The changing face of pulmonary hypertension diagnosis: a historical perspective on the influence of diagnostics and biomarkers

Jenny L. Hewes¹,², Ji Young Lee²,³,⁴, Karen A. Fagan¹,²,³ and Natalie N. Bauer¹,²

¹Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, AL, USA; ²Center for Lung Biology, College of Medicine, University of South Alabama, Mobile, AL, USA; ³Division of Pulmonary and Critical Care Medicine, University Hospital, University of South Alabama, Mobile, AL, USA; ⁴Department of Physiology and Cell Biology, College of Medicine, University of South Alabama, Mobile, AL, USA

Abstract
Pulmonary hypertension is a complex, multifactorial disease that results in right heart failure and premature death. Since the initial reports of pulmonary hypertension in the late 1800s, the diagnosis of pulmonary hypertension has evolved with respect to its definition, screening tools, and diagnostic techniques. This historical perspective traces the earliest roots of pulmonary hypertension detection and diagnosis through to the current recommendations for classification. We highlight the diagnostic tools used in the past and present, and end with a focus on the future directions of early detection. Early detection of pulmonary hypertension and pulmonary arterial hypertension and the proper determination of etiology are vital for the early therapeutic intervention that can prolong life expectancy and improve quality of life. The search for a non-invasive screening tool for the identification and classification of pulmonary hypertension is ongoing, and we discuss the role of animal models of the disease in this search.

Keywords
extracellular vesicles, right heart catheterization, echocardiography

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Introduction
From the earliest observations of pulmonary hypertension (PH), it has been a conundrum to physicians and scientists alike. Beginning with merely anatomic descriptions during post-mortem, PH was at first a hypothetical disease process. Patients presented with symptoms relatively common to other pulmonary or cardiac diseases including dyspnea and cyanosis, and without sophisticated diagnostic tools, PH was not described until death. From the late 1800s until the 1950s, conflicting reports of the etiology of PH are reflected in the literature, and with no true diagnostic test for the pulmonary artery pressure, this would remain the case until the acceptance of right heart catheterization (RHC) into clinical practice.

Clinical use of RHC had rocky early beginnings, but establishment of safety and efficacy made the technique the gold standard in diagnosis of pulmonary arterial hypertension (PAH). RHC changed the face of diagnosis and unmasked a prevalence of what was once considered an orphaned disease. Combinations of RHC and imaging provided the first diagnoses of a PAH epidemic with the Aminorex crisis in the late 1960s. This crisis set the stage for the first classifications of PH. These classifications have evolved over the years based on clinical findings and basic research; however, one area that has been suggested at each world congress is the identification of biomarkers for earlier diagnosis and treatment. Unfortunately, patients with early symptoms such as dyspnea and shortness of breath can be indistinguishable from other lung or heart diseases, or complicated by underlying issues even to-date. This historical
review and perspective shine light on the complicated history of diagnosis and classification of PH and PAH and provide an insight into future directions of disease mechanisms and biomarker research.

**PH: the discovery**

This history of PH is appropriately divided into two eras. PH prior to the use of heart catheterization in humans and the era of post-cardiac catheterization. In the pre-cardiac catheterization era of PH history, there exists only the hypothesis of PH based on symptoms and autopsy evidence. The first evidence of PH began in the late 19th century. Prior to the use of the heart catheter, the rise in the pressure of the pulmonary vascular system was immeasurable. Physicians could only speculate on increased pressure as evidenced by autopsy findings of vascular remodeling, complex lesions of the pulmonary vessels, and right ventricular hypertrophy. The literature suggested multiple etiological origins, but similarities existed in the descriptions of severe dyspnea and cyanosis. One of the first cases of PH reported was in 1891, by the renowned German physician, Dr. Ernst von Romberg. Romberg described a 24-year-old patient that suffered from severe dyspnea, chronic drowsiness, and cyanosis prior to death. Romberg’s autopsy report of the patient revealed vascular lesions of the small pulmonary arteries and severe right ventricular hypertrophy. Romberg, however, was unable to identify a pathological cause for the pulmonary artery lesions and ultimately described them as “pulmonary vascular sclerosis” of unknown origin.1,2

Due to a lack of sophisticated diagnostic tools, early descriptions of PH took many twists and turns. In 1901, Dr. Ayerza of the University of Buenos Aires described a male patient who suffered from chronic cough with sputum production, dyspnea, severe cyanosis, and manifestations of right heart failure. Patient history detailed repeat episodes of pneumonia and chronic respiratory symptoms with wet crackles and wheezing observed in both lungs. Ayerza’s patient died 24 days after admission, and autopsy revealed a hypertrophied right ventricle as well as thickening of the bronchial walls and abundant secretions. Histological findings featured medial hyperplasia of the pulmonary arteries and multiple thrombus obstructions. Ayerza felt his clinical case was of a unique origin and termed the pathological course of pulmonary disease and right heart failure “cardiaco negros” (black cardiac), a literal name taken from the cyanotic discoloration of the patient’s skin.3,4

It is evident today that Ayerza’s patient had PH associated with chronic lung disease or possibly chronic thrombotic embolisms. Ayerza’s hypothesis at the time was that pulmonary vascular remodeling was secondary to the pulmonary parenchymal disease; however, this idea would later be swayed by opposing views.

Soon after Ayerza’s description, physician Escudera published reports of black cardiac disease and noted the coexistence of pulmonary arterial sclerosis and bronchial syphilis in his patients. These observations suggest evidence of syphilitic origin, rather than chronic lung disease.3,5 Later, Arillaga, in his 1913 doctoral thesis, described a collective of 11 patients with severe cyanosis and right heart failure similar to Ayerza’s original case report. However, these patients came from various etiologies. Arillaga named the collective disorders after his mentor, Dr. Ayerza. International use of the term “Ayerza’s Disease” was used to describe patients presenting with common symptoms and pulmonary vascular sclerosis upon autopsy. Among these patients, Arillaga identified a subset of patients with pulmonary lesions of seemingly unknown origin and no evidence of pulmonary disease. This puzzling discovery would begin a search for an etiological cause for these patients that would persist for decades.3,6

The first example of Ayerza’s Disease in the United States was presented at the 34th Annual Meeting of the American Society for Medicine in 1919 by Dr. Aldred Scott Warthyn. Warthyn, a physician with a diverse legacy throughout multiple disciplines of biology, even obtaining a gastropod namesake, is perhaps best known for his contributions to cancer genetics.7 However, Warthyn published over 30 papers on the pathology of syphilis in the early 1900s and became a leading expert in the field.8 At the meeting in 1919, Dr. Warthyn reported observations of a patient for over five years and described a clinical course of dyspnea, cyanosis, increasing weakness, edema, and drowsiness which matched the Spanish description of Ayerza’s Disease. Postmortem autopsy and microscopic examination revealed characteristic “syphilitic mesenteritis” of the pulmonary arteries.9 This report coincided with Escudera’s work and the growing body of literature on syphilis. Further, a patient of Ayerza’s died in Escudera’s clinic and at autopsy was reported to have evidence of pulmonary arterial remodeling of syphilitic origin.2,5 Thus, at this time, there was significant confusion in the literature over the unstandardized meaning of Ayerza’s Disease, which led to a supposition that what we now call PH was induced by syphilis.

So, what was Ayerza’s Disease that caused debilitating symptoms, and death? This was finally answered by a British pathologist, Dr. Oscar Brenner of Massachusetts General Hospital in 1935. Dr. Brenner performed a study of over 100 cases of patients with PH symptoms; however, very few of these patients displayed the severity of symptoms and pathological findings to match those reports of Ayerza’s Disease. Ayerza’s Disease had been published in American, English, French, and Brazilian literature for over three decades, but Brenner remarked upon the different interpretation across multiple reports. Further, Brenner identified a subset of patients with idiopathic or primary pulmonary arterial lesions and remodeling and noted the absence of syphilis. In fact, Brenner went on to state that the 1921 publication by Arillaga had an inadequate description of the spirochetes isolated from the patient and could have been any type more commonly found in the mouth and bronchi of humans.6 Brenner, however, could not offer an
explanation for the cause of the primary or idiopathic pulmonary lesions described by Arillaga and himself. Indeed, these primary lesions would later be termed primary pulmonary hypertension (PPH) and now the present-day term of idiopathic pulmonary arterial hypertension (IPAH). Brenner suggested that the term Ayerza’s Disease was not a separate entity of PH, but a description of the severe end-stage result of PH and right heart failure and recommended its removal from use.6

“So-called” PH and the hilar dance

Following Brenner’s publication, PH became a widely used term used across literature to identify patients previously associated with Ayerza’s Disease. However, inconsistencies and debate of proper nomenclature still persisted. While Brenner had methodically examined and reported evidence of PH of multiple etiological causes, without the ability to directly measure pulmonary pressures, the term pulmonary hypertension was still considered by many as being hypothetical. Despite this, advances in the field of PH took place following Brenner’s publications due to advances in clinical technology. For example, Ayerza’s original descriptions had only offered systolic blood pressure taken by a crude device called a Potain apparatus,3 but the development and increasing availability of blood pressure apparatus, chest X-rays, and electrocardiogram (ECG) offered unique insight into symptoms and signs of PH.

A report in 1940 from the cardiology department of London’s King College Hospital presented three similar cases of women with severe dyspnea. Dr. Terrance East described a very loud second heart sound, an ECG with right heart axis deviation, and an enlarged pulmonary artery and right ventricle on chest X-ray. Autopsy revealed right ventricular hypertrophy by comparing weights of the right heart to Vierordt’s Tabellen. Vierordt’s Tabellen was developed by Dr. Hermann Vierordt, a German professor of internal medicine, which provided weights of internal organs through human development consisting of both normal and abnormal, perhaps a passion taken from his father who published texts of childhood development in the late 19th century, and considered the standard of measure at the time.10

An interesting aspect to East’s 1940 report was the mention of the outbreak of the Second World War. The report featured no citations and the author stressed that “the subsequent outbreak of war has made it impossible to survey the literature, so there are no references.” Although unable to review any literature, the report, due to the absence of pulmonary disease and heart defects, hypothesized the patients to have IPH or PPH.11 Dr. East titled his report “Pulmonary Hypertension,” although urgently addressing the term as a “hypothetical” cause of his patient outcomes.

A further report in 1940 from the pathology department of Guy’s Hospital described three cases of patients with severe cyanosis and dyspnea. Post-mortem right ventricular hypertrophy was indicated, and no other pathological changes were found. While accepting that it must be Brenner’s “so-called” PPH, this pathology report recommended the new term of idiopathic right ventricular hypertrophy.12 Both clinical reports from King College Hospital and Guy’s Hospital are an indication of the authors’ hesitancy to accept Brenner’s findings in PH. More broadly, these reports represent the reluctance to accept the hypothesis of PH prior to the ability to measure pulmonary arterial pressure.

Observations of the pulmonary trunk and arteries by X-ray in PH around the time of Dr. East’s report, and including his descriptions, often report observations of a “hilar dance” which is the pulsing or “dancing” motion with increased distensibility of the pulmonary artery. On X-ray film, the process is observed via shadows created from the rapid “hilar dance” movement. The term entered the cardiology literature in 1925 and had been described commonly in cases of septal defects, Eisenmenger’s complex, mitral stenosis, and patent ductus arteriosus (PDA). The hilar dance was even used as a grading system based on the extent of visible pulsation to evaluate multiple cardiac conditions.13 Significant debate has occurred over whether this hilar dance was a result of increased idiopathic pulmonary arterial pressure or increased blood flow to the pulmonary artery due to a left to right shunt physiology. This physiology would take on the term hyperkinetic PH and would exist into the 1950s. One experiment designed to answer the question required two healthy young men to run up three flights of stairs above the X-ray room five times. Analysis followed to test if the hilar dance was evident after increased blood flow, and the investigators found no obvious difference before and after exercise.13 Thus, under normal conditions, the increase in flow does not directly induce the “hilar dance”; however, this experiment alone does not take into account the alterations in distensibility and pressure in the presence of underlying co-morbidities or idiopathic PAH. The hilar dance is a term that has fallen from clinical use due to more accurate methods of identifying vascular changes in PH.

PH of various etiologies: historical reports

Despite the initial focus of literature on PPH or IPH, other sources of pulmonary arterial sclerosis were recognized around the world. Beginning with Parker and Weiss in 1936, studies on mitral stenosis demonstrated that left heart diseases stimulate changes in the pulmonary arteries and arterioles.14,15 Using 20 cases of well-established mitral stenosis, examinations of the pulmonary arteries revealed that half of them showed intimal fibrosis and medial hypertrophy of the small pulmonary arteries and arterioles. Further, this study emphasized the need for evaluating the extent of vascular pathology and remodeling to determine whether a patient has reversible or irreversible lesions that would interfere with the benefits of surgical repair of the mitral valve.14
Reports of PH, due to parasitic infection, emerged early in the history of PH from Egypt. Egyptian scientist Bilharz discovered the etiological cause of “bilharzia” a multiorgan disease, and as early as 1885, bilharzia ova (schistosomiasis) were known to be deposited in the lungs. Briefly, the adult male and female worms live within the veins of the host and fertilized eggs are shed into the environment. Eggs can also remain in various host tissues and induce inflammation. Pulmonary endarteritis and right heart failure due to schistosomiasis were identified in Egypt where the parasite is endemic. In 1928, the Faculty of Medicine of Cairo uncovered lesions of the pulmonary artery due to interactions of the bilharzial ova and reports of pulmonary bilharzias in post-mortem autopsy were common. Leading research from Shaw and Ghareeb at the University of Cairo estimated in 1938 that roughly 60 to 70% of the inhabitants of Egypt were infected with schistosomiasis. Shaw and Ghareeb reported autopsies of 282 schistosomiasis infection cases and determined that pulmonary involvement occurs in 33% of bilharzia cases. The report further described the arterial lesions and the pathological development of “Ayerza’s Disease.” Schistosomiasis is estimated to affect at least 230 million people worldwide and at present endemic areas offer preventative treatment every one or two years.

A second common geographical region for PH in the early 1900s was Brazil. The Brazilians primarily affected were gold-miners working in the valleys in the Andes Mountains at an altitude of over 14,000 feet. An important observation was made by a local physician, Dr. Courtney. He observed that fatality was common in cardiac failure patients being brought down from altitude by train. The train trip down the mountain actually involved an increased ascent to 16,000 feet before beginning decent of the mountain. However, patients brought down by air directly from the mountain showed improvement in their cyanosis dramatically when they reached the plain. The link between high-altitude and PH was speculative at best, and it would be many years before data on high-altitude medicine would begin to be published.

In the early 1900s, the emergence of the concept of chronic thromboembolic disease resulting in cardiopulmonary failure appeared in medical literature. The earliest descriptions of the disease were from Hart in 1916, Moller in 1920, and Ljungdahl in 1928. In 1931, Means and Mallory of the Massachusetts General Hospital reported cases of six patients with postmortem discoveries of thrombotic occlusions of the pulmonary vessels. While the disease was related to cardiopulmonary failure postmortem, the occurrence of thrombotic occlusions in the pulmonary vessels was not directly associated with PH until the development of cardiac catheterization.

The earliest descriptions of PH were based on symptoms such as dyspnea and cyanosis and postmortem exam findings of right heart hypertrophy and pulmonary vessel sclerosis. In the era of PH history prior to the use of cardiac catheterization, PH was clearly linked to heart disorders such as mitral stenosis and other congenital heart diseases, schistosomiasis infection, high altitude, lung disease, and idiopathic etiologies, but without direct measures of pulmonary vascular pressure, it remained challenging to define PH.

### Cardiac catheterization: an enduring diagnostic method

At the start of the 20th century, studies of pulmonary hemodynamics were largely confined to measurements of pulmonary arterial pressures in anesthetized animals on artificial respiratory devices. Studies were largely pioneered by French physiologists Claude Bernard and Marey who published experiments catheterizing a horse heart. The introduction of RHC in patients would ultimately change the field of PH and become the gold standard of diagnosis. RHC adoption into clinical use had a rocky beginning. In 1929, German surgeon in training, Werner Forssman urged physicians to adopt heart catheterization in the hospital but was firmly told not to attempt the procedure on patients. The bizarre story of Forssman deceiving a nurse to gain access to equipment and inserting a urinary catheter to his heart was told in his autobiography years later titled Experiments on Myself. His colleagues watched in fear as Forssman repeatedly walked up stairs to demonstrate the safety of the procedure. The stunt was considered irresponsible and unsafe. Following rejection from the medical community, Forssman abandoned his experiments. Dr. Forssman was terminated from his position in Germany for the feat, but his reports of his self-experimentation would go on to spark the interest of two American scientists over a decade later.

The adoption of heart catheterization to human patients was pioneered not by cardiologists, but by two American pulmonologists, André Cournand and Dickinson Richards. These pulmonologists were interested in obtaining mixed blood from the right side of the heart to use Fick’s principle to calculate cardiac output. Adolf Fick, in an 1870 short commentary at his local medical society in Würzburg Germany, stated that the cardiac output could be solved by simply obtaining an accurate respiratory gas of the mixed venous blood and arterial blood as well as the total oxygen intake into the lungs or the carbon dioxide elimination by the lungs. Mathematically, Fick’s equation was simple enough, but there was no direct method to access a sample of the mixed venous blood. Multiple attempts were made to gain respiratory gases of the mixed venous blood, most predominately rebreathing techniques that attempted to equilibrate the respiratory gases between the lungs and blood, but all were unsuccessful. Heart catheterization provided the needed access into the right atrium or the pulmonary artery for the blood sample, and after its recognition, for other uses, a Hamilton manometer was added to obtain cardiac and pulmonary pressures.
Cournand and Richards would take Forssman’s early attempt of heart catheterization and improve its clinical acceptance in the medical community. In their 1945 publication, the procedure was performed more than 260 times, often with the catheter left in place for up to 24 h with no complications. To demonstrate Fick’s principle of calculating cardiac output, a urinary catheter with a single opening at the tip was inserted into a large vein of the patient, most frequently the antecubital fossa. A flow of 1 to 2 drops per second of saline ran through the catheter, and the catheter was expertly guided under fluoroscopic control. Expired air was collected for 1 to 2 min, and 15 s after the collection of expired air, a collection of mixed venous blood from the right atrium followed by a sample of arterial blood 15 s later. Measurement of carbon dioxide and oxygen contents of the mixed venous blood and arterial blood was done on the Van Slyke-Neill apparatus and expired air samples were analyzed using the Haldane apparatus. This was the first utilization of Fick’s principle, but perhaps more importantly, the development of a safe procedure of heart catheterization in human patients. The two scientists were awarded the Nobel Prize in 1956 for adapting RHC to patient care. Dr. Forssman would share of third of the Nobel Prize for his contributions. Forssman responded to joint ownership of the Nobel Prize by stating “I feel like a village parson who has just learned he has been made bishop.”

PH: RHC brings new etiologies to our attention

Paul Wood, prior to the adoption of RHC into clinical use, outlined, in a 1959 review, the three basic types of PH at the time. Wood described passive PH, which is any condition that raises the pulmonary venous pressure, hyperkinetic PH caused by an increase in pulmonary blood flow induced by left to right shunting, and lastly vaso-occlusive PH. Vaso-occlusive PH was then subdivided into four major classes: (1) oblitative, defined by pulmonary vessel lesions or remodeling of the vessel itself, (2) vasoconstrictive, due to hyper-reactive muscular artery contraction, (3) obstructive evidenced by foreign or thromboembolic origin, and (4) polygenic PH due to many causes. Further complicating the PH categories was the use of term reactive PH which he stated was “the term used with advantage when it is not desired to beg the question of mechanism.”

One of the earliest reports of hypoxic vasoconstriction arose from the work by a Swedish physiologist, Ulf von Euler. Observing the pulmonary arterial pressure of an anaesthetized cat it was found that if the cat was ventilated with pure oxygen, the pulmonary arterial pressure was lowered and ventilation with lower oxygen levels raised it. In 1947, Motley et al. translated this discovery of hypoxic pulmonary vasoconstriction to humans. Motley, working in the Cournand laboratory, generated the first report of the effect of acute hypoxia on the pulmonary artery blood pressures. In this study, five male patients were given 10% oxygen in nitrogen, and right heart pressures were determined using RHC. The study found that PH was rapidly induced in the normal patients when breathing in hypoxic conditions and rapidly returned to normal when breathing was returned to normoxia. Pulmonary vascular resistance was doubled during hypoxia breathing conditions. The cause, however, was speculative at the time. Motley states that observations indicate that rapid changes of pulmonary arterial pressure are chiefly caused by variations in blood flow. However, the patients under hypoxia did not undergo increases in cardiac output, and in fact, the greater the decrease in cardiac output, the higher the corresponding change in pulmonary arterial pressure rise.

With the development and relative safety of RHC, physicians now had a tool to directly diagnose PH. In the 1960s, the first reports of large spikes of pre-capillary PH occurred across Europe. Aminorex is an appetite-suppressing drug that was widely available in several European countries in the 1960s. In countries where aminorex was available, there was a sudden 20-fold increase in PH occurrences. The link between patients with pre-capillary PH was the ingestion of aminorex for weight loss. Following the suspected link between aminorex and PH, Kay et al. administered aminorex to rats and dogs for up to 43 and 20 weeks, respectively. A detailed examination of the pulmonary vasculature, however, showed no evidence of pulmonary vascular disease. The exact mechanism of the anorexigen drug family and the pathogenesis of PH remain unknown. The current hypothesis is that since these drugs are 5-HT transporter substrates, the resulting increased blood 5-HT levels leads to increases in pulmonary blood pressure and arterial smooth muscle growth. Although devastating to the affected populations, this epidemic helped to spur discussions and interventions among physicians and scientists leading to the first World Health Organization meeting focused on PH.

World Health Organization: Geneva 1975

The World Health Organization held a meeting in 1975 following the epidemic in Geneva, Switzerland. Discussion of the classification of PH was a central topic of the meeting to address concerns with etiological classifications used by clinicians and morphological classifications developing in the field by morphologists. Clinicians identify and classify patients based on bedside and catheterization findings. Largely, clinicians were classifying patients with conditions known to cause right heart failure. These included chronic pulmonary thromboembolism and veno-occlusive disease. Heart diseases, which also cause PH, such as congenital heart disease that cause left-to-right shunting and mitral stenosis, were excluded from that category. If a patient remained uncategorized based on etiology of PH, they were known as PPH. However, morphologists classified PH based on changes of the lung architecture. PH was classified into three categories that included primary pulmonary
vascular disease (i.e. concentric intimal fibrosis, necrotizing arteritis, and plexiform lesions), primary veno-occlusive disease of the pulmonary veins and venules, and lastly pulmonary thromboembolism.\textsuperscript{33} Obviously, a large issue in nomenclature at the time was the commonly used term ‘primary pulmonary hypertension’, which was interpreted as both the clinical observation of PH of unknown etiology and PH caused by pulmonary vascular concentric intimal fibrosis, necrotizing arteritis, and plexiform lesions. The suggestion at the time was to convert the morphological term primary pulmonary hypertension into plexogenic pulmonary arteriopathy.\textsuperscript{32}

Under this established international clinical classification of PPH, this report outlined multiple causes of PPH. These include vasoconstriction or hyper-reactivity of the pulmonary arteries, hypoxia or high altitude, drug or toxin-induced PH, recurrent thromboembolisms, and connective tissue disorders.\textsuperscript{32} The clinical definition for the diagnosis of PPH was ambiguously defined as a pulmonary arterial pressure of $\geq 25$ mmHg.

In the WHO 1975 report, the clinical features of PPH were established. Importantly, it is stated that recognizing a patient in the early stages of the disease is difficult. Using the heart catheterization for the measurement of the pulmonary artery pressure was and still remains to be the gold standard diagnostic tool. However, as stated in the WHO report in the initial stages of PH, a higher pressure reading may only appear on exertion. Lastly of note, the report detailed the need for a non-invasive technique for the identification of early stage patients suspected of PH. Other benefits include monitoring of borderline patients and screening relatives. The development of non-invasive techniques to diagnose pulmonary hypertensive patients in early stage disease would provide the best opportunity for prolonging their lifespan.\textsuperscript{32}

**Current PH classifications**

Subsequent World Health Organization meetings have been held, and the nomenclature and grouping of vastly complex PH have been altered. What was once PPH is group 1 which consists predominately of the IPAH of unknown cause, the heritable PAH from known gene mutations, drug-induced PAH and lastly a few other distinct groups such as PH associated with HIV and connective tissue disease.

As the understanding of the molecular pathways involved in vascular function improved, a handful of drug treatments have been developed for the treatment of PAH. These treatments target the prostacyclin pathway, the nitric oxide pathway, and the endothelin pathway. Prior to the development of these PAH disease-specific drugs, the mean survival post-diagnosis of a patient was 2.8 years, but this has improved with therapeutic advances. PAH in particular remains a disease with high mortality rates and patients of different PAH etiologies have different life expectancies, primarily dependent on resolution of an underlying co-morbidity. Early diagnosis and treatment would certainly improve patient outcomes.

The most recent changes to diagnostic approaches for PAH were presented at the 6th World Symposium on Pulmonary Hypertension. It was discussed that recent published data indicate that normal mean pulmonary arterial pressure (mPAP) is $14.0 \pm 3.3$ mmHg. Two standard deviations above normal (97.5th percentile) would be $20$ mmHg. The standard $25$ mmHg was initially recognized to allow for clinicians to separate PAH from other causes of PH such as lung disease which typically have lower values of mPAP. However, it is possible that lowering the clinical definition to $\geq 20$ mmHg could help identify pulmonary hypertensive patients earlier in disease progression. The meeting has moved to also include pulmonary vascular resistance to the definition of diagnosis to determine whether a vascular disease is present with vascular resistance $\geq 3$ Woods Units.\textsuperscript{34}

Table 1 features the most current classifications of PH.

Recent publications and meeting proceedings have intensely debated the value of including mPAP during exercise as a clinically relevant diagnostic technique. The rationale for including mPAP during exercise relates to the microvascular recruitment that occurs in the lungs during increased cardiac outputs that may identify pulmonary arterial hypertensive patients earlier. Some data suggest that an occlusion of the majority ($>60\%$) of the microcirculation occurs before a patient becomes symptomatic.\textsuperscript{35} This is supported by the observations in patients with unilateral pulmonary artery absence or hypoplasia with normal pulmonary arterial pressures. Rises in pulmonary arterial pressures were only seen during exercise or further occlusion of existing pulmonary vasculature with a balloon-tipped catheter.\textsuperscript{36} While examining exercise PH could be beneficial for early diagnosis, the complexity of variables effecting patient change in pulmonary arterial pressure during exercise has complicated its use clinically for diagnosis and will likely continue to be reevaluated.\textsuperscript{34}

If a patient is misdiagnosed or undiagnosed for several months or years before receiving RHC, the opportune time for disease treatment may have already passed. The inclusion of stress mPAP in determining PH diagnosis has been brought forth and retracted throughout the history of WHO meetings. While it offers the exciting possibility of an earlier diagnosis, especially in borderline cases, patient safety during heart catheterization with exercise remains an issue. A study of 228 patients with systemic sclerosis identified 86 with borderline mPAP (21–24 mmHg). Patients with borderline mPAP were shown to be more likely develop PH than non-borderline patients. Also, the patients with borderline mPAP that developed PAH had a mortality of 18% within three years of diagnosis.\textsuperscript{37} It is likely that improvements of echocardiography on estimates of pulmonary pressures could reestablish the use of stress mPAP and offer insight into the progression of PAH in at risk patients. Figure 1 illustrates a time line of the major events leading us into the current age of PH classifications and diagnosis.
Table 1. Updated clinical classification of pulmonary hypertension (2019).

| Group 1: Pulmonary arterial hypertension | Group 2: PH associated with left heart disease | Group 3: PH-associated with lung disease/hypoxia | Group 4: PH due to pulmonary arterial obstructions | Group 5: Miscellaneous |
|-----------------------------------------|----------------------------------------------|-------------------------------------------------|-------------------------------------------------|----------------------|
| 1.1 Idiopathic PAH (IPAH)               | 2.1 PH heart failure with preserved LVEF     | 3.1 Obstructive lung disease                     | 4.1 Chronic thromboembolic PH (CTEPH)           | 5.1 Haematological disorders |
| 1.2 Heritable PAH (HPAH)                | 2.2 PH heart failure with reduced LVEF       | 3.2 Restrictive lung disease                     | 4.2 Other pulmonary obstructions                | 5.2 Systemic and metabolic disorders |
| 1.3 Drug/toxin-induced PAH              | 2.3 Valvular heart disease                   | 3.3 Other lung disease (mixed restrictive/obstructive) | 4.4 Hypoxia without lung disease                | 5.3 Others |
| 1.4 PAH associated with:                | 2.4 Congenital/acquired cardiovascular conditions leading to post capillary PH | 4.5 Hypoxia without lung disease                | 5.5 Developmental lung disorders               |
| Connective tissue disease (PH-CTD)     |                                              |                                                 |                                                 |                      |
| HIV infection (PH-HIV)                  |                                              |                                                 |                                                 |                      |
| Portal hypertension                     |                                              |                                                 |                                                 |                      |
| Congenital heart disease (PH-CTD)       |                                              |                                                 |                                                 |                      |
| Schistosomiasis                         |                                              |                                                 |                                                 |                      |
| 1.5 PAH long-term responders to calcium channel blockers | | | | |
| 1.6 PAH with venous/capillary involvement (PVID/PCH) | | | | |
| 1.7 Persistent PH of the newborn syndrome | | | | |

Fig. 1. Timeline of major events in the discovery and pathogenesis of pulmonary hypertension.
PH: pulmonary hypertension; HPV: Hypoxic Pulmonary Vasoconstriction; mPAP: mean pulmonary arterial pressure; PVR: Pulmonary Vascular Resistance.
Imaging in PAH: the past and future

Chest X-rays have long been used to evaluate the clinical status of patients since its introduction to medicine over a century ago. Chest X-rays of patients with severe PAH, identified by the appearance of an enlarged right heart border and enlarged pulmonary artery, are commonly seen in the early literature. Ninety percent of PAH patients have chest X-ray abnormalities at the time of their diagnosis, and some experimental studies have reported accuracy of chest X-ray use in PAH diagnosis. However, due to its limited sensitivity and specificity, chest X-ray plays little role in making an early diagnosis. Chest radiography can rule out moderate-to-severe lung disease and pulmonary venous hypertension due to left heart failure, but a lack of radiographic changes does not rule out PH or PAH. X-ray abnormalities also offer little information on disease progression or assessment of reversibility of pulmonary vascular remodeling in PAH.

The discovery of piezoelectricity by Curie and Curie in 1880 led to the prospect of creating ultrasonic waves. SONAR (sound navigation and ranging) technology utilized ultrasound technology which became useful in the First World War for detecting enemy submarines. The use of ultrasound was widespread into the 1930s, but unlike its main use in the present-day clinical setting for imaging, ultrasound was typically used early on for therapeutic purposes in diseases that heat was thought to be beneficial. In the 1940s, physicists and physicians alike began considering the ultrasound techniques used for exploring the ocean depths and detecting flows in constructions to produce images of the interior body structure. German physicist, Wolfe Keidel, was perhaps the first to imagine the use of ultrasound for obtaining information of the structure of the heart. However, multiple attempts at first images of the heart using an echocardiograph were unsuccessful and Kleidel determined his work to have “immense technical problems.” No progress would be made until Swedish physician Dr. Inge Edler, the director of the cardiovascular laboratory, became interested in using ultrasound to non-invasively evaluate mitral valve stenosis preoperatively. Kleidel along with physicist Hellmuth Hertz produced the first Amplitude-mode (A-mode) images on isolated water-filled hearts. Importantly, they would later use a continuous recording to record the changing depths from moving cardiac structures in time-motion ultrasound (M-mode) creating the first moving images of the heart. Kleidel is now acknowledged as the “Father of Echocardiography” and developments to the technique including 2D, Doppler, Color Doppler, and 3D echocardiography follow from Kleidel’s original pioneering work.

The transthoracic echocardiogram (TTE) is the initial test of choice and is most valuable in detecting left heart dysfunction as a potential cause of PH, and in examining the degree of right heart involvement by assessing right heart chamber size, wall thickness and function. Although TTE can estimate pulmonary artery systolic pressure by using tricuspid regurgitation velocity (TRV) and right atrial pressure, the angle-dependent nature of Doppler signals and limitations of right atrial pressure measurement, which itself is another indirect estimation based on inferior vena cava size and respiratory variation. RHC is still required to obtain accurate pulmonary artery pressure and diagnose PAH.

Improvements in computed tomography (CT) scanning and magnetic resonance imaging (MRI) have led to their use in pulmonary analysis. CT scanners were first introduced in the 1960s and were an exciting step for clinical imaging improvement. Even the Beatles were associated with the fundraising effort, famously donating record sales at the time to the cause. While improving the image of patients for clinicians by both MRI and CT technological advances, screening for PH by these methods is reliant upon pulmonary artery enlargement and right heart hypertrophy seen in later disease states. The techniques are useful for enhanced views of lung parenchymal disease, identifying potential pulmonary venous occlusive disease, and can clarify location and surgical accessibility of thromboembolisms in potential CTEPH patients.

Ventilation-perfusion (V/Q) scanning does not require iodine contrast and plays a unique role in diagnosing CTEPH with high sensitivity and specificity, differentiating it from IPAH. However, due to a high rate of indeterminate findings and inability to assess specific vascular or parenchymal pathology, its use is limited to initial screening of suspected PAH or estimation of postoperative pulmonary reserve in surgical candidates.

18-Flurodeoxyglucose positron emission tomography (18FDG-PET/CT) has, in the last decade, become important in the diagnosis and follow-up for cancer patients. 18-Fluorodeoxyglucose is a glucose analogue, readily taken up into highly proliferative cells, such as cancer. PET utilization with 18-fluorodeoxyglucose allows for imaging of areas with altered metabolism. A handful of studies have used this same technique to determine if lesions of PAH can be detected in a similar way. One study looked at IPAH patients, CTEPH and control patients. Hagan et al. evaluated the use of 18-fluorodeoxyglucose PET imaging in IPAH and CTEPH patients. IPAH patients had significantly higher lung parenchymal and right ventricular FDG uptake compared to controls. Further, NT-ProBNP levels were correlated with RV uptake in both IPAH and CTEPH patients. This indicates a potential for this technique to screen patients for arterial lesions forming early in the disease pathogenesis of PAH. However, the study has only a few patient samples and further work is needed to support the evidence of its potential in PAH screening.

A summary of imaging principles and current imaging research in the field of PH can be found in Table 2. The diagnostic algorithm to distinguishing PH patients...
Biomarkers

One of the most commonly used non-invasive and cost-effective approaches to evaluating patients is biomarkers. Biomarkers have been used clinically and in basic research for decades. The NIH definition for a biological marker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic process, or pharmacological responses to a therapeutic intervention.”\(^4^{10} \)

Clinically, the only biomarkers of PH used today are the natriuretic peptides. The natriuretic peptides of cardiac origin are synthesized and secreted into the group classifications is shown in Fig. 2. This figure is meant to be a simplified diagnostic algorithm and for a more detailed review of diagnosing PH, see elsewhere.\(^4^{8} \, 4^{9} \)

### Table 2. Investigating imaging principles in pulmonary hypertension.

| Imaging principle | Modality | Application | Reference |
|-------------------|----------|-------------|-----------|
| **Radiation**     | Chest X-ray | 90% of PAH patients have abnormal chest radiograph  
|                   |          | Allows identification of mild-to-severe lung diseases or pulmonary venous hypertension due to left-heart disease  
|                   |          | Degree of PH does not correlate with radiographic abnormalities  
|                   |          | Normal radiograph does not rule out PH  
| CT scan           |          | Improved identification of interstitial lung disease from Chest X-ray  
|                   |          | Can provide evidence in PVOD  
|                   |          | Can improve clarification of CTEPH  
| **Ultrasound**    | M-Mode Echo | Allows fine measurements with higher temporal and spatial resolutions  
|                   | 2D Echo  | Allows structures to be viewed in real time in a cross-section of the heart  
|                   | 3D Echo  | 3D views of structural design  
| Doppler echo      |          | Allows estimation of key hemodynamic and functional parameters such as mPAP and tricuspid annular plane systolic excursion (TAPSE), respectively  
|                   |          | Offers estimation of mPAP by measuring PAAT and CO* (with limitations)  
|                   |          | Estimation of tricuspid regurgitation  
|                   |          | Echo is not sensitive to mild and asymptomatic PH  
| **Magnetic resonance** | MRI | Good spatial and high temporal resolution and multiplanar imaging useful in diagnosis and etiology  
|                   |          | Offers methods of assessing pulmonary blood flow, PFA (pulmonary blood flow artifact) and vortical blood flow  
| **Nuclear imaging** | FDG-PET | Images indicating regions of altered metabolism  
|                   |          | Studies show increased FDG uptake in RV and lung parenchyma in PAH patients  

PH: pulmonary hypertension; PAH: pulmonary arterial hypertension; PVOD: Pulmonary Vascular Obstructive Disease; CTEPH: chronic thromboembolic pulmonary hypertension; mPAP: mean pulmonary arterial pressure; FDG: 18-flurodeoxyglucose; RV: Right Ventricle; PAAT: pulmonary artery acceleration time; MRI: magnetic resonance imaging; FDG-PET: 18-flurodeoxyglucose positron emission tomography.
circulation by the heart during ventricular overload and dysfunction. There are two major types of natriuretic peptides: brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP); the main source of BNP is the heart ventricles, suggesting that BNP is more useful for evaluating ventricular dysfunction.51 Indeed, several studies have now shown that BNP is a more reliable biomarker of right ventricular overload in PH than ANP.51,52

BNP was isolated from the porcine brain in 1988 by Sudoh et al. and was later found in the porcine heart. Human studies of BNP were lacking a monoclonal antibody until 1991.53 Mukoyama et al. began human BNP studies using radioimmunoassay to link increased levels of serum BNP with patients undergoing left ventricular overload in congestive heart failure (CHF).53 Relevance to BNP in PH and consequential right ventricular overload was established by Nagaya et al.51 Nayagaya et al. evaluated patients with primary PAH (now known as idiopathic PAH), chronic thromboembolic PH (CTEPH), and patients with atrial septal defect. Compared to control volunteers, all patients evaluated had increased plasma levels of both ANP and BNP, and these levels correlated with ventricular dysfunction. Further, the study looked at ANP and BNP outcomes to short-term treatment Nitric Oxide (NO) therapy and long-term treatment (long-term vasodilator response). While ANP was affected by short-term therapy and was easily influenced by variables such as blood pressure, age, and sodium intake, BNP was shown to be a more reliable biomarker of long-term therapy. BNP is also a non-invasive marker for efficacy of pulmonary thromboendarterectomy in CTEPH patients.54 Unfortunately, this fundamental biomarker offers little insight into early PH diagnosis prior to the development of right ventricular hypertrophy. Despite the current limitations of clinically used biomarkers of PH, basic research has identified several biomarkers of interest awaiting to be vetted clinically.

Certainly, accessible cells have been of interest in the biomarker field for years. Pool and Dunlop detected non-hematological cells in the blood of cancer patients as early as 1934 (Pool and Dunlop, 1934). Early in the field, a lack of a method to isolate circulating cells in the blood samples of humans resulted in dependency on immunohistochemistry of peripheral blood smears.55,56

Circulating cells of primary interest as biomarkers are circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPCs). CECs represent a population of detached differentiated mature endothelial cells. Since the improvement of isolation methodologies, such as immunomagnetic isolation, an increase in CECs has been associated with vascular injury.55 Interest in CECs in PAH arose from the hypothesis that they are derived from the damaged endothelial barrier and particularly from plexogenic lesions.57

A second source of circulating cells was first identified in 1997 by Dr. Jefferey Isner of the Tufts University. Dr. Isner isolated a population of mononuclear blood cells, that after seven days of incubation in endothelial growth medium, displayed endothelial morphology.58,59 This subset of

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**Fig. 2.** Diagnostic algorithm for pulmonary hypertension. A simplistic representation of the diagnostic methods used currently to distinguish patients into the most recent classifications (2019) of the five groups of pulmonary hypertension (PH). *Subgroup 3.4 Hypoxia-associated PH patients lack lung disease. **Subgroup 1.6 PAH with venous/capillary involvement and may display unmatched perfusion defects. ***Subgroup 1.5 PAH is differentiated as long-term responders to calcium channel blockers (CCB) following approximately one year of treatment. ECG: electrocardiogram; TTE: transthoracic echocardiography; HRCT: high-resolution computed tomography; PFT: pulmonary function test; RHC: right heart catheter.
circulating cells called CEPCs arise from the bone marrow and can be stimulated to participate in postnatal neovascularization. These CEPCs were characterized by their expression of CD34, VEGFR2, and CD133.99 However, the accepted characterization of CECs and CEPCs remains a controversial field. Unsurprisingly, the use of circulating cells as biomarkers in PH remains controversial with often conflicted data. In 2003, Bull et al. found that the number of CECs (E-selectin and CD36+) in both primary and secondary PH patients correlated with a rise in pulmonary arterial pressure. Smadja et al. investigated CECs (CD146+) and CEPCs (CD34+CD133+) across PAH patients, CTEPH patients, and normal control volunteers.57 No difference in CEP counts was found across all three groups. CECs (CD146+) were found to be significantly increased in PAH patients. Smadja et al. examined PAH patients with congenital heart disease and investigated CEPCs (146+) in PAH secondary to congenital heart disease (CHD).60 In CHD-associated PAH, it is clinically beneficial to identify CHD with reversible or irreversible vascular remodeling to predict a patient’s reversibility of PAH pathology after surgical correction. Currently, this is not directly possible and can only be suggested by lung biopsy. The study determined CEC levels to be the only relevant biomarker for discrimination of PAH reversibility.60 A separate study examined CECs and CEPCs (CD34+CD133+) of PAH and CTEPH patients. CECs were found to be significantly higher in PAH patients but not in CTEPH patients and CEPCs did not differ across all patient samples.61 In contrast, CEPC decreases have been reported in several studies,62,63 and CEPC increases were found in others.64,65 The key to the contradicting findings could be a result of differing detection methods (flow cytometry, immunohistochemistry, versus immunomagnetic separations) and differing patient PH populations and the timeline of development of the disease.

Endothelial dysfunction attributable to reduced bioavailability of nitric oxide (NO) plays an important role in PH pathogenesis. Alterations in NO signaling influence pulmonary vasoreactivity. As a biomarker, players in the regulation of NO synthesis and degradation could be very valuable clinically. Asymmetric dimethylarginine (ADMA) is the most abundant endogenous NO synthesis inhibitor first identified in 1992 by Vallance et al.66,67 Gorenflo et al. evaluated patients with congenital heart disease with and without PH. The study found that ADMA was increased in CHD patients with PH compared to both control patients and CHD patients without PH.68 Further studies have shown increases in plasma ADMA in CTEPH and IPAH patients.69 Telo et al. investigated high serum levels of ADMA for predicting the presence of PH in chronic obstructive lung disease (COPD) patients, finding a significantly positive correlation between ADMA and pulmonary arterial pressures.70 Further, Siques et al. determined ADMA to be a useful predictor of hypoxic PH.67 Limitations of these studies include population size and lack of patient diversity. However, ADMA shows promise as a clinical marker for PH, and evidence warrants further controlled clinical trials to investigate its biomarker implications for early diagnosis.67

While the role that inflammation plays in different etiologies of PH is controversial, there is no question to its presence. Since Tuder et al. examined PAH vessels for CD45+ leukocytes, the important question of the role that localized inflammatory leukocytes play has been ever present in the field.71 Several studies have found evidence of inflammation in IPAH, PAH associated with connective tissue disease, HIV-associated PAH, and drug-induced PAH. Tuder et al.’s histological studies of plexogenic lesions in 1994 also identified the presence of CD45+ leukocyte perivascular clusters around the plexiform lesions; more specifically, T cells and macrophages populations were identified.71 Studies suggest that PAH in systemic sclerosis patients have more pronounced inflammatory processes than in other PAH subtypes.72 Humbert et al. identified increased serum levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6).73 Thus, markers of inflammation may shed light on progression or specifically to vascular injury. While this is not a comprehensive review of biomarkers in PH and PAH, which can be found elsewhere,74,75 this section is meant to highlight major areas of biomarker research ongoing in the field. Table 3 contains a selection of biomarker studies in both PH and PAH.

An emerging player on the biomarker forefront, extracellular vesicles (EVs) are a heterogenous population of exosomes, microparticles (MPs), and apoptotic vesicles.76 The initial identification of so-called “Platelet Dust” by Peter Wolf in 1967 identified MPs of platelet origin, thought to be cellular trash.77 More than 30 years passed before it was recognized that these intact vesicles are released by a variety of cell types.78–80 A key characteristic of EVs is that they contain cytosolic proteins, RNA, and DNA and cellular membrane characteristics based on both their cell of origin and type of stimulus. The field has largely been hindered by a lack of standardization across naming and isolation procedures of EVs. However, characterization of isolated vesicles has been at the forefront of the field for a decade now, and due to publications recommending standard expectations for publication, which most journals are implementing, there have been significant improvements in data reliability and consistency.81,82

EVs are primarily characterized by size, under 1 μm, and membrane markers for identification of the parent cell type. Thus, in the literature prior to around 2016, the terms MP and microvesicle were used interchangeably, while exosomes were considered smaller with unique markers. A history of classification and isolation disagreement has plagued the field of EVs, but the formation of scientific societies such as the American Society for Exosomes and Microvesicles and the International Society of Extracellular Vesicles has improved international EV science and worked to
| Biomarker | Patient sample | Control | Key findings | Study |
|-----------|----------------|---------|--------------|-------|
| **Cardiac** | | | | |
| Atrial natriuretic peptide (ANP) | 18 Atrial septal defects | Control patients | †ANP in PH Less reliable than BNP | Nagaya et al.51 |
| | 10 IPAH | | | |
| | 16 CTEPH | | | |
| | 9 PPH | Six healthy control | †ANP in PH No correlation between ANP and hemodynamic variables | Morice et al.52 |
| | 18 CHD-PH | 21 CHD w/o PH | †ANP in CHD-PH compared to CHD w/o PH | Gorenflo et al.149 |
| Brain natriuretic peptide (BNP) | 18 Atrial septal defects | Control patients | †BNP levels in proportion to extent of RV dysfunction in PH | Nagaya et al.51 |
| | 10 IPAH | | | |
| | 16 CTEPH | | | |
| | 34 CTEPH | 12 Control | †BNP significantly increased in CTEPH compared to control BNP levels decreased in survivors after PEA | Nagaya et al.54 |
| N-terminal-pro-b-type natriuretic peptide (NT-proBNP) | 55 Severe PH (36 IPAH) | 9 Control | †NT-pro-BNP identified patients with poor prognosis | Fijalkowska et al.150 |
| | 65 PAH | None | †NT-pro-BNP associated with worse WHO functional class and increased mortality | Al-Naamani et al.151 |
| | 69 PAH | 41 sScI w/o PH | NT-ProBNP levels correlate with severity of PAH and increased risk of sScI developing PAH | Williams et al.152 |
| | 40 PAH | None | †NT-pro-BNP across all PH groups Independent predictor of mortality Decreased in survivors medically treated | Andreassen et al.153 |
| | 19 CTEPH | | | |
| | 2 Miscellaneous (sarcoidosis) | | | |
| | 76 IPAH | None | †NT-pro-BNP in IPAH significant predictor of adverse outcome | Nickel et al.154 |
| Troponin T | 97 PAH | 56 Normal | †NT-pro-BNP in PAH | Malhotra et al.155 |
| | 56 PAH | None | †Cardiac TnT independent marker of increased mortality | Torbicki et al.156 |
| | 5 CTEPH | | | |
| | 20 IPAH | None | Correlation between TnT and 6MWD/DT distance, RV systolic strain. | Filusch et al.157 |
| | 30 CTEPH | | | |
| | 5 LD-PH | | | |
| Heart-Fatty Acid Binding Protein (H-FABP) | 93 CTEPH | None | †H-FABP independent predictor of adverse outcome Patients with elevated H-FABP had lower probability of event free survival after PEA | Lankeit et al.158 |

(continued)
### Table 3. Continued

| Biomarker | Patient sample | Control | Key findings | Study |
|-----------|----------------|---------|--------------|-------|
| **Endothelial dysfunction** | | | | |
| Asymmetric  | 135 CTEPH | 40 Control | ↑ADMA in CHD | Skoro-Sajer et al.\(^{159}\) |
| Dimethylarginine (ADMA) | | 9 Healthy control | Lowered in CTEPH after surgery | |
| (children) | 30 PAH-CHD | 20 CHD without PH | ↑ADMA in PH-CHD | Sanli et al.\(^{160}\) |
| | 12 PAH | 35 Controls | ↑ADMA in CTEPH and PAH | Zhang et al.\(^{161}\) |
| | 23 CTEPH | 20 Healthy | | |
| Nitric oxide synthase | 46 Various PH | 23 Control | ↓NO synthase in PH | Giaid et al.\(^{162}\) |
| **Inflammation** | | | | |
| Interleukin-1Beta (IL-1β) | 29 PPH | 9 COPD-PH | ↑IL-1β in PPH | Humbert et al.\(^{73}\) |
| | 15 normal control | | Not in COPD-PH | |
| | 60 PAH | 21 Control | ↑IL-1β in PAH predicted patient survival | Soon et al.\(^{163}\) |
| Interleukin-2 (IL-2) | 60 PAH | 21 Control | ↑ in PAH | Soon et al.\(^{163}\) |
| | | | Predicted patient survival | |
| Interleukin-4 (IL-4) | 60 PAH | 21 Control | ↑IL-4 in PAH | Soon et al.\(^{163}\) |
| Interleukin-6 (IL-6) | 29 PPH | 15 Control | ↑IL-6 in PPH | Humbert et al.\(^{73}\) |
| | 60 PAH | 21 Control | ↑IL-6 in PAH | Soon et al.\(^{163}\) |
| | | | Predicted patient survival | |
| Interleukin-8 (IL-8) | 60 PAH | 21 Control | ↑IL-8 in PAH | Soon et al.\(^{163}\) |
| | | | Predicted patient survival | |
| Interleukin-10 (IL-10) | 60 PAH | 21 control | ↑IL-8 in PAH | Soon et al.\(^{163}\) |
| | | | Predicted patient survival | |
| Interleukin-12p70 (IL-12p70) | 60 PAH | 21 control | ↑IL-8 in PAH | Soon et al.\(^{163}\) |
| | | | Predicted patient survival | |
| CD40 Ligand (CD40L) | 10 sScl-PAH | 20 Control and 40 sScl w/o PH | ↑sCD40L in PAH associated with sScl | Allanore et al.\(^{164}\) |
| | | | sCD40L correlated with mPAP (Doppler Echo) | |
| | 13 PPH | 8 Normal | ↑CD40L in PPH and SPH but not CTEPH compared to control | Damás et al.\(^{165}\) |
| | 11 Secondary PH | | | |
| | 8 CTEPH | | | |
| B-cell lymphoma 2 (BCL2) | 35 PAH (children) | 38 Control children | sBcl-2 were increased in patients with lower 6MWD test | Akin et al.\(^{166}\) |
| Tumor necrosis factor-alpha (TNF-α) | 60 PAH | 21 Control | ↑TNF-α in PAH | Soon et al.\(^{163}\) |
| Growth Differentiation Factor-15 (GDF-15) | 76 IPAH | None | ↑GDF-15 in IPAH is an independent predictor of adverse outcomes | Nickel et al.\(^{154}\) |
| | 30 SSc-PAH | 24 SSc w/o PAH | ↑GDF-15 in SSc-PAH | Meadows et al.\(^{167}\) |
| | 44 IPAH | 13 normal | patients compared with SSc patient w/o PAH | |
| | | | GDF-15 correlated with estimated right ventricular systolic pressure (echo-cardiogram) and associated with reduced survival | |
## Table 3. Continued

| Biomarker                        | Patient sample | Control                  | Key findings                                                                 | Study                        |
|----------------------------------|----------------|--------------------------|------------------------------------------------------------------------------|------------------------------|
| **Pro-coagulation**              |                |                          |                                                                              |                              |
| Plasma P-selectin                 | 32 PPH         | 31 PVH                   | † plasma P-selectin in PPH and sPAH compared to PVH and control               | Sakamaki et al.168           |
|                                  | 25 secondary PH| 17 Control               |                                                                              |                              |
|                                  | 44 CTEPH       | 22 Control               | † plasma P-selectin in CTEPH                                                | Sakamaki et al.169           |
|                                  |                | 22 after APTE            |                                                                              |                              |
| Thrombomodulin (TM)              | 32 PPH         | 25 sPAH                  | ‡ TM in PPH compared to other groups                                         | Sakamaki et al.168           |
|                                  | 25 sPAH        | 31 PVH                   |                                                                              |                              |
|                                  |                | 17 Control               |                                                                              |                              |
|                                  | 44 CTEPH       | 22 Healthy subjects      | ‡ TM in CTEPH                                                                | Sakamaki et al.169           |
|                                  |                | 22 After APTE            |                                                                              |                              |
| Thromboxa B2                     | 65 PAH         | None                     | Higher TX B2 associated with worse WHO functional class                      | Al-Naamani et al.151         |
| CX3CR1 T-lymphocyte expression   | 7 PAH          | 7 Healthy                | † CX3CR1 T lymphocyte expression                                            | Balabanian et al.170         |
| and sfkn (CX3CL1)                |                |                          |                                                                              |                              |
| vonWillebrand Factor (vWF)       | 65 PAH         | None                     | Higher vWF associated with lowered 6MWD test, worse WHO functional class and increased mortality and transplant | Al-Naamani et al.151         |
| **Growth factors**               |                |                          |                                                                              |                              |
| Platelet Derived Growth Factor   | 13 PPH         | 5 Control lung samples   | † PDGF positive cells in PPH and PPH-HIV                                     | Humbert et al.73             |
| (PDGF)                           | 2 PPH-HIV      |                          |                                                                              |                              |
| Vascular Endothelial Growth     | 97 PAH         | 56 normal                | † sVEGFR1 in PAH Correlated with NYHA class                                  | Malholtra et al.155          |
| Factor Receptor I (Soluble VEGFR1) |             |                          |                                                                              |                              |
| **Pro-angiogenic**               |                |                          |                                                                              |                              |
| soluble endoglin (sEng)          | 97 PAH         | 56 Normal                | † sEng in PAH Independent predictor of survival Correlated with NYHA class   | Malholtra et al.155          |
| Soluble fms-like tyrosine kinases (flt-1) |       | 62 IPAH                 | † sFlt-1 in all PH groups except PV-PH compared to control                   | Tiede et al.171              |
|                                  | 14 APAH        | 40 Normal                |                                                                              |                              |
|                                  | 21 CVD         |                          |                                                                              |                              |
|                                  | 67 LD-PH       |                          |                                                                              |                              |
|                                  | 26 PV-PH       |                          |                                                                              |                              |
| Soluble Placental Growth Factor  | 62 IPAH        | 40 Normal                | † sPIGF in all PH groups compared to control                                 | Tiede et al.171              |
| (PiGF)                           | 14 APAH        |                          |                                                                              |                              |
|                                  | 21 CVD         |                          |                                                                              |                              |
|                                  | 67 LD-PH       |                          |                                                                              |                              |
|                                  | 26 PV-PH       |                          |                                                                              |                              |
| **Circulating cells**            |                |                          |                                                                              |                              |
| Circulating Endothelial         | 16 IPAH        | 16 Control               | † CEPC (+CD34+) In PAH                                                        | Asosingh et al.64            |
| Progenitor Cell (CEPC) (CD34+/CD133+) |            |                          |                                                                              |                              |
| Circulating Endothelial Cell (CEC) (CD 146+) |         |                          |                                                                              | Smadja et al.172             |
|                                  | 26 CHD-PH      | 5 Control children       | No increase in CEPC in CHD-PH                                                |                              |
|                                  | 10 irreversible CHD-PH | | † CEC in irreversible CHD-PH                                                | Smadja et al.172             |
|                                  |                |                          |                                                                              |                              |
standardize EV protocols. However, issues with classification between EV populations still arise; thus, the accepted current terminology is the extracellular vesicle, to encompass all such intact, circulating vesicles. Research in EVs in PH was spurred by evidence of their association with several cardiovascular diseases. EVs of endothelial origin are indicators of endothelial dysfunction or injury, and this dysfunction plays a role in PH pathogenesis. Amabile et al. found MPs of endothelial and leukocyte origin to be increased in patients with precapillary PH. Populations of MPs with PECAM+, VE-cadherin+, and E-selectin of endothelial origin were further shown to positively correlate with mPAP and PVR. Amabile’s group also performed a longer study following 21 PH patients for one year after their initial RHC and diagnosis. The seminal finding was that the initial baseline values of CD62+ MPs (endothelial in origin) predicted patient outcomes. Participants with higher values of CD62+ MPs had increased clinical complications and a significantly worse prognosis compared to patients with lower baseline levels of CD62+ MPs. These data indicate that endothelial MPs are a potential biomarker for predicting patient outcome. Endoglin-positive MPs, also considered of endothelial origin, were increased in PH patients compared to healthy controls. Further, this group sampled both the pulmonary arterial blood and the venous circulation in PAH patients and found that the endoglin-positive MPs were significantly increased in the pulmonary arterial blood which may be

| Biomarker | Patient sample | Control | Key findings | Study |
|-----------|----------------|---------|--------------|-------|
| Oxidative stress | Urinary F2-Isoprostane | 110 PAH | None | †Urinary F2-Isoprostane associated with increased mortality | Cracowski et al. |
| | | | | | |
| Miscellaneous | cyclic Guanosine Monophosphate (cGMP) | 18 CHD-PH | 21 CHD w/o PH | †cGMP in CHD-PH | Gorenflo et al. |
| | Uric acid | 99 PPH | None | Correlation btw natural logarithm of serum Uric Acid and mPAP | Voelkel et al. |
| | | 93 Secondary PH | | | |
| | | 90 PPH | 30 Normal | †UA in PPH compared to control | Nagaya et al. |
| | | | | Serum UA correlated with total pulmonary resistance and is independently related to mortality | |
| Bilirubin | 37 PAH | None | | †Bilirubin associated with worse functional classification and increased mortality | Takeda et al. |
| | Homocysteine | 30 PAH-CHD (children) | 20 CHD w/o PAH | †Homocysteine in CHD-PAH | Sanli et al. |
| | | | 20 Normally | | |
| C-reactive protein | 97 PAH | 56 Normal | | †CRP in PAH | Malholtra et al. |
| | 104 PAH | 95 Normal | | †CRP in CTEPH and PAH compared to control | Quarck et al. |
| | 79 CTEPH | | | CRP correlated with NYHA functional class, right atrial pressure, and 6MWDT | |
| | | | | CRP significantly lowered after successful PEA | |
| Red blood cell distribution width (RDW) | 162 PH | None | | †RDW is independently associated with increased mortality in PH | Hampole et al. |

PAH: Pulmonary Arterial Hypertension; PH: Pulmonary Hypertension; PAH-CHD: Pulmonary Arterial Hypertension associated w/ Congenital Heart Disease; CTEPH: Chronic Thromboembolic Pulmonary Hypertension; PPH: Primary Pulmonary Hypertension; IPAH: Idiopathic Pulmonary Arterial Hypertension; CVD: Collagen Vascular Disease; LD-PH: Lung Disease associated w/ Pulmonary Hypertension; PV-PH: Pulmonary Venous Pulmonary Hypertension; APAH: Associated Pulmonary Hypertension; PPH-HIV: Primary Pulmonary Hypertension associated w/ Human Immunodeficiency virus; SSc-PAH: Systemic Sclerosis associated w/ Pulmonary Arterial Hypertension.
reflective of the importance of endoglin signaling in the pathogenesis of PAH. More recently, a study focused on patients with PH associated with systemic sclerosis found that CD144⁺ MPs were significantly higher in patients with PH associated with systemic sclerosis compared to both healthy patients and systemic sclerosis patients without PH. Considering the important role of the pulmonary endothelium in PH, altogether, these data would indicate that EVs, especially of an endothelial origin, have the potential to be a valuable biomarker.

Reports of EVs from platelets in PH patients have been contradictory. Amabile found the levels of platelet MPs of (CD41⁺) to be unaltered in PH patients. In contrast, other studies have found increased levels in platelet MPs. Platelet and RBC EVs are of particular interest in patients with β-thalassemia-induced PH. The mechanism of PH development in this subset of patients is hypothesized to result from chronic activated platelets and abnormalities of the RBC membrane. Both platelet and RBC EVs were shown to be increased in PH patients with β-thalassemia.

In addition to markers indicating the cell of origin, other characteristics of EVs have been investigated as potential biomarkers. MPs, a subset of EVs, are thought to be derived from cell membranes that have exposed phosphatidylserine (PS), and a common approach to quantifying or identifying MPs is through FACS analysis following annexin V staining. However, reports of MP populations lacking PS on their membrane complicate the MP field. MPs of platelet and EV origin display procoagulant molecules such as PS and active tissue factor. Both characteristics can contribute to a procoagulant environment in PH; in fact, active tissue factor EVs were shown to be present at increased levels in patients with higher WHO functional class and low 6 Minute Walking Distance Test (6MWDT) distance.

Circulating intravascular adenosine nucleotides that participate in cell signaling pathways can induce vascular changes with similarities to IPAH pathology. The dephosphorylation of adenosine triphosphate (ATP) in the extracellular environment is primarily regulated by the ectonucleotidase (ENTPD1) CD39. CD39⁺ MPs contain nucleotidase activity that could importantly alter extracellular ATP-related signaling and contribute to PH pathology. CD39 is found on the cellular membranes of leukocytes, platelet, and endothelial cells, and importantly for its role as a biomarker, CD39 may act as an EV marker characteristic to vascular dysfunction. CD39 + EVs have indeed been found to be increased in patients with IPAH and awaits further research to determine not only its functional role in PH pathogenesis but its potential as a biomarker.

Where can animal models take us? Historical perspective and future directions

While PH biomarkers are increasingly needed to evaluate and screen for PH and PAH, a core issue lies with finding

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**Table 4. Extracellular vesicle markers in pulmonary hypertension patient populations.**

| EV marker | Patient population | Findings | Reference |
|-----------|--------------------|----------|-----------|
| **EV origin marker** | | | |
| CD105 + MP (Endoglin) | 20 PAH | ↑CD105 + MPs in PAH | Bakouboula et al.87 |
| CD62E + MP (E-selectin+) | 6 CTEPH | ↑CD105 + MPs in CTEPH | Belik et al.179 |
| CD105 + MP (Endoglin) | 24 PH | ↑CD62 + MPs in PH | Amabile et al.85 |
| CD144 + MP (VE-cadherin+) | 24 PAH | Baseline increased CD63 + MPs associated with adverse clinical complications | Amabile et al.86 |
| CD31+/CD41- MP (PECAM+) | 24 PH | ↑CD31 + MPs in PH Predicted hemodynamic severity | Amabile et al.85 |
| CD31+/CD61 + (Platelet) | 19 PH | ↑CD31+/CD61 + in PH | Diehl et al.89 |
| CD144 + MP (VE-cadherin+) | 24 PH | ↑CD144 + MPs in PH Predicted hemodynamic severity | Amabile et al.85 |
| CD45 + (leukocyte) | 24 PH | ↑CD45 + in PH | Amabile et al.85 |
| CD41 + MPs (platelet) | 24 PH | No change in CD41 + MP in PH | Amabile et al.85 |
| **EV characteristics** | | | |
| TF + MP | 20 PAH | ↑TF + MPs in PAH Correlated with PH severity | Bakouboula et al.87 |
| CD39 + (CD31+CD42b-) (CD31+CD42b+) | 10 IPAH | ↑CD39 + in IPAH | Visovatti et al.97 |

PH: pulmonary hypertension; PAH: pulmonary arterial hypertension; IPAH: idiopathic pulmonary arterial hypertension; MP: microparticle.
patient samples of early PH disease pathology. To examine biomarkers of early disease pathology and disease mechanisms, research has relied heavily on pulmonary hypertensive animal models. Rodents are increasingly used in research due to ease of housing, costs, and relatively short gestational period. While many studies have identified rodent PH models with human characteristics, an appropriate single model of human PH remains controversial.\textsuperscript{98,99}

Perhaps the most prevalent method used for inducing PH in animal models has been hypoxia exposure. Evidence of hypoxia-induced PH in animals arose from Colorado cattle farms where cattle began dying of high altitude-induced PH and right heart failure, termed Brisket disease. Grover and Reeves of the Cardiovascular Pulmonary Research Laboratory (CVP) at the University of Colorado pioneered the first experimental induction of PH in cattle at high altitudes for the study of Brisket disease. The team examined the effect of high-altitude exposures in cattle comparing pulmonary arterial pressure and right ventricular hypertrophy to cattle from low elevation found that 4 out of 10 animals at high altitude developed significant PH. Further, evidence of right ventricular hypertrophy was seen in the high-altitude cattle and was strongly correlated with mPAP.\textsuperscript{100}

Use of hypoxic chambers for induction of rodent PH is now a common procedure, and today they are combined with various secondary genetic, drug treatment, or surgical alterations to invoke varying degrees of PH and some PAH pathophysiology. Rats were exposed to simulated hypoxia as early as 1978.\textsuperscript{101} Following the spontaneous cattle PH research in Denver Colorado, Reeves and colleagues subjected rats to simulated high-altitude conditions. Lungs subjected to high altitude had hyporeactive responses to hypoxia compared to lungs subjected to lower altitude exposure, and this led to a better understanding between the mechanisms of hypoxic pulmonary vasoconstriction and the causative relationship with PH.\textsuperscript{102} Hill et al. began looking at potential biomarkers by exposing male Sprague-Dawley rats to hypoxia in hypoxic chambers for three weeks and evaluating plasma levels of ANP and BNP.\textsuperscript{102} Importantly, the study concluded that both ANP and BNP levels were increased by 70% during chronic hypoxia, an increase that coincides with human patient studies and highlights a similar pathology. The typical process of chronic hypoxia-induced PH is an exposure of 10% O$_2$ for up to four weeks in either normobaric or hypobaric atmospheric environments. Hypoxia-induced PH does represent a true human pathological stimulus of human PH. It is representative of clinical disease in patients with lung parenchymal disease (COPD, emphysema, etc.), obstructive sleep apnea, and high-altitude-induced PH. However, hypoxia-induced PH in rodent models exhibits reversible vascular remodeling that is repaired when returned to normoxia, strain variance in their response to hypoxia exposure, and relatively mild remodeling and inflammation.\textsuperscript{103}

The fawn-hooded rat is a genetic strain that develops severe PH spontaneously when raised in the mild high altitude. Potential for fawn-hooded rats commonly studied in Denver altitudes of 5280 ft., where PAH develops within one month of birth, for studying IPAH and hereditary PH is promising.\textsuperscript{104} The original strain was developed at the University of Michigan from “German Brown,” albino rats and Long Evans rats for studies on the strain’s platelet abnormalities and systemic hypertension. The exact mechanism of the development of PH in the fawn-hooded rat is unknown but is shown to be hereditary with 68% of offspring developing PH.\textsuperscript{104} The ongoing hypothesis is that fawn-hooded rats have an abnormality in mitochondrial pathways that disrupt oxygen sensing.\textsuperscript{105} Drawbacks of the fawn-hooded rat are the characteristic decreased alveolarization and lung hypoplasia due to abnormal lung development.\textsuperscript{106} So, while this model may provide some insight into the mechanisms and biomarkers of PH that develop later in life such as in bronchopulmonary dysplasia, there are concerns with its use strictly as a hereditary model of PAH.

\textit{Crotalaria}, a toxic legume, was used as early as the 1960s to induce PH in rats. Kay et al., in 1967, orally administered finely ground \textit{Crotalaria spectabilis} seeds to female rats demonstrating an increase in right ventricular hypertrophy and medial thickness of the pulmonary arteries.\textsuperscript{107} The exact mechanism of the seeds inducing PH was unknown at the time, but the causative agent was identified as the pyrrolizidine alkaloid, monocrotaline (MCT). MCT injection on its own and combined with hypoxia are a commonly used model of PH. MCT alone is an inexact model of IPAH due to acute lung injury and provides more evidence of parenchymal lung involvement and perivascular inflammation, but can model toxin-induced PAH. MCT treatment was shown to impair endothelial-dependent relaxation and decrease relaxation induced by carbachol or ionomycin in the MCT rat artery.\textsuperscript{108}

The addition of hypoxia or pneumectomy in MCT rats leads to the development of a more robust PAH with neoimatal formation in most of the distal pulmonary arteries.\textsuperscript{109} MCT combined with pneumectomy increases turbulent blood flow in the pulmonary circulation. The altered hemodynamic conditions of MCT following pneumonectomy led to the formation not only of distal neoointimal lesions but also more severe right ventricular hypertrophy when compared to MCT alone.\textsuperscript{110} The MCT model can provide us with valuable information about both biomarkers and mechanisms of disease. Chen et al. found that treating MCT-induced PH in rats with hepatocyte growth factor (HGF) decreased IL-6 and endothelial MPs (CD31+, CD42b+) compared to untreated MCT-induced PH rats.\textsuperscript{111} These and future experiments could point to important therapeutic indexes valuable for evaluating clinical trials.

The small molecule Sugen5416 was initially developed as a chemotherapeutic for cancer that inhibits the vascular endothelial growth factor (VEGF) receptor.\textsuperscript{112} The use of Sugen5416 in animal models of lung disease first began in
the study of emphysema. Sugen5416 induced alveolar septal cell apoptosis and rats displayed evidence of enlarged air spaces indicative of emphysema. Later work, using Sugen5416 to develop PAH models, clearly showed recapitulation of plexiform lesions similar to PAH patients in the form of increased gene transcript and protein of both VEGF and its receptor VEGF receptor-2. Over the next decade, the use of Sugen 5416 in conjunction with hypoxia exposure became common in both rat and mouse models. Several promising studies in Sugen/Hypoxia rats examined at various stages revealed that these rats had virtually indistinguishable plexogenic lesions and arteriopathy to human PAH, and further that the model has progressive disease as indicated by worsening cardiac index and increased density and complexity of pulmonary vascular lesions. This disease progression occurs following the return to normoxia. In essence, the pathology of early PAH can be studied in the SU/Hx model, an advantage that has remained unlikely in human PH cohorts. Thus, the Sugen/hypoxia model can be vital for our understanding of earlier stage biomarkers as well as the mechanisms responsible for disease progression.

Perhaps the most important link in heritable PAH is the bone morphogenetic protein type 2 (BMPR2) mutations. BMPR2 is a member of a family of receptors for transforming growth factor β (TGF-β), an important regulator of vascular remodeling and inflammation in the lung. The principle studies of BMPR2 emerged in 1997 and featured the critical chromosome localization of a PH “critical region” (Locus: PPH1) through the haplotype analysis of multiple families with relatively high prevalence of PH. A year later, the culprit for autosomal dominant disorder with reduced penetrance of PPH was identified as mutations in the gene encoding BMPR2. The majority of BMPR2 mutations causing PH in patients have largely been identified as causing BMPR2 haploinsufficiency, but the exact mechanism of pathology remains complex. BMPR2 mutations show sexual dimorphism with female penetrance at 42% and male penetrance of 14%. Also, there is considerable heterogeneity between BMPR2 mutation carriers with respect to their age and onset of PAH, and reduced patient penetrance values indicate a variety of other factors involved in PAH pathogenesis in BMPR2 mutation pathogenesis.

The ease of genetic manipulation of mice has led to multiple BMPR2 mutant mice models. In mice with different BMPR2 mutations, Frump et al. were able to identify BMPR2 mutations that lead to the development of more severe PAH. However, rat models are the preferred rodent for PH models due to their more robust vascular remodeling and right ventricular hypertrophy. More recently, a transgenic BMPR2 rat has been shown to develop spontaneous PAH. BMPR2 mutant rats will potentially shed light on a cast of players in disease pathogenesis that can give vital clues into biomarkers and mechanisms of early disease. Further, BMPR2’s role in TGF-β pathways can shed light on TGF-β signaling in areas beyond heritable PAH. A reduced expression or function of BMPR2 signaling alters the TGF-β signaling pathway. TGF-β signaling is involved in proliferation, inflammation, angiogenesis, and fibrosis of both the lungs and heart in PH and is immensely complex. Further evaluation of genetically manipulated rodent models like BMPR2 can offer clues into the TGF-β signaling pathway of PH.

While the exact role of inflammation in PAH has been controversial, there is little doubt that inflammatory mediators are present and contribute to the formation of PAH. Genetic manipulations in rodent models have further highlighted the influence of inflammation in PAH pathogenesis. A transgenic mouse with lung-specific over-expression of IL-6 was observed to exhibit elevated right ventricular systolic pressures and hypertrophy accompanied with pulmonary vasculopathy including increased muscularization of the pulmonary arteries. All of these characteristics were further exacerbated in hypoxia conditions. The IL-6 transgenic mouse was observed to have an accompaniment of increased pro-inflammatory and anti-apoptotic factors that could provide clues for the role IL-6 plays in human PH.

Fifty years ago, chronic hypoxia was the only model of PH which minimally contributes to PH pathogenesis and is reversible when re-exposed to normoxia. Clearly, many advances have been made in our animal models of PH and PAH, but there remains many unanswered questions. Evaluating and classifying PH animal models into the most relevant human WHO classifications have improved with advances in small animal catheterization and imaging techniques. While many animal models recapitulate some or all of the aspects of group 1 PAH, current models of groups 2, 4, and 5 PH are still not as robust. Group 2, as stated before is an increased in pulmonary venous pressure due to high filling pressures of the left heart, and is the most common type of PH in humans. While the main method of treatment in group 2 PH patients is treatment of the underlying left heart disease, the association of PH with LHD is a major determinant of patient outcome. Developing specific non-invasive screening procedures for group 2 PH using animal models may accelerate the identification of PH in LHD patients.

Other benefits of animal models include evaluating imaging modalities – most recently, echocardiography and PET imaging. The use of echocardiography in rodent models has been attractive to the field because the use of heart catheterization to monitor PH hemodynamics is a terminal process. A non-invasive evaluation of changes to cardiac function allows for repetitive measurements to monitor disease progression and importantly identify predictive characteristics of early disease. Pulmonary artery acceleration time (PAAT) measures by echocardiogram and MRI were shown in MCT PH rats to highly correlate with invasive measures of mPAP taken invasively by cardiac catheterization. Coronary artery ligation, although a common left heart failure model, has rarely been used as a model of group 2 left heart failure-induced PH. Recently, Dayeh...
et al. sought to determine a non-invasive measurement using echocardiography to predict the presence and severity of PH development in these animals. The study found that the measurement of LV wall motion score index (WMSI) is a reliable indicator of PH and RV dysfunction in the heart failure rat model.130

Each of these animal models of PH and PAH, although imperfect, have offered valuable insight into the diagnostics and mechanisms of this devastating disease. Following along those lines, although EVs are often thought of as a biomarker, studies have suggested that they play significant mechanistic roles in PAH development and progression. As an example, the exosome population from MCT-induced PAH mice were able to induce PAH in healthy mice following tail vein injections of the diseased EVs.133 EVs from chronically hypoxic rats decrease NO production and increase oxidative stress selectively in pulmonary endothelium when compared to aorta, and interestingly, EVs from the Sugen/hypoxia rat model selectively induce inflammatory molecule expression on the pulmonary artery.134,135 These data suggest that powerful information exists in animal models for EV research as not only biomarkers but also drivers of the progressive disease. Table 5 is a summary of current research in the field of EVs in rodent models of PH and PAH. Figure 3 is a timeline of the emergence of major animal models in the field of PAH.

| EV marker | Animal model | Findings | Reference |
|-----------|--------------|----------|-----------|
| CD31+/CD42b- MP | Monocrotaline PH rat | ↑CD31+/CD42b- in PH model Reduced after therapeutic treatment | Chen et al.111 |
| CD61 + MP | Chronic hypoxia | ↑CD61 + in hypoxia vs. normoxia rats | Tual-Chalot et al.134 |
| Erythrocyte+ MP | Chronic hypoxia | ↑Erythrocyte-derived MP in hypoxia vs. normoxia rats | Tual-Chalot et al.134 |
| CD45 + MP | Chronic hypoxia | No change in CD45 + MP | Tual-Chalot et al.134 |
| CD54+ | Chronic hypoxia | No change in CD45 + MP | Tual-Chalot et al.134 |
| Total EVs | Sugen5416/hypoxia PAH rat | ↑Total EV | Blair et al.135 |
| Total EVs | Chronic hypoxia | ↑Total EV | Tual-Chalot et al.134 |
| Total EVs | Monocrotaline PH mice | ↑Total EV | Aliotta et al.133 |

PH: pulmonary hypertension; EV: extracellular vesicle; MP: microparticle; PAH: pulmonary arterial hypertension.

Fig. 3. Timeline of major events in pulmonary hypertension experimental models.
In all, although not every model is completely reflective of disease, each of the animal models provides valuable insight into the different components of PAH and PH. Diagnostic, mechanistic, and prognostic data developed from progressive models, such as Sugen/hypoxia, will help guide both clinical and basic science investigations, while we work toward models more indicative of human disease.

Summary and conclusions
The two eras of PH, before and after clinical use of RHC, have provided physicians and scientists with valuable information and multiple conundrums. Prior to RHC, findings in autopsy specimens from patients with common symptoms such as dyspnea, fatigue, and chest pain revealed damage to the pulmonary circulation described as “pulmonary atherosclerosis.” Further, investigations into these lesions to determine etiology sent investigators down a twisting path including the suggestion that syphilis was the underlying cause. We cannot discount the value of these early studies, despite some wrong turns, for discoveries early on that at least some form of the disease was directly linked to pulmonary vascular damage.

With decades left before RHC would become useful, or certainly the gold standard, use of imaging technologies gave new indications on how PH could be identified. The hilar dance, first observed as shadows on chest X-ray film and later with angiography, was an indicator of changes in the pulmonary artery, whether due to increased pressure or flow is still unclear and the term is no longer used. However, this ushered in the use of newer technologies for the analysis of heart (ECG), pulmonary hilar (chest X-ray) and improved availability and reliability of blood pressure apparatus.

Despite a lack of sophisticated diagnostics, progress was made in identifying significant etiologies of PH. From the 1800s to 1930s descriptions in the literature of “bilharzia,” a multiorgan disease induced by the parasitic flat worm, Schistosoma, included patients symptomatic and with autopsy findings of PH. We now recognize this as a WHO priority and it is the ova of the parasite that induces PH. Further, in the 1930s, work by Parker and Weiss indicated that mitral valve stenosis would induce PH. This was some of the first work to suggest PH etiology from a left heart condition. Also, in the 1900s, were the first recognition in patients that hypoxia is sufficient to induce PH following observations of miners in the mountains of South America. To-date, there are still ongoing studies to actually understand how some members of the Andean societies have adapted to high-altitude living and work. In the mid-1900s, the first autopsy reports of chronic thromboembolic disease in the pulmonary circulation came to light; however, the fact that these severe clots contributed to an actual increase in pulmonary vascular pressure would not be described until the advent of RHC.

The clinical use of RHC has a rich and storied history beginning with Forssman’s self-experimentation and the pioneering work of Cournand and Richards. This tool alone changed the playing field for diagnosis of PH in any form by providing the first direct measure of the pulmonary arterial pressure. Unfortunately, the use of RHC also revealed that there were significantly more cases of PH than ever previously recognized.

The aminorex epidemic, its discovery aided by the widespread use of RHC, brought the first monumental change to our view, diagnosis and characterization of PH. Countries where aminorex was available experienced a 20-fold increase in the occurrence of PH, and this ultimately drove the founding of the first WHO congress on pulmonary hypertension (World Symposium on Pulmonary Hypertension – WSPH). From the Geneva meeting, we gained the clinical definition of PPH as pulmonary arterial pressure of \( \geq 25\text{mmHg} \). Over the intervening 40 years, the classifications have evolved based on clinical and morphometric data, leading us to the current hemodynamic classifications published in 2019. The major change suggested by this Task Force of the 6th WSPH is that we include pulmonary vascular resistance \( \geq 3 \) Wood Units in the definition of all forms of pre-capillary PH associated with mPAP \( \geq 20\text{mmHg} \). In all, the diagnostic improvements, supported by a number of physiologic studies to understand pulmonary hemodynamics, are bringing us closer to the ability to clearly define patients, which is especially useful for therapy and clinical trial design. However, the most recent symposium reiterated statements from the first symposium that there is a significant need to identify patients earlier in the disease progression; yet, we still do not have the key to this puzzle.

There has been no meaningful decrease in the time from symptom onset to diagnosis of PH in the past 20 years. This could be improved vastly by first developing non-invasive cost-effective screening with standardized methods for PH and identifying patients that would receive the most benefit from screening. Recent evidence suggests that WHO functional class I or II patients have significantly better long-term survival rates than patients with higher WHO functional classes, highlighting the need for earlier diagnosis and treatment of PAH. Patients now known to be at risk for developing PAH are patients with a familial history due to a known or unknown genetic mutation, drug and toxins exposure (methamphetamine, aminorex, cocaine, toxic rapeseed oil, etc.), connective tissue disease, schistosomiasis, HIV infection, portal hypertension, and congenital heart disease. Currently, recommendations are for a small population of people at risk to undergo a yearly echocardiogram to screen for PAH. A cheaper and faster but accurate technique allowing for screening access to a larger subset of patients at risk is biomarkers. Biomarkers can be seen from a simple blood test that indicates early PAH disease presence. However, this ideal biomarker, to identify patients early in the disease or even responsive to treatment, has yet to emerge for clinical use.
Some of the first biomarkers utilized in PH were the atria and BNP. While both provide valuable information about cardiac injury, put in the simplest terms, they still only give physicians information after the heart has been damaged. In order to look forward to understand the developing pulmonary vasculopathy, some have turned to circulating endothelial cells or endothelial progenitor cells. While there is some promising information suggesting that CECs in multiple forms of PH correlate with increased pulmonary artery pressure and may be predictive of the reversibility of CHD-induced PH. Alterations in NO signaling, specifically increases in ADMA, and inflammatory molecules such as IL-1β and IL-6 may provide valuable opportunities; however, expansion of all of these studies to large-scale populations will be necessary to vet them in patients.

An emerging emphasis on novel biomarkers for a number of vascular diseases has led to studies on circulating EVs in PH. At the forefront of this work were seminal studies that determined that MPs, a member of the EV family, of endothelial and leukocyte origin increased in patients with precapillary PH. Further, endothelial MPs positively correlated with mPAP and PVR. Lastly, increased CD62+MPs predicted worse patient outcomes. Systemic sclerosis patients with PH have elevated CD144+MPs compared to healthy and systemic sclerosis patients without PH. This is all extremely promising; however, there is controversy in the field related to isolation and characterization of populations of EVs. Over the last decade, significant work has been put into standardization of the EV protocols, and data are emerging that are clearly reproducible. While EVs may be able to give the physician insight into the workings of the vasculature and circulating elements, what remains, as with all of the biomarkers reported in patients, is that they show potential for evaluating severity or patient outcomes, but a major limitation is whether we can identify patients earlier. This is likely one area, among many, that animal models of the disease are highly valuable.

Rodent models of PH and PAH are the most commonly used today and include hypoxia exposure, genetic manipulations, MCT treatment, MCT with either hypoxia or pneumectomy, and the SU5416/hypoxia model. While no model is absolutely perfect, each model has significant value towards understanding mechanisms and aspects of the disease. Critics of animal models will point out that many therapies developed with animal studies have not been highly successful in patients; however, there have been a number of discoveries made, including the role of endothelin, NO signaling, and basic calcium channel signaling that are clearly reproducible. While EVs may be able to give the physician insight into the workings of the vasculature and circulating elements, what remains, as with all of the biomarkers reported in patients, is that they show potential for evaluating severity or patient outcomes, but a major limitation is whether we can identify patients earlier. This is likely one area, among many, that animal models of the disease are highly valuable.

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