explored.

Aims

The aims of the present project were to investigate the presence and potential alterations of different inflammatory cell-derived MPs in the blood of patients with various clinical forms of PH compared to donors and to analyze the correlation between circulating inflammatory cell-derived MPs levels with different clinical parameters, such as mPAP

and PVR. In addition, several endothelial cell-derived MPs were analyzed in different clinical forms of PH.

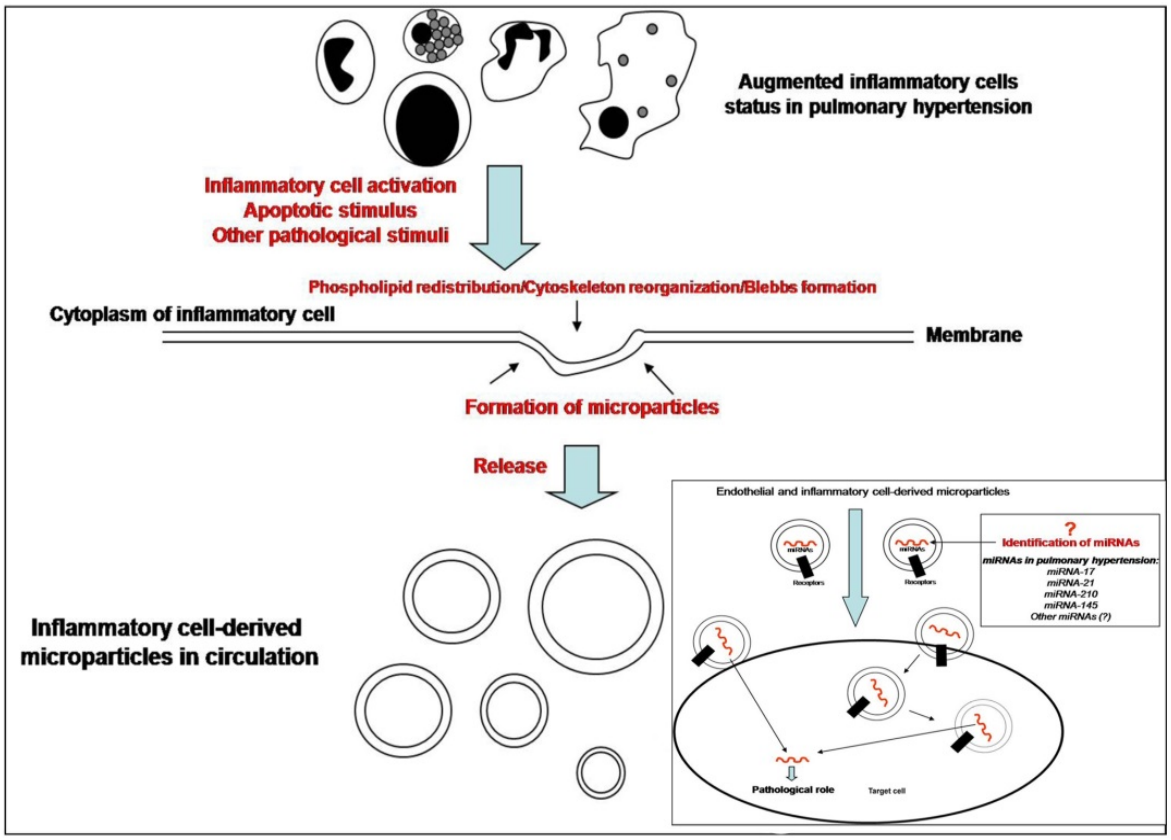


Figure 3. Hypothesis: Formation and release of inflammatory cell derived EVs from circulating inflammatory cells in PH which might carry the pathogenic information via different miRNAs.

Materials and methods

2. Materials and methods

2.1. **MATERIALS**

2.1.1. **Substances, reagents and medicines**

Substances, reagents and medicines	Company name
Right heart catheterization lab:	
Local anesthesia (Xylocain®)	Rotexmedica GmbH
Normal saline (0,9% NaCl)	B. Braun Melsungen, Germany
Flow cytometry:	
HBSS/BSA solution	Sigma-Aldrich, Germany
Distilled water	Thermo Fischer, Germany
Antibodies:	
CD68 PE	RD Systems, Germany
CD41 APC	BD Pharmingen, Germany
CD209 PE	BD Pharmingen, Germany
CD144 PE	BD Pharmingen, Germany
CD62E APC	BD Pharmingen, Germany
CD3 PE	BD Pharmingen, Germany
CD31 PE	Beckman Coulters, Germany
CD45 PE	Beckman Coulters, Germany

2.1.2. **Consumables**

Consumable articles	Company name
Gloves (Nitra-Tex®)	Ansell Ltd., UK
Napkins	Tork, Germany
Needles (BD Microlance 3®) 20G (0.9mm x 40mm), 26G (0.45mm x 13mm)	Becton Dickinson GmbH, Germany
Syringes (Injekt®-F) (1ml, 2ml, 5ml, 20ml)	B.Braun Melsungen AG, Germany
Cannula for vein catheter support 22G (Vasocan Braunüle®)	B.Braun Melsungen AG, Germany
Instrument for venous catheterization (Intradyn ™ Venous Hemostasis Introducer)	B.Braun Melsungen AG, Ger Many
Silicone catheter for right heart cath. (Vasofix® Safety®, 22G)	B.Braun Melsungen AG, Germany
Eppendorf tubes (Microtubes 1.5ml)	Sarstedt, Germany

Tips automatic pipettes 200µl, 1000µl, 10µl	Sarstedt, Germany
Automatic pipettes 10-100µl, 1-10µl	Eppendorf AG, Germany
Scalpels (Feather Disposable Scalpel)	Feather Safety Razor Co. LTD, Japan
Medical adhesive bands, 5cm/9.2m (3M™ Durapore™ Surgical Tape)	3M Health Care, USA
Gauze 4x5 cm (Purzellin®)	Lohmann und Rauscher, Germany
Gauze balls size 6, unsteril	Fuhrmann Verrbandstoffe GmbH, Ger
Surgical instruments	Fine Science Tools GmbH, Germany
Polyethylene cannula for insertion into the Jugular venes	B.Braun Melsungen AG, Germany

2.1.3. **Systems and technical devices**

Name of device(machine)	Company name
Ultrasound device	Philips electronics, Germany
FACS machine (BD Fortessa)	BD Biosciences, Ger
Micro 200R Centrifuge machine	Hettich centrifuge, UK
Rotina 420R Centrifuge machine	Hettich centrifuge, UK
Micro Star 17R	VWR International GmbH, Darmstadt
VWR Lab dancer	VWR International GmbH, Darmstadt
Perfectspin Mini	Peq Lab, Germany
Vortex Genie 2	Scientific Industries Inc. Bohemia, USA
Pipetus	Hirschmann Laborgeräte, Germany
Eppendorf Research plus	Sigma-aldrich, Germany

2.2 **Methods**

2.2.1 **Blood collection, plasma separation and EV isolation and measurement**

EV measurement and isolation performed in this study was based on previously published work with modifications (115, 116, 129, 133). The study was approved by the local ethical committee. The file number mentioned in the ethics commission is 111/14. Basically, human blood samples were prospectively collected from non-PH patients and patients with different forms of PH like I,hPAH, associated PAH, LHD-PH, COPD-PH, fibrosis-PH and CTEPH during the right heart catheterization from the pulmonary circulation before the PAWP was measured. Briefly 10-15 ml of whole blood from each

patient was drawn into citrated tubes, followed by successive centrifugation at 500 x g for 15 min and finally at 10.000 x g for 5 min at room temperature to obtain platelet-free plasma (PFP). 3 ml of PFP was stored at -80˚C**.** These samples were further used for Flow Cytometry characterization of different inflammatory- and endothelial-cell derived EVs. For the analysis of EVs the PFP samples were incubated for 20 min in the dark at room temperature with different fluorochrome labeled antibodies or corresponding isotype-matched IgG, including: anti-CD31-PE (Phycoerythrin); anti-CD144-PE; anti-CD62E-APC (Allophycocyanin); anti-CD45-PE; anti-CD14-PE; anti-CD209-PE; anti-CD3-PE and anti-CD68-PE. EVs (including the apoptotic bodies and MPs) were identified as events with a 0.5-3 μm diameter on forward light scatter (FSC) and side-angle light scatter (SSC) intensity dot plot representation with their specific fluorescence analysis, by flow cytometer (BD Fortessa) (Photography 1) which was calibrated with megamix beads. The results were successively expressed as events per microliter of plasma (ev/μl).

For the study of different EVs potentially altered in PH, following endothelial and inflammatory cell-derived targets were analyzed:

a) CD14 (monocyte)-EVs
b) CD68 (macrophage)-EVs
c) CD45 (leukocyte common antigen)-EVs
d) CD62E (E-selectin/endothelial cells)-EVs
e) CD31 (PECAM/endothelial cells, monocytes, neutrophils)-EVs
f) CD144 (VE-Cadherin/endothelial cells)-EVs
g) CD3 (T Cells)-EVs
h) CD209 (dendritic cells)-EVs

2.2.2 **Flow cytometry analysis of EVs**

From each analysis, 50 μL of freshly thawed plasma samples from PH patients and non-PH controls were obtained to identify the cellular origin of EVs, either of the following fluorescent monoclonal antibodies (mAbs) to specific cell surface markers were added with PE conjugated or Allophycocyanin (APC)-conjugated anti-human antibodies. After 20 minutes of incubation at room temperature in the dark, 1ml of FACS buffer was added to each labelled sample and washed at centrifuge. Finally, the pellet was re-suspended in 500μL of FACS buffer. These labeled plasma samples were analyzed immediately using a BD LSRFortessa™ (GERMANY) cell analyser equipped with BD FACSDiva™ software (v. 6.2).

Photography 1. Flow cytometry machine (BD LSRFortessa)

The samples were measured with a flow rate (FR) at medium (35µl per min), and forward and side scatter threshold, photomultiplier tube voltage, and window extension were optimized to detect sub-micron particles. For each time that samples were analyzed, 1 tube containing only 0.5-3 µm of polystyrene size calibration beads was measured at a fixed concentration. The following instrument settings were used for data acquisition: threshold 200; FSC voltage 55 V, SSC voltage 200 V; FSC and SSC in log scale. The acquisition was stopped when a fixed number of events (usually 10,000 to 20,000) were recorded (Figure 4). For each sample, data were acquired for all 8 markers as listed above. Finally, the EV number per µL of plasma was calculated according to the following formula: the calculation procedure from flow rate (FR) and time taken to measure each sample (t):

$$\mathbf{V\ (\mu l) = FR \times t}.$$

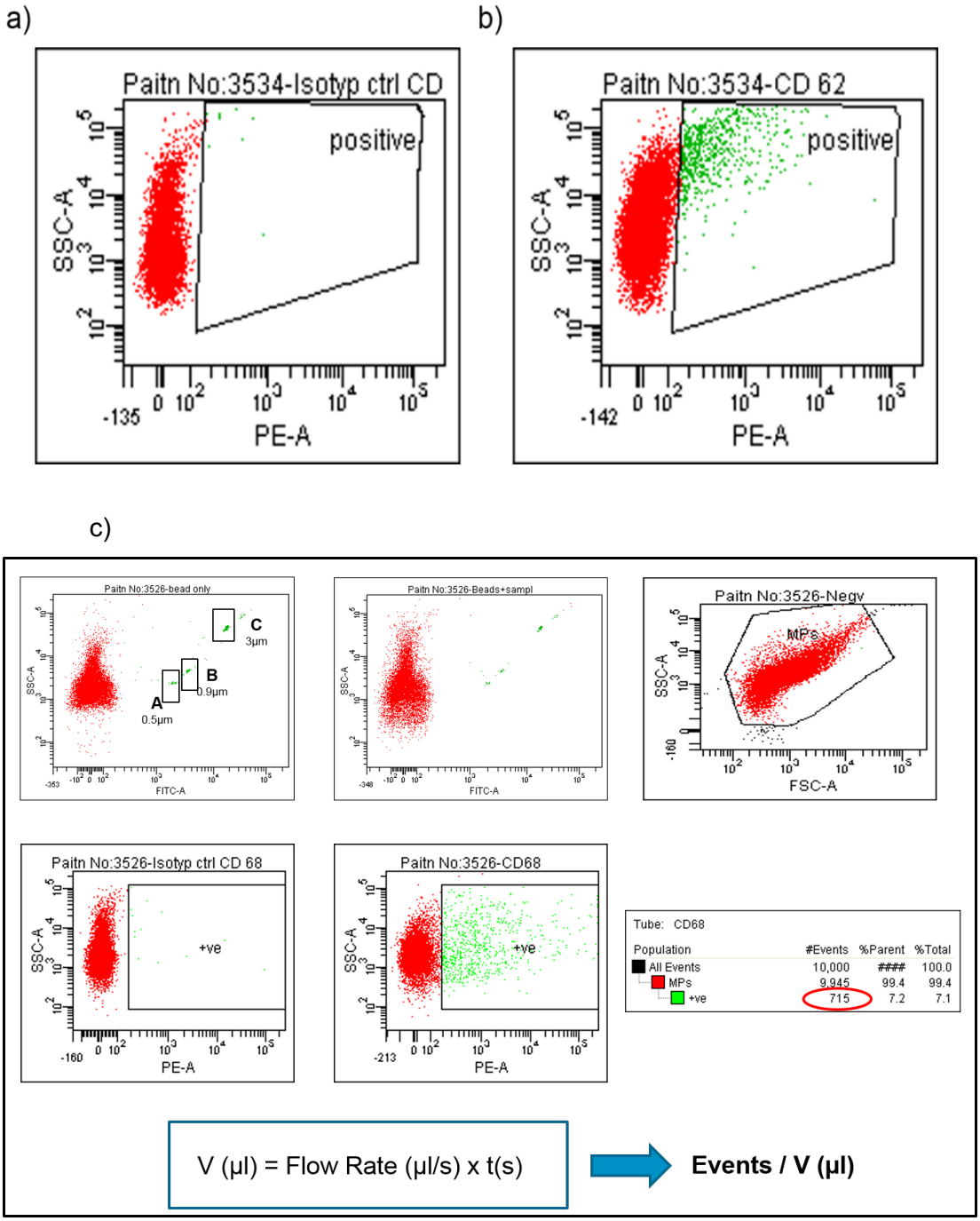


Figure 4. Flow cytometry characterization and measurement of various EVs. a) Isotype control for EVs derived from endothelial cells (CD62E). b) Example of the measurement for EVs from endothelial cells (CD62E). c) Results showing different populations which can be differentiated using only megamix beads; megamix beads with the sample; negative control; isotype control and example of measurement for EVs derived from macrophages (CD68) and total number of positive events.

2.2.3 **Statistical analysis**

Initially, the ROUT test was used for identification of outliers. Further, the unpaired T test with Welch's correction in the case of normally distributed values or Mann-Whitney test when values were not normally distributed, were used to compare non-PH control with respective PH groups. Spearman test was used for analyses of the correlations. $P<0.05$ was considered statistically significant. For the data and statistical analysis, the GraphPad Prism software was used.

Results

3.Results

The flow cytometric measurement of EVs was performed in different PH groups. I,hPAH has been shown in the first group in the table below (Table no. 4), whereas PH associated with a porto-pulmonary shunt, systemic to pulmonary shunt, and CTD has been analyzed under the second group named as associated PAH. Other forms of PH categorized are due to lung diseases like COPD, lung fibrosis, left heart disease, and thromboembolism.

3.1 **Clinical data, gender ratio, and age of patients with different forms of PH**

The table below (Table 4) shows different clinical parameters of the patients (age, gender ratio, New York Heart Association Functional Classification (NYHA), and pulmonary hemodynamics in various PH groups. The first group includes idiopathic and heritable pulmonary arterial hypertension (I,hPAH). Most of the patients belonging to this group are female, with age of around 45–50 years and show the most elevated mPAP and PVR values in all the study groups. The second group with associated PAH in the present study includes CTD, porto-pulmonary shunt, and systemic to pulmonary shunt, which shows the same prevalence in men and women in the age group of 36–56 years with second highest values of mPAP after I,h PAH in this study.

PH group	Age (years)	Gender ratio (f/m)%	mPAP (mmHg)	PVR (dyn*s*cm^{-5})	NYHA
Non-PH (n = 8)	62±5	50/50	17.0±1.0	162±38	na
i,hPAH (n = 6–11)	47±5	82/18	54.7±4.2	1033±232	I–IV
Associated PAH (n = 4-6)	45±9	50/50	38.5±6.9	450±159	II–IV
LHD-PH (n = 11-14)	69±3	57/43	33.8±3.7	326±71	II–IV
COPD-PH (n = 7)	66±4	29/71	37.0±2.9	470±38	III–IV
Fibrosis-PH (n = 9-13)	67±2	8/92	32.7±3.5	430±57	III–IV
CTEPH (n = 4-12)	67±4	75/25	37.7±6.3	487±125	II–IV

Table 4: This table shows different factors like age, gender and clinical PH parameters like mPAP and PVR in various PH groups like I, h PAH, Associated PAH, LHD-PH, COPD-PH, Fibrosis-PH, CTEPH. Available results with the number of patients/values for each PH group are given as mean+SEM.

The third group includes PH due to left heart disease and shows the prevalence of more females than males in the age group of 65–70 years with increased mPAP and PVR values. COPD and fibrosis-PH patients have been studied in the fourth and fifth groups and show the prevalence of more males of the age group 60–70 years with increased mPAP and PVR. The last group with CTEPH patients includes more females in the age group of 6570 years with increased mPAP and PVR.

3.2. **Endothelial cell-derived EVs in pulmonary circulation**

3.2.1. **CD62E (E-selectin/endothelial) cell-derived EVs profile in pulmonary circulation**

Circulating CD62E EVs were stained and measured by flow cytometry. The obtained results revealed a significant increase in CD62E cell-derived EVs in CTEPH patients. Furthermore, there was a strong tendency to an increase in EVs in COPD-PH patients and associated PAH patients in comparison to non-PH controls (Figure 5).

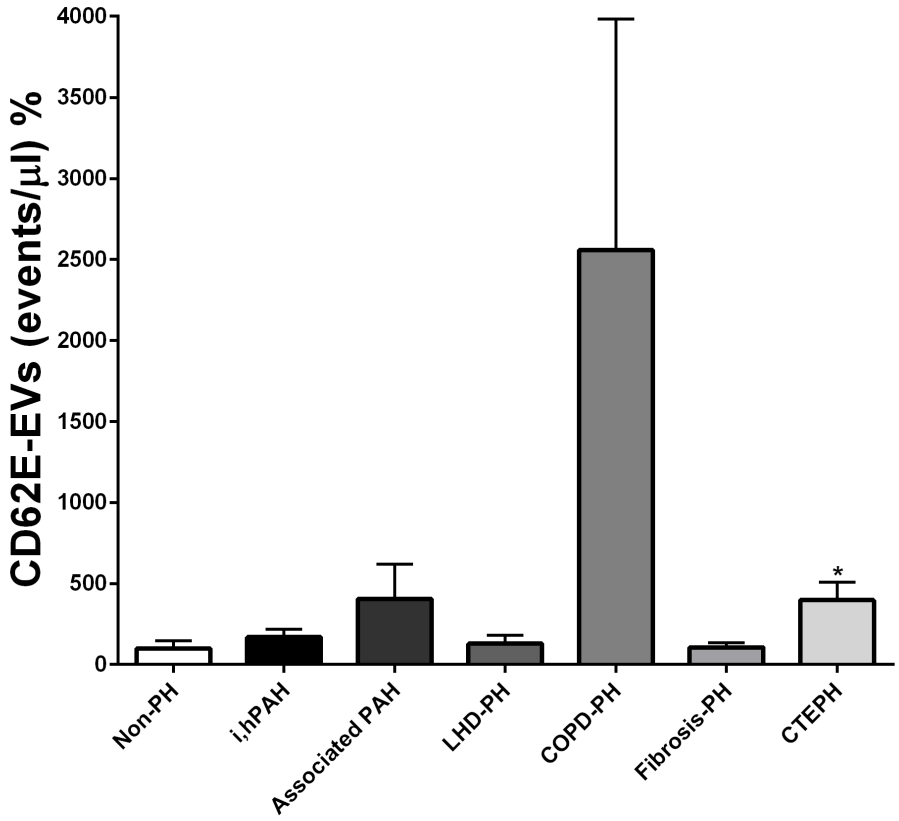


Figure 5. As mentioned in the method part, CD62E extracellular vesicles (EV), which are E-selectin/ endothelial cell-derived EVs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n=6-14). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension. *p <0.05.

3.2.2 **CD144 (VE-Cadherin/endothelial) cell-derived EVs profile in pulmonary circulation**

Circulating CD144 EVs were stained and measured by flow cytometry. The results show that there was a slight tendency to an increase in CD144 cell-derived EVs in some PH groups, mostly in COPD-PH patients, as compared to non-PH controls (Figure 6).

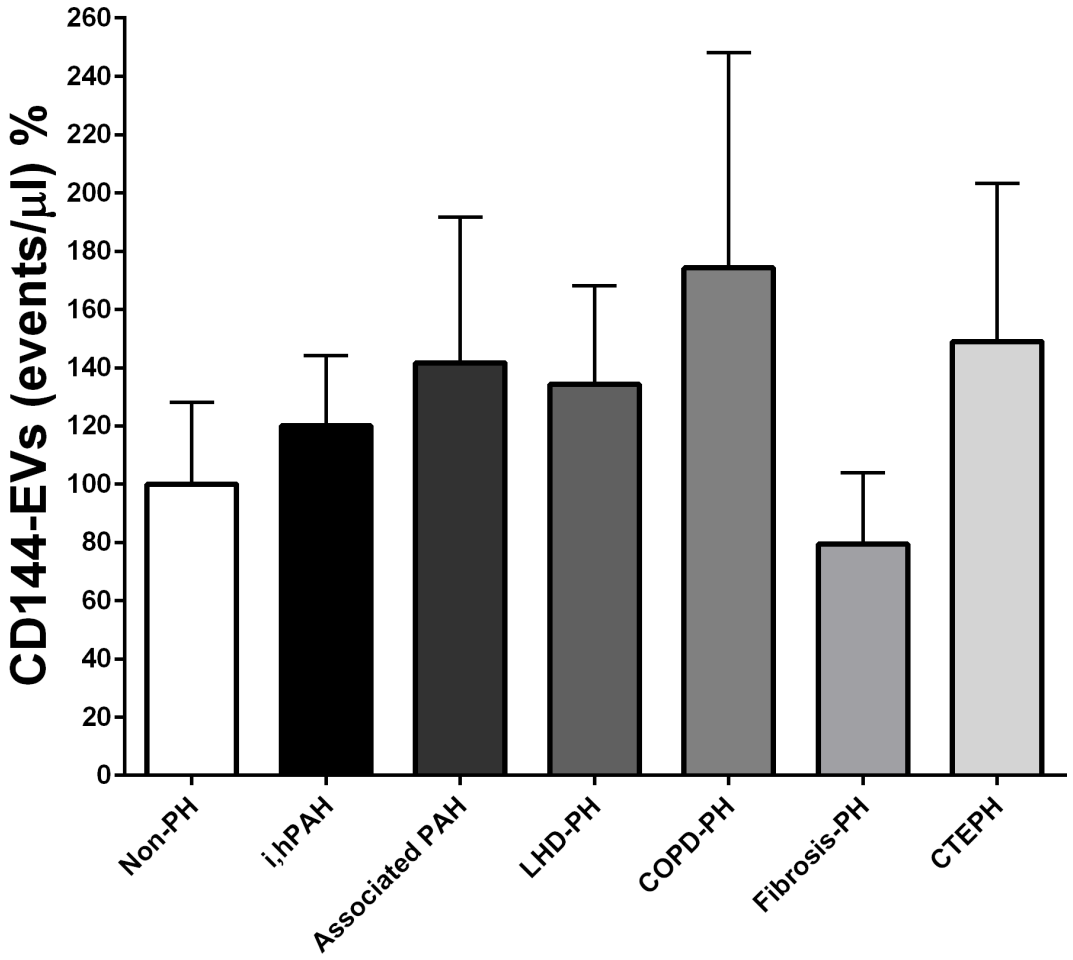


Figure 6. CD144 EVs, which are endothelial cell-derived EVs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n= 6-12). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension.

3.2.3 **CD31 (PECAM/endothelial cells, monocytes, neutrophils) cell-derived EVs profile in pulmonary circulation**

Circulating CD31 EVs were stained and measured by flow cytometry. The results report that there was a significant decrease in CD31 cell-derived EVs in LHD-PH patients and a tendency to decrease in COPD-PH, associated PAH and idiopathic and heritable-PH and fibrosis PH patients, whereas remained same in CTEPH patients in comparison to non-PH controls (Figure 7).

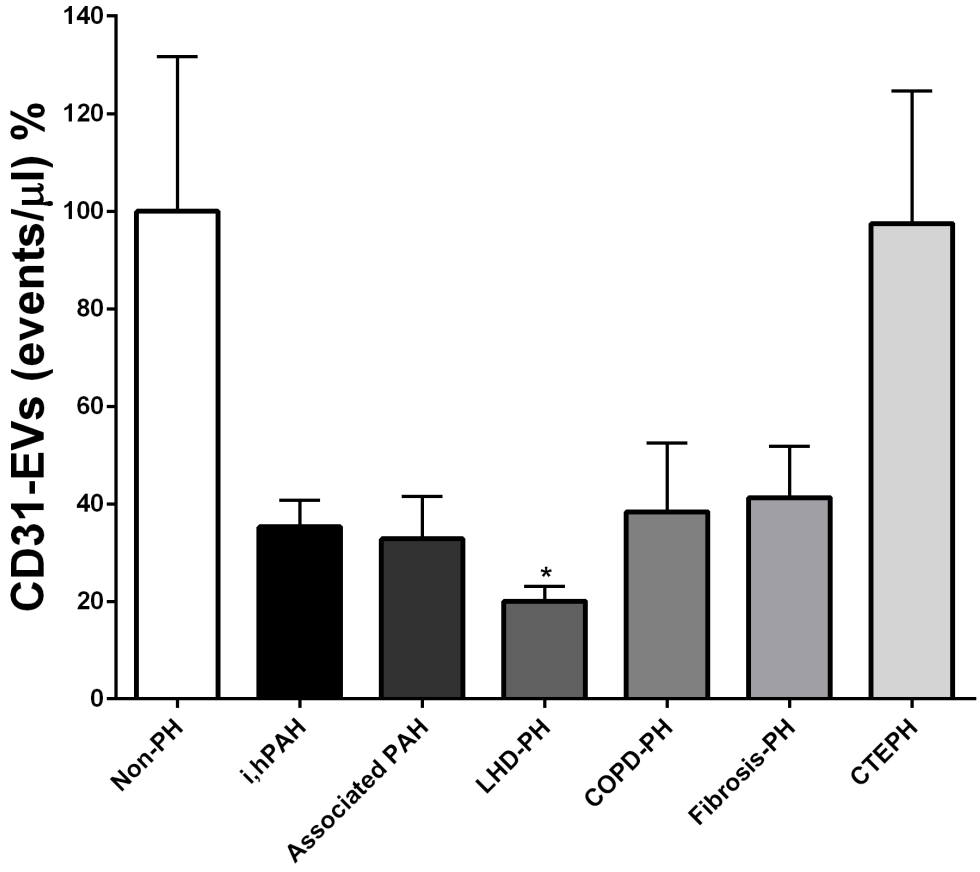


Figure 7. CD31 EVs derived from PECAM/endothelial cells, monocytes, neutrophils, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n=6-12). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension. *p <0.05.

3.3 **Inflammatory cell-derived EVs in pulmonary circulation**

3.3.1 **CD3 (T cell)-derived Evs profile in pulmonary circulation**

Flow cytometry analysis revealed that there was a significant increase of circulating CD3 derived Evs in the CTEPH and I, h PAH groups, and a strong tendency to increase in Associated PH, LHD-PH, COPD-PH and fibrosis PH groups in comparison to non-PH controls (Figure 8).

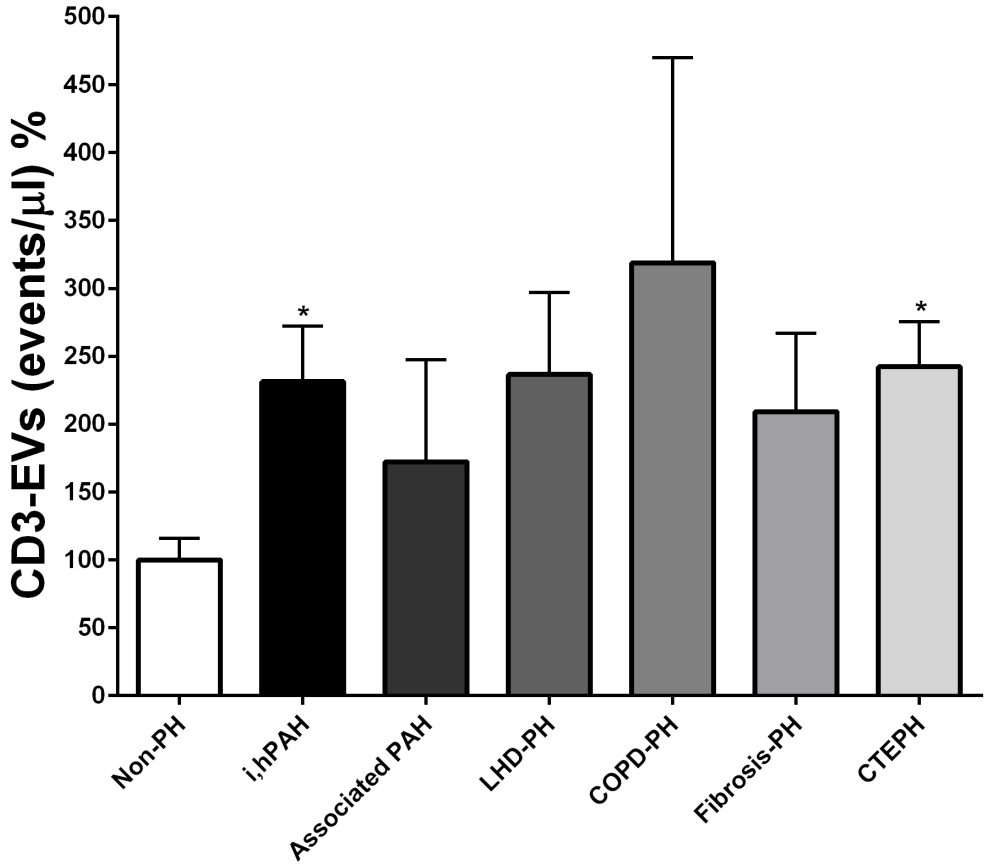


Figure 8. CD3 Evs, which are T cell-derived Evs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n= 6-14). Legend: I,h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension. *p <0.05.

3.3.2 **CD14 (monocyte) derived EVs profile in pulmonary circulation**

It has been shown in flow cytometry analysis that there was a slight tendency to an increase in circulating CD14 cell-derived EVs in associated PAH patients and a slight tendency to a decrease in idiopathic, heritable PAH and COPD-PH patients, whereas it remained the same in other groups in comparison to non-PH controls (Figure 9).

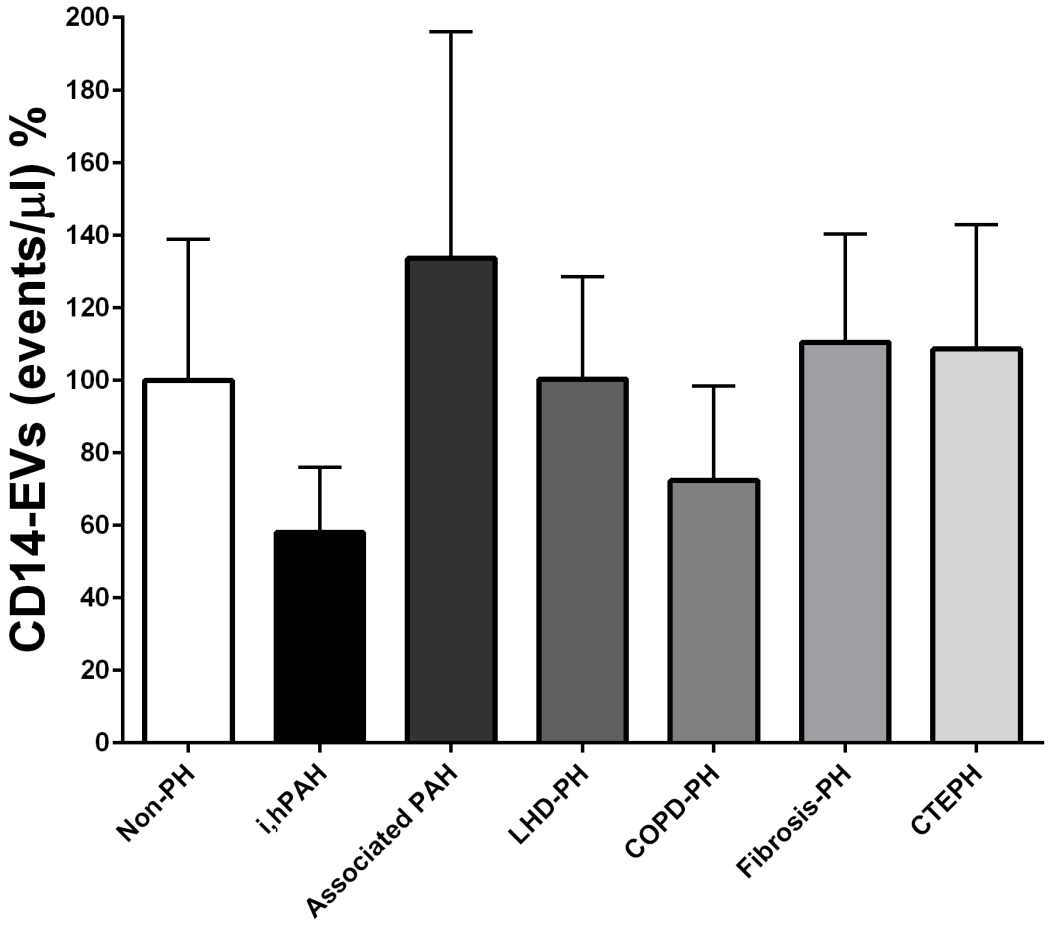


Figure 9. CD14 EVs, which are monocytes derived EVs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n= 6-14). Legend: I, h PAH: idiopathic, heritable Pulmonary arterial hypertension; Associated PAH – pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension.

3.3.3 **CD 45 (leukocyte common antigen) derived EVs profile in pulmonary circulation**

Circulating CD45 EVs were stained and measured by flow cytometry. The following results show that there was a tendency towards a decrease in CD45 cell-derived EVs in all PH groups as compared to non-PH control (Figure 10).

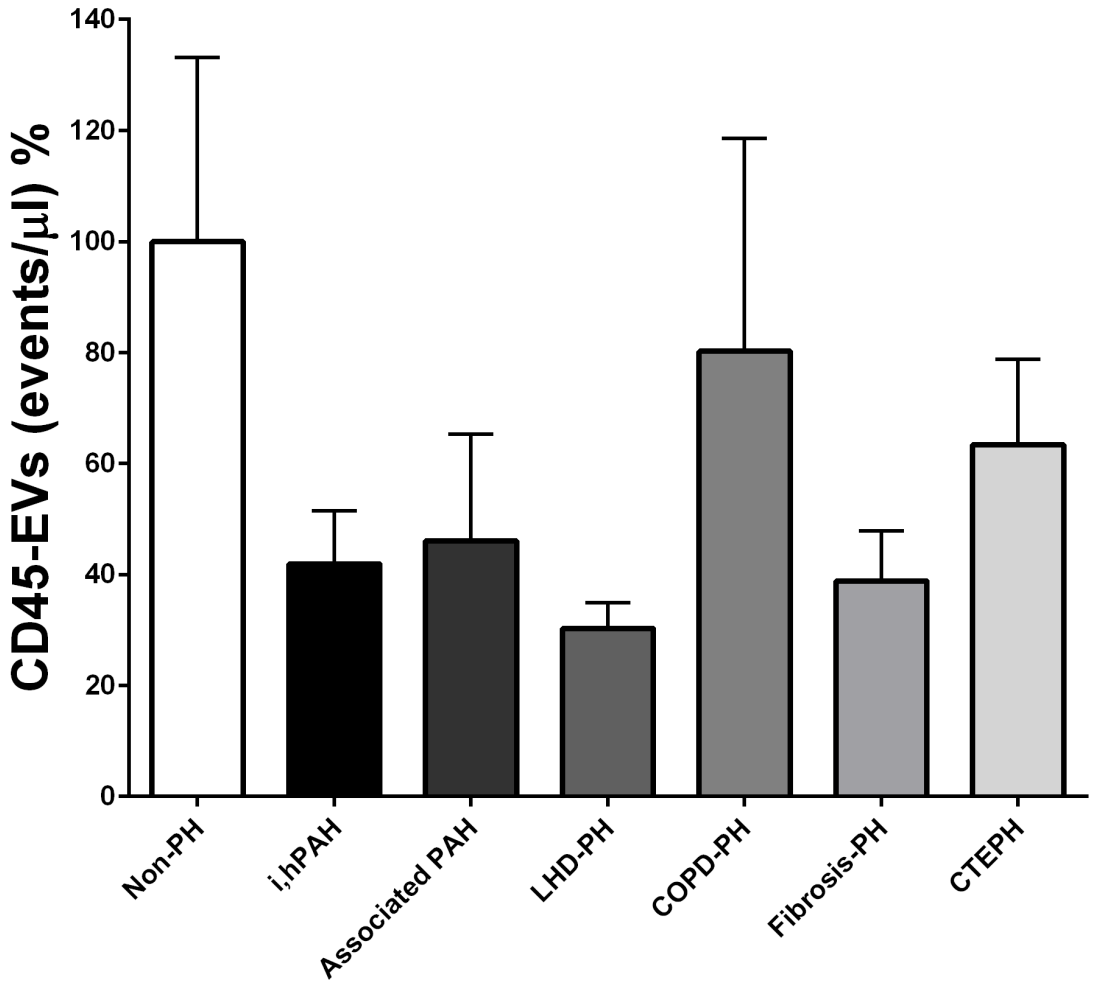


Figure 10. CD45 EVs, which are leukocyte common antigen derived EVs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n= 6-13). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension.

3.3.4 **CD68 (macrophages) derived EVs profile in pulmonary circulation**

Flow cytometry analysis showed that there was a tendency to an increase in circulating CD68 (macrophages)-derived EVs in CTEPH and I, h PAH, and a tendency to decrease in Associated PAH, whereas it remained the same in other groups compared to non-PH controls (Figure 11).

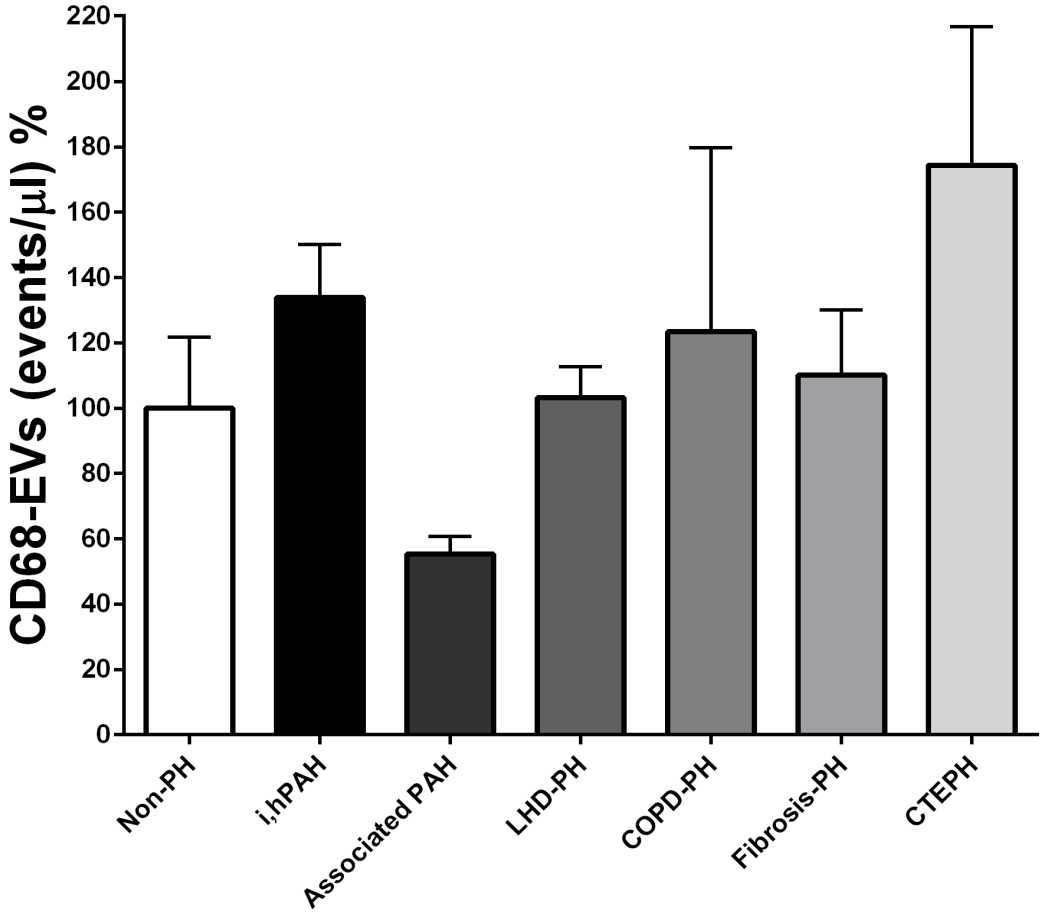


Figure 11. CD68 EVs, which are macrophages derived EVs, were compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100**%.** Results were presented as mean ± SEM (n= 5-13). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension.

3.4 **CD209 (dendritic cell) derived EVs profile in pulmonary circulation**

CD209 EVs were stained and analyzed with flow cytometry. It shows that circulating CD209, which are dendritic cell-derived EVs, were significantly decreased in LHD-PH, Fibrosis-PH and CTEPH, whereas their levels remained the same in other groups as compared to the non-PH control group (Figure 12).

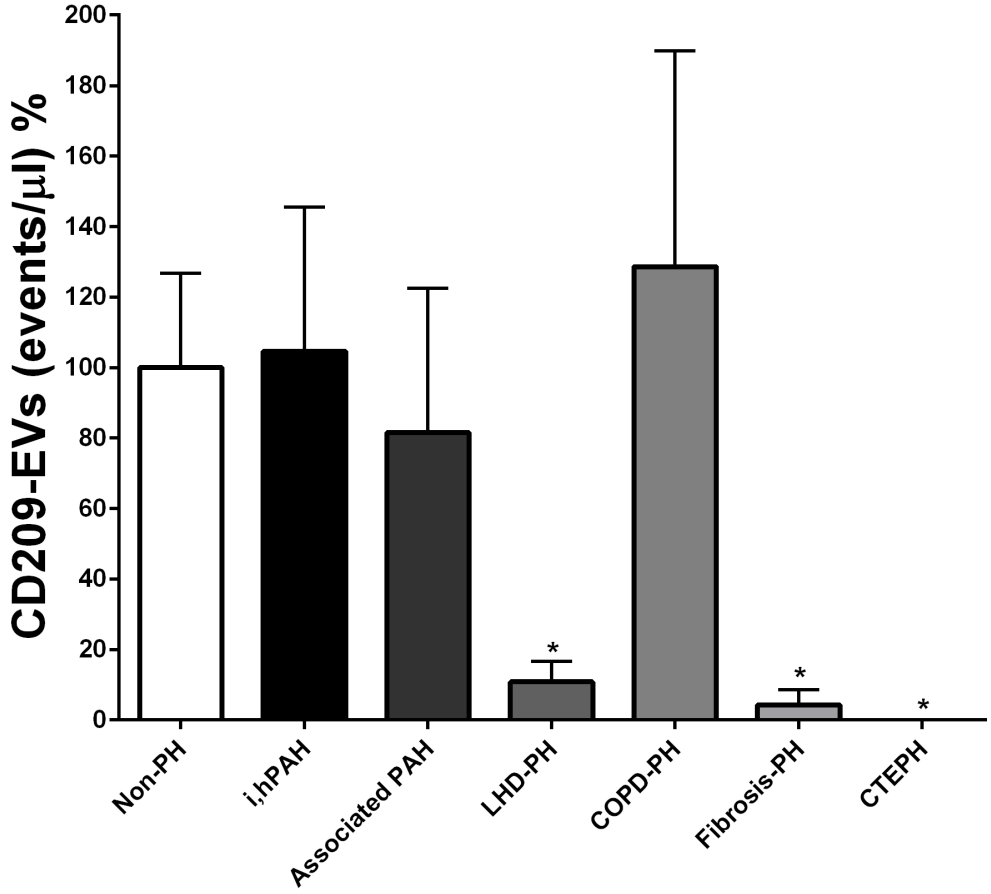
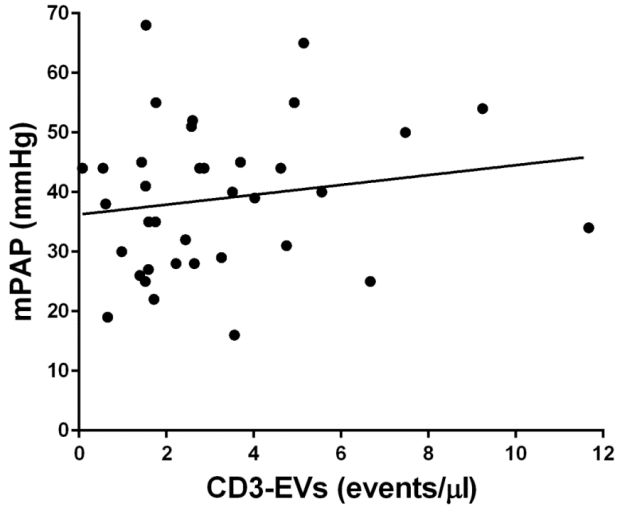


Figure 12. CD209 EVs, which are dendritic cell-derived EVs, are compared in different PH subgroups analyzed by flow cytometry and results were expressed as events/µl in percentage, where the mean value of the non-PH control was considered to be 100%. Results were presented as mean ± SEM (n= 6-12). Legend: I, h PAH: idiopathic, heritable pulmonary arterial hypertension; Associated PAH – Pulmonary arterial hypertension associated with different diseases; LHD-PH – Pulmonary hypertension due to left heart disease; COPD-PH – PH due to COPD; Fibrosis-PH – PH in lung fibrosis; CTEPH – Chronic thromboembolic pulmonary hypertension. *p<0.05.

3.5 **Correlation between different cell-derived EVs and important PH parameters like mPAP and PVR**

3.5.1 **Correlation between the inflammatory cell-derived CD3-EVs (events/µl) and the mPAP (mmHg)**

The correlation between the inflammatory cell-derived CD3 EVs, which are shown in events/µl, and the mPAP in mmHg revealed that there was a tendency to an increase in mPAP with increasing numbers of circulating CD3 EVs (Figure 13). However, the correlation did not reach statistical significance.

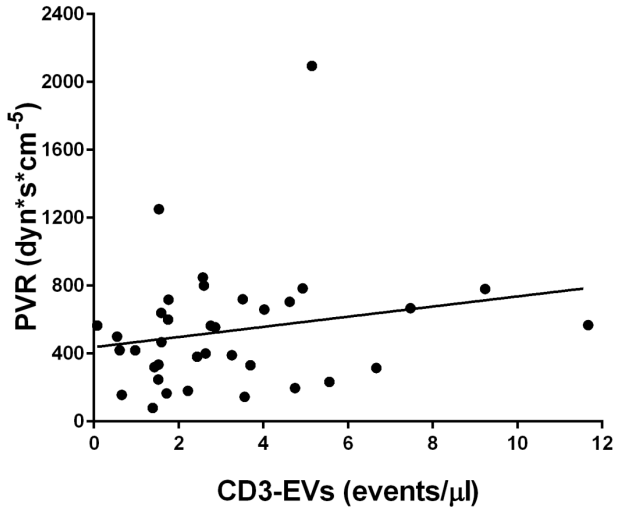


r=0.2074

Figure 13. Correlation between the inflammatory cell-derived CD3-EVs (events/µl) and the mPAP (mmHg).

3.5.2 **Correlation between the inflammatory cell-derived CD3 EVs (events/µl) and the PVR (dyn.s.cm^{-5})**

The correlation between the inflammatory cell-derived CD3 EVs (events/µl) and the PVR (dyn.s.cm^{-5}) has been shown below (Figure 14). There was a positive correlation between inflammatory cell-derived CD3 EVs and PVR, however, it did not reach statistical significance.

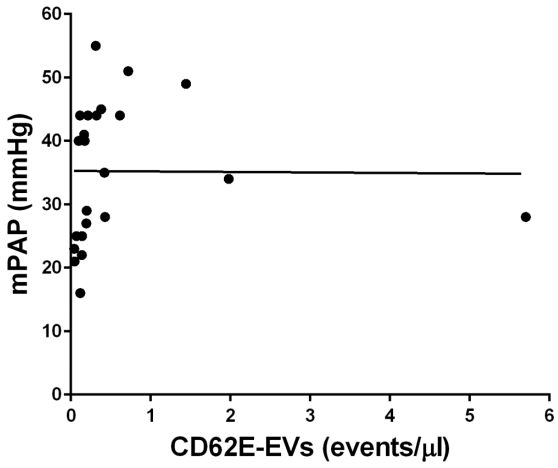


r=0.2525

Figure 14. Correlation between inflammatory cell-derived CD3-EVs (events/µl) and the PVR (dyn x s x cm^{-5}).

3.5.3 **Correlation between the endothelial cell-derived CD62E EVs (events/µl) and the mPAP (mmHg)**

The correlation between the endothelial cell-derived CD62E EVs, shown in events/µl, and the mPAP in mmHg is shown below (Figure 15). This correlation exposed that there was no tendency to an increase in mPAP with increasing numbers of CD62E EVs.

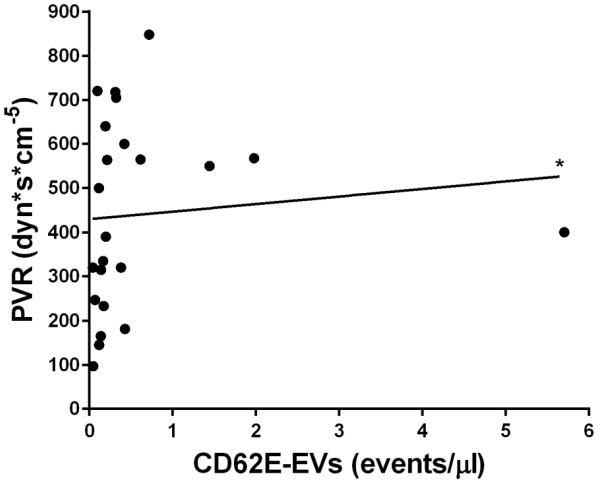


r=0.5274

Figure 15. Correlation between the endothelial cell-derived CD62E-EVs (events/µl) and the mPAP (mmHg).

3.5.4 **Correlation between the endothelial cell-derived CD62E EVs (events/µl) and the PVR (dyn.s.cm^{-5})**

The correlation between the endothelial cell-derived CD62E EVs, which is shown in events/µl, and the PVR in dyn.s.cm^{-5} is shown below (Figure 16). This analysis confirmed that there was a significant correlation to an increase in PVR with increasing numbers of circulating CD62E EVs.



r=0.4453

Figure 16. Correlation between the endothelial cell-derived CD62E-EVs (events/µl) and the PVR (dyn x s x cm^{-5}) *p<0.05.

Discussion

4. Discussion

In general, this study demonstrated an alteration in circulating levels of EVs from different endothelial and inflammatory cell origins in patients with various clinical forms of PH. Among the study of different inflammatory cell-derived EVs, the most important finding in this study was the enhanced profile of CD3-derived circulating EVs in PH Patients. Furthermore, it acknowledged us about the significant increase of CD3-derived EVs in CTEPH and I, h PAH patients and a strong tendency to increase in associated PAH, LHD-PH, COPD-PH and fibrosis-PH patients.

Results of this study showed that there was a prominent enhancement of CD3-derived EVs in the central blood circulation in different clinical forms of PH. This might explain the pathogenesis of PH induced by inflammation. The respective correlation analysis indicated a slight tendency towards an increase in mPAP and PVR values, with an increasing profile of circulating CD3-derived EVs, suggesting that this inflammatory cell-derived EV could have potential biomarker properties.

CD62E (E-selectin/endothelial cells) cell-derived EVs, analyzed in the scope of this study, showed a significant increase in CTEPH patients as well as a strong tendency to an increase in COPD-PH and associated PAH patients. Furthermore, it was concluded from results of this study, that there was a positive correlation in PVR, but no change in mPAP, with increasing CD62E-derived EVs. Therefore, it is suggestive that future studies should consider a deeper analysis of the promising biomarker attributes of this endothelial cell-derived EVs.

Other endothelial and inflammatory cell-derived EVs did not show the consistent profiles in different PH forms. Briefly, analytical description of CD144-derived EVs, shows a modest tendency to increase in some PH subgroups, especially in COPD-PH patients. The results of CD31-derived EVs in this study, demonstrated that there was a significant decrease in LHD-PH patients and a tendency to decrease in COPD-PH, associated PAH, idiopathic and heritable-PAH and fibrosis-PH patients. We further observed, a tendency to increase in CD14-derived EVs in associated PAH and tendency to decrease in I, h PAH, and COPD-PH patients, whereas there was no change in other groups. The investigation of CD45-derived EVs, demonstrated a tendency to decrease in all PH groups. In addition, it was observed that CD68-derived EVs seem to show a tendency to increase in CTEPH and I, h PAH and a trend to decrease in associated PAH. However, they remained same in other PH groups. Finally, the study of CD209-derived EVs profiles, exposes a significant decrease in LHD-PH, fibrosis-PH and CTEPH, whereas

no changes in other groups was observed. The EVs from the control group (non-PH), where all clinical forms of PH have been excluded, were taken as a reference in this study.

The increase in the inflammatory CD3-EVs is one of the important findings, which supports a report by Savai *et al*. which explained that the inflammatory processes are prominent in the context of PAH (134). Also, the increase in endothelial cell-derived (CD62E)-EVs, observed in the present study, complies with the findings reported by Amabile *et al*. that the CD62E cell-derived MPs are increased in PH (126).

4.1 **EVs in other cardiovascular diseases**

MPs are a type of EVs, which represent shed membrane vesicles that are released during apoptosis and/or activation of different cell types (115, 124). MPs are pro-inflammatory vesicles with diameters smaller than 1.1 µm and are released by different cells like endothelial cells types, for example, platelets, leucocytes, etc., which have cytoplasm and surface markers of their origin (129, 135, 136). After their release into circulation, MPs bind and fuse with their target cells by various mechanisms like receptor-ligand communication and thus play a vital role in cardiovascular diseases by mediating vascular inflammation and coagulation (124, 129, 137-139). It has also been reported that the MPs transport mRNA and miRNA by altering the protein expression of their target cells. Hence, the inhibition of miRNA degradation by RNase in the blood circulation can be explained (129, 131).

EVs are suggested to be active carriers in the pathogenesis of PH and other cardiovascular diseases, acting as a transport vehicles for different miRNAs (129). It has already been explained that the circulating EVs in patients with acute coronary syndrome might be associated with endothelial dysfunction implicating their clinical relevance (140). As mentioned by Amabile *et al*., there is high importance for the endothelial dysfunction of endothelial MPs in cardiovascular disorder in patients with ESRD (116). The possibility of MPs originating from plaque rupture in patients with acute myocardial infarction has been hypothesized to be less, depending on the non-similar structure of cellular origin between plaque and the plasma MPs (114, 141). Furthermore, there is an augmentation of circulating MPs in patients with cardiovascular risk factors, like hypertension, obesity, or cholesterol (142-144). Long-term treatment with antioxidants, such as vitamin C or carvedilol, suppresses the circulating endothelial MPs in patients with heart failure (145, 146). It has also been demonstrated that statins decrease the

endothelial MPs formation, whereas platelet-derived MPs remain unaffected by aspirins but can be downregulated by ticlopidine, abciximab or cilostazol (147-151).

4.2 **EVs in pulmonary hypertension**

Circulating endothelial cell-derived MPs are suggested to be involved in the pathogenesis of PH. Therefore, some MPs might function as biomarkers to assess the severity of a disease. It was already explained by Amabile *et al.* and Bakouboula *et al*. that there are increased numbers of endothelial cell-derived MPs in PH and that there is a correlation with the clinical parameters like mPAP (133) (115). It has been presented in the literature, that some MPs subgroups (CD31/ CD41/ CD144) are associated with hemodynamic severity of precapillary PH, whereas other subgroups (CD62E) are linked to inflammation (115). Indeed, MPs measurement has been suggested to assess the prognosis and monitoring the the endothelium-protective therapy in PH (152). It has been demonstrated that the increase in circulating procoagulant EVs is associated with mPAP, and that EVs with tissue factor (TF) are inversely proportional to the 6MWD (133). Interestingly, it has been reported that the platelet-derived EVs can upregulate the SMCs proliferation and, therefore my decrease the cross-sectional area of the vascular lumen (52). It has been explained that the elevated circulating procoagulant EVs with endoglin are key players in the pulmonary microvasculature (133).

4.2.1 **Endothelial cell derived EVs**

It was reported that endothelial cell-derived EVs might be represented as markers for endothelial injury and vascular remodeling leading to endothelial dysfunction (116). The correlation between the endothelial cell-derived EVs and endothelial dysfunction has been shown in patients with chronic renal failure (116) or coronary artery disease (117). The augmentation of endothelial cell-derived EVs has been shown to be linked with severe cardiovascular diseases (114, 152). Furthermore, it was also reported that there is an enhancement of endothelial cell-derived EVs in PH, which correlates with clinical parameters like mPAP (115).

In this study, it was demonstrated for the first time that the alteration of endothelial cell-derived EVs in different PH groups, as previous studies did not analyze the profiles in various clinical subgroups. Nonetheless, increase in CD62E, endothelial cell-derived EVs in precapillary PH, have already been reported (115, 153). It is noteworthy in this study that the endothelial CD62E cell-derived EVs were enhanced in various clinical PH forms such as associated PAH, COPD-PH, and CTEPH. In contrast to the results of this

work, the positive correlation between endothelial cell-derived CD62E-EVs and mPAP has been observed in PH patients (115). However, this study indicated a positive correlation between another important clinical parameter, that is, PVR and increasing numbers of CD62E-EVs in PH patients. It was also observed that there was no alteration of CD144 cell-derived EVs in most of the PH subgroups, however, there was a tendency to increase in some clinical forms of PH.

4.2.2 **Inflammatory cell-derived EVs**

Although the current knowledge gives enough evidence about the possible predictive nature of endothelial cell-derived MPs, there is very little known about inflammatory cell-derived MPs**.** It was suggested that inflammatory cell-derived MPs might be involved in the pathogenesis of other inflammatory diseases (125). Tuder *et al.* (154) suggested that increased inflammation with infiltration of T cells, B cells, and macrophages in plexiform lesions of PAH and association of perivascular inflammation with intima and media redesign in PAH, is indeed an important phenomenon found in PH (155). As mentioned by Savai *et al*., there is an accumulation and infiltration of inflammatory and immune cells in the pathogenesis of PH (134). Analysis of the obtained results revealed for the first time, that there are higher amount of inflammatory CD3-derived EVs in different forms of PH. There was also a tendency for a positive correlation between increased CD3 cell-derived EVs and clinical parameters such as mPAP and PVR. However, other inflammatory cell-derived EVs like CD14 and CD68-derived EVs did not show any significant and consistent profile change in different clinical forms of PH, although their importance in the pathogenesis of PH has been mentioned before (134). Therefore, it is conclusive from this study that the enhanced T cell-derived EVs are potential players in the plethora of various clinical forms of PH (Figure 17).

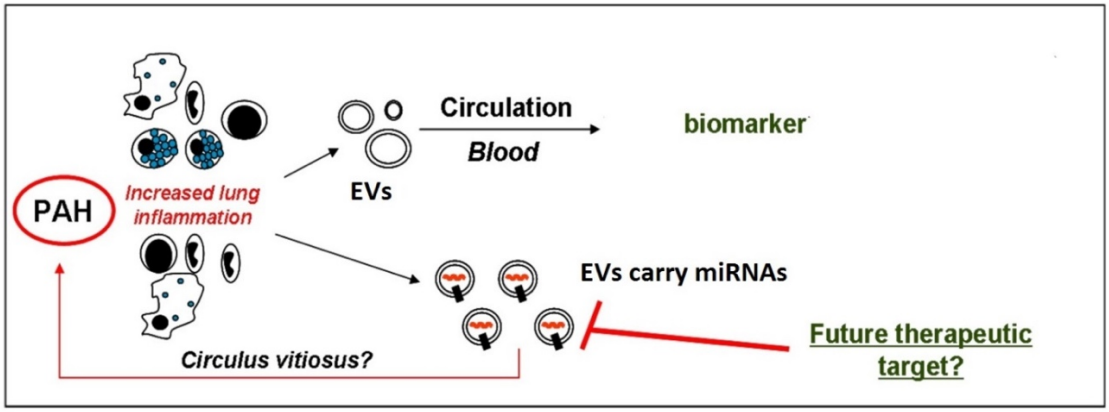


Figure 17. Inflammatory cell-derived EVs may represent potential biomarkers and active players in the pathology of PAH.

4.3 **Limitations**

Discussion of the limitations

PH patients have comorbidity with different cardiovascular diseases, which may also cause the elevation of EVs and MPs, as mentioned in the literature. Therefore, on the one hand, the conclusion that the altered EVs are attributable to PH pathology has to be taken with caution. On the other hand, the non-PH control group represents the patients excluded from PH, not healthy controls, who still may suffer from other diseases that may be associated with altered EVs. Therefore, it is expected that altered EVs profile would be even higher or more significant when compared to healthy controls.
The smaller number of patients included in each subgroup in analysis of results in this study is also considered limitation.

Right heart catheterization and diagnosis

Right heart catheterization remains the gold-standard method for the diagnosis of PH and for measuring ventricular elastance, arterial elastance, and ventriculoarterial coupling (156-158). There are different challenges for the diagnosis of PH, including the most frequent discussion on how to separate PAH and PH due to left heart failure with HFpEF (1). Patients with normal PAWP can also show PH due to heart failure with preserved ejection fraction (1). It is challenging to diagnose PH in the early stage in asymptomatic patients. In patients with systemic sclerosis, it is important to observe all the clinical symptoms, pulmonary function tests, and diffusion capacity for carbon monoxide, biomarkers, and echocardiography (1). Exercise-induced PH should be standardized according to the exercise criteria in different age groups and their effect on the therapy and prognosis (1).

Preparation and extraction of samples for EVs analysis

There are many methods used for the measurement of EVs, including MPs, but due to the lack of standardized methods and pre-analytical preparation of samples, there are variabilities in the number of MPs shown by different studies (159). Many successful studies on MPs have been performed by the use of flow cytometric analysis. Other assays analyzed MPs with electron and fluorescence confocal laser scan microscopy (159-162), capture-based assays (Elisa-based solid phase capture assays) (159, 163, 164), functional assays on isolated MPs (159, 165-168), proteomics analysis (159, 169, 170), impedance-based flow cytometry (159, 171), dynamic light scattering (DLS) (159,

172), nanoparticle tracking analysis (NTA) (159, 172), and atomic force microscopy (159, 173).

A frequently used assay for MPs in most studies is flow cytometry, which can be influenced by various pre-analytical and analytical issues. The pre-analytical issues, which cause the variability within the assay are as followings: more time difference between venipuncture and centrifugation as well as freezing of samples results in increased number of MPs; washing samples, double centrifugation and long-term storage of samples leads to decrease in MPs; thawing temperature does not play a role (159, 174).

The best pre-analytical condition to measure and analyze more accurately with a flow cytometer would be to prepare the sample directly after the blood sample was taken and quantify with flow cytometer (159, 174). Analytical conditions that influence the quantification of MPs are specific surface markers, labeling of MPs, creating a MP gate, settings of the used flow cytometer, quality of the antibodies, and the use of calibration and counting beads (159, 174). Some assays which are able to show the exact number of MPs without their further characters are fluorescence nanoparticle tracking analysis and atomic force microscopy (159, 174, 175).

4.4 **Future perspectives**

The Literature suggests that, the MPs act as a transport vehicle for micro RNAs, which are active agents in the pathogenesis of PH as well as other cardiovascular diseases (129). Further studies are needed to establish the association between alterations of various endothelial and inflammatory cell-derived MPs and up/down-regulation of different miRNAs in clinical forms of PH. This might might help understanding further, the complex pathogenesis of PH. Furthermore, future investigations should focus in the characteristics of inflammatory/endothelial cell-derived MPs, and also assess whether they fulfill the criteria to be novel promising biomarkers in the pathophysiology of PH.

Summary and conclusion

5. Summary and conclusion

MPs are classified as EVs, which represent shed-membrane structures released into the blood circulation during different cellular processes, such as apoptosis and/or cellular activation (113, 115). EVs can be categorized as exosomes (45 – 100 nm), MPs (100 – 1000 nm), and apoptotic bodies (1000 – 4000 nm), depending on their size and mechanisms of formation and release (113). It was reported that circulating endothelial cell-derived and procoagulant MPs, which are a type of EVs, are enhanced in patients with PH (2, 115, 129, 133, 153). The literature suggests that circulating endothelial cell-derived MPs are significantly augmented in precapillary PH and that they show a positive correlation with clinical parameters such as mPAP (115). Endothelial cell-derived EVs are elucidated to be promising biomarkers, as they fulfil many of the criteria as good biomarkers, and are also considered to play a pivotal role in the development of pulmonary vascular diseases by acting as transport vehicles for miRNAs (129). Thus, strong evidence about endothelial cell-derived MPs in the field of PH, could provide knowledge about their importance in the therapeutic field. However, very little is known about the inflammatory cell-derived MPs (115). It was shown by Savai *et al*. that there is a massive accumulation and infiltration of inflammatory/immune cells in PH (134).

Therefore, it was hypothesized that, inflammatory cell-derived EVs may play an important role in the pathogenesis of different pulmonary vascular diseases, since it is known that PH is an inflammatory disease. In this study, analysis of the endothelial cell-derived MPs in various clinical PH subgroups was done. Therefore, this study was designed to analyze circulating profiles of various inflammatory (CD3 (T cells), CD14 (monocytes), CD45 (leukocyte common antigen), CD68 (macrophages), endothelial (CD62E (E-selectin), CD31 (PECAM, monocytes, neutrophils), CD144 (VE-Cadherin), and CD209 (dendritic) cell-derived EVs in different clinical forms of PH.

The central blood was obtained during right heart catheterization from patients with different forms of PH (I,h PAH, associated PAH, COPD-PH, Fibrosis-PH, LHD-PH and CTEPH, and non-PH controls . The central blood from each patient during right heart catheterization was collected into citrated tubes which were further centrifuged at 500xg for 15 minutes, followed by 10.000xg for 5 minutes to obtain the platelet free plasma (PFP), as described by Amabile *et al*. (115). The PFP was used for flow cytometry analysis with a BD LSRFortessa^{tm} flow cytometer.

Focusing on inflammatory cells-derived EVs, the flow cytometry analysis revealed that there was an augmentation of circulating CD3 (T cell) cells-derived EVs (events/µl) in all of the above-mentioned clinical groups of PH, compared to the non-PH controls. However, this was only statistically significant in the case of I,h PAH and CTEPH.

Interestingly, only a moderate and non-significant positive correlation between circulating levels of CD3 cells-derived EVs and the relevant clinical parameters of PH, such as mPAP (mmHg) and PVR (dyn*s*cm^{-5}) was observed. Following the analysis of endothelial cell-derived EVs, it was identified that the circulating levels of CD62E-derived-EVs were increased in associated the PAH, COPD-PH and CTEPH groups. Conversely, there was no visible alterations in other groups, as compared to non-PH donors. A statistical significance was observed in the CTEPH group. Importantly, a positive correlation between CD62E cell-derived EVs and clinical parameters like PVR, but no correlation with mPAP was observed.

The present study shows for the first time that enhanced levels of circulating T cells-derived EVs are present in several forms of PH. This major finding supports the knowledge from the literature, that there is an accumulation and infiltration of inflammatory / immune cells, such as T Cells in redesigned pulmonary vasculature and the lung tissue of IPAH patients (134). Even though, the literature states a role for monocytes/macrophages in the pathogenesis of PH, a consistent alteration in the inflammatory monocytes/macrophages derived-EVs was not observed in the present study. The enhancement of endothelial cell derived-EVs in the pathophysiology of precapillary PH has been explained in the following literature (115, 126). This study also observed the augmentation of endothelial cell-derived CD62E-EVs in the associated PAH, COPD-PH and CTEPH groups.

Finally, it can be concluded from this study that there is an enhancement of circulating inflammatory cell-derived EVs originating from T cells in various PH subgroups. Thus, these inflammatory T cell-derived EVs may represent novel promising biomarkers in the pathogenesis of severe pulmonary vascular diseases. Future studies should target the precise roles of T cells-derived EVs, which may act as novel players in the disease development and/or progression, since EVs are known as carriers of mediators, such as miRNAs.

Zusammenfassung

6. Zusammenfassung

Mikropartikel (MPs), die als Abschnürung der Zellmembran während zellulärer Prozesse, wie der Zell aktivierung und/oder Apoptose in den Blutkreislauf freigesetzt werden, zählen zu den extrazellulären Vesikeln (EVs) (113, 115). Je nach Größe und Mechanismen der Bildung und Freisetzung, werden die extrazellulären Vesikel in Exosomen (45–100 nm), Mikropartikel (100-1000 nm) und apoptotische Körperchen (1000–4000 nm) klassifiziert (113). Es wird berichtet, dass im Blut zirkulierende endotheliale und pro-koagulierende Mikropartikeln bei Patienten mit pulmonaler Hypertonie erhöht sind (2, 115, 129, 133, 153). Es wird in der Literatur erwähnt, dass die zirkulierenden endothelialen MPs in der präkapillären PH signifikant erhöht sind und auch ihre positive Korrelation mit klinischen Parametern wie mPAP wurde gezeigt (115). Die endothelialen EVs werden als wichtige Biomarker angesehen, da sie alle Eigenschaften eines guten Biomarkers erfüllen und als Träger der Mikro RNAs eine entscheidende Rolle bei den pulmonalen vaskulären Krankheiten spielen (129). Da endotheliale MPs als wichtige Faktoren im Bereich der PH anzusehen sind, haben Sie auch eine wesentliche Bedeutung im therapeutischen Bereich. Jedoch ist sehr wenig über die inflammatorischen MPs bekannt (115). Savai et al. Wiesen nach, dass es zu einer massiven Akkumulation und Infiltration von Entzündungs- / Immunzellen in der PH kommt (134).

Aufgrund dieser Erkenntnisse stellten wir die Hypothese auf, dass inflammatorische EVs eine wichtige Rolle in der Pathogenese verschiedener Lungengefäßerkrankungen spielen könnten, da der bisherige Wissenstand zeigt, dass die PH eine entzündliche Erkrankung ist. Unsere Studie analysiert zum ersten Mal die endothelialen MPs in verschiedenen klinischen PH-Untergruppen. Daher wurde unsere Studie entwickelt, um die zirkulierenden Profile von verschiedenen inflammatorischen (CD3 (T-Zellen), CD14 (Monozyten), CD45 (Leukocyte Common Antigen), CD68 (Makrophagen)) und endothelialen (CD62E (E-Selectin), CD31 (PECAM, Monozyten, Neutrophile), CD144 (VE-Cadherin)) und CD209 (dendritische) EVs in verschiedenen klinischen Formen der PH zu untersuchen.

Das zentrale Blut wurde während der Rechtsherzkatheter von Patienten mit verschiedenen Formen der PH (idiopathische / erbliche pulmonale arterielle Hypertonie (i, h PAH), assoziierte PAH, PH aufgrund der chronisch obstruktiver Lungenerkrankung (COPD-PH), PH assoziiert mit pulmonaler Fibrose, PH aufgrund einer Linksherzerkrankung (LHD-PH) und chronischer thromboembolischer PH (CTEPH) und PH-Ausschluss gruppe/Kontrollgruppe entnommen. Das zentrale Blut von jedem Patienten wurde während der Rechtsherzkatheterisierung wurde in Citrat Röhrchen

gesammelt und dann 15 Minuten bei 500 × g und anschließend 5 Minuten bei 10.000 × g zentrifugiert um das Plättchen freie Plasma (PFP) zu erhalten, wie von Amabile et al. Erläutert (115). Für die Durchflusszytometrieanalyse wurde das PFP mit einem BD LSRFortessa^{tm} Durchfluss-zytometer gemessen.

Die Analyse der Durchflusszytometrie welche sich auf inflammatorische EVs konzentrierte zeigte, dass in allen oben erwähnten klinischen PH-Gruppen eine Zunahme der zirkulierenden CD3 (T-Zell) -abgeleiteten EVs (Ergebnisse: Events/ µl) im Vergleich zur Kontrollgruppe auftrat, wobei sie in der Gruppe von i, h PAH und CTEPH statistisch signifikant waren. Interessanterweise beobachteten wir nur eine moderate und keine signifikante positive Korrelation zwischen zirkulierenden Spiegeln von CD3-Zellen und den relevanten klinischen Parametern der PH, wie dem mittleren pulmonalen arteriellen Druck (mmHg) und dem pulmonalen Gefäßwiderstand (dyn * s * cm^{-5}). Nach der Analyse der endothelialen EVs identifizierten wir den CD62E, dessen zirkulierende Spiegel in assoziierten PAH-, COPD-PH- und CTEPH-Gruppen erhöht waren. Es traten allerdings keine sichtbaren Veränderungen in anderen Gruppen im Vergleich zur Kontrollgruppe auf, und wir beobachteten statistische Signifikanz in der CTEPH-Gruppe. Wichtig ist, dass wir auch eine positive Korrelation zwischen CD62E-Zell-abgeleiteten EVs und klinischen Parametern wie dem PVR gefunden haben, aber überraschenderweise trat keine Korrelation mit dem mPAP auf.

Unsere Studie zeigte zum ersten Mal erhöhte Spiegel von zirkulierenden T-Zellen abgeleiteten EVs in verschiedenen Formen der PH. Dieses wichtige Ergebnis unserer Studie unterstützt den Kenntnisstand der Literatur, dass eine Akkumulation und Infiltration von Entzündungs- / Immunzellen wie T-Zellen in neu gestalteten Lungengefäßen und Lungengewebe von IPAH-Patienten auftritt (134). In der Literatur wurde auch über die Rolle von Monozyten / Makrophagen in der Pathogenese der PH berichtet. Wir beobachteten überraschenderweise keine konsistente Veränderung in den von entzündlichen Monozyten / Makrophagen abgeleiteten EVs. Die Erhöhung der endothelialen EVs in der Pathophysiologie der präkapillären PH wurde bereits in der Literatur beschrieben (115, 126). Wir beobachteten in unsere Studie auch die Vermehrung von Endothelzellen-abgeleiteten CD62E-EVs in assoziierten PAH, COPD-PH und CTEPH-Gruppen.

Das Fazit unserer Forschungsergebnisse ist die Erkenntnis, dass es in verschiedenen PH-Untergruppen zu einer Zunahme von zirkulierenden inflammatorischen EVs aus T-Zellen kommt. Daher könnten diese entzündlichen T-Zell-abgeleiteten EVs neue wichtige Biomarker in der Pathogenese der schweren Lungengefäßerkrankungen darstellen. Zukünftige Studien sollten auf die genaue Rolle von T-Zell-abgeleiteten EVs abzielen, welche als wichtige Faktoren in der Krankheitsentwicklung und / oder -

progression dienen könnten, da die Literatur zeigt, dass EVs als Träger von Mediatoren wie Mikro-RNAs dienen.

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8. Supplements

8.1 List of figures

8.2 List of tables:

8.3 List of graphics and photographs:

8.4 List of abbreviations

5-HT	5 – hydroxytryptamine
6-MWD	6- min walk distance
ALK-1	Activin receptor-like Kinase-1
Ang -1	Angiopoietin
APC	Allophycocyanin
bFGF	Basic fibroblast growth factor
BGA	Blood gas analysis
BMPR2	Bone morphogenetic protein receptor type 2
BSA solution	Bovine serum albumin solution
CAV1	Caveolin-1
CD	Cluster of differentiation
CHD	Congenital heart disease
CML	Chronic myeloproliferative leukemia
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
COPD-PH	Pulmonary hypertension due to COPD
CTEPH	Chronic thromboembolic pulmonary hypertension
CTD	Connective tissue disease
CT	Computer tomography
CT angiography	Computer tomography angiography
CTEPH	Chronic thromboembolic pulmonary hypertension
DLCO	Diffusion capacity of the lung for carbon monoxide

DLS	Dynamic light scattering
DPLD	Diffuse parenchymal lung disease
DPAH	Drugs induced pulmonary arterial hypertension
ECG	Electrocardiogram
Eng	Endoglin
eNOS synthase	Endothelial Nitric oxide
EPCs	Endothelial progenitor cells
ERA	Endothelin receptor -A antagonist
ESRF	End stage renal failure
E-selectin selectin	Endothelial
EVs	Extracellular vesicles
FACS	Flow activated cell sorting
Fibrosis-PH	Pulmonary hypertension due to fibrosis
FKN	Fractalkine
FR	Flow rate
FSC	Forward light scatter
GOLD	Global initiative for chronic obstructive lung disease
HBSS solution	Hank's balanced salts solution
HFpEF	Heart failure with preserved ejection fraction
HGF	Hepatocyte growth factor
HHV	Human herpes virus
HIF1a	Hypoxia inducible factor 1a
HIV	Human immunodeficiency virus
HPAH	Heritable pulmonary arterial hypertension
HR-CT	High-resolution computer tomography
IL-6	Interleukins -6
IPAH	Idiopathic pulmonary arterial hypertension
IPF	Idiopathic pulmonary fibrosis
KCNK 3	Potassium channel subfamily K member 3
LHD-PH	Pulmonary hypertension due to Left heart disease
LVED	Left ventricular end diastolic pressure
mAbs	monoclonal antibodies
miRNAs RNAs	micro
MMP2	Matrix metalloproteinases
MPs	Microparticles
mPAP	mean pulmonary artery pressure
MVB	Multivesicular bodies
NaCl	Sodium (Natrium) chloride
NO	Nitric oxide
NT-proBNP	N-terminal pro B-type natriuretic peptide
NTA	Nanoparticle tracking analysis
PA	Pulmonary angiography
PAWP	Pulmonary artery wedge pressure
PAP	Pulmonary arterial pressure
PAH	Pulmonary arterial hypertension
PCH	Pulmonary capillary hemangiomatosis
PDGFR	Platelet derived growth factor receptor
PDE-5	Phosphodiesterase-5
PDE-5i	Phosphodiesterase-5 inhibitor
PE	Phycoerythrin
PEA	Pulmonary endarterectomy
PECAM	Platelet and endothelial cell adhesion molecule

PFP	Platelet free plasma
PFT	Pulmonary function test
PH-LHD	Pulmonary hypertension due to left heart disease
PH	Pulmonary hypertension
PPHN	Persistent pulmonary hypertension of the newborn
PPARs	Peroxisome proliferator activated receptors
PVR	Pulmonary vascular resistance
PVOD	Pulmonary veno-occlusive disease
RHC	Right heart catheterization
ROCK	Rho associated protein Kinase
RV	Right ventricle
sGC	Soluble guanylate cyclase
SMC cells	Smooth muscle
SSC	Side-angle light scatter
TGF-β1	Tumor growth factor β1
THBS1	Thrombospondin 1
Tie -1/2	Tyrosine kinase with immunoglobulin-like and EGF-like domains ½
TRPC	Transient receptor potential channels
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGF- α	Vascular endothelial growth factor – α
VEGF-1/2	Vascular endothelial growth factor -1/2
V/Q	Ventilation/ Perfusion
WU	Wood units
WSPH	World symposium of pulmonary hypertension
WHO-FC	World health organization functional class
X-ray	Chest radiography

9. List of Publications and poster presentations:

1. Enhanced circulating levels of CD3 cells-derived extracellular vesicles in different forms of pulmonary hypertension
Djuro Kosanovic,[1,2] Ujjwal Deo,[1] Henning Gall,[1] Balachandar Selvakumar,[1] Susanne Herold,[1] Astrid Weiss,[1] Aleksandar Petrovic,[1] Akylbek Sydykov,[1] Hossein Ardeschir Ghofrani,[1] and Ralph Theo Schermuly[1] Pulm Circ. 2019 Jul-Sep; 9(3): 2045894019864357. Published online 2019 Jul 22. Doi: 10.1177/2045894019864357
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2. MBML Annual Retreat, Rauischholzhausen, 2016 and 2017

3. American Thoracic Society meeting, ATS, San Diego 2018

10. Erklärung zur Dissertation

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

_____ _____

Ort, Datum Unterschrift

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Research Letter

Pulmonary Circulation

Enhanced circulating levels of CD3 cells-derived extracellular vesicles in different forms of pulmonary hypertension

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Abstract

It has been shown previously that increased circulating endothelial cells-derived extracellular vesicles represent an important pathological attribute of pulmonary hypertension. Although it is a well-known fact that inflammatory cells may also release extracellular vesicles, and pulmonary hypertension is a disease associated with abnormal inflammation, there is no profound knowledge with regard to the role of inflammatory cells-derived extracellular vesicles. Therefore, our study demonstrated that circulating levels of extracellular vesicles derived from T-cells are enhanced in various pulmonary hypertension forms and that endothelial cells-derived extracellular vesicles may have distinctive profiles in different clinical subgroups of pulmonary hypertension, which still remains as a poorly treatable and life-threatening disorder.

Keywords

extracellular vesicles, pulmonary hypertension, biomarkers, T-cells, endothelial cells

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To the Editor,

Microparticles (MPs) are a type of extracellular vesicles (EVs) and represent shed membrane structures, mostly found in the blood circulation, which originate from different cellular sources during apoptosis or/and activation.[1,2] In the previous years, some studies described the altered circulating profiles of endothelial cells-derived and procoagulant MPs in the context of pulmonary hypertension (PH).[2–5] Amabile et al. have demonstrated that patients with precapillary PH had significantly higher levels of circulating endothelial cells-derived MPs and some MPs correlated with increased mean pulmonary arterial pressure (mPAP).[2] In addition, EVs may also be active pathological players in pulmonary vascular disease development/progression, considering the fact that they represent carriers for various micro-RNAs.[6] Although there are evidences about the potential involvement of endothelial cells-derived MPs in the PH pathology, there is insufficient knowledge with regard to the inflammatory cells-derived MPs.[2] It is well known that massive accumulation of inflammatory/immune cells is a characteristic of PH, and inflammatory cells-derived MPs were found to play a pathogenic role in some lung disorders.[7,8] Finally, the levels of circulating endothelial cells-derived MPs in different clinical forms of PH are still not analyzed in detail.

Therefore, our study aimed to investigate the circulating profiles of different inflammatory (CD3 (T-cells), CD14

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(macrophages/monocytes), CD68 (macrophages)) and endothelial (CD62E (E-selectin) and CD144 (VE-cadherin)) cells-derived EVs in various clinical subgroups of PH.

Human blood samples were prospectively collected during right heart catheterization from non-PH subjects as controls and patients with different forms of PH: idiopathic/heritable PAH (i,hPAH), associated PAH (connective tissue disease, portal hypertension and congenital heart disease), PH due to left heart disease (LHD-PH), PH due to chronic obstructive pulmonary disease (COPD-PH), PH associated with lung fibrosis (fibrosis-PH) and chronic thromboembolic PH (CTEPH). As a limitation of the study, it is important to mention that non-PH control is based on patients that were excluded from any form of PH, but still can suffer from other health disorders which may be associated with altered EVs as well. Different available clinical parameters of the patients (age, gender ratio, New York Heart Association Functional Classification (NYHA) and hemodynamics) are summarized in Table 1. The study was approved by the local ethical committee of the Justus-Liebig University in Giessen. Three milliliters of the platelet-free plasma (PFP) was obtained from the blood taken from each patient and drawn into citrated tubes, by successive centrifugation (500 *g*/15 min, followed by 10,000 *g*/5 min at room temperature) as previously reported.[2] PFP samples were initially stored at −80°C and used later for flow cytometry (BD LSRFortessa^{tm}) quantification of different inflammatory (CD3, CD14 and CD68) and endothelial (CD144 and CD62E) cell-derived EVs, similarly as described in the literature.[2,9,10] Briefly, for each analysis, 50 µl of freshly thawed PFP samples were incubated for 20 min in the dark at room temperature with different fluorochrome-labeled antibodies or corresponding isotype-matched IgG, including: anti-CD3-PE (Phycoerythrin) (BD Pharmingen), anti-CD14-PE (R&D Systems), anti-CD68-PE (R&D Systems), anti-CD144-PE (BD Pharmingen) and anti-CD62E-Allophycocyanin (BD Pharmingen). EVs were identified as events with a 0.5–3 µm diameter on forward light scatter and side-angle light scatter intensity dot-plot representation, by comparison to flow cytometry calibration beads and analyzed for their specific fluorescence. Due to this size determination, we have used the term EVs, which is also considered to be the preferred generic term, as indicated in the literature.[11]

Results were expressed as events per microliter of plasma (events/µl) and presented as mean ± SEM in percentage, considering the average values of each non-PH group for all analyzed EVs as 100%. Due to the technical reasons, not all values for all analyzed targets and for all enrolled patients are available. ROUT test was used for identification of outliers. Further, unpaired T-test with Welch's correction in the case of normally distributed values or Mann–Whitney test when values were not normally distributed were performed to compare non-PH control with respective PH groups. Finally, Spearman test was used for analyses of the correlations.

Our results revealed that there was a prominent increase in the levels of circulating CD3 (T-cell)-EVs in all analyzed clinical forms of PH compared to the non-PH control, with the profiles for i,hPAH and CTEPH being statistically significant (Table 2). In contrast to the T-cells-derived EVs, there was no convincing change in the circulating profiles for macrophages/monocytes, as evident from the comparable levels of CD14-EVs and CD68-EVs in the most of clinical PH forms in comparison to the non-PH control (Table 2). Only the slight tendencies of augmented or even decreased levels of CD68-EVs were noticed in the case of i,hPAH and CTEPH, and associated PAH, respectively, compared to their controls. With regard to the endothelial cells-derived EVs, there was no substantial alteration in the levels of circulating CD144-EVs, except slight tendencies to increase in most of the PH subgroups, as compared to the non-PH control (Table 2). But CD62E-EVs demonstrated more conclusive information about the endothelial cells-derived EVs. There were enhanced levels of circulating CD62E-EVs in associated PAH, COPD-PH and CTEPH

Table 1. Available clinical data of the patients with different forms of pulmonary hypertension (PH).

PH group	Age (years)	Gender ratio (f/m) %	mPAP (mmHg)	PVR (dyn × s × cm^{-5})	NYHA
Non-PH (n = 8)	62 ± 5	50/50	17.0 ± 1.0	162 ± 38	na
i,hPAH (n = 6–11)	47 ± 5	82/18	54.7 ± 4.2	1033 ± 232	I–IV
Associated PAH (n = 4–6)	45 ± 9	50/50	38.5 ± 6.9	450 ± 159	II–IV
LHD-PH (n = 11–14)	69 ± 3	57/43	33.8 ± 3.7	326 ± 71	II–IV
COPD-PH (n = 7)	66 ± 4	29/71	37.0 ± 2.9	470 ± 38	III–IV
Fibrosis-PH (n = 9–13)	67 ± 2	8/92	32.7 ± 3.5	430 ± 57	III–IV
CTEPH (n = 4–12)	67 ± 4	75/25	37.7 ± 6.3	487 ± 125	II–IV

Note: The patients' characteristics/clinical parameters, such as age, gender ratio, mean pulmonary arterial pressure (mPAP), pulmonary vascular resistance (PVR) and New York Heart Association Functional Classification (NYHA) classes are given. Available values with the numbers of patients for each PH group are presented as mean ± SEM. f: female; m: male; non-PH: control (excluded PH); i,hPAH: idiopathic/heritable pulmonary arterial hypertension; LHD-PH: PH due to left heart disease; COPD-PH: PH due to chronic obstructive pulmonary disease; CTEPH: chronic thromboembolic pulmonary hypertension; na: not available.

Table 2. Profiles of circulating inflammatory and endothelial cells-derived extracellular vesicles (EVs) in different forms of pulmonary hypertension (PH).

	Inflammatory cells-derived (events/μl (%))			Endothelial cells-derived (events/μl (%))	
PH group	CD3-EVs	CD14-EVs	CD68-EVs	CD144-EVs	CD62E-EVs
Non-PH (n = 6–8)	100 ± 16	100 ± 39	100 ± 22	100 ± 28	100 ± 46
i,hPAH (n = 10–12)	231 ± 41[a]	58 ± 18	134 ± 16	120 ± 24	169 ± 49
Associated PAH (n = 5–6)	172 ± 75	134 ± 62	55 ± 5	142 ± 50	404 ± 217
LHD-PH (n = 12–14)	237 ± 60	100 ± 28	103 ± 10	134 ± 34	129 ± 52
COPD-PH (n = 6–7)	319 ± 151	72 ± 26	123 ± 56	174 ± 74	2558 ± 1423
Fibrosis-PH (n = 12–13)	209 ± 58	110 ± 30	110 ± 20	79 ± 24	106 ± 29
CTEPH (n = 11–12)	242 ± 33[a]	107 ± 34	174 ± 42	149 ± 54	398 ± 110[a]

Note: The flow cytometry characterization and quantification of different inflammatory (CD3, CD14 and CD68) and endothelial (CD144 and CD62E) cells-derived EVs are presented (events/μl in %). Available results with the numbers of patients/values for each PH group are given as mean ± SEM (n = 5–14). Non-PH: control (excluded PH); i,hPAH: idiopathic/heritable pulmonary arterial hypertension; LHD-PH: PH due to left heart disease; COPD-PH: PH due to chronic obstructive pulmonary disease; CTEPH: chronic thromboembolic pulmonary hypertension.
[a]$p < 0.05$ compared to the respective non-PH control.

in comparison to the non-PH control group, with statistically significant difference in the case of CTEPH (Table 2). Interestingly, there were no prominent changes in other PH forms, such as i,hPAH, LHD-PH and fibrosis-PH. Furthermore, there were positive correlations between the circulatory levels of CD3-EVs and mPAP and pulmonary vascular resistance (PVR) values, respectively. However, the correlations did not reach the statistical significance (data not shown). Finally, there was no correlation between the levels of CD62E-EVs and mPAP, but there was a significant positive correlation between CD62E-EVs circulatory levels and PVR (data not shown).

We have demonstrated for the first time the augmented circulatory levels of EVs derived from T-cells. This finding may fit in the current paradigm of PH as an "inflammatory disorder", since various inflammatory cells, including T-cells, are accumulated in the remodeled pulmonary vasculature and lungs of idiopathic PAH patients.[8] Interestingly, EVs derived from monocytes/macrophages, inflammatory cells which are also described in the context of this disease,[8] did not show an alteration in the profile. In the past, several studies indicated the involvement of endothelial cells-derived MPs in the pathology of PH.[2–5] Finally, the literature suggested the augmentation of circulatory levels of CD62E-MPs in precapillary PH.[2,5] We have analyzed the profile of CD62E-EVs in different forms of PH, and found a prominent increase in associated PAH, COPD-PH and CTEPH.

In conclusion, we have found the increased levels of the circulating EVs which originate from T-cells in different clinical forms of PH. Therefore, future studies should focus to identify whether there are promising biomarker properties as well as to reveal a potentially active role of this inflammatory cells-derived EVs in the pathogenesis and progression of PH. Finally, endothelial cells-derived EVs may have distinctive circulating profiles in different clinical PH subgroups.

Some data from this study have been previously reported in the form of abstract during the ATS conference in 2018.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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