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Review Article

Beyond Thrombosis: the Role of Platelets in Pulmonary Hypertension

Salina Nicoleau [a], Beata Wojciak-Stothard [a*]

[a] *National Heart and Lung Institute, Imperial College London, Hammersmith Campus, Du Cane Road W12 0NN, London, United Kingdom.*

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Abstract

Pulmonary Hypertension (PH) is a multifactorial and lethal disease, characterised by elevated pulmonary arterial pressure and progressive right heart failure. PH pathobiology rests on four pillars: vascular remodelling, vasoconstriction, inflammation and thrombosis. While vascular and inflammatory cells have been the focus of PH research over the past decades, platelets have received relatively less attention, despite their associations with key pathophysiological processes of the disease. Platelets contain a wide range of vasoactive, inflammatory and pro-thrombotic mediators, likely to promote PH development and progression. There is currently no cure for PH, and platelet-associated pathways may help identify new therapeutic strategies. This review summarises available evidence on the role of platelets in different forms of PH, and comments on the current state of platelet-targeting therapies. It also describes the latest advances in the *in vitro* technologies that enable exploration of platelet function under dynamic and physiologically relevant conditions.

Keywords: Pulmonary Hypertension; Platelets; Thrombosis; Vascular.

1. Introduction

Pulmonary Hypertension (PH) is a severe lung disease, characterised by elevated pulmonary arterial pressure (PAP), ultimately leading to right heart failure and death. The rise in lung blood pressure results from increased pulmonary vascular resistance (PVR) due to vasoconstriction, vascular remodelling [1] and thromboembolism [2]. PH is a recognised comorbidity of a variety of conditions, including left-sided heart and lung diseases [3].

The current classification, based on disease aetiology, divides PH into five groups: 1) Pulmonary Arterial Hypertension (PAH), 2) PH due to Left Heart Disease (LHD), 3) PH due to lung diseases and/or hypoxia, 4) Chronic Thromboembolic PH (CTEPH), and 5) PH with unclear multifactorial mechanisms (Figure 1).

While usually considered a rare disease, PH is becoming a common health issue across the world. PAH has an incidence of 2-10 cases per million adults per year in developed countries [4], with idiopathic PAH (IPAH) being the predominant subtype (50-60% of PAH cases) as per registries from Europe and the USA [4-6]. PH related to LHD or chronic hypoxia is more widespread than PAH [7], with 50.2% of patients with chronic obstructive pulmonary disease (COPD) experiencing mild PH [8]. Additionally, many studies have reported that at least 50% of patients with heart failure are diagnosed with PH [9, 10], with increasing reports of cases amongst African cohorts [11]. CTEPH prevalence and incidence remain hard to determine, due to the high number of patients undergoing pulmonary thromboendarterectomy (PTE), which is largely curative, and to the similarities between acute pulmonary embolism (PE) and pre-existing CTEPH symptoms [12]. Nevertheless, the crude annual CTEPH incidence has been estimated to be around 5 and 104 cases per 100 000 population per year in Europe and the USA, respectively [13], with approximately 3% of acute PE survivors developing the disease [14].

* Corresponding author: b.wojciak-stothard@imperial.ac.uk

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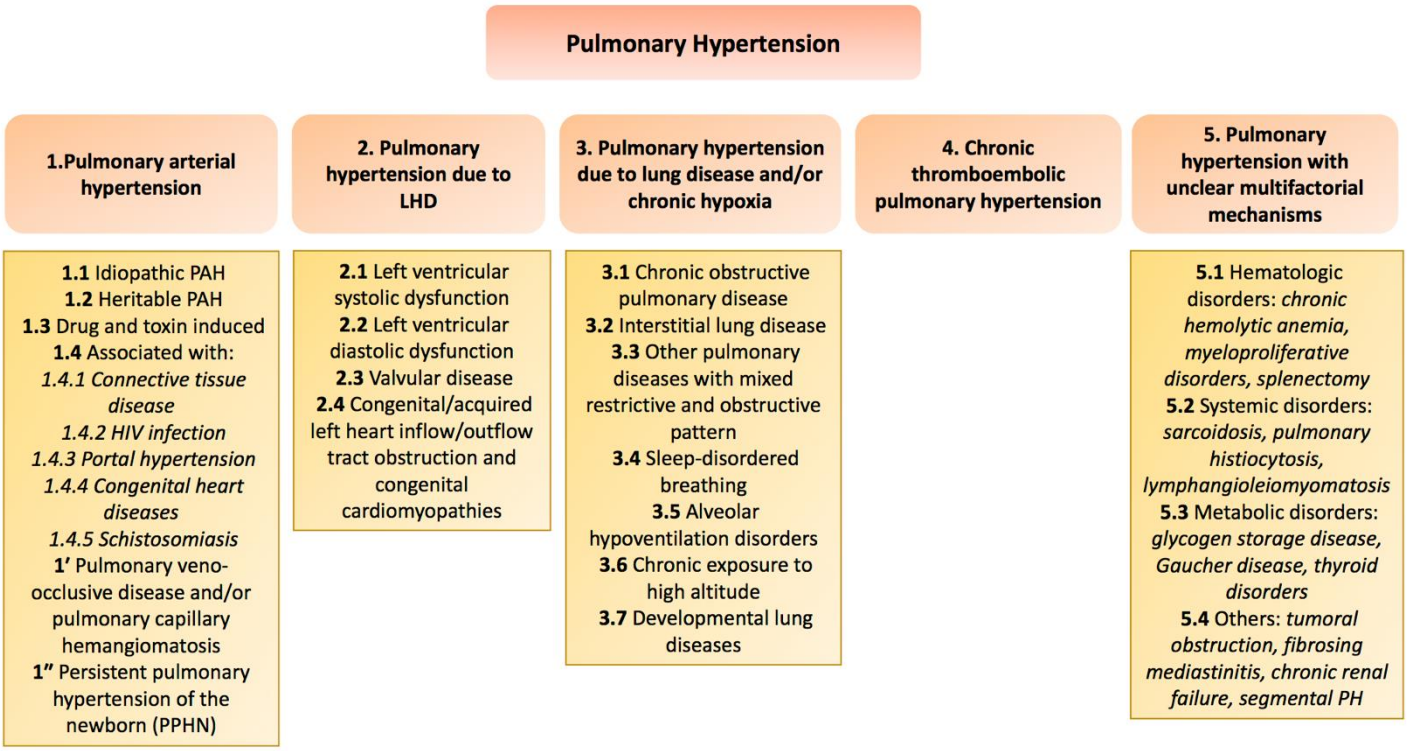

Figure 1. Latest classification of pulmonary hypertension (HIV: human immunodeficiency virus; LHD: Left-heart disease [15]

Considering the multifactorial nature of PH, it is often challenging to identify the disease mechanisms and to design appropriate therapies [7]. However, regardless of the underlying conditions, the vast majority of PH patients display abnormalities in their pulmonary vascular bed, manifested by remodelling of all or some of the vascular layers (adventitia, media and intima) and a decreased luminal cross-sectional area [16, 17].

PH research has significantly evolved over the last century, since the first autopsy report of IPAH by Ernst von Romberg in 1891 [18]. The role of endothelial (ECs) and smooth muscle cells (SMCs) has been the main focus of research in the past decades. Medial hypertrophy is a common feature to all functional classes of PH, with major phenotypic [19], genetic [20-22] and epigenetic [23] alterations noted in pulmonary vascular smooth muscle cells (PVSMCs). ECs are also widely recognised as essential contributors to vascular remodelling and vasoconstriction, through dysregulated production of vasoactive mediators [24-26] and a switch towards a hyper-proliferative, anti-apoptotic phenotype [27, 28]. Endothelial damage accompanied by loss of barrier function and followed by increased pro-thrombotic, pro-inflammatory, pro-proliferative and pro-angiogenic activation represent key events in initiation and progression of PH [29]. Inflammation plays an important role in disease development, although PH is not considered as an inflammatory disease *per se*. Indeed, PAH patients display elevated serum levels of cytokines and chemokines [30], and show increased perivascular immune cells infiltration in plexiform lesions in the lung [31].

While vascular and immune cells have been studied in great detail, platelets have received relatively less attention, despite their involvement in the main pathophysiological processes leading to PH. Platelets, through their ability to aggregate and to produce, store and release a wide range of growth factors, vasoactive mediators and cytokines [32], are likely to augment vascular remodelling and vasoconstriction, in addition to the formation of pulmonary thrombotic lesions observed in severe forms of PH [33].

Although there are clear associations between platelet dysfunction, thrombogenesis and vascular remodelling in PH, the nature of their interdependent relationships is not fully understood. This review presents the current evidence for the role of platelets in PH pathobiology, first by looking at the upstream events leading to platelet activation, and then focusing on the interactions of platelets with the pulmonary vasculature. Current platelet-targeting therapies and pre-clinical models of platelet function are discussed. The latest advances in the *in vitro* technologies enabling observations of platelet behaviour under dynamic, physiologically relevant conditions, are also reviewed.

PH is a serious and currently incurable health condition [34] and more effective therapeutic strategies need to be developed. Targeting platelets and thrombotic processes could help manage the disease groups most affected by platelet dysfunction, and improve patients' quality of life.

2. Pulmonary Hypertension

2.1. Definitions and Symptoms

PH is a haemodynamic and pathological condition defined by an increase in mean pulmonary arterial pressure (mPAP) to or above 25mmHg at rest, as measured by right heart catheterization [35].

Different haemodynamic definitions of PH are also available, based on combinations of PAP, pulmonary artery wedge pressure, cardiac output, diastolic pressure gradient and PVR, and are recommended to be used when distinction between isolated post-capillary hypertension and combined post- and pre-capillary hypertension is required[(4)]. Pre-capillary PH usually encompasses PAH, CTEPH, PH due to lung disease, and idiopathic PH, while post-capillary PH includes PH due to LHD [36].

Common symptoms of PH are non-specific, usually related to progressive right ventricular dysfunction, and initially manifest upon exertion [7]. They include dyspnea, fatigue, chest pain, syncope and palpitations, which can also be observed at rest in severe cases [8]. Progressive lower extremity oedema, liver failure, and ascites may also develop due to chronically elevated right atrial (RA) pressure [8].

Because of the complex aetiology of PH, and the variety of associated conditions, diagnosis requires careful planning and a multi-disciplinary approach. Right heart catheterization is considered the gold standard for PH diagnosis [36], but its invasiveness makes it unsuitable for routine use. Alternatives include electrocardiogram, chest radiography, echocardiography, pulmonary function test, computed tomography, and cardiac magnetic resonance imaging [36].

It is worth noting that elevated mPAP may not always have pathological cardiopulmonary causes, and can be observed in pregnancy, anaemia or sepsis. In such cases, the pulmonary bed remains unaffected, and PH resolves upon normalisation of the cardiac output [7].

PH prognosis remains poor, especially for PAH, despite the available therapies. A mortality rate of more than 10% has been estimated in high-risk PAH patients one year after diagnosis [36], although recent evidence derived from the SERAPHIN and GRIPHON studies suggests that patients experiencing a morbidity event (eg worsening of symptoms) within the first 3 months of their enrolment had a mortality rate of 30-40% [37].

2.2. Pathobiology of Pulmonary Hypertension

The complexity of PH can be attributed to a wide range of cellular and molecular alterations underlying its pathogenesis. Early hypotheses for the origin of vascular lesions included congenital thinning of the pulmonary artery (PA) media, and abnormal endothelial phenotypic modifications [38]. In 1958, Heath and Edwards published an extensive histological study of PH lungs, which provided the first detailed description of vascular remodelling [16]. It was in this study that increased PAP and PVR were linked to the formation of plexiform and dilation lesions, observed in advanced forms of the disease [16]. Another histological report by Yi and colleagues [17] described the obstructive intimal lesions in PH as ‘intermediate forms between plexiform and thrombotic lesions’, containing cells displaying a predominant myofibroblastic phenotype and different stages of differentiation.

Endothelial dysfunction is a hallmark of PH, characterised by reduced production of vasorelaxants, such as prostacyclin (PGI_2) [24] and nitric oxide (NO) [25], and increased production of vasoconstrictors, such as endothelin-1 (ET-1) [23]. Additionally, increased release of transforming growth factors (TGFs) [39] and vascular endothelial growth factor (VEGF) [40] promote medial hypertrophy and intimal hyperplasia (Figure 2).

Phenotypic switches are commonly observed in both pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs). PH PAECs typically demonstrate a hyper-proliferative, pro-angiogenic and anti-apoptotic phenotype [27, 28], with evidence of endothelial-to-mesenchymal transition (EMT) [41] and compromised barrier function [42]. PH PASMCs show transition from contractile to synthetic phenotype, which promotes their hyperplastic growth [19]. SMCs arising from EMT [43], circulating fibrocytes or mesenchymal progenitor cells are thought to play a role in hypoxia-induced vascular remodelling [44]. Endothelial progenitor cells (EPCs) may also contribute to disease development [45], though some evidence suggests a protective role [46, 47].

In addition to vascular stresses induced by hypoxia, inflammation, drugs and toxins, genetic changes have also been shown to contribute to PH pathobiology, notably mutations in the TGF-β receptors family. Indeed, bone morphogenic protein receptor II (BMPRII) mutations are found in approximately 53-86% of hereditary PAH (HPAH), and 14-35% of IPAH patients [48], while other forms of PAH are characterised by reduced BMPRII function due to decreased expression [49] or increased degradation of the receptor expression [50]. Less commonly, mutations in the activin-like kinase-type 1 (*ALK1*), endoglin (*ENG*) and *SMAD* genes have been implicated in increased susceptibility to PAH [48]. Interestingly, only 20% of the *BMPR2* mutations carriers develop the disease, suggesting that a “second hit” created by hypoxia, inflammation, drugs or toxins is required for disease progression [51, 52] (Figure 2).

No specific genetic mutations have been linked to the other forms of PH [53, 54], but there is evidence of a decreased expression of BMPR-1A, a transmembrane protein required for BMPRII signalling, in the lungs of CTEPH and other PH patients [55].

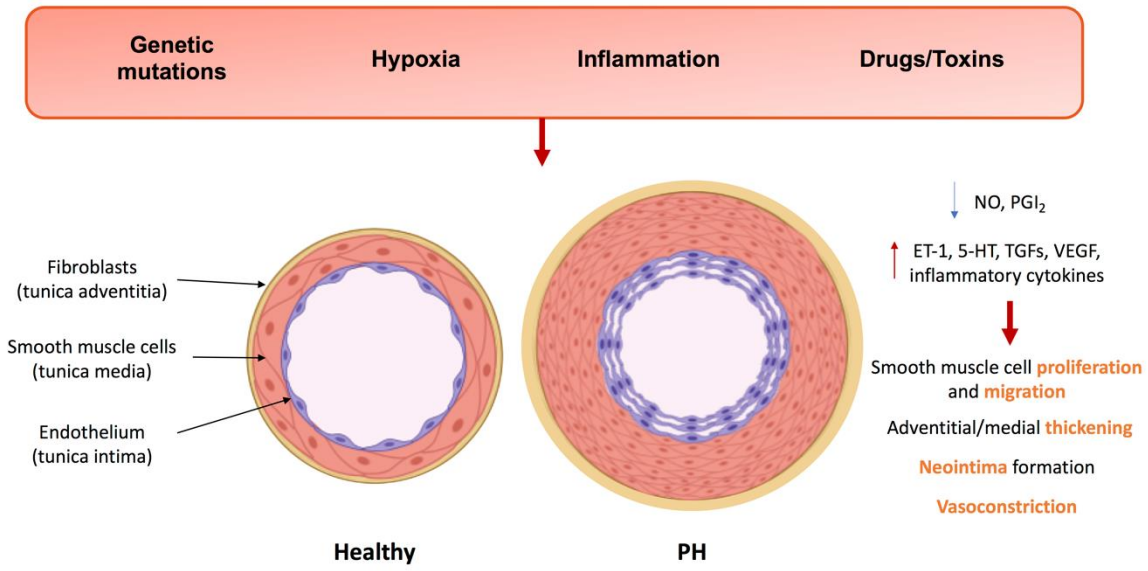

Figure 2. Abnormal vascular remodelling in pulmonary arterial hypertension

Genetic mutations, hypoxia, inflammation, drugs/toxins can cause endothelial dysfunction, leading to imbalances in the production and release of vasoactive mediators, mainly nitric oxide (NO), prostacyclin (PGI_2), serotonin (5-HT) and endothelin-1 (ET-1), growth factors (transforming growth factors (TGFs), vascular endothelial growth factor (VEGF)), and inflammatory cytokines. These all contribute to vasoconstriction, adventitial and medial thickening, as well as neointima formation, by promoting endothelial cells hypertrophy and hyperplasia, as well as smooth muscle cells and fibroblasts proliferation and migration. Such vascular changes ultimately cause an increase in pulmonary vascular resistance and pulmonary arterial pressure (Figure adapted from Schermuly et al, 2013 and Thompson and Lawrie, 2017) [1, 56].

2.3. Pulmonary Hypertension and Thrombosis

The potential role of thrombosis in the pathobiology of PH emerged in the 1970s, when Inglesby and colleagues first reported reduced plasma fibrinolysis in familial PH [57]. Many other studies have since found thrombotic lesions in the pulmonary vasculature of PAH patients [33, 58]. *In situ* thrombosis is now widely recognised as a common pathological feature of PAH, and large central thrombi can also develop in severe IPAH [59].

CTEPH results from an incomplete resolution of pulmonary thromboemboli, which leads to the formation of organised, fibrotic occlusions within the vasculature, limiting blood flow and increasing PAP [12]. Histological examination of PTE specimens consistently showed intimal thickening with collagen deposition, inflammatory cells infiltration, atherosclerosis and calcification [60]. In contrast with PAH, which affects <300 μm diameter vessels, CTEPH mostly involves the major pulmonary vessels [61] and can cause very severe PH if left untreated [62].

However, viewing CTEPH solely as a thrombotic obliteration of the central PA is too simplistic. Indeed, most of patients with acute pulmonary embolism do not develop CTEPH [14], which can persist even after PTE [63], suggesting that additional factors are required to induce the disease.

Many controversies remain around the pathobiology of CTEPH. Although less than 50% of PTE patients were found to have coagulation or haematologic abnormalities in a retrospective study [60], the European CTEPH Registry reported that previous PE and deep venous thrombosis were present in 74.8% and 56.1% of CTEPH patients, respectively [64]. Furthermore, risk factors for recurrent venous thromboembolism are observed in CTEPH, including lupus anticoagulant and antiphospholipid antibodies [65], elevated plasma factor VIII and von Willebrand Factor (VWF):Antigen levels [66].

Ineffective fibrinolysis may also play a key role in CTEPH, as suggested by the presence of lysis-resistant fibrin variants in CTEPH patients [67, 68]. Additionally, CTEPH is often associated with chronic infection or inflammation [69], and elevated levels of inflammatory cytokines (eg Interleukin-6 (IL-6), Monocyte Chemoattractant Protein-1 (MCP-1)) have been reported in patients [70]. Splenectomy has also been associated with higher CTEPH incidence

and worse prognosis [69]. These observations suggest that inflammation, abnormal platelet function and fibrinolysis create a pro-thrombotic environment, promoting vascular occlusion.

The hypothesis that increased PAP and PVR in CTEPH are caused not only by arterial obstruction, but also by vascular remodelling and vasoconstriction, is supported by the absence of correlation between the increased PAP and the degree of vascular bed obstruction [71]. Indeed, CTEPH patients have much higher PVR than acute PE patients with similar vascular bed obstruction levels [63]. This suggests that PE may be an initiating event, but is not sufficient to cause CTEPH, which actually results from the secondary pulmonary vasculopathy that follows thromboembolism.

Histological studies of CTEPH lungs revealed vascular changes similar to those seen in other forms of PH, including the formation of plexiform lesions [17, 71]. Interestingly, vascular remodelling was also observed in distal PAs which were not directly affected by thrombi [17], but were exposed to high PAP and shear stress due to chronic obstruction of the main arteries. A "dual vascular bed compartment" theory for CTEPH was first suggested by Moser and colleagues [72], whereby patients display an obstructed compartment subjected to chronic ischemia, and an unobstructed one subjected to increased blood flow. Both regions display vasculopathy, but induced by different causes.

CTEPH therefore appears as a multifaceted disease, in which major vessel thrombosis and remodelling is accompanied by pulmonary arteriopathy, characterised by endothelial dysfunction and excessive ECs and SMCs proliferation [72]. EMT [73] and progenitor cells [74] have also been suggested to participate in CTEPH progression.

Whilst the predominant view is that PE, followed by progressive vascular remodelling leads to CTEPH, it has also been argued that primary pulmonary arteriopathy can induce secondary *in situ* thrombosis and vascular occlusion [75]. Similarities between CTEPH and other forms of PH, such as pulmonary thrombosis and plexiform lesion formation [33, 58], are indicators of a complex aetiology of the disease.

3. Platelet Dysfunction in Pulmonary Hypertension

The role of platelets and thrombosis in PH remains controversial. Platelet activation and aggregation can either be regarded as an active regulator of vascular remodelling, or it can be seen as a passive bystander, secondary to endothelial dysfunction and pulmonary arteriopathy. This section discusses the physiological role of platelets and describes the mechanisms leading to platelet aggregation and dysfunction in PH.

3.1. The Physiological Role of Platelets in the Human Body

Discovered by Giuilio Bizzozero in 1882 [76], platelets are small, anucleate cell fragments (2-3 μm diameter) derived from megakaryocytes [77] that circulate in the bloodstream and have an essential role in the regulation of haemostasis and vascular integrity. Platelet dysfunction has been implicated in a wide range of diseases, including cardiovascular diseases (CVDs) and cancer [78].

Thousands of platelets can be produced by a single megakaryocyte, and around 10^{11} platelets are made every day in a healthy adult [79], with old platelets being destroyed in the spleen and liver by Kupffer cells [80]. Platelets express a variety of surface receptors, regulating their interactions with the endothelium and other platelets, and the release of their granule contents [78].

Under normal physiological conditions, interaction of platelets with vascular cells and soluble coagulation proteases (called coagulation factors) maintains the haemostatic balance, preventing thrombosis and haemorrhage [81]. Coagulation, initially described as a cascade of events initiated by two distinct pathways, known as the "extrinsic" and "intrinsic" pathways [81], is now viewed as a three-phase model of overlapping initiation, amplification and propagation of the response [82]. Coagulation ultimately leads to the formation of a fibrin mesh, which stabilises the pre-formed platelet plug [82]. Detailed mechanisms of this process have been reviewed elsewhere [83].

The tissue factor (TF)/factor VIIa complex is essential in initiating haemostasis [82]. TF is highly expressed in the vascular adventitia, but less so in ECs and SMCs in healthy blood vessels [84]. However, changes in blood flow [85], hypoxia [86], growth factors (including platelet-derived growth factors (PDGF)) [87] and inflammation [88] can affect endothelial TF expression. An overview of the coagulation cascade, initiated by endothelial TF, is provided in Figure 3. TF can also be found in circulation, in association with platelet-derived microparticles (PMPs) [89].

Upon tissue injury and sub-endothelial collagen exposure, platelets are captured by VWF, which binds the platelet GPIb/V/IX receptor complex [90]. This leads to platelet activation and shape changes, followed by GPIIb/IIIa surface expression, which mediates inter-platelet interactions via VWF or fibrinogen binding [91]. VWF usually substitutes for fibrinogen under high shear stress (HSS) conditions [92]. Activated platelets then recruit additional ones through the release of agonists, such as adenosine 5′-diphosphate (ADP), serotonin or thromboxane A-2 (TXA-2) [32], consolidating the formation of the platelet plug. Platelets may adhere to the endothelium in the absence of evident

endothelial injury, a process which will be described later in this review.

Platelets also act as an important link between primary and secondary haemostasis, which involves stabilisation of the pre-formed platelet plug by insoluble fibrin, the end-product of the coagulation cascade [81]. Platelets contribute to this process by exposing a negatively charged phospholipid surface, required for the catalytic activity of coagulation factor complexes [93].

Many pro-inflammatory, pro-thrombotic, and pro-angiogenic mediators are stored in platelets' α- and dense (δ) storage granules, and are released upon platelet activation [32]. P-selectin, TGFβ-1, VWF, PDGF, tumour necrosis factor-α (TNF-α), interleukins, coagulation Factor V and fibrinogen are found in platelets' α-granules [94]. Dense granules contain many vasoactive molecules, including serotonin (5-HT), ADP, adenosine 5′-triphosphate (ATP), calcium and catecholamines [95]. All these molecules are known to induce endothelial inflammation, SMC migration and proliferation, platelet aggregation and leukocyte migration [1], suggesting a potential role of platelets in PH pathogenesis.

The role of platelets therefore extends beyond thrombosis and haemostasis, with strong evidence of their involvement in vascular inflammation, atherosclerosis [96], arthritis [97] and PH associated with inflammatory and connective tissue diseases, including systemic sclerosis [98] and systemic lupus erythematosus [99].

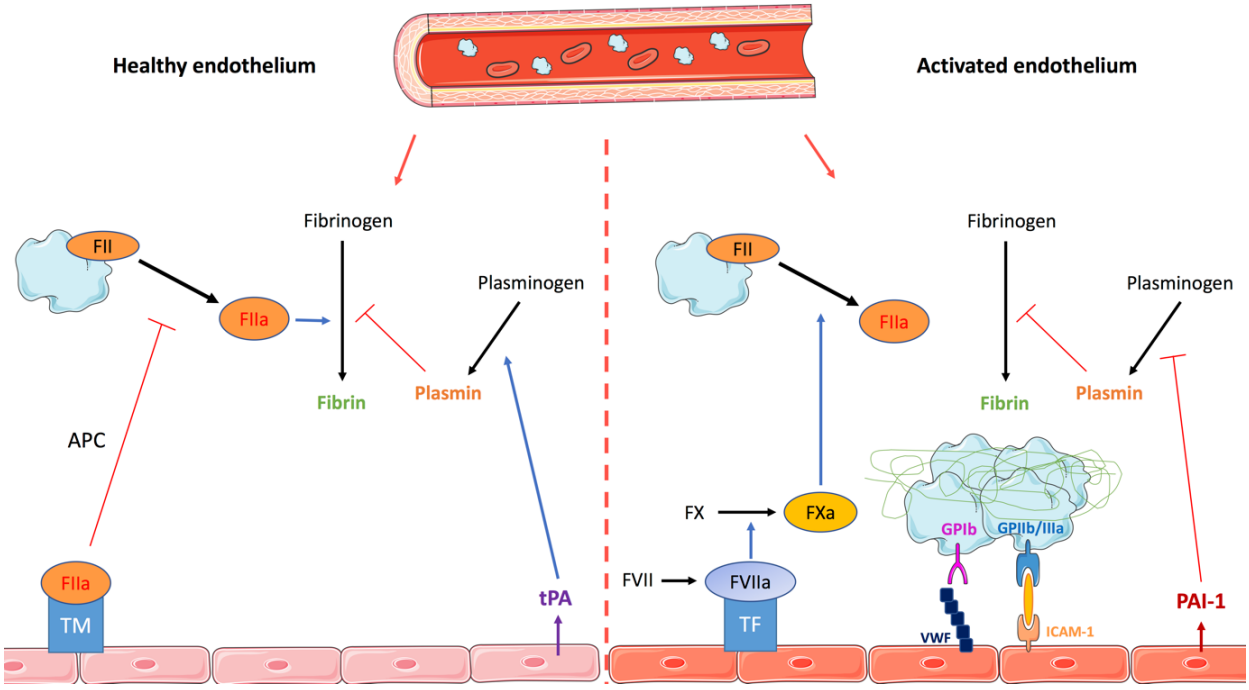

Figure 3. Overview of the coagulation cascade initiated by endothelial tissue factor

When activated, the endothelium expresses increases expression of tissue factor (TF), which binds and activates factor VII (FVII), leading to factor X (FX) activation. FXa then cleaves platelet- and endothelium-bound pro-thrombin (FII) into thrombin (FIIa), which in turn cleaves fibrinogen into fibrin. Platelets adhere to the activated endothelium by binding to von Willebrand factor (VWF) through the GPIb receptor, and to intercellular adhesion molecule-1 (ICAM-1) via GPIIb/IIIa, an interaction mediated by extracellular matrix proteins such as fibrinogen. Fibrin forms a mesh and stabilises the pre-formed platelet plug. The coagulation cascade is tightly regulated by endothelium-derived factors. Secreted tissue plasminogen activator (tPA) promotes the activation of plasminogen into plasmin, an inhibitor of fibrin formation. This process is inhibited by plasminogen activator inhibitor-1 (PAI-1), which is increased in pulmonary arterial hypertension. Additionally, thrombomodulin (TM) binds to thrombin, converting it into an anti-coagulant catalysing the activation of protein C (APC), which in turn inhibits thrombin production.

3.2. Platelet-vessel Wall Interactions

The endothelium maintains vascular integrity and regulates haemostasis, preventing excessive coagulation and thrombus formation. An intact, healthy endothelium displays an anti-thrombotic surface, mainly through the expression of thrombomodulin (TM), which binds thrombin and prevents fibrinogen cleavage [100] (Figure 3). ECs can also produce tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), therefore potentiating or inhibiting fibrinolysis [101]. Additionally, the endothelium is a major source of NO [102] and PGI_2 [103], which are key inhibitors of platelet aggregation and activation[104], in addition to their vasodilatory and anti-proliferative effects [102, 103].

However, inflammation, chronic hypoxia or shear stress can lead to endothelial activation and a switch towards a pro-thrombotic phenotype [86, 105, 106], resulting in imbalanced production of haemostatic and vasoactive mediators. PH patients typically display a hypercoagulable state, characterised by increased thrombin activity [107], increased plasma VWF [108] and PAI-1 [107] levels and reduced TM levels [109]. Aberrant TF expression has also been observed in vessels and plexiform lesions of PAH lungs[110], with higher levels of VWF associated with worse disease outcomes [108, 111]. Furthermore, impaired endothelial function leads to reduced NO [25] and PGI_2 [24] production, which not only promotes vasoconstriction [112], but also platelet activation and aggregation [113].

Platelets can directly bind to the vascular endothelium, in addition to their interactions with the sub-endothelial matrix and other platelets. This direct interaction is particularly important during inflammation. Under physiological conditions, platelets roll on the vascular wall, and their binding is enhanced upon endothelial activation, leading to firm adhesion [114]. Platelet-endothelial interactions are mediated by integrins and selectins, and comprise tethering, rolling and adhesion, bearing similarities to the leukocyte adhesion process [115] (Figure 4). P-selectin is essential for platelet rolling, particularly on the inflamed endothelium [114]. While P-selectin is expressed both by platelets [94] and ECs [116], only endothelial P-selectin is involved in platelet-endothelial interactions under stimulated conditions [114]. Like P-selectin, endothelial VWF is also stored in Weibel-Palade bodies and is released upon stimulation [117], binding to the GPIbα subunit of the GPIb/V/IX complex [118]. VWF is key for promoting platelet adhesion under high shear stress, enabling platelet-VWF interactions to sustain shear rates above $500s^{-1}$ [105, 119]. Particularly, ultra-large VWF multimers, released by activated ECs [120], have the ability to form very strong bonds with platelet GPIbα [121]. However, due to its fast dissociation rate [122], GPIbα-VWF binding is considered to be involved in initial and reversible platelet adhesion, rather than irreversible binding to the endothelium [123]. Interestingly, long-lasting GPIbα-VWF interactions appear to occur under abnormally high shear rates, above $10{,}000s^{-1}$ [124].

P-selectin glycoprotein ligand 1 (PSGL-1) [125] and GPIbα [126] are main P-selectin ligands on platelets. ECs can also express PSGL-1, which binds platelets' P-selectin after endothelial TNF-α stimulation [127]. The exact roles of endothelial and platelet PSGL-1 and P-selectin under physiological and pathological conditions remain to be explored.

In contrast with short-lived, selectin-mediated interactions, integrins are involved in stable platelet adhesion (Figure 4). Stable adhesions are initiated by GPIIb/IIIa on platelets, and αvβ3, Intercellular Adhesion Molecule-1 (ICAM-1) and VWF on ECs [128, 129]. Fibrinogen and vitronectin can bind to αvβ3 and ICAM-1, therefore acting as a bridge between platelets and ECs [130]. ICAM-1[131] and αvβ3 [132] expression and VWF production levels [120] are low under resting conditions, but can increase significantly upon endothelial activation, promoting platelet adhesion. Elevated ICAM-1 levels have been found in PAH [133] and CTEPH [134] patients, suggesting an increased risk for platelet-mediated pathological effects.

Of relevance to PH, an impairment of endothelial barrier function due to BMPRII mutations [135], hypoxia or other PH factors, may lead to sub-endothelial collagen and laminin exposure, promoting platelet adhesion and aggregation.

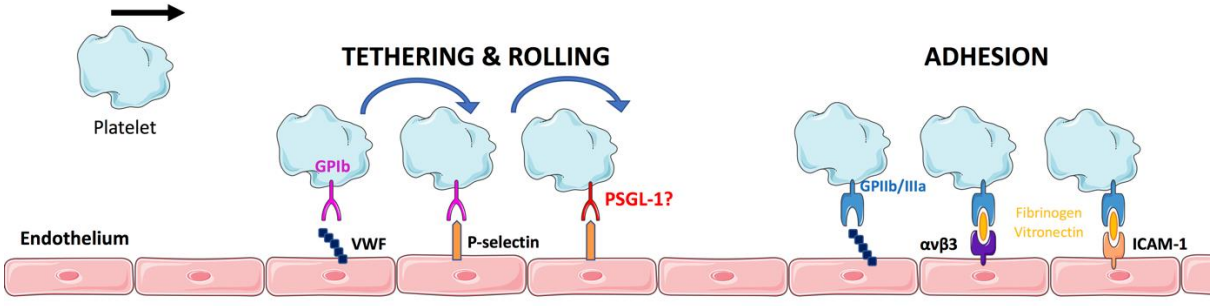

Figure 4. Platelet rolling and adhesion to the vascular endothelium

Platelets can directly bind to the vascular endothelium through tethering, rolling and adhesion, enhanced upon endothelial activation. Under high shear stress conditions, immobilised von Willebrand Factor (VWF) captures platelets and initiates their reversible binding via Glycoprotein Ib (GPIb). P-selectin also binds to GPIb, and potentially to P-selectin glycoprotein ligand 1 (PSGL-1), promoting platelet rolling. Stable adhesion of platelets is mediated by platelet and endothelial integrins, notably αvβ3 and Intercellular Adhesion Molecule-1 (ICAM-1) on endothelial cells, and Glycoprotein IIb/IIIa (GPIIb/IIIa) on platelets. Extracellular matrix proteins, such as fibrinogen or vitronectin, act as a bridge between platelets and the endothelium by binding their respective integrins. VWF is also required for stable platelet adhesion, especially under high shear stress.

3.3. Platelet Abnormalities in Pulmonary Hypertension

Platelet dysfunctions are commonly found in PH, and can range from metabolic alterations to phenotypic changes and defective aggregation.

Platelet metabolic abnormalities, such as increased mitochondrial reserve capacity, have been correlated with mPAP, PVR and RV stroke work index in PAH patients [136]. Thrombocytopenia has also been observed in patients with severe forms of PAH [137], likely due to microangiopathic haemolysis, a process in which flow through fibrin clots and plexiform lesions results in platelets shearing [138]. In contrast, increased levels of the thrombopoiesis-stimulating hormone thrombopoietin have been reported in pulmonary vessels of PH patients [139]. Additionally, increased mean platelet volume (MPV), indicative of platelet activation [140], was found in IPAH and in conjunction with decreased platelet count in CTPEH patients, suggesting increased platelet turnover [141]. Platelet production and destruction therefore seem to be altered in PH.

Platelet activation is higher in PH patients compared with healthy individuals. In CTEPH patients, platelets show hyperresponsiveness to thrombin stimulation, as well as increased activation of the small GTPase RalA, which is involved in degranulation [142]. Interestingly, platelet RhoA but not RalA was found to be increased in PAH patients [142], likely to reflect different pathophysiology of both diseases. RhoA plays a role in cytoskeletal reorganisation [143] and platelet aggregation [144].

Greater platelet agonist-induced aggregation was also found in CTEPH [141] and PAH patients [145]. Tyrosine phosphorylation, used as a marker of *in vivo* platelet activation, is increased by 79% in PAH patients, compared with controls [146]. Genome-wide RNA expression analyses of IPAH lung tissues showed that gene expression of proteins involved in coagulation, platelet activation and degranulation, including platelet factor 4 (*PF4*), P2Y purinoreceptor 1 (*P2RY1*), and coagulation factor II (thrombin) receptor-like 3 (*F2RL3*) was elevated, suggesting abnormal endogenous activation in platelets [147]. Additionally, platelets from PAH patients release soluble CD40L (sCD40L), a key pro-inflammatory molecule, at a higher level in response to thrombin receptor-activating peptide (TRAP), compared with controls [148].

Platelets can produce NO via eNOS and iNOS expression, though their role in this process is debatable [149, 150]. Reduced expression of platelet eNOS is reduced in PAH patients [151] can potentially lower their activation threshold. NO regulates platelets' intracellular Ca^{2+} (Ca^{2+}_i), which controls platelet activation and aggregation [152]. Indeed, abnormalities in Ca^{2+}_I homeostasis have been associated with reduced NO levels both in experimental models of arterial hypertension [153] and hypertensive human patients [154], and elevated [Ca^{2+}_I] was found to enhance platelets' sensitivity to agonist stimulation [153]. These observations may, at least in part, may explain the correlation between platelet hyper-aggregability and reduced NO levels observed in PH.

3.4. Shear Stress and Thrombotic Responses

Platelet aggregation is largely influenced by shear stress and changes in blood flow. In particular, shear acceleration and deceleration, also known as shear micro-gradients, create a pro-thrombotic environment [105, 155]. As previously mentioned, ECs orchestrate vascular responses to haemodynamic stimuli, through their ability to sense wall shear stress (WSS) via specific cell-surface mechanosensors [156]. While laminar, pulsatile flow with physiological WSS (mean 10-15 dyne/cm^2, peak<100 dyne/cm^2) promotes endothelial quiescence, disturbed flow and low WSS (<4 dyne/cm^2) have been shown to induce endothelial dysfunction, including high EC turnover, random actin alignment [157], increased permeability [157], increased expression of pro-inflammatory markers, such as MCP-1 [158] and ICAM-1 [157, 158], and increased secretion of VWF [105].

Shear stress also regulates VWF function, by causing important conformational changes in its A1, A2 and A3 domains and regulating its interactions with platelets [159]. Especially, high shear stress exposes the A1 domain, allowing it to form a stable complex with platelet GPIbα [160].

In vivo and *in vitro* studies, using stenosed microfluidic chambers, have previously shown that high WSS at the stenosis apex promotes reversible GPIb-mediated platelet recruitment, while low WSS downstream the stenotic region led to stable platelet aggregation via GPIIb/IIIa activation and in a VWF-dependent manner [105, 155]. Elongational flow was found to activate soluble VWF, high WSS to promote TXA-2 and ADP release from platelets, and decreasing WSS to induce endothelial VWF secretion and accumulation at the stenosis outlets [105]. Interestingly, platelet aggregation did not occur in straight microfluidic channels, even at very high shear rates (up to 20000s^{-1}) [105, 155], indicating a key role for stenotic geometries in thrombogenesis. This is of relevance in the context of vascular remodelling in PH, as luminal obstruction could create flow perturbations, therefore promoting *in situ* platelet aggregation.

4. The Role of Platelets in Pulmonary Hypertension Pathogenesis

Platelets, through their aggregation into thrombi and the production of a variety of vasoactive mediators, growth factors and inflammatory cytokines, contribute to vascular remodelling, vasoconstriction and the formation of occlusive thrombotic vascular lesions. This section will explore how platelets are related to each of these events.

4.1. Vascular Remodelling

Disordered angiogenesis is found in severe PH, usually in the form of angioproliferative, plexiform lesions [161]. A plethora of pro- and anti-angiogenic factors, including VEGF, PDGF, PF4 and angiostatin are released by platelets [94] (Figure 5). Beside angiogenesis, VEGF controls many fundamental functions of vascular cells, from apoptosis to NO production [162]. However, the role of VEGF in PH remains controversial. While some studies found that VEGF overexpression in MCT-[163] and chronic hypoxia-[164] induced PH rats led to haemodynamic improvements, others showed that inhibition of VEGF signalling attenuates PH [165]. Furthermore, despite the evidence for increased VEGF receptor-2 (VEGFR-2) expression in plexiform lesions of PH patients [161], VEGFR blockade causes severe PH when combined with chronic hypoxia in rats [166]. In humans, one study found increased platelet VEGF content in PH patients [167], but other studies observed no differences in plasma [168] and platelet [169] VEGF levels between IPAH and controls. VEGF administration after ECs accumulation in the pulmonary vasculature of chronic hypoxia rats had a slightly negative impact on PAP [166], suggesting that VEGF may have a dual role in PH, providing early protection before contributing to vascular remodelling. VEGF has also been shown to promote TF expression in ECs [170].

PDGF acts as a mitogen [171] and chemoattractant[172] for PASMCs, and PDGF receptor (PDGFR) expression is increased in PAH lungs [173, 174]. Consistently, inhibition of PDGFRs reverses experimental PH [175].

Angiostatin is another platelet-derived factor released upon aggregation [176] (Figure 5), which promotes EC apoptosis by antagonising VEGF [177]. Indeed, higher levels of platelet angiostatin in IPAH patients have been associated with increased EC apoptosis [169]. Additionally, adenoviral overexpression of angiostatin was shown to aggravate PAH in chronically hypoxic mice [178].

Platelet activating factor (PAF) has been shown to induce IL-6/8 [179] and VEGF [180] expression in pulmonary fibroblasts and vascular SMCs, and has been associated with increased disease severity in primary pulmonary hypertension of the newborn (PPHN) [181] and chronic hypoxia-induced PH [182]. In foetal lambs exposed to chronic high-altitude *in utero*, increased PAF levels and PAF-induced PASMCs proliferation were observed [183], while PAF-receptor antagonists attenuated hypoxia-induced PH and vascular remodelling in rat models [182].

Serotonin produced by the central nervous system is initially taken up by platelets [184] to prevent its vasoconstrictive [185], pro-proliferative [20] and pro-thrombotic [186] effects. While some studies found increased circulating serotonin levels in PH patients [187, 188], others did not [189, 190]. Higher serotonin release from platelets was also observed in PH patients compared to controls [187]. Secreted upon platelet activation, serotonin has a powerful vasoconstricting effect (Figure 5) and acts on many vascular cells through the serotonin transporter (SERT) and three serotonin receptors: 5-HT-1BR, 5-HT-2AR and 5-HT-2BR. Elevated levels of all these receptors have been found in PH lungs[191, 192], although only the expression of SERT was increased in cultured patient-derived PASMCs, and accounted for the serotonin-induced PASMCs growth response [192].

The importance of serotonin in vascular remodelling was first suggested in the 1960s, when an increased number of female PAH cases was associated with the intake of the anorectic drug Aminorex [193], known to affect serotonin transport in the lungs [194]. Increased SERT activity was shown to promote Ca^{2+}-dependent, Rho-associated protein kinase (ROCK)-mediated PASMC proliferation *in vitro* [195], as well as contraction through voltage-gated Kv channels inhibition [196]. Additionally, *SERT* gene polymorphism was found in 65% of PH patients, compared to 27% of controls [20].

Serotonin may also be involved in EPC differentiation [191], fibroblasts proliferation [197] and metabolic alterations in PAH [198]. Furthermore, it could promote inflammatory cells transmigration by increasing endothelial permeability [199], and platelet aggregation mediated by the 5-HT-2AR [200].

Angiopoietin-1, also released from platelets upon thrombin stimulation [201], has been proposed to stimulate endothelial serotonin production by activating TIE-2 receptors, further promoting PASMC proliferation [202].

4.2. Inflammation

Platelets release many mediators increasing endothelial dysfunction, inflammation, and leukocyte recruitment, which promote vascular remodelling. There is a close link between coagulation and vascular inflammation, and CTEPH patients typically display increased levels of inflammation markers, including C-Reactive Protein (CRP) [203] and TNF-α [204].

CD40L is an important pro-inflammatory molecule, expressed on the surface of activated platelets, and released as sCD40L [205] (Figure 5). Elevated levels of sCD40L have been found in PAH patients, alongside increased MCP-1 and IL-8 [148], and sCD40L has been shown to induce EC [206] and fibroblast [207] chemokine production, promoting leukocytes infiltration. Interactions between sCD40L and its endothelial receptor CD40 are known to induce vascular remodelling in other diseases, including atherosclerosiss [208], and could therefore participate in the development of PAH.

The pro-angiogenic chemokine stromal-derived factor-1a (SDF-1a) (also known as CXCL12) has been shown to induce monocyte chemotaxis [209] (Figure 5), and promote vascular remodelling through its receptors CXCR4 [210] and CXCR7 [211], notably by recruiting bone marrow-derived progenitor cells [210]. Increased CXCR7 [211, 212] and SDF-1a [212] levels were found both in PH patients' and mice models' lungs, and have been associated with poorer PAH survival [213].

Lymphotoxin-like inducible protein that competes with glycoprotein D for Herpesvirus entry mediator on T lymphocytes (LIGHT) is a platelet-derived TNF-α superfamily member [214], known to promote EC- and monocytes-mediated vascular inflammation [215] (Figure 5). Elevated serum LIGHT levels have been found in the femoral arteries of IPAH, CTEPH and secondary PAH patients, and correlated with increased mortality [216]. LIGHT receptors were also detected on PASMCs, PAECs and alveolar macrophages in PAH patients' lungs, and PAECs showed increased TF and PAI-1, as well as decreased TM expression levels after stimulation with recombinant LIGHT [216].

4.3. Vasoconstriction

The major vasoconstrictors released by platelets and elevated in PH patients' lungs are TXA-2 and serotonin [26, 188] (Figure 5). Because activated platelets also reduce endothelial NO and PGI_2 production, they exacerbate the imbalances between vasodilators and vasoconstrictors, promoting vasoconstriction.

There is also evidence that tetrahydrobiopterin (BH4), a cofactor and regulator of eNOS function, is produced by platelets [217]. Mice deficient in BH4 showed increased susceptibility to hypoxia-induced PH, while BH4 supplementation reduced vascular tone and remodelling, preventing disease development [218]. Decreased BH4 production due to platelet dysfunction may therefore contribute to impaired NO production and vasoconstriction.

4.4. Platelet Microparticles

Microparticles (MPs) are vesicle fragments (0.1-1μm in size) derived from plasma membranes of many cell types, usually upon cellular activation or stress, such as apoptosis [219]. Increased platelet MPs (PMPs) levels have been found in various types of PH, compared with healthy controls [220], and their membranes were shown to be 50 to 100 times more pro-coagulant than activated platelets' surfaces [221]. Indeed, increased TF and CD40L expression were found on PMP surfaces in PAH patients, who also displayed increased phosphatidylserine-positive MPs in their Pas [222]. PMPs were shown to regulate vascular tone through TXA-2 production [223], induce *in vitro* vascular SMCs proliferation [224], as well as VEGF- and PDGF-mediated *in vitro* and *in vivo* angiogenesis in a rat aortic ring model [225] (Figure 5). Additionally, incubation of rat PAECs with PMPs from hypoxic PH animals reduced endothelial NO production and increased oxidative stress, while *in vivo* injections of those PMPs impaired endothelium-dependent relaxation in rat Pas [226]. In PAH patients, PMPs displayed elevated CD39 nucleotidase activity [227], which may promote vasoconstriction and platelet activation through increased ADP production.

PMPs have also been shown to induce endothelial production of IL-1β, IL-6, and TNFα, as well as ICAM-1 expression [228], indicating that PMPs may promote further platelet aggregation and local inflammation. Interestingly, PMPs shedding was found to be induced by shear stress, with evidence of increased PMPs levels in patients with severe aortic valve stenosis [229]. This could be relevant in conditions of increased shear stress and flow disturbances caused by arterial luminal narrowing and/or thrombotic occlusion in PH.

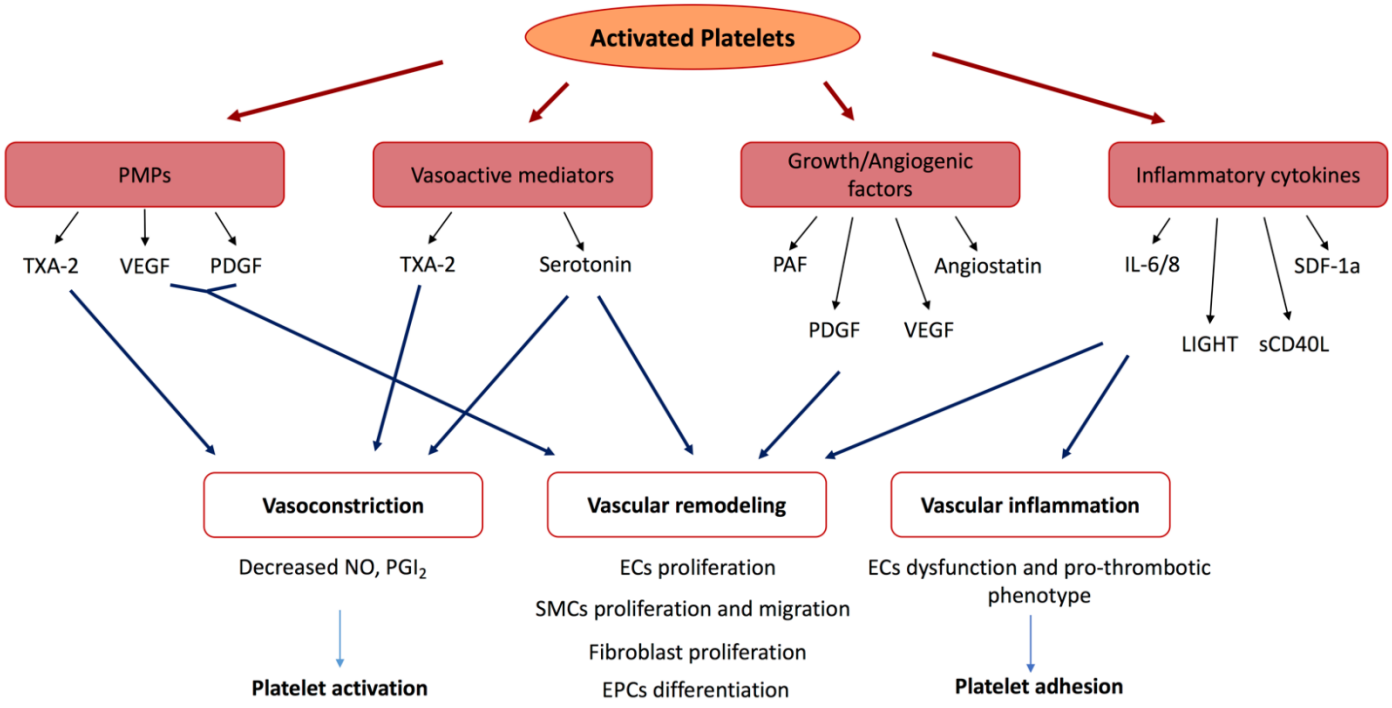

Figure 5. The contribution of platelet-derived factors to PH pathobiology

This figure summarises the contribution of platelet-derived factors to vasoconstriction, vascular remodelling and inflammation in pulmonary hypertension (PH). Vasoactive mediators, growth and angiogenic factors, inflammatory cytokines and platelet-derived microparticles (PMPs) are released upon platelet activation. All these factors induce endothelial dysfunction, leading to imbalances in nitric oxide (NO) and prostacyclin (PGI_2) production, and the switch towards an endothelial pro-thrombotic phenotype, which enhance platelet adhesion, activation and aggregation. This in turn causes further release of vasoactive factors from platelet, creating a vicious cycle of vascular remodelling and platelet activation. Many of the growth and angiogenic factors released by platelets promote endothelial cells (ECs) and fibroblast proliferation, smooth muscle cells (SMCs) proliferation and migration, as well as endothelial progenitor cells (EPCs) differentiation, which are major features of the vascular remodelling observed in PH. (IL-6/8: interleukin-6/8; LIGHT: lymphotoxin-like inducible protein that competes with glycoprotein D for Herpesvirus entry mediator on T lymphocytes; PAF: platelet activating factor; PDGF: platelet-derived growth factor; sCD40L: soluble CD40 ligand; SDF-1a: stromal-derived factor-1a; TXA-2: thromboxane A-2; VEGF: vascular endothelial growth factor)

5. Targeting Platelets in Pulmonary Hypertension Treatment

Most therapeutic strategies in PAH have been focused on targeting the endothelin, NO and prostacyclin pathways [36]. Available drug classes include prostacyclin analogues, endothelin receptors antagonists (ERAs), and phosphodiesterase (PDE)-5 inhibitors [36]. PH due to lung disease and/or hypoxia, and CTEPH may be treated with PAH-approved drugs under specific conditions, but there are no randomised controlled trials supporting the use of PAH drugs in other forms of PH [36].

Current therapies successfully target vasoconstriction, but have a modest effect on vascular remodelling. Mortality rate in PH is high (>20% within 1 year of diagnosis in high risk patients) [230], and novel, more effective and better targeted therapies are needed. As previously described, platelets show strong links with PH pathobiology, providing a rationale for the use of anticoagulation or antiplatelet drugs in disease therapy. This section will describe the therapeutic potential of new anti-platelet drugs and comment on beneficial, anti-thrombotic effects of current PH treatments.

5.1. Anticoagulation and Anti-platelet Drugs

Most recent treatment guidelines recommend warfarin for IPAH, HPAH and anorexigen-induced PAH, and for CTEPH patients, even after PTE [36]. However, the main evidence is derived from observational and registry studies [6, 231-233], and there is an urgent need for randomised controlled trials specifically evaluating the benefits of anticoagulants in PAH and other forms of PH. In the SERAPHIN trial, which assessed the therapeutic effects of a novel ERA, macitentan, in concomitance with other treatments, no significant difference in mortality and morbidity was found between the placebo and warfarin-treated patients [36]. In the COMPERA registry, anticoagulation use was associated with a 21% increase in IPAH patient survival [6]. A recent meta-analysis, which included 12 non-

randomised studies, demonstrated that anticoagulation therapy significantly reduced mortality in IPAH patients, but increased mortality in scleroderma-associated PAH patients [234], indicating that anticoagulation should be carefully tailored to the PAH subtype.

Anti-platelet therapy has also been assessed in PAH. A randomised controlled trial of 19 IPAH patients found that aspirin and clopidogrel reduced platelet aggregation, with aspirin but not clopidogrel reducing urinary TXA-2 metabolites levels, as well as the TXA-2:PGI_2 ratio [235]. Another controlled study of aspirin and simvastatin in 92 PAH patients found reduced TXA-2 production, but no effects on platelet aggregation and 6-minutes walking distance test (6MWDT) results compared to controls [236]. Interestingly, aspirin reduced PAP, RV hypertrophy and improved survival in MCT rat models, which was associated with decreased plasma levels of platelet-derived serotonin [237].

The efficacy of anti-platelet therapies therefore remains inconclusive. Other drugs interfering with platelet-endothelial interactions, such as monoclonal antibodies against the VWF A1 domain [238] or platelet GPIIb/IIIa [239] may be of relevance in PH treatment.

5.2. Prostacyclin Analogues

As previously mentioned, PGI_2 is a key inhibitor of platelet aggregation[104], beside its vasodilatory and anti-proliferative effects [102, 103]. Many PGI_2 analogues are used in PAH treatment, including epoprostenol or iloprost [36]. Iloprost was shown to directly inhibit agonist-induced platelet activation [240], while continuous epoprostenol infusion increased TM, decreased P-selectin levels [241], and normalised platelet aggregation [242] in primary and secondary PH patients. Epoprostenol was also shown to decrease LIGHT serum levels in PAH patients [216], and inhibit PMPs formation in whole blood [243]. These data suggest that the benefits of PGI_2 therapy in PH patients could partially be explained by their inhibitory action on platelets.

5.3. Phosphodiesterase Inhibitors

PDE-5 is one of the most active PDEs in the pulmonary vasculature, and promotes vasoconstriction by inhibiting cGMP in the NO/cGMP pathway [244]. Sildenafil, vardenafil and tadalafil are all PDE-5is approved for the treatment of erectile dysfunction, and have been shown to induce significant pulmonary vasodilation in PAH patients [245]. Sildenafil reduced platelet activation in sickle-cell disease-associated PAH [246], while vardenafil blocked platelet Ca^{2+} channels, reducing Ca^{2+} mobilisation and influx in thrombin-stimulated, rabbit washed platelets [247].

Milirone is a PDE-3 inhibitor used for PPHN[248] and post-operative PH [249] treatment. While it has been shown to inhibit platelet activation [250], it also has a side effect of thrombocytopenia [251]. It currently remains unclear whether the benefits of milirone are due to its antiplatelet or vasodilatory actions.

5.4. PDGFR Inhibitors

Imatinib is a multi-kinase (including PDGFR tyrosine kinase) inhibitor, which has been shown to reverse vascular remodelling in MCT rats and chronic hypoxia mice [175]. When combined with other PAH-approved drugs, including epoprostenol or sildenafil, imatinib improved the haemodynamics and functional capacities of severe PAH patients [252]. Sorafenib is another PDGFR inhibitor, demonstrated to prevent vascular remodelling and improve cardio-pulmonary functions in experimental PH[165]. PDGFR inhibitors may therefore exert their beneficial actions by counteracting the mitogenic [171] and chemoattractant [172] effects of platelet-derived PDGF on PASMCs.

5.5. Serotonin Antagonists

Serotonin transport and signalling could be a promising therapeutic target in PH. Indeed, inhibition of the 5HT-1BR attenuated hypoxia-induced PH in rat models [253], while PH patients receiving selective serotonin reuptake inhibitors (SSRIs) had a decreased mortality compared to those not receiving SSRIs [254]. Another study demonstrated that SERT inhibition decreased serotonin-induced proliferation of PASMC from IPAH patients *in vitro* by interfering with the ROCK signalling pathway [195]. A selective 5-HT-2BR antagonist (PRX-8066) was assessed in a randomised controlled trial of 72 COPD-associated PAH patients (trial no. NCT00677872), but only modest reductions in systolic PAP were observed, and the trial was discontinued. After encouraging pre-clinical results in MCT rat models [255], Terguride, a dual 5-HT-2A/BRs antagonist, was also tested in IPAH and PAH with connective tissue disease patients, but no improvements in haemodynamics, 6MWDT or time to clinical worsening were observed [256]. Only patients on background ERAs therapy showed improvements in PVR [256]. Interestingly, SERT, but not 5-HT receptors antagonists, limited PH patients-derived PASMCs growth responses to serotonin [192], suggesting that SERT may be a better therapeutic target than 5HTRs.

5.6. Other Therapies

NO is a potent inhibitor of platelet activation and aggregation [257], which explains the benefits of inhaled NO

administration in acute PH after cardiac surgery [258] and in PPHN [259]. Inhibition of TXA-2 synthesis with furegrelate sodium preserved vascular integrity and lowered PVR in chronic hypoxia piglets [260], and a recent study demonstrated that a novel antagonist of the thromboxane prostanoid receptor, NTP42, reduced mPAP, RV systolic pressure, vascular remodelling, inflammation and fibrosis in MCT-induced PAH rat models [261].

Determining whether platelet dysfunction and aggregation are a cause or consequence of PH pathobiology constitutes a challenge. While platelets are appealing therapeutic targets, their effects on the vasculature are complex, and a better understanding of how platelet content and degranulation are modulated is required. Some factors may be protective in PH, such as soluble tumour necrosis factor-like weak inducer of apoptosis (sTWEAK), whose reduced platelet storage levels are associated with worse prognosis in PAH [262]. Therefore, simply inhibiting platelet activity may not be the solution in the inflammatory context of PH, and therapeutic strategies must be carefully designed.

6. Investigating the Role of Platelets in Pulmonary Hypertension: Current Models

6.1. In Vivo Models

Intravital microscopy has been used since the 19th century [76], and the latest advances in the field have allowed highly precise, real-time *in vivo* monitoring of platelet adhesion and thrombus formation [263]. Current techniques mostly use murine systems, and involve inducing platelet aggregation via mechanical [264], electrical laser [265], chemical ($FeCl_3$) [266] or photochemical [267] injury to the vascular wall. Other methods using lipopolysaccharide [268] or calcium ionophores [123] have been used to promote platelet-endothelial interactions, but these usually do not result in full thrombogenesis.

These methods all cause thrombus formation through different mechanisms, and should therefore be chosen according to the experimental question to be addressed. For instance, while $FeCl_3$-induced injury involves endothelial denudation and subendothelial platelet adhesion [266], photochemical techniques do not deteriorate the endothelium, and thrombus formation is initiated by platelet-endothelial interactions [267].

Although useful for studying the mechanisms involved in platelet aggregation and thrombogenesis, these models may introduce artefacts and deviate from physiological relevance. To overcome this, mice transgenic for genes involved in haemostasis and fibrinolysis have been developed [269], but the major differences between human and mice vascular systems make murine findings challenging to translate into clinical practice [270].

Animal models of CTEPH have been developed since the 1990s, although none of them are representative of all human CTEPH features [271, 272]. One piglet model of CTEPH, developed by primary left PA ligation via sternotomy, followed by weekly transcatheter embolizations of Histoacryl into the right lower lobe for 5 weeks, was able to reconstruct most aspects of the disease, notably its dual pulmonary vascular bed component [273]. However, no model currently replicates the impaired fibrinolysis characteristic of CTEPH.

Many other models of PH exist, with MCT-[274], chronic hypoxia-[275] and Sugen-induced PH rats [166] and mice [276] being the most commonly used. Rats are usually better than mice in reflecting the degree of vascular remodelling observed in PAH patients [166, 274-276], although none of the existing models can accurately recreate all the features of human PH.

6.2. In Vitro Models

Many standardised *in vitro* assays exist to evaluate platelet function and aggregation, including platelet aggregometry [141, 151] and flow cytometry [142]. Platelet aggregometry involves exposing platelets to agonists (eg ADP, thrombin or TXA-2) *in vitro,* and has been used to identify platelet function abnormalities in CTEPH [141] and PAH [151].

However, considering the importance of haemodynamics and other blood components in thrombus formation, the static and isolated nature of these assays makes them physiologically irrelevant. Additionally, most of them require large amounts of whole blood [141, 142], a major limiting factor in many clinical situations.

Microfluidic devices, where blood or isolated platelets are perfused through microscopic-size channels, address some of these limitations, and their development has boomed over the past decades. First designed 50 years ago [277], these devices have enabled the investigation of many cellular and molecular mechanisms involved in haemostasis and thrombosis in a reproducible manner. Technological advancements have also greatly reduced the blood volumes required for experiments (<1mL) [278, 279].

Extracellular matrix (for example collagen)-coated devices are one of the simplest models, widely used to simulate thrombotic responses to vascular injury [280], or to evaluate dose-responses to antiplatelet drugs [279]. However, such models do not fully replicate the important dynamics between ECs, platelets and shear stress and therefore poorly correlate with the *in vivo* observations [263]. More complex, endothelium-lined devices have since been developed

and used in modelling thrombosis modelled under physiological shear conditions [278, 281]. A wide range of parallel or Y-shaped flow chambers are commercially available, and new software programmes help the design of customised channels. These usually provide a better reconstitution of the *in vivo* vessel geometries, such as stenosis or branch points, and have been used to model shear gradient-dependent platelet aggregation [105, 155]. More recently, Costa and colleagues [282] have used 3D-printing and computed tomography angiography data to recreate healthy and stenosed blood vessel chambers, lined with human umbilical vein endothelial cells (HUVECs).

Nevertheless, the use of cellular monolayer does not reflect the true dynamic, multi-cellular vascular microenvironment. Organs-on-chips (OOCs) are microfluidic platforms within which one or more living cell population(s) can be cultured simultaneously under flow conditions, aiming to mimic tissue- and organ-level physiological environments [283]. A few models of thrombosis-on-chips have been published, for instance by Jain and colleagues [106], who showed that tissue-tissue interactions between the alveolar epithelium and vascular endothelium were required for lipopolysaccharide-mediated intravascular thrombosis. However, despite the availability of microfluidic arterial wall [284], biomimetic PA models [285], and a recently published PA-on-a-chip model [286] , the role of platelet aggregation in the context of PH pathobiology has not been assessed using OOC technologies.

7. Conclusion

Platelets are key players in the pathobiology of PH, through their involvement in thrombosis, vascular remodelling and vasoconstriction. While the true nature of platelets' contribution to this complex, multifactorial disease is not fully understood, the degree of platelet contribution to the disease aetiology is likely to vary among functional classes of PH. Nevertheless, in all forms of PH, endothelial dysfunction, inflammation, NO/PGI_2 reduction, and disturbed flow caused by luminal narrowing, favours a pro-thrombotic environment, leading to platelet aggregation. Activated and aggregated platelets are likely to promote further vascular remodelling and inflammation through the release of vasoactive and inflammatory mediators, worsening the clinical outcome.

While the existing therapies are effective in reducing vasoconstriction, they fail to reverse vascular remodelling. Targeting platelet adhesion, aggregation and activation may be beneficial in PH and therapeutic strategies should be tailored to the individual patient's needs. Despite the advances made in modelling platelet function, more physiologically relevant *in vitro* systems, utilising patient-derived cells and accounting for both vascular remodelling and the resultant flow disturbances, need to be designed

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9. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

10. Ethical Approval

The manuscript does not contain experiments on animals and humans; hence ethical permission not required.

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