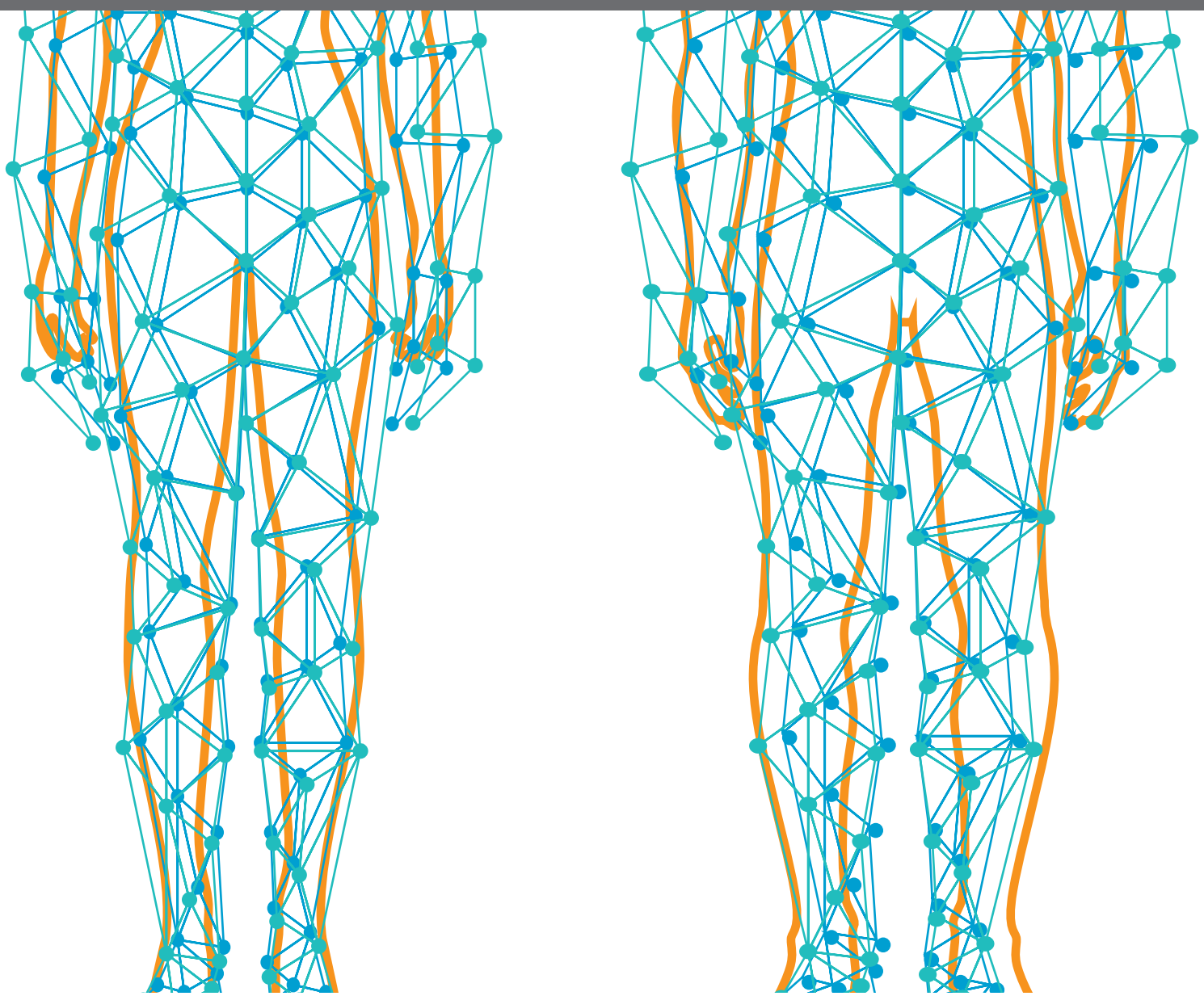
PULMONARY HYPERTENSION IN THE MODERN ERA: SCIENCE AND CLINICAL PRACTICE

EDITED BY: Elena Goncharova, Vinicio De Jesus Perez and Harm Jan Bogaard
PUBLISHED IN: Frontiers in Medicine



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-905-1
DOI 10.3389/978-2-88971-905-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

PULMONARY HYPERTENSION IN THE MODERN ERA: SCIENCE AND CLINICAL PRACTICE

Topic Editors:
Elena Goncharova, University of California, Davis, United States
Vinicio De Jesus Perez, Stanford University, United States
Harm Jan Bogaard, Amsterdam University Medical Center, Netherlands

Citation: Goncharova, E., De Jesus Perez, V., Bogaard, H. J., eds. (2021). Pulmonary Hypertension in the Modern Era: Science and Clinical Practice. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-905-1

Table of Contents

frontiers in Medicine

EDITORIAL
published: 28 October 2021
doi: 10.3389/fmed.2021.785181

Editorial: Pulmonary Hypertension in the Modern Era: Science and Clinical Practice

Elena A. Goncharova[1]*, *Harm J. Bogaard*[2] and *Vinicio A. de Jesus Perez*[3]

[1] Division of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, Lung Center, School of Medicine, University of California, Davis, Davis, CA, United States, [2] Department of Pulmonary Medicine, Amsterdam University Medical Center, Amsterdam, Netherlands, [3] Division of Pulmonary, Allergy and Critical Care Medicine, Stanford University Medical Center, Stanford, CA, United States

Keywords: pulmonary hypertension, group 1 PH, group 2 PH, group 5 PH, genetics and genomics, endothelial dysfunction

Editorial on the Research Topic

Pulmonary Hypertension in the Modern Era: Science and Clinical Practice

Pulmonary hypertension (PH) comprises five groups of progressive cardiopulmonary diseases defined as mean pulmonary arterial (PA) pressure (mPAP) $\geq$ 20 mmHg (1), leading to right heart failure and premature death. Despite past advances, the pathobiology of different groups of PH is incompletely understood, and diagnostic and treatment options are limited.

Pulmonary arterial hypertension (PAH, Group I PH) is a rare group of conditions that includes familiar and idiopathic forms. Multiple genetic and genomic risk factors have been identified (2), but much remains to discover. Using whole genome sequencing of two unrelated families with PAH, Pienkos et al. identified two novel rare variants in *TNIP2* and *TRAF2*, which could promote an altered immune response, pulmonary vascular remodeling and PH by permitting overactivation of NF-κB. In parallel studies, Rathinasabapathy et al. created transgenic mice harboring the human *Cav1* mutation. These researchers found that the *Cav1* mutation results in metabolic deficiencies, development of mild PH, reduced spontaneous exercise, and an inflammatory phenotype. This study adds to emerging evidence suggesting a role for insulin deficiency and exercise intolerance, and further supports the role of inflammation in PAH.

Other critical players in PAH are PA endothelial cells (PAECs), and the mechanisms by which PAECs regulate immune function and inflammation in PAH continue to attract attention. In this issue of the Journal, Bhagwani et al. provide a comprehensive review of the role of toll-like receptors, central players in innate immunity, in the immune and non-immune functions of endothelial cells in PH. One important but under-used tool in PAH pre-clinical research is high-resolution imaging, which was successfully used to study pathobiology of other diseases (3). In this special issue, Klouda et al. combined super-resolution microscopy with precision-cut lung slices and clearing technology to develop a novel evolutionary method to study cell behavior *ex vivo*, trace and reconstruct pulmonary vasculature, and address fundamental questions relevant to a wide variety of vascular disorders including PAH.

In addition to idiopathic and familiar forms, group 1 PH includes portopulmonary hypertension (PoPH) that afflicts ∼6–8% of patients with portal hypertension and is associated with high morbidity and mortality, particularly in the context of liver transplantation. In this issue, Thomas et al. review the most recent advances in our understanding of the pathogenesis and clinical course of PoPH. The last decade has seen exciting developments in the management of PoPH, with large clinical trials demonstrating the efficacy of several PH-specific therapies in improving

OPEN ACCESS

Edited and reviewed by:
Laurent Pierre Nicod, University of Lausanne, Switzerland

***Correspondence:**
Elena A. Goncharova
eagoncharova@ucdavis.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *28 September 2021*
Accepted: *08 October 2021*
Published: *28 October 2021*

Citation:
Goncharova EA, Bogaard HJ and de Jesus Perez VA (2021) Editorial: Pulmonary Hypertension in the Modern Era: Science and Clinical Practice. Front. Med. 8:785181. doi: 10.3389/fmed.2021.785181

hemodynamics and clinical outcomes. While there is more work to be done, the authors' state-of-the-art summary leaves us enthusiastic for future progress in the field of PoPH research.

As new oral prostacyclin therapies and prostacyclin agonists have become available for the treatment of PAH, there is an increasing need for methods for transitioning between oral, inhaled and parenteral formulations. Pan et al. describe their experience of successful transitioning between different prostanoid therapies. Particularly because a standardized approach in transitioning safely between different formulations without compromising treatment efficacy is lacking, their case series gives valuable insight into sensible clinical practice and the pharmacokinetics of various prostanoid formulations. In a parallel report, Kopp et al. share their experience with one particular method of prostanoid therapy: intravenous treprostenil administration *via* a Lenus Pro® Implantable Pump. While this method has clear advantages, the authors witnessed unexpected acceleration in treprostinil delivery in two patients. The flow rate delivered by the pumps in these 2 patients progressively exceeded the manufacturer admitted margin of error with increases of more than 50% of the expected flow rate at the end of follow up. Spontaneous flow increase from such an implantable pump is a potentially major pitfall, which needs to be identified and actively managed by the responsible clinicians. Prospective studies are needed to assess the long-term safety and efficacy of treprostinil administration by this device.

One of the most common and lethal forms of PH is PH due to left heart disease (PH-LHD, group 2 PH) (4). In their review article, Todd and Lai discuss the challenges and opportunities in developing biomarkers for diagnosing and managing PH-LHD. Presently, the only biomarkers available are B-type natriuretic peptide and N-terminal proBNP; however, data for these biomarkers comes mainly from studies in non-PH-LHD patients, which may not necessarily capture the complex phenotypes of PH-LHD patients. In their paper, the authors discuss exciting studies pointing at endothelin-1, vascular endothelial growth factor-D, and microRNA-206 as new potential circulating biomarkers for patients with PH-LHD and how they could serve as the basis of future risk stratification tools for making management decisions in PH-LHD patients.

A significant challenge for improving the quality of life and survival of PH-LHD patients is the lack of effective treatments. To date, there is no definite evidence that PH-specific therapies can be routinely used to treat patients with PH-CHD. Given this problem, there is an active interest in investigating experimental treatments that could target mechanisms inherent to the pathogenesis of PH-LHD. In this special issue, Mulkareddy and Simon provide a comprehensive review of the evidence that supports metformin as a prime candidate for clinical trials in PH-LHD. Given the strong association between metabolic dysregulation, insulin resistance, and cardiovascular diseases, investigators have identified the anti-diabetic drug metformin as a potential treatment for PH-LHD. Besides counteracting insulin resistance in type 2 diabetes mellitus, metformin has a range of beneficial effects that could potentially halt the progression of cardiopulmonary remodeling in PH-LHD and improve cardiac function, a major source of morbidity and mortality in these patients. Repurposing metformin for PH-LHD also offers the advantage of improving access to a potentially life-saving drug since the availability of generic formulations could help make this drug affordable and more accessible to patients around the world.

Group 5 PH comprises heterogeneous group of PH secondary to multifactorial mechanisms.

Al-Qadi et al. provide a thorough review of the epidemiology, pathogenesis and clinical approach of group 5 PH, with a specific focus on hematologic disorders, sarcoidosis and chronic renal failure. These forms of PH are caused by a mix of pathophysiologic drivers and can present with precapillary as well as postcapillary disease. The pathogenesis of most forms of group 5 PH includes hypercoagulability, altered vasomotor tone and varied degrees of cardiomyopathy due to a chronic high flow state or intrinsic involvement of the myocardium. In addition, specific to sarcoidosis are advanced interstitial lung disease, granulomatous obliteration of arterioles or venules and extrinsic compression of pulmonary arteries (and venous stenosis) by enlarged lymph nodes or fibrosing mediastinitis. Cardiotoxic and pulmonary toxic effects of medication deserve mention too, particularly the use of tyrosine kinase and alkylating agents in myeloproliferative disorders. Management of group 5 PH primarily involves optimizing treatment of the underlying disease and general measures such as supplemental oxygen, cautiously used diuretics and anticoagulation. PH-targeted therapy is usually reserved for patients with moderate to severe (based on functional class) RHC-confirmed pre-capillary PH with relatively reduced cardiac output.

In conclusion, this special issue highlights continuous interest in pulmonary hypertension research, spanning genetics and genomics, molecular mechanisms of PH pathogenesis, novel imaging techniques, and clinical studies. Together, efforts of many researchers continue to improve our understanding of disease pathobiology and treatment options for patients with PH.

AUTHOR CONTRIBUTIONS

EG, HB, and VJ drafted and edited the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respirat J.* (2019) 53:1801913. doi: 10.1183/13993003.01913-2018

2. Morrell NW, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, et al. Genetics and genomics of pulmonary arterial hypertension. *Eur Respirat J.* (2019) 53:1801899. doi: 10.1183/13993003.01899-2018

3. Ochoa LF, Kholodnykh A, Villarreal P, Tian B, Pal R, Freiberg AN, et al. Imaging of murine whole lung fibrosis by large scale 3D microscopy aided by tissue optical clearing. *Sci Rep.* (2018) 8:13348. doi: 10.1038/s41598-018-31182-2

4. Wijeratne DT, Lajkosz K, Brogly SB, Lougheed MD, Jiang L, Housin A, et al. Increasing incidence and prevalence of world health organization groups 1 to 4 pulmonary hypertension. *Circulation.* (2018) 11:e003973. doi: 10.1161/CIRCOUTCOMES.117.003973

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Medicine

CASE REPORT
published: 31 March 2020
doi: 10.3389/fmed.2020.00081

Transitioning Between Prostanoid Therapies in Pulmonary Arterial Hypertension

*Irene Z. Pan[1], Jessica R. Carey[1], Joshua A. Jacobs[1], John Dechand[1], Joshua J. Sessions[1], Teshia Sorensen[1], Brittany A. Penn[2], Jennalyn D. Mayeux[3], Nathan D. Hatton[3] and John J. Ryan[2]**

[1] Department of Pharmacy, University of Utah Health, Salt Lake City, UT, United States, [2] Division of Cardiovascular Medicine, Department of Medicine, University of Utah, Salt Lake City, UT, United States, [3] Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Utah, Salt Lake City, UT, United States

Background: New oral prostacyclin therapies and prostacyclin agonists have become available for the treatment of pulmonary arterial hypertension (PAH). However, methods for transitioning between oral, inhaled, and parenteral formulations are not well-established, except in the form of case reports and case series. Collectively, these emphasize the lack of a standardized process and approach in transitioning patients between PAH prostanoid therapies. In this case series, we report our experience at an accredited Pulmonary Hypertension center in transitioning between various oral, inhaled, and parenteral prostanoids to offer additional guidance on safe transitions in therapy.

Methods: All cases of prostanoid transitions at an accredited Pulmonary Hypertension center from March 2018 to September 2019 were included in this report. The transition approach for each case was developed through a review of the literature, extrapolation of available pharmacokinetic data, and collaboration between pharmacists and clinicians.

Results: This case series describes the transition of 3 patients from selexipag to parenteral treprostinil; 1 patient transitioning from parenteral treprostinil to selexipag; 1 patient transitioning from oral treprostinil to parenteral treprostinil; and 1 patient transitioning from inhaled treprostinil to selexipag. Four of the 6 patients presented here were transitioned to an alternate prostanoid on account of clinical worsening, while the remaining 2 patients transitioned due to intolerance of parenteral therapy and poor medication adherence. This case series includes patients with various etiologies of PAH including idiopathic PAH, methamphetamine-associated PAH, and scleroderma-associated PAH. All patients successfully completed each transition without serious adverse events.

Conclusions: With the increasing utilization and availability of prostanoids, there is a critical need for a standardized approach in transitioning safely between different formulations without compromising treatment efficacy. In this case series, we present our clinical experiences, guided by available pharmacokinetic data, in transitioning between various prostanoid formulations.

Keywords: pulmonary hypertension, therapeutics, right heart failure, pharmacology, prostaglandin, prostacyclin

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez, Stanford University, United States

Reviewed by:
Roberto Bernardo, Stanford University, United States
Peter Korsten, Nephrology and Rheumatology University Medical Center Göttingen, Germany

***Correspondence:**
John J. Ryan
john.ryan@hsc.utah.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *03 December 2019*
Accepted: *28 February 2020*
Published: *31 March 2020*

Citation:
Pan IZ, Carey JR, Jacobs JA, Dechand J, Sessions JJ, Sorensen T, Penn BA, Mayeux JD, Hatton ND and Ryan JJ (2020) Transitioning Between Prostanoid Therapies in Pulmonary Arterial Hypertension. Front. Med. 7:81. doi: 10.3389/fmed.2020.00081

BACKGROUND

In recent years, new oral prostanoid therapies such as selexipag and oral treprostinil have become globally available for the treatment of pulmonary arterial hypertension (PAH). These agents are becoming increasingly utilized due to their efficacy, oral route of administration, and potentially favorable side effect profile based on data from the FREEDOM trials with oral treprostinil (1–4) and the GRIPHON trial with selexipag (5). Established guidance on transitions between various formulations within this drug class have not been standardized. A number of case reports and case series have been published describing such transitions but lack consistent processes (6–13).

The most common method of determining dose equivalency between parenteral treprostinil and oral treprostinil is derived from a small phase 2 trial consisting of 33 patients. This dose conversion strategy can be found in the package insert for oral treprostinil and is summarized by the following equation (14):

Oral treprostinil total dose/day (mg) = parenteral treprostinil dose (ng/kg/min) × weight (kg) × 0.0072

During the transition period, the package insert recommends reducing parenteral treprostinil by up to 30 ng/kg/min per day while also increasing the dose of oral treprostinil by up to 6 mg per day as tolerated (14). However, despite sharing similar dosing strategies based on these recommendations, there are still numerous variations between different institutional practices. Most notable are the durations over which the transitions are implemented (6–10).

There is no standardized dose conversion strategy for transitioning from oral treprostinil to inhaled treprostinil, and no recommendations are provided in the package insert (14). There is published data for patients on doses of parenteral treprostinil ranging from 22.5 to 111 ng/kg/min and inhaled treprostinil ranging from 9 to 54 mcg four times daily, who have subsequently been transitioned to oral treprostinil 1 mg twice daily to 14 mg three times daily (7, 9, 10). Given the linear pharmacokinetic relationship between treprostinil plasma concentrations and doses up to 125 ng/kg/min, relative dose equivalencies between different formulations can be estimated (15). Parenteral treprostinil 10 ng/kg/min results in plasma concentrations of ∼1–2 ng/mL, which is roughly equivalent to inhaled treprostinil 54 mcg four times daily (15–17). Assuming inhaled treprostinil 54 mcg four times daily is approximately equivalent to parenteral treprostinil 5–10 ng/kg/min, the corresponding dose of oral treprostinil (for an average 70 kg patient) would be ∼1–2 mg three times daily. Other studies have described successful transitions between inhaled and parenteral treprostinil using parenteral doses of up to 25 ng/kg/min (18, 19).

For transitions between parenteral or inhaled prostanoids and selexipag, there is considerably less guidance available. Selexipag is an oral selective IP receptor agonist, and the pharmacokinetic relationship between dose and equivalent plasma concentrations are unknown. In the TRANSIT-1 study, 34 PAH patients on inhaled treprostinil were transitioned to selexipag over 8 weeks with a 94.1% success rate. The mean dose of inhaled treprostinil at baseline was 59.3 mcg four times daily (13). In a case series of 4 patients on parenteral treprostinil, the highest dose upon initiation of the transition was 46.5 ng/kg/min, which was successfully transitioned to selexipag 1,600 mcg twice daily (11). In another case series of 5 patients, parenteral treprostinil doses up to 96 ng/kg/min were successfully transitioned to selexipag 1,600 mcg twice daily (20). It is important to note the lack of information beyond 6 months following the transition, particularly in patients previously on high treprostinil doses.

In this case series, we report our experience in transitioning between oral, inhaled, and parenteral prostaglandin products at an accredited Pulmonary Hypertension (PH) center (**Table 1**).

METHODS

Approval of this study was obtained through the University of Utah Institutional Review Board. All cases that underwent transition between different prostanoid formulations at the University of Utah Pulmonary Hypertension Comprehensive Care Center from March 2018 to September 2019 were included in this report. No cases were omitted, and all available relevant data were included in this report. Written informed consent was obtained from the participants for the publication of this case report. The consecutive cases were collected prospectively. The different methodologies for transition were created after an extensive literature review and with collaboration between pharmacists and clinicians at University of Utah Health.

CASE PRESENTATION

Case #1: Selexipag to Parenteral Treprostinil

A 66-year-old female with World Health Organization (WHO) group 1, functional class (FC) III scleroderma-associated PAH underwent a planned admission to transition from selexipag to parenteral treprostinil due to declining clinical status. Prior to starting any PAH therapies, her right heart catheterization (RHC) showed a right atrial pressure (RAP) of 25 mmHg, pulmonary artery pressure (PAP) of 61/26 mmHg with a mean PAP of 43 mmHg, pulmonary capillary wedge pressure (PCWP) of 9 mmHg, pulmonary vascular resistance (PVR) of 6.7 Wood Units (WU), and cardiac index (CI) (Fick) of 2.3 L/min/m^2. PAH medications prior to the transition were: ambrisentan 10 mg daily, sildenafil 20 mg three times daily, and selexipag 600 mcg twice daily. The patient had received selexipag for ∼7 months. Her other medications included apixaban 5 mg twice daily, bumetanide 2 mg twice daily, and metolazone 2.5 mg twice weekly. Prior to the transition, while on PAH therapies, the patient had a 6 minute walk distance (6MWD) of 213 m; brain natriuretic peptide (BNP) of 288 pg/mL; and a transthoracic echocardiogram (TTE) demonstrating an estimated right ventricular systolic pressure (RVSP) of 61 mmHg, a moderate pericardial effusion, and normal right ventricle (RV) size and function. On admission, her total body weight was 101.4 kg (BMI 38.4 kg/m^2). She was hemodynamically stable with an oxygen saturation of 99% on her baseline oxygen requirement of 1L. Selexipag total daily dose was decreased by 400 mcg per day (split between the morning and evening

TABLE 1 | Baseline characteristics.

	Case #1	Case #2	Case #3	Case #4	Case #5	Case #6
Age (yr)	66	41	40	32	20	56
Type of PAH	Scleroderma-associated	Idiopathic	Methamphetamine-associated	Methamphetamine-associated	Idiopathic	Methamphetamine-associated
Transitioning agents	Selexipag to SQ Treprostinil	Selexipag to SQ Treprostinil	Selexipag to SQ Treprostinil	SQ Treprostinil to Selexipag	Oral Treprostinil to SQ Treprostinil	Inhaled Treprostinil to Selexipag
6MWD (m)	213	213	383	671	520	312
BNP (pg/mL)	288	579	NT-proBNP 599 pg/mL	21	43	189
Hemodynamics	RAP 25 mmHg PAP 61/26 mmHg mPAP 43 mmHg PCWP 9 mmHg PVR 6.7 WU CI 2.3 L/min/m^2	RAP 19 mmHg PAP 80/40 mmHg mPAP 54 mmHg PCWP 18 mmHg PVR 8.6 WU CI 2.56 L/min/m^2	RAP 5 mmHg PAP 87/32 mmHg mPAP 57 mmHg PCWP 12 mmHg PVR 9.3 WU CI 2.46 L/min/m^2	RAP 3 mmHg PAP 120/57 mmHg mPAP 78 mmHg PCWP 17 mmHg PVR 9.2 WU CI 3.7 L/min/m^2	RAP 10 mmHg PAP 100/55 mmHg mPAP 79 mmHg PCWP 21 mmHg PVR 13.68 WU CI 2.35 L/min/m^2	RAP 6 mmHg PAP 63/25 mmHg mPAP 44 mmHg PCWP 7 mmHg PVR 11.7 WU CI 1.6 L/min/m^2

TABLE 2 | Selexipag to parenteral treprostinil transitions (Cases #1-#3).

Drug Titration	Case 1	Case 2	Case 3
Selexipag dose prior to transition	600 mcg twice daily	1,600 mcg twice daily	1,400 mcg twice daily
Selexipag dose at time of treprostinil initiation	400 mcg	1,200 mcg	1,200 mcg
Treprostinil initiation dose	5 ng/kg/min	5 ng/kg/min	5 ng/kg/min
Selexipag dose down-titration	Total daily dose decreased by 400 mcg every 24 h	Total daily dose decreased by 800 mcg every 24 h until 400 mcg dose reached, then decreased by 400 mcg every 24 h	Decreased by 200 mcg per dose every 12 h
Treprostinil dose up-titration	Increased by 5 ng/kg/min every 24 h until selexipag off, then increased by 1 ng/kg/min every 24 h as tolerated	Increased by 3–5 ng/kg/min every 24 h until selexipag off, then increased by 1 ng/kg/min every 24 h as tolerated	Increased by 5 ng/kg/min every 24 h until selexipag off, then increased by 1 ng/kg/min every 24 h as tolerated
Treprostinil dosing weight used	54.2 kg (IBW)	70 kg (IBW)	50.1 kg (IBW)
Duration of transition	3 days	5 days	3 days
Treprostinil dose reached at hospital discharge	13 ng/kg/min	20 ng/kg/min	20 ng/kg/min

doses) starting on the day of hospital admission. Intravenous treprostinil was initiated at 5 ng/kg/min using an ideal body weight of 54.2 kg on day 1 of admission and increased by increments of 5 ng/kg/min every 24 hours to 10 ng/kg/min by the time of selexipag discontinuation. The transition was completed over 3 days (**Table 2**). The patient tolerated the transition well with no reports of headaches, flushing, flu-like symptoms, or symptomatic hypotension. Throughout the transition, she required 1 to 2 L/min of oxygen to maintain oxygen saturations >90% at rest. Her hospital stay was complicated by fluid overload and acute kidney injury likely secondary to cardiorenal syndrome, despite an adequate cardiac output shown on RHC. Following the transition, her RHC hemodynamics showed a RAP of 23 mmHg, PAP of 67/29 mmHg with a mean PAP of 44 mmHg, PCWP of 27 mmHg (increased PCWP secondary to right ventricular compression of the left ventricle), PVR of 2.2 WU, and CI (Fick) of 3.8 L/min/m^2. Prior to hospital discharge, the patient was transitioned from IV to SQ treprostinil and was instructed to continue up-titrating by 1 ng/kg/min every 2–4 days until goal of 40 ng/kg/min. At a 7-month follow-up, she was on SQ treprostinil at a rate of 43 ng/kg/min. Her BNP was stable at 261 pg/mL, and her TTE was unchanged. However, her 6MWD had decreased to 168 m, and she required 3 L/min of oxygen to maintain an oxygen saturation of >88%. Repeat RHC showed a RAP of 19 mmHg, PAP of 61/25 mmHg with a mean PAP of 37 mmHg, PCWP of 22 mmHg, PVR of 1.7 WU, and CI (Fick) of 4.14 L/min/m^2.

Case #2: Selexipag to Parenteral Treprostinil

A 41-year-old female with WHO group 1, FC IV idiopathic PAH underwent a planned admission to transition from selexipag to parenteral treprostinil. She had worsening functional class on ambrisentan 10 mg daily, sildenafil 20 mg three times daily, and selexipag 1,600 mcg twice daily. Her concomitant medications included torsemide 40 mg daily, spironolactone 25 mg daily, digoxin 250 mcg daily, and apixaban 5 mg twice daily. Prior to the transition, while on PAH therapies, the patient had a

6MWD of 213 m, BNP of 579 pg/mL, and a TTE demonstrating an estimated RVSP of 69 mmHg and a severely enlarged RV with mildly reduced RV systolic function. Her RHC on admission showed a RAP of 19 mmHg, PAP of 80/40 mmHg with a mean PAP of 54 mmHg, PCWP of 18 mmHg, PVR of 8.6 WU, and CI (Fick) of 2.56 L/min/m^2. On admission, her total body weight was 107 kg (BMI 44.6 kg/m^2). She was hemodynamically stable with an oxygen saturation of 92% on her baseline oxygen requirement of 4L. Selexipag was reduced to 1,200 mcg twice daily (75% of her home dose) on the day of admission and subsequently decreased by 800 mcg per day (split between the morning and evening doses). Once 400 mcg was reached, the dose was further decreased to 200 mcg after 24 h before stopping. Intravenous treprostinil was initiated on hospital day 1 at 5 ng/kg/min using an ideal body weight of 70 kg and was increased by 3–5 ng/kg/min every 24 h to 20 ng/kg/min by the time of selexipag discontinuation. The transition took place over the course of 5 days (**Table 2**). The patient tolerated the transition with no complaints. The patient did develop a rash during the admission, which was thought to be contact dermatitis secondary to an adhesive. Throughout the transition, her oxygen saturations remained >88% on 4 to 6 L/min NC, and her renal function remained unchanged. Prior to discharge, the patient was transitioned from IV to SQ treprostinil. She was continued on a gradual up-titration as an outpatient with a goal of 40 ng/kg/min and had reached 30 ng/kg/min by the time of her 1 month follow up.

Case #3: Selexipag to Parenteral Treprostinil

A 40-year-old female with WHO group 1, FC III methamphetamine-associated PAH underwent a planned admission for transition from oral selexipag to parenteral treprostinil. She initially presented to our PH clinic with progression of disease and worsening functional status despite treatment with tadalafil 40 mg daily, macitentan 10 mg daily, and selexipag 1,400 mcg twice daily for nearly 3 years. Her other medications included furosemide 40 mg in the morning and 20 mg in the afternoon and spironolactone 25 mg daily. Prior to her transition, the patient had a 6MWD of 382 m, NT-proBNP of 599 pg/mL, and a TTE revealing an estimated RVSP of 91 mmHg and a severely enlarged RV with mildly reduced RV systolic function. Her RHC showed a RAP of 5 mmHg, PAP of 87/32 mmHg with a mean PAP of 57 mmHg, PCWP of 12 mmHg, PVR of 9.3 WU, and CI (Fick) of 2.46 L/min/m^2. On admission, her total body weight was 94.5 kg (BMI 38.1 kg/m^2). She was hemodynamically stable with an oxygen saturation of 90% on her baseline oxygen requirement of 4L. Selexipag was decreased by 200 mcg every 12 h from her home dose of 1,400 mcg twice daily. IV treprostinil was initiated at 5 ng/kg/min using an ideal body weight of 50.1 kg at the time of the first selexipag dose decrease and subsequently uptitrated by 5 ng/kg/min every 24 h to 15 ng/kg/min by the time of selexipag discontinuation. The transition took place over a period of 3 days (**Table 2**). After selexipag discontinuation, treprostinil was increased to 20 ng/kg/min prior to hospital discharge. The patient tolerated the transition well with some mild nausea but had no other complaints. Her oxygen saturations remained >88% on 4–6 L/min of oxygen at night and on 1–3 L/min during the day throughout the transition. She was discharged on her home oxygen requirement of 4 L/min at night. On the day of discharge, the patient was transitioned from IV to SQ treprostinil with the plan to increase treprostinil by 3 ng/kg/min every 3–6 days to a goal of 35 ng/kg/min. At the time of publication, the patient is doing well but has not yet had follow up studies performed after her transition.

Case #4: Parenteral Treprostinil to Selexipag

A 32-year-old male with WHO group 1, FC II methamphetamine-associated PAH was admitted for transition from subcutaneous treprostinil to oral selexipag. The reason for transition was patient preference as his quality of life had significantly declined due to being limited by daily pump management. This transition was strongly discouraged by his PH care team, but after several conversations regarding his perspective and the risks associated, he chose to transition with close follow up. The patient was diagnosed with PAH 10 months prior to admission and was originally started on epoprostenol at the time of diagnosis but was quickly transitioned to subcutaneous treprostinil. His dose of treprostinil at the time of this transition was 32 ng/kg/min using a dosing weight of 70.6 kg. He was also on tadalafil 40 mg daily and macitentan 10 mg daily. Prior to the transition, while on PAH therapies, the patient had a 6MWD of 671 m, BNP of 21 pg/ml, and a TTE with an RVSP of 71.6 mmHg with normal RV systolic function and mild enlargement. His most recent RHC measurements immediately prior to the transition demonstrated a RAP of 3 mmHg, PAP of 120/57 mmHg with a mean PAP of 78 mmHg, PCWP of 17 mmHg, PVR of 9.2 WU, and CI (Fick) of 3.7 L/min/m^2. On admission, his total body weight was 65.5 kg (BMI 23.3 kg/m^2). The patient was hemodynamically stable with an oxygen saturation of 96% on room air while on his home treprostinil, tadalafil, and macitentan. The patient underwent a simultaneous down-titration of treprostinil by 4 ng/kg/min and up-titration of selexipag by 200 mcg every 12 h. The transition took place over a period of 5 hospital days (**Table 3**). The patient had no complaints of side effects during the transition. The patient's renal function remained stable during the transition, and his oxygen saturation remained >88% on his baseline requirement of 3L nocturnal oxygen and room air throughout the day. The patient successfully reached 1,600 mcg BID at the end of the transition and was discharged home on tadalafil, macitentan, and selexipag. At a 3-month follow-up, the patient had a 6MWD of 649 m, a BNP of 29 pg/mL, and a TTE that demonstrated an RVSP of 94.4 mmHg and severe RV enlargement and normal RV systolic function. He had complaints of flushing and jaw pain on selexipag 1,600 mcg BID, and thus his dose was reduced to 1,400 mcg daily in the morning and 1,600 mcg daily at bedtime.

TABLE 3 | Parenteral treprostinil to selexipag transition (Case #4).

	Treprostinil dose titration	Selexipag dose titration
Day 1 AM	32 ng/kg/min	0 mcg
Day 1 PM	28 ng/kg/min	200 mcg
Day 2 AM	24 ng/kg/min	400 mcg
Day 2 PM	20 ng/kg/min	600 mcg
Day 3 AM	16 ng/kg/min	800 mcg
Day 3 PM	12 ng/kg/min	1,000 mcg
Day 4 AM	8 ng/kg/min	1,200 mcg
Day 4 PM	4 ng/kg/min	1,400 mcg
Day 5 AM	0 ng/kg/min	1,600 mcg

Case #5: Oral Treprostinil to Parenteral Treprostinil

A 20-year-old male with WHO group 1, FC III idiopathic PAH was admitted for transition from oral treprostinil to subcutaneous treprostinil. The patient had a diagnosis of PAH since the age of 4 and had been on PAH-specific therapies prior to presentation to our center. The reason for transition was declining functional status on tadalafil 40 mg daily, macitentan 10 mg daily, and treprostinil diolamine 4 mg three times daily. Other medications included aspirin 81 mg daily and digoxin 125 mcg daily. His 6MWD at baseline on PAH therapies was 520 m, with a desaturation to 69%, requiring 25L via nasal cannula to bring his oxygen saturation to 82% at the end of 6 minutes. His initial BNP was 43 pg/mL. His TTE showed an RVSP of 97.9 mmHg, moderate RV enlargement, severe RV wall thickness, and normal RV systolic function. His RHC immediately prior to his transition to IV treprostinil showed a RAP of 10 mmHg, PAP of 100/55 mmHg with a mean PAP of 79 mmHg, PCWP of 21 mmHg, PVR of 13.68 WU, and CI (Fick) of 2.35 L/min/m^2. On admission, the patient's total body weight was 69.9 kg (BMI 23.4 kg/m^2). The patient was hemodynamically stable on his home treprostinil and had an oxygen saturation of 90% on room air. The patient underwent a simultaneous down-titration of oral treprostinil by 1 mg three times daily every 24 h while initiating and increasing IV treprostinil by 6 ng/kg/min every 24 h. The transition took place over a period of 4 hospital days (**Table 4**). The patient had no complaints of side effects during the transition, and the patient's renal function remained stable during the transition. His oxygen saturation at rest remained >90% throughout the entire transition without supplemental oxygen and was 94% on room air at the time of discharge. The patient successfully reached 24 ng/kg/min at the end of the transition and was converted to subcutaneous treprostinil and discharged home without any problems. At 2-month follow-up, the patient was WHO-FC II and had reached a dose of 40 ng/kg/min. His 6MWD had improved to 613 m, but he still required 25L of oxygen to achieve an oxygen saturation of 83%. His treprostinil was gradually increased to 61 ng/kg/min. At his 6-month follow-up, there was no new RHC data, his BNP was stable at 52 pg/mL and a TTE demonstrated normal RV size, severe RV wall thickness, and normal RV function.

TABLE 4 | Oral Treprostinil to Parenteral Treprostinil (Case #5).

	Oral treprostinil down-titration	IV treprostinil dose titration
Day 1 AM	4 mg	
Day 1 Mid-day	4 mg	6 ng/kg/min
Day 1 PM	3 mg	6 ng/kg/min
Day 2 AM	3 mg	6 ng/kg/min
Day 2 Mid-day	3 mg	12 ng/kg/min
Day 2 PM	2 mg	12 ng/kg/min
Day 3 AM	2 mg	12 ng/kg/min
Day 3 Mid-day	2 mg	18 ng/kg/min
Day 3 PM	1 mg	18 ng/kg/min
Day 4 AM	1 mg	18 ng/kg/min
Day 4 Mid-day	1 mg	24 ng/kg/min
Day 4 PM	OFF	24 ng/kg/min
	Discharged	

Case #6: Inhaled Treprostinil to Selexipag

A 56-year-old male with WHO group 1 methamphetamine-associated PAH, diagnosed 3.5 years previously, presented to our PH clinic after being lost to follow-up for 15 months. At the time of reestablishing care, the patient was WHO-FC III and was directly admitted to the hospital for intravenous diuresis for right heart failure and re-initiation and optimization of PAH-specific therapies. The patient was experiencing intolerable cough and poor adherence to inhaled treprostinil, and the decision was made to transition him to selexipag. The patient was also non-adherent to a regimen of tadalafil 40 mg daily and macitentan 10 mg daily. His other medications included torsemide 80 mg daily, metolazone 5 mg as needed, potassium chloride 20 mEq three times daily, spironolactone 100 mg daily, and aspirin 81 mg daily. Prior to admission, the patient had a 6MWD of 312 m and resting oxygen saturation of 90% on room air. His initial BNP was 189 pg/mL. His TTE showed an RVSP of 65.9 mmHg, severe RV enlargement, and severely reduced RV systolic function. RHC showed a RAP of 6 mmHg, PAP of 63/25 mmHg with a mean PAP of 44 mmHg, PCWP of 7 mmHg, PVR of 11.7 WU, and CI (Fick) of 1.6 L/min/m^2. On admission to the hospital, the patient was restarted on macitentan 10 mg daily, and treprostinil was changed to 6 puffs four times daily for medication optimization in anticipation of transitioning him from inhaled treprostinil to selexipag. He was WHO-FC II upon discharge from the hospital, but due to a delay in access had an interruption in macitentan for 6 days after discharge. The decision was then made to begin the transition from inhaled treprostinil to selexipag 2 weeks later as an outpatient. At the time of transition, the patient was using inhaled treprostinil 6 puffs three times daily. The plan was to initiate selexipag 200 mcg twice daily for 1 week while maintaining inhaled treprostinil at 6 puffs three times daily, then to continue the transition as outlined in **Table 5**. However, during the second week of the transition, the patient complained of increased cough related to inhaled treprostinil and wanted to discontinue use. The patient had also not increased selexipag as planned. The transition plan was modified as shown in **Table 5**.

TABLE 5 | Inhaled treprostinil to selexipag (Case #6).

Planned transition			Actual transition		
	Inhaled treprostinil down-titration	**Selexipag up-titration**		**Inhaled treprostinil down-titration**	**Selexipag up-titration**
Week 1	6 puffs three times daily	200 mcg twice daily	Week 1	6 puffs three times daily	200 mcg twice daily
Week 2	3 puffs three times daily	400 mcg twice daily	Week 2	3 puffs three times daily	400 mcg in the morning and 200 mcg in the evening
Week 3	OFF	600 mcg twice daily Increase every 1–2 weeks as tolerated to max of 1,600 mcg twice daily	Week 3	OFF	400 mcg twice daily Increase every 1–2 weeks as tolerated to max of 1,600 mcg twice daily

The patient reached a maintenance dose of 1,600 mcg twice daily approximately 6 weeks after starting selexipag. At a 6-month follow-up, he remained on selexipag 1,600 mcg twice daily. His 6MWD improved to 431 m, and his BNP was 164 pg/mL. His TTE at 8 months showed a reduction in RVSP to 53.4 mmHg, severe RV enlargement, and moderately to severely reduce RV function.

DISCUSSION

These 6 cases provide real world experience of transitioning patients between oral, inhaled, and parenteral prostanoids at a specialized PAH center over an 18-month period. This series illustrates one of the many challenges in contemporary PAH management where there is a lack of consensus regarding transitions between different agents. As prescriptions for oral prostanoid products continue to rise and the number of specialized PAH centers and providers continue to expand, it is increasingly essential to share experiences and institutional practice patterns. The purpose of this case series was to provide additional insight into transition strategies and serve as a resource for other practitioners faced with the challenge of transitioning patients between alternative prostanoid formulations.

We created individualized approaches for transitioning between prostanoid formulations based on reviews of the available literature and extensive discussion amongst our PAH providers. The absence of standardized protocols to facilitate prostanoid transitions provides a challenge to those caring for PAH patients. The risks of providing a suboptimal dose with prostanoid transitions includes acute right ventricular failure and death. The risk of transitioning patients to significantly higher doses too quickly include hypotension resulting in organ failures, as well as significant side effects. This in turn may go on to limit the amount prescribed, thus leading to inadequate treatment of patients with this severe, progressive disease.

When faced with the clinical scenario of transitioning patients between various prostanoid agents, we found the limited number of published reports describing real-world experiences to be challenging. The goal of this paper is to serve as a practical guide to our approach and details the challenges associated with these transitions. This paper is distinct from prior reports because it is comprised of different strategies and methods of delivering prostanoids, namely oral, inhaled, intravenous, and subcutaneous. Prior reports largely focused on one type of transition.

Within this case series, the predominant reason for transitioning between prostanoid formulations was clinical deterioration. Less commonly, patients were transitioned due to intolerance or poor adherence to the previous delivery system. In addition to studying how best to safely implement transitions, it would also be worthwhile to further examine reasons to switch between different prostanoid analogs from both the patients' and providers' perspectives. In the era of shared-decision making, it is important to ensure patients have been thoroughly educated on initial prostanoid therapy options, and in turn, that transition between agents is feasible, as demonstrated in this case series (21).

More prostanoid analogs are currently under clinical investigation, including a dry powder inhaled form of treprostinil (22) and ralinepag (23), a novel prostacyclin IP receptor agonist. Unless efforts are made to study the optimum manner in which PAH providers and their patients can transition between agents, this area will become increasingly complex. It will be important for drug developers and those working in the field of PAH to study and develop clinical standards in transitioning between agents.

LIMITATIONS

The cases presented here are small in number and come from a single center. Other reports have described more standardized protocols, with larger sample sizes and with some homogeneity between cases (7, 13).

In clinical practice there are well-known difficulties in categorizing patients with pulmonary hypertension. For example, in this series, several patients displayed a PCWP >15 mmHg at various time points during their clinical care. This reflects the difficulty with interpreting hemodynamics in PAH patients and emphasizes one of the challenges in PH management. All patients met WHO group I PAH criteria at the time of therapy initiation. However, over time, several patients developed

progressive heart failure with elevated PCWP likely due to left ventricular compression from an oversized right ventricle. As is routine in our and other PH programs, these cases are reviewed thoroughly and repeatedly in order to have a confirmatory diagnosis of PAH.

During the period under study, no patients were transitioned from or to epoprostenol. Therefore, such transitions are not presented in this paper. Although our transitions were well-tolerated clinically, it remains difficult to compare approaches between different institutions.

CONCLUSIONS

With the increased use of oral prostanoids, there is a critical need to provide evidence- and pharmacokinetic-based guidance on how to transition between oral and parenteral prostanoids. In this case series, we present our approach for this practice in the hope to generate discussion and to determine best practice in this area.

ETHICS STATEMENT

Written informed consent was obtained from the participants for the publication of this case report.

AUTHOR CONTRIBUTIONS

All authors contributed to reviewing the source data and crafting the manuscript.

ACKNOWLEDGMENTS

The authors wish to acknowledge Matt Bowen and Angela Chea who helped coordinate the clinical care during transitions. The authors also wish to acknowledge Erin Fox, PharmD who reviewed an earlier version of this manuscript. John J. Ryan and his research was supported by funding from The Reagan Corporation, The Gordon Family, and The Cushman Family.

REFERENCES

1. Tapson VF, Torres F, Kermeen F, Keogh AM, Allen RP, Frantz RP, et al. Oral treprostinil for the treatment of pulmonary arterial hypertension in patients on background endothelin receptor antagonist and/or phosphodiesterase type 5 inhibitor therapy (the FREEDOM-C study): a randomized controlled trial. *Chest.* (2012) 142:1383–90. doi: 10.1378/chest.11-2212
2. Tapson VF, Jing ZC, Xu KF, Pan L, Feldman J, Kiely DG, et al. Oral treprostinil for the treatment of pulmonary arterial hypertension in patients receiving background endothelin receptor antagonist and phosphodiesterase type 5 inhibitor therapy (the FREEDOM-C2 study): a randomized controlled trial. *Chest.* (2013) 144:952–8. doi: 10.1378/chest.12-2875
3. Jing ZC, Parikh K, Pulido T, Jerjes-Sanchez C, White RJ, Allen R, et al. Efficacy and safety of oral treprostinil monotherapy for the treatment of pulmonary arterial hypertension: a randomized, controlled trial. *Circulation.* (2013) 127:624–33. doi: 10.1161/CIRCULATIONAHA.112.124388
4. White RJ, Jerjes-Sanchez C, Bohns Meyer GM, Pulido T, Sepulveda P, Wang KY, et al. Combination therapy with oral treprostinil for pulmonary arterial hypertension: a double-blind, placebo-controlled study. *Am J Respir Crit Care Med*. (2019) doi: 10.1164/rccm.201908-1640OC
5. Sitbon O, Channick R, Chin KM, Frey A, Gaine S, Galie N, et al. Selexipag for the treatment of pulmonary arterial hypertension. *N Engl J Med.* (2015) 373:2522–33. doi: 10.1056/NEJMoa1503184
6. Coons JC, Miller T, Simon MA, Ishizawar DC, Mathier MA. Oral treprostinil for the treatment of pulmonary arterial hypertension in patients transitioned from parenteral or inhaled prostacyclins: case series and treatment protocol. *Pulm Circ.* (2016) 6:132–5. doi: 10.1086/685111
7. Chakinala MM, Feldman JP, Rischard F, Mathier M, Broderick M, Leedom N, et al. Transition from parenteral to oral treprostinil in pulmonary arterial hypertension. *J Heart Lung Transplant.* (2017) 36:193–201. doi: 10.1016/j.healun.2016.06.019
8. AbuHalimeh BJ, Parambil JG, Tonelli AR. Different efficacy of inhaled and oral medications in pulmonary hypertension. *Heart Lung.* (2017) 46:334–7. doi: 10.1016/j.hrtlng.2017.04.010
9. Ackerbauer KA, Tandon R. Transition from subcutaneous or inhaled treprostinil to oral treprostinil at home in patients with pulmonary arterial hypertension: a retrospective case series. *J Pharm Pract.* (2018) 31:163–6. doi: 10.1177/0897190017703507
10. Smith ZR, Kelly B, Awdish RL, Hegab S. Transitioning parenteral or inhaled treprostinil to oral treprostinil diolamine: case series and review of the literature. *J Pharm Pract.* (2019) 32:599–604. doi: 10.1177/0897190018764585
11. Fanous SM, Janmohamed M. Transition from treprostinil to selexipag in patients with pulmonary arterial hypertension: case series. *Am J Health Syst Pharm.* (2018) 75:1877–81. doi: 10.2146/ajhp170814
12. Thurber KM, Williams BM, Bates RE, Frantz RP. Transition of intravenous treprostinil to oral therapy in a patient with functional class IV chronic thromboembolic pulmonary hypertension. *Pharmacotherapy.* (2017) 37:e76–81. doi: 10.1002/phar.1951
13. Frost A, Janmohamed M, Fritz JS, McConnell JW, Poch D, Fortin TA, et al. Safety and tolerability of transition from inhaled treprostinil to oral selexipag in pulmonary arterial hypertension: results from the TRANSIT-1 study. *J Heart Lung Transplant.* (2019) 38:43–50. doi: 10.1016/j.healun.2018.09.003
14. *Orenitram Package Insert*. Research Triangle Park, NC: United Therapeutics Corp.
15. McSwain CS, Benza R, Shapiro S, Hill N, Schilz R, Elliott CG, et al. Dose proportionality of treprostinil sodium administered by continuous subcutaneous and intravenous infusion. *J Clin Pharmacol.* (2008) 48:19–25. doi: 10.1177/0091270007309708
16. *Remodulin Package Insert*. Research Triangle Park, NC: United Therapeutics Corp.
17. *Tyvaso Package Insert*. Research Triangle Park, NC: United Therapeutics Corp.
18. Enderby CY, Soukup M, Al Omari M, Zeiger T, Burger C. Transition from intravenous or subcutaneous prostacyclin therapy to inhaled treprostinil in patients with pulmonary arterial hypertension: a retrospective case series. *J Clin Pharm Ther.* (2014) 39:496–500. doi: 10.1111/jcpt.12170
19. Preston IR, Feldman J, White J, Franco V, Ishizawar D, Burger C, et al. Safety and efficacy of transition from inhaled treprostinil to parenteral treprostinil in selected patients with pulmonary arterial hypertension. *Pulm Circ.* (2014) 4:456–61. doi: 10.1086/677360
20. Holthaus N, Prins K, Rose L, Prisco S, Pritzker M, Thenappan T. EXPRESS: transition from parental prostacyclin to selexipag: a case series of five pulmonary arterial hypertension patients. *Pulm Circ.* (2019) 9:2045894019862167. doi: 10.1177/2045894019862167

21. McGoon MD, Ferrari P, Armstrong I, Denis M, Howard LS, Lowe G, et al. The importance of patient perspectives in pulmonary hypertension. *Eur Respir J.* (2019) 53:1801919. doi: 10.1183/13993003.01919-2018
22. ClinicalTrials.gov identifier: NCT03399604. Available online at: www.clinicaltrials.gov/ct2/show/NCT03399604
23. Torres F, Farber H, Ristic A, McLaughlin V, Adams J, Zhang J, et al. Efficacy and safety of ralinepag, a novel oral IP agonist, in PAH patients on mono or dual background therapy: results from a phase 2 randomised, parallel group, placebo-controlled trial. *Eur Respir J.* (2019) 54:1901030. doi: 10.1183/13993003.01030-2019

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

METHODS
published: 16 July 2020
doi: 10.3389/fmed.2020.00343

From 2D to 3D: Promising Advances in Imaging Lung Structure

Timothy Klouda[1†], David Condon[2†], Yuan Hao[1], Wen Tian[2,3], Maria Lvova[1], Ananya Chakraborty[2], Mark R. Nicolls[2,3], Xiaobo Zhou[4], Benjamin A. Raby[1,4] and Ke Yuan[1]*

[1] Divisions of Pulmonary Medicine, Boston Children's Hospital, Boston, MA, United States, [2] Division of Pulmonary, Allery and Critical Care Medicine, Stanford University, Stanford, CA, United States, [3] VA Palo Alto Health Care System, Department of Medicine, Stanford University, Stanford, CA, United States, [4] Division of Pulmonary and Critical Care Medicine, Channing Division of Network Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States

The delicate structure of murine lungs poses many challenges for acquiring high-quality images that truly represent the living lung. Here, we describe several optimized procedures for obtaining and imaging murine lung tissue. Compared to traditional paraffin cross-section and optimal cutting temperature (OCT), agarose-inflated vibratome sections (aka precision-cut lung slices), combines comparable structural preservation with experimental flexibility. In particular, we discuss an optimized procedure to precision-cut lung slices that can be used to visualize three-dimensional cell-cell interactions beyond the limitations of two-dimensional imaging. Super-resolution microscopy can then be used to reveal the fine structure of lung tissue's cellular bodies and processes that regular confocal cannot. Lastly, we evaluate the entire lung vasculature with clearing technology that allows imaging of the entire volume of the lung without sectioning. In this manuscript, we combine the above procedures to create a novel and evolutionary method to study cell behavior *ex vivo*, trace and reconstruct pulmonary vasculature, address fundamental questions relevant to a wide variety of vascular disorders, and perceive implications to better imaging clinical tissue.

Keywords: lung structure, vibratome, precision cut lung slices, optical clearing, confocal, STED

OPEN ACCESS

Edited by:
Elena Goncharova, University of Pittsburgh, United States

Reviewed by:
Charly Lai, Indiana University, United States
Rebecca R. Vanderpool, University of Arizona, United States

***Correspondence:**
Ke Yuan
ke.yuan@childrens.harvard.edu

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *30 March 2020*
Accepted: *09 June 2020*
Published: *16 July 2020*

Citation:
Klouda T, Condon D, Hao Y, Tian W, Lvova M, Chakraborty A, Nicolls MR, Zhou X, Raby BA and Yuan K (2020) From 2D to 3D: Promising Advances in Imaging Lung Structure. Front. Med. 7:343. doi: 10.3389/fmed.2020.00343

INTRODUCTION

High resolution imaging of intact tissue is vital to understanding the anatomy and physiology underlying human disease. Especially in the lung, where cells and tissues interact with the different environmental and internal stimuli, robust visualization of tissue throughout the intact lung helps identify the patterns and characteristics of diseases from asthma to fibrosis to COPD (1).

Detailed imaging of murine lung tissue that preserves the structure and cellular components helps advance the knowledge and understanding of diseases so therapeutic approaches can be applied to human subjects. The ability to label protein, RNA, and other biological compounds with a high signal-to-noise ratio while maintaining physiological structure broadens the understanding of pathology and can demonstrate the spatial approximation of different biological compounds and cell types *in situ*.

Despite sophisticated advancements in imaging technology, acquiring high-quality, clinically relevant data remains challenging. The inherent difficulties in lung tissue preparation are the technical bottleneck. Sub-optimally prepared lung tissue can lead to inaccurate alveolar architecture, light scattering and difficulties with fluorescent staining, which may potentially produce an inaccurate model.

The natural respiratory cycle is driven by negative intrathoracic pressure generated by the respiratory muscles (2). Without this pressure gradient, as when a lung is harvested, the delicate pulmonary tissue collapses and no longer resembles its physiological structure. Preparing lung tissue for imaging requires inflating the collapsed airway and perfusing the vasculature, which changes the dynamic lung into a static fixture, maintaining structural integrity and molecular components. Given the different configuration and composition in alveolar and endothelial tissue, along with the rate at which the airways and vasculature taper in the lung, distinct techniques must be used to prepare tissue as realistically as possible.

Numerous different strategies and techniques exist for preparing animal and human tissue for visualization (1, 3, 4). The standard of practice for pathologists and histologists is preparing tissue in thin slices for microscopic visualization. However, advances in biomedical photonics has allowed advanced viewing of lung tissue, improving structure, resolution and clarity of samples. Precision-cut lung slices (PCLS) provides the advantage of maintaining the three-dimensional architecture of the lung compared to thin slices, allowing for dynamic views similar to physiological conditions. Advances in microscopy, including stimulated mission depletion microscopy (STED), bypass the diffraction limit of light microscopy providing super resolution images. Clearing optical techniques, such as solvent-based clearing protocols, has significantly improved the viewing of these biological specimens by decreasing the amount of light scattering (5). Current techniques focus on optimizing tissue clarity, retaining anatomical structure, and maintaining cellular molecules for fluorescence staining.

The primary purpose of this manuscript is to describe a novel, evolutionary method to prepare thin-sectioned, thick-sectioned, and whole-organ murine lung tissue that combines OCT tissue clearing, PCLS and tissue clearing into one protocol. The optimized sample provides high-quality images of preserved structures that can be fluorescence-stained. A secondary purpose of this manuscript is to review commonly used tissue preparation methods, discussing the pros and cons of each strategy.

MATERIALS AND METHODS

Please refer to **Table 1** for instruments and equipment, chemicals and reagents and solutions used during methods.

Animals

Animals used were 10–12 weeks, 22–25 g, male C57BL6 or NG2-Cre-ER/ROSA26RtdTomato (NG2-tdT) mice, as previously published (6). The NG2-tdT mouse line exhibits selective pericyte labeling by tdTomato when B6.Cg-Tg (Cspg4-Cre/Esr1*) BAkik/J (NG2-Cre-ER) mice (https://www.jax.org/strain/008538) are crossed with with tdTomato Ai14 (https://www.jax.org/strain/007908). NG2-tdT mice were treated with 2 mg tamoxifen dissolved in corn oil (20 mg/ml) for two consecutive injections. After tamoxifen treatments, mice rested for 7 days before tissue harvest.

TABLE 1 | Equipment, Chemicals and reagents, Instruments and solutions used in the materials and methods.

Equipment

- Cryostat slicer (HM525NX, ThermoFisher)
- Vibration slicer (Leica VT1000s, Nussloch, Germany)
- Confocal microscope (Zeiss 880 with Airyscan)
- Light Sheet microscope (LaVision)
- Facility Line Confocal/STED microscopy (Abberior Instruments America LLC)

Chemicals and reagents

- UltraPure Low Melting Point Agarose (2%, Invitrogen,16520050)
- Mouse-anti-mouse/human SMA-Cy3 (1:300, Sigma, C6198-.2ML)
- Rat-anti-mouse CD31 (1:100; BD-Pharmingen, 553370 or 550274)
- Rabbit-anti-mouse-RFP (1:100, Rockland, 600-401-379)
- Goat anti-rabbit STAR RED (1:100, Abberior Instruments GmbH, STRED)

Instruments and solutions

- Flushing buffer (1X PBS + 0.1% heparin)
- PLP perfusion buffer (0.075 M lysine, 0.37 M sodium phosphate pH 7.2, 2% formaldehyde, and 0.01 M $NaIO_4$, a detailed receipt can be found at Cold Spring Harbor Protocols)
- Inflation solution (½ volume of 100% OCT mixed with ½ volume of 30% sucrose)
- Expansion solution (2% agarose in 1XPBS)
- Blocking buffer (5% serum (goat, determined by species of secondary antibodies), 0.3% Triton X100 in 1XPBS)
- Antibody dilution buffer (1% BSA, 0.3% Triton X-100 in 1XPBS)
- MPBS (Percentage of Methanol in 1XPBS)
- BABB solution (1 volume of Benzyl Alcohol mix with 2 volume of Benzyl Benzoate)

Harvest, Cannulation, and Perfusion of Mouse Lung

Anesthetize the animal with 3% isoflurane to ensure the lack of pain reflex, then sacrifice by neck isolation. Open the chest and create a small nick through the suprarenal abdominal aorta. Insert a 25–30 g butterfly needle* (**Figures 1A,A′**) connected to a 30 cc syringe into the right ventricle. Flush with a 20 ml flushing buffer slowly but steadily until the fluid extravasating from the abdominal aorta is clear and colorless. *Note: use of the butterfly needle prevents fluid leakage due to multiple cannulations.

(1) OCT Tissue Preparation

Switch syringe at infusion port of butterfly needle and flush with Periodate-Lysine-Paraformaldehyde (PLP) buffer. Cannulate trachea with 22 g blunt-ended needle and inflate with 2.5–4 ml OCT inflation solution. Immediately following inflation, separate and put each lobe in the tissue mold completely covered with OCT. Place the mold on a metal block and submerge in liquid nitrogen. After the OCT is completely frozen, store at −80°. Proceed with cryosectioning, immunolabeling, counterstaining, and imaging (each step is summarized in **Figure 2**).

(2) Vibratome

Cannulate trachea with 22 g blunt-ended needle and inflate with 2.5–4 ml 2% agarose solution. Incubate the dissected lungs in 4% paraformaldehyde solution overnight at 4° on an agitator.

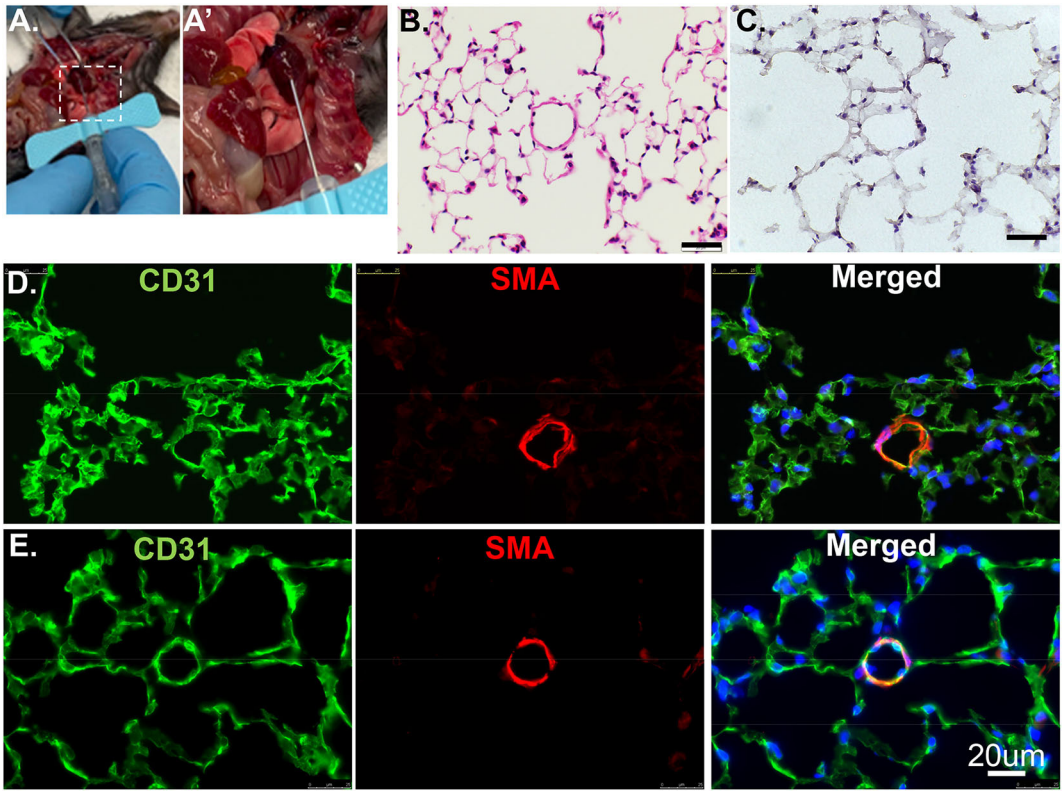


FIGURE 1 | Cross section of lung tissues using H&E or IF. **(A)** The RV is cannulated with a 25G butterfly needle to facilitate switching solutions from flushing buffer to PLP for pulmonary vascular perfusion. **(A')** Enlarged image of dashed rectangle in **A**. **(B)** Wildtype murine lung structure using H&E. Scale bar: 20 um. **(C)** Wildtype murine lung structure using H&E after inflation with 100% OCT. Scale bar: 20 um. **(D)** The same procedure as in C but applied IF staining. CD31: green, stained for endothelial; SMA: red, stained for smooth muscle layer; DAPI: blue, stained for nuclei. **(E)** PLP buffered murine lung described in manuscript providing improved structural integrity.

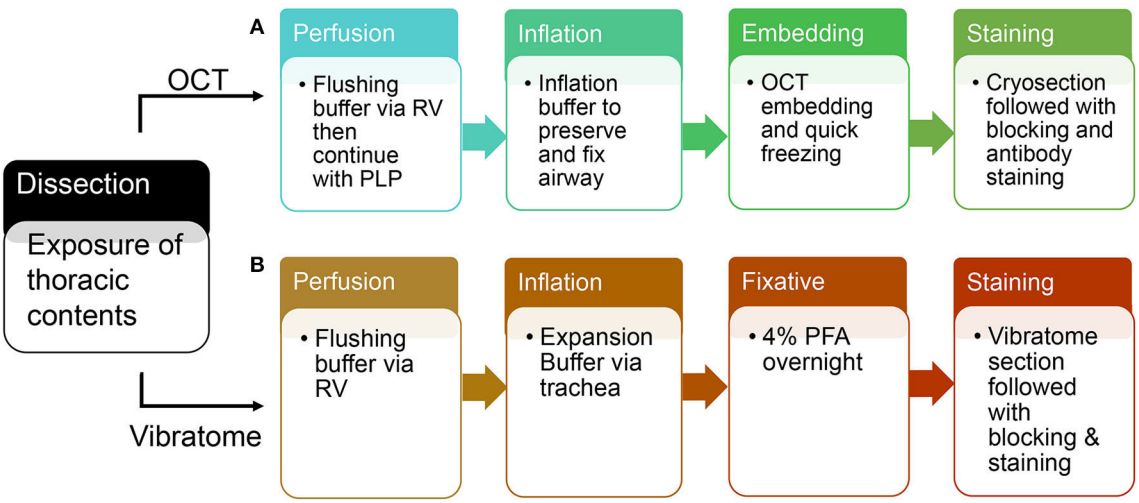


FIGURE 2 | A schematic flow of OCT tissue preparation and vibratome tissue preparation. **(A)** An overview of steps in tissue preparation using OCT. **(B)** An overview of steps in tissue preparation using low melting agarose.

The next day, wash with 1XPBS at 4° and store for future use. Separate and section each lobe with Vibration Slicer at 300 um thickness. Block slices in serum overnight and incubate with primary/secondary antibodies in antibody dilution buffer, and lastly image with Zeiss confocal 880 (each step is summarized in **Figure 2**). Vibratome-sliced sections prepared for STED imaging

will use the same protocol as Confocal, but incubate with STED designated secondary antibody (7), such as STAR Red.

(3) Tissue Clearing

Tissue clearing is effective with any thickness of tissue slice, including the whole lobe. Wash with PBS, then the following serial dilutions of MPBS: 25, 50, 75, 100% (dehydration) to 75, 50, 25%, PBS (rehydration), with an overnight incubation at each step. Incubation time will vary based on the thickness of the sample being prepared, with a minimum of 30 min recommended for thin samples (300 um) or overnight for whole lung lobes. The rest of the staining will be performed at 4°. Incubate in serum blocking buffer (the selected serum depends on the species of the secondary antibody, e.g., 5% Goat serum) overnight. Incubate primary antibodies in blocking buffer overnight. Wash slices in the blocking buffer at least three times. Incubate in secondary antibodies at least overnight. Wash slices in the blocking buffer at least three times. After a brief wash in PBS, dehydrate with serial dilutions of MPBS and stop at 100% methanol. Submerge tissue in BABB* and gently agitate until the tissue is colorless. The organ must be as dehydrated as possible, as BABB will fail to clear tissue in the presence of water. Proceed with imaging with Confocal or Light-Sheet Microscopy. *Note: Once combined, BABB is extremely volatile and toxic on skin contact.

RESULTS

Paraffin embedding is the traditional technique to preserve and evaluate lung structure. Paraffin sections are a valuable resource to reveal structural and matrix components details, such as procedures Movat, Trichrome, H&E staining (**Figure 1B**). However, paraffin embedding is suboptimal for immunofluorescence imaging. In this method, the lung is infused through airways with 3% formalin to preserve the structure and prevent artifacts that can preclude accurate histological evaluation. The fixative also cross-links certain proteins in and on the cells. Thus, antigen retrieval to "unmask" cross-linked antigens is often essential and required. Unfortunately, some antigens can be destroyed by the high-temperature processing used in "unmasking" and are no longer recognized by their designated antibodies. Sometimes, high antibody concentration throughout the tissue may cause non-specific binding as false-negative results.

Embedding in OCT (aka frozen sections) compound, a method with minimal antigen damage is much better for two-dimensional immunofluorescence (IF) imaging. OCT compound was developed specifically to facilitate tissue cryo-sectioning and permeability. Tissue preparation with OCT allows for faster start-to-finish tissue processing, thinner sections (10 um), with more consistent antigen penetration for immunolabeling when compared to paraffin preservation and sectioning.

Previously, we used PBS to flush blood from the harvested tissue, 3% formalin as a fixative, then inflated through airways with 100% OCT. After H&E and antibody staining, we found discontinuous layers of alveolar pneumocytes and transparent/matrix basement membrane, which failed to reflect the real structure (**Figures 1C,D**). To optimize this procedure, we used Periodate-Lysine-Paraformaldehyde (PLP) and inflation solution to preserve structural integrity. After a flushing buffer, PLP solution fixes the vasculature from the inside out while further clearing the tissue of blood. The inflation buffer optimizes the structural preservation of the alveolar/capillary interface even at the periphery and reduces autofluorescence (**Figure 1E**). We recommend performing the inflation step as quickly as possible to facilitate rapid coating with OCT and freezing. This step will exclude air bubbles and optimize the overall structure appearance.

To avoid regional sampling bias when analyzing lung structure in a whole-mount context, we applied the expansion solution via trachea and sectioned into 300–600 um vibratome slices (the slice thickness can be determined by the working distance of confocal objective lens), or as known as Precision Cut Lung Slices (PCLS). These slices can be easily permeabilized and stained with antibodies CD31 for endothelium and SMA for smooth muscle. We then used confocal Z-stack/reconstitution to assess CD31/SMA-positive cell coverage on individual vessels (**Figure 3**). Quantification of our results can be achieved by counting the number of overlapped nuclei with or without positive antibody staining (8).

As proof of principle, we used a previous created a NG2-selective reporter mouse and showed that intraperitoneal tamoxifen selectively marks NG2 promoter-driven cells with red fluorescence in capillaries (6). The vibratome slices collected from this transgenic line was stained with RFP and imaged using Stimulated emission depletion (STED) microscopy (**Figure 4A**). Compared to the out-of-focus blur and an artifact seen by confocal (**Figure 4B**), STED allowed high resolution and distinction of single positive RFP on the cytoplasm at high spatial density (**Figure 4C**).

Molecular and optical interrogation of large-sized biological tissue (>1 mm) can be achieved by combining optical tissue clearing and light-sheet microscopy (**Figure 5**). It enables a true representation of cell location in a whole organ level that allows the immunolabeling of proteins that are essential under normal and pathologic conditions. BABB clearing after systematic tissue dehydration, rehydration, labeling, and then second dehydration provides for a relatively fast protocol for this visualization. Positive SMA stain area quantification can be calculated by processing with the "Surface and Filament Tracer" tool of Imaris (Bitplane) (9).

DISCUSSION

In this manuscript, we outline new, enhanced methodologies to produce high-resolution images that preserve the structure and optimize experimental flexibility on thin-section, thick-sections, and whole organ images. The combination of tissue clearing preparation with lung inflation enables an unparalleled system-based visualization of biological pathways within the whole organ. Inflating the lung gives us a better perspective of distal vs. proximal structures and provides superior spatial detail compared to methods not used in combination.

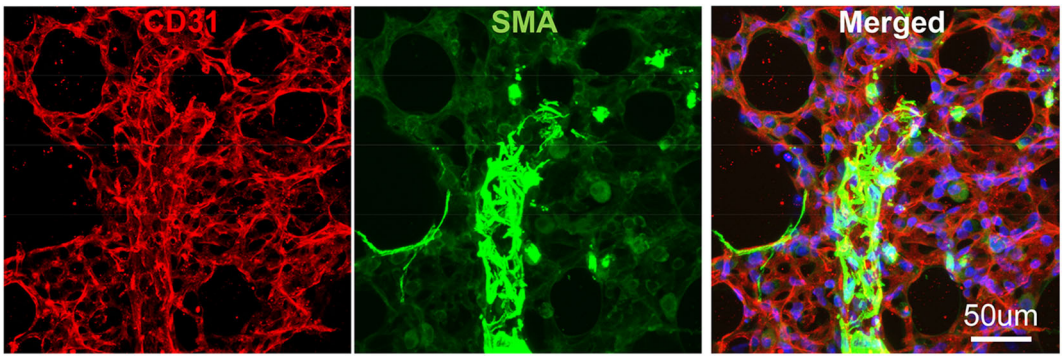


FIGURE 3 | Vibratome section of lung tissues using IF. The wildtype murine lung after flushing was inflated with agarose and proceed with fixative. CD31: green, stained for endothelial; SMA: Red, stained for smooth muscle layer; DAPI: blue, stained for nuclei.

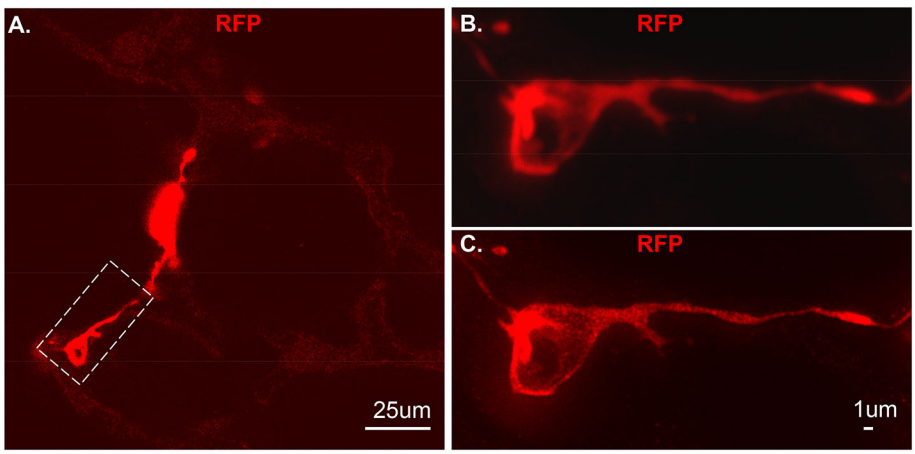


FIGURE 4 | An individual tdT positive cell from vibratome sections. The section was stained using RFP and the secondary antibody used is anti-rabbit-STAR RED. **(A)** An overall view of NG2-tdT lung using STED. A RFP positive cell in dash rectangle was captured using Confocal **(B)** or captured using STED **(C)**. RFP: red fluorescence protein.

The preparation of tissue microslices is a technique that uses a vibratome, or vibrating blade, to produce precision-cut organotypic slices. This allows for increased accuracy and reproducibility when generating precision-cut lung slices (PCLS) compared to alternate methods (10). Tissue slices are time- and cost-effective compared to other *in vivo* work (11, 12). PCLS has been used in human and animal studies focusing on lung anatomy, toxic exposures, infectious disease and immunological studies (13, 14). They can maintain the structural framework at both the tissue and cellular level, making them useful for studying anatomical abnormalities seen in diseases, such as asthma or COPD (15). This makes PCLS the ideal platform to study tissue-specificity and cancer cell selectivity of gene therapy vectors prior to *in vivo* trials (4).

However, PCLS does not come without limitations. PCLS displays a snapshot of the cells and molecules residing in lung tissue at the time of removal and does not capture the heterogeneity seen in some pathological processes. The PCLS model is also static, which means that any testing involving mechanical stress, such as barotrauma, is limited. Samples have a viability of 3–6 days, limiting the extend that PCLS can model *in vivo* situations (12). While a PCLS is an excellent model for physiologic and toxicologic studies, future investigation is needed to describe methods for improved viability incorporation of immune components to enhance PCLS.

Confocal imaging was patented in 1957 and aimed to overcome the limitations of traditional fluorescence microscopes when viewing thin-cut samples (16). It is superior to conventional microscopy by focusing light inside tissue and limiting the emitted light returned, allowing the specimen to be imaged at a single "point" at a time. This allows the reconstruction of high-resolution, high contrast three-dimensional images without the artifacts that limit conventical microscopy. However, confocal imaging is restricted by its prolonged scanning time, photobleaching secondary to long scan times, causing reduced signal-to-noise ratios, and the inability to simultaneously visualize multiple specimen layers (17).

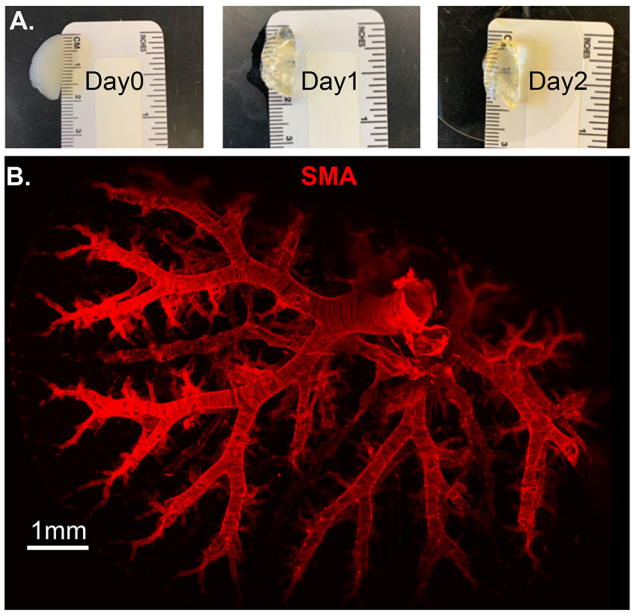


FIGURE 5 | Lung structure staining by smooth muscle actin (SMA) staining. Wildtype mouse whole lung lobe was gradually and optically cleared (became transparent) in 2 days with organic solvent BABB **(A)** and imaged using 3D Light Sheet **(B)**.

Due to these limitations, techniques were developed which split fluorescence light into multiple planes. This method, or light sheet microscopy, significantly reduced scanning time and some of the limitations seen with confocal imaging. Light sheet microscopy has been employed to investigate inflammation, cancer, hemopoiesis and infection in lung and other tissues (18–21). It has also has been used to investigate the distribution of *Aspergillosis fumigatus* and identify the local inflammatory response secondary to pulmonary infections (22). However, this technique is limited by optical aberrations and a point-by-point scanning technique (17).

Stimulated emission depletion (STED) microscopy creates super-resolution images at a molecular level. This overcomes the diffraction limitations seen in confocal microscopy to improve resolution and provide nanoscale visualization of individually labeled molecules (23). This technique has been recently used in neuroscience to better visualize dendritic cells and understand their structure and function (24). STED has been applied to lung tissue by detecting filamentous human respiratory syncytial virus particles, providing a better understanding and mechanism of how the virus spreads cell to cell (e.g., microbiota activity) (25). STED microscopy can potentially aid in understanding the molecular mechanisms of receptors and their specific ligands in disease, such as pulmonary hypertension, fibrosis and malignancies (26, 27). An understanding of these molecules at their response can lead to the development of possible pharmaceutical therapies.

Tissue optical clearing is a chemical process aiming to improve light penetration throughout intact tissue, rendering them transparent and allowing fluorescent microscope imaging (28). Organic-solvent based clearing protocols, such as 3DISCO can achieve high levels of tissue transparency, allowing imaging of large samples, such as brain, tumors and embryos (5, 29). However, the inability to preserve fluorescent protein emission with solvent-based clearing techniques has led to a pursue with aqueous-based solutions. Current techniques include passive immersion, hyperhydration, or hydrogel embedding. While these techniques traditionally do not clear as well as solvent-based methods, they are easy to implement and useful in samples where a wide range of fluorescent dyes and proteins are required. CLARITY is a method for chemically transforming intact biological tissue into a hydrogel-tissue hybrid (3). This hybrid model is amendable to light and macromolecular labels while also retaining structure and biological molecules, such as proteins and nucleic acids. CLARITY has the additional benefit of being plastic-safe, making materials easier to select for processing and imaging safer on most institutional equipment due to lack of necessity for imaging while submerged within an organic solvent. The method has also been successfully applied to numerous animal and human brain models, with the potential to be applied to different, large organ systems. For lung tissue mainly, we found the solvent-based method is more stable and less time-consuming than CLARITY due to variable lipid and protein contents.

In summary, our study provides novel and insightful methods to improve and enhance experimental procedures to preserve pulmonary vasculature with minimal change from regional to a whole-organ large scale. Additionally, all presented methods can have a broader and more comprehensive application, such as assessment of lung pathology in clinical samples.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC at Boston Children's Hospital and Stanford University.

AUTHOR CONTRIBUTIONS

KY: conception and design, acquisition of data, analysis and interpretation of data, drafted and revised the manuscript. TK and DC: analysis and interpretation of data, drafted and revised the manuscript. YH, WT, ML, AC, MN, XZ, and BR made substantial contributions to the acquisition of data, analysis and interpretation of data. All authors contributed to the article and approved the submitted version.

FUNDING

KY was supported by American Heart Association Scientist Development grant 15SDG25710448, the Parker B. Francis

Fellowship; the Pulmonary Hypertension Association Aldrighetti Research Award. Harvard Neurobiology Imaing Facility was supported by Havard Medical School/Boston Children's Hospital Center for Neuroscience research #NS072030.

ACKNOWLEDGMENTS

The authors thank Dr. Abinaya Nathan, Ms. Joyce Yu, and Mr. Elya Shamskhou for their technical support.

REFERENCES

1. Ochoa LF, Kholodnykh A, Villarreal P, Tian B, Pal R, Freiberg AN, et al. Imaging of murine whole lung fibrosis by large scale 3D microscopy aided by tissue optical clearing. *Sci Rep.* (2018) 8:13348. doi: 10.1038/s41598-018-31182-2
2. Sparrow D, Weiss ST. Respiratory physiology. *Annu Rev Gerontol Geriatr.* (1986) 6:197–214.
3. Tomer R, Ye L, Hsueh B, Deisseroth K. Advanced clarity for rapid and high-resolution imaging of intact tissues. *Nat Protoc.* (2014) 9:1682–97. doi: 10.1038/nprot.2014.123
4. Rosales Gerpe MC, van Vloten JP, Santry LA, de Jong J, Mould RC, Pelin A, et al. Use of precision-cut lung slices as an *ex vivo* tool for evaluating viruses and viral vectors for gene and oncolytic therapy. *Mol Ther Methods Clin Dev.* (2018) 10:245–56. doi: 10.1016/j.omtm.2018.07.010
5. Richardson DS, Lichtman JW. Clarifying tissue clearing. *Cell.* (2015) 162:246–57. doi: 10.1016/j.cell.2015.06.067
6. Yuan K, Liu Y, Zhang Y, Nathan A, Tian W, Yu J, et al. Mural cell SDF1 signaling is associated with the pathogenesis of pulmonary arterial hypertension. *Am J Respir Cell Mol Biol.* (2020) 62:747–59. doi: 10.1165/rcmb.2019-0401OC
7. Nagerl UV, Willig KI, Hein B, Hell SW, Bonhoeffer T. Live-cell imaging of dendritic spines by STED microscopy. *Proc Natl Acad Sci USA.* (2008) 105:18982–7. doi: 10.1073/pnas.0810028105
8. Volz KS, Jacobs AH, Chen HI, Poduri A, McKay AS, Riordan DP, et al. Pericytes are progenitors for coronary artery smooth muscle. *Elife.* (2015) 4:e10036. doi: 10.7554/eLife.10036.024
9. Lagerweij T, Dusoswa SA, Negrean A, Hendrikx EML, de Vries HE, Kole J, et al. Optical clearing and fluorescence deep-tissue imaging for 3D quantitative analysis of the brain tumor microenvironment. *Angiogenesis.* (2017) 20:533–46. doi: 10.1007/s10456-017-9565-6
10. Sanderson MJ. Exploring lung physiology in health and disease with lung slices. *Pulm Pharmacol Ther.* (2011) 24:452–65. doi: 10.1016/j.pupt.2011.05.001
11. Held HD, Martin C, Uhlig S. Characterization of airway and vascular responses in murine lungs. *Br J Pharmacol.* (1999) 126:1191–9. doi: 10.1038/sj.bjp.0702394
12. Rosner SR, Ram-Mohan S, Paez-Cortez JR, Lavoie TL, Dowell ML, Yuan L, et al. Airway contractility in the precision-cut lung slice after cryopreservation. *Am J Respir Cell Mol Biol.* (2014) 50:876–81. doi: 10.1165/rcmb.2013-0166MA
13. Liu R, An L, Liu G, Li X, Tang W, Chen X. Mouse lung slices: an *ex vivo* model for the evaluation of antiviral and anti-inflammatory agents against influenza viruses. *Antiviral Res.* (2015) 120:101–11. doi: 10.1016/j.antiviral.2015.05.008
14. de Graaf IA, Olinga P, de Jager MH, Merema MT, de Kanter R, van de Kerkhof EG, et al. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nat Protoc.* (2010) 5:1540–51. doi: 10.1038/nprot.2010.111
15. Liu G, Betts C, Cunoosamy DM, Aberg PM, Hornberg JJ, Sivars KB, et al. Use of precision cut lung slices as a translational model for the study of lung biology. *Respir Res.* (2019) 20:162. doi: 10.1186/s12931-019-1131-x
16. Nwaneshiudu A, Kuschal C, Sakamoto FH, Anderson RR, Schwarzenberger K, Young RC. Introduction to confocal microscopy. *J Invest Dermatol.* (2012) 132:e3. doi: 10.1038/jid.2012.429
17. Mohan K, Purnapatra SB, Mondal PP. Three dimensional fluorescence imaging using multiple light-sheet microscopy. *PLoS ONE.* (2014) 9:e96551. doi: 10.1371/journal.pone.0096551
18. Hulsdunker J, Ottmuller KJ, Neeff HP, Koyama M, Gao Z, Thomas OS, et al. Neutrophils provide cellular communication between ileum and mesenteric lymph nodes at graft-versus-host disease onset. *Blood.* (2018) 131:1858–69. doi: 10.1182/blood-2017-10-812891
19. Wertheimer T, Velardi E, Tsai J, Cooper K, Xiao S, Kloss CC, et al. Production of BMP4 by endothelial cells is crucial for endogenous thymic regeneration. *Sci Immunol.* (2018) 3:eaal2736 doi: 10.1126/sciimmunol.aal2736
20. Brede C, Friedrich M, Jordan-Garrote AL, Riedel SS, Bauerlein CA, Heinze KG, et al. Mapping immune processes in intact tissues at cellular resolution. *J Clin Invest.* (2012) 122:4439–46. doi: 10.1172/JCI65100
21. D. Stegner, vanEeuwijk JMM, Angay O, Gorelashvili MG, Semeniak D, Pinnecker J, et al. Thrombopoiesis is spatially regulated by the bone marrow vasculature. *Nat Commun.* (2017) 8:127. doi: 10.1038/s41467-017-00201-7
22. Amich J, Mokhtari Z, Strobel M, Vialetto E, Sheta D, Yu Y, et al. Three-dimensional light sheet fluorescence microscopy of lungs to dissect local host immune-aspergillus fumigatus interactions. *mBio.* (2020) 11:e02752-19. doi: 10.1128/mBio.02752-19
23. Blom H, Brismar H. STED microscopy: increased resolution for medical research? *J Intern Med.* (2014) 276:560–78. doi: 10.1111/joim.12278
24. Blom H, Ronnlund D, Scott L, Spicarova Z, Widengren J, Bondar A, et al. Spatial distribution of Na^+-K^+-ATPase in dendritic spines dissected by nanoscale superresolution STED microscopy. *BMC Neurosci.* (2011) 12:16. doi: 10.1186/1471-2202-12-16
25. Mehedi M, Smelkinson M, Kabat J, Ganesan S, Collins PL, Buchholz UJ. Multicolor stimulated emission depletion (STED) microscopy to generate high-resolution images of respiratory syncytial virus particles and infected cells. *Bio Protoc.* (2017) 7:e2543. doi: 10.21769/BioProtoc.2543
26. Oczypok EA, Perkins TN, Oury TD. All the "RAGE" in lung disease: the receptor for advanced glycation endproducts (RAGE) is a major mediator of pulmonary inflammatory responses. *Paediatr Respir Rev.* (2017) 23:40–49. doi: 10.1016/j.prrv.2017.03.012
27. Oczypok EA, Perkins TN, Oury TD. Alveolar epithelial cell-derived mediators: potential direct regulators of large airway and vascular responses. *Am J Respir Cell Mol Biol.* (2017) 56:694–9. doi: 10.1165/rcmb.2016-0151PS
28. Cai R, Pan C, Ghasemigharagoz A, Todorov MI, Forstera B, Zhao S, et al. Panoptic imaging of transparent mice reveals whole-body neuronal projections and skull-meninges connections. *Nat Neurosci.* (2019) 22:317–7. doi: 10.1038/s41593-018-0301-3
29. Qi Y, Yu T, Xu J, Wan P, Ma Y, Zhu J, et al. FDISCO: advanced solvent-based clearing method for imaging whole organs. *Sci Adv.* (2019) 5:eaau8355. doi: 10.1126/sciadv.aau8355

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 31 July 2020
doi: 10.3389/fmed.2020.00352

When Innate Immunity Meets Angiogenesis—The Role of Toll-Like Receptors in Endothelial Cells and Pulmonary Hypertension

Aneel Bhagwani[1,2], *A. A. Roger Thompson*[3] *and Laszlo Farkas*[1]*

[1] Division of Pulmonary, Critical Care and Sleep Medicine, Department of Internal Medicine, Davis Heart & Lung Research Institute, The Ohio State University, Columbus, OH, United States, [2] Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, United States, [3] Department of Infection, Immunity & Cardiovascular Disease, Faculty of Medicine, Dentistry & Health, University of Sheffield, Sheffield, United Kingdom

Toll-like receptors serve a central role in innate immunity, but they can also modulate cell function in various non-immune cell types including endothelial cells. Endothelial cells are necessary for the organized function of the vascular system, and part of their fundamental role is also the regulation of immune function and inflammation. In this review, we summarize the current knowledge of how Toll-like receptors contribute to the immune and non-immune functions of the endothelial cells.

Keywords: toll-like receptors, endothelial cells, angiogenesis, pulmonary hypertension, immunology

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez,
Stanford University, United States

Reviewed by:
Zhiyu Dai,
University of Arizona, United States
Feng-Ming Yang,
Taipei Medical University, Taiwan

***Correspondence:**
Laszlo Farkas
Laszlo.Farkas@osumc.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *28 April 2020*
Accepted: *12 June 2020*
Published: *31 July 2020*

Citation:
Bhagwani A, Thompson AAR and Farkas L (2020) When Innate Immunity Meets Angiogenesis—The Role of Toll-Like Receptors in Endothelial Cells and Pulmonary Hypertension. Front. Med. 7:352. doi: 10.3389/fmed.2020.00352

TOLL-LIKE RECEPTORS

The human body is constantly exposed to exogenous immunological triggers and reacts to these triggers after recognizing their associated molecules. Janeway proposed over 20 years ago that invading microorganisms, such as viruses and bacteria, have specific molecular patterns, so-called pathogen-associated molecular patterns (PAMPs), that trigger recognition by the immune system and named these pattern recognition receptors (PRRs) (1). The PAMPs are sensed by germline-encoded evolutionary conserved host sensors called pathogen recognition receptors or PRRs and form a key element of the innate immune system (2, 3). PRRs are not only present in typical immune cells, such as monocytes, macrophages, and T lymphocytes, but also are expressed in non-immune cells, including endothelial cells (4). Four classes of PRRs are known today: (1) the Toll-like receptors (TLRs) which we will focus on in this review; (2) C-type lectin receptors (CLRs): this large family of cell surface transmembrane receptors bind carbohydrates via specific recognition domains and are important for the immune response to fungal pathogens (5); (3) retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR) which are cytoplasmic sensors of viral RNA or self-processed RNA (6, 7); and (4) nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) which are cytoplasmic receptors that recognize PAMPs. In contrast to TLRs, NLRs mainly signal via the formation of a multimeric protein complex called the inflammasome (8). Of these four receptor families, TLRs are the most extensively studied class. Although TLRs are an integral part of the innate immune system, their expression is not limited to immune cells but their presence can also be detected in non-immune cells such as endothelial cells, which are further discussed in this review article. The name "Toll-like receptor" originates from the structural homology of TLRs with the Toll transmembrane protein which is important for embryonic development in *Drosophila melanogaster* (9). Toll has an important role for antibacterial defense in Drosophila

(10), because mutations in the Toll gene decrease the antimicrobial and antifungal response, leading to increased susceptibility to infection and death (11). The cytoplasmic domain of the Toll protein shares homology with human interleukin-1 (IL-1) receptors (IL-1Rs), leading to similar biochemical signal transduction by IL-1Rs and Toll protein (12). Human transmembrane receptors with structural homology to the Drosophila Toll protein were categorized as Toll-like receptors (TLRs) (13). At present, 10 TLRs have been identified in humans (TLR1–10) and 12 (TLR1–9, 11, and 13) in mice (14). TLRs 1–9 are highly conserved in mammals, TLR10 is non-functional in mice, and the human TLR11 gene contains a stop codon that results in lack of production of functional TLR11 (15, 16). The TLRs are localized to either the cell surface membrane or the membranes of intracellular compartments. TLRs 1, 2, 4, 5, 6, and 11 are found at the cell surface and detect extracellular PAMPs, whereas TLR 3, 7, 8, and 9 bind intracellular PAMPs localized to intracellular vesicles such as endosomes and lysosomes, or vesicles derived from the endoplasmic reticulum (17).

In addition to recognizing extracellular and intracellular PAMPs, the immune system plays an important role in the response to non-pathogenic conditions, such as trauma, ischemia, and autoimmune disorders. Polly Matzinger proposed a danger signal model paralleling the concept of PAMPs and suggested that any molecule that is normally not secreted from the cell could activate an immune response if released from the cell in response to injury (18). This damage model further evolved to the concept of damage-associated molecular patterns (DAMPs) (19). Similar to the recognition of PAMPs by PRRs, certain PRRs, including TLR2 and TLR4, bind DAMPs (20). A list of the most common PAMPs (pathogen-associated molecules) and DAMPs (self-molecules) that activate the different TLRs is shown in **Table 1**.

TABLE 1 | Ligands for various toll like receptors.

TLR	Ligand	
	PAMP	**DAMP**
TLR1	Bacterial lipoproteins (21, 22)	
TLR2	Soluble peptidoglycan (SPGN) (23)	Biglycan (24)
	Lipoteichoic acid (LTA) (23, 25)	High Mobility Group Box 1 HMGB1(26)
	Pam_3CSK_4 (27)	Monosodium urate crystals (28, 29)
		Calcium Pyrophosphate Dihydrate (28)
		Human cardiac myosin and C0C1f fragment of cardiac myosin binding protein-C (30, 31)
TLR3	Viral dsRNA and Polyinosinic:polycytidylic acid (poly(IC)) (32)	dsRNA from necrotic cells (33)
	siRNA (34)	mRNA (35),
TLR4	Lipopolysaccharide LPS (36)	Biglycan (24)
		High-mobility group box 1 (HMGB1) (26)
		Fibrinogen (37)
		Heparan sulfate (38, 39)
		C0C1f fragment of cardiac myosin binding protein-C (30)
TLR5	Flagellin (40)	
TLR6	Diacylated lipoproteins (41)	
TLR7	Guanosine and uridine-containing ssRNA (42)	Human cardiac myosin (31)
TLR8	Single-stranded RNA (ssRNA), bacterial RNA (43–45)	
TLR9	Unmethylated CpG oligodinucleotides (ODNs) from bacterial DNA (46)	Mitochondrial CpG-ODN (47)
TLR10	Human immunization virus-1 (HIV-1) proteins (48)	

Ligands for various Toll-like receptors differentiated into PAMPs and DAMPs. PAMP, pathogen-associated molecular patterns; DAMP, damage-associated molecular patterns; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; ODN, oligodinucleotides; mRNA, messenger RNA.

TOLL-LIKE RECEPTOR SIGNALING

TLRs are evolutionary conserved type 1 transmembrane glycoprotein receptors with an ectodomain and a cytosolic domain. The ectodomain contains varying numbers of leucine-rich repeats (LRRs) that are required for ligand binding. Vertebrate TLRs contain 16–28 LRRs and human TLRs 19–25 LRRs. These LRRs form a continuous structure and adopt a horseshoe shape, which facilitates ligand binding (49). After ligand binding, the receptor forms an m-shaped dimer that sandwiches the ligand, bringing the cytoplasmic and transmembrane domains together to initiate signaling (50). Ligand binding causes either homodimerization or heterodimerization of TLRs and the formation of heterodimers promotes ligand diversity, whereas homodimerization increases ligand specificity (51). For example, TLR2 and TLR4 homodimerize but only the TLR4 homodimer can initiate TNFα signaling, whereas TLR1, TLR2, and TLR6 need to heterodimerize to initiate TNFα signaling (52). Differences in LRRs in the extracellular domains along with combinations of different TLRs not only promote ligand diversity to recognize a large number of PAMPs but also determine differences in the downstream signaling (53, 54).

Ligand binding then initiates a cascade of downstream signaling via the cytosolic Toll/interleukin-1 receptor (TIR) domain, which is a conserved domain shared by TLRs and the interleukin 1 receptor (IL-1R) superfamily (55). The TIR domains of TLRs dimerize upon ligand binding. This self-association leads to the recruitment of intracellular TIR-containing adaptor proteins via TIR–TIR interactions, forming a trimer. The recruitment of additional intermediates then elongates this trimer. The sequential and cooperative

binding of the TIR domains amplifies the signal and results in a highly sensitive response (56). The main TIR-containing adaptor proteins are myeloid differentiation factor 88 (MyD88), Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (MAL or TIRAP), and TIR domain-containing adapter molecules (TICAM), such as TIR-domain-containing adapter-inducing interferon-β (TRIF or TICAM-1) and translocating chain-associated membrane protein (TRAM or TICAM-2). These adaptor proteins bind to TLRs and initiate major downstream signaling pathways, including nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), and interferon-regulatory factor (IRF) (56, 57).

All TLRs except TLR3 follow the MyD88-dependent pathway-activating mitogen-activated protein kinase kinase kinase 7 (MAPKKK7 or TAK1) which is downstream of TRAF6. TAK1 then leads to activation of both enzyme IκB kinase (IKK)-activating NF-κB and MAPK family c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 leading to AP-1 activation and proinflammatory cytokine production (58). However, endosomal TLR3 and TLR4 signal via a MyD88-independent pathway involving TRIF. TLR3 associates with TRIF directly (59), whereas TLR4 associates with TRIF through TRAM (60). TRIF then activates TRAF3 activating the IRF3/7 signaling pathway leading to type 1 IFN production (61) and TRAF6 activating the NF-κB and AP-1 signaling response (62, 63).

Activation of these pathways results in the release of cytokines such as interleukin-4 (IL-4), IL-13, tumor necrosis factor-α (TNF-α), IL-1β, chemokines such as IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), and type I interferons (IFNs), such IFN-α

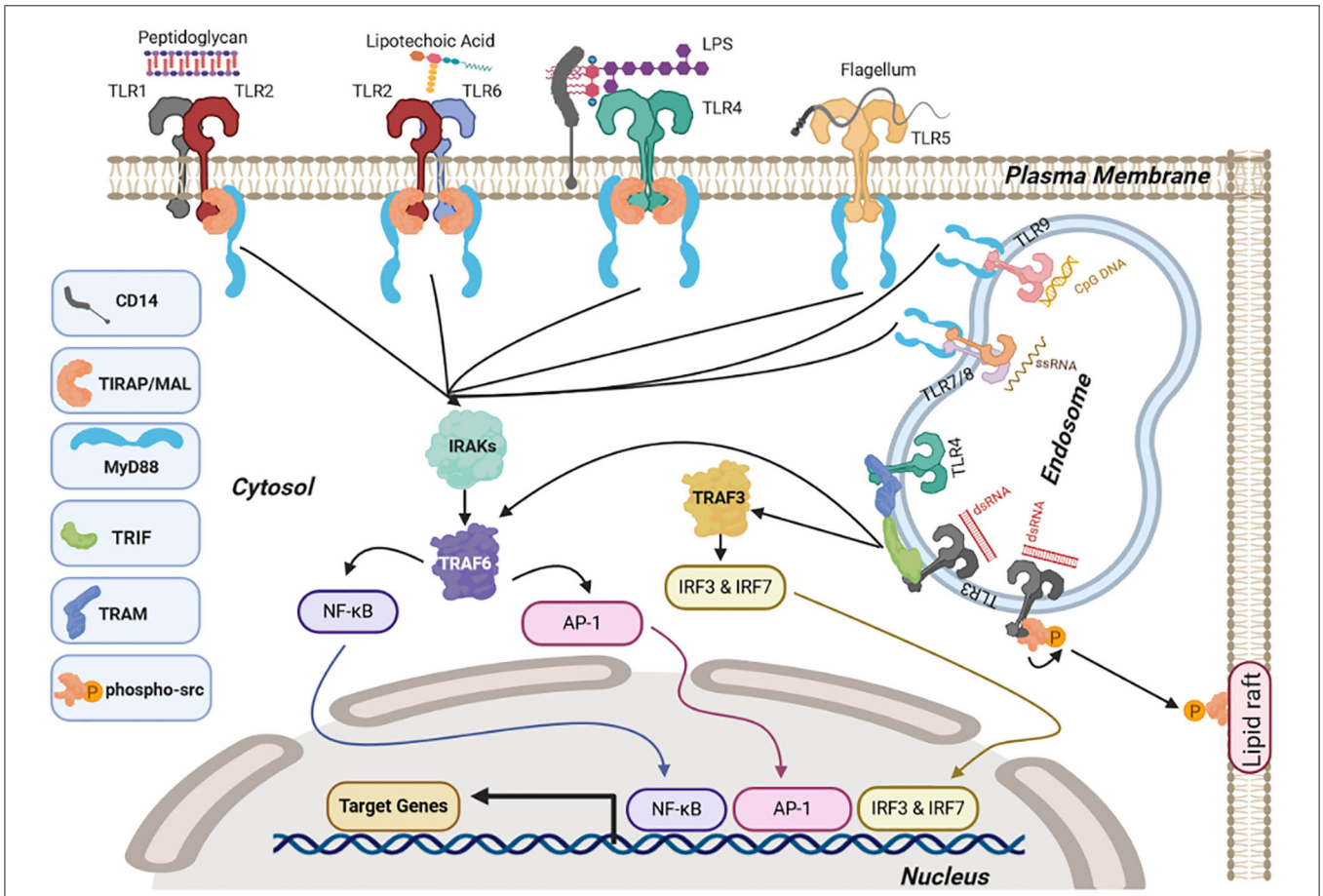


FIGURE 1 | Toll-like receptor (TLR) signaling. Upon ligand binding at the cell surface, TLR1/2, TLR2/6, and TLR4 interact with myeloid differentiation factor 88 (MyD88) via the adaptor molecule Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (MAL or TIRAP) whereas TLR5 and endosomal TLRs TLR7/8 and TLR9 interact directly through MyD88. This causes activation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1), signaling via interleukin-1 receptor-associated kinase 4 (IRAK1 and 2) and tumor necrosis factor receptor (TNFR)-associated factor 6 TRAF6. Endosomal TLR3 and TLR4 induce NF-κb and AP-1 via TRIF associating with TRAF6 along with interferon regulatory factor IRF3/7 signaling via TRIF and TRAF3. Once NF-κB, AP-1, and IRFs are activated they translocate to the nucleus and activate transcription of target genes, including pro-inflammatory cytokines (NF-κB and Ap-1) and type I interferons (IRFs). TLR3 also induces auto-phosphorylation of the proto-oncogene Src, causing sequestration of Src in lipid rafts. Figures were drawn via biorender.com.

and IFN-β (64). The release of the cytokines varies and depends on receptor, cell type, and species (65, 66).

Not all TLRs signal via the same adaptor proteins; double-stranded RNA (ds-RNA) activation of TLR3 also initiates non-canonical signaling via recruitment and autophosphorylation of the proto-oncogene tyrosine-protein kinase Src. This leads to inhibition of cell migration, proliferation, and cell adhesion via functional sequestration of Src to lipid rafts (67). A summary of the signaling mechanisms downstream of TLRs is shown in **Figure 1**.

EXPRESSION AND ROLE OF TLRs IN ENDOTHELIAL CELLS

Endothelial cells (ECs) form the inner layer of the blood vessel, the tunica intima, and have contact to circulating cells in the lumen and vascular smooth muscle cells in the adjacent tunica media of the blood vessel. Therefore, ECs are uniquely positioned to maintain hemostasis and regulate the vascular tone via release of vasoactive mediators (68). However, ECs also respond to injury via secretion of cytokines, chemokines, and growth factors, as well as via expression of adhesion molecules (69). They hereby regulate the recruitment and activation of immune cells at the site of injury (70). ECs further recognize PAMPs and DAMPs through PRRs, particularly the TLRs (71). ECs from different species and tissues vary in the expression of TLRs. For example, human umbilical cord ECs (HUVECs) express high levels of TLR1-4, but low levels of TLR5-10, whereas human aortic ECs have high levels of all TLRs except for TLR3 and TLR9 compared to human peripheral blood mononuclear cells (PBMCs) as controls (72).

Similarly, the expression of TLRs varies between the different sections of the vascular tree, as major blood vessels have low endothelial expression of TLR2 and TLR4 (73). TLR expression is different among macrovascular and microvascular ECs. For example, baseline TLR4 levels are higher in dermal microvascular ECs than in aortic macrovascular ECs and elevated TLR4 levels correlated with increased chemokine and cytokine expression (74). In addition to mature ECs, TLRs are also found in precursor ECs, such as endothelial colony forming cells (ECFCs). ECFCs are a promising source for postnatal vascularization strategies and tissue repair (75) and are isolated from different sources (cord blood, peripheral blood, and lung) by outgrowth of EC colonies via limiting dilution from blood or tissues (76–79). ECFCs from cord blood and peripheral blood expressed mRNA for all TLRs, and umbilical cord ECFCs showed higher TLR4 expression than peripheral blood ECFCs, HUVECs, and PBMCs (80).

TLRs are also important for the differentiation and reprogramming of cells. Forced expression of a set of transcription factors is a common method to induce pluripotent stem cells. These transcription factors are octamer-binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-master regulator of cell cycle entry and proliferative metabolism (c-Myc). Lentiviral expression of these transcription factors increased the efficiency of generating induced pluripotent stem cells via TLR3/TRIF compared to non-viral expression methods (81). In addition, activation of TLR3 by its ligand double-stranded RNA (dsRNA) combined with endothelial growth factors promoted differentiation of human fibroblasts to ECs (82).

ROLE OF TLRs IN BLOOD VESSEL FORMATION AND EXTENSION

The network of blood vessels expands via two main mechanisms: vasculogenesis is *de novo* formation of blood vessels, and angiogenesis is sprouting or splitting of existing blood vessels (83). Sprouting angiogenesis occurs in five steps: (A) After action of angiogenic growth factor (e.g., vascular endothelial growth factor, VEGF) on a quiescent blood vessel, there is (B) degradation of the capillary basement membrane followed by (C) EC proliferation and (D) selection of tip and stalk cells. (E) ECs then migrate from the existing blood vessel to form new vascular tubes (tubulogenesis) and finally (F) connect to another blood vessel by fusion (**Figure 2**) (84).

TLR signaling regulates the complex process of sprouting angiogenesis at different stages of the process: The initial step of angiogenesis requires the degradation of the ECM via matrix metalloproteinases (MMPs) 2 and 9 to facilitate EC migration and tube formation (85) along with degrading endothelial tight junctions (86). Tight junctions (TJ) seal the intercellular gap to maintain endothelial barrier function, and several proteins including occludins, claudins, and zonula occludens form these tight junctions (87). The endothelial tight junction protein claudin-5 increases EC proliferation (88) but decreases EC migration and permeability, hence reducing vascular sprouting (89) similar to ECs lacking zonula occludens 1 (ZO-1), which show less migration and vascular sprouting (90). MMP9 reduced ZO-1, occludin, and claudin-5 levels via proteolysis, resulting in enhanced endothelial permeability, migration, and tube formation, and this effect was reversed with MMP9 gene silencing (86, 91). Cytokines increase MMP9 expression in an NF-κB-dependent manner via a NF-κB-binding site on the MMP9 promoter (92). TLRs promote MMP9 expression via NF-κB and extracellular signal-regulated kinase (ERK1/2) signaling pathways. TLR2 signaling influences endothelial MMP9 expression via ERK1/2 and c-Jun N-terminal kinase (JNK) signaling pathways along with MMP9-mediated reduction in tight junction proteins (93). Chlamydia pneumoniae infection promoted expression of VEGF and MMP9 in HUVECs via TLR2 and TLR4 (94). TLR4 results in EC hyperpermeability via NF-κB and AP-1-induced stromal interaction molecule 1 (STIM1) expression in human lung microvascular ECs (HLMVECs) (95). TLR2/6 activation augments EC permeability via reduced claudin-5 expression and disappearance of tight junctions, which was partly mediated by ERK1/2 (96).

In addition to tight junctions, TLRs also influence the adherens junctions between ECs. Adherens junctions are cell–cell junctions with cadherins, such as vascular endothelial (VE-) cadherin, connecting neighboring plasma membranes via homophilic interactions (97). TLR2 stimulation reduced the expression of VE-cadherin resulting in increased EC detachment

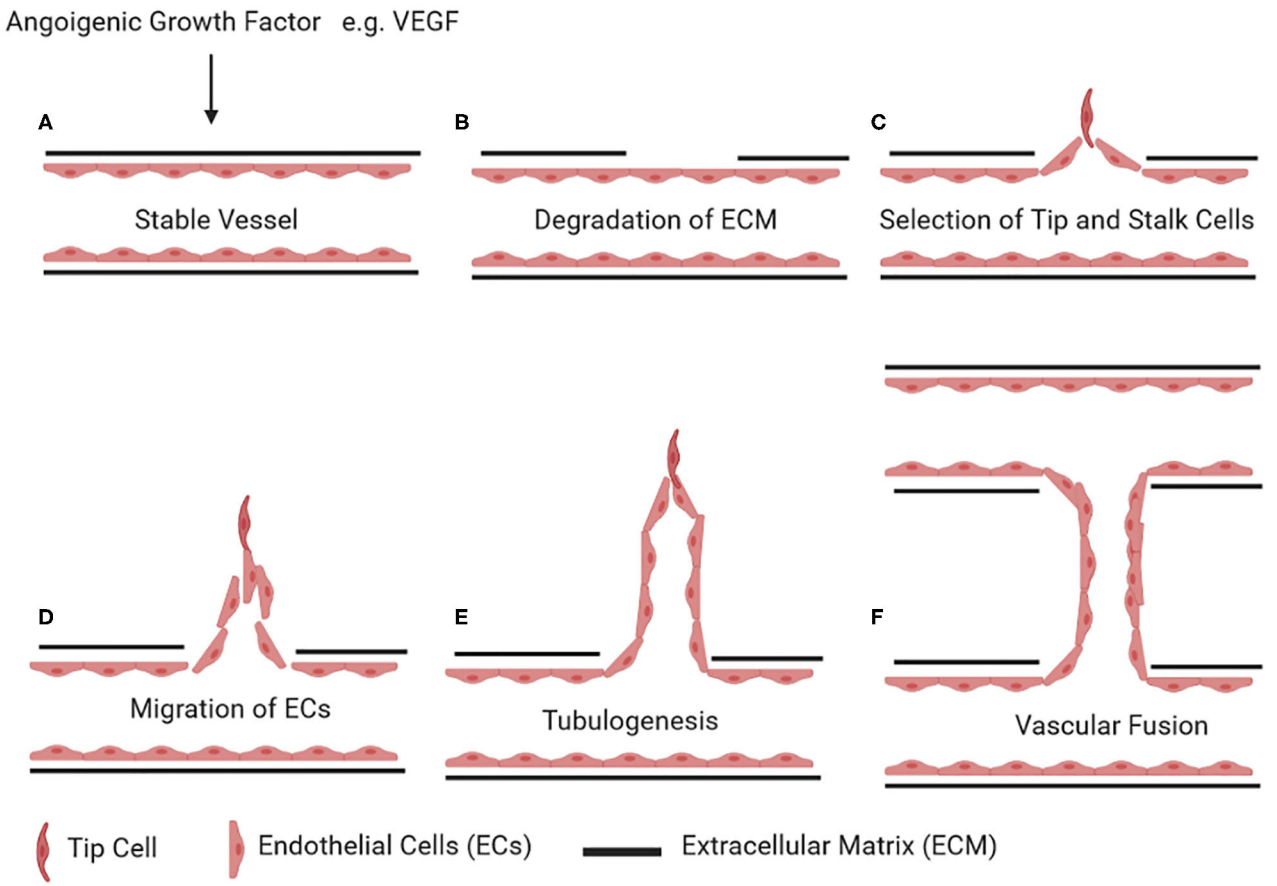


FIGURE 2 | Stages of angiogenesis. Upon stimulus by an angiogenic growth factor (e.g., vascular endothelial growth factor, VEGF) on a quiescent vessel **(A)**, ECs degrade the basement membrane and the surrounding extracellular matrix **(B)**. ECs then differentiate into tip and stalk cells **(C)**. Tip cells start invading the extracellular matrix **(D)**. The stalk cells behind the tip cells continue to proliferate and form vascular tubes **(E)**. Once a tip cell fuses with the tip cell of the adjacent sprouting vessel, this results in the formation of a new connecting lumen **(F)**. Figures were drawn via biorender.com.

and reduced EC migration while increasing MMP2/9 activity and EC apoptosis in human saphenous vein ECs (98). Similarly, in HLMVECs, TLR3 activation reduced the expression of claudin-5, ZO-1, and VE-cadherin (97).

TLR signaling further influences the expression of growth factors with a central role in regulating vascular growth. In human intestinal microvascular cells, bacterial ligands via TLRs increased angiogenic factors such as VEGF, VEGF receptor 2 (VEGFR-2), IL-8, and phosphorylation of focal adhesion kinase (p-FAK) (99). Of note, FAK promotes EC survival, angiogenesis, and vascular network stability (100). Among these growth factors, VEGF-A is a central angiogenic molecule that directs migration of endothelial tip cells during angiogenesis (101). Biglycan-stimulated TLR2 and TLR4 signaling increases VEGF-A levels, resulting in endothelial proliferation, migration, and tube formation. TLR2/4 stimulation activates NF-κB, which interacts with the hypoxia-inducible factor-1α (HIF-1α) promoter to augment HIF-1α levels. HIF-1α in turn binds to the VEGF-A, promoter increasing VEGF-A levels (102).

Activation of TLR5 by the bacterial ligand Flagellin promotes endothelial tube formation in human microvascular ECs and HUVECs through activation of the phosphoinositide 3-kinase (PI3K)/AKT1 signaling pathway (103). In rat, aortic cell culture TLR5 activation increased microvessel formation along with vessel survival but had no effect on *de novo* blood vessel formation (104).

However, TLRs also promote angiogenesis in a VEGF-independent manner. Mycoplasma lipopeptide MALP-2 in HUVECs and human monocytes/macrophages via TLR2/TLR6 promoted activation of granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF promoted angiogenesis in these endothelial cells while no enhanced VEGF levels in study were seen, suggesting that VEGF was not responsible for this angiogenesis in this regard (105). The end product of lipid oxidation, ω-(2-carboxyethyl)pyrrole (CEP), is generated during inflammation, wound healing, and aging. CEP interacted with TLR1/TLR2 heterodimer, promoting angiogenesis via MyD88-dependent NF-κB signaling in multiple EC lineages from human umbilical vein, mouse lung, or aorta independent

of VEGF as CEP-mediated effects were unaffected by VEGFR kinase inhibition (106). In lung ECs, TLR4 signaling increases ERK-mediated activation of the transcription factor Forkhead box protein C2 (FoxC2), a transcription factor associated with lymph angiogenesis and endothelial specification by promoting delta-like 4 (DLL4) expression (107). DLL4 is a NOTCH ligand, which in angiogenic vasculature is associated with filipodia-rich endothelial tip cell formation responsible for guiding new sprouts (108).

Anti-TLR2 antibodies promoted angiogenesis in HUVECs in a manner similar to stromal cell-derived factor 1 (SDF1 also known as C–X–C motif chemokine 12 or CXCL12). This effect was mediated by Gi-protein-coupled receptor C–X–C motif chemokine receptor 4 (CXCR4) via ERK 1/2 and AKT, which was completely abolished by blocking G-protein and CXCR4, indicating a potential cross talk between TLR2 and CXCR4 pathways (109).

In contrast, TLR3 activation inhibited EC migration and tube formation in HUVECs via phosphorylation of Src independent of TRIF, TRAF3, MyD88, and IRF3 signaling (67). In primary choroidal ECs, TLR3 stimulation was anti-angiogenic and a TLR3 agonist reduced corneal neo-vascularization (34). Similarly, dsRNA of viral origin and polyinosinic:polycytidylic acid (poly(I:C)) promoted apoptosis and inhibited angiogenesis and migration in HUVECs (110). Our group has shown that TLR3 deficiency impaired migration of human pulmonary-artery ECs via caspase-3-dependent apoptosis (111). Similar to TLR3, TLR9 reduces angiogenesis, because the TLR9 agonist ODN1826 suppresses aortic angiogenesis, inhibits migration of tip cells in aortic ring assay, and suppresses corneal neovascularization *in vivo* (112). Based on these findings, activation of some TLRs appears to promote angiogenesis by downregulation of EC tight junction and adherens junction proteins, induction of angiogenic growth factors, increased proliferation, and salvation from apoptosis, but other TLR3s, such as TLR3, seem to have anti-angiogenic effects following stimulation. However, further in-depth studies are required to fully understand how signaling through the different TLRs contributes to the various stages of angiogenesis and to identify the context-dependent effects of TLR signaling on angiogenesis.

ENDOTHELIAL TLRs AND INFLAMMATION

Blood vessels are the main highway for inflammatory cells to travel to the site of injury. Inflammation and angiogenesis are therefore intricately linked processes, and signaling pathways that promote inflammation frequently also facilitate angiogenesis by inducing EC proliferation and migration. It is therefore not surprising that many proinflammatory cytokines stimulate angiogenesis, including IL-6. In mouse aortic ring assays and lung endothelial cells and HUVECs, IL-6 stimulation increased angiogenesis independent of VEGF (113). One important pathway driving IL-6 expression is the NF-κB pathway, and every member of the TLR family can signal via NF-κB (114).

TLR expression varies between different EC populations. TLR4 levels were found to be higher in microvascular ECs compared to macrovascular ECs, resulting in higher NF-κB and IL-6 levels in microvascular ECs (74). Similarly, TLR2 activation promotes in human lung microvascular EC expression of cytokines and adhesion molecules, leading to adhesion of neutrophil granulocytes (115, 116). Similar to TLR2 and TLR4, TLR9 activation causes NF-κB-mediated increase in tissue factor levels favoring a procoagulant phenotype in human coronary-artery ECs (117). In rat PAECs, TLR9 activation leads to NF-κB-mediated IL-6 production (118). In human intestinal microvascular ECs, flagellin-mediated TLR5 activation promotes expression of leukocyte adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), leading to increased transendothelial migration of leukocytes (119). TLR9 activation further enhances neutrophil adhesion on HUVECs (120). These data show that TLR signaling in ECs also promotes cross talk between ECs and inflammatory cells.

As with the foreign molecules eliciting TLR responses, the internal molecules of the body also act as DAMPs to promote immune responses via TLRs (**Table 1**). One of the DAMPs is high-mobility group box 1 (HMGB1). HMGB1 is an endogenous inflammatory mediator released from dying and activated cells during many pathogenic conditions including pancreatitis, cancers, atherosclerosis, and myocardial infarction (121, 122). An interesting mechanism of inflammatory signaling in ECs is the TLR-high-mobility group box 1 (HMGB1) axis. HMGB1 is an alarmin that is released in response to injury from necrotic cells, triggering an inflammatory response (123). HMGB1 signals via TLRs 2, 3, 4, 7, and 9 (26, 123, 124) to induce expression of pro-inflammatory cytokines through MyD88/NF-κB signaling (124), which in turn increases secretion of HMGB1, creating a positive feedback loop to amplify the HMGB1-mediated inflammatory effect (125). Likewise, extracellular histone-mediated activation of TLR2/TLR4 increases tissue factor expression via NF-κB and AP-1, promoting thrombus formation in human coronary artery ECs (126). TLR-mediated inflammatory signaling can also utilize G proteins. The intercellular domains of TLR2, TLR3, and TLR4 contain a consensus motif for binding of pertussis toxin-sensitive heterotrimeric G proteins Gαi/o. Gαi/o activates MAPK and Akt and interferons downstream of TLR2, TLR3, and TLR4 in ECs while having no effect on NF-κB signaling (127).

TLR 4 is the prototypical (128) and most studied TLR in literature. The functions of TLR4 as previously discussed not only are limited to its immunological role but also extend to various aspects of the vascular biology. One of the major cardiovascular disorders and the top most cause of death in the developing world is the coronary artery disease (CAD) or the ischemic heart disease (IHD), accounting for >9 million deaths globally in 2016 (129). In the heart, all the TLRs are expressed with the expression of TLR4 being the highest (130) and hence is a very important for the molecular pathogenesis of IHD. TLR4 along with TLR1 and TLR2 is highly expressed in human atherosclerotic plaques (131), with the highest expression in the shoulder regions of the plaques in the coronary arteries where the incidence of plaque rupture is highest (132). TLR4 significantly raised the levels of tissue factor (TF), a critical initiator of blood clotting from endothelial cells actively contributing to arterial thrombus formation (133). LPS-mediated TLR4 activation of human coronary artery endothelial

cells resulted in increased IL-1β and TNF-α which are elevated in congestive heart failure (CHF) and CAD, hence contributing directly to their pathogenesis. Furthermore, TLR4 activation resulted in reduced cardiac function in mice whereas TLR4 deficiency promoted survival and reduction in septic shock and myocardial ischemia-induced cardiac dysfunction (134).

In addition to TLR4, endosomal TLR7 and TLR9 also play an important role in atherosclerotic lesions. Under normal conditions, major arteries of the body have a negligible expression of TLR7 and TLR9 (73). However, TLR7 levels were increased in endothelial cells, smooth muscle cells, and macrophages of mouse atherosclerotic lesions of the aortic arch (135). In apoE*3-Leiden mouse restenosis model, TLR7/9 activation significantly led to femoral artery cuff intimal hyperplasia and accelerated atherosclerosis and blockade of TLR7/9 significantly reduced neointima formation, atherosclerosis, and macrophage cytokine production (136). TLR7 activation may differentially respond according to disease condition. In patients with carotid endarterectomy, TLR7 was higher in atherosclerotic plaques, yet elevated TLR7 expression in the plaques was associated with better outcomes by production of anti-inflammatory cytokines (137).

RNA is another important DAMP that activates TLR3 (35, 138). We and others have shown that TLR3 expression and signaling are important in vascular biology and pulmonary hypertension (PH) (111, 139).

TLRs IN PULMONARY HYPERTENSION

Pulmonary hypertension (PH) is a chronic, progressive disorder of the lung vasculature characterized by abnormal pulmonary-artery vasoconstriction and remodeling, leading to right heart failure and death (140). Idiopathic pulmonary arterial hypertension (iPAH) shows dysregulated EC differentiation and growth (141), and EC dysfunction is now recognized as a central process in the initiation and progression of PH (142). There are multiple aspects of EC dysfunction, which include the emergence of apoptosis-resistant, hyperproliferative ECs, dysregulated release of mediators from ECs, and endothelial-to-mesenchymal transition. One concept suggests that during development of PAH, EC apoptosis results in the selection of these apoptosis-resistant, hyperproliferative ECs. In our recent publication, we showed TLR3 deficiency in pulmonary-artery ECs from PAH patients and in lungs from a rat model of severe PH with occlusive arteriopathy (111). Treatment with the TLR3 agonist and poly(I:C) reduced severe PH and occlusive arteriopathy in rats and increased endothelial TLR3 expression in an interleukin-10 (IL-10)-dependent manner (111). Our results mirror the protective role of TLR3 signaling after balloon injury in large systemic arteries (139). However, other conflicting results seemingly contradict a protective role of TLR3 in the vasculature, but these findings may be due to a more severe degree of endothelial injury in the model system (143). Because knockout of type I IFN receptor and type I IFN treatment have frequently been associated with mostly reversible PH in highly preselected patient groups with significant comorbidities (144, 145), further evaluation of TLR3-targeted therapy is required in PAH. However, type IFN therapy seems to reduce severe angioocclusive PH in rats (146).

Recently, the HMGB1–TLR4 axis has emerged as a potential driver of pulmonary vascular remodeling in PAH. HMGB1 was elevated in concentric and plexiform lesions from patients with iPAH and in the lungs of mice exposed to chronic hypoxia (147). In addition, monocrotaline (MCT), an EC toxin causing severe PH, enhances the release of HMGB1 from injured ECs (148). Pro-inflammatory cytokines promote HMGB1 secretion, and therefore HMGB1 secretion and TLR4 may be part of a positive feedback loop enhancing inflammation and lung vascular remodeling in PAH. In addition, activation of the HMGB1/TLR4 axis in rats exposed to chronic hypoxia caused a significant decline in bone morphogenic protein receptor 2 (BMPR2), connecting HMGB1 with a well-known pathway hypomorphism in PAH pathobiology (149, 150). An inhibitor of HMGB1–TLR4 interaction has been characterized as a novel potential therapeutic, translating the findings with the TLR4–HMGB1 pathway to a potential clinical treatment in PAH, although clinical evaluation is necessary as a next step after the initial preclinical study (151, 152). Therapeutic strategies targeting TLRs in PH could be a potential avenue where both agonists and antagonists can improve pathogenesis of PH, depending on the TLR in question. In contrast to reduced PH following treatment of PH rats with the TLR3 agonist poly(I:C) (111), P5779, a blocker of the HMGB1–TLR4 interaction, reduces PH in rats (151, 152). Although other different TLR agonists and antagonists are available, there is a need for further study of TLR signaling in PH to identify additional targets depending on the role of the particular TLR in PH pathobiology.

In addition, TLR signaling can also contribute to another pathogenic mechanism in PAH, endothelial-to-mesenchymal transition (EndMT). EndMT is a process by which ECs acquire a mesenchymal cell phenotype. There is growing evidence that EndMT contributes to development and progression of pulmonary vascular remodeling and severe PAH (153–156). TLR4 activation promoted EndMT and expression of the progenitor cell marker c-kit (CD117) in mouse pulmonary ECs, indicating a potential connection between TLR4 and EndMT (157). Our group has recently shown that clonally expanded $CD117^+$ ECs promote the formation of occlusive arteriopathy in rats exposed to chronic hypoxia and that $CD117^+$ ECs undergo EndMT *in vitro* and *in vivo* (158). Hence, growing literature indicates that $CD117^+$ ECs represent a stem-like EC population in the developing and adult lung (159, 160) and that $CD117^+$ ECs contribute to lung vascular remodeling in PAH (158).

In PAH, not only ECs but also smooth muscle cells (SMCs) play a central role in the pathogenesis of the disease (161). Normal pulmonary-artery SMCs most abundantly expressed TLR3, TLR4, and TLR6. TLR3 and TLR4 co-activation resulted in IL-8 release whereas TLR3 activation alone promoted IP10 and endothelin 1 release (162). HMGB1-mediated activation of TLR4 in pulmonary-artery SMCs augmented SMC proliferation and migration along with a decline in bone morphogenic protein receptor 2 (BMPR2) signaling. Based on the dysfunctional

BMPR2 pathway in PAH, it is likely that the HMGB1/TLR4 pathway has a pathogenic role in PAH (149). BMPR2-deficient mice had elevated levels of TLR4 in pulmonary-artery SMCs and lungs. LPS stimulation then leads to a significant increase in IL-6 and IL-8 production (163). These data indicate that TLR4-mediated downregulation of BMPR2 plays an auto-enhancer role driving TLR4 expression in the form of a vicious circle, which contributes to the pathogenesis of PAH. However, hypoxia reduces TLR4 expression and TLR4 knockout mice spontaneously develop PH, suggesting a protective role of TLR4 in PAH (164). This work coincided with a similar observation in ECs, which showed that hypoxia exposure reduced the expression of TLR4 and downstream nuclear translocation of AP-1 in cultured HUVECs and HPAECs (165). In stark contrast, some studies suggest that TLR4-deficient mice were protected from chronic hypoxia-induced PH (166, 167).

In summary, the existing literature indicates that TLRs have functions that exceed by far the induction of an innate immune response in ECs. Instead, TLR signaling is tightly connected with crucial elements of EC function, such as proliferation, apoptosis, angiogenic sprouting, and migration. Potential therapies targeting TLR–ligand interactions or using TLR agonists have emerged in multiple vascular diseases, in particular in PH, but careful preclinical and clinical evaluation is required when modulating TLR signaling because of the highly conserved and multifaceted effects of TLR signaling in ECs.

AUTHOR CONTRIBUTIONS

AB, AT, and LF conceived the manuscript. AB and LF wrote the initial draft of the manuscript. All authors edited the manuscript and approved the final version.

FUNDING

AB received support from The Fulbright Scholarship. AT was funded by a British Heart Foundation Intermediate Clinical Fellowship to AT (FS/18/13/3328). LF received support from the National Heart Lung & Blood Institute of the National Institutes of Health under award number HL139881. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

The figures in this publication are created with BioRender.com.

REFERENCES

1. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol.* (1989) 54:1–13. doi: 10.1101/SQB.1989.054.01.003
2. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol.* (2011) 30:16–34. doi: 10.3109/08830185.2010.529976
3. Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* (2002) 20:197–216. doi: 10.1146/annurev.immunol.20.083001.084359
4. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022
5. Hoving JC, Wilson GJ, Brown GD. Signalling C-type lectin receptors, microbial recognition and immunity. *Cell Microbiol.* (2014) 16:185–94. doi: 10.1111/cmi.12249
6. Onoguchi K, Yoneyama M, Fujita T. Retinoic acid-inducible gene-I-like receptors. *J Interf Cytokine Res.* (2011) 31:27–31. doi: 10.1089/jir.2010.0057
7. Loo YM, Gale M. Immune signaling by RIG-I-like receptors. *Immunity.* (2011) 34:680–92. doi: 10.1016/j.immuni.2011.05.003
8. Motta V, Soares F, Sun T, Philpott DJ. Nod-like receptors: versatile cytosolic sentinels. *Physiol Rev.* (2015) 95:149–78. doi: 10.1152/physrev.00009.2014
9. Hashimoto C, Hudson KL, Anderson KV. The toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell.* (1988) 52:269–79. doi: 10.1016/0092-8674(88)90516-8
10. Rosetto M, Engström Y, Baldari CT, Telford JL, Hultmark D. Signals from the IL-1 receptor homolog, toll, can activate an immune response in a drosophila hemocyte cell line. *Biochem Biophys Res Commun.* (1995) 209:111–16. doi: 10.1006/bbrc.1995.1477
11. Lemaitre B, Nicolas E, Michaut L, Reichhart J, Hoffmann JA. The dorsoventral regulatory gene cassette. *Cell.* (1996) 86:973–83. doi: 10.1016/S0092-8674(00)80172-5
12. Gay NJ, Keith FJ. Drosophila toll and IL-1 receptor. *Nature.* (1991) 351:355–6. doi: 10.1038/351355b0
13. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to drosophila toll. *Proc Natl Acad Sci USA.* (1998) 95:588–93. doi: 10.1073/pnas.95.2.588
14. Dowling JK, Dellacasagrande J. Toll-like receptors: ligands, cell-based models, and readouts for receptor action. *Methods Mol Biol.* (2016) 1390:3–27. doi: 10.1007/978-1-4939-3335-8_1
15. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, et al. A toll-like receptor that prevent infection by uropathogenic bacteria. *Science.* (2004) 303:1522–6. doi: 10.1126/science.1094351
16. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol.* (2005) 17:1–14. doi: 10.1093/intimm/dxh186
17. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol.* (2010) 11:373–84. doi: 10.1038/ni.1863
18. Matzinger P. Tolerance, danger, danger, and extended family. *Annu Rev.lmmunol.* (1994) 12:991–1045. doi: 10.1146/annurev.iy.12.040194.005015
19. Seong S-Y, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol.* (2004) 4:469–78. doi: 10.1038/nri1372
20. Schaefer L. Complexity of danger: the diverse nature of damage-associated molecular patterns. *J Biol Chem.* (2014) 289:35237–45. doi: 10.1074/jbc.R114.619304
21. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell.* (2007) 130:1071–82. doi: 10.1016/j.cell.2007.09.008
22. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol.* (2002) 169:10–14. doi: 10.4049/jimmunol.169.1.10
23. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J Biol Chem.* (1999) 274:17406–9. doi: 10.1074/jbc.274.25.17406
24. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J Clin Invest.* (2005) 115:2223–33. doi: 10.1172/JCI23755
25. Hashimoto M, Tawaratsumida K, Kariya H, Aoyama K, Tamura T, Suda Y. Lipoprotein is a predominant toll-like receptor 2 ligand in

Staphylococcus aureus cell wall components. *Int Immunol.* (2006) 18:355–62. doi: 10.1093/intimm/dxh374
26. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem.* (2004) 279:7370–7. doi: 10.1074/jbc.M306793200
27. Brandt KJ, Fickentscher C, Kruithof EKO, De Moerloose P. TLR2 ligands induce NF-κB activation from endosomal compartments of human monocytes. *PLoS ONE.* (2013) 8:e80743. doi: 10.1371/journal.pone.0080743
28. Liu-Bryan R, Pritzker K, Firestein GS, Terkeltaub R. TLR2 signaling in chondrocytes drives calcium pyrophosphate dihydrate and monosodium urate crystal-induced nitric oxide generation. *J Immunol.* (2005) 174:5016–23. doi: 10.4049/jimmunol.174.8.5016
29. Kono H, Chen CJ, Ontiveros F, Rock KL. Uric acid promotes an acute inflammatory response to sterile cell death in mice. *J Clin Invest.* (2010) 120:1939–49. doi: 10.1172/JCI40124
30. Lipps C, Nguyen JH, Pyttel L, Lynch TL, Liebetrau C, Aleshcheva G, et al. N-terminal fragment of cardiac myosin binding protein-C triggers pro-inflammatory responses *in vitro*. *J Mol Cell Cardiol.* (2016) 99:47–56. doi: 10.1016/j.yjmcc.2016.09.003
31. Zhang P, Cox CJ, Alvarez KM, Cunningham MW. Cutting edge: cardiac myosin activates innate immune responses through TLRs. *J Immunol.* (2009) 183:27–31. doi: 10.4049/jimmunol.0800861
32. Alexopoulou L, Holt AC, Medzhitov R FR. Recognition of double-stranded RNA and activation of NF-kappaB by Toll. *Nature.* (2001) 413:732–8. doi: 10.1038/35099560
33. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis Rheum.* (2005) 52:2656–65. doi: 10.1002/art.21273
34. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature.* (2008) 452:591–7. doi: 10.1038/nature06765
35. Karikó K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for toll-like receptor 3. *J Biol Chem.* (2004) 279:12542–50. doi: 10.1074/jbc.M310175200
36. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med.* (2013) 45:e66. doi: 10.1038/emm.2013.97
37. Al-ofi E, Coffelt SB, Anumba DO. Fibrinogen, an endogenous ligand of Toll-like receptor 4, activates monocytes in pre-eclamptic patients. *J Reprod Immunol.* (2014) 103:23–28. doi: 10.1016/j.jri.2014.02.004
38. Brennan TV, Lin L, Huang X, Cardona DM, Li Z, Dredge K, et al. Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation. *Blood.* (2012) 120:2899–908. doi: 10.1182/blood-2011-07-368720
39. Akbarshahi H, Axelsson JBF, Said K, Malmström A, Fischer H, Andersson R. TLR4 dependent heparan sulphate-induced pancreatic inflammatory response is IRF3-mediated. *J Transl Med.* (2011) 9:1–8. doi: 10.1186/1479-5876-9-219
40. Iqbal M, Philbin VJ, Withanage GSK, Wigley P, Beal RK, Goodchild MJ, et al. Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar typhimurium. *Infect Immun.* (2005) 73:2344–50. doi: 10.1128/IAI.73.4.2344-2350.2005
41. Buwitt-Beckmann U, Heine H, Wiesmüller KH, Jung G, Brock R, Akira S, et al. Toll-like receptor 6-independent signaling by diacylated lipopeptides. *Eur J Immunol.* (2005) 35:282–9. doi: 10.1002/eji.200424955
42. Zhang Z, Ohto U, Shibata T, Krayukhina E, Taoka M, Yamauchi Y, et al. Structural analysis reveals that toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA. *Immunity.* (2016) 45:737–48. doi: 10.1016/j.immuni.2016.09.011
43. Forsbach A, Nemorin J-G, Montino C, Müller C, Samulowitz U, Vicari AP, et al. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. *J Immunol.* (2008) 180:3729–38. doi: 10.4049/jimmunol.180.6.3729
44. Cervantes JL, La Vake CJ, Weinerman B, Luu S, O'Connell C, Verardi PH, et al. Human TLR8 is activated upon recognition of *Borrelia burgdorferi* RNA in the phagosome of human monocytes. *J Leukoc Biol.* (2013) 94:1231–41. doi: 10.1189/jlb.0413206
45. Eigenbrod T, Pelka K, Latz E, Kreikemeyer B, Dalpke AH. TLR8 senses bacterial RNA in human monocytes and plays a nonredundant role for recognition of streptococcus pyogenes. *J Immunol.* (2015) 195:1092–9. doi: 10.4049/jimmunol.1403173
46. Mogensen TH, Paludan SR, Kilian M, Ostergaard L. Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J Leukoc Biol.* (2006) 80:267–77. doi: 10.1189/jlb.1105626
47. De Dios R, Nguyen L, Ghosh S, McKenna S, Wright CJ. CpG-ODN mediated TLR9 innate immune signaling and calcium dyshomeostasis converge on the NFκB inhibitory protein IκBβ to drive IL1α and IL1β expression. *Immunology.* (2020) 160:64–77. doi: 10.1111/imm.13182
48. Henrick BM, Yao XD, Zahoor MA, Abimiku A, Osawe S, Rosenthal KL. TLR10 senses HIV-1 proteins and significantly enhances HIV-1 infection. *Front Immunol.* (2019) 10:482. doi: 10.3389/fimmu.2019.00482
49. Matsushima N, Tanaka T, Enkhbayar P, Mikami T, Taga M, Yamada K, et al. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics.* (2007) 8:1–20. doi: 10.1186/1471-2164-8-124
50. Botos I, David MS, Davies DR. Structural biology of TLRs. *HHS Public Acces.* (2011) 19:447–59. doi: 10.1016/j.str.2011.02.004
51. Christmas P. Toll-like receptors: sensors that detect infection. *Nat Educ.* (2010) 3:85. Available online at: https://www.nature.com/scitable/topicpage/toll-like-receptors-sensors-that-detect-infection-14396559/#
52. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc Natl Acad Sci USA.* (2000) 97:13766–71. doi: 10.1073/pnas.250476497
53. Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, et al. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J Immunol.* (2001) 166:15–19. doi: 10.4049/jimmunol.166.1.15
54. Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* (2003) 24:528–33. doi: 10.1016/S1471-4906(03)00242-4
55. Nimma S, Ve T, Williams SJ, Kobe B. Towards the structure of the TIR-domain signalosome. *Curr Opin Struct Biol.* (2017) 43:122–30. doi: 10.1016/j.sbi.2016.12.014
56. Ve T, Vajjhala PR, Hedger A, Croll T, Dimaio F, Horsefield S, et al. Structural basis of TIR-domain-assembly formation in MAL- and MyD88-dependent TLR4 signaling. *Nat Struct Mol Biol.* (2017) 24:743–51. doi: 10.1038/nsmb.3444
57. Dunne A, Ejdebäck M, Ludidi PL, O'Neill LAJ, Gay NJ. Structural complementarity of toll/interleukin-1 receptor domains in toll-like receptors and the adaptors mal and MyD88. *J Biol Chem.* (2003) 278:41443–51. doi: 10.1074/jbc.M301742200
58. Roy A, Srivastava M, Saqib U, Liu D, Faisal SM, Sugathan S, et al. Potential therapeutic targets for inflammation in toll-like receptor 4 (TLR4)-mediated signaling pathways. *Int Immunopharmacol.* (2016) 40:79–89. doi: 10.1016/j.intimp.2016.08.026
59. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the toll-like receptor signaling. *J Immunol.* (2002) 169:6668–72. doi: 10.4049/jimmunol.169.12.6668
60. Tanimura N, Saitoh S, Matsumoto F, Akashi-Takamura S, Miyake K. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun.* (2008) 368:94–99. doi: 10.1016/j.bbrc.2008.01.061
61. Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, et al. Specificity in toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature.* (2006) 439:204–7. doi: 10.1038/nature04369
62. Jiang Z, Mak TW, Sen G, Li X. Toll-like receptor 3-mediated activation of NF-κB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-β. *Proc Natl Acad Sci USA.* (2004) 101:3533–8. doi: 10.1073/pnas.0308496101
63. Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BRG, Li X. Poly(dI·dC)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFκB and MAP kinase is through an interleukin-1 receptor-associated

kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. *J Biol Chem.* (2003) 278:16713–19. doi: 10.1074/jbc.M300562200
64. Ghosh TK, Mickelson DJ, Fink J, Solberg JC, Inglefield JR, Hook D, et al. Toll-like receptor (TLR) 2-9 agonists-induced cytokines and chemokines: I. Comparison with T cell receptor-induced responses. *Cell Immunol.* (2006) 243:48–57. doi: 10.1016/j.cellimm.2006.12.002
65. Sandig H, Bulfone-Paus S. TLR signaling in mast cells: common and unique features. *Front Immunol.* (2012) 3:185. doi: 10.3389/fimmu.2012.00185
66. Lundberg AM, Drexler SK, Monaco C, Williams LM, Sacre SM, Feldmann M, et al. Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems. *Blood.* (2007) 110:3245–52. doi: 10.1182/blood-2007-02-072934
67. Yamashita M, Chattopadhyay S. A TRIF-Independent Branch of TLR3 Signaling. *J Immunol.* (2020) 188:2825–33. doi: 10.4049/jimmunol.1103220
68. Sandoo A, Veldhuijzen van Zanten JJC., Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. *Open Cardiovasc Med J.* (2015) 4:302–12. doi: 10.2174/1874192401004010302
69. Galley HF, Webster NR. Physiology of the endothelium. *Br J Anaesth.* (2004) 93:105–13. doi: 10.1093/bja/aeh163
70. Sturtzel C. Endothelial Cells. In: Sattler S, Kennedy-Lydon T, editors. *The Immunology of Cardiovascular Homeostasis Pathology*. Cham: Springer International Publishing (2017). p. 71–91. doi: 10.1007/978-3-319-57613-8_4
71. Al-Soudi A, Kaaij MH, Tas SW. Endothelial cells: from innocent bystanders to active participants in immune responses. *Autoimmun Rev.* (2017) 16:951–62. doi: 10.1016/j.autrev.2017.07.008
72. Wang W, Deng M, Liu X, Ai W, Tang Q, Hu J. TLR4 activation induces nontolerant inflammatory response in endothelial cells. *Inflammation.* (2011) 34:509–18. doi: 10.1007/s10753-010-9258-4
73. Pryshchep O, Ma-Krupa W, Younge BR, Goronzy JJ, Weyand CM. Vessel-specific toll-like receptor profiles in human medium and large arteries. *Circulation.* (2008) 118:1276–84. doi: 10.1161/CIRCULATIONAHA.108.789172
74. Lu Z, Li Y, Jin J, Zhang X, Lopes-Virella MF, Huang Y. Toll-like receptor 4 activation in microvascular endothelial cells triggers a robust inflammatory response and cross talk with mononuclear cells via interleukin-6. *Arterioscler Thromb Vasc Biol.* (2012) 32:1696–706. doi: 10.1161/ATVBAHA.112.251181
75. Banno K, Yoder MC. Tissue regeneration using endothelial colony-forming cells: promising cells for vascular repair. *Pediatr Res.* (2018) 83:283–90. doi: 10.1038/pr.2017.231
76. Shafiee A, Patel J, Hutmacher DW, Fisk NM, Khosrotehrani K. Meso-endothelial bipotent progenitors from human placenta display distinct molecular and cellular identity. *Stem Cell Rep.* (2018) 10:890–904. doi: 10.1016/j.stemcr.2018.01.011
77. Alphonse RS, Vadivel A, Zong S, McConaghy S, Ohls R, Yoder MC, et al. The isolation and culture of endothelial colony-forming cells from human and rat lungs. *Nat Protoc.* (2015) 10:1697–708. doi: 10.1038/nprot.2015.107
78. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood.* (2004) 104:2752–60. doi: 10.1182/blood-2004-04-1396
79. Lin Y, Solovey A, Hebbel RP, Lin Y, Weisdorf DJ, Solovey A, et al. Origins of circulating endothelial cells and endothelial outgrowth from blood find the latest version : origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest.* (2000) 105:71–77. doi: 10.1172/JCI8071
80. Mazzucchelli I, Lisini D, Garofoli F, Dragoni S, Angelini M, Pozzi M, et al. Expression and function of toll-like receptors in human circulating endothelial colony forming cells. *Immunol Lett.* (2015) 168:98–104. doi: 10.1016/j.imlet.2015.09.014
81. Lee J, Sayed N, Hunter A, Au KF, Wong WH, Mocarski ES, et al. Activation of innate immunity is required for efficient nuclear reprogramming. *Cell.* (2012) 151:547–58. doi: 10.1016/j.cell.2012.09.034
82. Sayed N, Wong WT, Ospino F, Meng S, Lee J, Jha A, et al. Transdifferentiation of human fibroblasts to endothelial cells role of innate immunity. *Circulation.* (2015) 131:300–9. doi: 10.1161/CIRCULATIONAHA.113.007394
83. Naito H, Iba T, Takakura N. Mechanisms of new blood vessel formation and proliferative heterogeneity of endothelial cells. *Int Immunol.* (2020) 32:295–305. doi: 10.1093/intimm/dxaa008
84. Adair TH, Montani J-P. Angiogenesis. *Colloq Ser Integr Syst Physiol From Mol to Funct.* (2010) 2:1–84. doi: 10.4199/C00017ED1V01Y201009ISP010
85. Sun C, Feng S Bin, Cao ZW, Bei JJ, Chen Q, Xu XJ, et al. Up-regulated expression of matrix metalloproteinases in endothelial cells mediates platelet microvesicle-induced angiogenesis. *Cell Physiol Biochem.* (2017) 41:2319–32. doi: 10.1159/000475651
86. Chen F, Ohashi N, Li W, Eckman C, Nguyen JH. Disruptions of occludin and claudin-5 in brain endothelial cells *in vitro* and in brains of mice with acute liver failure. *Hepatology.* (2009) 50:1914–23. doi: 10.1002/hep.23203
87. Cong X, Kong W. Endothelial tight junctions and their regulatory signaling pathways in vascular homeostasis and disease. *Cell Signal.* (2020) 66:109485. doi: 10.1016/j.cellsig.2019.109485
88. Morita K, Sasaki H, Furuse M, Tsukita S. Endothelial claudin: Claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol.* (1999) 147:185–94. doi: 10.1083/jcb.147.1.185
89. Ma S, Li Q, Zhouwen JPJ, Xi JN, Guan WX, Jia W. Claudin- 5 regulates blood-brain barrier permeability by modifying brain microvascular endothelial cell proliferation, migration, and adhesion to prevent lung cancer metastasis. *CNS Neurosci Ther.* (2017) 23:947–60. doi: 10.1111/cns.12764
90. Tornavaca O, Chia M, Dufton N, Almagro LO, Conway DE, Randi AM, et al. ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. *J Cell Biol.* (2015) 208:821–38. doi: 10.1083/jcb.201404140
91. Mahajan SD, Aalinkeel R, Reynolds JL, Nair B, Sykes DE, Bonoiu A, et al. Suppression of MMP-9 expression in brain microvascular endothelial cells (BMVEC) using a gold nanorod (GNR)-siRNA nanoplex. *Immunol Invest.* (2012) 41:337–55. doi: 10.3109/08820139.2011.604863
92. Bond M, I RPF, Baker AH, Newby AC. Synergistic upregulation of metalloproteinase-9 by growth factors and in £ ammatory cytokines : an absolute requirement for transcription factor NF- U B. *FEBS Lett.* (1998) 435:29–34. doi: 10.1016/S0014-5793(98)01034-5
93. Zhu H, Dai R, Zhou Y, Fu H, Meng Q. TLR2 ligand Pam3CSK4 regulates MMP-2/9 expression by MAPK/NF-κB signaling pathways in primary brain microvascular endothelial cells. *Neurochem Res.* (2018) 43:1897–904. doi: 10.1007/s11064-018-2607-7
94. Paolillo R, Iovene MR, Carratelli CR, Rizzo A. Induction of vegf and MMP-9 expression by toll-like receptor 2 / 4 in human endothelial cells infected with chlamydia pneumoniae. *Int J Immunopathol Pharmacol.* (2012) 25:377–86. doi: 10.1177/039463201202500207
95. DebRoy A, Vogel SM, Soni D, Sundivakkam PC, Malik AB, Tiruppathi C. Cooperative signaling via transcription factors NF-κB and AP1/c-Fos mediates endothelial cell STIM1 expression and hyperpermeability in response to endotoxin. *J Biol Chem.* (2014) 289:24188–201. doi: 10.1074/jbc.M114.570051
96. Nagyoszi P, Wilhelm I, Farkas AE, Fazakas C, Dung NTK, Haskó J, et al. Expression and regulation of toll-like receptors in cerebral endothelial cells. *Neurochem Int.* (2010) 57:556–64. doi: 10.1016/j.neuint.2010.07.002
97. Meng W, Takeichi M. Adherens junction : molecular. *Cold Spring Harb Perspect Biol.* (2009) 1:a002899. doi: 10.1101/cshperspect.a002899
98. Quillard T, Araújo HA, Franck G, Shvartz E, Sukhova G, Libby P. TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J.* (2015) 36:1394–404. doi: 10.1093/eurheartj/ehv044
99. Schirbel A, Kessler S, Rieder F, West G, Rebert N, Asosingh K, et al. Pro-angiogenic activity of TLRs and NLRs: a novel link between gut microbiota and intestinal angiogenesis. *Gastroenterology.* (2013) 144:613–23.e9. doi: 10.1053/j.gastro.2012.11.005
100. Braren R, Hu H, Kim YH, Beggs HE, Reichardt LF, Wang R. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. *J Cell Biol.* (2006) 172:151–62. doi: 10.1083/jcb.200506184
101. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol.* (2003) 161:1163–77. doi: 10.1083/jcb.200302047

102. Hu L, de Zang M, Wang HX, Li JF, Su LP, Yan M, et al. Biglycan stimulates VEGF expression in endothelial cells by activating the TLR signaling pathway. *Mol Oncol.* (2016) 10:1473–84. doi: 10.1016/j.molonc.2016.08.002
103. Kim SJ, Chen Z, Chamberlain ND, Volin MV, Swedler W, Volkov S, et al. Angiogenesis in rheumatoid arthritis is fostered directly by toll-like receptor 5 ligation and indirectly through interleukin-17 induction. *Arthritis Rheum.* (2013) 65:2024–36. doi: 10.1002/art.37992
104. Aplin AC, Ligresti G, Fogel E, Zorzi P, Smith K, Nicosia RF. Regulation of angiogenesis, mural cell recruitment and adventitial macrophage behavior by Toll-like receptors. *Angiogenesis.* (2014) 17:147–61. doi: 10.1007/s10456-013-9384-3
105. Grote K, Schuett H, Salguero G, Grothusen C, Jagielska J, Drexler H, et al. Toll-like receptor 2/6 stimulation promotes angiogenesis via GM-CSF as a potential strategy for immune defense and tissue regeneration. *Blood.* (2010) 115:2543–52. doi: 10.1182/blood-2009-05-224402
106. West XZ, Malinin NL, Merkulova AA, Tischenko M, Kerr BA, Borden EC, et al. Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. *Nature.* (2010) 467:972–6. doi: 10.1038/nature09421
107. Xia S, Menden HL, Korfhagen TR, Kume T, Sampath V. Endothelial immune activation programmes cell-fate decisions and angiogenesis by inducing angiogenesis regulator DLL4 through TLR4-ERK-FOXC2 signalling. *J Physiol.* (2018) 596:1397–417. doi: 10.1113/JP275453
108. Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell.* (2009) 137:1124–35. doi: 10.1016/j.cell.2009.03.025
109. Wagner NM, Bierhansl L, Nöldge-Schomburg G, Vollmar B, Roesner JP. Toll-like receptor 2-blocking antibodies promote angiogenesis and induce ERK1/2 and AKT signaling via CXCR4 in endothelial cells. *Arterioscler Thromb Vasc Biol.* (2013) 33:1943–51. doi: 10.1161/ATVBAHA.113.301783
110. Guo Z, Chen L, Zhu Y, Zhang Y, He S, Qin J, et al. Double-stranded RNA-induced TLR3 activation inhibits angiogenesis and triggers apoptosis of human hepatocellular carcinoma cells. *Oncol Rep.* (2012) 27:396–402. doi: 10.3892/or.2011.1538
111. Farkas D, Roger Thompson AA, Bhagwani AR, Hultman S, Ji H, Kotha N, et al. Toll-like receptor 3 is a therapeutic target for pulmonary hypertension. *Am J Respir Crit Care Med.* (2019) 199:199–210. doi: 10.1164/rccm.201707-1370OC
112. Wu J, Cui H, Dick AD, Liu L. TLR9 agonist regulates angiogenesis and inhibits corneal neovascularization. *Am J Pathol.* (2014) 184:1900–10. doi: 10.1016/j.ajpath.2014.03.001
113. Gopinathan G, Milagre C, Pearce OMT, Reynolds LE, Hodivala-Dilke K, Leinster DA, et al. Interleukin-6 stimulates defective angiogenesis. *Cancer Res.* (2015) 75:3098–107. doi: 10.1158/0008-5472.CAN-15-1227
114. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol.* (2009) 1:1–10. doi: 10.1101/cshperspect.a001651
115. Wilhelmsen K, Mesa KR, Prakash A, Xu F, Hellman J. Activation of endothelial TLR2 by bacterial lipoprotein upregulates proteins specific for the neutrophil response. *Innate Immun.* (2012) 18:602–16. doi: 10.1177/1753425911429336
116. Wilhelmsen K, Mesa KR, Lucero J, Xu F, Hellman J. ERK5 protein promotes, whereas MEK1 protein differentially regulates, the toll-like receptor 2 protein-dependent activation of human endothelial cells and monocytes. *J Biol Chem.* (2012) 287:26478–94. doi: 10.1074/jbc.M112.359489
117. El Kebir D, Damlaj A, Makhezer N, Filep JG. Toll-like receptor 9 signaling regulates tissue factor and tissue factor pathway inhibitor expression in human endothelial cells and coagulation in mice. *Crit Care Med.* (2015) 43:e179–89. doi: 10.1097/CCM.0000000000001005
118. Loomis Z, Eigenberger P, Redinius K, Lisk C, Karoor V, Nozik-Grayck E, et al. Hemoglobin induced cell trauma indirectly influences endothelial TLR9 activity resulting in pulmonary vascular smooth muscle cell activation. *PLoS ONE.* (2017) 12:e0171219. doi: 10.1371/journal.pone.0171219
119. Maaser C, Heidemann J, von Eiff C, Lugering A, Spahn TW, Binion DG, et al. Human intestinal microvascular endothelial cells express toll-like receptor 5: a binding partner for bacterial flagellin. *J Immunol.* (2004) 172:5056–62. doi: 10.4049/jimmunol.172.8.5056
120. El Kebir D, József L, Pan W, Wang L, Filep JG. Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. *J Immunol.* (2009) 182:4386–94. doi: 10.4049/jimmunol.0803044
121. Aucott H, Sowinska A, Harris HE, Lundback P. Ligation of free HMGB1 to TLR2 in the absence of ligand is negatively regulated by the C-terminal tail domain. *Mol Med.* (2018) 24:1–10. doi: 10.1186/s10020-018-0021-x
122. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol.* (2011) 29:139–62. doi: 10.1146/annurev-immunol-030409-101323
123. Branco-Madeira F, Lambrecht BN. High mobility group box-1 recognition: the beginning of a rageless era? *EMBO Mol Med.* (2010) 2:193–95. doi: 10.1002/emmm.201000077
124. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock.* (2006) 26:174–9. doi: 10.1097/01.shk.0000225404.51320.82
125. Wu H, Chen Z, Xie J, Kang LN, Wang L, Xu B. High mobility group box-1: a missing link between diabetes and its complications. *Mediators Inflamm.* (2016) 2016:3896147. doi: 10.1155/2016/3896147
126. Yang X, Li L, Liu J, Lv B, Chen F. Extracellular histones induce tissue factor expression in vascular endothelial cells via TLR and activation of NF-κB and AP-1. *Thromb Res.* (2016) 137:211–18. doi: 10.1016/j.thromres.2015.10.012
127. Dauphinee SM, Voelcker V, Tebaykina Z, Wong F, Karsan A. Heterotrimeric G i /G o proteins modulate endothelial TLR signaling independent of the MyD88-dependent pathway. *Am J Physiol Hear Circ Physiol.* (2011) 301:2246–53. doi: 10.1152/ajpheart.01194.2010
128. Ghosh M, Subramani J, Rahman MM, Shapiro LH. CD13 Restricts TLR4 endocytic signal transduction in inflammation. *J Immunol.* (2015) 194:4466–76. doi: 10.4049/jimmunol.1403133
129. Nowbar AN, Gitto M, Howard JP, Francis DP, Al-Lamee R. Mortality from ischemic heart disease: analysis of data from the world health organization and coronary artery disease risk factors from NCD risk factor collaboration. *Circ Cardiovasc Qual Outcomes.* (2019) 12:1–11. doi: 10.1161/CIRCOUTCOMES.118.005375
130. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biol Pharm Bull.* (2005) 28:886–92. doi: 10.1248/bpb.28.886
131. Edfeldt K, Swedenborg J, Hansson GK, Yan Z. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation.* (2002) 105:1158–61. doi: 10.1161/circ.105.10.1158
132. Holloway JW, Yang IA, Ye S. Variation in the toll-like receptor 4 gene and susceptibility to myocardial infarction. *Pharmacogenet Genomics.* (2005) 15:15–21. doi: 10.1097/01213011-200501000-00003
133. Ren M, Li R, Luo M, Chen N, Deng X, Yan K, et al. Endothelial cells but not platelets are the major source of Toll-like receptor 4 in the arterial thrombosis and tissue factor expression in mice. *Am J Physiol Regul Integr Comp Physiol.* (2014) 307:R901–7. doi: 10.1152/ajpregu.00324.2014
134. Fallach R, Shainberg A, Avlas O, Fainblut M, Chepurko Y, Porat E, et al. Cardiomyocyte toll-like receptor 4 is involved in heart dysfunction following septic shock or myocardial ischemia. *J Mol Cell Cardiol.* (2010) 48:1236–44. doi: 10.1016/j.yjmcc.2010.02.020
135. Liu CL, Santos MM, Fernandes C, Liao M, Iamarene K, Zhang JY, et al. Toll-like receptor 7 deficiency protects apolipoprotein E-deficient mice from diet-induced atherosclerosis. *Sci Rep.* (2017) 7:847. doi: 10.1038/s41598-017-00977-0
136. Karper JC, Ewing MM, Habets KLL, De Vries MR, Peters EAB, Van Oeveren-Rietdijk AM, et al. Blocking toll-like receptors 7 and 9 reduces postinterventional remodeling via reduced macrophage activation, foam cell formation, and migration. *Arterioscler Thromb Vasc Biol.* (2012) 32:72–80. doi: 10.1161/ATVBAHA.112.249391
137. Karadimou G, Folkersen L, Berg M, Perisic L, Discacciati A, Roy J, et al. Low TLR7 gene expression in atherosclerotic plaques is associated withmajor adverse cardioand cerebrovascular events. *Cardiovasc Res.* (2017) 113:30–39. doi: 10.1093/cvr/cvw231
138. Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, et al. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med.* (2008) 205:2609–21. doi: 10.1084/jem.20081370
139. Cole JE, Navin TJ, Cross AJ, Goddard ME, Alexopoulou L, Mitra AT, et al. Unexpected protective role for toll-like receptor 3 in the arterial wall. *Proc Natl Acad Sci USA.* (2011) 108:2372–7. doi: 10.1073/pnas.1018515108

140. Frump AL, Lahm T. The basic science of metabolism in pulmonary arterial hypertension. *Adv Pulm Hypertens.* (2018) 17:95–102. doi: 10.21693/1933-088X-17.3.95
141. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol.* (1994) 144:275–85.
142. Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. *Circulation.* (2004) 109:159–65. doi: 10.1161/01.CIR.0000102381.57477.50
143. Zimmer S, Steinmetz M, Asdonk T, Motz I, Coch C, Hartmann E, et al. Activation of endothelial toll-like receptor 3 impairs endothelial function. *Circ Res.* (2011) 108:1358–66. doi: 10.1161/CIRCRESAHA.111.243246
144. Papani R, Duarte AG, Lin YL, Kuo YF, Sharma G. Pulmonary arterial hypertension associated with interferon therapy: a population-based study. *Multidiscip Respir Med.* (2017) 12:1–7. doi: 10.1186/s40248-016-0082-z
145. George PM, Oliver E, Dorfmuller P, Dubois OD, Reed DM, Kirkby NS, et al. Evidence for the involvement of type I interferon in pulmonary arterial hypertension. *Circ Res.* (2014) 114:677–88. doi: 10.1161/CIRCRESAHA.114.302221
146. Bauer EM, Zheng H, Lotze MT, Bauer PM. Recombinant human interferon alpha 2b prevents and reverses experimental pulmonary hypertension. *PLoS ONE.* (2014) 9:e96720. doi: 10.1371/journal.pone.0096720
147. Bauer EM, Shapiro R, Zheng H, Ahmad F, Ishizawar D, Comhair SA, et al. High mobility group box 1 contributes to the pathogenesis of experimental pulmonary hypertension via activation of toll-like receptor 4. *Mol Med.* (2012) 18:1509–18. doi: 10.2119/molmed.2012.00283
148. Sadamura-Takenaka Y, Ito T, Noma S, Oyama Y, Yamada S, Kawahara KI, et al. HMGB1 promotes the development of pulmonary arterial hypertension in rats. *PLoS ONE.* (2014) 9:e102482. doi: 10.1371/journal.pone.0102482
149. Wang J, Tian XT, Peng Z, Li WQ, Cao YY, Li Y, et al. HMGB1/TLR4 promotes hypoxic pulmonary hypertension via suppressing BMPR2 signaling. *Vascul Pharmacol.* (2019) 117:35–44. doi: 10.1016/j.vph.2018.12.006
150. Morrell NW. Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? *Proc Am Thorac Soc.* (2006) 3:680–6. doi: 10.1513/pats.200605-118SF
151. Goldenberg NM, Hu Y, Hu X, Volchuk A, Zhao YD, Kucherenko MM, et al. Therapeutic targeting of high-mobility group box-1 in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2019) 199:1566–9. doi: 10.1164/rccm.201808-1597LE
152. Grinnan D, Farkas L. A novel peptide for immunomodulation in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2019) 199:1460–61. doi: 10.1164/rccm.201902-0388ED
153. Suzuki T, Carrier EJ, Talati MA, Rathinasabapathy A, Chen X, Nishimura R, et al. Isolation and characterization of endothelial-to-mesenchymal transition-cells in pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol.* (2017) 314:L118–126. doi: 10.1152/ajplung.00296.2017
154. Ranchoux B, Antigny F, Rucker-Martin C, Hautefort A, Péchoux C, Bogaard HJ, et al. Endothelial-to-mesenchymal transition in pulmonary hypertension. *Circulation.* (2015) 131:1006–18. doi: 10.1161/CIRCULATIONAHA.114.008750
155. Hopper RK, Moonen JRAJ, Diebold I, Cao A, Rhodes CJ, Tojais NF, et al. In pulmonary arterial hypertension, reduced bmpr2 promotes endothelial-to-mesenchymal transition via hmga1 and its target slug. *Circulation.* (2016) 133:1783–94. doi: 10.1161/CIRCULATIONAHA.115.020617
156. Good RB, Gilbane AJ, Trinder SL, Denton CP, Coghlan G, Abraham DJ, et al. Endothelial to mesenchymal transition contributes to endothelial dysfunction in pulmonary arterial hypertension. *Am J Pathol.* (2015) 185:1850–8. doi: 10.1016/j.ajpath.2015.03.019
157. Suzuki T, Tada Y, Nishimura R, Kawasaki T, Sekine A, Urushibara T, et al. Endothelial-to-mesenchymal transition in lipopolysaccharide-induced acute lung injury drives a progenitor cell-like phenotype. *Am J Physiol Lung Cell Mol Physiol.* (2016) 310:L1185–98. doi: 10.1152/ajplung.00074.2016
158. Bhagwani AR, Farkas D, Harmon B, Authelet KJ, Cool CD, Kolb M, et al. Clonally selected primitive endothelial cells promote occlusive pulmonary arteriopathy and severe pulmonary hypertension in rats exposed to chronic hypoxia. *Sci Rep.* (2020) 10:1136. doi: 10.1038/s41598-020-58083-7
159. Fang S, Wei J, Pentinmikko N, Leinonen H, Salven P. Generation of functional blood vessels from a single c-kit+ adult vascular endothelial stem cell. *PLoS Biol.* (2012) 10:e1001407. doi: 10.1371/journal.pbio.1001407
160. Suzuki T, Suzuki S, Fujino N, Ota C, Yamada M, Suzuki T, et al. c-Kit immunoexpression delineates a putative endothelial progenitor cell population in developing human lungs. *Am J Physiol Lung Cell Mol Physiol.* (2014) 306:855–65. doi: 10.1152/ajplung.00211.2013
161. Gao Y, Chen T, Raj JU. Endothelial and smooth muscle cell interactions in the pathobiology of pulmonary hypertension. *Am J Respir Cell Mol Biol.* (2016) 54:451–60. doi: 10.1165/rcmb.2015-0323TR
162. George PM, Badiger R, Shao D, Edwards MR, Wort SJ, Paul-Clark MJ, et al. Viral toll like receptor activation of pulmonary vascular smooth muscle cells results in endothelin-1 generation; relevance to pathogenesis of pulmonary arterial hypertension. *Biochem Biophys Res Commun.* (2012) 426:486–91. doi: 10.1016/j.bbrc.2012.08.106
163. Soon E, Crosby A, Southwood M, Yang P, Tajsic T, Toshner M, et al. Bone morphogenetic protein receptor type II deficiency and increased inflammatory cytokine production: a gateway to pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2015) 192:859–72. doi: 10.1164/rccm.201408-1509OC
164. Ma L, Ambalavanan N, Liu H, Sun Y, Jhala N, Bradley WE, et al. TLR4 regulates pulmonary vascular homeostasis and remodeling via redox signaling. *Front Biosci Landmark.* (2016) 21:397–409. doi: 10.2741/4396
165. Ishida I, Kubo H, Suzuki S, Suzuki T, Akashi S, Inoue K, et al. Hypoxia diminishes toll-like receptor 4 expression through reactive oxygen species generated by mitochondria in endothelial cells. *J Immunol.* (2002) 169:2069–75. doi: 10.4049/jimmunol.169.4.2069
166. Young KC, Hussein SMA, Dadiz R, Demello D, Devia C, Hehre D, et al. Toll-like receptor 4deficient mice are resistant to chronic hypoxia-induced pulmonary hypertension. *Exp Lung Res.* (2010) 36:111–19. doi: 10.3109/01902140903171610
167. Bauer EM, Chanthaphavong RS, Sodhi CP, Hackam DJ, Billiar TR, Bauer PM. Genetic deletion of toll-like receptor 4 on platelets attenuates experimental pulmonary hypertension. *Circ Res.* (2014) 114:1596–600. doi: 10.1161/CIRCRESAHA.114.303662

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

MINI REVIEW
published: 19 August 2020
doi: 10.3389/fmed.2020.00425

Metformin in Pulmonary Hypertension in Left Heart Disease

Vinaya Mulkareddy[1] *and Marc A. Simon*[1,2,3,4,5,6*]

[1] *Heart and Vascular Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, United States,* [2] *Division of Cardiology, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA, United States,* [3] *Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, United States,* [4] *Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA, United States,* [5] *McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, United States,* [6] *Clinical and Translational Science Institute, University of Pittsburgh, Pittsburgh, PA, United States*

Metformin is ubiquitously used in the management of Type II Diabetes Mellitus (DMII). Over the years, our growing knowledge of its therapeutic potential has broadened its use to the treatment of infertility in polycystic ovarian syndrome, gestational diabetes, and even obesity. Recently, it has been suggested as a novel therapy in cardiovascular disease (CVD). Given that CVD is the leading cause of death in patients with DMII, with ∼ 75% dying from a cardiovascular event, the intersection of DMII and CVD provides a unique therapeutic target. In particular, pulmonary hypertension (PH) related to CVD (Group II PH) may be an optimal target for metformin therapy. The objective of this review article is to provide an overview of the pathophysiology of PH related to left heart disease (PH-LHD), outline the proposed pathophysiologic mechanism of insulin resistance in heart failure and PH-LHD, and evaluate the role metformin may have in heart failure and PH-LHD.

Keywords: pulmonary hypertension, heart failure, therapeutics, metformin, HFpEF

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez, Stanford University, United States

Reviewed by:
Eric Douglas Austin, Vanderbilt University, United States
Bradley Maron, Harvard Medical School, United States

****Correspondence:***
Marc A. Simon
simonma@upmc.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *29 April 2020*
Accepted: *01 July 2020*
Published: *19 August 2020*

Citation:
Mulkareddy V and Simon MA (2020) Metformin in Pulmonary Hypertension in Left Heart Disease. Front. Med. 7:425. doi: 10.3389/fmed.2020.00425

METFORMIN AND CARDIOVASCULAR DISEASE

Several large observational cohort studies have outlined the merits of metformin in CVD in diabetics, including improved outcomes in coronary artery disease (CAD), myocardial infarction (MI), and heart failure (HF). Furthermore, data suggests that metformin may avert the progression and/or development of HF (1). The most current data corroborating this is from supplemental analysis of the EMPA-REG OUTCOME trial, in which patients were randomly assigned to receive empagliflozin 10 mg or 25 mg or placebo daily. Outcomes from this trial include CV death, non-fatal MI or stroke, and hospitalizations. Metformin use, even in the placebo arm of the trial, was associated with improved cardiovascular (CV) outcomes including CV death, HF hospitalizations, major adverse cardiac events, and all-cause mortality (2). The benefit of metformin in CVD is so profound that the American College of Cardiology (ACC) has delineated its benefits in its 2018 ACC consensus, "New Diabetic Drugs to Reduce CV Outcomes" (3). Despite this growing evidence, data on metformin's potential therapeutic role in specific CVD processes is limited.

PULMONARY HYPERTENSION DUE TO CARDIOVASCULAR DISEASE

Pulmonary hypertension is a complex, heterogeneous, multisystem syndrome that affects 10–20% of the general population (4). It results from a panvasculopathy that restricts blood flow in the distal pulmonary arteries with eventual maladaptive consequences on the right ventricle. It is

a rapidly progressive, debilitating disease process that leads to right ventricular failure, and ultimately death. With its heterogeneous pathophysiology and increasing prevalence, the World Health Organization (WHO) created the Dana Point Clinical Classification, most recently updated by the 6th World Symposium on Pulmonary Hypertension which catalogs the disease into 5 categories based on etiology and pathophysiology (5). The most commonly encountered form of PH is WHO group 2, also known as pulmonary venous hypertension due to left heart disease (4, 6).

PH is estimated to affect up to 35% of patients with left heart disease (LHD). PH-LHD is defined as a mean pulmonary artery pressure of > 20 mmHg with a concomitant pulmonary artery wedge pressure of > 15 mmHg (7). As we uncover more about PH-LHD, it is becoming increasingly apparent that the pathophysiologic aberrations and the phenotypic variations are highly dependent on the underlying cardiovascular disease process, e.g., heart failure with reduced ejection fraction (HFrEF) vs. heart failure with preserved ejection fraction (HFpEF) (4, 7, 8).

HFrEF is characterized by signs and symptoms of HF in the setting of abnormal left ventricle (LV) systolic function. It is defined by a LV ejection fraction (LVEF) of ≤ 40%, progressive LV dilation, eccentric remodeling, reduced LV systolic elastance, and increased arterial elastance. Regardless of the etiology, there are a number of beneficial therapeutic interventions available including beta-adrenergic receptor blockers, aldosterone antagonists, ace-inhibitors, angiotensin receptor blockers, hydralazine and isosorbide dinitrate, intracardiac defibrillators, and cardiac resynchronization therapy. Unfortunately, the benefits of these therapies do not translate to PH-HFrEF, as HFrEF patients with PH continue to have worse outcomes than those without (9–12).

In contrast, HFpEF is a complex, heterogeneous, systemic syndrome characterized by signs, and symptoms of HF in the setting of a normal left ventricular ejection fraction. A number of cardiac and extracardiac aberrations and comorbidities result in HFpEF's heterogeneity and phenotypic variation (13, 14). Unlike in HFrEF, there are a paucity of beneficial therapeutic interventions available in HFpEF raising the question of whether targeting specific HFpEF sub-phenotypes may lead to, as of yet, elusive successful therapies. One such unique phenotype is pulmonary hypertension (PH).

PH is prevalent in ∼ 60–70% of HFrEF patients and 80% of HFpEF patients and is also associated with a higher morbidity and mortality (15). The mechanism and pathophysiology of PH in HF, as alluded to previously, is complex and incompletely understood. Initially elevated LV end-diastolic pressure and secondary mitral regurgitation leads to chronically elevated left atrial pressure and increased pulmonary artery wedge pressure. This contributes to both pulmonary vasoconstriction and alveolar capillary stress failure. Eventually over time there is increased pulmonary vascular remodeling, endothelial dysfunction, intimal fibrosis, and venous hypertension. The venous congestion from left atrial hypertension in conjunction with reactive arterial vasoconstriction and remodeling is referred to as combined pre- and post-capillary PH, defined hemodynamically as a mean pulmonary artery pressure >20 mmHg with a pulmonary artery wedge pressure > 15 mmHg and pulmonary vascular resistance ≥ 3 Woods units (5, 7). Other pathophysiologic mechanisms have also been implicated in PH-LHD as well-including intrinsic myocyte stiffness, extracellular matrix alterations, microvascular dysfunction, inflammation, and metabolic dysfunction (7, 16). Given the lack of available therapies, investigating alternative pathophysiologic mechanisms such as metabolic syndrome or insulin resistance as novel therapeutic targets is paramount.

METABOLIC SYNDROME, INSULIN RESISTANCE, PH-LHD

Heart failure (HF) is a heterogeneous syndrome with regards to demographics, underlying etiology, disease course, and prognosis. To better categorize and risk stratify this complex syndrome, different schema have been developed including the American Heart Association (AHA) stages as well as the New York Heart Association classification (17). Over the last several years, greater efforts have been made to sub-phenotype HF in order to improve risk stratification and facilitate therapeutic interventions. This stratification has led to a greater awareness of the role of metabolic syndrome in HF as well as PH-LHD.

DM is closely associated with HF. A meta-analysis of HF clinical trial populations uncovered that the prevalence of DMII in HF patients approximates 30% (18). This increases to 40% in African Americans. Additionally, patients with concomitant HF and DM have increased morbidity and mortality. In the Candesartan in Heart Failure Assessment of Reduction in Mortality and Morbidity trial (CHARM), DM was an independent predictor of cardiovascular death and hospitalization in patients with HFpEF (19). More recently, DMII has been linked as an independent risk factor in the development of HF as publicized by data derived from the Framingham Risk Study. Both men and women with DMII were 5x more likely to develop HF even after taking into account incidence of CAD (20). Furthermore, 60% of HF patients with normal fasting glucose and insulin levels were found to have abnormal responses to oral glucose tolerance testing—implicating insulin resistance as an underlying pathophysiologic contributor to HF (20). Given this, traditional and novel glycemic control agents are being increasingly studied as a therapeutic agent in the management of HF. A list of such agents is summarized in **Table 1**.

It is hypothesized that insulin resistance directly affects myocardial structure and function through lipotoxicity, free fatty acid oxidation, oxidative stress, impaired nitric oxide bioavailability, and mitochondrial dysfunction and fibrosis (27–31). Impaired insulin signaling diminishes myocardial glucose extraction and uptake resulting in increased fatty acid oxidation (20). Free fatty oxidation in turn leads to accumulation of lipid storage molecules including triglycerides, ceramide, and diacylglycerol in cardiomyocytes. Ceramide induces apoptosis and impaired mitochondrial function, which eventually results in ventricular dysfunction and remodeling. The histologic outcome is similar to what is observed in pressure overload, volume overload, or myocardial infarction (20).

TABLE 1 | Antiglycemic agents and cardiovascular outcomes.

Agent	Trial	CV death	All-cause death	HF hospitalizations
Empagliflozin	EMPA-REG (2)	0.62 (0.49–0.77)	0.68 (0.57–0.82)	0.65 (0.50–0.85)
Canagliflozin	CANVAS (21)	0.87 (0.72–1.06)	0.87 (0.74–1.01)	0.67 (0.52–0.87)
Saxagliptin	SAVOR-TIMI53 (22)	1.03 (0.87–1.22)	1.11 (0.96–1.27)	1.27 (1.07–1.51)
Sitagliptin	TECOS (23)	1.03 (0.89–1.19)	1.01 (0.90–1.14)	1.00 (0.83–1.20)
Liraglutide	LEADER (24)	0.78 (0.66–0.93)	0.85 (0.74–0.97)	0.87 (0.73–1.05)
Semaglutide	SUSTAIN-6 (25)	0.98 (0.65–1.48)	1.05 (0.74–1.50)	1.11 (0.77–1.61)
Exenatide	EXSCEL (26)	0.88 (0.76–1.2)	0.86 (0.77–0.97)	0.94 (0.78–1.13)

All numerical values including cardiovascular death, all cause death, and heart failure hospitalizations are presented as hazard ratios with the 95% confidence interval. CV indicates cardiovascular; HF indicates heart failure.

The role of insulin resistance in HF and PH-LHD has been well-documented in preclinical models. For example, altered glucose metabolism involving downregulation of 5′adenosine monophosphate-activated protein kinase (AMPK) has been implicated in the pathogenesis of PH-LHD. Mice exposed to a high fat diet and L-NAME (NO synthase suppression) to induce metabolic syndrome and hypertension were found to have many of the hallmarks of HFpEF including exercised intolerance, lung congestion, LV hypertrophy, cardiac fibrosis, reduced myocardial capillary density, increased left ventricular filling pressure, reduced LV global longitudinal strain despite preserved LV ejection fraction (EF), and decreased contraction velocity with impaired relaxation (32).

In another preclinical model, metabolic syndrome, and left ventricular dysfunction were induced in mice through supracoronary aortic banding and a high fat diet supplemented by olanzapine. In this model, metabolic syndrome exacerbated group 2 PH via vascular remodeling. There was increased macrophage accumulation, interleukin 6 (IL-6), and signal transducer and activator of transcription 3 (STAT3). These findings were associated with increased proliferation of smooth muscle cells in pulmonary arteries, remodeling of distal pulmonary arteries, and PH (33).

Metabolic changes resulting from abnormal glucose handling and insulin resistance were found to precede the structural and functional aberrations in cardiac muscle. This was demonstrated through glucose-6-phosphate (G6P) via mammalian target of rapamycin (mTOR) and endoplasmic reticulum stress in *ex vivo* animal models, *in vivo* animal models, and in humans with HF (34). While under stress such as increased inotropic demand, cardiac myocytes undergo both metabolic, and structural remodeling. Metabolically, carbohydrates become the main energy source. Structurally, cardiac hypertrophy has been linked to increased activity of mTOR, a regulator myocardial protein synthesis (35). Rats pretreated with various energy sources including glucose, non-carbohydrate energy substrate, or glucose analog underwent *ex vivo* analysis of cardiac muscle exposed to high workloads. There was increased G6P accumulation, mTOR activity, endoplasm reticulum stress, and impaired contractility regardless of energy source. This was not appreciated in rats pretreated with rapamycin (an mTOR inhibitor) or metformin (an AMPK activator). This was re-demonstrated with *in vivo* rat models. Rats exposed to aortic constriction had increased glucose tracer analog uptake and contractile dysfunction utilizing micro-PET imaging (34). These changes preceded LV dilation. Furthermore, humans with heart failure were found to have decreased G6P and endoplasmic reticulum stress markers once the heart was mechanically unloaded utilizing left ventricular assist devices (34). Pathophysiologically, it is hypothesized that increasing workload surpasses cardiomyocyte glucose uptake capability and oxidative capacity. This results in G6P accumulation, a crucial mediator in load-induced mTOR activation. mTOR impairs cardiac power and induces ER stress leading to contractile dysfunction and hypertrophy. Rapamycin, metformin, and mechanical unloading reversed this metabolic cascade (34).

Impact

HF is a worldwide epidemic, affecting ∼ 6 million people in the United States and 23 million people worldwide (36). Approximately 1 in 5 adults over the age of 40 will develop HF and survival estimates are as low as 70% at 1 year and 50% at 5 years. Furthermore, it costs the United States roughly 30 billion dollars per year. This is expected to more than double by 2030 (36). Morbidity and mortality of HF patients with concomitant PH is significantly higher (10, 11, 37–40). It is also apparent that there is continuum of risk with even borderline PH associated with higher mortality and morbidity (41). Unfortunately, once HF patients develop PH there are no therapies to reverse or even cease progression.

Thus far, treatment of PH-LHD is geared toward managing volume status with diuretics and dietary modifications, implementing goal directed medical therapy for left ventricular dysfunction, and treating comorbidities such as systemic hypertension. Despite medical optimization with the aforementioned therapies, elevated pulmonary pressures persist, and often worsen. Traditional PAH therapies have been studied, but unfortunately with generally negative outcomes. Endothelin Receptor Antagonists (ERA) trials such as ENABLE (Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure) reported no benefit in regards to mortality and in fact found increased heart failure exacerbations and heart failure hospitalizations secondary to fluid retention (42). Prostacyclins have been associated with improved cardiac output and decreased pulmonary vascular resistance but have had a

strong trend toward higher mortality (43). Phosphodiesterase-5 inhibitors in clinical trials have had disappointing results. For example, RELAX (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Diastolic Heart Failure) showed no significant improvement in clinical outcomes (44). Uncovering novel therapies to prevent the development of PH-LHD from HF or even halt the progression of PH-LHD is paramount given the growing public health concern that HF and PH-LHD poses. Given the association of insulin resistance with HF and PH-LHD, metformin is of high interest as a novel treatment.

PRECLINICAL MODELS: METFORMIN'S MECHANISTIC EVIDENCE

Mechanistically, metformin exerts its benefit on the CV system in a pleotropic manner (**Figure 1**). It is hypothesized that these pleotropic effects are due to its role in directly activating AMPK, which is a cellular energy sensor that is intricately involved in numerous metabolic processes not limited to liver and skeletal muscle glucose metabolism (45).

In skeletal muscle, metformin enhances glucose-mediated glucose uptake resulting in improved insulin sensitivity, oxidative metabolism, mitochondrial function (46). Dysregulation of AMPK and its downstream signaling network have both been implicated in metabolic syndrome, HF, and PH. This may be further mediated upstream via sirtuin-3 (SIRT3), a mitochondrial deacetylase. When downregulated, SIRT3 contributes to the development of insulin resistance, and eventually diabetes. Furthermore, SIRT3-AMPK downregulation in pulmonary artery smooth muscle cells is associated with the development of PH in leptin deficient, obese, diabetic rats with metabolic syndrome, and was reversed with metformin (47). Metformin restored SIRT 3 in skeletal muscles, improved insulin sensitivity, and was associated with decreased pulmonary pressures (47).

Metformin upregulates the SIRT3-AMPK-Glut4 pathway. Glucose transporter type 4 (Glut 4), an insulin-regulated glucose transporter in adipose tissue and striated muscle, improves insulin action and glucose uptake. Myokines, such as fibroblast growth factor 21 (FGF21) which regulates simple sugar intake, may be a critical link in crosstalk between skeletal muscle metabolism and pulmonary vascular smooth muscle cells, further activating AMPK, and preventing pulmonary vascular remodeling. Additional mechanisms of action of metformin on vascular remodeling include AMPK-mediated inhibition of estrogen and aromatase synthesis, inhibition of leptin secretion, and downstream STAT3 activation—all crucial mediators of group 2 PH (48).

Additionally, metformin augments weight loss, directly treating the adverse effects of obesity (19). Over 80% of HF patients in clinical trials and epidemiologic cohorts are overweight or obese. Mechanistically, obesity is hypothesized to have pleiotropic effects on HF and PH-LHD as a result of abnormal endocrine, cellular, and cardiometabolic signaling. Important mediators of this include adipokines including leptin and adiponectin. Leptin directly affects the sympathetic nervous system, aldosterone receptor signaling, janus kinase (JAK) signal transducer, STAT proteins, and p38 mitogen-activated protein kinase, all of which have been implicated in deleterious cardiac remodeling, impaired calcium handling, and impaired relaxation. Adiponectin decreases with obesity and is linked

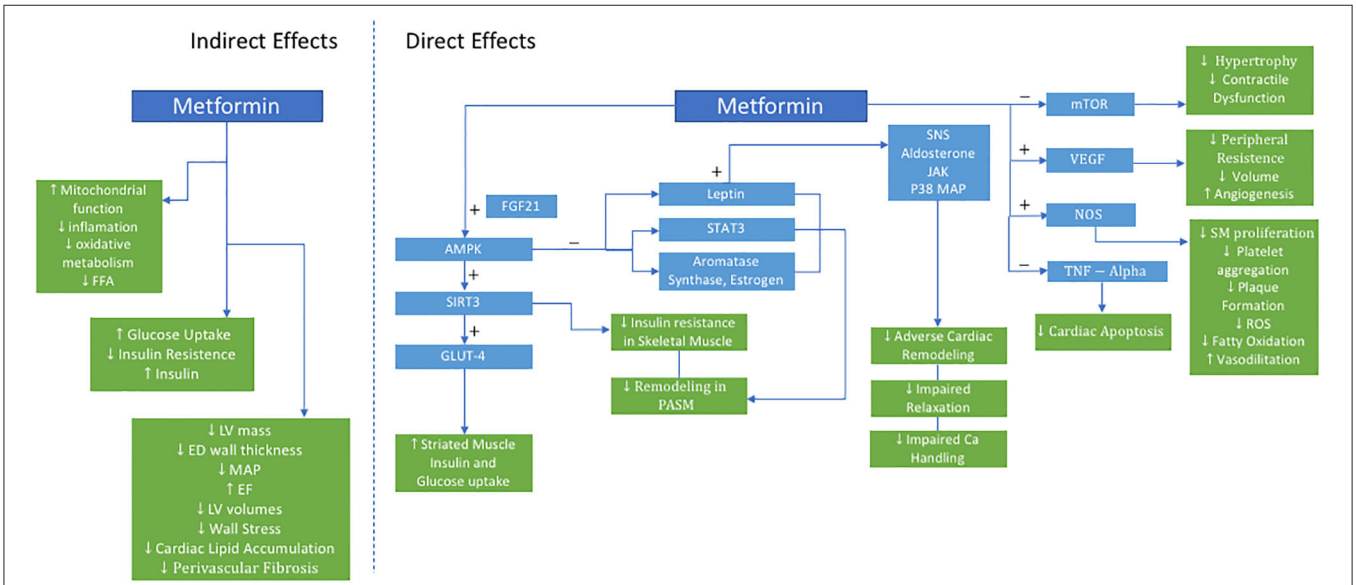


FIGURE 1 | Proposed Actions of Metformin in PH-HFpEF. AMPK indicates 5′-AMP-activated protein kinase; Ca, calcium; ED, end-diastolic; EF, ejection fraction; FGF21, fibroblast growth factor 21; FFA, free fatty acid; LV, left ventricle; GLUT4, glucose transporter type 4; JAK, janus kinase; LV, left ventricle; MAP, mean arterial pressure; mTOR, mammalian target of rapamycin; NOS, nitric oxide synthase; P38 MAP, P38 mitogen-activated protein; PASM, pulmonary artery smooth muscle; ROS, reactive oxygen species; SIRT3, sirtuin-3; SM, smooth muscle; SNS, sympathetic nervous system; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

to increasing inflammation, oxidative stress, as well as adverse cardiac remodeling (49). While the effect of obesity on HF has been previously described, obesity's impact on the pulmonary vasculature in the PH-LHD phenotype is still unknown.

Metformin has also been shown to have a beneficial role in chronic systemic hypertension preventing the development and progression of hypertensive LHD. Spontaneously hypertensive rats were noted to have significant myocardial metabolic changes even prior to developing cardiac dysfunction. Metformin treatment in this model lowered mean arterial pressure, lowered glucose uptake, improved ejection fraction, and decreased left ventricular mass and end-diastolic wall thickness. Furthermore, free fatty acid levels and mTOR activity were reduced as well (50). In another model, spontaneous hypertensive, insulin-resistant rats (SHHF) treated with metformin had decreased LV volumes, wall stress, cardiac lipid accumulation, and perivascular fibrosis—all markers of LV remodeling. Additional improvements were seen in indices systolic and diastolic ventricular function. Metformin activated AMPK, vascular endothelial growth factor, and nitric oxide synthase, while reducing myocyte apoptosis and tumor necrosis factor-alpha expression—all have which been implicated in HF pathogenesis. These metabolic, molecular, and structural changes translated to functional cardiomyocyte improvements and prevention of HF (51).

CLINICAL TRIALS

Will preclinical, observational, and *post-hoc* data translate into distinct measurable benefit for HF or PH-LHD? This has yet to be determined, but there is certainly reasonable evidence to support clinical trials (52). In a compelling pilot study recently completed, Mohan et al. (53) assessed the therapeutic benefit of metformin on LV hypertrophy in 68 patients with coronary artery disease without diabetes. They showed metformin decreased LV mass, systolic blood pressure, body weight, and oxidative stress as measured by thiobarbituric acid reactive substances in patients with preserved LVEF (53). Another trial in 62 non-diabetic insulin resistant HFrEF patients found improvements in functional class and minute ventilation/carbon dioxide production (VE/VCO2 slope) (54). We are currently pursuing a prospective phase II clinical trial evaluating the therapeutic efficacy of metformin in PH-HFpEF (Clinicaltrials.gov identifier NCT03629340) which will evaluate exercise hemodynamics, functional capacity, skeletal muscle signaling, and insulin sensitivity.

METFORMIN IN PAH AND HFPEF

Briefly, although beyond the scope of this review, the therapeutic role of metformin is also being investigated in both pulmonary arterial hypertension (PAH) and HFpEF. There is a growing body of evidence that PAH is associated with a number of systemic metabolic derangements including metabolic syndrome, insulin resistance, and glucose intolerance. Alterations in aerobic glycolysis, tricarboxyclic acid metabolism, and fatty oxidation have all been implicated in the pathophysiology. Given these findings, there is at least one currently ongoing clinical trial (NCT01884051) looking at the role of metformin in PAH (55). While in HFpEF there is significant clinical heterogeneity, there is increasing evidence illustrating metformin's therapeutic benefit across the varying phenotypes. Halabi et al. (56) recently published a systematic review and meta regression analysis analyzing the role of metformin in HFpEF. Metformin reduced mortality and morbidity in HFpEF patients even after adjustment for other HF and glycemic control therapies including beta blockers, angiotensin converting enzyme inhibitors, and insulin (56).

AUTHOR CONTRIBUTIONS

All authors contributed to all aspects of the manuscript.

FUNDING

MS was supported by the NIH grants 1R01AG058659, 2P01HL103455, and UL1 TR001857.

REFERENCES

1. Nichols GA, Koro CE, Gullion CM, Ephross SA, Brown JB. The incidence of congestive heart failure associated with antidiabetic therapies. *Diabetes Metab Res Rev.* (2005) 21:51–7. doi: 10.1002/dmrr.480
2. Inzucchi SE, Fitchett D, Jurisic-Erzen D, Woo V, Hantel S, Janista C, et al. Are the cardiovascular and kidney benefits of empagliflozin influenced by baseline glucose-lowering therapy? *Diabetes Obes Metab.* (2020) 22:631–9. doi: 10.1111/dom.13938
3. Das SR, Everett BM, Birtcher KK, Brown JM, Cefalu WT, Januzzi JL Jr, et al. 2018 ACC expert consensus decision pathway on novel therapies for cardiovascular risk reduction in patients with type 2 diabetes and atherosclerotic cardiovascular disease: a report of the American college of cardiology task force on expert consensus decision pathways. *J Am Coll Cardiol.* (2018) 72:3200–23. doi: 10.1016/j.jacc.2018.09.020
4. Rose-Jones LJ, McLaughlin VV. Pulmonary hypertension: types and treatments. *Curr Cardiol Rev.* (2015) 11:73–9. doi: 10.2174/1573403X09666131117164122
5. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J.* (2019) 53:1801913. doi: 10.1183/13993003.01913-2018
6. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol.* (2013) 62(Suppl. 25):D34–41. doi: 10.1016/j.jacc.2013.10.029
7. Vachiery JL, Tedford RJ, Rosenkranz S, Palazzini M, Lang I, Guazzi M, et al. Pulmonary hypertension due to left heart disease. *Eur Respir J.* (2019) 53:1801913. doi: 10.1183/13993003.01897-2018
8. Zelt JGE, Chaudhary KR, Cadete VJ, Mielniczuk LM, Stewart DJ. Medical therapy for heart failure associated with pulmonary hypertension. *Circ Res.* (2019) 124:1551–67. doi: 10.1161/CIRCRESAHA.118.313650

9. Ghio S, Raineri C, Scelsi L, Asanin M, Polovina M, Seferovic P. Pulmonary hypertension and right ventricular remodeling in HFpEF and HFrEF. *Heart Fail Rev.* (2020) 25:85–91. doi: 10.1007/s10741-019-09810-4
10. Ghio S, Gavazzi A, Campana C, Inserra C, Klersy C, Sebastiani R, et al. Independent and additive prognostic value of right ventricular systolic function and pulmonary artery pressure in patients with chronic heart failure. *J Am Coll Cardiol.* (2001) 37:183–8. doi: 10.1016/S0735-1097(00)01102-5
11. Lam CS, Roger VL, Rodeheffer RJ, Borlaug BA, Enders FT, Redfield MM. Pulmonary hypertension in heart failure with preserved ejection fraction: a community-based study. *J Am Coll Cardiol.* (2009) 53:1119–26. doi: 10.1016/j.jacc.2008.11.051
12. Aronson D, Eitan A, Dragu R, Burger AJ. Relationship between reactive pulmonary hypertension and mortality in patients with acute decompensated heart failure. *Circ Heart Fail.* (2011) 4:644–50. doi: 10.1161/CIRCHEARTFAILURE.110.960864
13. Shah SJ. Precision medicine for heart failure with preserved ejection fraction: an overview. *J Cardiovasc Transl Res.* (2017) 10:233–44. doi: 10.1007/s12265-017-9756-y
14. Shah SJ, Kitzman DW, Borlaug BA, van Heerebeek L, Zile MR, Kass DA, et al. Phenotype-specific treatment of heart failure with preserved ejection fraction: a multiorgan roadmap. *Circulation.* (2016) 134:73–90. doi: 10.1161/CIRCULATIONAHA.116.021884
15. Senni M, Paulus WJ, Gavazzi A, Fraser AG, Diez J, Solomon SD, et al. New strategies for heart failure with preserved ejection fraction: the importance of targeted therapies for heart failure phenotypes. *Eur Heart J.* (2014) 35:2797–815. doi: 10.1093/eurheartj/ehu204
16. Tromp J, Westenbrink BD, Ouwerkerk W, van Veldhuisen DJ, Samani NJ, Ponikowski P, et al. Identifying pathophysiological mechanisms in heart failure with reduced versus preserved ejection fraction. *J Am Coll Cardiol.* (2018) 72:1081–90. doi: 10.1016/j.jacc.2018.06.050
17. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al. ACC/AHA 2005 guideline update for the diagnosis and management of chronic heart failure in the Adult: a report of the American college of cardiology/American heart association task force on practice guidelines (writing committee to update the 2001 guidelines for the evaluation and management of heart failure): developed in collaboration with the American college of chest physicians and the International society for heart and lung transplantation: endorsed by the heart rhythm society. *Circulation.* (2005) 112:e154–235. doi: 10.1161/CIRCULATIONAHA.105.167586
18. Cohen-Solal A, Beauvais F, Logeart D. Heart failure and diabetes mellitus: epidemiology and management of an alarming association. *J Card Fail.* (2008) 14:615–25. doi: 10.1016/j.cardfail.2008.04.001
19. Alpert MA, Terry BE, Mulekar M, Cohen MV, Massey CV, Fan TM, et al. Cardiac morphology and left ventricular function in normotensive morbidly obese patients with and without congestive heart failure, and effect of weight loss. *Am J Cardiol.* (1997) 80:736–40. doi: 10.1016/S0002-9149(97)00505-5
20. Velez M, Kohli S, Sabbah HN. Animal models of insulin resistance and heart failure. *Heart Fail Rev.* (2014) 19:1–13. doi: 10.1007/s10741-013-9387-6
21. Mahaffey KW, Neal B, Perkovic V, de Zeeuw D, Fulcher G, Erondu N, et al. Canagliflozin for primary and secondary prevention of cardiovascular events: results from the CANVAS program (Canagliflozin cardiovascular assessment study). *Circulation.* (2018) 137:323–34. doi: 10.1161/CIRCULATIONAHA.117.032038
22. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med.* (2013) 369:1317–26. doi: 10.1056/NEJMoa1307684
23. Green JB, Bethel MA, Armstrong PW, Buse JB, Engel SS, Garg J, et al. Effect of Sitagliptin on cardiovascular outcomes in type 2 diabetes. *N Engl J Med.* (2015) 373:232–42. doi: 10.1056/NEJMoa1501352
24. Marso SP, Daniels GH, Brown-Frandsen K, Kristensen P, Mann JFE, Nauck MA, et al. Liraglutide and cardiovascular outcomes in type 2 diabetes. *N Engl J Med.* (2016) 375:311–22. doi: 10.1056/NEJMoa1603827
25. Marso SP, Bain SC, Consoli A, Eliaschewitz FG, Jódar E, Leiter LA, et al. Semaglutide and cardiovascular outcomes in patients with type 2 diabetes. *N Engl J Med.* (2016) 375:1834–44. doi: 10.1056/NEJMoa1607141
26. Holman RR, Bethel MA, Mentz RJ, Thompson VP, Lokhnygina Y, Buse JB, et al. Effects of once-weekly exenatide on cardiovascular outcomes in type 2 diabetes. *N Engl J Med.* (2017) 377:1228–39. doi: 10.1056/NEJMoa1612917
27. Willemsen S, Hartog JW, Hummel YM, van Ruijven MH, van der Horst IC, van Veldhuisen DJ, et al. Tissue advanced glycation end products are associated with diastolic function and aerobic exercise capacity in diabetic heart failure patients. *Eur J Heart Fail.* (2011) 13:76–82. doi: 10.1093/eurjhf/hfq168
28. Pieske B, Wachter R. Impact of diabetes and hypertension on the heart. *Curr Opin Cardiol.* (2008) 23:340–9. doi: 10.1097/HCO.0b013e3283031ab3
29. van Heerebeek L, Somsen A, Paulus WJ. The failing diabetic heart: focus on diastolic left ventricular dysfunction. *Curr Diab Rep.* (2009) 9:79–86. doi: 10.1007/s11892-009-0014-9
30. Borbely A, Papp Z, Edes I, Paulus WJ. Molecular determinants of heart failure with normal left ventricular ejection fraction. *Pharmacol Rep.* (2009) 61:139–45. doi: 10.1016/S1734-1140(09)70016-7
31. Milsom AB, Jones CJ, Goodfellow J, Frenneaux MP, Peters JR, James PE. Abnormal metabolic fate of nitric oxide in Type I diabetes mellitus. *Diabetologia.* (2002) 45:1515–22. doi: 10.1007/s00125-002-0956-9
32. Schiattarella GG, Altamirano F, Tong D, French KM, Villalobos E, Kim SY, et al. Nitrosative stress drives heart failure with preserved ejection fraction. *Nature.* (2019) 568:351–6. doi: 10.1038/s41586-019-1100-z
33. Ranchoux B, Nadeau V, Bourgeois A, Provencher S, Tremblay E, Omura J, et al. Metabolic syndrome exacerbates pulmonary hypertension due to left heart disease. *Circ Res.* (2019) 125:449–66. doi: 10.1161/CIRCRESAHA.118.314555
34. Sen S, Kundu BK, Wu HC, Hashmi SS, Guthrie P, Locke LW, et al. Glucose regulation of load-induced mTOR signaling and ER stress in mammalian heart. *J Am Heart Assoc.* (2013) 2:e004796. doi: 10.1161/JAHA.113.004796
35. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol.* (2002) 4:648–57. doi: 10.1038/ncb839
36. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, et al. Heart disease and stroke statistics-2020 update: a report from the American heart association. *Circulation.* (2020) 141:e139–596. doi: 10.1161/CIR.0000000000000746
37. Vanderpool RR, Saul M, Nouraie M, Gladwin MT, Simon MA. Association between hemodynamic markers of pulmonary hypertension and outcomes in heart failure with preserved ejection fraction. *JAMA Cardiol.* (2018) 3:298–306. doi: 10.1001/jamacardio.2018.0128
38. Bursi F, McNallan SM, Redfield MM, Nkomo VT, Lam CS, Weston SA, et al. Pulmonary pressures and death in heart failure: a community study. *J Am Coll Cardiol.* (2012) 59:222–31. doi: 10.1016/j.jacc.2011.06.076
39. Salamon JN, Kelesidis I, Msaouel P, Mazurek JA, Mannem S, Adzic A, et al. Outcomes in World Health Organization group II pulmonary hypertension: mortality and readmission trends with systolic and preserved ejection fraction-induced pulmonary hypertension. *J Card Fail.* (2014) 20:467–75. doi: 10.1016/j.cardfail.2014.05.003
40. Kjaergaard J, Akkan D, Iversen KK, Kjoller E, Kober L, Torp-Pedersen C, et al. Prognostic importance of pulmonary hypertension in patients with heart failure. *Am J Cardiol.* (2007) 99:1146–50. doi: 10.1016/j.amjcard.2006.11.052
41. Maron BA, Hess E, Maddox TM, Opotowsky AR, Tedford RJ, Lahm T, et al. Association of borderline pulmonary hypertension with mortality and hospitalization in a large patient cohort: insights from the veterans affairs clinical assessment, reporting, and tracking program. *Circulation.* (2016) 133:1240–8. doi: 10.1161/CIRCULATIONAHA.115.020207
42. Kalra PR, Moon JC, Coats AJ. Do results of the ENABLE (Endothelin antagonist bosentan for lowering cardiac events in heart failure) study spell the end for non-selective endothelin antagonism in heart failure? *Int J Cardiol.* (2002) 85:195–7. doi: 10.1016/S0167-5273(02)00182-1
43. Califf RM, Adams KF, McKenna WJ, Gheorghiade M, Uretsky BF, McNulty SE, et al. A randomized controlled trial of epoprostenol therapy for severe congestive heart failure: the Flolan International randomized survival trial (FIRST). *Am Heart J.* (1997) 134:44–54. doi: 10.1016/S0002-8703(97)70105-4
44. Redfield MM, Chen HH, Borlaug BA, Semigran MJ, Lee KL, Lewis G, et al. Effect of phosphodiesterase-5 inhibition on exercise capacity and clinical status in heart failure with preserved ejection fraction: a randomized clinical trial. *JAMA.* (2013) 309:1268–77. doi: 10.1001/jama.2013.2024
45. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia.* (2017) 60:1577–85. doi: 10.1007/s00125-017-4342-z

46. Bailey CJ, Puah JA. Effect of metformin on glucose metabolism in mouse soleus muscle. *Diabete Metab.* (1986) 12:212–8.
47. Goncharov DA, Goncharova EA, Tofovic SP, Hu J, Baust JJ, Pena AZ, et al. Metformin therapy for pulmonary hypertension associated with heart failure with preserved ejection fraction versus pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2018) 198:681–4. doi: 10.1164/rccm.201801-0022LE
48. Lai YC, Tabima DM, Dube JJ, Hughan KS, Vanderpool RR, Goncharov DA, et al. SIRT3-AMP-activated protein kinase activation by nitrite and metformin improves hyperglycemia and normalizes pulmonary hypertension associated with heart failure with preserved ejection fraction. *Circulation.* (2016) 133:717–31. doi: 10.1161/CIRCULATIONAHA.115.018935
49. Oh A, Okazaki R, Sam F, Valero-Munoz M. Heart failure with preserved ejection fraction and adipose tissue: a story of two tales. *Front Cardiovasc Med.* (2019) 6:110. doi: 10.3389/fcvm.2019.00110
50. Li J, Minczuk K, Massey JC, Howell NL, Roy RJ, Paul S, et al. Metformin improves cardiac metabolism and function, and prevents left ventricular hypertrophy in spontaneously hypertensive rats. *J Am Heart Assoc.* (2020) 9:e015154. doi: 10.1161/JAHA.119.015154
51. Cittadini A, Napoli R, Monti MG, Rea D, Longobardi S, Netti PA, et al. Metformin prevents the development of chronic heart failure in the SHHF rat model. *Diabetes.* (2012) 61:944–53. doi: 10.2337/db11-1132
52. Rena G, Lang CC. Repurposing metformin for cardiovascular disease. *Circulation.* (2018) 137:422–4. doi: 10.1161/CIRCULATIONAHA.117.031735
53. Mohan M, Al-Talabany S, McKinnie A, Mordi IR, Singh JSS, Gandy SJ, et al. A randomized controlled trial of metformin on left ventricular hypertrophy in patients with coronary artery disease without diabetes: the MET-REMODEL trial. *Eur Heart J.* (2019) 40:3409–17. doi: 10.1093/eurheartj/ehz203
54. Wong AKF, Symon RS, Ang DSC, Alzadjali MA, Choy AM, Petrie J, et al. Metformin in insulin resistant LV dysfunction, a double-blind, placebo controlled trial (TAYSIDE trial). *Eur Heart J.* (2010) 31:849. doi: 10.1093/eurheartj/ehq289
55. Assad TR, Hemnes AR. Metabolic dysfunction in pulmonary arterial hypertension. *Curr Hypertens Rep.* (2015) 17:20. doi: 10.1007/s11906-014-0524-y
56. Halabi A, Sen J, Huynh Q, Marwick TH. Metformin treatment in heart failure with preserved ejection fraction: a systematic review and meta regression analysis. *J Am Coll Cardiol.* (2020) 75(11 Suppl. 1):952. doi: 10.1016/S0735-1097(20)31579-5

Conflict of Interest: MS: Research support from Novartis, Aadi. Consultancy fees from Complexa, Actelion, United Therapeutics, Acceleron.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer BM has declared a past co-authorship with one of the author MS to the handling Editor.

frontiers in Medicine

ORIGINAL RESEARCH
published: 11 September 2020
doi: 10.3389/fmed.2020.00540

Expression of a Human Caveolin-1 Mutation in Mice Drives Inflammatory and Metabolic Defect-Associated Pulmonary Arterial Hypertension

Anandharajan Rathinasabapathy[1], Courtney Copeland[2], Amber Crabtree[1], Erica J. Carrier[1], Christy Moore[1], Sheila Shay[1], Santhi Gladson[1], Eric D. Austin[3], Anne K. Kenworthy[4†], James E. Loyd[1], Anna R. Hemnes[1] and James D. West[1]**

[1] Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [2] Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States, [3] Pediatrics, Vanderbilt University Medical Center, Nashville, TN, United States, [4] Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, United States

Background: In 2012, mutations in Cav1 were found to be the driving mutation in several cases of heritable pulmonary arterial hypertension (PAH). These mutations replaced the last 21 amino acids of Cav1 with a novel 22-amino-acid sequence. Because previously only Cav1 knockouts had been studied in the context of PAH, examining the *in vivo* effects of this novel mutation holds promise for new understanding of the role of Cav1 in disease etiology.

Methods: The new 22 amino acids created by the human mutation were knocked into the native mouse Cav1 locus. The mice underwent hemodynamic, energy balance, and inflammatory measurements, both at baseline and after being stressed with either a metabolic or an inflammatory challenge [low-dose lipopolysaccharide (LPS)]. To metabolically challenge the mice, they were injected with streptozotocin (STZ) and fed a high-fat diet for 12 weeks.

Results: Very little mutant protein was found *in vivo* (roughly 2% of wild-type by mass spectrometry), probably because of degradation after failure to traffic from the endoplasmic reticulum. The homozygous mutants developed a mild, low-penetrance PAH similar to that described previously in knockouts, and neither baseline nor metabolic nor inflammatory stress resulted in pressures above normal in heterozygous animals. The homozygous mutants had increased lean mass and worsened oral glucose tolerance, as previously described in knockouts. Novel findings include the preservation of Cav2 and accessory proteins in the liver and the kidney, while they are lost with homozygous Cav1 mutation in the lungs. We also found that the homozygous mutants had a significantly lower tolerance to voluntary spontaneous exercise than the wild-type mice, with the heterozygous mice at an intermediate level. The mutants also had higher circulating monocytes, with both heterozygous and homozygous animals having higher pulmonary MCP1 and MCP5 proteins. The heterozygous animals also lost weight at an LPS challenge level at which the wild-type mice continued to gain weight.

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez, Stanford University, United States

Reviewed by:
Stephen Y. Chan, University of Pittsburgh Medical Center, United States
Sebastien Bonnet, Laval University, Canada
Hyung J. Chun, Yale University, United States

***Correspondence:**
Anandharajan Rathinasabapathy anandharajan.rathinasabapathy@vumc.org
James D. West j.west@vumc.org

†Present address:
Anne K. Kenworthy, Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, VA, United States

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *01 June 2020*
Accepted: *30 July 2020*
Published: *11 September 2020*

Citation:
Rathinasabapathy A, Copeland C, Crabtree A, Carrier EJ, Moore C, Shay S, Gladson S, Austin ED, Kenworthy AK, Loyd JE, Hemnes AR and West JD (2020) Expression of a Human Caveolin-1 Mutation in Mice Drives Inflammatory and Metabolic Defect-Associated Pulmonary Arterial Hypertension. Front. Med. 7:540. doi: 10.3389/fmed.2020.00540

Conclusions: The Cav1 mutation identified in human patients in 2012 is molecularly similar to a knockout of Cav1. It results in not only metabolic deficiencies and mild pulmonary hypertension, as expected, but also an inflammatory phenotype and reduced spontaneous exercise.

Keywords: Cav1, pulmonary hypertension, exercise, inflammation, monocyte, metabolism

INTRODUCTION

Caveolae are flask-shaped invaginations in the plasma membrane, typically about 50–100 nm in diameter. They are abundant in endothelial cells, smooth muscle, and adipocytes but also present in many other cell types (1). They serve many cellular functions, including buffering mechanical stress, maintaining membrane integrity, and regulating endocytosis (2). They are also involved in many signal transduction pathways, although their exact role is still under investigation.

Caveolin-1 (Cav1) is an essential component of caveolae in most tissues. It is a 178-amino-acid integral membrane protein that inserts into the membrane in a hairpin structure, generally as part of a complex of 14–16 caveolin monomers (generally incorporating both caveolin 1 and 2). The caveolae assembly also involves several accessory proteins, including cavins, PACSIN2, and EHD-2 (3).

Until 2012, the disease with the clearest association with Cav1 mutations had been lipodystrophy (4), although associations between caveolae function and disease had been drawn for multiple other conditions, including cancer, muscular dystrophy, and cardiovascular disease (5, 6). There was an extensive literature on Cav1 knockout driving pulmonary hypertension in mice (7–10), but this was largely theoretical since it had not been seen in humans. Overall, though, Cav1 knockout mice are viable and fertile, and their health problems are unobvious until in advanced age.

However, in 2012, Cav1 mutations were found as the likely causative mutations in a familial pulmonary hypertension family (11), and since then, causative mutations have been found in additional families, although it remains a rare cause. Other examples include a F160X Cav1 frame shift mutation associated with both pulmonary arterial hypertension (PAH) and congenital generalized lipodystrophy that causes premature truncation of the protein (12–14). In addition, in a recent whole-exome sequencing of 2,572 cases, potentially causative CAV1 mutations were found in 10 individuals, representing seven families or isolated cases (15).

The mutation identified in that first family was a frameshift mutation near the end of the protein P158P fsX22. This replaced the final 21 amino acids, which were known to make up a trafficking signal (16), with an entirely novel 22 amino acids. That this resulted in pulmonary hypertension in humans as heterozygous was interesting since, in mice, only homozygous knockouts developed the disease. This implied the possibility of a dominant negative effect—since caveolins assemble into oligomers, one might imagine that the mutant proteins within these structures either retain the wild-type protein in the endoplasmic reticulum (ER) or Golgi or are trafficked to the plasma membrane and form caveolae with the wild-type, resulting in some dysfunction of the caveolae. We thus set to work in building a mouse with a knock-in of the human mutation into the native mouse Cav1 locus shortly after its initial publication in 2012.

In the interim, both we and others took a closer look at how the mutation worked in cell culture models. On its own, the P158P fsX22 mutation was trapped in the endoplasmic reticulum; however, when co-expressed with wild-type Cav1, it appeared to act as a partial dominant negative, with fewer caveolae and with compromised function as membrane reservoir, in addition to potential signaling defects (17, 18). The goal of the present study was to determine whether these defects were recapitulated *in vivo* in mice and their physiological consequences.

MATERIALS AND METHODS

Reagents and Chemicals

Streptozotocin (STZ), lipopolysaccharide (LPS), and all the other fine chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). High-fat diet (HFD) was purchased from Bio-Serv (Flemington, NJ, USA). Caveolin (610060) and Caveolin 2 (610684) antibodies were purchased from BD Biosciences (San Jose, CA, USA). Cavin1 (ab48824), EHD2 (ab23935), β-actin (ab8227), and CD68 (ab125212) antibodies were obtained from Abcam (Cambridge, MA, USA), α-smooth muscle actin was from Dako (Santa Clara, CA, USA), and PACSIN2 (AP8088b) antibody was from Abcepta (San Diego, CA, USA). Proteome Profiler Mouse Cytokine Array Kit was purchased from R&D systems (Minneapolis, MN, USA).

Animals

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Vanderbilt University Medical Center (approval numbers M/12/106 and M/11/207), in compliance with the National Institutes of Health guidelines. All these studies are conducted in accordance with the ARRIVE guidelines for reporting experiments involving animals (19).

Abbreviations: AUC, area under the curve; CAV1, caveolin-1; CAV2, caveolin-2; CAVIN1, caveolae-associated protein 1; ECHO, echocardiography; EDA, end diastolic area; EF, ejection fraction; EHD2, EH domain containing 2; ER, endoplasmic reticulum; HFD, high-fat diet; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharides (from bacteria); MCP-1, monocyte chemoattractant protein 1; MCP-5, monocyte chemoattractant protein 5; PACSIN2, protein kinase C and casein kinase substrate in neurons 2; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RVH, right ventricular hypertrophy; RVSP, right ventricular systolic pressure; STZ, streptozotocin.

Generation of Transgenic Mice Expressing Human CAV1 Mutation

Heterozygous ($Cav1^{+/P}$) and homozygous ($Cav1^{P/P}$) animals expressing the frame shift human caveolin 1 mutation (P158P fsX22) were generated on a mixed C57/Bl6J and FVB/N background. This was accomplished with a traditional knock-in into embryonic stem cells, with the mutation inserted into the native locus of the mouse CAV1 exon 3 and with the recombination arms extending into intron 2 and the 3′ genomic sequence. Selection markers were included in intron 2 but excised during the final creation of the mouse.

Study Design

Two different animal studies were performed apart from a pilot phenotyping investigation. In the phenotyping study, we had two sets of groups. Nine- to 11-weeks-old ($Cav1^{+/+}$, $n = 4$; $Cav1^{+/P}$, $n = 4$; $Cav1^{P/P}$, $n = 2$) and 28- to 31-weeks-old ($Cav1^{+/+}$, $n = 8$; $Cav1^{+/P}$, $n = 15$) animals in the first (younger) and second (older) groups were subjected to hemodynamic assessments. In the subsequent studies, the animals were either exposed to high-fat diet + streptozotocin (diet study) or lipopolysaccharide (LPS study). Unless specified, all animals throughout the studies were exposed to 12:12-h dark/light cycle with unlimited access to regular chow and water. Both sexes (male and female) were used in all studies.

a. Diet study

In this study, 25- to 58-weeks-old animals were fed either on regular chow (13.5% fat) or 60% high-fat, high-calorie diet for 4 months. This study included six groups in two subsets. Each subset had three groups each of wild-type, heterozygous, and homozygous Cav1 animals—one set on regular chow and the other on HFD. In the first subset (HFD), after 4 weeks of HFD exposure, the animals were fasted for 4 h and streptozotocin (STZ) was administered (150 mg/kg, i.p. route). Following 3 days of STZ administration, blood glucose was estimated to confirm diabetes (blood glucose, >250 mg/dl). A second dose (half-dose) of STZ was administered (on the 5th week) to the animals, which did not develop diabetes following a single dose of STZ. Subsequent to STZ administration, the animals were continued on HFD for another 8 weeks. Blood glucose was monitored in all animals every 4 weeks—week 0 and end of weeks 4, 8, and 12. After a week of acclimatization in individual metabolic cages (week 10), all animals underwent an extensive indirect calorimetry energy expenditure assessment for a continuous 72 h in the Vanderbilt Mouse Metabolic Phenotypic Center. On the 11th week, an oral glucose tolerance test (OGTT) was performed on all animals, followed by 4 h of fasting. Following OGTT, the animals were continued on HFD until the experiments were terminated. Transthoracic echocardiography was performed on all animals before the hemodynamic assessment. This HFD subset included three groups, $Cav1^{+/+}$ ($n = 13$), $Cav1^{+/P}$ ($n = 15$), and $Cav1^{P/P}$ ($n = 16$). All animals, $Cav1^{+/+}$ ($n = 10$), $Cav1^{+/P}$ ($n = 8$), and $Cav1^{P/P}$ ($n = 20$), in the next subset were exposed to regular chow throughout the study and subjected to the same experimental parameters, as mentioned previously.

b. LPS study

Similar to the diet study, we had six groups of animals spread in this study between two subsets, aged between 42 and 76 weeks. In the first subset (LPS), the animals fed on high-energy chow (25.4% fat) were challenged with LPS (7.5 μg/dose/intratracheal route) every 4 days, for a cumulative dose of 45 μg over 3 weeks. All the animals were provided with saline and supplemental gel food and eventually HFD, if they were sick. This subset included the following groups: $Cav1^{+/+}$ ($n = 3$), $Cav1^{+/P}$ ($n = 10$), and $Cav1^{P/P}$ ($n = 4$). After 21 days of LPS exposure, the study was terminated following a hemodynamic assessment. In the second subset (vehicle), all the experimental parameters as explained above were adopted, except LPS, which was replaced with the vehicle (PBS). This subset had three groups: $Cav1^{+/+}$ ($n = 3$), $Cav1^{+/P}$ ($n = 3$), and $Cav1^{P/P}$ ($n = 2$).

Energy Expenditure Assessment by Indirect Calorimetry Method

For the energy expenditure assessment, the animals were individually placed in metabolic cages (identical to home cages with bedding) in a 12-h light/dark cycle, temperature/humidity-controlled dedicated room in the Vanderbilt Mouse Metabolic Phenotyping Core. Energy expenditure measures were obtained by indirect calorimetry (Promethion, Sable Systems, Las Vegas, NV, USA). The calorimetry system consisted of cages identical to home cages, with the bedding equipped with water bottles and food hoppers connected to load cells for food and water intake monitoring. All animals had *ad-libitum* access to either regular diet or HFD and water. The air within the cages was sampled through microperforated stainless steel sampling tubes that ensure uniform cage air sampling. Promethion utilizes a pull-mode, negative pressure system with an excurrent flow rate set at 2,000 ml/min. Water vapor was continuously measured, and its dilution effect on O_2 and CO_2 was mathematically compensated for the analysis stream (20). O_2 consumption and CO_2 production were measured for each mouse every 5 min for 30 s. Incurrent air reference values were determined every four cages. The respiratory quotient was calculated as the ratio of CO_2 production over O_2 consumption. Energy expenditure was calculated using the Weir equation: EE (kcal/h) = 60 * [0.003941 * VO_2 (ml/min) + 0.001106 * VCO_2 (ml/min)] (21). Ambulatory activity was determined every second with XYZ beams. Data acquisition and instrument control were coordinated by MetaScreen v2.2.18, and the raw data were processed using ExpeData v1.7.30 (Sable Systems). Finally, the fat and the lean masses were estimated by NMR on the Bruker Minispec (Bruker Biospin Corporation, Bellerica, MA, USA). This study included the following randomly chosen animals: (i) regular chow subset—$Cav1^{+/+}$ ($n = 10$), $Cav1^{+/P}$ ($n = 8$), and $Cav1^{P/P}$ ($n = 8$) and (ii) HFD subset—$Cav1^{+/+}$ ($n = 9$), $Cav1^{+/P}$ ($n = 10$), and $Cav1^{P/P}$ ($n = 8$).

Oral Glucose Tolerance Test

On the 11th week of the study, oral glucose tolerance test (OGTT) was performed on the same animals discussed in the energy balance study. For OGTT, all animals fasted for 4 h were administered with clinical-grade dextrose (per oral, 1.5 g/kg), and blood glucose was monitored at 0, 10, 20, 30, 45, 60, 90, and 120 min after the sugar load. Almost all animals in the HFD group demonstrated more than 600 mg/dl blood glucose, and therefore the OGTT area under the curve could not be calculated for this group.

Transthoracic Echocardiography and Hemodynamic Assessment

All the animals were subjected to transthoracic echocardiography on the penultimate day of the study using Vivo 770 high-resolution image systems (Visual Sonics, Toronto, Canada). Briefly, the animals were anesthetized with 2% isoflurane–oxygen mixture, and all ventricular and pulmonary dimensions and functions were recorded. Subsequently, invasive hemodynamic assessment was performed as explained previously (22). Following the hemodynamic measurement, the animals were euthanized, blood was withdrawn, a thoracotomy was performed to exsanguinate the heart and the lung, and finally, all other required organs were collected and tissue samples were processed for RNA, protein, and histology assessments. Right ventricular hypertrophy or Fulton's index [RVH = RV / (LV + S)] was calculated as the ratio of wet weight of the right ventricle (RV) to the left ventricle + intra-ventricular septum (LV + S). A complete blood count analysis was performed at Vanderbilt University Medical Center Pathology Core.

Western Blot and Dot Blot Analyses

After euthanization, the superior lobe of the right lung was frozen for protein work and homogenized in radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Following the protein estimation, an equal amount (30 μg) of protein was resolved on 4–12% NuPAGE gel (Thermo Fisher Scientific, Waltham, MA, USA) and transferred electrophoretically onto a polyvinylidene fluoride membrane, and the membranes were blocked with 5% non-fat milk solution in Tris-buffered saline with 0.1% Tween 20 for 1 h. Subsequently, the membranes were probed with the following antibodies: Caveolin (1:10,000), Caveolin 2 (1:500), Cavin 1 (1:1,000), EHD2 (1:1,000), PACSIN2 (1:1,000), and β-actin (1:5,000) overnight at 4°C. The blots were developed on the following day and visualized on the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA). For the dot blot experiment, 200 μg of protein was used as per the manufacturer's instruction.

Immunohistochemical Analysis

After euthanization, the left lobe of the lung was processed and paraffin-embedded, and immunohistochemical staining was performed as explained previously (23). CD45 (1:100), CD68 (1:100), and α-smooth muscle actin (1:200) antibodies were used for the assessment of macrophage and pulmonary vessel muscularization (24), and the images were analyzed as explained previously.

Mass Spectrometry Identification of Mutant Cav1 in Mouse Tissues

Cav1 was immunoprecipitated from mouse tissues using Dynabeads® Protein G Immunoprecipitation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, 5 μg pAb h1-97 IgG (20 μl) was cross-linked to 50 μl Dynabeads® Protein G by BS 3 (Sulfo-DSS; Thermo Scientific™ Pierce™) according to the manufacturer's suggested protocol. Tissues were lysed at 4°C with 1,000 μl CelLytic™ M buffer (Sigma-Aldrich), and the lysed cells were centrifuged for 15 min at 12,000 × *g* at 4°C to pellet the cellular debris. The protein-containing supernatant was transferred into a new tube containing Dynabeads®-Ab complex and incubated with rotation for 10 min at room temperature. The tube was placed on a magnet, and the supernatant ("unbound") was transferred to a clean tube for further analysis. The Dynabeads®-Ab-antigen complex was washed four times, using 200 μl washing buffer for each wash. The final wash was conducted in a new clean tube. The wash solution ("wash") was collected for further analysis. The Dynabeads®-Ab-antigen complex was resuspended with 20 μl elution buffer, 10 μl premixed NuPAGE® LDS sample buffer, and NuPAGE sample reducing agent and heated for 10 min at 70°C. The supernatant was loaded onto the gel for western blot and mass spectrometry identification. Bands corresponding to caveolin-1 were excised and subjected to in-gel trypsin digestion. The resulting peptides were analyzed by a 70-min data-dependent liquid chromatography–tandem mass spectrometry (MS/MS) analysis. Briefly, the peptides were autosampled onto a 200 × 0.1-mm (Jupiter 3 μm, 300 A) self-packed analytical column coupled directly to an LTQ (ThermoFisher) using a nanoelectrospray source and resolved using an aqueous-to-organic gradient. A series of full scans followed by five data-dependent MS/MS was collected throughout the separation. Dynamic 17 exclusion was enabled to minimize the acquisition of redundant peptide spectra. MS/MS spectra were searched *via* SEQUEST against a mouse database (UniprotKB—reference proteome set) to which the mutant caveolin sequence had been appended and that also contained a reversed version for each of the entries (25). The identifications were filtered and collated at the protein level using Scaffold (Proteome Software).

Statistics

Graph Pad Prism, version 8.4.2 (La Jolla, CA, USA) was used for the statistical analysis. One-way ANOVA data analysis followed by Bonferroni's test for multiple comparison was performed for end-point parameters. Two-way ANOVA followed by Tukey's multiple-comparison *post-hoc* analysis was performed to compare the interaction between genotype and treatment. Pearson correlation coefficient was performed to compare the correlation between variables. The values are represented as means ± SEM; $p \leq 0.05$ were considered as statistically significant.

RESULTS

Creation of the Cav1^{P158Pfsx22} Mice

Although Caveolae have been studied in the context of PAH for many years, it was studied in the context of knock-out (7–10). Before 2012, CAV1 mutations had never been identified as causal in human PAH. The initial mutations identified, a P158P fsX22 mutation, was not obviously a complete loss-of-function mutation. The mutation impacted only the final 20 amino acids, replacing the existing termination of the protein with a new rather different termination of 21 amino acids.

Our goal here was to study the physiologic impact of the human CAV1 mutations as closely as possible in a mouse model. Although the mouse and the human CAV1 sequences are very well-conserved—both are 178 amino acids, with 169/178 amino acids identical (95%) and 175/178 conserved—third base redundancy means that just inserting the frameshift would have produced a different amino acid sequence in mice. We thus replaced the end of the mouse CAV1 with the mutated human sequence rather than merely inserting the frameshift (**Figure 1A**). The final amino acid sequence of our knock-in of the human mutation into the mouse is depicted in **Figure 1B**.

These Cav1^{P15PfsX22} mice were created through routine embryonic stem cell methods with homologous recombination. The resulting mice were born with normal Mendelian proportions and were apparently healthy and bred well, even as homozygous.

Cav1^{P15PfsX22} Protein Is Largely Degraded in Mice, With an Organ-Specific Pattern of Loss of Accessory Proteins

First, we examined the expression of both Cav1 protein in knock-in mice as well as with other proteins which are part of caveolae. These include Cav2, a homolog with which Cav1 normally hetero-oligomerizes, Cavin1, a protein that stabilizes caveolae, and EHD2 and PACSIN, which form an actin cytoskeleton-interacting complex as part of caveolae (26).

By quantitative RT-PCR using primers that spanned exons 2 and 3, we found that the spliced RNA expression of the knock-in was comparable to that of the wild-type, as expected (not shown). However, in the lungs, we found that the overall Cav1 levels were reduced by about 20% in heterozygous animals and undetectable in homozygous ones (**Figure 2A**). The levels of Cav2 were decreased at approximately the same extent as Cav1, in accord with prior literature indicating that the expression of Cav1 is required for the stability of Cav2 (27, 28). We also saw a decrease in accessory proteins EHD2 and Cavin1, but not PACSIN2, which increased as Cav1 decreased.

Since the native and the mutant proteins are of the same size, and the antibody recognizes both (there is no antibody to the mutant section of the protein, and we did not tag it in an attempt to preserve the function), we could not readily determine how much of this was from the native and how much was from the mutant protein. Although the homozygous animal had no visible Cav1 band by western blot, this is presumably because it is retained in the ER and degraded, as has been previously reported (17, 18). However, at least when overexpressed, it has been reported that native Cav1, by complexing with a mutant, can assist with the transport to caveolae, which implies that perhaps the mutant might be stabilized in the heterozygous mice. We thus assessed the levels of mutant compared to wild-type protein by using mass spectrometry. We assessed the levels of four different peptides common between the mutant and the wild-type versions of Cav1 and three different peptides specific to the mutant version. We found that, even in heterozygous animals, the expression of the mutant peptides was very low—on average only 1.4% of that of the wild-type Cav1 (**Figure 2B**).

The pattern in other organs was distinctive—in the heart, most proteins were similar to the lung, but there was no increase in PACSIN2 levels in the homozygous animals (**Figure 2C**). In the liver, there was no decrease in Cav2 or EHD2, but Cavin1 was still decreased and PACSIN2 appeared to trend down (**Figure 2D**). The kidney was similar to the liver, except that Cav2 decreased moderately in the homozygous animals (**Figure 2E**). This preservation of Cav2 in some tissues when Cav1 is missing

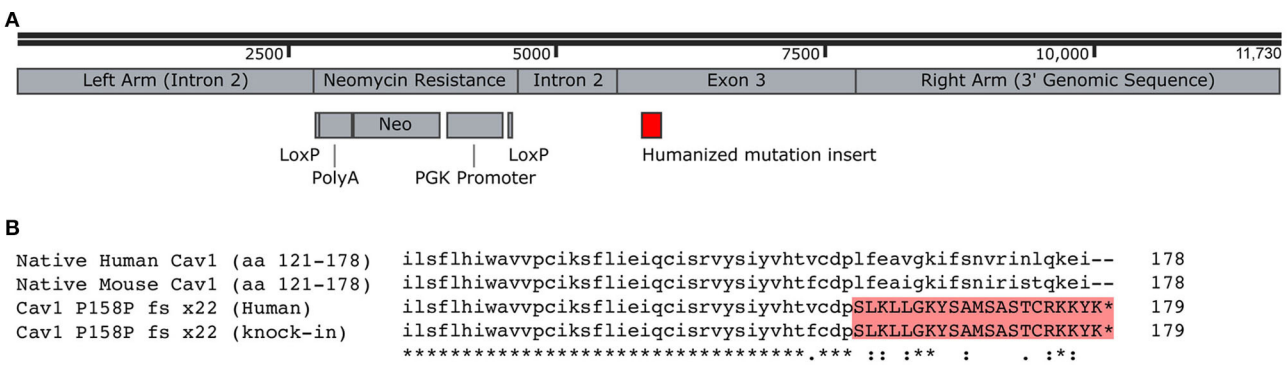


FIGURE 1 | Creation of Cav1^{P15PfsX22} mice. **(A)** Recombination construct used for homologous recombination in embryonic stem cells. The LoxP sites were recombined, removing the neomycin resistance, before the creation of mice. **(B)** Alignment of human and mouse C-termini of Cav1, demonstrating the addition of a novel termination sequence with the human mutation and the inclusion of an identical sequence in the murine knock-in. The asterisk denotes identity, the dot denotes a semi-conservative substitution, and the colon denotes a conservative substitution.

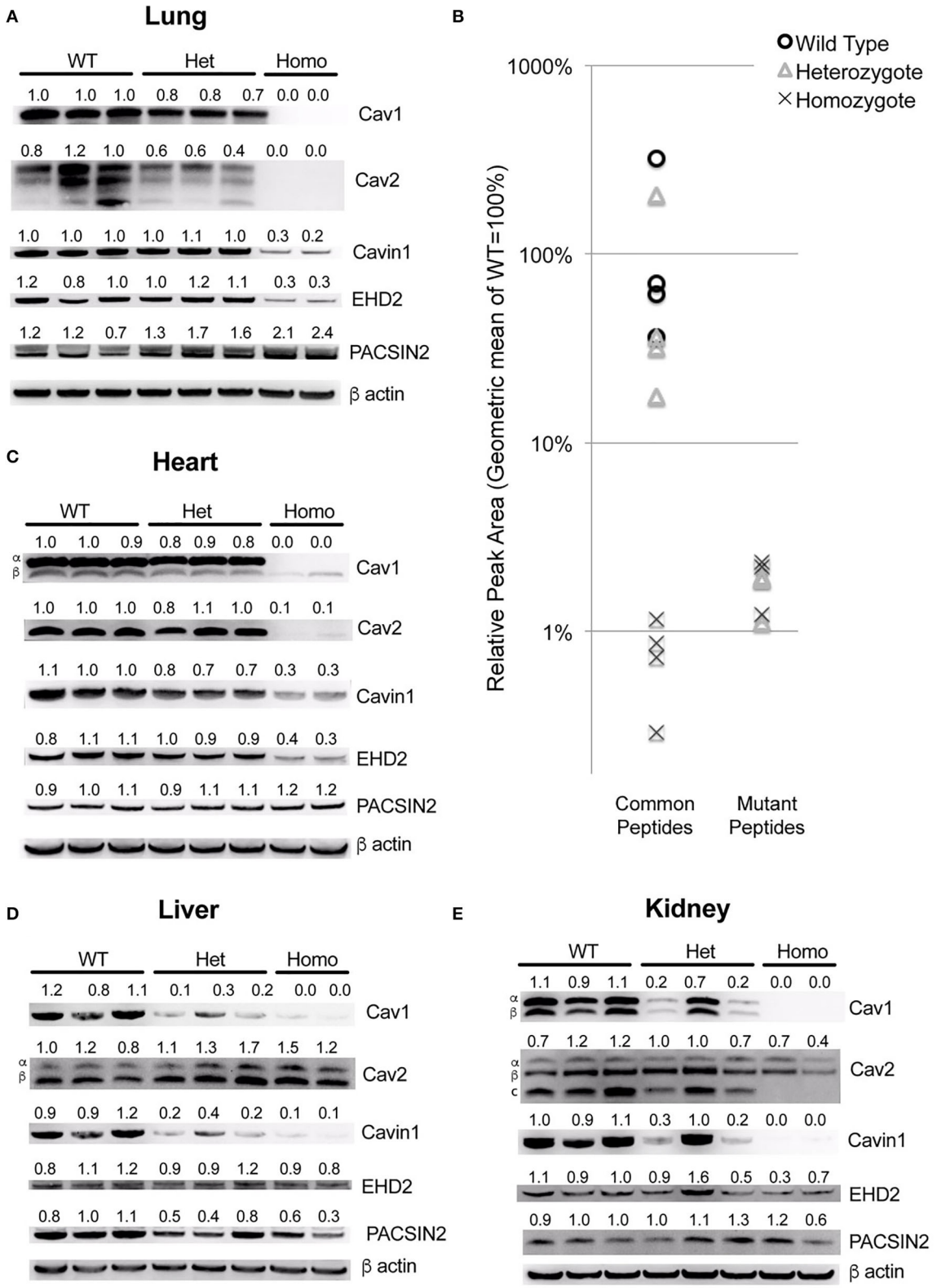


FIGURE 2 | The Cav1^{P15PfsX22} protein is largely degraded in mice, with an organ-specific pattern of loss of accessory proteins. **(A)** Western blots for the indicated proteins from whole mouse lung from wild-type, heterozygous and homozygous mice for the Cav1^{P15PfsX22} mutation—the numbers indicate densitometry normalized to wild type and to beta-actin. **(B)** The mass spectrometry quantification results of proteins from wild-type, heterozygous, and homozygous mice suggest that very little mutant protein is retained even in heterozygous mice. **(C)** Western blots for the indicated proteins from whole mouse heart. **(D)** Western blots for the indicated proteins from whole mouse liver. **(E)** Western blots for the indicated proteins from whole mouse kidney.

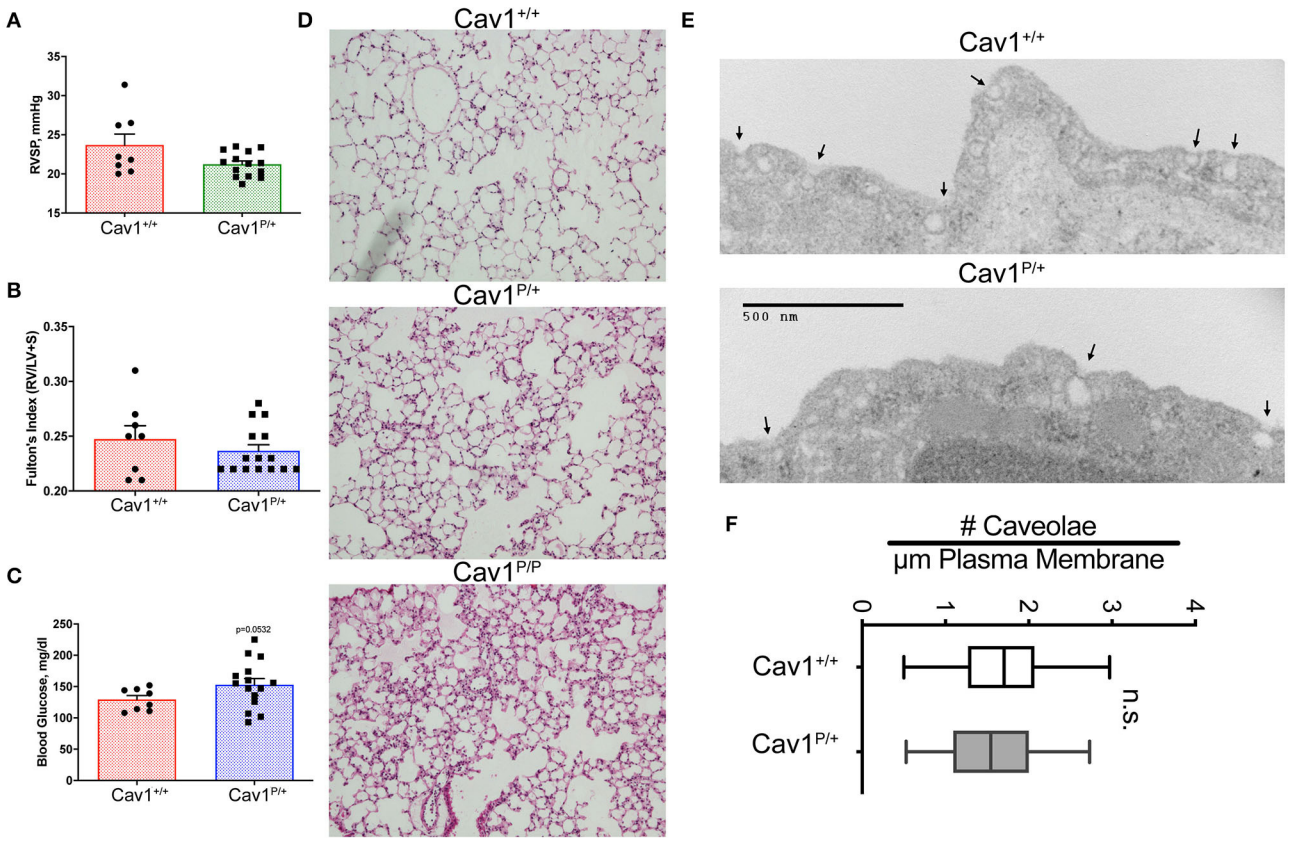


FIGURE 3 | Cav1^{P15PfsX22} mice have a mild phenotype, with no change in the number of caveolae in heterozygous mice. No difference in **(A)** right ventricular systolic pressure or **(B)** right ventricle hypertrophy, and only subtle differences in **(C)** blood glucose in older heterozygous (27–30 weeks) Cav1 mutant mice. **(D)** Some Cav1 mutant animals appeared to have an increased number of pulmonary inflammatory cells, but the phenotype was inconsistent. Lung architecture was observed under ×100 magnification. **(E)** Example of transmission electron microscopy of wild-type (top) and heterozygous (bottom) Cav1 mutants. **(F)** Based on counts of 30–38 fields from three separate mice of each genotype, there is no significant difference in the number of caveolae between wild-type and heterozygous endothelium for Cav1^{P15PfsX22} (referred to as Cav1^{P} in the figure).

contradicts some literature but is supported by others, which have found that Cav2 can form homo-oligomers (29). Broadly, these data suggest that, in all circumstances, the mutant Cav1 is rapidly degraded, and the effect on accessory proteins is very organ specific.

Initial Phenotyping of Cav1^{P158Pfsx22} Mice Showed a Moderate Phenotype Consistent With Prior Reports on Cav1 Knockout Mice

As an initial examination of the phenotype in these mice, we performed hemodynamic and histologic phenotyping on 27- to 30-weeks-old heterozygous Cav1 mutant and littermate control animals. The heterozygous were our focus because the heterozygous mutation results in pulmonary arterial hypertension in human patients. We found no significant difference in right ventricular systolic pressure (RVSP) (**Figure 3A**) or right ventricular hypertrophy (**Figure 3B**) and only a slight trend toward increased blood glucose (**Figure 3C**, $p = 0.0532$, mean 153 vs. 129 mg/dl). The results in a smaller experiment with younger animals (8- to 10-weeks-old mice) which included a few homozygous animals showed a slight increase in RVSP in homozygous of about 30 mmHg, consistent with previous reports (**Supplemental Figure 1A**), but neither Fulton index (right ventricular hypertrophy) nor blood glucose changed in the mutant mice (**Supplemental Figures 1B,C**). By histology, the Cav1 mutant animals (Cav1^{P158Pfsx22}, hereafter referred to as Cav1^{P}), appeared to have increased inflammatory cell burden in the lungs (**Figure 3D**, **Supplemental Figure 2** for higher-resolution pictures), but this pattern was inconsistent and not apparent in the older mice initially. We performed a transmission electron micrography of lung sections and found no difference in the number of caveolae between wild-type and heterozygous endothelium (**Figures 3E,F**).

Stressing Cav1^{P158Pfsx22} Mice With a Diabetes Model Results in Only Subtle Changes in Hemodynamic Phenotype

Cav1 mutation in humans is more commonly associated with lipodystrophy, and Cav1 knockout in mice has previously been shown to cause broader metabolic dysfunctions (30), in addition

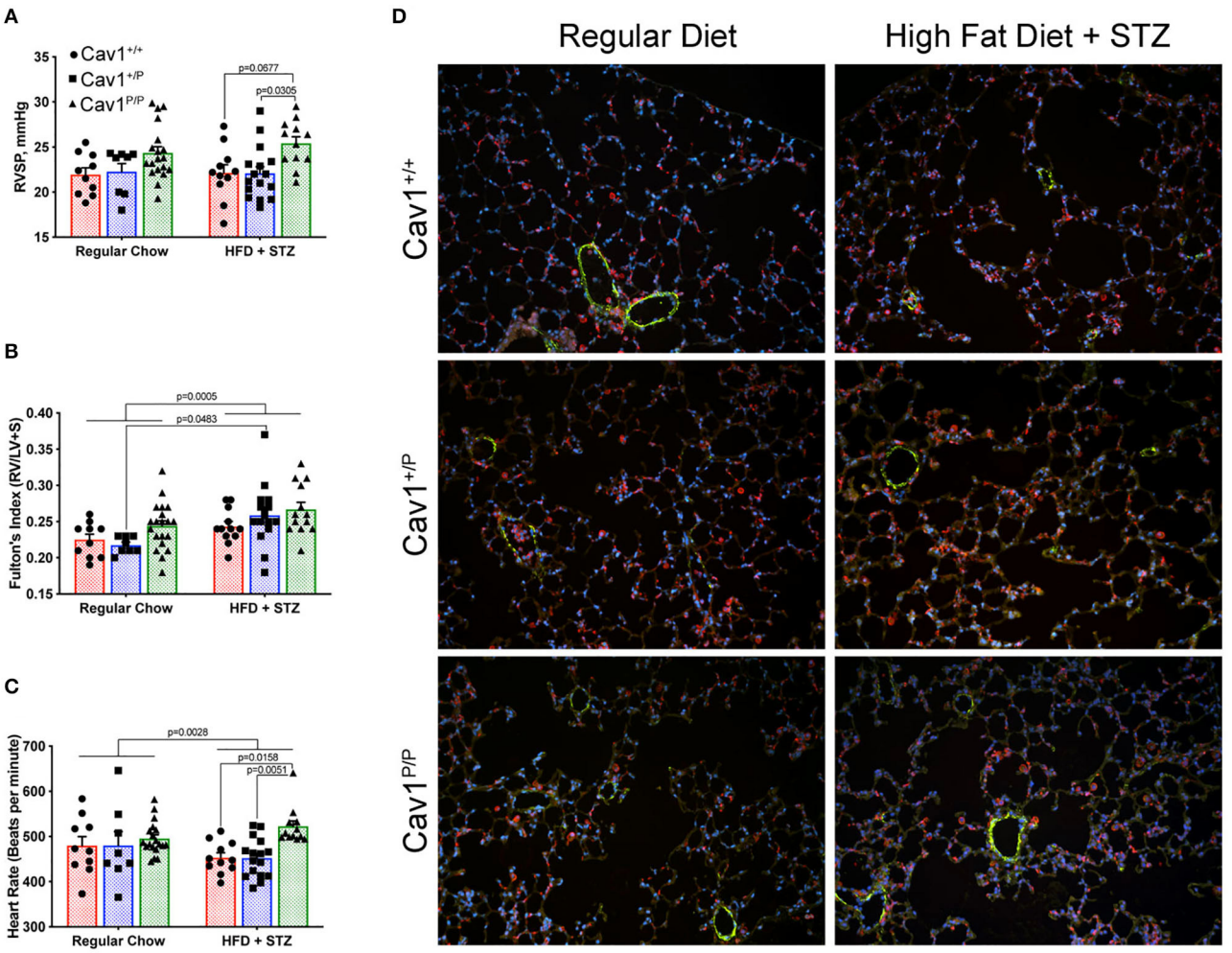


FIGURE 4 | A diabetes model, high-fat diet plus streptozotocin (HFD + STZ), does not bring out a more dramatic hemodynamic phenotype in Cav1^{P15PfsX22} mice. **(A)** Right ventricular systolic pressure was not changed by diet; Cav1 homozygous mutant mice (Cav1$^{P/P}$) showed a slight increase in pressure overall. **(B)** Right ventricular hypertrophy was slightly higher in HFD + STZ mice, but this was not genotype dependent. **(C)** Heart rate under anesthesia was slightly altered by HFD + STZ as shown. **(D)** Lung histology was fairly normal in all groups. DAPI (blue), smooth muscle actin (green), and CD45 (red) immunofluorescence. Pulmonary vasculature was observed under ×200 magnification.

to a failure to gain weight under a high-fat diet (31). We thus hypothesized that the pulmonary phenotype might be more strongly brought out by metabolically stressing the mice. We used a high-fat diet with streptozotocin (STZ) to metabolically stress the Cav1^{P158Pfsx22} mice. STZ is a naturally occurring alkylating agent which is extremely toxic to insulin-producing beta cells; two doses of STZ should thus result in insulin deficiency, whereas the high-fat diet results in insulin resistance.

The inclusion of high-fat diet plus streptozotocin (HFD + STZ) made a little difference to the hemodynamics. Although the mice homozygous for mutation once again had slightly higher pressures, the increase was lower than what we had previously seen in younger mice; HFD + STZ did not cause meaningful changes in the pressures of any group (**Figure 4A**). HFD + STZ slightly increased RV mass across groups—there was no genotype-specific effect (**Figure 4B**). There were small but statistically different changes in heart rate under anesthesia, with the control and the heterozygous mice moving downwards slightly and the homozygous mice moving upwards slightly (**Figure 4C**), but the changes probably are not large enough to be meaningful. The lung histology across groups was fairly normal (**Figure 4D**)—these pressures are not high enough to drive muscularization (see **Supplemental Figure 3** for detailed counts of muscularized vessels, which did not change). We also observed a slight increase in CD45^{+} inflammatory cells in the homozygous group, but it was too patchy within and between mice to be statistically significant (not shown).

We also measured a large number of cardiac indices—left ventricular fractional shortening, left ventricular ejection fraction, left ventricular cardiac output, cardiac index, pulmonary artery area, right ventricular cardiac output, and right ventricle internal dimension during diastole. There were no meaningful

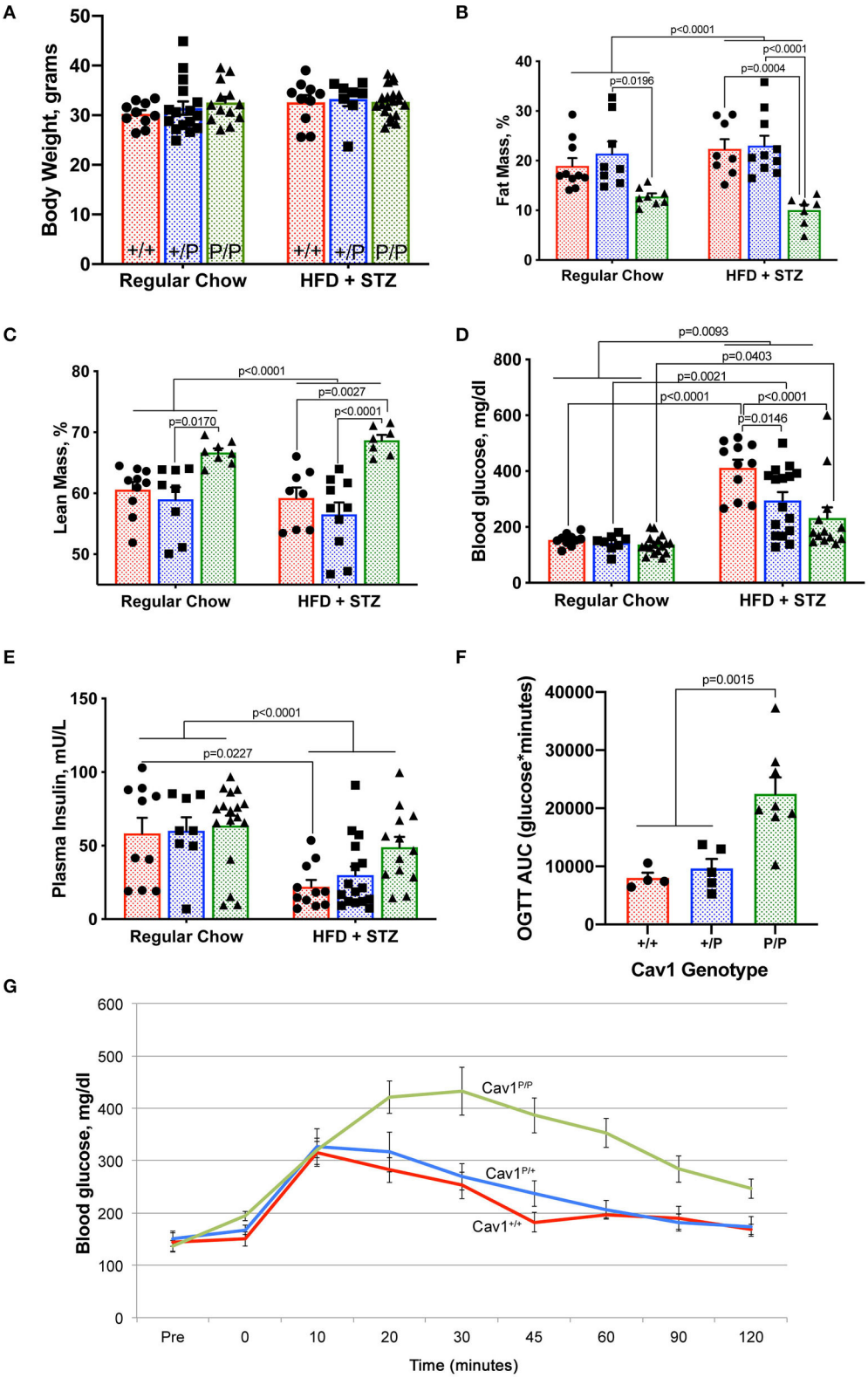


FIGURE 5 | Cav1^{P158Pfsx22} homozygous mice exhibit altered body composition and glucose tolerance. For all figure parts, red shading/circles indicate wild-type mice, blue shading/squares indicate heterozygous mice, and green shading/triangle indicates homozygous mice. Each symbol is the measurement from one animal, with

(Continued)

FIGURE 5 | the bar showing mean and SEM, except for **(G)**. Statistics were performed by ANOVA with *post-hoc* tests. **(A)** Body weight is not impacted by genotype or diet. **(B)** Homozygous mice have a lower percentage of body fat. **(C)** Homozygous mice have a higher percentage lean mass. **(D)** Although blood glucose is increased significantly in all groups with high-fat diet plus streptozotocin (HFD + STZ), it is increased to a lower extent in homozygous Cav1^{P15PfsX22} mice. **(E)** Insulin is reduced in HFD + STZ mice but to a lesser extent in Cav1 homozygous mice. **(F)** Although they can produce more insulin, homozygous mice take a much longer time to clear oral glucose. OGTT, oral glucose tolerance test; AUC, area under the curve. These are regular chow-fed mice (HFD + STZ are off-scale for OGTT). **(G)** Average blood glucose at each time point during the oral glucose tolerance test by genotype. The error bars are standard error of the mean.

differences between groups in any of these measurements (not shown).

Cav1^{P158Pfsx22} Homozygous Mice Have Altered Body Composition and Glucose Tolerance Compared to Wild-Type or Heterozygous Mice

Mice on either regular diet or high-fat diet plus streptozotocin were subjected to metabolic phenotyping. Although mice on high-fat diet alone tend to have an increased body weight, HFD + STZ did not result in significant differences in body mass, either by diet or by genotype (**Figure 5A**). However, mice on HFD + STZ had a slightly higher body fat on average, except for Cav1$^{P/P}$ mice, which had a significantly lower proportion of body fat and a higher proportion of lean mass (**Figures 5B,C**) at the same overall weight. Caveolae are abundant in adipose tissues, and adipose tissues in Cav1 knockout mice lack perilipin, which is necessary to stabilize adipocyte oil droplets (32). In contrast, caveolae in the skeletal muscle is produced by Cav3 (28), whose action is independent of Cav1 and thus likely still functional in these mice. These findings are thus consistent with the literature.

By measurements of blood glucose (**Figure 5D**) and plasma insulin (**Figure 5E**), we found that the Cav1$^{P/P}$ mice retained greater insulin production than the other genotypes. It is possible that streptozotocin is reliant on caveolae for its function. The heterozygous mice appeared to have an intermediate phenotype. Cav1$^{P/P}$ mice had a significantly delayed glucose clearance, with the area under the curve of the oral glucose tolerance test being nearly three times as high in homozygous as in heterozygous or wild-type mice (**Figures 5F,G**). The data shown are from regular chow-fed animals; OGTT was not possible in HFD + STZ mice because their glucose readings were higher than the measurable level throughout the first 90 min of the OGTT test, regardless of genotype. This lower insulin sensitivity is consistent with earlier studies on caveolin 1, which directly regulates insulin receptors (33, 34).

Cav1^{P158Pfsx22} Mice Have Much Lower Waking Locomotion but Are Similar to Wild-Type in Energy Balance Measurements

We measured a variety of metabolic parameters in heterozygous and homozygous Cav1^{P158Pfsx22} mice, both during the night (waking) and during the day (sleeping). These include food uptake, locomotion, energy expenditure, O_2 consumption, CO_2 production, and respiratory coefficient (**Figure 6**). For most of these, there was a diet-specific effect but no genotype-specific effect. The exception to this was locomotion—homozygous Cav1^{P158Pfsx22} mice move less than half as much on average as wild-type mice during their waking hours (**Figure 6D**). The heterozygous mice were intermediate—this is one of only a small number of parameters in which the heterozygous mice show any difference compared to the wild-type mice. This is consistent with previously published results, in which Cav1 knockout mice had reduced endurance in a forced swim test (27); however, the current data indicate that this reduced physical ability is true of normal activity as well.

The respiratory coefficient, the ratio of CO_2 exhaled to O_2 consumed, is indicative of the preferred metabolic substrate, with pure carbohydrate metabolism producing a number of "1" and pure fat metabolism producing a number of "0.7." That similarity of respiratory coefficient between homozygous and wild-type animals (**Figures 6K,L**) is moderately unexpected—cav1 depletion might be expected to interfere only with certain metabolic pathways. In shifting to a high-fat diet, though, it appears that Cav1^{P158Pfsx22} mice shift to a higher proportion of fat burning in the same proportion as wild-type mice.

Mice Homozygous for the Cav1^{P158Pfsx22} Mutation Have Elevated Circulating Monocytes and Elevated Monocyte Chemoattractant Proteins in the Lungs

We performed complete blood counts on mice from the dietary study (**Figure 7**). We found that circulating monocytes were increased in homozygous Cav1^{P158Pfsx22} mice overall, but without a clear effect of diet (**Figure 7A**). In contrast, there were no significant changes in lymphocytes, overall white blood count, or neutrophils (**Figures 7B–D**). We tested the cytokine levels in plasma and the lung by dot blot array. Although we found no significant differences in cytokines in plasma (not shown), we found increases in monocyte chemoattractant proteins 1 and 5 (MCP1/CCL2, MCP5/CCL12) in the lung (**Figure 7E**). There were otherwise few differences between groups (**Figure 7F**). There are a few previous publications linking Cav1 to monocyte attraction or function (35, 36), but this is not typically thought of as a primary role for caveolae.

These data, combined with the trend toward increased CD45$^+$ cells that we saw in previous figures (**Figure 4D**), suggest that Cav1^{P158Pfsx22} mutation in homozygous mice is associated with moderate infiltration of inflammatory cells in the lung, but more specifically the circulating monocytes. CD68 staining for monocytes/macrophages found a trend toward an increase in the lungs of Cav1 mutant mice, but the level of variability was too high at the current *n* for it to be significant (**Supplemental Figures 4A,B**). In particular, there

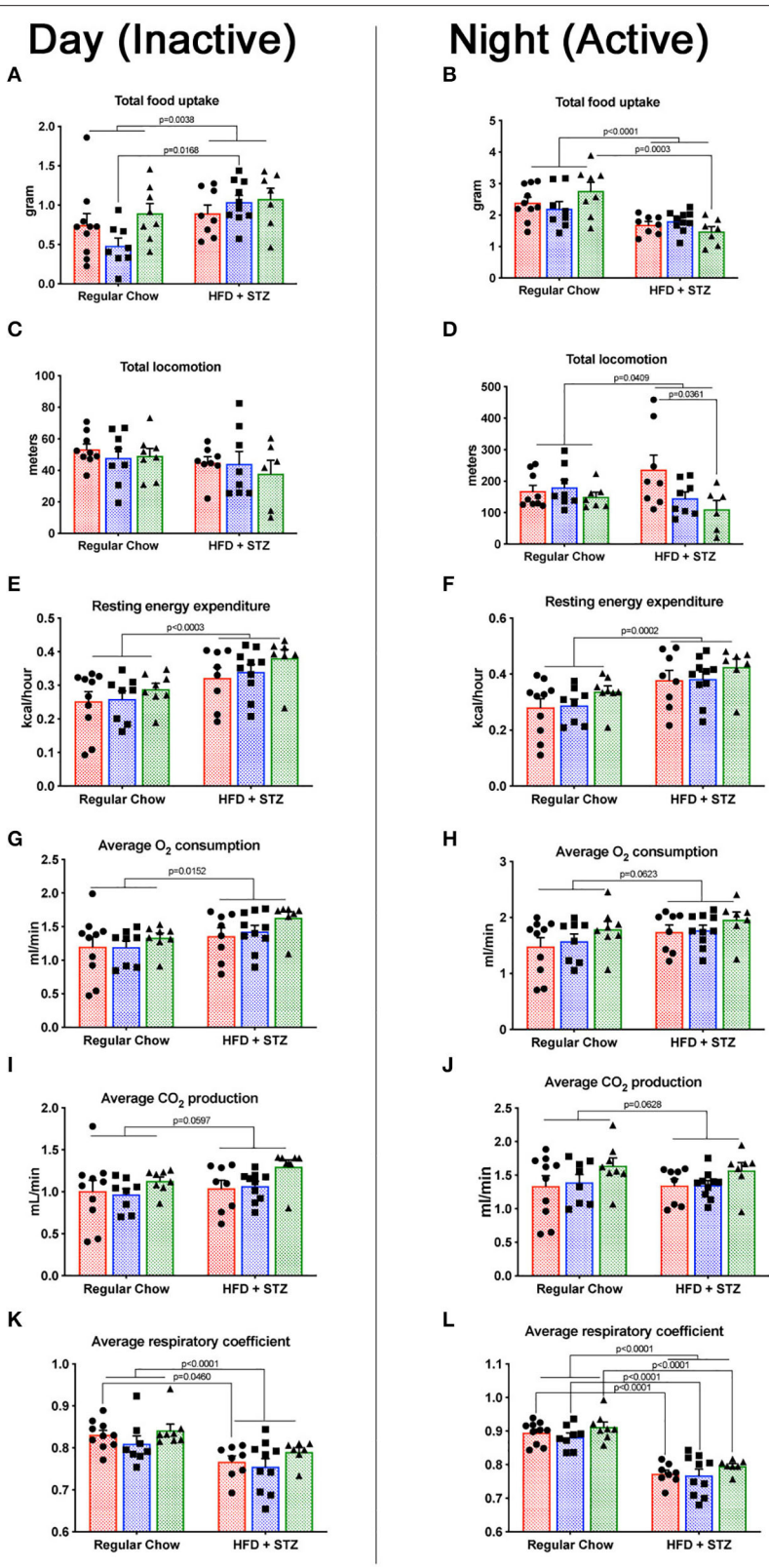


FIGURE 6 | Cav1^{P158Pfsx22} mice have lower waking locomotion but are similar to wild-type mice in energy balance measurements. For all figure parts, red shading/circles indicate wild-type mice, blue shading/squares indicate heterozygous mice, and green shading/triangles indicate homozygous mice. Each symbol is

(Continued)

FIGURE 6 | the measurement from one animal, with the bar showing the mean and SEM. The left half of the figure **(A,C,E,G,I,K)** are measurements made during the day, when mice were inactive; the right half of the figure **(B,D,F,H,J,L)** are measurements made during the night, when mice were active. **(A,B)** Food consumed during the day and the night, respectively. **(C,D)** Total locomotion (this includes climbing as well as horizontal locomotion but is nearly identical to horizontal locomotion alone. **(E,F)** Resting energy expenditure is significantly higher with high-fat diet plus streptozotocin (HFD + STZ) in both day and night. **(G,H)** The oxygen consumption trends were higher. **(I,J)** CO_2 production. **(K,L)** The respiratory coefficient is significantly lower in HFD + STZ, as expected.

were frequently large numbers immediately surrounding large vessels (**Supplemental Figure 4C**).

Heterozygous Cav1^{P158Pfsx22} Mice Do Not Appear to Have a Hemodynamic Susceptibility to LPS but May Have a Susceptibility to Body Weight

The trends in histology shown in **Figures 3**, **4**, combined with the mild suggestion of increased circulating monocytes and increased lung monocyte chemoattractant proteins in **Figure 7**, made us wonder if the Cav1^{P158Pfsx22} mice would be particularly susceptible to an inflammatory insult. We thus performed a small study using low-dose intratracheal installation of LPS, focusing on heterozygous animals, since human patients with this mutation are heterozygous.

We utilized a low dose of LPS with repeated dosing, which we had previously determined in other models to have a little effect on wild-type mice but which brought out a phenotype in susceptible animals (37). It did not, however, appear to have an impact on RVSP in either heterozygous or homozygous mice (**Figure 8A**—compare to **Figure 4A**). Fulton index is a little higher in all LPS-treated animals, but this did not differ by genotype (**Figure 8B**). Heart rate under anesthesia was similar across groups (**Figure 8C**). The only element here in which there was an apparent susceptibility was body weight; we would have anticipated that body weight would increase after 3 weeks on breeder chow, and it generally did for WT mice. However, the weight stayed the same or decreased in LPS-treated heterozygous mice (**Figure 8D**). By ANOVA, genotype was significant at $p = 0.0446$.

DISCUSSION

This study established that the new human CAV1 termination sequence, when knocked in to the native locus in mice, results in the degradation of a large majority of mutant proteins, in both heterozygous and homozygous animals, resulting in the loss of CAV2 and accessory proteins in some, but not all, tissues (**Figure 2**). Heterozygous mutants have no change in their hemodynamic phenotype, and their number of caveolae in the pulmonary vascular endothelium is unchanged (**Figure 3**)—there appears to be a compensatory upregulation of wild-type Cav1. Furthermore, they have no particular hemodynamic susceptibility to either metabolic challenge (**Figure 4**) or inflammatory challenge (**Figure 8**). For most energy balance measurements, the heterozygous mice were more similar to the wild-type mice rather than to the homozygous—they did not share the homozygous increase in lean mass or their poor oral glucose tolerance (**Figure 5**). In other aspects, though, the heterozygous animals did show interesting differences. They appeared to have an intermediate phenotype in blood glucose and plasma insulin between wild-type and homozygous mice (**Figures 5D,E**). This was also the first study to show that the loss of Cav1 results in reduced spontaneous locomotion; the heterozygous mice appeared to have an intermediate phenotype (**Figure 6D**). Finally, the heterozygous Cav1 mutants appeared to have an intermediate phenotype in the increase in circulating monocytes seen in homozygous animals (**Figure 7A**), and they had the same increase in monocyte chemoattractant proteins (**Figure 7E**). While the LPS challenge did not increase their right ventricular systolic pressure, it did appear to make them susceptible to weight loss (**Figure 8D**).

The substantial degradation of the mutant protein in the homozygous mice is consistent with prior findings in cell culture that this mutation causes the protein to accumulate in the endoplasmic reticulum as the result of the introduction of an ER retention signal (17). Notably, the levels of Cav1 protein detected in heterozygous animals varied across tissues; they were only modestly decreased in the lung and the liver, whereas substantially less Cav1 was detected in the liver and the kidney. This suggests that in some tissues the wild-type copy of Cav1 may assist in the translocation of the mutant copy to the plasma membrane, as has been observed in cell culture studies (17, 18). Alternatively, the compensatory upregulation of WT Cav1 may occur in a tissue- or cell type-specific manner.

The metabolic findings in this study are largely supportive of the existing literature on the effects of the loss of Cav1. Both the lower fat mass (32) and the increased lean mass (28) are consistent with prior reports in mice and with the known association with lipodystrophy in human patients (4). The poor insulin sensitivity and the oral glucose tolerance AUCs are also consistent with earlier reports (33, 34). The previous publication showing that Cav1 knockout mice had difficulty with the forced swim test (27) makes sense because of their reduced ability to rapidly internalize glucose; the present study is the first to show that this is true even for a routine activity—in homozygous and, to a lesser extent, in heterozygous animals. The mechanism is presumably the same.

The inflammatory phenotype that we observed, while mild, is particularly interesting in the context of a recent publication in which reciprocal bone marrow transplants between wild-type and Cav1$^{-/-}$ animals suggested that the

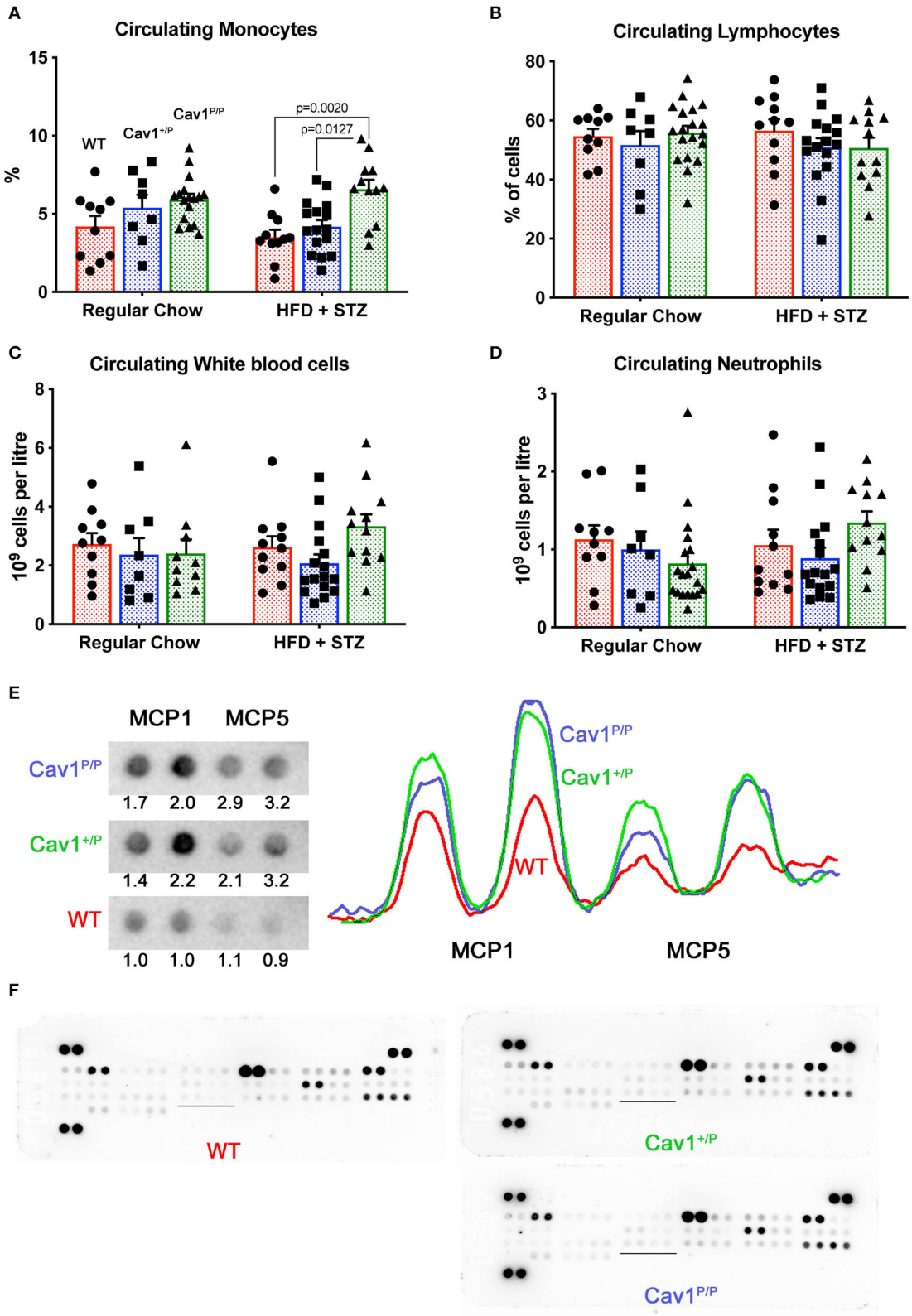


FIGURE 7 | Mice homozygous for the Cav1^{P158Pfsx22} mutation have elevated circulating monocytes and elevated monocyte chemoattractant proteins in the lungs. For figure parts **(A–D)**, red shading/circles indicate wild-type (WT) mice, blue shading/squares indicate heterozygous mice, and green shading/triangles indicate

(Continued)

FIGURE 7 | homozygous mice. Each symbol is the measurement from one animal, with the bar showing the mean and SEM. The statistics are derived by ANOVA, with *post-hoc* tests to compare individual groups. **(A)** The percentage of circulating monocytes is increased in homozygous Cav1 mutant animals, although diet has no significant effect. **(B)** The percentage of circulating lymphocytes is not altered by genotype or diet. **(C)** White blood count is not altered by genotype or diet. **(D)** Circulating neutrophils are not altered by genotype or diet. **(E)** MCP1 and MCP5 protein are increased in both heterozygous and homozygous mutant animals. The tracing is densitometry for each of the blots at the left; the numbers are areas under each curve, normalized to the average WT level of each protein. **(F)** Overall cytokine blots on lung proteins from WT, heterozygous, and homozygous mutants have few differences aside from MCP1 and MCP5 (the underlined section is blown up in **(E)** above).

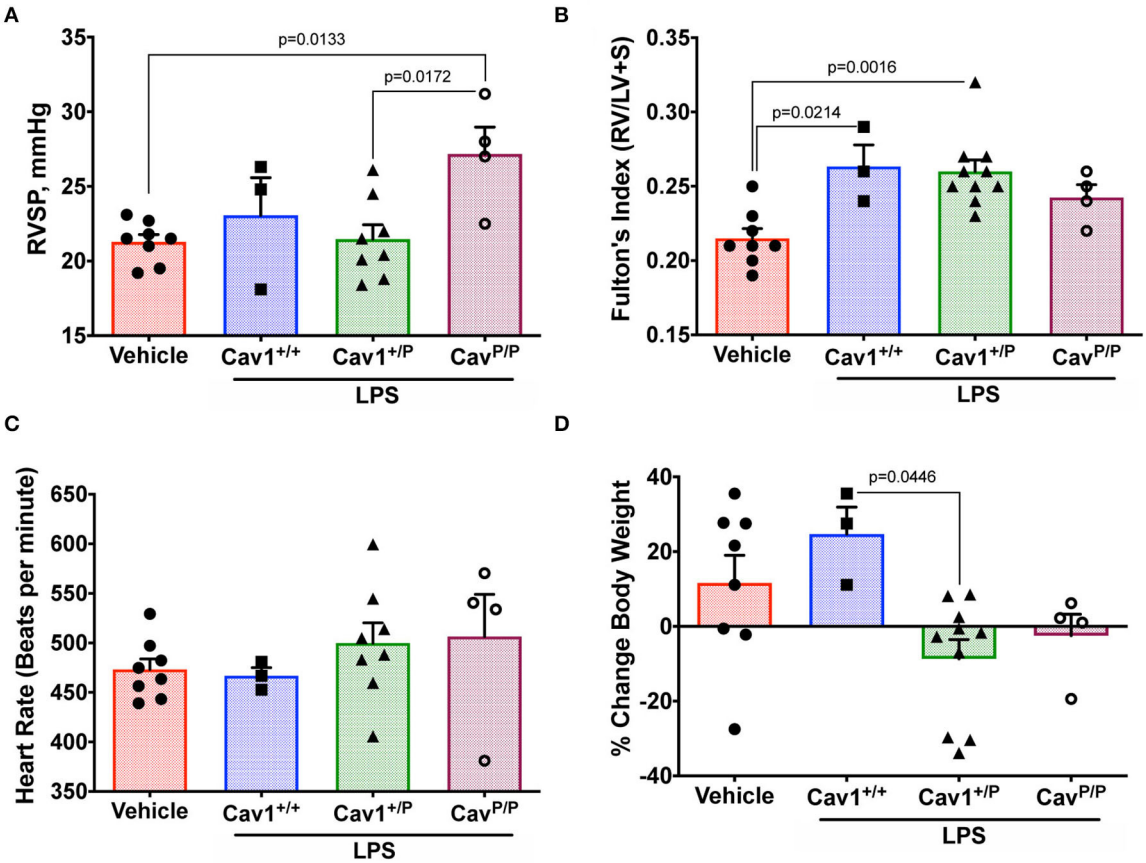


FIGURE 8 | Heterozygous Cav1^{P158Pfsx22} mice do not appear to have a hemodynamic susceptibility to lipopolysaccharide (LPS) but may have a susceptibility to body weight. For all figure parts, blue shading/squares indicate wild-type mice, green shading/triangles indicate heterozygous mice, and maroon shading/open circle indicate homozygous mice in the LPS group. Red shading/circles in vehicle control mice indicate a mixture of genotypes (three wild type, three heterozygous, and two homozygous mice). Each symbol is the measurement from one animal, with the bar showing the mean and SEM. The mice given a chronic low-dose of LPS through installation for 3 weeks had **(A)** no changes in right ventricular systolic pressure (compared to **Figure 4A**). **(B)** No genotype-dependent changes in right heart hypertrophy. **(C)** No differences in heart rate under anesthesia. **(D)** Heterozygous animals may be sensitive to LPS in weight gain, by ANOVA.

pulmonary hypertensive phenotype of Cav1$^{-/-}$ was being driven by circulating cells (38). We are not the first to see an association between macrophages and Cav1. Cav1 knockouts showed increased adventitial macrophages in aortic transplants (39). The overexpression of Cav1 reduced the infiltration of monocytes/macrophages in a mouse model of pulmonary fibrosis (40). Reduced Cav1 levels in monocytes specifically lead to increased chemotaxis activity (36). Furthermore, the loss of Cav1 in psoriatic skin as well as peripheral blood mononuclear cells and CD14^{+} monocytes results in skin inflammation and an increase in monocyte–macrophage lineage activities (36). In scleroderma patients, the decreased levels of Cav1 serve as a risk factor for the differentiation of monocytes to spindle-shaped fibrocytes (41). Potentially, these and other studies suggest that the loss of Cav1 may have a direct impact on monocyte/macrophage recruitment, function, or both.

Even the homozygous mice in the present study had less of a pulmonary hypertensive phenotype than had previously been published for Cav1 knockouts, although the level of pulmonary hypertension in other studies has also been variable, with some studies showing no increased pressure at baseline (42). These

differences are probably just strain differences—the original knockouts were created on a mixed 1295SVJ × Black Swiss background, whereas ours were on a mixed C57/Bl6J × FVB/N background. Differences in background genotype can make a large difference in the pulmonary hypertensive phenotype. The same is true, really, of the patients. Mutations that seem functionally similar in other individuals produce lipodystrophy, not pulmonary hypertension, which makes it seem likely that the reason that we did not see pulmonary hypertension in these mice is that they lacked the underlying polymorphisms associated with disease in the patients, although it could be due to differences between human and mouse anatomy or Cav1 function.

Hypoxia is a standard "second hit" for pulmonary hypertension genetic models, and Cav1 has previously been extensively associated with vasoreactivity, particularly through nitric oxide (10). However, the susceptibility of Cav1 knockout mice to hypoxia has been previously published, with results suggesting no particular pulmonary susceptibility but an increased rate of right ventricular failure (42). Because this model is likely to be molecularly similar to knockouts (**Figure 2**), we did not pursue susceptibility to hypoxia in this model.

Whether the inflammatory or the metabolic abnormalities seen in these mice are actually drivers of the hemodynamic phenotype is currently unknown. However, these defects are causally associated in some other models, and so overall, the present work adds to the body of literature associating insulin resistance and increased inflammation with pulmonary hypertension.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Vanderbilt University Medical Center.

AUTHOR CONTRIBUTIONS

AK, AH, AR, CC, EA, EC, and JW conceived and designed the experiment. AC, AR, CC, CM, SG, and SS performed the experiment. AR, AK, AH, CC, and JW analyzed the data. AR and JW wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH grants R01 HL111259, R01 HL095797, R01HL135011, P01 HL092870, DK059637, 1S10RR028101-01, and VUMC Faculty Research Scholars.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00540/full#supplementary-material

REFERENCES

1. Ariotti N, Parton RG. SnapShot: caveolae, caveolins, and cavins. *Cell.* (2013) 154:704–e701. doi: 10.1016/j.cell.2013.07.009
2. Parton RG, Del Pozo MA. Caveolae as plasma membrane sensors, protectors and organizers. *Nat Rev Mol Cell Biol.* (2013) 14:98–112. doi: 10.1038/nrm3512
3. Parton RG. Caveolae: structure, function, and relationship to disease. *Annu Rev Cell Dev Biol.* (2018) 34:111–36. doi: 10.1146/annurev-cellbio-100617-062737
4. Patni N, Garg A. Congenital generalized lipodystrophies–new insights into metabolic dysfunction. *Nat Rev Endocrinol.* (2015) 11:522–34. doi: 10.1038/nrendo.2015.123
5. Razani B, Lisanti MP. Caveolin-deficient mice: insights into caveolar function human disease. *J Clin Invest.* (2001) 108:1553–61. doi: 10.1172/JCI200114611
6. Lee H, Park DS, Razani B, Russell RG, Pestell RG, Lisanti MP. Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. *Am J Pathol.* (2002) 161:1357–69. doi: 10.1016/S0002-9440(10)64412-4
7. Zhao YY, Liu Y, Stan RV, Fan L, Gu Y, Dalton N, et al. Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc Natl Acad Sci USA.* (2002) 99:11375–80. doi: 10.1073/pnas.172360799
8. Murata T, Lin MI, Huang Y, Yu J, Bauer PM, Giordano FJ, et al. Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice. *J Exp Med.* (2007) 204:2373–82. doi: 10.1084/jem.20062340
9. Maniatis NA, Shinin V, Schraufnagel DE, Okada S, Vogel SM, Malik AB, et al. Increased pulmonary vascular resistance and defective pulmonary artery filling in caveolin-1-/- mice. *Am J Physiol Lung Cell Mol Physiol.* (2008) 294:L865–873. doi: 10.1152/ajplung.00079.2007
10. Zhao YY, Zhao YD, Mirza MK, Huang JH, Potula HH, Vogel SM, et al. Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration. *J Clin Invest.* (2009) 119:2009–18. doi: 10.1172/JCI33338
11. Austin ED, Ma L, Leduc C, Berman Rosenzweig E, Borczuk A, Phillips JA 3rd, Palomero T, et al. Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. *Circ Cardiovasc Genet.* (2012) 5:336–43. doi: 10.1161/CIRCGENETICS.111.961888
12. Garg A, Kircher M, Del Campo M, Amato RS, Agarwal AK, University of Washington Center for Mendelian G. Whole exome sequencing identifies de novo heterozygous CAV1 mutations associated with a novel neonatal onset lipodystrophy syndrome. *Am J Med Genet A.* (2015) 167A:1796–806. doi: 10.1002/ajmg.a.37115
13. Schrauwen I, Szelinger S, Siniard AL, Kurdoglu A, Corneveaux JJ, Malenica I, et al. A frame-shift mutation in CAV1 is associated with a severe neonatal progeroid and lipodystrophy syndrome. *PLoS ONE.* (2015) 10:e0131797. doi: 10.1371/journal.pone.0131797
14. Han B, Copeland CA, Kawano Y, Rosenzweig EB, Austin ED, Shahmirzadi L, et al. Characterization of a caveolin-1 mutation associated with both pulmonary arterial hypertension and congenital generalized lipodystrophy. *Traffic.* (2016) 17:1297–312. doi: 10.1111/tra.12452
15. Zhu N, Pauciulo MW, Welch CL, Lutz KA, Coleman AW, Gonzaga-Jauregui C, et al. Novel risk genes and mechanisms implicated by exome sequencing of 2572 individuals with pulmonary arterial hypertension. *Genome Med.* (2019) 11:69. doi: 10.1186/s13073-019-0685-z

16. Hayer A, Stoeber M, Bissig C, Helenius A. Biogenesis of caveolae: stepwise assembly of large caveolin and cavin complexes. *Traffic.* (2010) 11:361–82. doi: 10.1111/j.1600-0854.2009.01023.x
17. Copeland CA, Han B, Tiwari A, Austin ED, Loyd JE, West JD, et al. A disease-associated frameshift mutation in caveolin-1 disrupts caveolae formation and function through introduction of a *de novo* ER retention signal. *Mol Biol Cell.* (2017) 28:3095–111. doi: 10.1091/mbc.e17-06-0421
18. Marsboom G, Chen Z, Yuan Y, Zhang Y, Tiruppathi C, Loyd JE, et al. Aberrant caveolin-1-mediated Smad signaling and proliferation identified by analysis of adenine 474 deletion mutation (c.474delA) in patient fibroblasts: a new perspective on the mechanism of pulmonary hypertension. *Mol Biol Cell.* (2017) 28:1177–85. doi: 10.1091/mbc.e16-11-0790
19. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW. Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. *J Gene Med.* (2010) 12:561–3. doi: 10.1002/jgm.1473
20. Lighton JRB. *Measuring Metabolic Rates: A Manual For Scientists/John R.B. Lighton.* Oxford: Oxford University Press (2008). doi: 10.1093/acprof:oso/9780195310610.001.0001
21. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol.* (1949) 109:1–9. doi: 10.1113/jphysiol.1949.sp004363
22. Bryant AJ, Robinson LJ, Moore CS, Blackwell TR, Gladson S, Penner NL, et al. Expression of mutant bone morphogenetic protein receptor II worsens pulmonary hypertension secondary to pulmonary fibrosis. *Pulm Circ.* (2015) 5:681–90. doi: 10.1086/683811
23. Rathinasabapathy A, Bryant AJ, Suzuki T, Moore C, Shay S, Gladson S, et al. rhACE2 therapy modifies bleomycin-induced pulmonary hypertension via rescue of vascular remodeling. *Front Physiol.* (2018) 9:271. doi: 10.3389/fphys.2018.00271
24. Rathinasabapathy A, Horowitz A, Horton K, Kumar A, Gladson S, Unger T, et al. The selective angiotensin II type 2 receptor agonist, compound 21, attenuates the progression of lung fibrosis and pulmonary hypertension in an experimental model of bleomycin-induced lung injury. *Front Physiol.* (2018) 9:180. doi: 10.3389/fphys.2018.00180
25. Yates JR 3rd, Eng JK, Mccormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem.* (1995) 67:1426–36. doi: 10.1021/ac00104a020
26. Parton RG, Tillu VA, Collins BM. Caveolae. *Curr Biol.* (2018) 28:R402–5. doi: 10.1016/j.cub.2017.11.075
27. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, et al. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science.* (2001) 293:2449–52. doi: 10.1126/science.1062688
28. Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, et al. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem.* (2001) 276:38121–38. doi: 10.1074/jbc.M105408200
29. Kwon H, Lee J, Jeong K, Jang D, Pak Y. A novel actin cytoskeleton-dependent non-caveolar microdomain composed of homo-oligomeric caveolin-2 for activation of insulin signaling. *Biochim Biophys Acta.* (2013) 1833:2176–89. doi: 10.1016/j.bbamcr.2013.05.003
30. Bosch M, Mari M, Herms A, Fernandez A, Fajardo A, Kassan A, et al. Caveolin-1 deficiency causes cholesterol-dependent mitochondrial dysfunction and apoptotic susceptibility. *Curr Biol.* (2011) 21:681–6. doi: 10.1016/j.cub.2011.03.030
31. Le Lay S, Kurzchalia TV. Getting rid of caveolins: phenotypes of caveolin-deficient animals. *Biochim Biophys Acta.* (2005) 1746:322–33. doi: 10.1016/j.bbamcr.2005.06.001
32. Martin S, Fernandez-Rojo MA, Stanley AC, Bastiani M, Okano S, Nixon SJ, et al. Caveolin-1 deficiency leads to increased susceptibility to cell death and fibrosis in white adipose tissue: characterization of a lipodystrophic model. *PLoS ONE.* (2012) 7:e46242. doi: 10.1371/journal.pone.0046242
33. Cohen AW, Razani B, Wang XB, Combs TP, Williams TM, Scherer PE, et al. Caveolin-1-deficient mice show insulin resistance and defective insulin receptor protein expression in adipose tissue. *Am J Physiol Cell Physiol.* (2003) 285:C222–35. doi: 10.1152/ajpcell.00006.2003
34. Haddad D, Al Madhoun A, Nizam R, Al-Mulla F. Role of caveolin-1 in diabetes and its complications. *Oxid Med Cell Longev.* (2020) 2020:9761539. doi: 10.1155/2020/9761539
35. Lee MK, Moore XL, Fu Y, Al-Sharea A, Dragoljevic D, Fernandez-Rojo MA, et al. High-density lipoprotein inhibits human M1 macrophage polarization through redistribution of caveolin-1. *Br J Pharmacol.* (2016) 173:741–51. doi: 10.1111/bph.13319
36. Takamura N, Yamaguchi Y, Watanabe Y, Asami M, Komitsu N, Aihara M. Downregulated Caveolin-1 expression in circulating monocytes may contribute to the pathogenesis of psoriasis. *Sci Rep.* (2019) 9:125. doi: 10.1038/s41598-018-36767-5
37. Rathinasabapathy A, Austin ED, Tanjore H, Sherrill T, Blackwell T, Muthian G, et al. Loss of KCNK3 in mice drives susceptibility to inflammatory pulmonary arterial hypertension. In: *D106. Pick Your Poison: Genes, Drugs, and PAH.* A7206. Available online at: https://www.atsjournals.org/doi/pdf/10.1164/ajrccm-conference.2019.199.1_MeetingAbstracts.A7206
38. Asosingh K, Wanner N, Weiss K, Queisser K, Gebreab L, Kassa B, et al. Bone marrow transplantation prevents right ventricle disease in the caveolin-1-deficient mouse model of pulmonary hypertension. *Blood Adv.* (2017) 1:526–34. doi: 10.1182/bloodadvances.2016002691
39. Mierke J, Christoph M, Augstein A, Pfluecke C, Jellinghaus S, Woitek F, et al. Influence of caveolin-1 and endothelial nitric oxide synthase on adventitial inflammation in aortic transplants. *Kardiol Pol.* (2020) 78:124–30. doi: 10.33963/KP.15079
40. Lin X, Barravecchia M, Matthew Kottmann R, Sime P, Dean DA. Caveolin-1 gene therapy inhibits inflammasome activation to protect from bleomycin-induced pulmonary fibrosis. *Sci Rep.* (2019) 9:19643. doi: 10.1038/s41598-019-55819-y
41. Tourkina E, Reese C, Perry B, Dyer S, Bonner M, Visconti RP, et al. Caveolin-1 Deficiency May Play a Role in the Predisposition of African Americans to SSc ILD.in: *2012 American College of Rheumatology Annual Meeting.* Washington, DC. (2012).
42. Cruz JA, Bauer EM, Rodriguez AI, Gangopadhyay A, Zeineh NS, Wang Y, et al. Chronic hypoxia induces right heart failure in caveolin-1-/- mice. *Am J Physiol Heart Circ Physiol.* (2012) 302:H2518–27. doi: 10.1152/ajpheart.01140.2011

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 07 October 2020
doi: 10.3389/fmed.2020.570016

Current Understanding of Circulating Biomarkers in Pulmonary Hypertension Due to Left Heart Disease

Noah Todd[1] *and Yen-Chun Lai*[1,2]*

[1] Division of Pulmonary, Critical Care, Sleep and Occupational Medicine, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [2] Department of Anatomy, Cell Biology and Physiology, Indiana University School of Medicine, Indianapolis, IN, United States

Pulmonary hypertension due to left heart disease (PH-LHD; Group 2), especially in the setting of heart failure with preserved ejection fraction (HFpEF), is the most frequent cause of PH. Despite its prevalence, no effective therapies for PH-LHD are available at present. This is largely due to the lack of a concise definition for hemodynamic phenotyping, existence of significant gaps in the understanding of the underlying pathology and the impact of associated comorbidities, as well as the absence of specific biomarkers that can aid in the early diagnosis and management of this challenging syndrome. Currently, B-type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) are guideline-recommended biomarkers for the diagnosis and prognosis of heart failure (HF) and PH. Endothelin-1 (ET-1), vascular endothelial growth factor-D (VEGF-D), and microRNA-206 have also been recently identified as new potential circulating biomarkers for patients with PH-LHD. In this review, we aim to present the current state of knowledge of circulating biomarkers that can be used to guide future research toward diagnosis, refine specific patient phenotype, and develop therapeutic approaches for PH-LHD, with a particular focus on PH-HFpEF. Potential circulating biomarkers identified in pre-clinical models of PH-LHD are also summarized here.

Keywords: pulmonary hypertension, group 2 PH, PH-HFpEF, HFpEF - heart failure with preserved ejection fraction, biomarkers

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez, Stanford University, United States

Reviewed by:
Sandeep Sahay, Weill Cornell Medical College of Cornell University, United States
Roberto J. Bernardo, University of Oklahoma, United States

***Correspondence:**
Yen-Chun Lai
yelai@iu.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *05 June 2020*
Accepted: *31 August 2020*
Published: *07 October 2020*

Citation:
Todd N and Lai Y-C (2020) Current Understanding of Circulating Biomarkers in Pulmonary Hypertension Due to Left Heart Disease. Front. Med. 7:570016. doi: 10.3389/fmed.2020.570016

INTRODUCTION

Pulmonary hypertension due to left heart disease (PH-LHD, Group 2) is the most common cause of PH and is a growing public health problem with high morbidity and mortality (1–3). In fact, the presence of PH in patients with LHD has been associated with up to 5.6 times higher mortality compared to patients without PH (4). Left ventricular (LV) systolic dysfunction and diastolic dysfunction, left-sided valvular disease (aortic and mitral valve disease), and metabolic dysregulation are all known contributing factors that lead to increased LV filling pressure and the subsequent development of PH. Among these, PH attributed to LV diastolic dysfunction, also referred to as PH associated with heart failure with preserved ejection fraction (PH-HFpEF), is the most common form, although the reported prevalence varies from 23 to 83% due to variable definitions and diagnostic methods used to date (5–7). In addition to the already existing ambiguity in definition of this challenging syndrome, the 6th World Symposium on PH (Nice, 2018) has

recently redefined PH-LHD as consisting of a mean pulmonary artery pressure (mPAP) greater than 20 mm Hg (revised from at least 25 mm Hg) and a pulmonary artery wedge pressure (PAWP) greater than 15 mm Hg (**Figure 1**) (8–11). Although this change reflects recent reports describing increased risk of disease progression in patients with a mild elevation in mPAP (21 to 24 mm Hg), this revision may lead to further confusion among clinicians when trying to diagnose PH in patients (9, 12).

Abnormally elevated LV filling pressures in HFpEF, heart failure with reduced ejection fraction (HFrEF, systolic heart failure), and valvular disease can all lead to elevated left atrium (LA) pressure, increased LA volume, and reduced LA compliance (5, 13). Basic cellular mechanisms affecting LV and LA remodeling include myocyte hypertrophy, up-regulated myocardial brain/B-type natriuretic peptide (BNP) expression in the failing ventricular and atrial myocardium in response to pressure increases and/or volume overload, endothelial dysfunction, vascular oxidative stress, inflammation, interstitial fibrosis, metabolic abnormalities, etc. (14–18). Increased LA pressure can then back up into the pulmonary circulation, leading to increased pulmonary venous pressure, which is in turn transferred to pulmonary capillaries, causing damage to the alveolar-capillary barrier (also known as alveolar-capillary stress failure). As the disease progresses, structural and functional changes regulated by chronic elevation in capillary pressure may trigger pulmonary vasoconstriction, reduce nitric oxide (NO) bioavailability, increase endothelin-1 (ET-1) production, and promote remodeling in the pulmonary arteries and veins, with various combinations of intimal proliferation, medial hypertrophy, and adventitial thickening (13, 17, 19–22). These pulmonary vascular abnormalities may then lead to a further increase in mPAP in addition to PAWP elevation, resulting in elevated right ventricular (RV) afterload and ultimately causing right-side heart failure. Depending on the extent of progressive pulmonary vascular abnormalities (also known as the pre-capillary component) to the underlying LHD, the backward transmission of elevated filling pressure can lead to an increase in pulmonary artery pressure proportionally (1:1 ratio) or disproportionately (> 1: 1 ratio) (17, 23–25). Patients with no significant pulmonary vasoconstriction or intrinsic pulmonary vasculopathy often exhibit proportional PH (isolated post-capillary PH, IpcPH). On the other hand, if chronically elevated LV filling pressure triggers pulmonary vasoconstriction and pathological pre-capillary remodeling to the point of having a high transpulmonary gradient (TPG, defined as mPAP–PAWP that exceeds 12 mmHg), elevated pulmonary vascular resistance (PVR, defined as TPG/cardiac output that exceeds 3 Wood units), and/or a high diastolic pulmonary gradient (DPG, defined as diastolic PAP–PAWP that equals or exceeds 7 mm Hg), patients exhibit out-of-proportion PH (combined pre-capillary and post-capillary PH, CpcPH). The severity of pre-capillary involvement can be established by the measured TPG, PVR, and/or DPG during right heart catheterization. In the past, TPG $\leq$ 12 mm Hg, PVR $\leq$ 3 Wood units, and/or DPG < 7 mm Hg suggested IpcPH, and when elevated (TPG > 12 mm Hg, PVR > 3 Wood units, and/or DPG $\geq$ 7 mm Hg), suggested CpcPH (8). These features have been associated with mortality and cardiac hospitalizations in patients with CpcPH associated with HFpEF (CpcPH-HFpEF) (26, 27). However, the new definition from the 6th World Symposium on PH only includes PVR of less than 3 Wood units for IpcPH and PVR of at least 3 Wood units for CpcPH (**Figure 1**) (9–11).

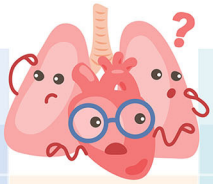
Despite its significant prevalence, along with high morbidity and mortality, there is no Food and Drug Administration (FDA)-approved treatment for PH-LHD at present. While the use of pulmonary vasodilators has been proven to be effective in the treatment of pulmonary arterial hypertension (PAH; Group 1), the use of these agents in patients with PH-LHD has been shown to be ineffective or even harmful (see Fernandez et al. and Vachiéry et al. for recent reviews in detail) (22, 28). This is at least in part due to the lack of a concise and uniform definition for hemodynamic phenotyping, poor understanding of the underlying pathology and the impact of associated comorbidities, as well as the absence of specific biomarkers that can aid in the early diagnosis and management of this heterogeneous disease. Currently, BNP and N-terminal proBNP (NT-proBNP) are guideline-recommended biomarkers for the diagnosis and prognosis of HF and PH (29–31). However, these are not specific for PH-LHD and vary significantly with age, sex, body mass index (BMI), and renal function (30, 31). Several biomarkers have also been shown to be complimentary to the established natriuretic peptides in guiding disease management and have proven to be diagnostically valuable in distinguishing between IpcPH and CpcPH. In this review, we aim to provide an updated overview of the current state of knowledge regarding circulating biomarkers that may be used to guide future research toward diagnosis, refinement of specific patient phenotypes and development of therapeutic approaches for PH-LHD, with a particular focus on PH-HFpEF. We will first focus on the well-established natriuretic peptides, then we will review known biomarkers proposed for PAH and their new roles in PH-LHD. Finally, we will highlight some of the emerging circulating biomarkers, including those identified recently in pre-clinical models of PH-LHD.

WELL-ESTABLISHED BIOMARKERS: BNP AND NT-proBNP

As LV dysfunction progresses, diastolic wall stress is the primary stimulus for myocardial BNP expression, which plays an important role in the regulation of cardiac remodeling, blood pressure, and intravascular volume (14–16). BNP is transcribed and produced primarily in the cardiomyocytes of the ventricles as prohormone proBNP, which is then cleaved into BNP (biologically active with a half-life of 20 min) and its N-terminal fragment NT-proBNP (biologically inactive with a half-life of 70 min). The precursor, BNP, and NT-proBNP are secreted directly into circulation in response to increased myocardial stretch mediated by pressure or volume overload (14–16).

BNP/NT-proBNP in HFpEF and HFrEF

Plasma levels of BNP and NT-proBNP are elevated in patients with HF and increase in proportion to the degree of LV dysfunction and the severity of symptoms of HFpEF, HFrEF, and



Old						New (Based on the 6th WSPH; Nice 2018)				
mPAP	PAWP	PVR	DPG	TPG		mPAP	PAWP	PVR	DPG	TPG
≥25 mm Hg	≤15 mm Hg	—	—	—	Pre-capillary PH	>20 mm Hg	≤15 mm Hg	≥3 WU	—	—
≥25 mm Hg	>15 mm Hg	≤3 WU	<7 mm Hg	≤12 mm Hg	Isolated post-capillary PH (IpcPH)	>20 mm Hg	>15 mm Hg	<3 WU	—	—
≥25 mm Hg	>15 mm Hg	>3 WU	≥7 mm Hg	>12 mm Hg	Combined post-capillary PH (CpcPH)	>20 mm Hg	>15 mm Hg	≥3 WU	—	—

FIGURE 1 | Updated hemodynamic definition and clinical classification of pulmonary hypertension (based on the 6th World Symposium on PH, Nice 2018). mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; TPG, transpulmonary gradient (defined as mPAP–PAWP); PVR, pulmonary vascular resistance (defined as TPG/cardiac output); DPG, diastolic pressure gradient (defined as diastolic PAP–PAWP).

valvular disease (15, 32, 33). In fact, BNP and NT-proBNP are among the first circulating biomarkers included in the current guidelines for the diagnosis and risk stratification of HF (8, 31). While definitive cutoffs of BNP and NT-proBNP are not well-established for HFpEF or HFrEF (*see* Ponikowski et al. and Pieske et al. for the most recent recommended values) (18, 30), and various studies have used different thresholds, it is generally recognized that their levels are, on average, lower in HFpEF compared to HFrEF (30, 33). This may be due to the high prevalence of obesity in HFpEF populations, as disproportionately low BNP and NT-proBNP levels have been reported in obese patients, which may be related to the mechanisms involving natriuretic peptide degradation in adipose tissue, insulin resistance, and enhanced pericardial restraint (32, 34–37). Adipocytes strongly express the natriuretic peptide receptors NPR-A and NPR-C. As BNP binds to both NPR-A and NPR-C, it has been suggested that increased adipose tissue can lead to more efficient clearance of BNP in obese patients. However, lower plasma NT-proBNP levels seen in patients with obesity-related HF cannot be explained by the same mechanism because NT-proBNP does not bind to NPR-A or NPR-C (38, 39). Additionally, Obokata et al. found that patients with obesity-related HFpEF display more concentric LV remodeling, greater epicardial fat thickness, higher PAWP, RV dilation and dysfunction, elevated right atrial (RA) pressure, and increased total heart volume accompanied by lower NT-proBNP levels compared to non-obese HFpEF patients (32). In this study, PAWP was higher for any given NT-proBNP level in patients with obesity-related HFpEF compared to non-obese HFpEF patients; however, the relationship between NT-proBNP and LV transmural pressure, defined as intracavitary pressure (PAWP) minus the external pressure applied to the LV from pericardium and the right side of the heart (RA pressure), did not change in both obese and non-obese patients with HFpEF (32). As increased epicardial fat and higher heart volume exacerbate pericardial restraint, which may reduce wall stress as external pressure applied to the ventricle increases, these data may provide an alternative mechanism for the lower concentration of NT-proBNP observed in patients with obesity-related HFpEF (32, 37). Nevertheless, even mild elevations in NT-proBNP have been found to correlate with an increased risk of HF among individuals with obesity (40).

BNP/NT-proBNP in PH-HFpEF and PH-HFrEF

Using a BNP cutoff value of > 100 pg/ml, an analysis from the Northwestern University HFpEF Program reported that up to 70% of patients with confirmed HFpEF have elevated BNP, which is associated with significantly higher mPAP, RA pressure, and PVR, along with higher rates of HF hospitalization, cardiovascular hospitalization, and death compared to HFpEF patients with "normal" BNP (≤ 100 pg/ml) (41). Elevated BNP levels were also found to be associated with an increase in PA systolic pressure (PASP) in PH patients associated with HFpEF, HFrEF, or valvular disease (42). Significantly higher BNP levels were observed in patients with CpcPH (50% of whom have LV ejection fraction <50%) compared to that of patients with PAH (25). Similarly, CpcPH-HFpEF patients were found to have about 1.8-fold higher levels of NT-proBNP compared to IpcPH-HFpEF patients, which was associated with an increased frequency of HF hospitalizations, reduced RV-vascular coupling (as measured by the ratio between tricuspid valve annular plane systolic

excursion and PASP; TAPSE/PASP), impaired RV function, and severely depressed exercise capacity (43). Hussain et al. reported that NT-proBNP values were overall higher in HFpEF patients with PH, as compared to patients without PH. NT-proBNP levels were also higher in HFpEF patients with RV dysfunction (decreased TAPSE) despite the absence of PH. A moderate negative correlation between NT-proBNP and TAPSE/PASP ratio was found in this study cohort (44). A retrospective analysis performed by Hoeper et al. in 108 patients diagnosed with PH-HFpEF also revealed that each 100 ng/l increase in NT-proBNP is associated with an increased risk of death (45). Guazzi et al. also demonstrated that NT-proBNP values were higher in non-survivors among PH-HFpEF and PH-HFrEF patients, as compared to survivors, although this difference was not deemed significant in the multivariate analysis of survival (46). **Table 1** summarizes recent findings of BNP/NT-proBNP in PH-LHD.

In contrast, Miller et al. reported that NT-proBNP levels were lower in systemic sclerosis patients with early PH due to LHD than that due to PAH (47). Mazurek et al. showed that NT-proBNP levels were similar in both PH-HFpEF and PAH patients (48). The reason for these discrepancies is not immediately clear. While the levels of BNP and NT-proBNP have not yet been compared within all PH-patient populations and cannot yet be used to differentiate between pre-capillary and PH-LHD, the increase in circulating levels of these biomarkers appears to correlate well with the worsening of clinical outcomes, particularly in PH-HFpEF patients. Whether or not the monitoring of either BNP or NT-proBNP would be meaningful and effective in identifying higher risk patients or in guiding specific therapy to improve outcomes in patients with PH-LHD needs to be further investigated.

KNOWN BIOMARKERS ASSOCIATED WITH LHD: ET-1

As mentioned above, chronic elevation in pulmonary capillary pressure may result in reduced pulmonary vascular compliance, structural abnormalities, and vasoconstriction in the pulmonary vasculature. Endothelial dysfunction, which leads to a reduction in NO production and an increase in ET-1 levels, is thought to be a significant contributor to these changes (13, 17, 19, 21, 22). ET-1 is the most potent endogenous vasoconstrictor known at present and has been frequently reported to be elevated in patients with HFpEF, HFrEF, and left-sided valvular disease (49–53). It is derived from prepro-ET-1, which is first proteolytically cleaved to yield a 39-amino acid intermediate Big ET-1, followed by a subsequent production of the 21-amino acid vasoactive peptide by endothelin converting enzymes (ECEs) (**Figure 2**) (54). The production and release of both Big ET-1 and ET-1 are promoted in response to increased myocardial stress, shear stress, low levels of estrogen, hyperglycemic conditions, oxidized LDL cholesterol, elevated proinflammatory cytokines, and other conditions that are commonly involved in the progression of LHD, though Big ET-1 has at least two orders of magnitude less vasoconstrictor potency than the mature ET-1 (55, 56). ET-1 acts at two different G protein-coupled receptors, ET_A and ET_B. ET_A receptors are located predominantly in vascular smooth muscle cells and myocytes, and are known for their potent and long-lasting vasoconstrictive and proliferative responses to ET-1. ET_B activation, on the other hand, exerts vasoconstriction in smooth muscle cells but induces transient vasodilation in endothelial cells by releasing NO and prostacyclin. ET-1 levels were found to be increased in blood of HF patients (51, 52). Increased plasma levels of Big ET-1 were reported in PAH patients as well (57). Recently, Meoli et al. reported that ET-1 is higher in plasma samples collected from the wedge position of CpcPH-HFpEF patients compared to HFpEF patients with IpcPH or without PH (58). Although the sample size was small, increased wedge plasma ET-1 concentration in this study was reported to correlate strongly with PVR in patients with CpcPH-HFpEF. A slightly larger prospective cohort study performed by Chowdhury et al. showed that regardless of being associated with CpcPH or IpcPH, the wedge plasma concentration of ET-1 in PH-HFpEF patients is higher and is associated with PH, PVR, and 1-year heart failure hospitalization compared to HFpEF patients without PH (59). Similarly, Obokata et al. reported that patients with confirmed HFpEF display elevated levels of C-terminal pro-ET-1 (CT-proET-1), a stable circulating precursor of ET-1, the magnitude of which is associated with higher pulmonary artery pressure and worse pulmonary artery compliance at rest and during exercise (52). Increased plasma CT-proET-1 levels in this study also showed high diagnostic accuracy in identifying patients with abnormal pulmonary vascular reserve during exercise. Although ET-1 may play a role in the pathophysiology of PH-HFpEF, the results of using FDA-approved endothelin receptor antagonists for the treatment of PAH have been rather disappointing in the treatment of patients with PH-HFpEF. The pilot study of bosentan, a dual ET_A and ET_B receptor antagonist, showed no signs of benefit in PH-HFpEF patients (60). Moreover, this treatment may even be detrimental in the combined populations of CpcPH- and IpcPH-HFpEF patients. Bosentan demonstrated similar harmful effects during a clinical trial for the treatment of PH-HFrEF patients (61). The MELODY-1 trial, which randomized patients with CpcPH (> 75% normal ejection fraction), to a 12-week treatment with macitentan, a dual ET_A and ET_B receptor antagonist, or a placebo showed an increased incidence of side effects in the macitentan group (mainly fluid retention), without any major improvements in any of the exploratory endpoints (62). Currently, the SERENADE trial, a phase IIb clinical trial, is recruiting patients with HFpEF associated with pulmonary vascular disease or RV dysfunction to evaluate the long-term (24–52 weeks) treatment effect of macitentan (NCT03153111).

EMERGING BIOMARKERS: VEGF-D/FIGF

Beyond vasoconstrictive and proliferative responses mediated by ET-1, increased secretion of inflammatory cytokines and growth factors (e.g., transforming growth factor alpha 1, TGF-α1; vascular endothelial growth factor, VEGF; and interleukin 1, IL1) have also been associated with structural and functional changes in response to the retrograde increase in pulmonary

TABLE 1 | Collected studies of BNP/NT-proBNP in PH-LHD.

Authors (ref #)	Biomarker	Patients (*n*)	Controls (*n*)	Primary findings	Correlation of BNP/NT-proBNP with hemodynamics
Anjan et al. (41)	BNP	HFpEF (113), w/ BNP > 100 pg/ml, prospectively enrolled	HFpEF (46), w/ BNP ≤ 100 pg/ml	Normal BNP associated w/ lower age, female gender, obesity; Elevated BNP predicts CV hospitalization/death	RV wall thickness, RV size, LA/RA size, mPAP, RAP, PVR↗ Diastolic function, RV systolic function↘
Jin et al. (42)	BNP	LHD (47), PH-LHD (35), prospectively enrolled	Healthy volunteers (36)	BNP elevated in PH-LHD patients compared to LHD patients	PASP ↗
Assad et al. (25)	BNP	CpcPH (364), IpcPH (1456), retrospective analysis	PAH (564)	BNP elevated in CpcPH and IpcPH patients compared to PAH patients	N/A
Gorter et al. (43)	NT-proBNP	IpcPH-HFpEF (46), CpcPH-HFpEF (30), prospectively enrolled	Non-PH HFpEF (21)	NT-proBNP elevated in PH-HFpEF patients compared to non-PH HFpEF patients. CpcPH-HFpEF patients have elevated NT-proBNP compared to IpcPH-HFpEF patients.	TAPSE/PASP ↘
Hussain et al. (44)	NT-proBNP	PH-HFpEF (76), retrospective analysis	Non-PH HFpEF (61)	NT-proBNP elevated in PH-HFpEF patients compared to HFpEF patients	PASP ↗ TAPSE ↘
Hoeper et al. (45)	NT-proBNP	PH-HFpEF w/ DLCO < 45% predicted value (52), retrospective analysis	PH-HFpEF w/ DLCO ≥ 45% predicted value (56)	Elevated NT-proBNP associated with increased risk of death in univariate model	N/A
Guazzi et al. (46)	NT-proBNP	Survivors of PH-HFpEF and PH-HFrEF (246), prospectively enrolled	Nonsurvivors of PH-HFpEF and PH-HFrEF (47)	Elevated NT-proBNP not associated with increased risk of death in multivariate model	N/A
Miller et al. (47)	NT-proBNP	SSc PH-LHD (15), SSc PAH (9), retrospective analysis w/ prospective followup	SSc (19)	NT-proBNP significantly higher at diagnosis of SSc PAH compared to SSc PH-LHD	N/A
Mazurek et al. (48)	NT-proBNP	PH-HFpEF (39), prospectively enrolled	PAH (37)	No significant difference in NT-proBNP between PH-HFpEF and PAH	N/A

BNP, B-type natriuretic peptide; CpcPH, combined pre- and post-capillary pulmonary hypertension; CV, cardiovascular; DLCO, diffusion capacity of the lung for carbon monoxide; HFpEF, Heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; IpcPH, isolated post-capillary pulmonary hypertension; LA, left atrium; LAD, left atrial diameter; LHD, left heart disease; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction; mPAP, mean pulmonary arterial pressure; NT-proBNP, N-terminal fragment of pro-B-type natriuretic peptide; PAH, pulmonary arterial hypertension; PASP, pulmonary arterial systolic pressure; PH, pulmonary hypertension; PH-LHD, pulmonary hypertension associated with left heart disease; PVR, pulmonary vascular resistance; RA, right atrium; RAP, right atrial pressure; RV, right ventricle; RVSP, right ventricular systolic pressure; SSc, systemic sclerosis; TAPSE, tricuspid annular plane systolic excursion. ↗ indicates positive correlation with the corresponding biomarker; ↘ indicates an inverse correlation with the corresponding biomarker.

capillary pressure (22). Among them, the role of vascular endothelial growth factor-D (VEGF-D) has been recently evaluated in PH-LHD. VEGF-D is a secreted factor that regulates angiogenesis, lymphangiogenesis and vascular permeability (63, 64). It is synthesized and secreted as a large precursor, which is subsequently proteolytically processed at both N- and C-termini to yield the mature forms. Unprocessed VEGF-D is selective for vascular endothelial growth factor receptor 3 (VEGFR-3), which is mainly expressed in lymphatic endothelial cells, whereas the mature VEGF-D activates both VEGFR-3 and VEGFR-2, the latter of which is found in both vascular and lymphatic endothelial cells (64). Besides modulating the growth of blood and lymphatic vessels, VEGF-D has been shown to induce cardiac fibrogenesis by stimulating myofibroblast growth, migration, and type I collagen synthesis (65). VEGF-D has also been shown to be up-regulated by mechanistic (formerly mammalian) target of rapamycin (mTOR), a master regulator of cell growth, proliferation, and survival that has been implicated in PAH, lymphangioleiomyomatosis (LAM), and cancer (66). While adenoviral delivery of a gene encoding mature VEGF-D has been

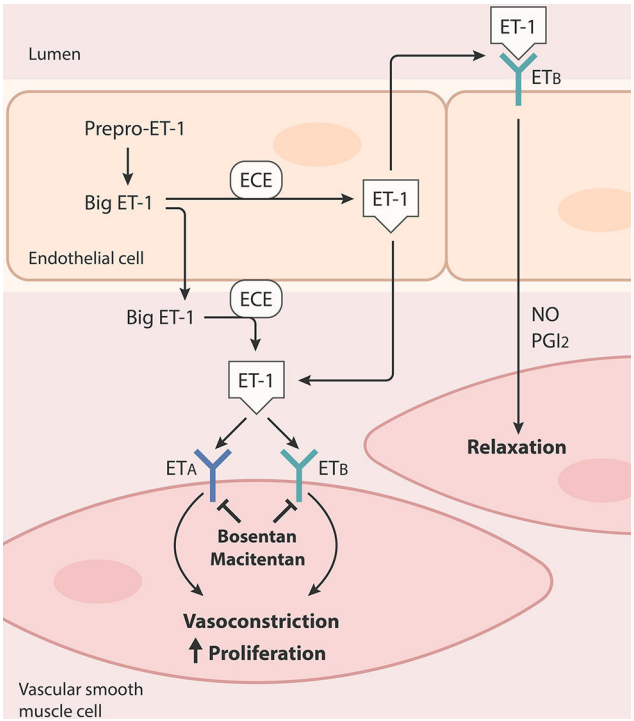


FIGURE 2 | Production and release of endothelin-1 (ET-1) and ET receptors-mediated actions in vascular smooth muscle cells and endothelial cells. ET-1 is derived from prepro-ET-1, which is first proteolytically cleaved to yield a 39-amino acid intermediate Big ET-1, followed by a subsequent production of the 21-amino acid vasoactive peptide by endothelin converting enzymes (ECEs). ET-1 can active endothelin receptors type A (ET_A) and type B (ET_B). ET_A is located predominantly in vascular smooth muscle cells, while ET_B resides in vascular smooth muscle cells and endothelial cells. Activation of ET_A or ET_B in vascular smooth muscle cells results in vasoconstriction and proliferation. Activation of ET_B induces transient vasodilation in endothelial cells by releasing nitric oxide (NO) and prostacyclin (PGI_2).

shown to improve myocardial perfusion in pigs and has now advanced through phase I/IIa studies in patients with refractory angina (67, 68), elevated circulating levels of VEGF-D have been reported in patients with HF, atrial fibrillation, ischemic stroke, PAH, chronic thromboembolic pulmonary hypertension (CTEPH) and LAM (69–73). Circulating VEGF-D levels were found to be higher in HF patients with pulmonary congestion (70). Elevated VEGF-D levels were also found to be a predictor of all-cause mortality in patients with coronary artery disease (CAD) and enabled differentiation of subjects with HF from patients with acute dyspnea (70, 74). Recently, Säleby et al. reported that VEGF-D levels were elevated in patients with PAH, CTEPH, PH-HFpEF, and PH-HFrEF compared to control subjects and HF patients without PH, with its levels in PH-HFrEF patients being significantly higher compared to all other etiologies of PH (72). Of note, plasma levels of VEGF-A were higher in patients with PAH, CTEPH, and PH-LHD compared to controls, although some controversy exists regarding the presence or absence of significant differences in VEGF-A between PH etiologies (71, 72). However, data are more consistent for VEGF-D, showing increased levels in PH-LHD compared to PAH and CTEPH patients. Furthermore, levels of soluble fms-like tyrosine kinase 1 (sFlt-1, also known as soluble VEGFR-1 or sVEGFR-1) were significantly elevated in PH-LHD patients compared to controls, PAH, and no-PH LHD patients (71). These results are in agreement with a study by Houston et al., in which the authors showed that higher VEGF-D levels correlate with increased PAWP, reduced cardiac output, and higher BNP levels in patients with heart failure (75). A lower ejection fraction (30 ± 18 vs. 49 ± 22%) was also found in subjects with higher VEGF-D levels and elevated PAWP compared to those with lower VEGF-D levels in this study cohort. While there is no data available regarding the effect of therapies targeting VEGF-D in HF, PH, and PH-LHD patients, early treatment with VEGFR3 inhibitors has been shown to improve the lumen obliteration and pulmonary pressures in Sugen/hypoxia rat models of PAH (76). Collectively, the available evidence suggests that VEGF-D could be a potential biomarker for distinguishing PH-HFrEF from other etiologies of PH, but this needs to be further evaluated in large multi-center studies.

POTENTIAL BIOMARKERS: microRNA-206

microRNAs (miRNAs) have been the subject of much excitement in molecular biology in the last few years. Composed of single-stranded non-coding RNAs that self-pair into a stem-and-loop structure, miRNAs act as post-transcriptional modifiers of their messenger RNA (mRNA), inducing their degradation and/or translational repression. While some controversy still exists as to their exact mechanism in identifying gene targets and roles in regulating diseases, miRNAs have emerged as promising biomarkers due to their relatively high stability (77). They can be detected extracellularly and have been implicated in a wide range of diseases. Several miRNA microarray profiling and quantitative PCR array studies have reported candidate miRNAs with potential utility as biomarkers in HF and PAH (*see* Fernandez et al., Wong et al., and Boucherat et al. for recent reviews in detail) (22, 78, 79); however, only a few miRNAs have been studied in PH-LHD. Among them, circulating levels of muscle-specific miR-204 were reported to be uniquely elevated across the pulmonary vasculature in patients with PAH, but not in patients with PH-LHD (80). On the other hand, muscle-specific miR-206 levels were found to be reduced in serum of patients with PH-HFpEF, PH-HFrEF, and valvular disease-associated PH (42). Notably, decreased miR-206 levels in this study cohort correlated with an increase in PASP, BNP, and left atrial longitudinal diameter (LAD). In addition, the authors found that the predictive value of miR-206 in detecting PH in LHD was greatly improved when combined with BNP and LAD. The exact role of miR-206 in PH-LHD is currently unknown; however, reduction of miR-206 has been shown to stabilize hypoxia-inducible factor-1α (HIF-1α) and to induce proliferation in cultured PASMCs (81, 82). In contrast, miR-206 levels were found to be elevated in lungs of rats with monocrotaline (MCT)-induced PAH, and cardiac-specific overexpression of miR-206 also led to cardiac hypertrophy (83, 84). While the reduction of

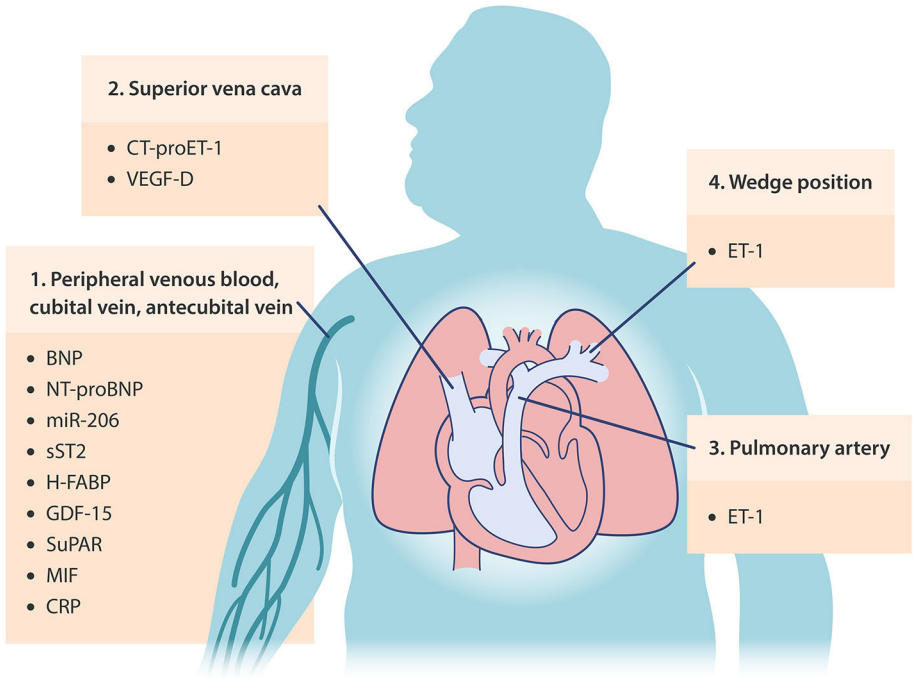


FIGURE 3 | Occurrence locations of potential circulating biomarkers in PH-LHD. BNP, brain/B-type natriuretic peptide; NT-proBNP, N-terminal proBNP; miR-206, microRNA-206; sST2, soluble suppression of tumorigenicity 2; H-FABP, heart type fatty acid binding protein; GDF-15, growth differentiation factor 15; suPAR, soluble urokinase plasminogen activator receptor; MIF, macrophage migration inhibitory factor; CRP, C-reactive protein; ET-1, endothelin-1; CT-proET-1, C-terminal pro-ET-1; VEGF-D, vascular endothelial growth factor-D.

circulating miR-206 levels may be compensated for by an increase in the intracellular miR-206 concentration, further research is needed to determine whether circulating miR-206 can be useful in the early identification of PH in LHD patients.

FUTURE CANDIDATE BIOMARKERS

In addition to the emerging biomarkers discussed above, several biomarkers of distinct processes in cardiopulmonary regulation may aid in the characterization, differentiation, and/or determination of disease management of PH-LHD (**Figure 3** and **Table 2**). Soluble suppression of tumorigenicity 2 (sST2), also known as interleukin-1 receptor-like 1 (IL1RL1), is one such example. sST2 is a marker of mechanical stress and ventricular remodeling, which identifies patients with HFrEF and has been shown recently to be elevated in plasma of patients with Group 2 PH, compared to subjects admitted for elective coronary angiography (in whom CAD was excluded), in a single-center retrospective study performed by Mirna et al. (85). While cautious interpretation of data is needed and detailed information regarding baseline characteristics, especially for Group 2 patients, is lacking, Mirna et al. also showed that Group 2 PH patients had higher circulating levels of growth differentiation factor 15 (GDF-15; a marker of cell injury and inflammation), heart type fatty acid binding protein (H-FABP; a marker of ongoing myocardial damage), and soluble urokinase plasminogen activator receptor (suPAR; a marker of ongoing inflammation) (85). In addition, higher plasma levels of macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, were found to be associated with greater PASP and natriuretic peptide levels in HFpEF patients (86). Mazurek et al. also showed a trend toward higher levels of galectin-3, a beta-galactoside-binding protein which has been implicated in inflammation and fibrosis, in serum collected from pulmonary artery of patients with PH-HFpEF relative to those with PAH (48). While this may be associated with greater systemic fibrosis and inflammation seen in HFpEF populations, no correlations between galectin-3 levels and any measured hemodynamic endpoints were found in PH-HFpEF or PAH patients in this study (48). The common biomarker of inflammation and cardiovascular distress, C-reactive protein (CRP), has also gained some interest as a potential biomarker of PH-LHD. Using a CRP cutoff value of > 3 mg/dl, the RELAX trial reported that approximately 60% enrolled HFpEF patients have elevated CRP (87). Higher CRP levels were associated with younger age, higher BMI, COPD, atrial fibrillation, RV dysfunction, reduced exercise tolerance, higher circulating levels of ET-1, aldosterone, and NT-pro BNP. While this led the authors to conclude that high CRP levels may identify a unique HFpEF phenotype that is associated with comorbidity-driven systemic inflammation, CRP levels were not associated with PH or LV function in this study cohort (87). Lastly, platelets from patients with PH-HFpEF have also been shown to exhibit a distinct metabolic phenotype compared to patients with PAH (88).

TABLE 2 | Collected studies of future candidate biomarkers in PH-LHD.

Authors (ref #)	Biomarkers	Patients (*n*)	Controls (n)	Primary findings	Correlation with hemodynamics
Mirna et al. (85)	sST2, GDF-15, H-FABP, suPAR, BNP	Group 1 PH (13), Group 2 PH (35), Group 3 PH (7), Group 4 PH (12), Group 5 PH (19), retrospective analysis	Elective coronary angiography w/o CAD (74)	BNP, GDF-15, H-FABP, and suPAR are elevated in Group 2 PH patients and correlate with some hemodynamic measurements	H-FABP w/ RVEDD, RAA, TR: ↗ sST2 w/ RVEDD, RAA, TR, PASP: ↗ sST2 w/ TAPSE: ↘ GDF-15 w/ RVA, RAA: ↗ suPAR w/ RVEDD, RAA, TR: ↗ suPAR w/ TAPSE: ↘
Luedike et al. (86)	MIF	HFpEF w/ MIF > 51.58 ng/ml (31), prospectively enrolled	HFpEF w/ MIF ≤ 51.58 ng/ml (31)	High MIF associated w/ 180-day mortality/hospitalization and more severe HFpEF symptoms	MIF w/ PASP: ↗
Mazurek et al. (48)	Galectin-3	PH-HFpEF (39), prospectively enrolled	PAH (37)	Galectin-3 positively correlated with mortality	N/A
DuBrock et al. (87)	hs-CRP	HFpEF w/ CRP > 3 mg/l (121), retrospective analysis	HFpEF w/ CRP ≤ 3 mg/l (93)	High CRP associated with increased comorbidity burden and presence of RV dysfunction	N/A
Nguyen et al. (88)	Platelet bioenergetics	PH-HFpEF (20), retrospective analysis	Healthy volunteers (20)	PH-HFpEF patient platelets display increased maximal OCR and reserve respiratory capacity	Reserve respiratory capacity w/ RV SWI: ↘

CAD, coronary artery disease; GDF-15, growth differentiation factor 15; H-FABP, heart-type fatty acid binding protein; hs-CRP, high-sensitivity C-reactive protein; MIF, macrophage inhibitory factor; OCR, platelet oxygen consumption rate; RAA, right atrial area; RVEDD, right ventricular end diastolic diameter; sST2, soluble suppression of tumorigenicity 2; suPAR, soluble urokinase plasminogen activator receptor; SWI, stroke work index; TR, severity of tricuspid regurgitation. ↗ indicates positive correlation with the corresponding biomarker; ↘ indicates an inverse correlation with the corresponding biomarker.

Unlike platelets from PAH patients, only an increase in maximal respiratory capacity, but not in glycolytic rate, was observed in platelets from PH-HFpEF patients relative to healthy controls. The enhancement of platelet maximal respiratory capacity was found to be associated with RV dysfunction, but not with mPAP or PVR in patients with PH-HFpEF (88). Whether or not a measurement of these factors can provide a method for distinguishing PH-LHD patient phenotypes, identifying at-risk subjects, and predicting PH-LHD patient outcomes requires additional research.

Apart from clinical studies, recent advances in pre-clinical models have provided new insights in the development of future biomarkers. Plasma levels of leptin, an adipokine known to induce secretion of proinflammatory cytokines and reactive oxygen species (ROS) generation, were found to be associated with PH severity in rats with PH-HFpEF induced by high-fat diet (HFD) in combination with supra-coronary aortic banding (SAB) and the antipsychotic olanzapine (89). Decreased plasma levels of the cardioprotective adipokine adiponectin after Sugen-mediated induction of PH-HFpEF were also observed in leptin receptor-deficient obese ZSF1 rats (90). Interestingly, metformin (the first-line drug for type 2 diabetes) was found to improve metabolic syndrome and pulmonary pressures in these models through modulation of leptin, adiponectin, as well as other mechanisms involving, at least in part, suppression of interleukin-6-associated inflammation and activation of sirtuin-3-mediated skeletal muscle glucose uptake (89, 90). Moreover, metformin has recently been shown to prevent RV dysfunction via improved insulin resistance and reduced RV lipid accumulation in HFD-treated mice (91). These findings have led to an ongoing trial designed to evaluate the effect of metformin in patients with PH-HFpEF (NCT03629340). Nitrite, a dietary precursor of NO, has displayed similar qualities in a rat model of PH-HFpEF where it functions to increase adiponectin levels and improve skeletal muscle insulin resistance (90). A recent study by Simon et al. reported that inhalation of nitrite reduced pulmonary, RA, and pulmonary capillary wedge pressures in patients with PH-HFpEF (92). A clinical trial is now underway to examine the effect of oral nitrite in PH-HFpEF patients (NCT03015402). Moreover, therapies targeting ET-1 and NO with a Rho-kinase (ROCK) inhibitor, fasudil, have also been shown to be effective in rats with end-stage PH-LHD induced by SAB and in patients with PH-HFpEF, with more favorable results in the CpcPH-HFpEF population (93, 94).

SUMMARY AND FUTURE PERSPECTIVES

While many challenges still exist, researchers in the field are encouraged to further expand the growing array of circulating

biomarkers to guide future research toward facilitating screening, diagnosis, refinement of specific patient phenotypes, and development of therapeutic approaches. Consensus to concise and standardized definition for hemodynamic phenotyping is required, along with longitudinal data consisting of multiple time points and evaluation in large, multi-center studies to validate the viability of the aforementioned potential biomarkers and to explore future candidates. The ongoing PVDOMICS study supported by the National Institutes of Health/National Heart, Lung, and Blood Institute (NIH/NHLBI) may also reveal new relevant information (95). Since HFpEF and PH are recognized as multiorgan and systemic disorders (96, 97), the quest toward identification of meaningful extra-cardiopulmonary biomarkers may aid in providing important new insights into this clinically challenging syndrome and driving PH-LHD research forward.

AUTHOR CONTRIBUTIONS

NT and Y-CL wrote the article.

FUNDING

The writing of this review is supported by National Heart, Lung, and Blood Institute (R01HL142638) and American Heart Association (17SDG33400233).

ACKNOWLEDGMENTS

We thank Dr. Sergei Snovida for helpful comments on the manuscript and Elfy Chiang for assistance and production of the figures.

REFERENCES

1. Hansdottir S, Groskreutz DJ, Gehlbach BK. WHO's in second?: a practical review of World Health Organization group 2 pulmonary hypertension. *Chest.* (2013) 144:638–50. doi: 10.1378/chest.12-2114
2. Strange G, Playford D, Stewart S, Deague JA, Nelson H, Kent A, et al. Pulmonary hypertension: prevalence and mortality in the Armadale echocardiography cohort. *Heart.* (2012) 98:1805–11. doi: 10.1136/heartjnl-2012-301992
3. Wijeratne DT, Lajkosz K, Brogly SB, Lougheed MD, Jiang L, Housin A, et al. Increasing incidence and prevalence of world health organization groups 1 to 4 pulmonary hypertension. *Circ Cardiovasc Qual Outcomes.* (2018) 11:e003973. doi: 10.1161/CIRCOUTCOMES.117.003973
4. Chatterjee NA, Lewis GD. What is the prognostic significance of pulmonary hypertension in heart failure? *Circ Heart Fail.* (2011) 4:541–5. doi: 10.1161/CIRCHEARTFAILURE.111.963785
5. Vachiery JL, Adir Y, Barbera JA, Champion H, Coghlan JG, Cottin V, et al. Pulmonary hypertension due to left heart diseases. *J Am Coll Cardiol.* (2013) 62(Suppl. 25):D100–8. doi: 10.1016/j.jacc.2013.10.033
6. Guha A, Amione-Guerra J, Park MH. Epidemiology of pulmonary hypertension in left heart disease. *Prog Cardiovasc Dis.* (2016) 59:3–10. doi: 10.1016/j.pcad.2016.07.001
7. Levine AR, Simon MA, Gladwin MT. Pulmonary vascular disease in the setting of heart failure with preserved ejection fraction. *Trends Cardiovasc Med.* (2019) 29:207–17. doi: 10.1016/j.tcm.2018.08.005
8. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J.* (2016) 37:67–119. doi: 10.1093/eurheartj/ehv317
9. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J.* (2019) 53:1801913. doi: 10.1183/13993003.01913-2018
10. Condon DF, Nickel NP, Anderson R, Mirza S, de Jesus Perez VA. The 6th World Symposium on Pulmonary Hypertension: what's old is new. *F1000Res.* (2019) 8:888. doi: 10.12688/f1000research.18811.1
11. Galie N, McLaughlin VV, Rubin LJ, Simonneau G. An overview of the 6th World symposium on pulmonary hypertension. *Eur Respir J.* (2019) 53:1802148. doi: 10.1183/13993003.02148-2018
12. Simon MA, Maron BA. Pulmonary hypertension in patients with heart failure with preserved ejection fraction. where to draw the line. *Am J Respir Crit Care Med.* (2019) 200:278–9. doi: 10.1164/rccm.201903-0689ED
13. Al-Omary MS, Sugito S, Boyle AJ, Sverdlov AL, Collins NJ. Pulmonary hypertension due to left heart disease: diagnosis, pathophysiology, and therapy. *Hypertension.* (2020) 75:1397–408. doi: 10.1161/HYPERTENSIONAHA.119.14330
14. Casaclang-Verzosa G, Gersh BJ, Tsang TS. Structural and functional remodeling of the left atrium: clinical and therapeutic implications for atrial fibrillation. *J Am Coll Cardiol.* (2008) 51:1–11. doi: 10.1016/j.jacc.2007.09.026
15. Bergler-Klein J, Gyongyosi M, Maurer G. The role of biomarkers in valvular heart disease: focus on natriuretic peptides. *Can J Cardiol.* (2014) 30:1027–34. doi: 10.1016/j.cjca.2014.07.014
16. Volpe M, Rubattu S, Burnett J Jr. Natriuretic peptides in cardiovascular diseases: current use and perspectives. *Eur Heart J.* (2014) 35:419–25. doi: 10.1093/eurheartj/eht466
17. Guazzi M, Naeije R. Pulmonary hypertension in heart failure: pathophysiology, pathobiology, and emerging clinical perspectives. *J Am Coll Cardiol.* (2017) 69:1718–34. doi: 10.1016/j.jacc.2017.01.051
18. Pieske B, Tschope C, de Boer RA, Fraser AG, Anker SD, Donal E, et al. How to diagnose heart failure with preserved ejection fraction: the HFA-PEFF diagnostic algorithm: a consensus recommendation from the Heart Failure Association (HFA) of the European Society of Cardiology (ESC). *Eur Heart J.* (2019) 40:3297–317. doi: 10.1093/eurheartj/ehz641
19. Borlaug BA. The pathophysiology of heart failure with preserved ejection fraction. *Nat Rev Cardiol.* (2014) 11:507–15. doi: 10.1038/nrcardio.2014.83
20. Rossi A, Gheorghiade M, Triposkiadis F, Solomon SD, Pieske B, Butler J. Left atrium in heart failure with preserved ejection fraction: structure, function, and significance. *Circ Heart Fail.* (2014) 7:1042–9. doi: 10.1161/CIRCHEARTFAILURE.114.001276
21. Dayeh NR, Ledoux J, Dupuis J. Lung capillary stress failure and arteriolar remodelling in pulmonary hypertension associated with left heart disease (Group 2 PH). *Prog Cardiovasc Dis.* (2016) 59:11–21. doi: 10.1016/j.pcad.2016.05.002
22. Fernandez AI, Yotti R, Gonzalez-Mansilla A, Mombiela T, Gutierrez-Ibanes E, Perez Del Villar C, et al. The biological bases of group 2 pulmonary hypertension. *Int J Mol Sci.* (2019) 20:5884. doi: 10.3390/ijms20235884
23. Naeije R, Vachiery JL, Yerly P, Vanderpool R. The transpulmonary pressure gradient for the diagnosis of pulmonary vascular disease. *Eur Respir J.* (2013) 41:217–23. doi: 10.1183/09031936.00074312
24. Assad TR, Brittain EL, Wells QS, Farber-Eger EH, Halliday SJ, Doss LN, et al. Hemodynamic evidence of vascular remodeling in combined post- and precapillary pulmonary hypertension. *Pulm Circ.* (2016) 6:313–21. doi: 10.1086/688516
25. Assad TR, Hemnes AR, Larkin EK, Glazer AM, Xu M, Wells QS, et al. Clinical and biological insights into combined post- and pre-capillary pulmonary hypertension. *J Am Coll Cardiol.* (2016) 68:2525–36. doi: 10.1016/j.jacc.2016.09.942
26. Gerges C, Gerges M, Lang MB, Zhang Y, Jakowitsch J, Probst P, et al. Diastolic pulmonary vascular pressure gradient: a predictor of prognosis

in "out-of-proportion" pulmonary hypertension. *Chest.* (2013) 143:758–66. doi: 10.1378/chest.12-1653
27. Vanderpool RR, Saul M, Nouraie M, Gladwin MT, Simon MA. Association between hemodynamic markers of pulmonary hypertension and outcomes in heart failure with preserved ejection fraction. *JAMA Cardiol.* (2018) 3:298–306. doi: 10.1001/jamacardio.2018.0128
28. Vachiery JL, Tedford RJ, Rosenkranz S, Palazzini M, Lang I, Guazzi M, et al. Pulmonary hypertension due to left heart disease. *Eur Respir J.* (2019) 53:1801897. doi: 10.1183/13993003.01897-2018
29. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Respir J.* (2015) 46:903–75. doi: 10.1183/13993003.01032-2015
30. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis treatment of acute chronic heart failure: The Task Force for the diagnosis treatment of acute chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur Heart J.* (2016) 37:2129–200. doi: 10.1093/eurheartj/ehw128
31. Leuchte HH, Ten Freyhaus H, Gall H, Halank M, Hoeper MM, Kaemmerer H, et al. Risk stratification strategy and assessment of disease progression in patients with pulmonary arterial hypertension: updated recommendations from the cologne consensus Conference 2018. *Int J Cardiol.* (2018) 272S:20–9. doi: 10.1016/j.ijcard.2018.08.084
32. Obokata M, Reddy YNV, Pislaru SV, Melenovsky V, Borlaug BA. Evidence supporting the existence of a distinct obese phenotype of heart failure with preserved ejection fraction. *Circulation.* (2017) 136:6–19. doi: 10.1161/CIRCULATIONAHA.116.026807
33. Gaggin HK, Januzzi JL Jr. Biomarkers and diagnostics in heart failure. *Biochim Biophys Acta.* (2013) 1832:2442–50. doi: 10.1016/j.bbadis.2012.12.014
34. Khan AM, Cheng S, Magnusson M, Larson MG, Newton-Cheh C, McCabe EL, et al. Cardiac natriuretic peptides, obesity, and insulin resistance: evidence from two community-based studies. *J Clin Endocrinol Metab.* (2011) 96:3242–9. doi: 10.1210/jc.2011-1182
35. Wang TJ. The natriuretic peptides and fat metabolism. *N Engl J Med.* (2012) 367:377–8. doi: 10.1056/NEJMcibr1204796
36. Madamanchi C, Alhosaini H, Sumida A, Runge MS. Obesity and natriuretic peptides, BNP and NT-proBNP: mechanisms and diagnostic implications for heart failure. *Int J Cardiol.* (2014) 176:611–7. doi: 10.1016/j.ijcard.2014.08.007
37. Borlaug BA, Reddy YNV. The Role of the Pericardium in Heart Failure: Implications for Pathophysiology and Treatment. *JACC Heart Fail.* (2019) 7:574–85. doi: 10.1016/j.jchf.2019.03.021
38. Nishikimi T, Nakagawa Y. Does impaired processing of pro-B-type (or brain) natriuretic peptide cause decreased plasma BNP levels in obese heart failure patients? *Ann Transl Med.* (2019) 7(Suppl 6):S221. doi: 10.21037/atm.2019.08.56
39. Mizuno Y, Harada E, Katoh D, Kashiwagi Y, Morikawa Y, Nakagawa H, et al. Cardiac production of B-type natriuretic peptide is inversely related to the plasma level of free fatty acids in obese individuals - possible involvement of the insulin resistance. *Endocr J.* (2013) 60:87–95. doi: 10.1507/endocrj.EJ12-0239
40. Ndumele CE, Matsushita K, Sang Y, Lazo M, Agarwal SK, Nambi V, et al. N-terminal pro-brain natriuretic peptide and heart failure risk among individuals with and without obesity: the Atherosclerosis Risk in Communities (ARIC) study. *Circulation.* (2016) 133:631–8. doi: 10.1161/CIRCULATIONAHA.115.017298
41. Anjan VY, Loftus TM, Burke MA, Akhter N, Fonarow GC, Gheorghiade M, et al. Prevalence, clinical phenotype, and outcomes associated with normal B-type natriuretic peptide levels in heart failure with preserved ejection fraction. *Am J Cardiol.* (2012) 110:870–6. doi: 10.1016/j.amjcard.2012.05.014
42. Jin P, Gu W, Lai Y, Zheng W, Zhou Q, Wu X. The circulating MicroRNA-206 level predicts the severity of pulmonary hypertension in patients with left heart diseases. *Cell Physiol Biochem.* (2017) 41:2150–60. doi: 10.1159/000475569
43. Gorter TM, van Veldhuisen DJ, Voors AA, Hummel YM, Lam CSP, Berger RMF, et al. Right ventricular-vascular coupling in heart failure with preserved ejection fraction and pre- vs. *post-capillary pulmonary hypertension*. *Eur Heart J Cardiovasc Imaging.* (2018) 19:425–32. doi: 10.1093/ehjci/jex133
44. Hussain I, Mohammed SF, Forfia PR, Lewis GD, Borlaug BA, Gallup DS, et al. Impaired right ventricular-pulmonary arterial coupling and effect of sildenafil in heart failure with preserved ejection fraction: an ancillary analysis from the phosphodiesterase-5 inhibition to improve clinical status and exercise capacity in diastolic heart failure (RELAX) trial. *Circ Heart Fail.* (2016) 9:e002729. doi: 10.1161/CIRCHEARTFAILURE.115.002729
45. Hoeper MM, Meyer K, Rademacher J, Fuge J, Welte T, Olsson KM. Diffusion capacity and mortality in patients with pulmonary hypertension due to heart failure with preserved ejection fraction. *JACC Heart Fail.* (2016) 4:441–9. doi: 10.1016/j.jchf.2015.12.016
46. Guazzi M, Bandera F, Pelissero G, Castelvecchio S, Menicanti L, Ghio S, et al. Tricuspid annular plane systolic excursion and pulmonary arterial systolic pressure relationship in heart failure: an index of right ventricular contractile function and prognosis. *Am J Physiol Heart Circ Physiol.* (2013) 305:H1373–81. doi: 10.1152/ajpheart.00157.2013
47. Miller L, Chartrand S, Koenig M, Goulet JR, Rich E, Chin AS, et al. Left heart disease: a frequent cause of early pulmonary hypertension in systemic sclerosis, unrelated to elevated NT-proBNP levels or overt cardiac fibrosis but associated with increased levels of MR-proANP and MR-proADM: retrospective analysis of a French Canadian cohort. *Scand J Rheumatol.* (2014) 43:314–23. doi: 10.3109/03009742.2013.854407
48. Mazurek JA, Horne BD, Saeed W, Sardar MR, Zolty R. Galectin-3 levels are elevated and predictive of mortality in pulmonary hypertension. *Heart Lung Circ.* (2017) 26:1208–15. doi: 10.1016/j.hlc.2016.12.012
49. McMurray JJ, Ray SG, Abdullah I, Dargie HJ, Morton JJ. Plasma endothelin in chronic heart failure. *Circulation.* (1992) 85:1374–9. doi: 10.1161/01.CIR.85.4.1374
50. Hulsmann M, Stanek B, Frey B, Sturm B, Putz D, Kos T, et al. Value of cardiopulmonary exercise testing and big endothelin plasma levels to predict short-term prognosis of patients with chronic heart failure. *J Am Coll Cardiol.* (1998) 32:1695–700. doi: 10.1016/S0735-1097(98)00437-9
51. Valero-Munoz M, Li S, Wilson RM, Boldbaatar B, Iglarz M, Sam F. Dual endothelin-A/Endothelin-B receptor blockade and cardiac remodeling in heart failure with preserved ejection fraction. *Circ Heart Fail.* (2016) 9:e003381. doi: 10.1161/CIRCHEARTFAILURE.116.003381
52. Obokata M, Kane GC, Reddy YNV, Melenovsky V, Olson TP, Jarolim P, et al. The neurohormonal basis of pulmonary hypertension in heart failure with preserved ejection fraction. *Eur Heart J.* (2019) 40:3707–17. doi: 10.1093/eurheartj/ehz626
53. Peltonen T, Taskinen P, Napankangas J, Leskinen H, Ohtonen P, Soini Y, et al. Increase in tissue endothelin-1 and ETA receptor levels in human aortic valve stenosis. *Eur Heart J.* (2009) 30:242–9. doi: 10.1093/eurheartj/ehn482
54. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* (1988) 332:411–5. doi: 10.1038/332411a0
55. Viljoen M, Koorts AM. A role for proinflammatory cytokines in the behavioral disturbances and cognitive decline in chronic renal failure patients. *Clin Nephrol.* (2004) 61:227–9. doi: 10.5414/CNP61227
56. Hirata Y, Kanno K, Watanabe TX, Kumagaye S, Nakajima K, Kimura T, et al. Receptor binding and vasoconstrictor activity of big endothelin. *Eur J Pharmacol.* (1990) 176:225–8. doi: 10.1016/0014-2999(90)90532-B
57. Rubens C, Ewert R, Halank M, Wensel R, Orzechowski HD, Schultheiss HP, et al. Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension. *Chest.* (2001) 120:1562–9. doi: 10.1378/chest.120.5.1562
58. Meoli DF, Su YR, Brittain EL, Robbins IM, Hemnes AR, Monahan K. The transpulmonary ratio of endothelin 1 is elevated in patients with preserved left ventricular ejection fraction and combined pre- and post-capillary pulmonary hypertension. *Pulm Circ.* (2018) 8:2045893217745019. doi: 10.1177/2045893217745019
59. Chowdhury MA, Moukarbel GV, Gupta R, Frank SM, Anderson AM, Liu LC, et al. Endothelin 1 is associated with heart failure hospitalization and long-term mortality in patients with heart failure with preserved

ejection fraction and pulmonary hypertension. *Cardiology.* (2019) 143:124–33. doi: 10.1159/000501100
60. Koller B, Steringer-Mascherbauer R, Ebner CH, Weber T, Ammer M, Eichinger J, et al. Pilot study of endothelin receptor blockade in heart failure with diastolic dysfunction and pulmonary hypertension (BADDHY-Trial). *Heart Lung Circ.* (2017) 26:433–41. doi: 10.1016/j.hlc.2016.09.004
61. Kaluski E, Cotter G, Leitman M, Milo-Cotter O, Krakover R, Kobrin I, et al. Clinical and hemodynamic effects of bosentan dose optimization in symptomatic heart failure patients with severe systolic dysfunction, associated with secondary pulmonary hypertension–a multi-center randomized study. *Cardiology.* (2008) 109:273–80. doi: 10.1159/000107791
62. Vachiéry J-L, Delcroix M, Al-Hiti H, Efficace M, Hutyra M, Lack G, et al. Macitentan in pulmonary hypertension due to left ventricular dysfunction. *Eur Resp J.* (2018) 51:1701886. doi: 10.1183/13993003.01886-2017
63. Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA.* (1998) 95:548–53. doi: 10.1073/pnas.95.2.548
64. Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, et al. VEGF-D Is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circul Res.* (2003) 92:1098–106. doi: 10.1161/01.RES.0000073584.46059.E3
65. Zhao T, Zhao W, Meng W, Liu C, Chen Y, Bhattacharya SK, et al. Vascular endothelial growth factor-D mediates fibrogenic response in myofibroblasts. *Mol Cell Biochem.* (2016) 413:127–35. doi: 10.1007/s11010-015-2646-1
66. Chen H, Guan R, Lei Y, Chen J, Ge Q, Zhang X, et al. Lymphangiogenesis in gastric cancer regulated through Akt/mTOR-VEGF-C/VEGF-D axis. *BMC Cancer.* (2015) 15:103. doi: 10.1186/s12885-015-1109-0
67. Rutanen J, Rissanen TT, Markkanen JE, Gruchala M, Silvennoinen P, Kivela A, et al. Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart. *Circulation.* (2004) 109:1029–35. doi: 10.1161/01.CIR.0000115519.03688.A2
68. Hartikainen J, Hassinen I, Hedman A, Kivela A, Saraste A, Knuuti J, et al. Adenoviral intramyocardial VEGF-DDeltaNDeltaC gene transfer increases myocardial perfusion reserve in refractory angina patients: a phase I/IIa study with 1-year follow-up. *Eur Heart J.* (2017) 38:2547–55. doi: 10.1093/eurheartj/ehx352
69. Berntsson J, Smith JG, Johnson LSB, Soderholm M, Borne Y, Melander O, et al. Increased vascular endothelial growth factor D is associated with atrial fibrillation and ischaemic stroke. *Heart.* (2019) 105:553–8. doi: 10.1136/heartjnl-2018-313684
70. Borne Y, Gransbo K, Nilsson J, Melander O, Orho-Melander M, Smith JG, et al. Vascular endothelial growth factor D, pulmonary congestion, and incidence of heart failure. *J Am Coll Cardiol.* (2018) 71:580–2. doi: 10.1016/j.jacc.2017.11.058
71. Saleby J, Bouzina H, Lundgren J, Radegran G. Angiogenic and inflammatory biomarkers in the differentiation of pulmonary hypertension. *Scand Cardiovasc J.* (2017) 51:261–70. doi: 10.1080/14017431.2017.1359419
72. Saleby J, Bouzina H, Ahmed S, Lundgren J, Radegran G. Plasma receptor tyrosine kinase RET in pulmonary arterial hypertension diagnosis and differentiation. *ERJ Open Res.* (2019) 5:00037-2019. doi: 10.1183/23120541.00037-2019
73. Seyama K, Kumasaka T, Souma S, Sato T, Kurihara M, Mitani K, et al. Vascular endothelial growth factor-D is increased in serum of patients with lymphangioleiomyomatosis. *Lymphat Res Biol.* (2006) 4:143–52. doi: 10.1089/lrb.2006.4.143
74. Wada H, Suzuki M, Matsuda M, Ajiro Y, Shinozaki T, Sakagami S, et al. Distinct characteristics of VEGF-D and VEGF-C to predict mortality in patients with suspected or known coronary artery disease. *J Am Heart Assoc.* (2020) 9:e015761. doi: 10.1161/JAHA.119.015761
75. Houston BA, Tedford RJ, Baxley RL, Sykes B, Powers ER, Nielsen CD, et al. Relation of lymphangiogenic factor vascular endothelial growth factor-D to elevated pulmonary artery wedge pressure. *Am J Cardiol.* (2019) 124:756–62. doi: 10.1016/j.amjcard.2019.05.056
76. Al-Husseini A, Kraskauskas D, Mezzaroma E, Nordio A, Farkas D, Drake JI, et al. Vascular endothelial growth factor receptor 3 signaling contributes to angioobliterative pulmonary hypertension. *Pulm Circul.* (2015) 5:101–16. doi: 10.1086/679704
77. Grasedieck S, Scholer N, Bommer M, Niess JH, Tumani H, Rouhi A, et al. Impact of serum storage conditions on microRNA stability. *Leukemia.* (2012) 26:2414–6. doi: 10.1038/leu.2012.106
78. Wong LL, Wang J, Liew OW, Richards AM, Chen YT. MicroRNA and heart failure. *Int J Mol Sci.* (2016) 17:502. doi: 10.3390/ijms17040502
79. Boucherat O, Potus F, Bonnet S. microRNA and pulmonary hypertension. *Adv Exp Med Biol.* (2015) 888:237–52. doi: 10.1007/978-3-319-22671-2_12
80. Estephan LE, Genuardi MV, Kosanovich CM, Risbano MG, Zhang Y, Petro N, et al. Distinct plasma gradients of microRNA-204 in the pulmonary circulation of patients suffering from WHO groups I and II pulmonary hypertension. *Pulm Circ.* (2019) 9:2045894019840646. doi: 10.1177/2045894019840646
81. Yue J, Guan J, Wang X, Zhang L, Yang Z, Ao Q, et al. MicroRNA-206 is involved in hypoxia-induced pulmonary hypertension through targeting of the HIF-1alpha/Fhl-1 pathway. *Lab Invest.* (2013) 93:748–59. doi: 10.1038/labinvest.2013.63
82. Jalali S, Ramanathan GK, Parthasarathy PT, Aljubran S, Galam L, Yunus A, et al. Mir-206 regulates pulmonary artery smooth muscle cell proliferation and differentiation. *PLoS ONE.* (2012) 7:e46808. doi: 10.1371/journal.pone.0046808
83. Sharma S, Umar S, Centala A, Eghbali M. Role of miR206 in genistein-induced rescue of pulmonary hypertension in monocrotaline model. *J Appl Physiol (1985).* (2015) 119:1374–82. doi: 10.1152/japplphysiol.00699.2014
84. Yang Y, Del Re DP, Nakano N, Sciarretta S, Zhai P, Park J, et al. miR-206 mediates YAP-induced cardiac hypertrophy and survival. *Circ Res.* (2015) 117:891–904. doi: 10.1161/CIRCRESAHA.115.306624
85. Mirna M, Rohm I, Jirak P, Wernly B, Baz L, Paar V, et al. Analysis of novel cardiovascular biomarkers in patients with Pulmonary Hypertension (PH). *Heart Lung Circ.* (2020) 29:337–44. doi: 10.1016/j.hlc.2019.03.004
86. Luedike P, Alatzides G, Papathanasiou M, Heisler M, Pohl J, Lehmann N, et al. Predictive potential of macrophage migration inhibitory factor (MIF) in patients with heart failure with preserved ejection fraction (HFpEF). *Eur J Med Res.* (2018) 23:22. doi: 10.1186/s40001-018-0321-1
87. DuBrock HM, AbouEzzeddine OF, Redfield MM. High-sensitivity C-reactive protein in heart failure with preserved ejection fraction. *PLoS ONE.* (2018) 13:e0201836. doi: 10.1371/journal.pone.0201836
88. Nguyen QL, Wang Y, Helbling N, Simon MA, Shiva S. Alterations in platelet bioenergetics in Group 2 PH-HFpEF patients. *PLoS ONE.* (2019) 14:e0220490. doi: 10.1371/journal.pone.0220490
89. Ranchoux B, Nadeau V, Bourgeois A, Provencher S, Tremblay E, Omura J, et al. Metabolic syndrome exacerbates pulmonary hypertension due to left heart disease. *Circ Res.* (2019) 125:449–66. doi: 10.1161/CIRCRESAHA.118.314555
90. Lai YC, Tabima DM, Dube JJ, Hughan KS, Vanderpool RR, Goncharov DA, et al. SIRT3-AMP-activated protein kinase activation by nitrite and metformin improves hyperglycemia and normalizes pulmonary hypertension associated with heart failure with preserved ejection fraction. *Circulation.* (2016) 133:717–31. doi: 10.1161/CIRCULATIONAHA.115.018935
91. Brittain EL, Talati M, Fortune N, Agrawal V, Meoli DF, West J, et al. Adverse physiologic effects of Western diet on right ventricular structure and function: role of lipid accumulation and metabolic therapy. *Pulm Circ.* (2019) 9:2045894018817741. doi: 10.1177/2045894018817741
92. Simon MA, Vanderpool RR, Nouraie M, Bachman TN, White PM, Sugahara M, et al. Acute hemodynamic effects of inhaled sodium nitrite in pulmonary hypertension associated with heart failure with preserved ejection fraction. *JCI Insight.* (2016) 1:e89620. doi: 10.1172/jci.insight.89620
93. Zhuang R, Wu J, Lin F, Han L, Liang X, Meng Q, et al. Fasudil preserves lung endothelial function and reduces pulmonary vascular remodeling in a rat model of endstage pulmonary hypertension with left heart disease. *Int J Mol Med.* (2018) 42:1341–52. doi: 10.3892/ijmm.2018.3728
94. Zhang X, Zhang X, Wang S, Luo J, Zhao Z, Zheng C, et al. Effects of fasudil on patients with pulmonary hypertension associated with left ventricular heart failure with preserved ejection fraction: a prospective intervention study. *Can Respir J.* (2018) 2018:3148259. doi: 10.1155/2018/3148259
95. Hemnes AR, Beck GJ, Newman JH, Abidov A, Aldred MA, Barnard J, et al. PVDOMICS: a multi-center study to improve understanding of

pulmonary vascular disease through phenomics. *Circ Res.* (2017) 121:1136–9. doi: 10.1161/CIRCRESAHA.117.311737

96. Shah SJ, Borlaug BA, Kitzman DW, McCulloch AD, Blaxall BC, Agarwal R, et al. Research priorities for heart failure with preserved ejection fraction: national heart, lung, and blood institute working group summary. *Circulation.* (2020) 141:1001–26. doi: 10.1161/CIRCULATIONAHA.119.041886
97. Nickel NP, Yuan K, Dorfmuller P, Provencher S, Lai YC, Bonnet S, et al. Beyond the lungs: systemic manifestations of pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2020) 201:148–57. doi: 10.1164/rccm.201903-0656CI

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

CASE REPORT
published: 30 October 2020
doi: 10.3389/fmed.2020.539707

Unexpected Acceleration in Treprostinil Delivery Administered by a Lenus Pro® Implantable Pump in Two Patients Treated for Pulmonary Arterial Hypertension

Garance Kopp[1], *Anne-Lise Hachulla*[2,3], *Stéphane Noble*[3,4,5], *Aurélien Bringard*[1,5,6], *Paola M. Soccal*[1,3,5], *Maurice Beghetti*[3,5,7] *and Frédéric Lador*[1,4,5]*

[1] *Division of Pneumology, University Hospitals of Geneva, Genève, Switzerland,* [2] *Division of Radiology, University Hospitals of Geneva, Genève, Switzerland,* [3] *Pulmonary Hypertension Program, University Hospitals of Geneva, Genève, Switzerland,* [4] *Division of Cardiology, University Hospitals of Geneva, Genève, Switzerland,* [5] *Medical Faculty, University of Geneva, Genève, Switzerland,* [6] *Division of Anesthesiology, Pharmacology, and Intensive Care, University Hospitals of Geneva, Genève, Switzerland,* [7] *Pediatric Cardiology Unit, Children's Hospital, University Hospitals of Geneva, Genève, Switzerland*

OPEN ACCESS

Edited by:
Elena Goncharova, University of California, Davis, United States

Reviewed by:
Soban Umar, University of California, Los Angeles, United States
Adriano Tonelli, Cleveland Clinic, United States

***Correspondence:**
Frédéric Lador
Frederic.Lador@hcuge.ch

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *02 March 2020*
Accepted: *28 September 2020*
Published: *30 October 2020*

Citation:
Kopp G, Hachulla A-L, Noble S, Bringard A, Soccal PM, Beghetti M and Lador F (2020) Unexpected Acceleration in Treprostinil Delivery Administered by a Lenus Pro® Implantable Pump in Two Patients Treated for Pulmonary Arterial Hypertension. Front. Med. 7:539707. doi: 10.3389/fmed.2020.539707

Intravenous treprostinil administration by an implantable pump is an attractive option for pulmonary arterial hypertension (PAH) treatment and is the subject of recent publications. Short-term studies are promising, but there is still a lack of long-term prospective data. We analyzed the treprostinil flow rate administered by the Lenus Pro® implantable pump in 2 patients suffering from PAH during follow-up times of respectively 4.2 and 3 years. The flow rate delivered by the pumps in these 2 patients exceeded the manufacturer admitted margin of error within 2 years and continued to increase to reach, respectively, 158 and 120% of the expected flow rate at the end of the follow up. In one case, the implantable pump had to be removed for this reason. The *ex-vivo* flow rate of the withdrawn pump determined in the laboratory reached 173% of the predicted value. This correlated with the *in-vivo* measurement, which suggests a continuous flow increase even after pump removal and without treprostinil use. Spontaneous flow increase from such an implantable pump is a potentially major pitfall, which needs to be identified and actively managed by the responsible clinicians.

Keywords: pulmonary arterial hypertension, prostacyclin analogs, implantable pump, treprostinil delivery, internal device

INTRODUCTION

The prostacyclin pathway is a major target for treatment of pulmonary arterial hypertension (PAH), recommended for high-risk patients (1). Due to their short half-lives, prostacyclin analogs are usually administrated by continuous intravenous (IV), subcutaneous (SC) or intermittent inhaled routes. Treprostinil has a longer half-life and is much more stable at room temperature than epoprostenol permitting oral, SC or IV route administration. Its use has shown improvement in 6-min walk distance, functional class and pulmonary hemodynamics compared to placebo (2–4). The use of oral treprostinil is limited to first line therapy in non-high risk patients, due to its lesser efficacy than parenteral treprostinil (4). Unfortunately SC treprostinil administration is often

limited by significant local side effects (2). Continuous IV administration is traditionally performed by central venous catheter connected to an external pump.

The development of an implantable pump for continuous IV treprostinil administration by Tricumed Medizintechnik GmbH (LenusPro® pump, approved in Europe in 2009) has attracted scientific attention as it may offer less restrictive use by patients as well as reducing catheter-related bloodstream infection rates (5–7). The Lenus Pro® pump contains a micro-infusion system that operates by pressurized gas storage in a titanium tank which is accessible by a puncturable silicone interface directly under the skin. Two pump models are available, one with a 20 ml capacity, the other with a 40 ml capacity. The device is usually implanted in the abdominal subcutaneous tissue and connected to a tunneled catheter accessing the superior vena cava (**Figure 1**). The flow is regulated by a glass capillary chip intended to guarantee a constant flow rate (1.3 ml/day), irrespectively of gas pressure. As the flow rate is fixed, drug titration is performed by adjusting treprostinil concentration. Percutaneous refills are usually performed every 14–28 days, depending on the size of the pump.

Two small prospective studies assessed periprocedural and 6-month safety, respectively, reporting good clinical results when using IV treprostinil administered by this pump (5, 6). We share here our experience with 2 cases receiving intravenous treprostinil via a LenusPro® implantable pump during a follow-up of, respectively, 4.2 and 3 years.

MATERIALS AND METHODS

The residual treprostinil volume in the pump was measured by a PAH specialized nurse before each refill procedure for the two patients treated by IV treprostinil administered by an implantable pump, as recommended by the manufacturer. The time between refill procedures and the volume administered were also reported in the registry by the nurse. Using these data we calculated the treprostinil flow rate between each refill procedure, for both patients during the follow-up period.

The *ex-vivo* flow rate of the withdrawn pump was determined in the laboratory by refilling it with saline solution (0.9 % NaCl) and keeping it in a constant 37°C water bath for 7 days. The flow rate was established by weighting (to 100 μg precision) at 24 h intervals a 5 ml polystyrene tube (352058 Becton-Dickinson) filled by the pump.

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

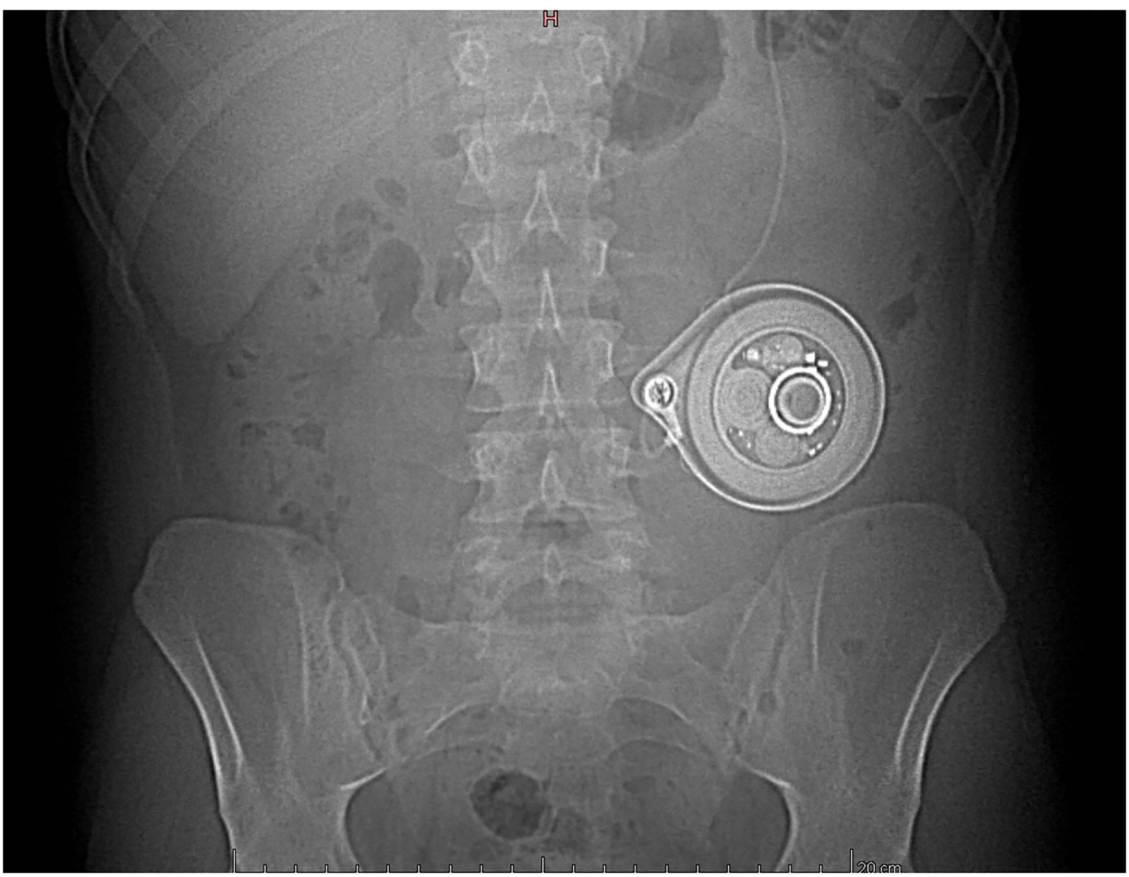


FIGURE 1 | The pump (Lenus Pro®, 20 ml) implanted in the left hypochondria for intravenous treprostinil delivery (patient 1).

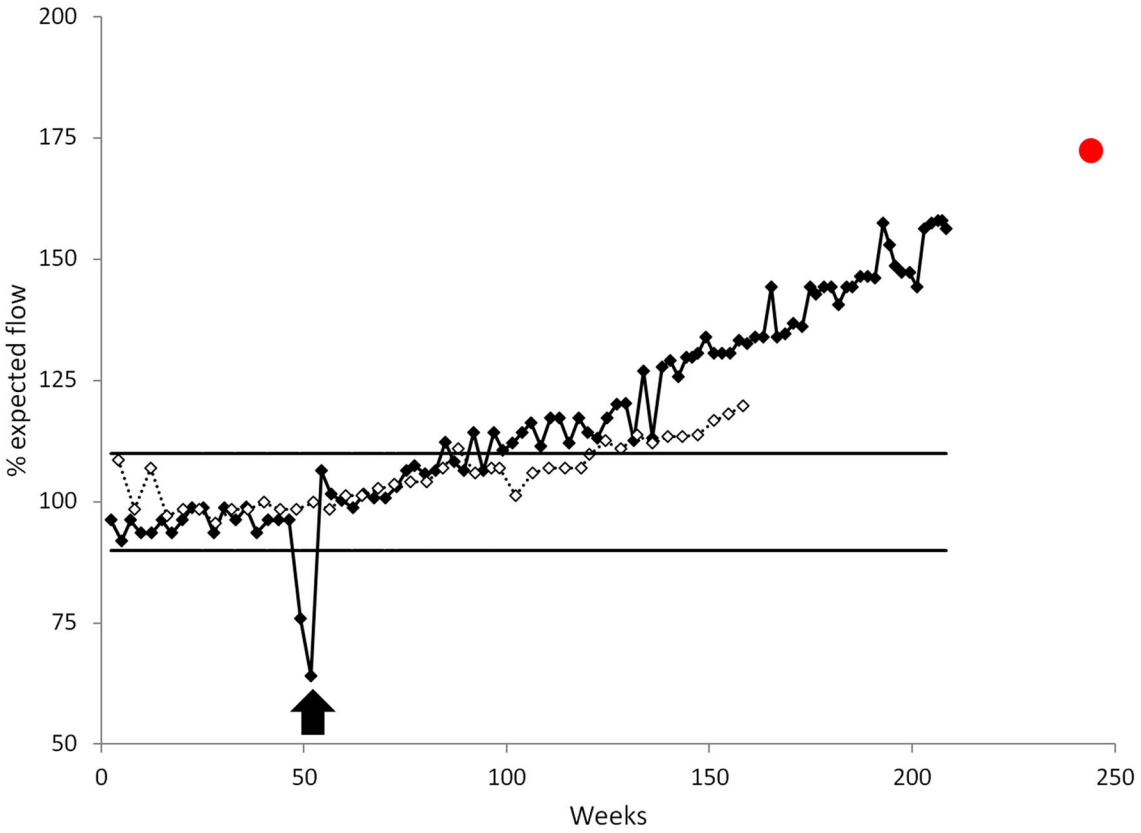


FIGURE 2 | Treprostinil delivery flow rate by the LenusPro® pump for patient 1 (black) and patient 2 (white) expressed relative to the expected calibrated flow. For patient 1, a catheter thrombosis resulted in a dramatic flow decrease that was treated by a standard repermeabilization procedure (black arrow). Horizontal lines represent the ±10% accepted flow error. The red dot represents the *ex-vivo* flow rate of the withdrawn pump determined in experimental condition by refilling it with a 20 ml saline solution.

CASES

The first case is a 47-year-old female patient suffering from drug-induced PAH. Under combined dual therapy (sildenafil, bosentan) the initial evolution was favorable. Due to clinical and hemodynamic deterioration, treatment of SC treprostinil was introduced later. The tolerance of the latter quickly became limited by pain at the injection site, justifying a switch to IV treatment. The patient being reluctant to IV access with external pump an implantable pump was proposed (LenusPro® 20 ml, **Figure 1**). The target dosage was reached in 2 months (47 ng/kg/min) while the clinical improvement allowed stabilization in functional class II. One year after implantation a central catheter thrombosis occurred resulting in a 40% flow rate decrease (see **Figure 2**). This was only discovered by the observation of an increased residual volume in the pump whereas the electronic flow rate sensor should have triggered an alarm in such a case. The catheter thrombosis was successfully treated by gentle rinsing with a heparin solution under fluoroscopic control by contrast injection, according to the manufacturer's guide. During the 4 years of follow-up we observed a gradual decrease in the residual treprostinil volume at refilling resulting in a shorter period between refill procedures.

The pump is calibrated by the manufacturer to a constant flow rate (1.04 ml/day ± 10%) whereas 4.2 years after implantation the observed flow rate had increased to 1.64 ml/day (77.4 ng/kg/min) which is a 58% flow increase above the predicted value (**Figure 2**). The increase in the treprostinil delivery flow rate resulted a proportional drug-dose increase that was, fortunately, well tolerated but induced a significant cost increase. The pump was removed and replaced by a 40 ml model due to the patient still being reluctant to other modalities of treatment and still being stabilized on this therapy. The *ex-vivo* flow rate of the withdrawn pump was established at 1.797 ± 0.017 ml/day, corresponding to a 73% flow increase over the expected value (**Figure 2**). This suggests a continuous flow increase even after pump removal and independent of treprostinil use. Considering this particular case, it was considered that the dose increase may have contributed to the progressive need of the PAH evolution and that it may have participated to maintain the patient in a stable condition.

The second case is a 22-year-old male patient followed since childhood for idiopathic PAH. The clinical course was initially favorable under inhaled iloprost monotherapy

sequentially combined with bosentan and, later, sildenafil due to hemodynamic deterioration. Despite the triple therapy the clinical and hemodynamic response was unsatisfactory. The iloprost treatment was successfully changed from inhaled to IV route delivered by a central venous catheter. Following that time the patient developed numerous catheter-related infections requiring hospitalizations and central line replacements. In order to reduce the risk of infection an implantable pump (LenusPro® 40 ml) was fitted.

As with the first case we observed a progressive increase in treprostinil flow reaching 1.52 ml/day which is a 20% increase over the calibrated value of 1.27 ml/day (± 10%) (**Figure 2**). The drug dilution was adjusted at each refill procedure in order to counterbalance the increased flow rate and maintain a stability of treatment at 35–41 ng/kg/min without any adverse event or side effect related to the treatment. Despite these measures, further hemodynamic evaluation concluded that the PAH was worsening under maximal treatment. The patient was referred for a lung transplant but died suddenly whilst still on the waiting list.

DISCUSSION

The currently available studies of the Lenus Pro® pump have drawn justifiable attention with good results reported and without significant short-term complications or safety issues (5, 6). Nonetheless the studies included small collectives and only two of them were prospective.

Interestingly a recent retrospective study including 129 patients with pulmonary hypertension (PH) treated by IV treprostinil administered by the Lenus Pro pump® has shown 82 complications after a median follow-up of 19 months. Most of these events were non-infectious catheter-related complications. As in the cases previously described, flow rate increases occurred in five patients. Four of them required pump replacement for this reason. Following treprostinil flow rate increase, two patients developed acute cardiac decompensation requiring intensive care unit admission. One patient underwent pump replacement. In the other patient, the treprostinil dose was adjusted to the higher flow rate without explantation (7). An unexpected increase in the flow rate administered by a LenusPro pump was also reported in a 14 years old patient (8).

In their multicentric study, Ewert et al. reported a septum defect of a Lenus Pro pump in one patient (6). This resulted in a severe overdose with subsequent hypotension, necessitating pump replacement. This septum defect was considered by the authors likely to be due to improper use of non-approved needles for refilling the pump.

This highlights the importance of maintaining a constant prostacyclin analog flow rate for patient stability and improvement as previously described in another study (9).

A warning has been published in 2013 by the manufacturer reporting occurrences of increased flow of the LenusPro® pump after several years of use. It is speculated that the molecule or its solvent provokes an increase in the cross-section of the glass capillary canal of the chip and therefore an augmentation of flow by 10%, especially when pumps are used for longer periods of time (2–4 years). The corrective action was introduced by the new development of a chip canal with a more resistant glass (10).

Nevertheless, an unexpected increase in the drug administration rate was reported with a Lenus Pro implantable pump coming from upgraded series, i.e., after the 2013 safety note of the manufacturer (11). This increase from 1.3 to 1.7 ml/day resulted in symptoms of deterioration of exercise capacity and fatigue without hemodynamic collapse. The second pump of the first described patient of the present work was also introduced after 2013, demonstrating that the corrective action introduced may not have been sufficient.

Recently, the variance of the fixed flow rate during long-term follow-up has been addressed on 126 patients (12) during a median follow-up of 12 months. Based on 2,853 refills, the relative flow rate deviation between each individual refill was between −10% and +10% in 94.5% of cases. However, three refill cases (0.1%) had a relative flow rate deviation of more than 40% from the previous refill (one case of −40% and two cases of +40%).

SynchroMed® II (Medtronic) is the second implantable pump currently approved for IV treprostinil administration in patients with PH. Unlike the Lenus Pro® pump, SynchroMed® II pump is battery-driven and has an adjustable flow rate, between 0.048 and 24 ml/day. Nonetheless, recent studies reported decreased flow rate and flow rate accuracy over time with this device (13, 14). Gomberg-Maitland et al. reported also eight pump failures events (13). One of them was undetected, leading to subsequent death of the patient. After this, pump design have been implemented, addressing many of the observed pump failures and motor stalls. Later, a programmable battery-driven model (Siromedes® pump) with adjustable flow has been developed by the German company Tricumed and approved in 2014. The three devices have similar use and implantation procedures.

The use of an implantable pump for IV treprostinil administration in PAH management is attractive, but the unexpected increase in the rate of treprostinil delivery by the Lenus Pro® pump is a major issue.

Such an increase is potentially life threatening and must be detected and actively managed by the responsible clinicians. This therapy should be administered in PH specialized centers by clinicians familiarized with the device. Reporting the time between refill procedures, the residual volume and the volume administered at each refill procedure should allow early detection of any derivation from the target flow rate and anticipate potential pump failure.

As an alternative option of removing and replacing the defective pump, adjustment of the drug dilution has to be considered, as long as the flow deviation from the factory setting can be anticipated.

This report is limited to the description of only two cases. Nevertheless, the frequency of adverse events such as previously described in our cases could have been underestimated in the studies actually available, due to the retrospective bias. Prospective studies are still needed to assess the long-term safety and efficacy of treprostinil administration by this device.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

We hereby confirm that written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

GK, MB, and FL contributed conception and design of the article. AB and FL performed the analysis of the withdrawn pump. GK wrote the first draft of the manuscript. MB, AB, and FL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Research funding from Actelion and Bayer-Schering received by MB and educational grant from GlaxoSmithKline received by FL had no relationship with the present manuscript and its content.

ACKNOWLEDGMENTS

Ewan Roche for his english language assistance.

REFERENCES

1. Galiè N, Channick RN, Frantz RP, Grünig E, Jing ZC, Moiseeva O, et al. Risk stratification and medical therapy of pulmonary arterial hypertension. *Eur Respir J.* (2019) 53:1801889. doi: 10.1183/13993003.01889-2018
2. Simonneau G, Barst RJ, Galie N, Naeije R, Rich S, Bourge RC, et al. Continuous subcutaneous infusion of treprostinil, a prostacyclin analogue, in patients with pulmonary arterial hypertension: a double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med.* (2002) 165:800–4. doi: 10.1164/ajrccm.165.6.2106079
3. Hiremath J, Thanikachalam S, Parikh K, Shanmugasundaram S, Bangera S, Shapiro L, et al. Exercise improvement and plasma biomarker changes with intravenous treprostinil therapy for pulmonary arterial hypertension: a placebo-controlled trial. *J Heart Lung Transplant.* (2010) 29:137–49. doi: 10.1016/j.healun.2009.09.005
4. Jing ZC, Parikh K, Pulido T, Jerjes-Sanchez C, White RJ, Allen R, et al. Efficacy and safety of oral treprostinil monotherapy for the treatment of pulmonary arterial hypertension: a randomized, controlled trial. *Circulation.* (2013) 127:624–33. doi: 10.1161/CIRCULATIONAHA.112.124388
5. Richter MJ, Ewert R, Warnke C, Gall H, Classen S, Grimminger F, et al. Procedural safety of a fully implantable intravenous prostanoid pump for pulmonary hypertension. *Clin Res Cardiol.* (2017) 106:174–82 doi: 10.1007/s00392-016-1037-2
6. Ewert R, Richter MJ, Steringer-Mascherbauer R, Grünig E, Lange TJ, Opitz CF, et al. Intravenous treprostinil infusion via a fully implantable pump for pulmonary arterial hypertension. *Clin Res Cardiol.* (2017) 106:776–83. doi: 10.1007/s00392-017-1114-1
7. Richter MJ, Harutyunova S, Bollmann T, Classen S, Gall H, Gerhardt F, et al. Long-term safety and outcome of intravenous treprostinil via an implanted pump in pulmonary hypertension. *J Heart Lung Transplant.* (2018) 37:1235–44. doi: 10.1016/j.healun.2018.06.006
8. Desole S, Richter MJ, Heine A, Ewert R. Intravenous treprostinil via an implantable pump in pediatric pulmonary arterial hypertension. *Pulm Circ.* (2018) 9:1–5. doi: 10.1177/2045894018788846
9. Kingman MS, Tankersley MA, Lombardi S, Spence S, Torres F, Chin KS, et al. Prostacyclin administration errors in pulmonary arterial hypertension patients admitted to hospitals in the United States: a national survey. *J Heart Lung Transplant.* (2010) 29:841–6. doi: 10.1016/j.healun.2010.03.008
10. Bülow M, Otto K-H. *Tricumed. Urgent Safety Information.* (2013). Available online at: https://www.swissmedic.ch/recalllists_dl/08938/Vk_20131223_02-e1.pdf
11. Kurzyna M, Małaczyńska-Rajpold K, Koteja A, Pawlak A, Chrzanowski Ł, Furdal M, et al. An implantable pump Lenus pro® in the treatment of pulmonary arterial hypertension with intravenous treprostinil. *BMC Pulm Med.* (2017) 17:162. doi: 10.1186/s12890-017-0474-7
12. Richter MJ, Harutyunova S, Bollmann T, Classen S, Fuge J, Gall H, et al. Flow rate variance of a fully implantable pump for the delivery of intravenous treprostinil in pulmonary arterial hypertension. *Pulm Circ.* (2020) 10:1–5. doi: 10.1177/2045894020910136
13. Gomberg-Maitland M, Bourge RC, Shapiro SM, Tarver III JH, Zwicke DL, Feldman JP, et al. Long-term results of the DelIVery for Pulmonary Arterial Hypertension trial. *Pulm Circ.* (2019) 9:1–9. doi: 10.1177/2045894019878615
14. Bourge RC, Waxman AB, Gomberg-Maitland M, Shapiro SM, Tarver III JH, Zwicke DL, et al. Treprostinil administered to treat pulmonary arterial hypertension using a fully implantable programmable intravascular delivery system: results of the DelIVery for PAH Trial. *Chest.* (2016) 150:27–34. doi: 10.1016/j.chest.2015.11.005

Conflict of Interest: MB has served as consultant and/or advisory board member for Actelion, Bayer-Schering, Lilly, GlaxoSmithKline, Novartis, MSD, and Pfizer Inc. and has received investigator-initiated research funding from Actelion and Bayer-Schering. FL has served as consultant and/or advisory board member for Novartis, MSD, and Bayer-Schering, has received conference fees from Actelion, Bayer-Schering, Lilly, GlaxoSmithKline, Novartis, and Pfizer Inc., and has received an educational grant from GlaxoSmithKline.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 03 November 2020
doi: 10.3389/fmed.2020.569413

Portopulmonary Hypertension: From Bench to Bedside

Christopher Thomas[1†], *Vladimir Glinskii*[1†], *Vinicio de Jesus Perez*[1] *and Sandeep Sahay*[2*]

[1] *Division of Pulmonary, Allergy & Critical Care Medicine, Stanford University School of Medicine, Stanford, CA, United States,*
[2] *Houston Methodist Hospital Lung Center, Houston Methodist Hospital, Houston, TX, United States*

Portopulmonary hypertension (PoPH) is defined as pulmonary arterial hypertension (PAH) associated with portal hypertension and is a subset of Group 1 pulmonary hypertension (PH). PoPH is a cause of significant morbidity and mortality in patients with portal hypertension with or without liver disease. Significant strides in elucidating the pathogenesis, effective screening algorithms, accurate diagnoses, and treatment options have been made in past 20 years. Survival of PoPH has remained poor compared to IPAH and other forms of PAH. Recently, the first randomized controlled trial was done in this patient population and showed promising results with PAH specific therapy. Despite positive effects on hemodynamics and functional outcomes, it is unclear whether PAH specific therapy has a beneficial effect on long term survival or transplant outcomes. In this review, we will discuss the epidemiology, pathophysiology, clinical and hemodynamic characteristics of PoPH. Additionally, this review will highlight the lacunae in our current management strategy, challenges faced and will provide direction to potentially useful futuristic management strategies.

Keywords: portopulmonary hypertension, pulmonary arterial hypertension, portal hypertension, liver transplant, MELD exception

OPEN ACCESS

Edited by:
Laurent Pierre Nicod,
University of Lausanne, Switzerland

Reviewed by:
Olivier Sitbon,
Université Paris-Saclay, France
John-David Aubert,
Centre Hospitalier Universitaire Vaudois (CHUV), Switzerland

***Correspondence:**
Sandeep Sahay
ssahay@houstonmethodist.org

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *04 June 2020*
Accepted: *23 September 2020*
Published: *03 November 2020*

Citation:
Thomas C, Glinskii V, de Jesus Perez V and Sahay S (2020) Portopulmonary Hypertension: From Bench to Bedside. Front. Med. 7:569413. doi: 10.3389/fmed.2020.569413

INTRODUCTION AND BACKGROUND

Pulmonary arterial hypertension (PAH) is a chronic progressive disease characterized by elevated pulmonary artery pressure and pulmonary vascular resistance (PVR) eventually leading to right heart failure and premature death. PAH associated with portal hypertension is called portopulmonary hypertension (PoPH), and is a subset of Group 1 PH (1). Similar to other causes of PAH, PoPH is characterized by the presence of intimal proliferation, medial hypertrophy, adventitial fibrosis of the muscular arteries and plexiform arteriopathy, and is thought to be histopathologically indistinguishable from other PAH phenotypes (2–4). Importantly, there does not seem to be a link between the presence or severity of PoPH with the degree of underlying liver dysfunction or hepatic venous pressure gradient (5, 6).

Patients with PoPH may be treated with PAH specific therapies, which may decrease the severity of the disease, improve functional parameters and hemodynamics, and allow for liver transplant (LT) (7). Despite a rapidly evolving understanding of PoPH, challenges remain in the diagnosis, treatment and transplantation of patients with PoPH. In this review, we will discuss the latest expert opinion in diagnosis and management of PoPH, as well as identify key areas of future research focus.

DEMOGRAPHICS AND CLINICAL CHARACTERISTICS OF PATIENTS WITH PoPH

The exact prevalence of PoPH is difficult to determine. The annual incidence of all types of PAH is <10 patients per million population, and PoPH patients are a subset of this group (8). PoPH was the third most common form of PAH in a population-based epidemiologic study in France (9). Historically, PoPH was thought to be 5–10% of all patients with PAH (10, 11). More recently, French authors have reported that the proportion of newly diagnosed (incident) patients with PoPH is as high as 15% of all patients with PAH, and is continuing to rise as wider screening practices are adopted (12). PoPH is thought to be present in anywhere between 2 and 10% of patients with portal hypertension (6, 13). Furthermore, while the vast majority of cases of PoPH are in patients with portal hypertension related to cirrhosis, non-cirrhotic causes of portal hypertension (including portal vein thrombosis, granulomatous disease, auto-immune disorders, drug reactions, infections, and congenital abnormalities) are also important contributors (14, 15). McDonnell et al. showed a prevalence of histopathologic changes of PAH of 0.61% in autopsies of patients with cirrhosis (16), and small cohort studies have shown prevalence of PoPH in patients with cirrhosis undergoing transplant evaluation to be between 5 and 6% (17–19).

Although the hemodynamic profile of patients with PoPH is better than in patients with idiopathic/familial PAH (IPAH/HPAH), their overall mortality is similar or worse (12, 20–22). The French Pulmonary Hypertension Registry (FHPR) shows a 1, 3, and 5-years survival of 84, 69, and 51%, respectively, for patients with PoPH (12), which is similar to the survival data for patients with IPAH, HPAH and anorexigen associated PAH (23). In contrast, analysis of the US based REVEAL registry showed that patients with PoPH had significantly worse survival when compared to patients with IPAH/FPAH: 67 vs. 85% at 2 years, and 40 vs. 64% at 5 years (**Figure 1**) (20). These findings were similar in the Spanish REHAP registry, where the 5 year mortality was 49 and 69%, for PoPH and IPAH/HPAH, respectively (**Figure 2**) (24, 25). Furthermore, severe PoPH is associated with significantly decreased survival in patients undergoing LT (7, 26), and severe PoPH precludes liver transplantation and significantly affects the course of hepatic failure in these patients (26, 27).

The lack of better outcomes in patients with PoPH as compared to their IPAH/FPAH counterparts is not entirely clear, especially in light of their relatively better hemodynamic profile and functional status (20, 25). One possible explanation is increased deaths from liver related events, which account for 25–33% of deaths in patients with PoPH, as compared to only 5% in patients with IPAH (22, 25). In patients with liver disease, higher Child-Pugh Scores are associated with worse outcomes (14, 22), which makes it clear that the severity of liver disease (rather than PoPH) is the main factor in worse outcomes in this population. Another possibility is a relative delay in PAH-specific treatments within the PoPH cohort. PoPH patients started on PAH-specific therapy live longer, despite worse hemodynamics, when compared to PoPH patients not on therapy (25). However, the percentage of PoPH patients on pulmonary vasodilators at both time of enrollment and at 90-days post enrollment into the REVEAL registry was significantly lower than their IPAH/FPAH counterparts (20). The cause of this delay is likely multifactorial, and includes milder symptoms, better hemodynamics, and lack of definitive data to suggest the ideal agent in patients with PoPH.

Likewise, the difference in survival seen between different PoPH cohorts are also likely due to a variety of factors, which include differences in regional screening practices for the presence of PoPH. Patients in the US and Spain with cirrhosis are typically screened at time of liver transplant evaluation, which likely skews the diagnosis of PoPH to patients with more severe liver disease. In contrast, all patients with cirrhosis are screened for PoPH in France, which likely leads to increased rates of diagnosis of PoPH in patients with milder liver disease, which in turn increases the overall survival rates. Rates of referrals of patients to PH specific centers may differ by region (the REVEAL registry included PAH patients treated in the community in addition to academic PH-centers). There may also be differences in baseline characteristics of patients enrolled in each respective registry (including New York Heart Association Functional Class [NYHA FC] and severity of underlying liver disease at the time of enrollment). Additional prospective studies are needed as PoPH-specific management guidelines are established and standardized, to see if this gap can be improved.

PATHOGENESIS OF PoPH

The underlying mechanisms for the development of PoPH are poorly understood and continue to be an area of active research. Liver cirrhosis and portal hypertension lead to splanchnic vasodilatation and formation of portosystemic shunts, which are thought to contribute to PoPH pathogenesis in multiple ways (**Figure 3**). Mechanically, increased intrahepatic resistance to flow from cirrhosis results in an increased portal pressure gradient and portosystemic collateralization through the reperfusion/dilation of preexisting vessels and through the generation of new vessels (28). On a molecular level, portosystemic shunting enables blood to bypass the liver, and thus evade hepatic metabolism of vasoactive substances. This direct reduction in the peripheral vascular resistance, combined with indirect vasodilation via intestinal vasoactive substances that are now able to bypass the liver and reach systemic circulation, ultimately result in a hyperdynamic state. This phenomenon has been seen in patients immediately after undergoing a transjugular intrahepatic portosystemic shunt (TIPS) placement, and persisted even a month out (29). Likewise, levels of vasoactive substances acting in the pulmonary circulation (including pulmonary vasodilators nitric oxide and prostacyclin, as well as pulmonary vasoconstrictors endothelin-1, thromboxane A2, and serotonin), are similarly dysregulated in cirrhosis (30–32). The imbalance of these vasoactive substances in the pulmonary vasculature results in net vasoconstriction and pulmonary vascular resistance elevation.

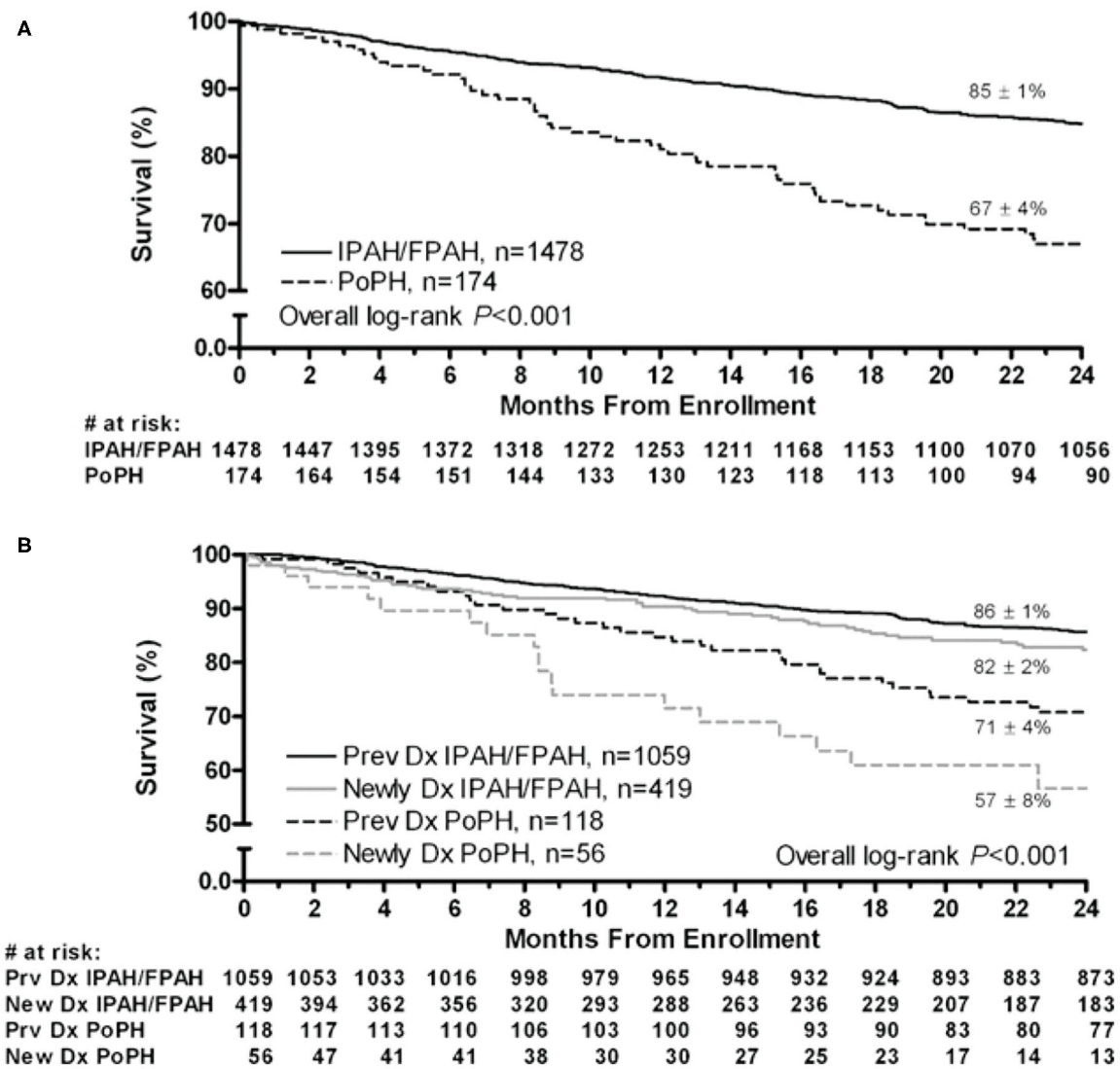
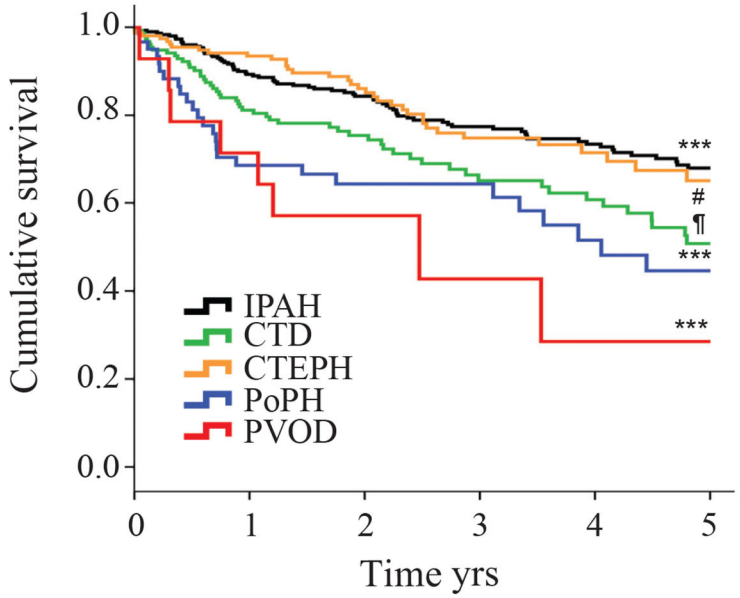


FIGURE 1 | Two-year survival from enrollment. **(A)** Patients with PoPH and IPAH/FPAH. **(B)** Stratified by duration of disease from enrollment. Dx, diagnosis; FPAH, familial pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; PoPH, portopulmonary hypertension. Reproduced with permission of Elsevier: Chest 141 (4) 906-915; doi: 10.1378/chest.11-0160 Epub 21 July 2011.

Shear stress from persistently high flows leads to endothelial cell injury and the activation and repression of genes that participate in the vascular remodeling process, and is believed to play a role in various forms of PAH (33–35). Exposure of and damage to the underlying arterial smooth muscle results in smooth muscle proliferation and thickening of the tunica intima, media, and adventitia within the pulmonary vasculature (4). The arterial wall thickening, in turn, can result in more sluggish pulmonary blood flow, platelet aggregation, and thrombus formation, which can then become recanalized over time (3). Autopsy data of patients with PoPH confirms that the end stage of this process is medial and intimal thickening, plexiform lesions and fibrotic venular obstruction (36). Together, these vascular lesions result in permanently elevated PVR that distinguishes PoPH from other causes of elevated mPAP in patients with portal hypertension (**Figure 5**).

Signaling mediated by bone morphogenic protein receptor 2 (BMPR2), a member of the TGF-β superfamily, has been shown to play a key role in familial pulmonary hypertension, and mutations in the gene encoding this receptor have also been found in 15–40% of idiopathic cases of PAH (37, 38). Bone morphogenic protein 9 (BMP9) is a circulating factor produced by hepatic stellate cells, which serves as a ligand for BMPR2 signaling, and has been shown to have a protecting effect against hepatic fibrosis (39). Recent studies have shown that patients with PoPH have significantly lower circulating levels of BMP9 as compared to control patients with advanced liver disease and no evidence of pulmonary hypertension (40). BMP9



At risk n							
IPAH	314	304	236	179	136	108	p-value[#]
CTD	157	149	98	67	46	37	0.023
CTEPH	162	153	117	82	51	34	0.95a1
PoPH	61	58	34	26	21	14	<0.001
PVOD	15	14	8	4	3	2	<0.001

FIGURE 2 | Kaplan-Meier estimates of 5-years survival from time of diagnosis in different pulmonary hypertension subtypes. IPAH, Idiopathic Pulmonary arterial hypertension; CTD, connective tissue disease; CTEPH, chronic thromboembolic pulmonary hypertension; PoPH, portopulmonary hypertension; PVOD, pulmonary veno-occlusive disease. The *p*-value for the overall comparison is <0.001. [#] compared with IPAH. Reproduced with permission of the © ERS 2020: European Respiratory Journal 40 (3) 596-603; doi: 10.1183/09031936.00101211 Published 31 August 2012.

levels were likewise significantly lower in patients with PoPH as compared to patients with other etiologies of group 1 PAH (41). Moreover, selective enhancement of endothelial BMPR2 with exogenous BMP9 has been shown to reverse the presence of PAH in multiple mouse models of PAH (42). Together, these findings suggest that BMP9 and BMPR2 associated downstream signaling pathways also likely play a role in the pathogenesis of PoPH.

More recently, activation of toll-like receptors (TLRs) by bacterial lipopolysaccharides (LPS) from bacterial translocation has been implicated in the pathogenesis of PAH. Mouse models deficient in BMPR2 exposed LPS were shown to develop evidence of pulmonary hypertension, which did not occur in their wild-type counterparts (43). The underlying mechanism for this is thought to be rooted in increased IL-6 production and activation of the STAT3 transcription factor, which is associated with increased expression of TLR4 (43). TLR4 is the main receptor for LPS and has been previously show to promote the development of PAH (44–46). Together, these data suggest that exposure to bacterial endotoxins, which occurs more frequently in cirrhotic patients due to recurrent gut bacterial translocations, may provide a "second hit" toward the development of PoPH in an otherwise predisposed host.

Lastly, there is growing evidence that altered sex hormone also contributes to the pathogenesis of PoPH. Estrogen binds to the promoter region of the BMPR2 gene and regulates its expression (47). Polymorphisms in CYP1B1 which metabolizes estrogen were associated with penetrance of PAH in women with BMPR2 mutations, but not men (48). Expression of CYP1B1 has also been shown to be increased in experimental models and human PAH (49). A candidate gene study to identify genetic risk factors in PoPH found single nucleotide polymorphisms (SNPs) in the genes coding for estrogen receptor 1 and aromatase, a critical enzyme in the estrogen metabolism (50). Recently, Al-Naamani et al. showed that in PoPH patients, the risk allele rs7175922 in CYP19A1 was associated with significantly higher levels of estradiol, urinary 2-hydroxyestrogen/16-α-hydroxyestrone (2-OHE/16α-OHE1), plasma levels of dehydroepiandrosterone-sulfate and plasma levels of 16-α-hydroxyestradiol (16α-OHE2) compared to the patients with liver disease but no PoPH (51). 16α-OHE1 leads to the development of PAH in animals via upregulation

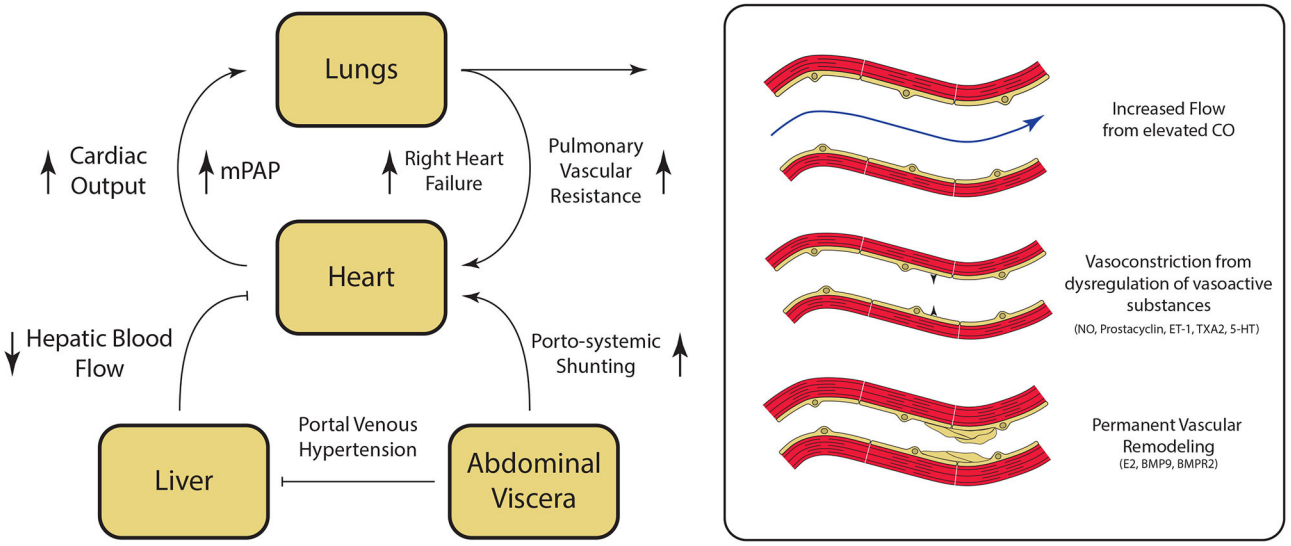


FIGURE 3 | Pathogenesis of Portopulmonary Hypertension (PoPH). Hepatic fibrosis results in portal venous hypertension, increased resistance to hepatic blood flow, and splanchnic vasodilation. Splanchnic vasodilation results in increase in overal circulating volume. and the diversion of blood flow from the liver to the heart via porto-systemic shunting resulting in an overall hyperdynamic state. Dysregulation of key regulator of pulmonary vascular tone associated with cirrhosis results in pulmonary vasoconstriction. At the same time, damage to the pulmonary endothelium and the underlying smooth muscle results in permanent vascular remodeling, which ultimately leads to the development of pulmonary hypertension. CO, cardiac output; NO, nitric oxide; ET-1, endothelin-1; TXA2, thromboxane A2; 5-HT, serotonin; E2, estrogen; BMP9, Bone Morphogenic Protein 9; BMPR2, Bone Morphogenic Receptor 2. Please refer to the text for additional details.

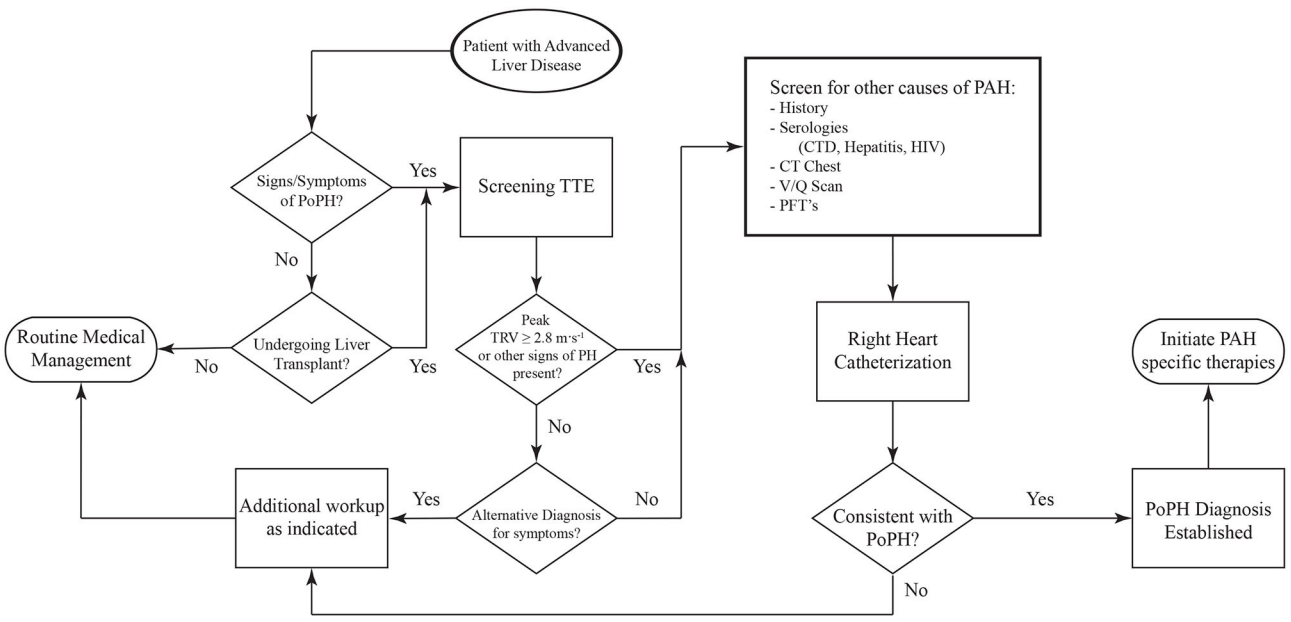


FIGURE 4 | Algorithm for the diagnosis of PoPH. PoPH, Portopulmonary Hypertension; TTE, Transthoracic Echocardiogram; TRV, Tricuspid Regurgitant Velocity; CTD, Connective Tissue Diseases; PFT's, Pulmonary Function Tests.

of miR-29 and has been associated with abnormal markers of insulin resistance (52). 16α-OHE1 has also been shown to increase oxidative-stress related proliferation in pulmonary arterial smooth muscle cells from PAH patients (53). Similarly, Sahay et al. performed qualitative immunohistochemical (IHC) staining of the explanted livers of PoPH patients and compared them with normal and cirrhotic livers. However, limited by the small sample size, they did not find any significant difference between the PoPH and cirrhotic livers, but aromatase expression was increased compared to the normal liver (54). More research is

TABLE 1 | Comon clinical and diagnostic findings in patients with Pulmonary Hypertension.

Symptoms	Physical exam	CT findings	TTE findings (57)
Dyspnea on exertion	Accentuated P2	PA diameter ≥29 mm	Peak TR velocity > 2.8 m/s
Fatigue	Right sided S3	PA to Ascending Aorta	RV/LV basal diameter > 1.0
Weakness	Right sided S4	diameter ratio of ≥1	Flattening of interventricular septum
Orthopnea	TR Murmur	RA Dilation	RV outflow Doppler acceleration
Near-syncope	Pulmonic Insufficiency Murmur	RV Dilation	<105 msec
Syncope	Jugular Venous Distension		Early diastolic pulmonary regurgitation
Palpitations	Ascites		velocity >2.2 m/s
Chest Pain	Lower Extremity Edema		PA diameter > 25 mm
	Cyanosis		IVC diameter >21 mm
			RA end-systole area >18 cm^2

TR, Tricuspid Regurgitation; PA, Pulmonary Artery; RA, Right Atrium; RV, Right Ventricle; LV, Left Ventricle; IVC, inferior vena cava.

needed to explore the links between altered estrogen metabolism and the development of PoPH in patients with liver disease.

SCREENING AND DIAGNOSIS OF PORTOPULMONARY HYPERTENSION

Screening for PoPH should begin with a detailed history and physical exam (**Figure 4**). Common symptoms of PoPH include unexplained dyspnea on exertion, fatigue, weakness, orthopnea, near-syncope, syncope, palpitations, and chest pain, with dyspnea being by far the most common. On physical exam, these patients often have an accentuated pulmonic component of the second heart sound (P2), a right sided S3, and a right sided S4. Additionally, the presence of a tricuspid regurgitant murmur or a pulmonic insufficiency murmur can be heard in 40% and 13% of patients, respectively (55). Other signs of right heart failure, including jugular venous distention, ascites, and bilateral lower extremity edema, may also be seen (56). Additional history looking for other known World Health Organization (WHO) Group 1 PAH risk factors should be taken. This includes use of drugs such as methamphetamines, use of dietary supplements, prior history of chemotherapy, and history of autoimmune diseases. Clinicians should also review the medical history for any other conditions known to be associated with PH, such as left heart disease, chronic lung disease, obstructive sleep apnea, venous thromboembolism, and hematologic disorders. If the history and physical arouses any suspicion for PoPH, the patient should undergo an echocardiogram.

Laboratory workup should include serological screening tests for connective tissue diseases, hepatitis and HIV. Additional evaluation with pulmonary function tests (including spirometry, lung volumes, diffusion capacity, and a 6 min walk test) and a CT scan of the chest should be obtained to screen for the presence of significant lung disease, and to establish a baseline which can then be used to track disease progression (11, 57). A ventilation/perfusion (V/Q) scan should also be done to screen for the presence of chronic thromboembolic pulmonary hypertension (CTEPH) (especially in the setting of portal vein thrombosis). See **Table 1** for more symptoms, physical exam and imaging findings.

Transthoracic echocardiography (TTE) is the best screening test for PoPH, as it is for other forms of PAH. All patients with portal hypertension should be screened for PoPH with a TTE. A list of the most common echocardiographic findings that are seen in patients with pulmonary hypertension can be seen in **Table 1**. Although traditionally used throughout the literature to screen for the presence of pulmonary hypertension, right ventricular systolic pressure (RVSP) calculations using the simplified Bernoulli equation are prone to error, stemming from the difficulty in accurately assessing a patient's right atrial pressure. In their analysis of echocardiographic screen of patients undergoing liver transplant evaluation, Krowka et al. demonstrated only moderate correlation between the RVSP and the PA systolic pressures that were measured on right heart catheterization (RHC) (19). Moreover, this discrepancy increased with increasing values of RVSP. Likewise, cutoff values of RVSP used for ruling out the presence of PoPH have differed between studies, and the ideal threshold value for a positive screen has been a matter of debate. As such, it has been suggested that the use of RVSP should be replaced by continuous wave Doppler measurements of peak tricuspid regurgitant velocity, with cutoff values of 2.8 $m{\cdot}s^{-1}$ for intermediate and 3.4 $m{\cdot}s^{-1}$ for high probability for the presence of PH (58). Issues with calculating RVSP notwithstanding, current guidelines from the American Association for the Study of Liver Diseases (AASLD) recommend evaluation with right heart catheterization in all patients being considered for LT with an RVSP ≥45 mmHg on screening echocardiogram (59). The International Liver Transplant Society (ILTS) guidelines recommend a RHC in patients with RVSP > 50 mmHg and/or evidence of right ventricular (RV) hypertrophy or dysfunction on TTE (60). Clinicians should understand that when lower RVSP cutoffs are used, the sensitivity will approach 100%, but the specificity will decrease, resulting in a significant number of false positives (17). However, given the high mortality associated with severe PoPH and LT, performing an unnecessary RHC is preferable to "missing" PoPH. In practice, all patients in whom there are TTE findings suspicious for PoPH (elevated RVSP or signs of RV dysfunction) should undergo RHC.

RIGHT HEART CATHETERIZATION HEMODYNAMICS INTERPRETATION IN PoPH

The diagnosis of PoPH includes hemodynamic evaluation of both the pulmonary and hepatic circulations. Portal hypertension can be challenging to diagnose non-invasively. Esophageal varices

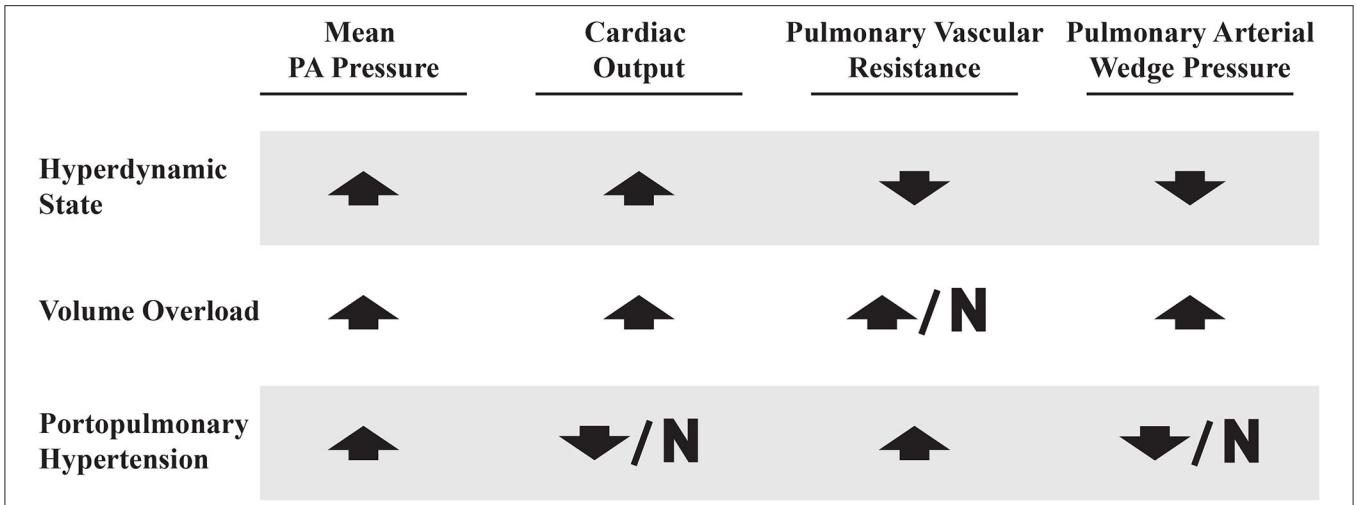


FIGURE 5 | Right heart catheterization profiles in patients with elevated mean PA pressures. Up arrow, increased; Down arrow, decreased; N, normal.

and skin collaterals are suggestive of portal hypertension. On ultrasound, decreased portal vein flow velocity, and portal vein biphasic or flow reversal are suggestive of portal hypertension (61). A hepatic venous pressure gradient (HVPG) (wedge hepatic vein pressure [WHVP]—free hepatic vein pressure [FHVP]) of >5 mmHg is diagnostic of sinusoidal portal hypertension (62).

On RHC, the patient must have both an elevated mPAP and PVR and a normal pulmonary artery wedge pressure (PAWP) for a positive diagnosis of PoPH. During the most recent World Symposium on Pulmonary Hypertension, the hemodynamic criteria for the diagnosis of pre-capillary pulmonary hypertension (which includes WHO Group 1) were revised. With this change, patients with a mPAP > 20 mmHg (previously > 25 mmHg), a PVR of ≥ 3 Wood units (WU) (≥240 dynes), and a PAWP < 15 mmHg meet the diagnostic criteria for the presence of PAH (1).

Careful evaluation of a patient's hemodynamic profile on RHC is crucial in order to distinguish PoPH from other clinical conditions that are common in patients with liver disease that can also result in elevated mPAPs. These conditions can be classified into three broad categories: hyperdynamic state, volume overload and true portopulmonary hypertension (63). See **Figure 5** for comparisons between these hemodynamic profiles.

Patients with decompensated liver disease may present with an elevated mPAP, PAWP, and PVR. This may be due to a combination of volume overload, hyperdynamic state, and PoPH (perhaps all three). Volume overload is managed with careful diuresis, as patients with liver disease are prone to kidney injury (64). A hyperdynamic state can be intrinsic to liver disease, but may also be due to other treatable medical comorbidities (such as anemia, arteriovenous shunts, and hyperthyroidism) (65–67). This common clinical scenario speaks to the challenge of diagnosing and treating PoPH, which is best done at expert centers which are most familiar with these complicated patients.

TREATMENT OF PoPH

The importance of screening for PoPH was established by Krowka et al. in a retrospective review of case reports and case series, which looked at the relationship between the presence of untreated PoPH and cardiopulmonary related mortality in patients undergoing LT (7). In this seminal work, Krowka et al. examined 43 patients with PoPH (confirmed by RHC) who were not on medical treatment and underwent LT. The patients were grouped based on severity of pulmonary hypertension. Patients with a mPAP between 25 and 35 mmHg were considered to have mild disease, those with a mPAP between 35 and 50 mmHg were considered moderate, and those with a mPAP >50 mmHg were considered severe. In these groups, 100% of the patients with severe and 50% of the patients with moderate PoPH died due to cardiopulmonary related events (the majority died during the transplantation hospitalization). No mortality was reported among the patients with mild PoPH (mPAP < 35 mmHg). As a result of this study, and others, routine screening for the presence of pulmonary hypertension is now recommended in all patients undergoing evaluation for LT.

After this landmark study established that PoPH patients with higher mPAP are at high risk of mortality with LT, it prompted the question of whether medical therapy could mitigate some of those risks. Treatment of PoPH prior to or instead of liver transplantation remains challenging and is fraught with a paucity of data and large knowledge gaps. Randomized controlled trials of pulmonary hypertension specific medical therapies for patients with portopulmonary hypertension have been limited by concerns regarding adverse hepatic effects of PAH specific medications (68). We will review select clinical trials of PAH-specific therapy in PoPH, as well as the indications and contraindications for liver transplantation.

Retrospective data on treatment of PoPH is quite heterogenous, with some studies showing improvement in hemodynamics and outcomes, and others showing no treatment effect. There is higher quality data from prospective non-randomized studies, from which a few themes emerge. A recently published prospective cohort study from the French Pulmonary Hypertension Registry (FPHR) examined data on 637 patients with PoPH, 90% of whom were started on PAH-specific therapies (12). These patients experienced significant improved WHO functional class, 6-min walk distance (6MWD) and hemodynamic parameters. The ANGEL study is a recent single arm prospective trial of ambrisentan in 31 patients with PoPH (69). This trial showed improved hemodynamics (PVR, mPAP, cardiac index [CI]) and functional class, but no change in 6MWD. From these prospective studies, it is reasonable to conclude that PAH directed therapy has favorable effects on hemodynamics and functional outcomes, but the effects on mortality and candidacy for LT are unclear.

As previously stated, the vast majority of randomized controlled trials of PAH-specific therapies in PAH patients have excluded patients with PoPH. One notable exception is the PATENT-1 trial by Ghofrani et al. (phase 3, double-blind, randomized controlled trial), which randomized 443 patients with various etiologies of WHO group 1 PAH to receive either placebo or two different doses of riociguat (70). This trial is significant in that it included 13 patients with PoPH, 11 of whom received the highest dose of study medication (2.5 mg daily). The primary end point of the PATENT-1 trial was 6MWD, which was significantly improved in the riociguat 2.5 mg group. Secondary hemodynamic (PVR, mPAP, and CO) and functional (WHO functional class, Borg dyspnea scale and time to clinical worsening) endpoints were also significantly improved in the riociguat 2.5 mg daily group. The PATENT-2 trial was an extension of the PATENT-1 study, in which all eligible patients from the first study were given riociguat and followed for 2 years. PATENT-2 showed sustained improvements in functional class and 6MWD (71). A recently published subgroup analysis of the 13 patients with PoPH who were enrolled in PATENT-1 and 2 showed similar improvements in WHO functional class, 6MWD and PVR (72). Importantly, riociguat was well-tolerated by the PoPH patients.

The first ever randomized controlled trial of PAH specific therapy in patients with PoPH was published in 2019. The PORTICO study, which randomized 85 patients with PoPH to receive either macitentan or placebo for 12 weeks, found that the macitentan group had a 35% reduction in PVR, the primary endpoint (73). While there were significant improvements in some secondary endpoints (mPAP and CI), there were no significant improvements in WHO functional class and 6MWD. Importantly, macitentan was well-tolerated in this population, with no hepatic side effects.

A notable limitation of PORTICO is that it excluded patients with Child-Pugh class C liver disease and patients with a MELD score of >19, which are groups with known poor survival (12).

It is still unclear whether this significant reduction in PVR could translate into more meaningful outcomes for patients (improved functional capacity, decreased mortality) or alter candidacy for liver transplant. However, a recently published *post-hoc* analysis of the PORTICO study found that patients in the macitentan treatment group had significantly decreased waitlist and perioperative mortality risk categories as compared with the placebo arm (74).

Taken together, these studies show that PAH specific therapies can improve hemodynamics and functional outcomes in patients with PoPH. Importantly, these medications seem to be well-tolerated in patients with liver disease and PoPH. By extension, they can help patients become candidates for LT and potentially live longer. In the absence of PoPH specific treatment guidelines, clinicians should follow the general treatment principles of PAH, with special attention to the unique considerations for patients with PoPH. More research is needed to elucidate the nuances of PAH specific therapies in the PoPH population.

LIVER TRANSPLANTATION

As previously discussed, the severity of PoPH is an established risk factor for liver transplant waitlist mortality (75) and poor perioperative outcomes (7). As such, decisions about candidacy for liver transplantation in patients with PoPH are complex and best done by experienced centers.

The following general principles are recommended by the AASLD (59) for management of patients with liver disease who are being considered for LT. All patients who are undergoing evaluation for LT should be screened for PoPH with transthoracic echocardiography, and patients with RVSP > 45 mmHg should be further evaluated by RHC. Patients with mPAP < 35 mmHg do not require treatment with PAH specific therapies and can proceed with further evaluation for LT. Patients with mPAP > 35 mmHg should be evaluated by an experienced PH specialist and be considered for PAH specific therapy. If the mPAP can be lowered to <35 mmHg and the PVR can be lowered to <5 WU with medical therapy, LT can be pursued.

For some patients with mild liver disease and PoPH, their higher risk for waitlist mortality is not accurately captured by the MELD score (76). LT candidates with PoPH are eligible for MELD exception points if the following criteria are met: initial mPAP > 35 and PVR > 3 WU; patients are treated with PAH specific therapy; and post-treatment mPAP is lowered to < 35 mmHg (76).

Giving MELD exception points to patients with PoPH is controversial for a number of reasons. The U.S. based Organ Procurement and Transplant Network (OPTN) requires a RHC and resubmission of full hemodynamic data every 3 months (76) to maintain the MELD exception points, which is burdensome and often impractical for both patients and institutions. Despite having to submit full hemodynamic data every 3 months, only mPAP < 35 mmHg is used to maintain eligibility MELD exception points. Some patients may achieve a lower PVR with PAH directed therapy, but continue to have an elevated mPAP from volume overload and/or a high flow state (60). In an analysis of patients with PoPH on the LT waitlist, initial PVR

was an independent predictor of waitlist mortality, while pre- and post-treatment mPAP were not (75). This suggests that a broader hemodynamic picture should be used to maintain MELD exception points eligibility.

In one analysis of 155 patients on the LT waitlist with approved PoPH MELD exception points, less than half of the patients actually met the standardized OPTN MELD exception criteria. Furthermore, almost one third of patients in this study lacked sufficient hemodynamic data to diagnose PoPH or had hemodynamic data that was inconsistent with PoPH (77). These researchers found that overall mortality was higher for patients given PoPH MELD exception points (as compared to patients listed without exception points), which shows the need for more research into methods of risk stratification for patients with PoPH.

In the era of the AASLD/ILTS guidelines and MELD exception points, data on the natural course of PoPH after LT is limited. In one retrospective cohort study, hemodynamics and survival data for 35 patients with PoPH were examined (78). After LT, 6 month, 1 year, and 3 year survival rates were 80, 77, and 77%, respectively. Of the 27/35 patients who survived more than 6 months after LT, all were able to be weaned from intravenous epoprostenol. Furthermore, most of the patients had improvement in their hemodynamics after LT, with 30% having a mPAP < 25 mmHg at last follow up. This data suggests that carefully selected patients with PoPH who undergo LT will have significant improvement in their hemodynamics.

CHALLENGES IN THE DIAGNOSIS AND MANAGEMENT OF PoPH

There are a number of barriers to the effective diagnosis and treatment of patients with PoPH. As previously discussed, accurate diagnosis of PoPH is challenging, and the differential for an elevated mPAP in the setting of liver disease is broad. There is only one randomized controlled trial for PAH specific therapy in patients with PoPH. Advanced liver disease symptoms have significant overlap with the side effects of PAH specific therapies (e.g., nausea, fatigue, and fluid overload), which can make the initiation and maintenance of treatment challenging (67).

Many of the drugs used to treat PoPH are metabolized in the liver, and drug metabolism is altered by liver disease and other medications that patients may be taking (67). Additionally, patients may have hepatic encephalopathy or lack the social support needed to safely administer parenteral therapy or take medications multiple times per day (67). More generally, many of the treatment barriers that apply to all patients with PAH apply to patients with PoPH: late referrals, lack of an expert center in close proximity to where the patient lives, and insurance issues. There is significant variability in the screening and management practices of PoPH, discordance between published guidelines and actual practice patterns, and disagreements about the role of LT in PoPH (79).

CONCLUSIONS AND AREAS OF FUTURE FOCUS

PoPH remains a challenging disease entity with many facets in need of further research. More research is needed into which types of PAH specific therapies will benefit patients with varying severities of PoPH. The first randomized control trial in PoPH was recently published, which showed that macitentan significantly lowered PVR in patients with PoPH. It unfortunately remains unclear if hemodynamics improvements translate into successful LT or substantial survival benefit. While it is clear that patients with severe disease have high mortality with or without LT, their long-term response to treatment is unknown. It is unknown if mild PoPH needs to be treated or given MELD exception points, and the risk for progression to more severe disease is unknown.

Pre-operative risk stratification of patients prior to LT remains a challenge, as the traditional cut off of a mPAP of 35 mmHg is somewhat arbitrary and is based on a small retrospective review of case reports and series. There are no published guidelines on initiation of PAH specific therapies in PoPH, or whether to continue PoPH treatment post-LT. Finally, there is no centralized national registry of PoPH patients who undergo LT; this would serve as a foundation for focusing on the aforementioned areas of need.

While there are indeed many barriers to and challenges in caring for patients with PoPH, it remains a field with many areas of active investigation. As the mechanisms and treatment continue to be explored, we are confident that care for these complex patients will continue to improve.

AUTHOR CONTRIBUTIONS

CT and VG: primarily responsible for writing the manuscript. VJ and SS: editing and revision and mentoring. All authors contributed to the article and approved the submitted version.

FUNDING

SS has grant funding from ACCP CHEST unrelated to the current work.

REFERENCES

1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J.* (2019) 53:1801913. doi: 10.1183/13993003.01913-2018
2. Jonigk D, Golpon H, Bockmeyer CL, Maegel L, Hoeper MM, Gottlieb J, et al. Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol.* (2011) 179:167–79. doi: 10.1016/j.ajpath.2011.03.040
3. Edwards BS, Weir EK, Edwards WD, Ludwig J, Dykoski RK, Edwards JE. Coexistent pulmonary and portal hypertension:

morphologic and clinical features. *J Am Coll Cardiol.* (1987) 10:1233–8. doi: 10.1016/S0735-1097(87)80123-7
4. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, et al. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J.* (2019) 53:1801887. doi: 10.1183/13993003.01887-2018
5. Krowka MJ. Portopulmonary hypertension. *Semin Respir Crit Care Med.* (2012) 33:17–25. doi: 10.1055/s-0032-1301731
6. Hadengue A, Benhayoun MK, Lebrec D, Benhamou JP. Pulmonary hypertension complicating portal hypertension: prevalence and relation to splanchnic hemodynamics. *Gastroenterology.* (1991) 100:520–8. doi: 10.1016/0016-5085(91)90225-A
7. Krowka MJ, Plevak DJ, Findlay JY, Rosen CB, Wiesner RH, Krom RA. Pulmonary hemodynamics and perioperative cardiopulmonary-related mortality in patients with portopulmonary hypertension undergoing liver transplantation. *Liver Transpl.* (2000) 6:443–50. doi: 10.1053/jlts.2000.6356
8. Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. *Eur Respir J.* (2007) 30:104–9. doi: 10.1183/09031936.00092306
9. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, et al. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med.* (2006) 173:1023–30. doi: 10.1164/rccm.200510-1668OC
10. Badesch DB, Raskob GE, Elliott CG, Krichman AM, Farber HW, Frost AE, et al. Pulmonary arterial hypertension: baseline characteristics from the REVEAL Registry. *Chest.* (2010) 137:376–87. doi: 10.1378/chest.09-1140
11. Zelniker TA, Huscher D, Vonk-Noordegraaf A, Ewert R, Lange TJ, Klose H, et al. The 6MWT as a prognostic tool in pulmonary arterial hypertension: results from the COMPERA registry. *Clin Res Cardiol.* (2018) 107:460–70. doi: 10.1007/s00392-018-1207-5
12. Savale L, Guimas M, Ebstein N, Fertin M, Jevnikar M, Renard S, et al. Portopulmonary hypertension in the current era of pulmonary hypertension management. *J Hepatol.* (2020) 73:130–9. doi: 10.1016/j.jhep.2020.02.021
13. Chen HS, Xing SR, Xu WG, Yang F, Qi XL, Wang LM, et al. Portopulmonary hypertension in cirrhotic patients: Prevalence, clinical features and risk factors. *Exp Ther Med.* (2013) 5:819–24. doi: 10.3892/etm.2013.918
14. Sithamparanathan S, Nair A, Thirugnanasothy L, Coghlan JG, Condliffe R, Dimopoulos K, et al. National Pulmonary Hypertension Service Research Collaboration of the United, and Ireland, Survival in portopulmonary hypertension: Outcomes of the United Kingdom National Pulmonary Arterial Hypertension Registry. *J Heart Lung Transplant.* (2017) 36:770–9. doi: 10.1016/j.healun.2016.12.014
15. Schouten JN, Garcia-Pagan JC, Valla DC, Janssen HL. Idiopathic noncirrhotic portal hypertension. *Hepatology.* (2011) 54:1071–81. doi: 10.1002/hep.24422
16. McDonnell PJ, Toye PA, Hutchins GM. Primary pulmonary hypertension and cirrhosis: are they related? *Am Rev Respir Dis.* (1983) 127:437–41. doi: 10.1164/arrd.1983.127.4.437
17. Colle IO, Moreau R, Godinho E, Belghiti J, Ettori F, Cohen-Solal A, et al. Diagnosis of portopulmonary hypertension in candidates for liver transplantation: a prospective study. *Hepatology.* (2003) 37:401–9. doi: 10.1053/jhep.2003.50060
18. Kawut SM, Taichman DB, Ahya VN, Kaplan S, Archer-Chicko CL, Kimmel SE, et al. Hemodynamics and survival of patients with portopulmonary hypertension. *Liver Transpl.* (2005) 11:1107–11. doi: 10.1002/lt.20459
19. Krowka MJ, Swanson KL, Frantz RP, McGoon MD, Wiesner RH. Portopulmonary hypertension: Results from a 10-year screening algorithm. *Hepatology.* (2006) 44:1502–10. doi: 10.1002/hep.21431
20. Krowka MJ, Miller DP, Barst RJ, Taichman D, Dweik RA, Badesch DB, et al. Portopulmonary hypertension: a report from the US-based REVEAL Registry. *Chest.* (2012) 141:906–15. doi: 10.1378/chest.11-0160
21. Swanson KL, Wiesner RH, Nyberg SL, Rosen CB, Krowka MJ. Survival in portopulmonary hypertension: Mayo Clinic experience categorized by treatment subgroups. *Am J Transplant.* (2008) 8:2445–53. doi: 10.1111/j.1600-6143.2008.02384.x
22. Le Pavec J, Souza R, Herve P, Lebrec D, Savale L, Tcherakian C, et al. Portopulmonary hypertension: survival and prognostic factors. *Am J Respir Crit Care Med.* (2008) 178:637–43. doi: 10.1164/rccm.200804-613OC
23. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, et al. Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. *Circulation.* (2010) 122:156–63. doi: 10.1161/CIRCULATIONAHA.109.911818
24. Escribano-Subias P, Blanco I, Lopez-Meseguer M, Lopez-Guarch CJ, Roman A, Morales P, et al. Survival in pulmonary hypertension in Spain: insights from the Spanish registry. *Eur Respir J.* (2012) 40:596–603. doi: 10.1183/09031936.00101211
25. Lázaro Salvador M, Quezada Loaiza C, Rodríguez Padial L, Barberá J, López-Meseguer M, López-Reyes R, et al. Portopulmonary hypertension: prognosis and management in the current treatment era. Results from the REHAP Registry. *Intern Med J.* (2020). doi: 10.1111/imj.14751
26. Krowka MJ, Mandell MS, Ramsay MA, Kawut SM, Fallon MB, Manzarbeitia C, et al. Hepatopulmonary syndrome and portopulmonary hypertension: a report of the multicenter liver transplant database. *Liver Transpl.* (2004) 10:174–82. doi: 10.1002/lt.20016
27. Sussman N, Kaza V, Barshes N, Stribling R, Goss J, O'Mahony C, et al. Successful liver transplantation following medical management of portopulmonary hypertension: a single-center series. *Am J Transplant.* (2006) 6:2177–82. doi: 10.1111/j.1600-6143.2006.01432.x
28. Bolognesi M, Di Pascoli M, Verardo A, Gatta A. Splanchnic vasodilation and hyperdynamic circulatory syndrome in cirrhosis. *World J Gastroenterol.* (2014) 20:2555–63. doi: 10.3748/wjg.v20.i10.2555
29. Van der Linden P, Le Moine O, Ghysels M, Ortinez M, Deviere J. Pulmonary hypertension after transjugular intrahepatic portosystemic shunt: effects on right ventricular function. *Hepatology.* (1996) 23:982–7. doi: 10.1002/hep.510230507
30. Huertas A, Guignabert C, Barbera JA, Bartsch P, Bhattacharya J, Bhattacharya S, et al. Pulmonary vascular endothelium: the orchestra conductor in respiratory diseases: highlights from basic research to therapy. *Eur Respir J.* (2018) 51:1700745. doi: 10.1183/13993003.00745-2017
31. Shenoda B, Boselli J. Vascular syndromes in liver cirrhosis. *Clin J Gastroenterol.* (2019) 12:387–97. doi: 10.1007/s12328-019-00956-0
32. Porres-Aguilar M, Altamirano JT, Torre-Delgadillo A, Charlton MR, Duarte-Rojo A. Portopulmonary hypertension and hepatopulmonary syndrome: a clinician-oriented overview. *Eur Respir Rev.* (2012) 21:223–33. doi: 10.1183/09059180.00007211
33. Rodriguez-Roisin R, Krowka MJ, Herve P, Fallon MB, E.R.S.T.F.P.-H.Committee VDS. Pulmonary-Hepatic vascular Disorders (PHD). *Eur Respir J.* (2004) 24:861–80. doi: 10.1183/09031936.04.00010904
34. Baeyens N, Bandyopadhyay C, Coon BG, Yun S, Schwartz MA. Endothelial fluid shear stress sensing in vascular health and disease. *J Clin Invest.* (2016) 126:821–8. doi: 10.1172/JCI83083
35. Pascall E, Tulloh RM. Pulmonary hypertension in congenital heart disease. *Future Cardiol.* (2018) 14:343–53. doi: 10.2217/fca-2017-0065
36. Furuta M, Sato T, Tsujino I, Tanino M, Watanabe T, Nishimura M. An autopsy case of portopulmonary hypertension associated with idiopathic portal hypertension. *Eur Resp J.* (2013) 42:P2649. Available online at: https://erj.ersjournals.com/content/42/Suppl_57/P2649
37. P.International PHC, Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, et al. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet.* (2000) 26:81–4. doi: 10.1038/79226
38. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, et al. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet.* (2000) 37:741–5. doi: 10.1136/jmg.37.10.741

39. Desroches-Castan A, Tillet E, Ricard N, Ouarne M, Mallet C, Belmudes L, et al. Bone morphogenetic protein 9 is a paracrine factor controlling liver sinusoidal endothelial cell fenestration and protecting against hepatic fibrosis. *Hepatology*. (2019) 70:1392–408. doi: 10.1002/hep.30655
40. Rochon ER, Krowka MJ, Bartolome S, Heresi GA, Bull T, Roberts K, et al. Pulmonary vascular complications of liver disease 2 study, bmp 9/10 in pulmonary vascular complications of liver disease. *Am J Respir Crit Care Med*. (2020) 201:1575–8. doi: 10.1164/rccm.201912-2514LE
41. Nikolic I, Yung LM, Yang P, Malhotra R, Paskin-Flerlage SD, Dinter T, et al. Bone morphogenetic protein 9 is a mechanistic biomarker of portopulmonary hypertension. *Am J Respir Crit Care Med*. (2019) 199:891–902. doi: 10.1164/rccm.201807-1236OC
42. Long L, Ormiston ML, Yang X, Southwood M, Graf S, Machado RD, et al. Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nat Med*. (2015) 21:777–85. doi: 10.1038/nm.3877
43. Soon E, Crosby A, Southwood M, Yang P, Tajsic T, Toshner M, et al. Bone morphogenetic protein receptor type II deficiency and increased inflammatory cytokine production. A gateway to pulmonary arterial hypertension. *Am J Respir Crit Care Med*. (2015) 192:859–72. doi: 10.1164/rccm.201408-1509OC
44. Bauer EM, Chanthaphavong RS, Sodhi CP, Hackam DJ, Billiar TR, Bauer PM. Genetic deletion of toll-like receptor 4 on platelets attenuates experimental pulmonary hypertension. *Circ Res*. (2014) 114:1596–600. doi: 10.1161/CIRCRESAHA.114.303662
45. Bauer EM, Shapiro R, Zheng H, Ahmad F, Ishizawar D, Comhair SA, et al. High mobility group box 1 contributes to the pathogenesis of experimental pulmonary hypertension via activation of Toll-like receptor 4. *Mol Med*. (2013) 18:1509–18. doi: 10.2119/molmed.2012.00283
46. Young KC, Hussein SM, Dadiz R, deMello D, Devia C, Hehre D, et al. Toll-like receptor 4-deficient mice are resistant to chronic hypoxia-induced pulmonary hypertension. *Exp Lung Res*. (2010) 36:111–9. doi: 10.3109/01902140903171610
47. Austin ED, Hamid R, Hemnes AR, Loyd JE, Blackwell T, Yu C, et al. BMPR2 expression is suppressed by signaling through the estrogen receptor. *Biol Sex Differ*. (2012) 3:6. doi: 10.1186/2042-6410-3-6
48. Austin ED, Cogan JD, West JD, Hedges LK, Hamid R, Dawson EP, Wheeler LA, Parl FF, Loyd JE, Phillips J3rd A, Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. Eur Respir J (2009) 34:1093-9. doi: 10.1183/09031936.00010409
49. White K, Johansen AK, Nilsen M, Ciuclan L, Wallace E, Paton L, et al. Activity of the estrogen-metabolizing enzyme cytochrome P450 1B1 influences the development of pulmonary arterial hypertension. *Circulation*. (2012) 126:1087–98. doi: 10.1161/CIRCULATIONAHA.111.062927
50. Roberts KE, Fallon MB, Krowka MJ, Brown RS, Trotter JF, Peter I, et al. Pulmonary Vascular Complications of Liver Disease Study, Genetic risk factors for portopulmonary hypertension in patients with advanced liver disease. *Am J Respir Crit Care Med*. (2009) 179:835–42. doi: 10.1164/rccm.200809-1472OC
51. Al-Naamani N, Krowka MJ, Forde KA, Krok KL, Feng R, Heresi GA, et al. Estrogen signaling and portopulmonary hypertension: the pulmonary vascular complications of liver disease study (PVCLD2). *Hepatology*. (2020). doi: 10.1002/hep.31314
52. Chen X, Talati M, Fessel JP, Hemnes AR, Gladson S, French J, et al. Estrogen metabolite 16alpha-hydroxyestrone exacerbates bone morphogenetic protein receptor type II-associated pulmonary arterial hypertension through microrna-29-mediated modulation of cellular metabolism. *Circulation*. (2016) 133:82–97. doi: 10.1161/CIRCULATIONAHA.115.016133
53. Hood KY, Montezano AC, Harvey AP, Nilsen M, MacLean MR, Touyz RM. Nicotinamide adenine dinucleotide phosphate oxidase-mediated redox signaling and vascular remodeling by 16alpha-hydroxyestrone in human pulmonary artery cells: implications in pulmonary arterial hypertension. *Hypertension*. (2016) 68:796–808. doi: 10.1161/HYPERTENSIONAHA.116.07668
54. Sahay S, Barrios R, Deavers M, Ren Y, Frost AE. aromatase expression of liver in portopulmonary hypertension, A63. In: Ride the Lightning: Basic and Translational Studies in Pulmonary Hypertension. San Diego, CA (2018). p. A2102–A2102.
55. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Primary pulmonary hypertension. A national prospective study. *Ann Intern Med*. (1987) 107:216–23. doi: 10.7326/0003-4819-107-2-216
56. Robalino BD, Moodie DS. Association between primary pulmonary hypertension and portal hypertension: Analysis of its pathophysiology and clinical, laboratory and hemodynamic manifestations. *J Am Coll Cardiol*. (1991) 17:492–8. doi: 10.1016/S0735-1097(10)80121-4
57. Frost A, Badesch D, Gibbs JSR, Gopalan D, Khanna D, Manes A, et al. Diagnosis of pulmonary hypertension. *Eur Respir J*. (2019) 53. doi: 10.1183/13993003.01904-2018
58. Lau EM, Tamura Y, McGoon MD, Sitbon O. The 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: a practical chronicle of progress. *Eur Respir J*. (2015) 46:879–82. doi: 10.1183/13993003.01177-2015
59. Martin P, DiMartini A, Feng S, Brown R Jr, Fallon M. Evaluation for liver transplantation in adults: 2013 practice guideline by the American Association for the Study of Liver Diseases and the American Society of Transplantation. *Hepatology*. (2014) 59:1144–65. doi: 10.1002/hep.26972
60. Krowka MJ, Fallon MB, Kawut SM, Fuhrmann V, Heimbach JK, Ramsay MA, et al. International liver transplant society practice guidelines: diagnosis and management of hepatopulmonary syndrome and portopulmonary hypertension. *Transplantation*. (2016) 100:1440–52. doi: 10.1097/TP.0000000000001229
61. Hernández DB, Serrano MA, Gabutti JA, Casanova IE, Guerrero M. Portal hypertension. What, when and how of portal gradients, TIPS and Neo-porta. *Europ Congress Radiol*. (2018). doi: 10.1594/ecr2018/C-3153
62. de Franchis R, Baveno VIF. Expanding consensus in portal hypertension: report of the Baveno VI Consensus Workshop: stratifying risk and individualizing care for portal hypertension. *J Hepatol*. (2015) 63:743–52. doi: 10.1016/j.jhep.2015.05.022
63. Cartin-Ceba R, Krowka MJ. Portopulmonary hypertension. *Clin Liver Dis*. (2014) 18:421–38. doi: 10.1016/j.cld.2014.01.004
64. Garcia-Tsao G, Parikh CR, Viola A. Acute kidney injury in cirrhosis. *Hepatology*. (2008) 48:2064–77. doi: 10.1002/hep.22605
65. Park SC, Beerman LB, Gartner JC, Zitelli BJ, Malatack JJ, Fricker FJ, et al. Echocardiographic findings before and after liver transplantation. *Am J Cardiol*. (1985) 55:1373–8. doi: 10.1016/0002-9149(85)90507-7
66. Reddy YNV, Melenovsky V, Redfield MM, Nishimura RA, Borlaug BA. High-output heart failure: a 15-year experience. *J Am Coll Cardiol*. (2016) 68:473–82. doi: 10.1016/j.jacc.2016.05.043
67. AbuHalimeh B, Krowka MJ, Tonelli AR. Treatment barriers in portopulmonary hypertension. *Hepatology*. (2019) 69:431–43. doi: 10.1002/hep.30197
68. Sitbon O, O'Callaghan DS, Savale L. Portopulmonary hypertension: light at the end of the tunnel? *Chest*. (2012) 141:840–2. doi: 10.1378/chest.11-2378
69. Preston IR, Burger CD, Bartolome S, Safdar Z, Krowka M, Sood N, et al. Ambrisentan in portopulmonary hypertension: A multicenter, open-label trial. *J Heart Lung Transplant*. (2020) 39:464–72. doi: 10.1016/j.healun.2019.12.008
70. Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, et al. Riociguat for the treatment of pulmonary arterial hypertension. *N Engl J Med*. (2013) 369:330–40. doi: 10.1056/NEJMoa1209655
71. Rubin LJ, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, et al. Riociguat for the treatment of pulmonary arterial hypertension: a long-term extension study (PATENT-2). *Eur Respir J*. (2015) 45:1303–13. doi: 10.1183/09031936.00090614
72. Cartin-Ceba R, Halank M, Ghofrani HA, Humbert M, Mattson J, Fritsch A, et al. Riociguat treatment for portopulmonary hypertension: a subgroup analysis from the PATENT-1/-2 studies. *Pulm Circ*. (2018) 8:2045894018769305. doi: 10.1177/2045894018769305

73. Sitbon O, Bosch J, Cottreel E, Csonka D, de Groote P, Hoeper MM,et al. Macitentan for the treatment of portopulmonary hypertension (PORTICO): a multicentre, randomised, double-blind, placebo-controlled, phase 4 trial. *Lancet Respir Med.* (2019) 7:594–604. doi: 10.1016/S2213-2600(19)30091-8
74. Krowka M, Cottreel E, Hoeper MM, Kim NH, Martin N, Sitbon O, et al. Macitentan improves risk categorization for liver transplant mortality in patients with portopulmonary hypertension: a portico study post hoc analysis. *Liver Transpl.* (2020) 26:935–40. doi: 10.1002/lt.25747
75. DuBrock HM, Goldberg DS, Sussman NL, Bartolome SD, Kadry Z, Salgia RJ, et al. Predictors of Waitlist Mortality in Portopulmonary Hypertension. *Transplantation.* (2017) 101:1609–15. doi: 10.1097/TP.0000000000001666
76. Guidance to Liver Transplant Programs and the National Liver Review Board for: Adult MELD Exception Review.
77. Goldberg DS, Batra S, Sahay S, Kawut SM, Fallon MB. MELD exceptions for portopulmonary hypertension: current policy and future implementation. *Am J Transplant.* (2014) 14:2081–7. doi: 10.1111/ajt.12783
78. Savale L, Sattler C, Coilly A, Conti F, Renard S, Francoz C, et al. Long-term outcome in liver transplantation candidates with portopulmonary hypertension. *Hepatology.* (2017) 65:1683–92. doi: 10.1002/hep.28990
79. DuBrock HM, Salgia RJ, Sussman NL, Bartolome SD, Kadry Z, Mulligan DC, et al. Portopulmonary hypertension: a survey of practice patterns and provider attitudes. *Transplant Direct.* (2019) 5:e456. doi: 10.1097/TXD.0000000000000900

Conflict of Interest: SS is a speaker and consultant for J&J, Bayer, United Therapeutics, Advisor for Liquidia, and Gossamer biotech. SS has received prior and current research grants from ACCP CHEST, and is a clinical trial end point adjudication committee member in a trial sponsored by GSK.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 25 March 2021
doi: 10.3389/fmed.2020.616720

Epidemiology, Pathogenesis, and Clinical Approach in Group 5 Pulmonary Hypertension

*Mazen Al-Qadi, Barbara LeVarge and H. James Ford**

Division of Pulmonary and Critical Care Medicine, Pulmonary Hypertension Program, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Pulmonary hypertension (PH) is recognized to be associated with a number of comorbid conditions. Based on these associations, PH is classified into 5 groups, considering common pathophysiologic drivers of disease, histopathologic features, clinical manifestations and course, and response to PH therapy. However, in some of these associated conditions, these characteristics are less well-understood. These include, among others, conditions commonly encountered in clinical practice such as sarcoidosis, sickle cell disease, myeloproliferative disorders, and chronic kidney disease/end stage renal disease. PH in these contexts presents a significant challenge to clinicians with respect to disease management. The most recent updated clinical classification schemata from the 6th World Symposium on PH classifies such entities in Group 5, highlighting the often unclear and/or multifactorial nature of PH. An in-depth review of the state of the science of Group 5 PH with respect to epidemiology, pathogenesis, and management is provided. Where applicable, future directions with respect to research needed to enhance understanding of the clinical course of these entities is also discussed.

Keywords: multifactorial, pulmonary hypertension, Group 5, hematologic, sickle cell, sarcoidosis, metabolic, CKD - chronic kidney disease

OPEN ACCESS

Edited by:
Vinicio De Jesus Perez, Stanford University, United States

Reviewed by:
Roberto J. Bernardo, University of Oklahoma, United States
Jeremy Mazurek, University of Pennsylvania, United States
Nicholas Kolaitis, University of California, San Francisco, United States

****Correspondence:***
H. James Ford
hjford@med.unc.edu

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: *13 October 2020*
Accepted: *17 December 2020*
Published: *25 March 2021*

Citation:
Al-Qadi M, LeVarge B and Ford HJ (2021) Epidemiology, Pathogenesis, and Clinical Approach in Group 5 Pulmonary Hypertension. Front. Med. 7:616720. doi: 10.3389/fmed.2020.616720

INTRODUCTION

Pulmonary hypertension (PH) is characterized by elevated mean pulmonary artery pressure (mPAP) of >20 mmHg as determined by right heart catheterization (RHC). PH is divided into 5 groups based on the underlying mechanism using the original World Health Organization classification system framework. In 2019, the proceedings of the World Symposium on Pulmonary Hypertension published the updated and revised classification based on the 6th World Symposium on PH (1). According to the new classification, causes of PH include pulmonary arterial hypertension (PAH, Group 1 PH), pulmonary venous hypertension due to elevated filling pressure of the left heart (Group 2 PH), PH due to chronic pulmonary disease or hypoxemia (Group 3 PH), chronic thromboembolic disease (Group 4), whereas PH that develops due to multiple or unclear mechanisms is referred to as Group 5 PH (**Table 1**). Within Group 5, several clinical disorders are implicated in association with the development of PH (**Table 2**). The most commonly seen clinical entities in Group 5 PH (Groups 5.1–5.3) are covered in detail: PH in the setting of hematologic disorders, sarcoidosis, and chronic renal failure. A brief overview of some of the remaining, less common but clinically relevant entities in Group 5 is also provided. Complex congenital heart disease (Group 5.4) is outside the scope of this manuscript.

TABLE 1 | Current clinical classification of pulmonary hypertension (1).

Group	Description
1	**Pulmonary Arterial Hypertension**
	1.1 Idiopathic PAH
	1.2 Heritable PAH
	1.3 Drug- and toxin-induced PAH
	1.4 PAH associated with:
	1.4.1 Connective tissue disease
	1.4.2 HIV infection
	1.4.3 Portal hypertension
	1.4.4 Congenital heart disease
	1.4.5 Schistosomiasis
	1.5 PAH long-term responders to CCB
	1.6 PAH with overt signs of venous/capillaries (PVOD/PCH) involvement
	1.7 Persistent PH of the Newborn syndrome
2	**PH Due to Left Heart Disease**
	2.1 PH due to heart failure with preserved EF
	2.2 PH due to heart failure with reduced EF
	2.3 Valvular heart disease
	2.4 Congenital/acquired CV conditions leading to post-capillary PH
3	**PH Due to Lung Diseases and/or Hypoxia**
	3.1 Obstructive lung disease
	3.2 Restrictive lung disease
	3.3 Other lung disease with mixed restrictive/obstructive pattern
	3.4 Hypoxia without lung disease
	3.5 Developmental lung disorders
4	**PH Due to Pulmonary Artery Obstruction**
	4.1 Chronic thromboembolic PH
	4.2 Other pulmonary artery obstructions
5	**PH With Unclear and/or Multifactorial Mechanisms**
	5.1 Hematologic disorders
	5.2 Systemic disorders
	5.3 Others
	5.4 Complex congenital heart disease

TABLE 2 | Subclassifications of group 5 pulmonary hypertension: PH with unclear and/or multifactorial mechanisms (1).

5.1 Hematologic disorders	Hemolytic anemias Myeloproliferative disorders
5.2 Systemic and metabolic disorders	Pulmonary Langerhans cell histiocytosis Gaucher disease Glycogen storage disease Neurofibromatosis Sarcoidosis
5.3. Others	Chronic renal failure with or without hemodialysis Fibrosing mediastinitis
5.4 Complex congenital heart diseases	Segmental pulmonary hypertension • Isolated pulmonary artery of ductal origin • Absent pulmonary artery • Pulmonary atresia with ventricular septal defect and major aorto-pulmonary collateral arteries • Hemitruncus • Other Single ventricle • Unoperated • Operated Scimitar syndrome

Considering the true upper limits of normal mPAP, the 6th World Symposium on PH introduced revised hemodynamic definitions, affirming PH in those with mPAP >20 mmHg as opposed to mPAP ≥25 mmHg as previously defined. A pulmonary vascular resistance (PVR) of at least 3 Wood Units was further mandatory to define pre-capillary pulmonary hypertension. These changes impact identification and management of all groups of PH, including group 5 PH. With updated review and discussion of the literature, the proceedings outlined additional changes specifically impacting group 5 PH, including removal of both splenectomy and thyroid disease as specific subtypes of PH (asserting these conditions as risk factors) and reclassification of lymphangioleiomyomatosis into Group 3 PH (1).

PULMONARY HYPERTENSION IN HEMATOLOGIC DISORDERS

Chronic Hemolytic Anemias

Prevalence

PH is increasingly recognized as a major source of morbidity and mortality in patients with chronic hemolysis, most notably in the context of sickle cell disease. The prevalence of PH in hemolytic anemia is variable depending upon the diagnostic criteria, methods used to diagnose PH, and population studied. In sickle cell disease (SCD, hemoglobin SS), 30–40% of patients have evidence of elevated pulmonary artery pressures based on tricuspid regurgitant jet velocity (TRV) ≥2.5 m/s on Doppler echocardiography. However, only 6–10.5% of patients have mPAP of ≥25 mmHg on invasive hemodynamic evaluation by RHC (2–5). This reflects the fact that estimation of pulmonary artery pressure is often inaccurate by echocardiogram. Additionally, elevated pulmonary artery pressures can be related to high cardiac output (due to anemia) and not always secondary to increased pulmonary vascular resistance (PVR). Using the new definition of PH (mPAP >20 mmHg), the prevalence of PH in SCD is probably higher than that reported in these studies.

PH has also been reported in association with other hemolytic anemias. In a study of 110 patients with β-thalassemia intermedia, 59% of patients had elevated peak systolic tricuspid gradient >30 mmHg on Doppler echocardiography, suggestive of PH. Only 6 patients had RHC and were found to have severe pre-capillary PH. All patients in this study had normal left ventricular function and high cardiac output (6). The prevalence of PH by echocardiography was even higher (75%) in a small study of 35 patients with homozygous β-thalassemia (7). More recently, Derchi et al. evaluated the prevalence of PH in a large multicenter cross-sectional study of 1,309 patients with β-thalassemia in Italy. Nine percent of patients had TRV >3 m/s; patients in the "PH likely" group with TRV ≥3.2 m/s underwent RHC. The prevalence of RHC-confirmed PH was 2.1%. Increased age and splenectomy were independent risk factors for PH in this study (8).

Notably, the prevalence of PH in patients with hereditary spherocytosis is much lower than reported with SCD and thalassemia. In two retrospective studies, no patients had

a TRV of ≥2.8 m/s on echocardiography to suggest PH (9, 10). Nonetheless, PH has been described in hereditary spherocytosis and stomatocytosis, with most cases related to chronic thromboembolic pulmonary hypertension (CTEPH) following splenectomy (11).

Pathogenesis

The pathogenesis of PH in chronic hemolysis is multifaceted and is most extensively studied in SCD. The vasomotor tone of the pulmonary circulation is normally regulated by nitric oxide (NO), a powerful vasodilator and modulator of endothelial proliferation. NO is produced by the pulmonary endothelium in response to shear stress on the surface of endothelial cells (12). The enzyme NO synthase catalyzes cleavage of the terminal amino group from the amino acid L-arginine, producing NO. Hemolysis causes the release of arginase-1, which depletes L-arginine eliminating the NO precursor (13). Additionally, studies have shown that free hemoglobin produced by intravascular hemolysis in SCD consumes NO (14, 15). Impaired NO production is also related to hemolysis-induced generation of asymmetric dimethylarginine, an endogenous NO synthase inhibitor (16). Production of heme due to oxidation of free hemoglobin also activates Toll-like receptor 4 promoting vaso-occlusive events (17, 18). Interestingly, a growing body of literature indicates a possible role for free hemoglobin in the pathogenesis of PAH in patients with no known hemolysis. Rafikova et al. found that free hemoglobin levels were significantly higher in patients with known PAH compared to patients with no PAH. Furthermore, the severity of PAH correlated with higher cell-free hemoglobin levels. In an animal model of PAH, inhibition of heme translocation using sulfasalazine prevented the development of PH (19).

Endothelin-1 (ET-1), a potent vasoconstrictor of pulmonary vascular bed, plays a role in the imbalance between vasodilators and vasoconstrictors in SCD. Plasma and urine levels of ET-1 are elevated in patients with SCD compared with matched controls (20). Furthermore, Phelan et al. showed that exposure of cultured human endothelial cells to previously sickled erythrocytes results in enhanced gene expression of ET-1 (21).

Hypercoagulability is another important factor that adds to pulmonary endothelial dysfunction. A hypercoagulable state results from reduced NO, asplenia (functional or surgical), and activation of thrombotic factors (tissue factor, platelets, and thrombin) in SCD (22, 23). This is supported by findings of pulmonary thromboemboli in 38–80% of postmortem lung examinations of deceased SCD patients (24, 25).

Proliferative arteriopathy seen in SCD-PH implies possible contribution of angiogenic factors to the pathogenesis. A recent case-control study of children with β-thalassemia major demonstrated a significantly higher serum level of vascular endothelial growth factor in patients with PH than those without PH or matched healthy children (26).

Finally, a significant number of patients with SCD have been found to have restrictive cardiomyopathy and diastolic dysfunction (27). In fact, 50% of patients with SCD and PH have pulmonary artery wedge pressure (PAWP) of >15 mmHg, consistent with pulmonary venous hypertension (i.e., post-capillary PH) (3, 5, 28). Vaso-occlusive events, systemic hypertension, iron overload (due to transfusions) and end-organ damage (e.g., renal failure) in patients with chronic hemolysis contribute to diastolic left ventricular dysfunction as well (29). Collectively, all these factors increase the risk of developing PH in patients with SCD and other hemolytic anemias.

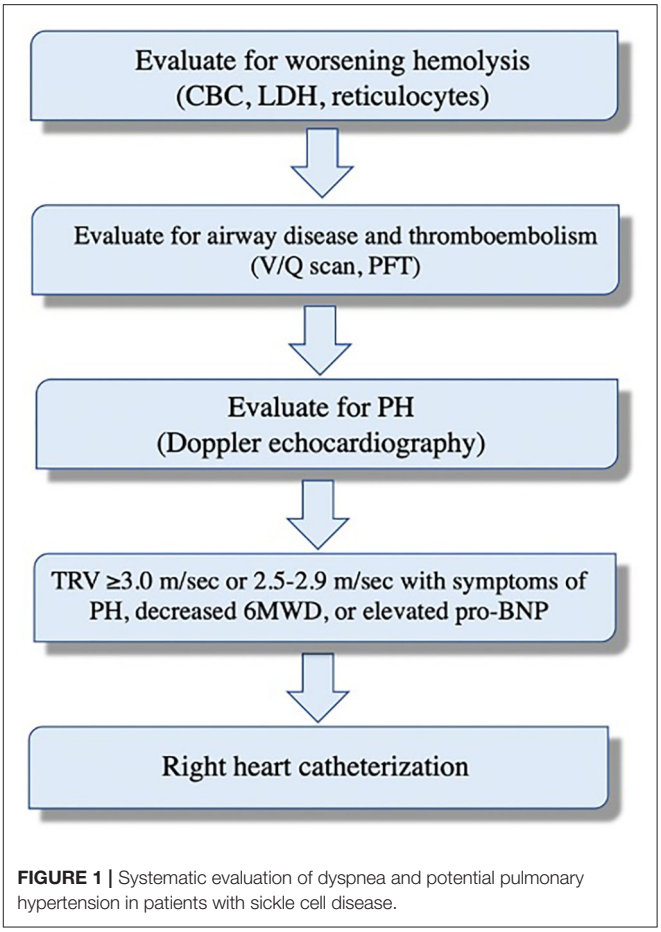


FIGURE 1 | Systematic evaluation of dyspnea and potential pulmonary hypertension in patients with sickle cell disease.

Evaluation

Pulmonary symptoms are common in patients with chronic hemolysis and often are multifactorial. Patients with SCD, for example, may have recurrent chest pains, fatigue, and chronic shortness of breath. These symptoms can be related to anemia, acute chest syndrome, obstructive airway disease, thromboembolism, and/or PH (30–32). Therefore, these should be systematically evaluated (**Figure 1**). Asymptomatic patients also remain at greater risk of developing PH; therefore, screening with Doppler echocardiography is recommended. The American Thoracic Society guidelines recommend screening patients with SCD with Doppler echocardiography every 1–3 years to assess mortality risk. Importantly, echocardiography should not be performed within 4 weeks of acute chest syndrome or within 2 weeks of acute vaso-occlusive crisis as these acutely increase the pulmonary artery systolic pressure (PASP) due to hypoxemia and worsening anemia (33). There are no screening guidelines

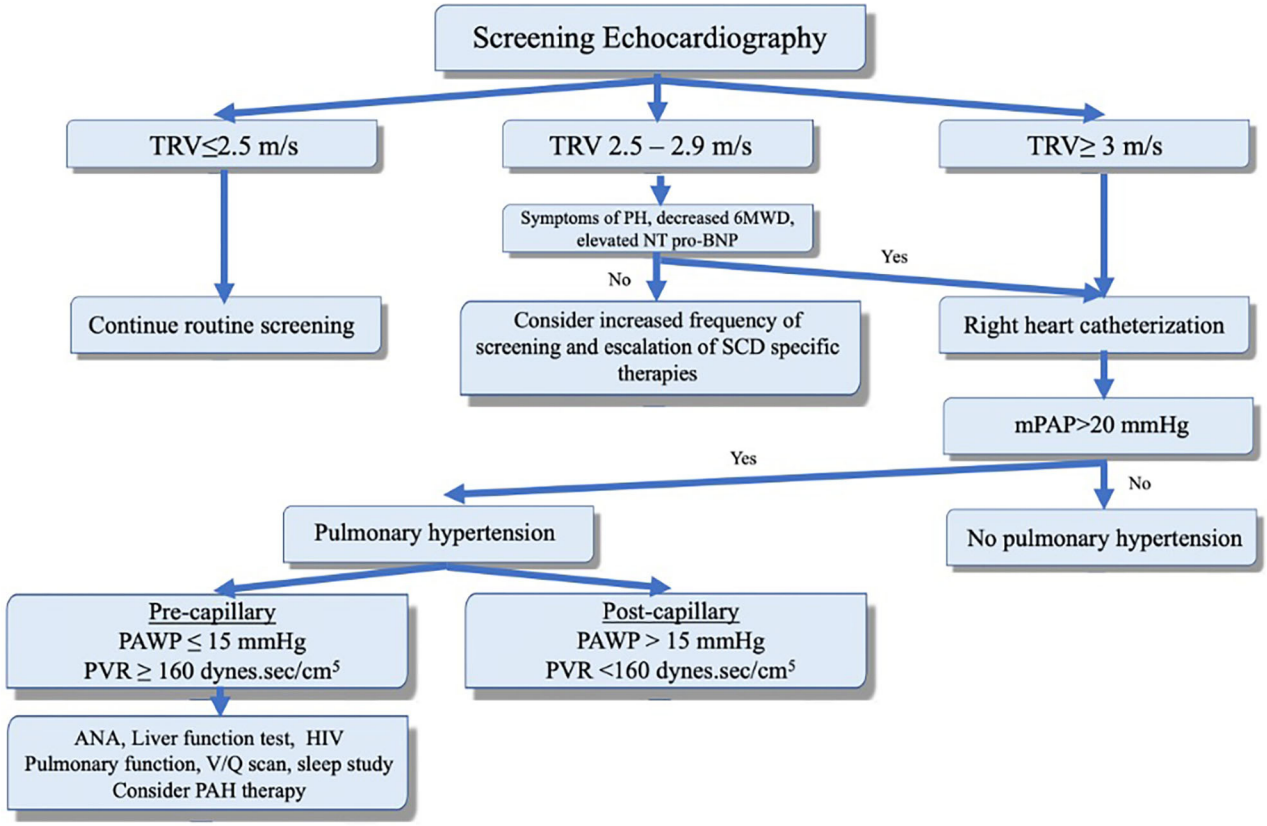


FIGURE 2 | Screening, diagnosis, and clinical classification to guide management of pulmonary hypertension in the context of sickle cell disease. Adapted with permission of the American Thoracic Society. Copyright © 2020 American Thoracic Society. All rights reserved. Klings et al. (33). The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society. Readers are encouraged to read the entire article for the correct context. The authors, editors, and The American Thoracic Society are not responsible for errors or omissions in adaptations.

for patients with other hemolytic diseases. Nonetheless, it is reasonable to obtain echocardiography on high-risk patients (e.g., severe hemolysis, asplenia, prior venous thromboembolism, and iron overload), especially those with unexplained pulmonary symptoms (34).

PH is suspected if the TRV on echocardiography is >2.5 m/sec. However, TRV ≥2.5 m/s has a low positive predictive value as only 31% of such patients had mPAP ≥25 mmHg on RHC (27). A TRV ≥3 m/s is 3 standard deviations above the population mean and is more specific (PH confirmed in 66–77% of patients on RHC) (4, 5). A TRV between 2.5 and 3 m/s combined with either an NT-proBNP > 164.5 pg/ml or 6-min walk distance of < 333 m increases the likelihood of PH and warrants further evaluation and RHC (**Figure 2**) (3, 5). PASP is commonly estimated indirectly by measuring the right ventricular systolic pressure which (in the absence of pulmonic stenosis) are equivalent. Using the modified Bernoulli equation ($P = 4V^2$), the PASP is calculated from the TRV. This means that minor variations in measurement of TRV can result in significant differences in estimated pulmonary pressures. Furthermore, even when accurate, elevated PASP can be related to high cardiac output often seen in patients with anemia. On the other hand, PH can be missed on echocardiography as the TRV is not always measurable (35). Thus, RHC remains the test of choice to confirm the presence of PH and assess the PAWP, cardiac output, and calculated PVR. It is worth mentioning that reduced blood viscosity and high cardiac output (due to anemia) result in a lower baseline PVR than healthy subjects. Thus, PVR of >2 Wood units is considered elevated in these patients (3–5).

Blood biomarkers can also be helpful in the evaluation of PH in chronic hemolytic anemia. Lactate dehydrogenase is a commonly used marker for hemolysis has been shown to correlate with NO resistance and severity of PH (36). NT-proBNP is another useful biomarker for PH and right ventricular dysfunction. In a study by Machado et al., elevated NT- proBNP (>160 pg/mL) in SCD patients correlated with TRV and was predictive of PH. Additionally, elevated NT- proBNP was an independent predictor of mortality (37).

Treatment

Management of PH in hemolytic anemias includes general measures such as supplemental oxygen to reverse hypoxemia and prevent its deleterious effect on the pulmonary vascular tone. Diuretics are used to treat volume overload but should

be used cautiously due to risk of sickling in patients with SCD. Lifelong anticoagulation is indicated in SCD patients with PH secondary to CTEPH. Although surgical pulmonary thromboendarterectomy (PTE) can be curative in CTEPH, it is undoubtedly more challenging in SCD patients due to the increased risk of sickling and occlusive crises during cardiopulmonary bypass. The best perioperative approach in these patients remains controversial. Avoidance of hypoxemia, hypothermia, and acidosis reduces the risk of perioperative complications. Additionally, exchange transfusion to reduce hemoglobin S to <20% prior to surgery is advised (38, 39). This aggressive transfusion regimen, however, has not been shown to reduce perioperative complications in patients with SCD (40). Inoperable patients are considered for balloon pulmonary angioplasty (BPA). In a recent study of PH in SCD, three patients with CTEPH underwent BPA with significant reduction in PVR in 2 patients and normalization of mPAP in the third patient (41).

Optimizing treatment of the underlying hemolytic disease is of paramount importance. Hydroxyurea is a myelosuppressive agent that has been shown to decrease sickle cell hemoglobin polymerization, reducing hemolysis and frequency of acute chest syndrome, and vaso-occlusive crises. Furthermore, hydroxyurea decreases hospitalization and mortality in SCD patients with HbSS and is currently recommended for patients with more than one episode of acute chest syndrome or >3 vaso-occlusive crises per year (33, 42–44). The risk of developing PH is also reduced with hydroxyurea in patients with thalassemia (45). Chronic transfusion is recommended in SCD patients who fail to respond or cannot tolerate hydroxyurea. This strategy is associated with improvement in pulmonary vascular changes in SCD patients (46). Similarly, adherence to transfusion and iron chelation therapy in patients with thalassemia prevented PH (47, 48).

SCD patients who remain symptomatic despite optimization of their SCD therapy (i.e., hydroxyurea, transfusion) are considered for PH-targeted therapy. This is usually reserved for patients with moderate to severe (based on functional class) RHC-confirmed pre-capillary PH with relatively reduced cardiac output. In general, endothelin receptor antagonists (ERAs) are recommended in patients with moderate PH (Functional class 2-3). Bosentan, an endothelin receptor antagonist, was evaluated in two randomized placebo-controlled trials of SCD patients with RHC-proven pre- and post-capillary PH. Both studies were terminated early due to slow enrollment but modest improvement in hemodynamic parameters was observed. Efficacy endpoints were not analyzed to draw definitive conclusions but bosentan was well-tolerated (49). Patients with more severe PH (class IV functional status, evidence of right-sided heart failure, or reduced cardiac output) can be treated with prostanoids. The efficacy of prostacyclins has been evaluated in a small retrospective study of 11 patients who received treprostinil or epoprostenol and showed improvement in right ventricular systolic pressure on echocardiography (50). Patients who need such therapy should be referred to centers with expertise in managing PH in SCD patients due to concerns of complications (e.g., high-cardiac output pulmonary edema, line thrombosis). Phosphodiesterase type 5 inhibitors (PDE-5Is) should generally be avoided in this population due to risk of increased hospitalizations for painful crises noted in a study of sildenafil (51). Riociguat is a soluble guanylate cyclase stimulator that does not rely on NO and, therefore, is an attractive agent for SCD-PH. Riociguat is currently approved for treatment of Group 1 PAH and Group 4 CTEPH. Weir et al. recently reported in a small case series tolerability of riociguat in four of six patients with sickle related CTEPH. Additionally, riociguat was associated with significant improvement in exercise capacity (mean 6MWD improvement of 56.8 m), functional class, NT-proBNP, and RVSP in 3 patients. Vaso-occlusive crisis developed in only one patient who was not on hydroxyurea (52). A phase 2, multi-multi-center, randomized, double-blind, placebo-controlled, study is now underway to evaluate the effectiveness and safety of riociguat in patients with sickle cell disease.

Myeloproliferative Disorders

Prevalence

Myeloproliferative disorders (MPDs) are characterized by clonal expansion of the multipotent hematopoietic progenitor cell with overproduction of one of the blood elements (i.e., erythrocytes, leukocytes, or platelets). The prevalence of PH in patients with MPDs has been evaluated in multiple small studies. Most of these studies used Doppler echocardiography (PASP>35 mmHg) to diagnose PH, with an estimated prevalence of 13–48% of PH in MPDs (53–56). Interestingly, in a larger study of 103 patients with MPDs, the prevalence of PH was much lower with only 5 patients found to have PH (57). Among MPDs, patients with chronic myeloid leukemia, polycythemia vera, essential thrombocytosis, and myelofibrosis are particularly at higher risk of developing PH. The presence of PH is associated with poor prognosis in MPDs.

Pathogenesis

PH in MPDs has two main phenotypes: chronic thromboembolic (CTEPH) and proliferative pulmonary arteriopathy. Thrombotic events (both venous and arterial) and microcirculatory disturbances are common in polycythemia vera and essential thrombocytosis patients. In a large study of 1,213 patients with polycythemia vera, 41% developed thrombotic complications (58). This translates into higher risk of CTEPH. In a retrospective small study of 10 patients with MPDs and RHC-confirmed PH, six patients were diagnosed with CTEPH at the time of MPD diagnosis. CTEPH was strongly associated with elevated hematocrit (59). Presence of cardiovascular comorbidities, history of thrombosis or splenectomy and increased age further increase the risk of thrombosis in these patients (60–62).

High hematocrit in polycythemia vera is associated with hypercoagulability as demonstrated by the progressive increase in blood viscosity that parallels higher hematocrit values (63). The major impact of hematocrit on blood viscosity is in the venous (low-shear) circulation as it decreases blood flow and increases the risk of venous thrombosis. In the arterial circulation, under high-shear, platelets are displaced to the periphery by the large red cell mass and come in contact with endothelial cells, causing platelets activation (release of thromboxane A2, platelet-derived growth factor, vascular endothelial growth factor) and thrombosis (64, 65). The abnormalities of erythrocytes and

platelets in MPDs also contribute to the aggregation of red blood cells and disturbed blood flow. Furthermore, chronically activated leukocytes (especially with JAK2 mutation) interact with platelets and endothelial cells and increase the risk of thrombosis (66–69). Splenectomy is used in the treatment of MPDs and can be associated with a hypercoagulable state as well (61). Notably, obstruction of pulmonary arteries in MPDs is not always related to thrombi but can be secondary to tumor emboli or circulating megakaryocytes with subsequent release of vasoactive substances that will further increase the tone in the pulmonary vascular bed (70, 71).

The second PH phenotype in MPDs is pulmonary arteriopathy which can result from different mechanisms. Portopulmonary hypertension may complicate chronic liver disease which is known to occur in MPDs (72). Medication-induced PH, fitting into Group 1 PH, is another potential cause of PH in these patients. Dasatinib, a tyrosine kinase inhibitor commonly used for treatment of chronic myeloid leukemia, has been associated with development of PH which seems to improve after discontinuation (73). Several TKIs other than dasatinib (e.g., bosutinib, ponatinib, lapatinib) have also been implicated in the development of PH. In a small study of 27 patients who received lapatinib, three patients were found to have PH by RHC with resolution of PH in all three after cessation of therapy (74).

Alkylating agents such as cyclophosphamide, melphalan, and busulfan (used for treatment of myelofibrosis) can cause PH with severely reduced diffusing capacity of the lung for carbon monoxide and abnormal findings on computed tomography of the chest (septal thickening, ground glass opacities, and lymph node enlargement) that are consistent with pulmonary veno-occlusive disease. Unlike dasatinib-induced PH, these patients have a poorer prognosis. Extramedullary hematopoiesis may complicate MPDs and result in infiltrative changes in the portal circulation with subsequent development of portal hypertension and portopulmonary hypertension. In cases of lung involvement, extramedullary hematopoiesis may lead to pulmonary vascular occlusion and PH. Proliferative changes in the pulmonary vessels may also occur due to increased circulating vascular endothelial growth factors and enhanced angiogenesis in MPDs (75). Finally, pre-capillary PH can be related to abnormal JAK2 signaling and depletion of NO. This is supported by improvement in PH and NO with JAK2 inhibitors.

Treatment

Since hematologic abnormalities contribute directly to the pulmonary vascular changes seen in MPDs, treatment of the underlying disease is a potential strategy. Indeed, allogeneic hematopoietic stem cell transplant and JAK inhibitors to treat MPDs have been shown to improve PH (76). For example, treatment with ruxolitinib (JAK1/JAK2 inhibitor) in 15 patients with myelofibrosis and PH resulted in improvement in right ventricular function and PASP by echocardiography in 66% of patients and was associated with significant reduction in NT-proBNP and increase in NO level (77). In cases of drug-induced PH, improvement in PH can be expected with discontinuation of the offending agent (especially in dasatinib-induced PH) although this alone may not be enough to reverse PH. When appropriate, CTEPH is treated with PTE or BPA. Riociguat, a guanylate cyclase stimulator, is associated with improved functional status and hemodynamics in patients with inoperable disease or suboptimal post-PTE hemodynamic outcomes (78, 79). External beam radiotherapy to the thorax has been used to treat extramedullary hematopoiesis in patients with MPDs and PH with improvement in pulmonary artery pressures (80). PAH-specific therapy has not been studied in MPD-associated PH.

PULMONARY HYPERTENSION IN SARCOIDOSIS

Prevalence

Sarcoidosis is characterized by granulomatous inflammation to an unknown trigger that primarily affects the lungs. The prevalence of sarcoidosis-associated PH (SAPH) is variable. This variation comes from inconsistency in methods used to diagnose SAPH. Autopsy studies have shown pulmonary vascular involvement in ∼5% of patients with significant parenchymal lung disease (81). Similar prevalence has been found in studies that evaluated SAPH based on clinical evidence of right ventricular failure (i.e., cor pulmonale) (82). However, studies that used hemodynamic assessment using RHC to diagnose SAPH reported higher prevalence up to 23% (83, 84). The prevalence is substantially higher in patients with pulmonary symptoms and advanced lung disease due to sarcoidosis. In a study of 15 patients with advanced fibrotic sarcoidosis, Emirgil et al. found evidence of PH on RHC in 10 patients (67%) (85). Another study of 363 patients with advanced sarcoidosis (listed for lung transplantation) found evidence of PH on RHC in 74% of these patients (86). Racial differences may significantly impact the course of disease and risk of developing PH. For example, in a recent large prospective study of 512 patients, predominantly Caucasian, with sarcoidosis, SAPH was found in 2.9% of those with intermediate or high probability for PH (by echocardiography) (87). It is important to note that some patients with sarcoidosis may have normal pulmonary artery pressure at rest with evidence of exercise-induced PH (88).

Pathogenesis

There are several mechanisms of PH in sarcoidosis. Each one of these may have a different response and outcome to a specific therapy. Pre-capillary PH may result from hypoxic pulmonary vasoconstriction and destruction of the distal vascular bed with advanced interstitial lung disease. This is supported by the finding that the majority of SAPH patients have advanced fibrotic lung disease (89–91). This usually leads to mild to moderate PH (mPAP <35 mmHg). Nevertheless, at least one third of sarcoidosis patients have no significant fibrosis which indicates that hypoxemia and fibrosis are not the only explanation for SAPH.

Granulomatous vasculopathy and angiitis with obliteration of arterioles or venules has been reported in autopsies of patients with pulmonary sarcoidosis (92–94). Occlusion of pulmonary vessels by granulomas causes PH and sometimes can be mistaken with thromboembolic disease. This pathology is associated with more severe PH than that related to lung fibrosis. Granulomatous

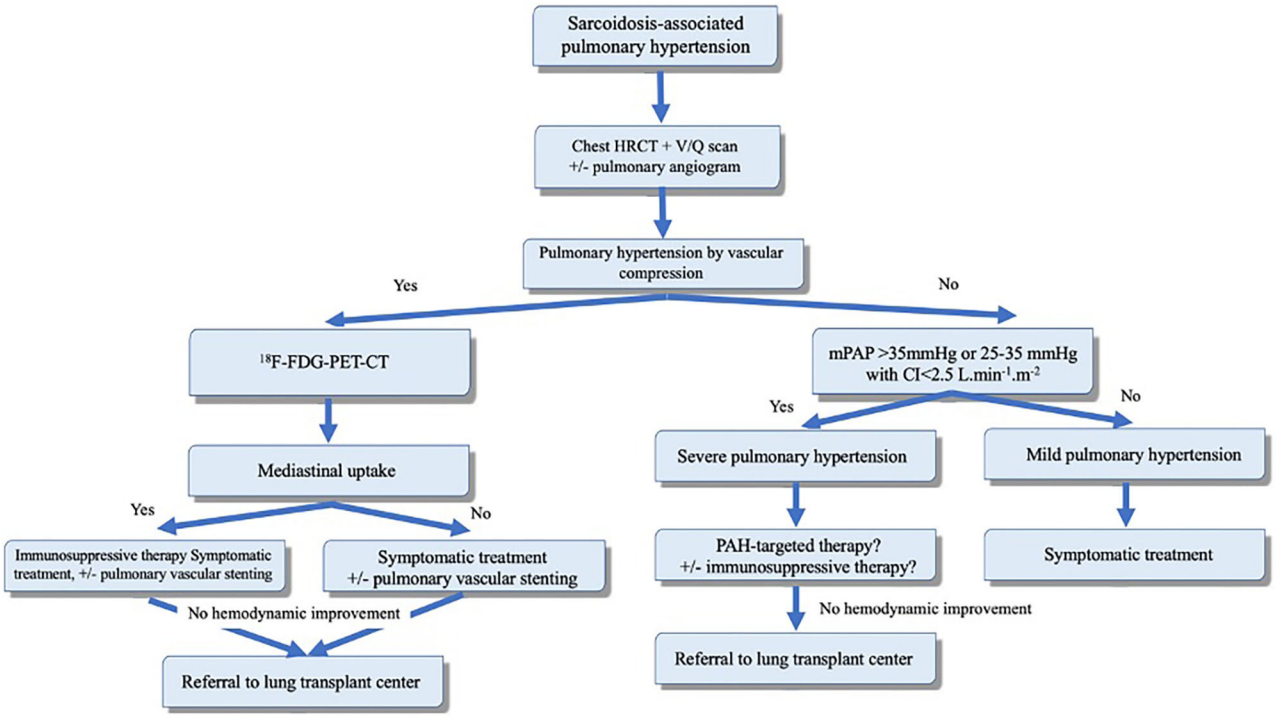


FIGURE 3 | Proposed algorithm for evaluation and management of sarcoidosis-associated pulmonary hypertension (SAPH). Adapted from Boucly et al. (101). Reproduced with permission of the © ERS 2020.

vasculitis can also affect the post-capillary vessels causing focal stenosis and a clinical pattern consistent with pulmonary veno-occlusive disease (95, 96).

Moreover, sarcoidosis is associated with increased risk of venous thromboembolism, which carries associated risk of developing CTEPH in some patients (97, 98). Sarcoidosis can be associated with mediastinal and hilar lymphadenopathy that can cause extrinsic compression of pulmonary arteries (and venous stenosis) or fibrosing mediastinitis, resulting in SAPH.

Cardiac involvement occurs in 20–25% of patients with sarcoidosis (99). Thus, post-capillary PH can develop in sarcoidosis due systolic or diastolic dysfunction with left ventricular involvement. In a retrospective study of 130 sarcoidosis patients with persistent dyspnea, 29% of patients with SAPH had an elevated PAWP >15 mmHg (89). Because cardiac involvement can be subtle, screening for cardiac sarcoidosis using magnetic resonance imaging and 18 F-fluorodeoxyglucose positron emission tomography is recommended (100). Finally, post-capillary SAPH can be due to extrinsic compression of the pulmonary veins.

Treatment

As outlined earlier, SAPH can be related to multiple mechanisms (**Figure 3**). However, some patients may have a prominent mechanism. For example, in the presence of advanced parenchymal (fibrotic) lung disease, PH with mPAP of <35 mmHg is likely related to hypoxemia and destruction of the pulmonary vascular bed associated with fibrotic changes. Treatment in these patients should focus on treatment of parenchymal lung disease and correction of hypoxemia. PAH-specific therapy carries the risk of worsening ventilation-perfusion mismatch and, therefore, should be avoided.

PAH-specific therapy can be considered in carefully selected patients with moderate to severe SAPH that is "out-of-proportion" to the degree of parenchymal lung disease and hypoxia (i.e., mPAP >35 mmHg). Such degree of PH suggests a component of vasculopathy that may respond to pulmonary vasodilators. Vasoreactivity has been demonstrated in a small prospective observational study of 8 patients with moderate-to-severe SAPH with improvement in mPAP and PVR to inhaled NO (102). This highlights the potential role of pulmonary vasodilators in these patients. Such therapy has been evaluated in multiple studies and shown to improve the hemodynamic parameters with no significant change in functional status or 6-min walk distance (**Table 3**). Similarly, in a double-blind placebo-controlled trial, bosentan improved mPAP and PVR, but failed to demonstrate benefit in 6-min walk distance (110).

Immunosuppressive therapy alone may result in hemodynamic improvement in patients with granulomatous vasculopathy or compressive lymphadenopathy (101). SAPH due to extrinsic compression can be treated with pulmonary artery or vein angioplasty and stenting (114). Overall, patients with SAPH have poor prognosis and should be evaluated early for lung transplantation.

TABLE 3 | Existing published studies evaluating treatment of sarcoid-associated pulmonary hypertension.

Publication	Study design (number of subjects)	Treatment (# of patients)	Outcome
Preston at al. (102)	Prospective observational (8)	Inh NO (5), inh NO with IV epo (1), CCB (2)	Short term 20% decreased PVR and mPAP; long term increased 6MWT
Culver et al. (103)	Retrospective chart review (7)	Bosentan (3), bosentan and IV epo (4)	Decreased mPAP at 6–18 mo in about 50% patients
Fisher et al. (104)	Retrospective case series (7)	IV epo (6), subcut trep (1)	Improved functional class
Milman et al. (105)	Retrospective chart review (12)	Sildenafil (12)	Decreased mPAP and PVR, increased CO, no change 6MWT
Barnett et al. (106)	Retrospective case series (22)	IV epo (1), bosentan (12), sildenafil (9)	Increased 6MWT and functional class, decreased mPAP and PVR
Baughman et al. (107)	Prospective open label 16 weeks (15)	Inh iloprost (15)	Decreased mPAP and PVR in 6 of 15 and increased 6MWT in 3 of 15 patients
Baughman et al. (89)	Retrospective chart review (5)	Bosentan (5)	Decreased mPAP in 3 of 5 patients at 4 mo
Judson et al. (108)	Prospective open label 12 weeks (25)	Ambrisentan (21)	No change 6MWT; 11 patients discontinued drug at 12 weeks
Dobarro et al. (109)	Retrospective chart review (8)	Sildenafil (9), bosentan (2); only 8 followed up with repeat RHC	Increased 6MWT and decreased NT-proBNP; non-statistically significant increase in CO/CI and decreased PVR
Baughman et al. (110)	Prospective placebo-controlled 16 weeks (35)	Bosentan (23), placebo (12)	Decreased mPAP and PVR; no change in 6MWT
Keir et al. (111)	Retrospective (33)	Sildenafil (29), bosentan (3)	Increased 6MWT, decreased NT-proBNP, improved TAPSE
Bonham et al. (112)	Retrospective case series (26)	Parenteral prostacyclin with epo (7) and trep (6), ERAs (12), PDE-5I (20), CCB (1)	Increased CI/CO, decreased PVR, improved functional class, decreased NT-proBNP
Ford et al. (113)	Prospective open label 24 weeks (12)	Tadalafil (12)	No change 6MWT at 24 weeks

Inh, inhaled; NO, nitric oxide; IV, intravenous; epo, epoprostenol; CCB, calcium channel blocker; mPAP, mean pulmonary arterial pressure; PVR, pulmonary vascular resistance; 6MWT, 6 min walk test; CO, cardiac output; CI, cardiac index; mo, months; NT-proBNP, N terminal pro brain natriuretic peptide; TAPSE, tricuspid annular plane systolic excursion.

PULMONARY HYPERTENSION IN CHRONIC KIDNEY DISEASE AND END STAGE RENAL DISEASE

The evaluation and management of PH in the context of chronic kidney disease (CKD), particularly end stage renal disease (ESRD), presents unique challenges to the clinician in terms of elucidating the main drivers of PH in this patient population. The clinical presentation of such patients is one in which PH is often multifactorial, hence the placement of this clinical entity into Group 5 of the PH classification schemata. As such, management relies in large part on attempts to correct and optimize multiple physiologic derangements often seen in CKD and ESRD.

Definition, Epidemiology, and Scope of the Problem

CKD is defined as abnormal renal function present for at least 3 months and is staged in severity based on the degree of reduction of glomerular filtration rate and the degree of albuminuria. ESRD is defined as renal failure requiring regular interval, long-term dialysis, or renal transplant to maintain survival. The true prevalence of PH amongst patients with CKD and ESRD is difficult to discern, due to the lack of robust data. Most of what is known is drawn from relatively small, retrospective, and almost exclusively echocardiography based studies. Amongst patients with CKD and not yet on dialysis, the prevalence of PH was observed to be upwards of 30% or more. Prevalence was highest among those with most advanced CKD (CKD 5) (115, 116). When considering those patients on hemodialysis (HD), a meta-analysis of known studies by Tang and colleagues (116) showed the prevalence of PH (based largely on echocardiography) between 19 and 56%. There is less data available with regard to the prevalence of PH in the setting of peritoneal dialysis (PD), but it appears to be lower than that of HD (117).

O'Leary et al. (118) recently published a retrospective analysis of a large cohort of patients with varying degrees of CKD that underwent RHC. PH was present in 1,267/1,873 (68%) of patients, using the accepted definition of PH at that time of mean PAP $\geq$ 25 mmHg. Of these, 76% had post-capillary PH and 24% had pre-capillary disease, using elevated PAWP ($\geq$ 15 mmHg) as the differentiating factor. The authors also noted that mortality was higher among patients with advanced stages of CKD, and further compounded by the presence of PH. The distribution of hemodynamic profiles seen underscores the significant role of Group 2 PH phenomenon related to elevated left ventricular filling pressures in driving PH in an overwhelming majority of patient with CKD. Comorbid left ventricular systolic and/or diastolic dysfunction, as well as a propensity to volume overload at times are the main drivers of this. However, many of those with CKD/ESRD with and without elevated PAWP have other factors driving PH as well. Among these are alterations in circulating

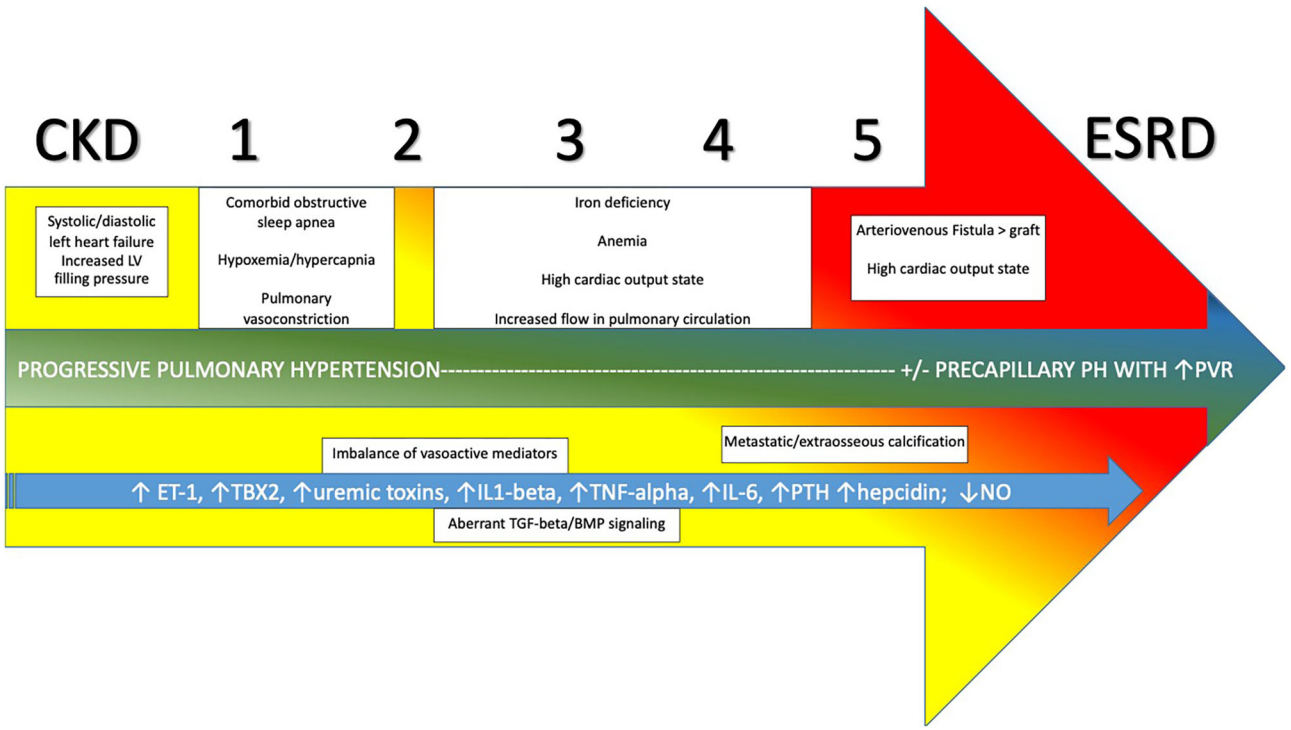


FIGURE 4 | Evolution and development of pulmonary hypertension in CKD progressing through stages 1–5 to ESRD and relevant contributors. CKD, Chronic Kidney Disease; ESRD, End Stage Renal Disease; PVR, pulmonary vascular resistance; ET-1, endothelin-1; TBX2, thromboxane 2; IL1-beta, interleukin-1-beta; TNG-alpha, tumor necrosis factor-alpha; IL-6, interleukin-6; PTH, parathyroid hormone; NO, nitric oxide; TGF-beta, transforming growth factor-beta; BMP, bone morphogenetic protein.

inflammatory and vasoactive mediators, endothelial dysfunction, alterations in cardiopulmonary flow related to anemia and arteriovenous fistula (AVF), factors related to dialysis itself, and comorbid Group 3 PH processes (see **Figure 4**).

Altered Inflammatory and Vasoactive Mediators and Related Endothelial Dysfunction

The presence of uremic toxins in patients with advanced CKD or ESRD can contribute to pulmonary vascular endothelial dysfunction. Specifically, mono-methyl L- Arginine, symmetric dimethyl arginine, and asymmetric dimethyl arginine are present in increased levels and inhibit NO synthase, impairing the intrinsic production and bioavailability of NO, the vasodilatory effects of which serve to maintain normal pulmonary vascular tone. There is also evidence that increased levels of uremic toxins are associated with increased inflammation, endothelial dysfunction, and oxidative stress (119). There is decreased availability of circulating NO in patients on HD with PH vs. those without PH. In addition, patients on HD with PH showed decreased ability to recover NO levels after HD, suggesting impairment in NO synthesis (120). As noted previously, ET-1 contributes to pulmonary vascular remodeling, is present in excess in patients with Group 1 PAH, and is also known to be elevated in patients with CKD and those on HD. Further, HD does not facilitate clearance of ET-1 and levels remain elevated after HD is performed (120). Angiopoietin 1 and 2 are additional mediators that modulate vascular smooth muscle growth via action on endothelial cells. There is evidence that both are important in the pathogenesis of CKD and PAH (121).

Cellular inflammation is a key driver of disease in both PH and CKD. In addition to the noted effects of uremic toxins, levels of circulating pro-inflammatory cytokines are increased in patients on HD with PH relative to those without, particularly IL-1beta, TNF-alpha, and IL-6. High-sensitivity c-reactive protein was also noted to be higher in the group with PH (122). The transforming growth factor beta (TGF-beta) and bone morphogenetic protein (BMP) signaling pathways modulate downstream gene expression that controls properties of vascular structure and tone. It has been demonstrated that this pathway is aberrant in CKD and PAH, leaning in a pro-inflammatory direction that favors vascular remodeling. Thus, there is a strong likelihood that the TGF-beta/BMP axis is relevant in patients with both PH and CKD/ESRD (123, 124).

Alterations in Cardiopulmonary Flow

Intentional arteriovenous shunting caused by the surgical creation of AVF or placement of a synthetic arteriovenous graft (AVG) contributes to a high cardiac output state. This leads initially to increased loading on the left ventricle. When this phenomenon is coupled with the increased output state, a

syndrome of high-output heart failure may manifest. With time, persistence of this high flow is thought to lead to increased shear forces on the pulmonary vascular endothelium that can cause pulmonary vascular remodeling and an intrinsic arteriopathy in some patients (125). The propensity to develop PH is higher in patients with AVF relative to AVG, as AVF can enlarge with time and develop progressively higher flows, which in turn increases flow through the pulmonary circulation to increasing degrees. It has been noted when flow through the fistula exceeds 2 L/min or more than 20% of cardiac output, the risk of pulmonary hypertension is higher. Furthermore, presence of the AVF on the upper arm presents increased likelihood of PH as compared to AVF of the lower arm (126).

Practices regarding the placement and maintenance of AVF for HD have come under a bit more scrutiny in recent years as a result of noted effects on circulatory and pulmonary vascular physiology. There has been deliberate advocacy on the part of the Centers for Medicare and Medicaid Services to utilize AVF over catheter-based HD in most patients, driven largely by legitimate concern for the risk of catheter related bloodstream infection and thrombosis (127). Since the inception of this initiative, it has been increasingly recognized that some patients with CKD/ESRD may have subclinical or mild PH before arterio-venous access for dialysis is placed. Some advocate for more systematic echocardiographic screening for PH prior to placement of AVF. If evidence of PH is present, then PD and/or expedited/prioritized renal transplant evaluation may be more appropriate (128). Further study is needed in this regard.

Anemia due to reduced renal production of erythropoietin, as well as iron deficiency and anemia of chronic disease is very common in patients with CKD and ESRD. This contributes to compensatory increase in cardiac output (much like that seen in SCD) and thus exposes the pulmonary circulation to higher than usual flow and the risks of vascular remodeling noted above. Iron deficiency in both PAH and CKD appears to be modulated largely by increased levels of hepcidin, which inhibits enteric iron transport and uptake (129, 130). Iron deficiency thus may ultimately affect pulmonary vascular regulation through downstream effects on hypoxia inducible factors. Risk for development of PH in the context of CKD/ESRD and anemia is most significant when hemoglobin is $<$10 g/dl (131).

Factors Related to Dialysis

As noted, HD presents a higher likelihood of developing PH, due in part to the use of AVF or AVG in many patients. In addition, it appears that the type of dialysis membrane used may contribute to presence of PH as well. Thromboxane B2, a pro-proliferative, pro-thrombotic, and pro-inflammatory molecule, is known to be elevated in patients with PAH compared to controls. In addition, it has been shown that thromboxane B2 levels are higher in patients on HD and/or with PH as compared to those not on HD and/or with no evidence of PH (132). The reason for elevated thromboxane B2 in some patients on HD appears to be related to increased release from circulating monocytes when cellulose dialysis filter membranes are utilized as compared to polysulfone membranes (133, 134).

The likelihood of PH is also directly proportional to older age and number of years on HD (131). The reason for this is likely simply related to the duration of exposure of the pulmonary circulation to the aforementioned pathophysiologic effects of HD. An additional phenomenon that may contribute to pulmonary vascular changes and PH is the showering of micro-bubbles from the dialysis machine with each HD session. These micro-bubbles have the potential to obstruct small pulmonary capillaries, leading to local inflammatory changes and microthrombosis, setting the stage for progressive pre-capillary arteriopathy to progress as HD continues over time (135, 136).

Comorbid Group 3 PH Processes

As noted previously, Group 3 PH is often driven by abnormal oxygenation and ventilation, commonly seen during sleep in patients with obstructive sleep apnea (OSA). There is a high prevalence of OSA amongst patients with CKD, independent of the need for HD (137, 138). The associated hypoxemia, and in some cases hypercapnia, contributes to pulmonary vasoconstriction and some degree of PH in many patients with CKD. Asymmetric dimethyl arginine is also noted to be elevated in patients with sleep disordered breathing, implicating impairment of NO production as at least one plausible molecular mechanism for the development of PH attributable to OSA (139). This pathway may be of particular relevance to patients with CKD. As such, screening for nocturnal hypoxemia and OSA with formal polysomnography is of paramount importance. Those found to have sleep disordered breathing should be titrated for and treated with nocturnal non-invasive positive airway pressure therapy.

In addition to OSA, it has been noted that secondary hyperparathyroidism seen in CKD/ESRD can lead to extraosseous calcium deposition in the interalveolar septae and pulmonary arterial system. Alveolar septal deposition can contribute to restrictive physiology on pulmonary function testing, as well as reduction in diffusing capacity (140). The overall clinical impact of these phenomenon with respect to the development of PH is not as clear compared to that of OSA. Additionally, it has been shown that parathyroid hormone levels do not differ between patients with and without PH in the context of CKD/ESRD (141).

PH Diagnosis and Invasive Hemodynamic Evaluation in Patients With CKD/ESRD

As with all forms of pulmonary hypertension, the initial preferred tool for screening is transthoracic echocardiography. This is especially important in CKD/ESRD patients with unexplained dyspnea. Timing of echocardiography is most optimal soon after the completion of a session of dialysis, with the patient at or very near their established dry weight. The presence of estimated PASP $\geq$ 50 mmHg and or significant right ventricular dilatation or dysfunction should prompt invasive hemodynamic measurement by RHC. As with echocardiography, RHC should be performed in the relative immediate time period after a session of dialysis, such that the left and right sided circulation are as volume and pressure off-loaded as possible, ensuring that hemodynamic

measurements are reflective largely of alterations in flow and/or PVR that may be present.

The PEPPER study (142) was an important endeavor that helped to understand the frequency of PH in patients with advanced CKD (stage 4 or 5) and unexplained dyspnea, as well as to understand the effect of HD on pulmonary hemodynamics. In the study, 31 CKD patients not on dialysis and 31 patients on HD with dyspnea underwent RHC. In the group on HD, 78% were found to have PH (13% pre-capillary PH, 65% post-capillary PH). Among those not on HD, 77% had PH (6% pre-capillary PH, 71% post-capillary PH). Further, in the group on HD, 25/31 underwent RHC pre and post dialysis. It was noted that mean PAP and PAWP were both significantly lower post-dialysis, underscoring the importance of timing of RHC relative to HD.

In patients that are on HD and have an AVF, it may be useful to study the effects of fistula occlusion during RHC, however there is scant literature to guide this practice. This must be done cautiously and for a relatively short time, as there is some risk of associated thrombosis of the AVF. Published studies looking at the hemodynamic effects of fistula compression are echocardiography based. Yigla et al. found that after compression of AVF for 1 min in four patients on HD with echocardiographic evidence of PH resulted in a 10% reduction in mean estimated PA systolic pressure and a 15% reduction in estimated cardiac output (reduction from supranormal values) (143).

Treatment of PH in the Context of CKD and ESRD

Given that the majority of PH in the context of ESRD/HD is pulmonary venous hypertension, the mainstay of treatment is optimizing volume status with diuretics or dialysis, controlling systemic blood pressure, and medical therapies that optimize systolic and diastolic function of the left ventricle. The importance of correcting hypoxemia and sleep disordered breathing when relevant was previously discussed. If clinical history or risk factors suggest that CTEPH may be a possibility, then consideration must be given to screening ventilation-perfusion lung scan. If there is evidence of CTEPH, then additional imaging and hemodynamic workup is indicated to determine if the patient is a candidate for PTE or BPA. For patients in whom there is exceedingly high AVF flow and elevation in cardiac output driving PH, consideration should be given to revision of the AVF via banding or ligation (144, 145).

In general, trials of off label use of PAH-specific therapies in group 5 PH related to CKD/ESRD should be reserved for a very select few patients for whom all other contributing factors to PH have been optimized but have persistent and significant RHC-proven PVR elevation, particularly in the context of reduced cardiac output reflective of RV failure. The presence of clear Group 1 PAH comorbidities such as connective tissue disease, HIV, or portal hypertension, may also make a case for treatment with PAH therapies more compelling in the setting of a hemodynamic profile consistent with clear pre-capillary disease. As with all group 5 PH, there is no randomized, controlled data regarding the use of PAH-specific therapy in CKD/ESRD associated PH. Though not absolutely contraindicated in renal failure, PDE-5 inhibitors and riociguat should be used with caution in patients with advanced CKD/ESRD due to potential alterations in pharmacokinetics. As such, dose adjustments may be needed in this context. (146, 147).

Renal transplant surgery is generally regarded as not presenting prohibitive levels of operative risk to patients with mild to moderate degrees of PH and RV dysfunction, particularly considering the procedure is not usually associated with large intravascular volume and hemodynamic shifts as seen for example in liver transplantation. Patients with significant PH, and particularly severe right ventricular dysfunction, are considered with an abundance of caution for renal transplantation, especially if interventions to attempt to improve pulmonary hemodynamics have failed. There is no clear evidence based data available to systematically risk stratify patients with ESRD and PH for renal transplant surgery (148). Independent of immediate perioperative surgical risk however, it is noted that pre-transplant PH (based on echocardiographic studies) is associated with a significant increased risk of graft failure (149). Furthermore, post-transplant survival is decreased in patients with elevated estimated PASP on echocardiogram $>$ 50 mmHg and in those with elevated PVR $>$ 3 Wood units (150, 151). In general, approach to renal transplantation candidacy in the setting of PH should be done in an individualized, multidisciplinary fashion, especially given the lack of robust studies to reliably predict outcomes.

PULMONARY HYPERTENSION ASSOCIATED WITH LESS COMMON COMORBIDITIES

Pulmonary Langerhans Cell Histiocytosis

Pulmonary Langerhans cell histiocytosis (PLCH) is an uncommon cystic lung disease characterized by pathologic dendritic cell infiltration of the walls of the distal bronchiole. The involved dendritic cell subset, the Langerhans cell, localizes within the airway epithelium and processes encountered inhaled antigens, becoming activated when additional molecular signals indicate a state of danger (152). Activated dendritic cells migrate to lymphoid tissue to induce the adaptive immune response. PLCH occurs almost exclusively in smokers (153), and smokers with or without pulmonary diseases have significantly greater presence of Langerhans cells in the lung (154, 155). Cigarette smoking further induces production of molecules such as TNF-alpha, TGF-beta, GM-CSF, and osteopontin; these factors promote development, activation, and survival of Langerhans cells, though migration out of the airway tissue may be impaired (156–158). The bronchiolocentric nodules characteristic of PLCH contain a mixed population of Langerhans and other inflammatory cell types (159). Factors predisposing the small population of smokers developing PLCH remain incompletely understood.

Relative to other forms of chronic lung disease, PLCH is associated with higher prevalence of PH, and the vascular component of disease can be great (160, 161). PH has been

found in 41–100% of PLCH patients, with prevalence particularly high (>90%) in those undergoing lung transplant evaluation (160, 162–164). Presence of PH has been consistently associated with increased mortality in PLCH (163, 165). While inverse correlation between pulmonary artery pressure elevation and spirometry has been noted in some studies (163, 166), others have not demonstrated this finding (160, 161). Examinations of tissue specimens have demonstrated typical vascular changes of PAH, including intimal fibrosis and medial hypertrophy of small and medium-sized pulmonary arteries (160). Observation of Langerhans cells with vessels is rare, and vascular involvement can also be noted in regions without parenchymal infiltration of Langerhans cells (160, 161). In contrast to most forms of PAH, capillary and venular pathology appears to be common in PLCH-PH (160, 165–168).

Management of PLCH, for those with or without associated PH, should most importantly center around smoking cessation. Stabilization and even substantial disease regression has been observed in those successful at quitting (169–172). While most case reports and series have focused on improvements in radiology or pulmonary function following smoking cessation, benefits on the pulmonary vascular component of disease should also be presumed. One case report of a patient with PLCH and associated PH has described PH resolution following smoking cessation (173). For patients with symptomatic PLCH refractory to smoking cessation, steroids, and chemotherapeutic agents have been used with mixed results (174–178). Use of PAH-specific therapy in those with PLCH-PH has been best described in a series by Le Pavec et al., where 14 patients were treated with an ERA, PDE-5I, and/or inhaled iloprost. Use of PAH-specific therapy was associated with improvements in mPAP and PVR on follow-up hemodynamic reassessments; a trend toward improved survival was also observed (167). With histologic observation of pulmonary venous involvement in PLCH-PH, the potential for development of pulmonary edema in response to PAH-specific therapy must be acknowledged, and cases consistent with this outcome have been documented after initiation of intravenous epoprostenol (160). Encouragingly, oxygenation of treated patients did not worsen in the series by Le Pavec (167). Given vascular complexity, lung transplant should be considered in those with associated PH and in others with severe PLCH. PLCH patients make up <1 percent of lung transplant recipients in the United States, with overall similar post-transplant outcomes to other patient groups (162, 164).

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder caused by mutation in NF1, the tumor suppressor gene encoding neurofibromin. Incidence is 1 in 3,000 births, with near complete penetrance; skin manifestations are usually prominent and early in onset. Major manifestations leading to diagnosis include café au lait macules, cutaneous and subcutaneous neurofibromas, axillary and inguinal freckling, and Lisch nodules of the iris (179). NF1-related parenchymal lung disease affects 10–20% of adults and is characterized by cysts, micronodules, and interstitial abnormalities (179–181). Arterial and venous vascular lesions, including arterio-venous malformations, arterial aneurysms, coarctation of the abdominal aorta, and renal artery stenosis, are important but under recognized sources of morbidity in NF1 (182, 183). Neurofibromin is expressed in endothelial and vascular smooth muscle cells, and it is thus suspected that absence of functional protein leads to dysregulation of growth and proliferation of intimal and medial layer cells, in systemic and pulmonary circulations (182, 184). Patients with NF1 also have increased malignancy risk, and cancer is the leading cause of death with life expectancy reduced by 10–15 years (185, 186).

Pulmonary hypertension in NF1 patients has been described only in case reports and series, thus prevalence of NF1-PH is difficult to estimate but thought to be rare. The largest series of PH in NF1 recently characterized 49 patients from the French PH National Referral Center. Despite NF1 affecting males and female equally, 80% of NF1-PH patients were female, and median age at diagnosis of PH was 62. No specific NF1 mutations were associated with PH, and others with NF1-PH were not observed in the families of most patients. Hemodynamics at diagnosis were consistent with severe pre-capillary PH (mean mPAP 45 with PVR 10.7 WU). The majority of patients had normal or mildly abnormal spirometry, with decreased diffusion capacity for carbon monoxide (median 30% predicted) and resting hypoxemia. Imaging was most frequently notable for diffuse lung cysts, ground glass opacities, and emphysema (187). Importantly, this series and another literature review have identified normal imaging or single radiologic abnormalities in nearly 30% of NF1-PH patients (184, 187). Lung tissue has been infrequently available; the few histologic studies have demonstrated arterial remodeling with muscularization of small arterioles as well as particular increase in intimal layer thickness (187, 188). Findings of thrombosis have not been noted, and presence of plexiform lesions has been variable (187, 189). Three patients undergoing transplant all had pulmonary venous wall thickening (187), and a patient with pulmonary capillary hemangiomatosis associated with NF1 has also been reported (190).

Prognosis of NF1-PH is poor, with transplant free survival of 87, 54, and 42% at 1, 3, and 5 years, respectively (187). Of patients in the large series by Jutant et al., 45/49 were started on PAH-specific therapy; ERAs, PDE-5Is, and prostacyclin derivatives were all used alone or in combination. Treated patients demonstrated improvement in cardiac index and PVR; however, oxygenation worsened slightly and clinical improvements were modest and not sustained (187). Lung transplantation has been successfully performed for NF1 patients (187, 188); however, concern for malignancy risk following transplant and immunosuppression can be a barrier (191). The tyrosine kinase inhibitor sorafenib, which is capable of suppressing kinases operating in pathways also shared by targets of neurofibromin, was used in a single patient with refractory NF1-PH. Hemodynamic and clinical improvements resulted after 3 months of use (192). Given neurofibromin's role in containment of cell proliferation, investigation of drugs impacting cell cycle regulation are particularly of interest in NF1-PH.

Gaucher Disease

Gaucher disease (GD) is an autosomal recessive lysosomal storage disease with an incidence of 1 in 50,000 births. GD

results from inadequate function of the lysosomal enzyme glucocerebrosidase, leading to accumulation of its substrate in macrophages, described as Gaucher cells. Accumulation of macrophages is particularly prominent in the bone marrow, liver, and spleen, with resulting hepatosplenomegaly, thrombocytopenia, anemia, osteopenia, and painful bone crises. GD is classified into three subtypes (GD 1, 2, and 3), with GD type 1, the non-neuronopathic form of GD, being the most common. Pulmonary involvement can be seen in all GD types, though those with GD1 are at most risk for developing pulmonary vascular complications of the disease (193). Enzyme replacement therapy for GD was first approved by the United States Food and Drug Administration in 1991 and has provided significant improvements in hematologic counts, spleen and liver volumes, and other clinical parameters for GD type 1 (194, 195). Importantly, use of enzyme replacement therapy has dramatically decreased need for splenectomy, previously performed for GD due to lack of other management options (195–197).

The prevalence of PH in adult GD type 1 patients, as estimated by echocardiography, ranges from 7 to 30% (198, 199). Mild PASP elevation (35–50 mmHg) is noted in the majority, with 0.76% documented to have PASP >50 (198). Treatment with enzyme replacement therapy is associated with lower prevalence of PH (7.4% in treated patients vs. 30% in untreated patients) (198). Though enzyme replacement therapy has been questioned to be causative of PH (200, 201), most current evidence suggests protection, and multiple examples of PH-related clinical improvements after initiation exist (198). In contrast, splenectomy has strong association with development of GD-PH. Severe PH complicating GD occurs predominantly in females who have undergone splenectomy as part of GD management (198, 199). The largest published series of GD-PH patients included 14 adult patients from a single referral center. Females made up 71% of the population, with a mean age of 36 at PH diagnosis, and all patients had previously undergone splenectomy at mean age 12. Other pertinent findings from this series includes lack of correlation between development of PH and GD severity by other metrics. Heteroallelic mutations (with one copy of the most common mutation N370S) were more prevalent than N370S homozygotes (199). Of interest, this report and others provide examples of patients also meeting criteria for hepatopulmonary syndrome; in these cases PH was either coexisting with hepatopulmonary syndrome or was unmasked after initiation of enzyme replacement therapy and resulting resolution of hepatopulmonary syndrome (199, 202). When tissue has been available, observed arterial changes have resembled those of PAH, including medial hypertrophy and intimal fibrosis, as well as plexiform lesions (203–205). In addition, intravascular Gaucher cells have been observed, though are not uniformly present (205–207).

If not previously initiated, most GD-PH patients will start on enzyme replacement therapy, with case reports of this alone leading to significant improvement (198, 199). ERAs, PDE-5Is, and prostanoids have all been used in individual patients with GD-PH, generally with positive outcomes (198, 199, 208). Given shared risk factor (splenectomy) with CTEPH, use of anticoagulation should be discussed particularly in splenectomized patients, though data about its role is lacking. Coumadin has been used in several patients in combination with enzyme replacement therapy and PAH-specific therapy (198). In cases of refractory GD-PH, imatinib has been used successfully in a single patient (209). Finally, though other comorbidities can pose a challenge to candidacy, lung transplant has been used as a treatment modality for a few cases of severe GD-PH (203, 205).

DISCUSSION

Group 5 pulmonary hypertension remains a varied and challenging group of clinical entities. Understanding of the pathogenetic factors underlying many of the clinical conditions in the group has advanced considerably, however the relative rarity and/or variable clinical presentation of many of these disease processes has made it challenging to identify unifying treatment principles that can be applied broadly. Greater understanding may be gained by enhanced prospective registries of the more common conditions associated with PH, particularly sarcoidosis, sickle cell disease, myeloproliferative disorders, and chronic kidney disease/ESRD. More robust randomized trials of PAH therapies will be needed to understand if any of the ever-increasing number of PAH treatments may be of benefit in any of these populations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

HF has received institutional research grant support from United Therapeutics/Lung Biotechnology, Actelion/Jannsen, Liquidia, and Eiger. He has received consulting honoraria from Actelion/Jannsen, United Therapeutics/Lung Biotechnology, Liquidia, Bayer, and Altavant. BL has received institutional research grant support from United Therapeutics and Actelion/Jannsen. MA-Q has received institutional research grant support from Bellerophon and Actelion/Jannsen.

REFERENCES

1. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J.* (2019) 53:1801913. doi: 10.1183/13993003.01913-2018
2. Rosenzweig EB, Abman SH, Adatia I, Beghetti M, Bonnet D, Haworth S, et al. Paediatric pulmonary arterial hypertension: updates on definition,

classification, diagnostics and management. *Eur Respir J*. (2019) 53:1801916. doi: 10.1183/13993003.01916-2018
3. Fonseca GH, Souza R, Salemi VM, Jardim CV, Gualandro SF. Pulmonary hypertension diagnosed by right heart catheterisation in sickle cell disease. *Eur Respir J*. (2012) 39:112–8. doi: 10.1183/09031936.00134410
4. Mehari A, Gladwin MT, Tian X, Machado RF, Kato GJ. Mortality in adults with sickle cell disease and pulmonary hypertension. *JAMA*. (2012) 307:1254–6. doi: 10.1001/jama.2012.358
5. Parent F, Bachir D, Inamo J, Lionnet F, Driss F, Loko G, et al. A hemodynamic study of pulmonary hypertension in sickle cell disease. *N Engl J Med*. (2011) 365:44–53. doi: 10.1056/NEJMoa1005565
6. Aessopos A, Farmakis D, Karagiorga M, Voskaridou E, Loutradi A, Hatziliami A, et al. Cardiac involvement in thalassemia intermedia: a multicenter study. *Blood*. (2001) 97:3411–6. doi: 10.1182/blood.V97.11.3411
7. Grisaru D, Rachmilewitz EA, Mosseri M, Gotsman M, Lafair JS, Okon E, et al. Cardiopulmonary assessment in beta-thalassemia major. *Chest*. (1990). 98:1138–42. doi: 10.1378/chest.98.5.1138
8. Derchi G, Galanello R, Bina P, Cappellini MD, Piga A, Lai ME, et al. Prevalence and risk factors for pulmonary arterial hypertension in a large group of β-thalassemia patients using right heart catheterization: a Webthal study. *Circulation*. (2014) 129:338–45. doi: 10.1161/CIRCULATIONAHA.113.002124
9. Crary SE, Ramaciotti C, Buchanan GR. Prevalence of pulmonary hypertension in hereditary spherocytosis. *Am J Hematol*. (2011) 86:E73–6. doi: 10.1002/ajh.22182
10. Das A, Bansal D, Ahluwalia J, Das R, Rohit MK, Attri SV, et al. Risk factors for thromboembolism and pulmonary artery hypertension following splenectomy in children with hereditary spherocytosis. *Pediatr Blood Cancer*. (2014) 61:29–33. doi: 10.1002/pbc.24766
11. Jaïs X, Ioos V, Jardim C, Sitbon O, Parent F, Hamid A, et al. Splenectomy and chronic thromboembolic pulmonary hypertension. *Thorax*. (2005) 60:1031–4. doi: 10.1136/thx.2004.038083
12. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries *in vivo*. *Circulation*. (1995) 91:1314–9. doi: 10.1161/01.CIR.91.5.1314
13. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, et al. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA*. (2005) 294:81–90. doi: 10.1001/jama.294.1.81
14. Liu, X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR Jr. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem*. (1998) 273:18709–13. doi: 10.1074/jbc.273.30.18709
15. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO III, Schechter AN, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. (2002). 8:1383–9. doi: 10.1038/nm1202-799
16. Kato GJ, Wang Z, Machado RF, Blackwelder WC, Taylor JG VI, Hazen SL. Endogenous nitric oxide synthase inhibitors in sickle cell disease: abnormal levels and correlations with pulmonary hypertension, desaturation, haemolysis, organ dysfunction and death. *Br J Haematol*. (2009) 145:506–13. doi: 10.1111/j.1365-2141.2009.07658.x
17. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. (2014) 123:377–90. doi: 10.1182/blood-2013-04-495887
18. Ghosh S, Adisa OA, Chappa P, Tan F, Jackson KA, Archer DR, et al. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. *J Clin Invest*. (2013) 123:4809–20. doi: 10.1172/JCI64578
19. Rafikova O, Williams ER, McBride ML, Zemskova M, Srivastava A, Nair V, et al. Hemolysis-induced lung vascular leakage contributes to the development of pulmonary hypertension. *Am J Respir Cell Mol Biol*. (2018) 59:334–45. doi: 10.1165/rcmb.2017-0308OC
20. Tharaux PL, Hagège I, Placier S, Vayssairat M, Kanfer A, Girot R et al. Urinary endothelin-1 as a marker of renal damage in sickle cell disease. *Nephrol Dial Transplant*. (2005) 20:2408–13. doi: 10.1093/ndt/gfi111
21. Phelan M, Perrine SP, Brauer M, Faller DV. Sickle erythrocytes, after sickling, regulate the expression of the endothelin-1 gene and protein in human endothelial cells in culture. *J Clin Invest*. (1995) 96:1145–51. doi: 10.1172/JCI118102
22. Berney SI, Ridler CD, Stephens AD, Thomas AE, Kovacs IB. Enhanced platelet reactivity and hypercoagulability in the steady state of sickle cell anaemia. *Am J Hematol*. (1992) 40:290–4. doi: 10.1002/ajh.2830400409
23. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in sickle cell anemia. *J Clin Invest*. (1998) 101:1899–904. doi: 10.1172/JCI1932
24. Carstens GR, Paulino BBA, Katayama EH, Amato-Lourenço LF, Fonseca GH, Souza R, et al. Clinical relevance of pulmonary vasculature involvement in sickle cell disease. *Br J Haematol*. (2019) 185:317–26. doi: 10.1111/bjh.15795
25. Graham JK, Mosunjac M, Hanzlick RL, Mosunjac M. Sickle cell lung disease and sudden death: a retrospective/prospective study of 21 autopsy cases and literature review. *Am J Forensic Med Pathol*. (2007) 28:168–72. doi: 10.1097/01.paf.0000257397.92466.50
26. Alkholy UM, Mohamed SA, Elhady M, Attar SE, Abdalmonem N, Zaki A. Vascular endothelial growth factor and pulmonary hypertension in children with beta thalassemia major. *J Pediatr*. (2019) 95:593–9. doi: 10.1016/j.jpedp.2018.06.015
27. Niss O, Quinn CT, Lane A, Daily J, Khoury PR, Bakeer N, et al. Cardiomyopathy with restrictive physiology in sickle cell disease. *JACC Cardiovasc Imaging*. (2016) 9:243–52. doi: 10.1016/j.jcmg.2015.05.013
28. Castro O, Hoque M, Brown BD. Pulmonary hypertension in sickle cell disease: cardiac catheterization results and survival. *Blood*. (2003) 101:1257–61. doi: 10.1182/blood-2002-03-0948
29. Sachdev V, Machado RF, Shizukuda Y, Rao YN, Sidenko S, Ernst I, et al. Diastolic dysfunction is an independent risk factor for death in patients with sickle cell disease. *J Am Coll Cardiol*. (2007) 49:472–9. doi: 10.1016/j.jacc.2006.09.038
30. Lisbona R, Derbekyan V, Novales-Diaz JA. Scintigraphic evidence of pulmonary vascular occlusion in sickle cell disease. *J Nucl Med*. (1997) 38:1151–3.
31. Santoli F, *Zerah* F, *Vasile* N, *Bachir* D, *Galacteros* F, *Atlan* G. Pulmonary function in sickle cell disease with or without acute chest syndrome. *Eur Respir J*. (1998) 12:1124–9. doi: 10.1183/09031936.98.12051124
32. Vichinsky EP, Styles LA, Colangelo LH, Wright EC, Castro O, Nickerson B. Acute chest syndrome in sickle cell disease: clinical presentation and course. cooperative study of sickle cell disease. *Blood*. (1997) 89:1787–92. doi: 10.1182/blood.V89.5.1787.1787_1787_1792
33. Klings ES, Machado RF, Barst RJ, Morris CR, Mubarak KK, Gordeuk VR, et al. An official American Thoracic Society clinical practice guideline: diagnosis, risk stratification and management of pulmonary hypertension of sickle cell disease. *Am J Respir Crit Care Med*. (2014) 189:727–40. doi: 10.1164/rccm.201401-0065ST
34. Morris CR, Kim HY, Trachtenberg F, Wood J, Quinn CT, Sweeters N, et al. Risk factors and mortality associated with an elevated tricuspid regurgitant jet velocity measured by Doppler-echocardiography in thalassemia: a Thalassemia Clinical Research Network report. *Blood*. (2011) 118:3794–802. doi: 10.1182/blood-2010-11-319152
35. Ristow B, Ali S, Ren X, Whooley MA, Schiller NB. Elevated pulmonary artery pressure by Doppler echocardiography predicts hospitalization for heart failure and mortality in ambulatory stable coronary artery disease: the Heart and Soul Study. *J Am Coll Cardiol*. (2007) 49:43–9. doi: 10.1016/j.jacc.2006.04.108
36. Kato GJ, McGowan V, Machado RF, Little JA, Taylor J VI, Morris CR, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood*. (2006) 107:2279–85. doi: 10.1182/blood-2005-06-2373
37. Machado RF, Anthi A, Steinberg MH, Bonds D, Sachdev V, Kato GJ, et al. N-terminal pro-brain natriuretic peptide levels and risk of death in sickle cell disease. *JAMA*. (2006). 296:310–8. doi: 10.1001/jama.296.3.310
38. Mahesh B, Besser M, Ravaglioli A, Pepke-Zaba J, Martinez G, Klein A, et al. Pulmonary endarterectomy is effective and safe in patients with haemoglobinopathies and abnormal red blood cells: the Papworth experience. *Eur J Cardiothorac Surg*. (2016) 50:537–41. doi: 10.1093/ejcts/ezw062

39. Yung GL, Channick RN, Fedullo PF, Auger WR, Kerr KM, Jamieson SW, et al. Successful pulmonary thromboendarterectomy in two patients with sickle cell disease. *Am J Respir Crit Care Med*. (1998) 157:1690–3. doi: 10.1164/ajrccm.157.5.9710032
40. Vichinsky EP, Haberkern CM, Neumayr L, Earles AN, Black D, Koshy M, et al. A comparison of conservative and aggressive transfusion regimens in the perioperative management of sickle cell disease. the preoperative transfusion in sickle cell disease study group. *N Engl J Med.* (1995) 333:206–13. doi: 10.1056/NEJM199512143332415
41. Savale L, Habibi A, Lionnet F, Maitre B, Cottin V, Jais X, et al. Clinical phenotypes and outcomes of precapillary pulmonary hypertension of sickle cell disease. *Eur Respir J*. (2019) 54:1900585. doi: 10.1183/13993003.00585-2019
42. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med.* (1995) 332:1317–22. doi: 10.1056/NEJM199505183322001
43. Ferster A, Vermylen C, Cornu G, Buyse M, Corazza F, Devalck C, et al. Hydroxyurea for treatment of severe sickle cell anemia: a pediatric clinical trial. *Blood*. (1996) 88:1960–4. doi: 10.1182/blood.V88.6.1960.bloodjournal8861960
44. Steinberg MH, Barton F, Castro O, Pegelow CH, Ballas SK, Kutlar A, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. *JAMA*. (2003) 289:1645–51. doi: 10.1001/jama.289.13.1645
45. Karimi, Borzouee M, Mehrabani A, Cohan N. Echocardiographic finding in beta-thalassemia intermedia and major: absence of pulmonary hypertension following hydroxyurea treatment in beta-thalassemia intermedia. *Eur J Haematol.* (2009) 82:213–8. doi: 10.1111/j.1600-0609.2008.01192.x
46. Detterich JA, Kato RM, Rabai M, Meiselman HJ, Coates TD, Wood JC. Chronic transfusion therapy improves but does not normalize systemic and pulmonary vasculopathy in sickle cell disease. *Blood*. (2015) 126:703–10. doi: 10.1182/blood-2014-12-614370
47. Aessopos A, Farmakis D, Hatziliami A, Fragodimitri C, Karabatsos F, Joussef J, Mitilineou E, et al. Cardiac status in well-treated patients with thalassemia major. *Eur J Haematol. (*2004) 73:359–66. doi: 10.1111/j.1600-0609.2004.00304.x
48. Taher AT, Musallam KM, Karimi M, El-Beshlawy A, Belhoul K, Daar S, et al. Overview on practices in thalassemia intermedia management aiming for lowering complication rates across a region of endemicity: the OPTIMAL CARE study. *Blood*. (2010) 115:1886–92. doi: 10.1182/blood-2009-09-243154
49. Barst RJ, Mubarak KK, Machado RF, Ataga KI, Benza RL, Castro O, et al. Exercise capacity and haemodynamics in patients with sickle cell disease with pulmonary hypertension treated with bosentan: results of the ASSET studies. *Br J Haematol.* (2010) 149:426–35. doi: 10.1111/j.1365-2141.2010.08097.x
50. Weir NA, Saiyed R, Alam S, Conrey A, Desai HD, George MP, et al. Prostacyclin-analog therapy in sickle cell pulmonary hypertension. *Haematologica.* (2017) 102:e163–5. doi: 10.3324/haematol.2015.131227
51. Machado RF, Barst RJ, Yovetich NA, Hassell KL, Kato GJ, Gordeuk VR, et al. Hospitalization for pain in patients with sickle cell disease treated with sildenafil for elevated TRV and low exercise capacity. *Blood*. (2011) 118:855–64. doi: 10.1182/blood-2010-09-306167
52. Weir NA, Conrey A, Lewis D, Mehari A. Riociguat use in sickle cell related chronic thromboembolic pulmonary hypertension: a case series. *Pulm Circ.* (2018) 8:2045894018791802. doi: 10.1177/2045894018791802
53. Altintas A, Karahan Z, Pasa S, Cil T, Boyraz T, Iltumur K, et al. Pulmonary hypertension in patients with essential thrombocythemia and reactive thrombocytosis. *Leuk Lymphoma.* (2007) 48:1981–7. doi: 10.1080/10428190701493928
54. Garypidou V, Vakalopoulou S, Dimitriadis D, Tziomalos K, Sfikas G, Perifanis V. Incidence of pulmonary hypertension in patients with chronic myeloproliferative disorders. *Haematologica.* (2004) 89:245–6.
55. Gupta R, Perumandla S, Patsiornik Y, Niranjan S, Ohri A. Incidence of pulmonary hypertension in patients with chronic myeloproliferative disorders. *J Natl Med Assoc.* (2006) 98:1779–82.
56. Reisner SA, Rinkevich D, Markiewicz W, Tatarsky I, Brenner B. Cardiac involvement in patients with myeloproliferative disorders. *Am J Med.* (1992) 93:498–504. doi: 10.1016/0002-9343(92)90576-W
57. Chebrek S, Aïssi K, Francès Y, Mercier C, Farnault L, Sébahoun G, et al. Pulmonary hypertension in patients with chronic myeloproliferative neoplasms. *Leuk Lymphoma*. (2014) 55:223–5. doi: 10.3109/10428194.2013.797083
58. Polycythemia vera: the natural history of 1213 patients followed for 20 years. Gruppo Italiano Studio Policitemia. *Ann Intern Med*. (1995) 123:656–64. doi: 10.7326/0003-4819-123-9-199511010-00003
59. Guilpain P, Montani D, Damaj G, Achouh L, Lefrère F, Le Pavec J, et al. Pulmonary hypertension associated with myeloproliferative disorders: a retrospective study of ten cases. *Respiration*. (2008) 76:295–302. doi: 10.1159/000112822
60. Marchioli R, Finazzi G, Landolfi R, Kutti J, Gisslinger H, Patrono C, et al. Vascular and neoplastic risk in a large cohort of patients with polycythemia vera. *J Clin Oncol*. (2005) 23:2224–32. doi: 10.1200/JCO.2005.07.062
61. Mohren M, Markmann I, Dworschak U, Franke A, Maas C, Mewes S, et al. Thromboembolic complications after splenectomy for hematologic diseases. *Am J Hematol*. (2004) 76:143–7. doi: 10.1002/ajh.20018
62. Wolanskyj AP, Schwager SM, McClure RF, Larson DR, Tefferi A. et al. Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. *Mayo Clin Proc*. (2006) 81:159–66. doi: 10.4065/81.2.159
63. Pearson TC, Wetherley-Mein G, Vascular occlusive episodes and venous haematocrit in primary proliferative polycythaemia. *Lancet*. (1978) 2:1219–22. doi: 10.1016/S0140-6736(78)92098-6
64. Huang PY, Hellums JD. Aggregation and disaggregation kinetics of human blood platelets: Part III. the disaggregation under shear stress of platelet aggregates. *Biophys J*. (1993) 65:354–61. doi: 10.1016/S0006-3495(93)81080-4
65. Turitto VT, Weiss HJ. Platelet and red cell involvement in mural thrombogenesis. *Ann N Y Acad Sci*. (1983) 416:363–76. doi: 10.1111/j.1749-6632.1983.tb35199.x
66. Arellano-Rodrigo E, Alvarez-Larrán A, Reverter JC, Villamor N, Colomer D, Cervantes F, et al. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. *Haematologica*. (2006) 91:169–75.
67. Carobbio A, Finazzi G, Guerini V, Spinelli O, Delaini F, Marchioli R, et al. Leukocytosis is a risk factor for thrombosis in essential thrombocythemia: interaction with treatment, standard risk factors, and Jak2 mutation status. *Blood*. (2007) 109:2310–3. doi: 10.1182/blood-2006-09-046342
68. Falanga A, Marchetti M, Evangelista V, Vignoli A, Licini M, Balicco M, et al. Polymorphonuclear leukocyte activation and hemostasis in patients with essential thrombocythemia and polycythemia vera. *Blood*. (2000) 96:4261–6. doi: 10.1182/blood.V96.13.4261.h8004261_4261_4266
69. Jensen MK, de Nully Brown P, Lund BV, Nielsen OJ, Hasselbalch HC. Increased circulating platelet-leukocyte aggregates in myeloproliferative disorders is correlated to previous thrombosis, platelet activation and platelet count. *Eur J Haematol*. (2001) 66:143–51. doi: 10.1034/j.1600-0609.2001.00359.x
70. Dot JM, Sztrymf B, Yaïci A, Dorfmüller P, Capron F, Parent F, et al. Pulmonary arterial hypertension due to tumor emboli. *Rev Mal Respir*. (2007) 24:359–66. doi: 10.1016/S0761-8425(07)91070-0
71. Marvin KS, Spellberg RD. Pulmonary hypertension secondary to thrombocytosis in a patient with myeloid metaplasia. *Chest*. (1993) 103:642–4. doi: 10.1378/chest.103.2.642
72. Toros AB, Gokcay S, Cetin G, Ar MC, Karagoz Y, Kesici B. et al. Portal hypertension and myeloproliferative neoplasms: a relationship revealed. *ISRN Hematol*. (2013) 2013:673781. doi: 10.1155/2013/673781
73. Montani D, Bergot E, Günther S, Savale L, Bergeron A, Bourdin A, et al. Pulmonary arterial hypertension in patients treated by dasatinib. *Circulation*. (2012) 125:2128–37. doi: 10.1161/CIRCULATIONAHA.111.079921
74. Alkhatib Y, Albashaireh D, Al-Aqtash T, Awdish R. The role of tyrosine kinase inhibitor "Lapatinib" in pulmonary hypertension. *Pulm Pharmacol Ther*. (2016) 37:81–4. doi: 10.1016/j.pupt.2016.03.002
75. Cortelezzi A, Gritti G, Del Papa N, Pasquini MC, Calori R, Gianelli U, et al. Pulmonary arterial hypertension in primary myelofibrosis is common and

associated with an altered angiogenic status. *Leukemia*. (2008) 22:646–9. doi: 10.1038/sj.leu.2404943
76. Faiz SA, Iliescu C, Lopez-Mattei J, Patel B, Bashoura L, Popat U. Resolution of myelofibrosis-associated pulmonary arterial hypertension following allogeneic hematopoietic stem cell transplantation. *Pulm Circ.* (2016) 6:611–13. doi: 10.1086/687291
77. Tabarroki A, Lindner DJ, Visconte V, Zhang L, Rogers HJ, Parker Y, et al. Ruxolitinib leads to improvement of pulmonary hypertension in patients with myelofibrosis. *Leukemia*. (2014) 28:1486–93. doi: 10.1038/leu.2014.5
78. Ghofrani HA, D'Armini AM, Grimminger F, Hoeper MM, Jansa P, Kim NH, et al. Riociguat for the treatment of chronic thromboembolic pulmonary hypertension. *N Engl J Med*. (2013) 369:319–29. doi: 10.1056/NEJMoa1209657
79. Ghofrani HA, Hoeper MM, Halank M, Meyer FJ, Staehler G, Behr J, et al. Riociguat for chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension: a phase II study. *Eur Respir J*. (2010) 36:792–9. doi: 10.1183/09031936.00182909
80. Steensma DP, Hook CC, Stafford SL, Tefferi A. Low-dose, single-fraction, whole-lung radiotherapy for pulmonary hypertension associated with myelofibrosis with myeloid metaplasia. *Br J Haematol.* (2002) 118:813–6. doi: 10.1046/j.1365-2141.2002.03695.x
81. Iwai K, Tachibana T, Takemura T, Matsui Y, Kitaichi M, Kawabata Y. Pathological studies on sarcoidosis autopsy. I. epidemiological features of 320 cases in Japan. *Acta Pathol Jpn.* (1993) 43:372–6. doi: 10.1111/j.1440-1827.1993.tb01148.x
82. Mayock RL, Bertrand P, Morrison CE, Scott JH. Manifestations of sarcoidosis. analysis of 145 patients, with a review of nine series selected from the literature. *Am J Med.* (1963) 35:67–89. doi: 10.1016/0002-9343(63)90165-7
83. Gluskowski J, Hawrylkiewicz I, Zych D, Zieliński J. Effects of corticosteroid treatment on pulmonary haemodynamics in patients with sarcoidosis. *Eur Respir J.* (1990) 3:403–7.
84. Rizzato G, Pezzano A, Sala G, Merlini R, Ladelli L, Tansini G, et al. Right heart impairment in sarcoidosis: haemodynamic and echocardiographic study. *Eur J Respir Dis.* (1983) 64:121–8.
85. Emirgil C, Sobol BJ, Herbert WH, Trout K. The lesser circulation in pulmonary fibrosis secondary to sarcoidosis and its relationship to respiratory function. *Chest.* (1971) 60:371–8. doi: 10.1378/chest.60.4.371
86. Shorr AF., Helman DL, Davies DB, Nathan SD. Pulmonary hypertension in advanced sarcoidosis: epidemiology and clinical characteristics. *Eur Respir J*. (2005) 25:783–8. doi: 10.1183/09031936.05.00083404
87. Huitema MP, Bakker ALM, Mager JJ, Rensing BJWM, Smits F, Snijder RJ, et al. Prevalence of pulmonary hypertension in pulmonary sarcoidosis: the first large European prospective study. *Eur Respir J.* (2019) 54:1900897. doi: 10.1183/13993003.00897-2019
88. Głuskowski J, Hawryłkiewicz I, Zych D, Wojtczak A, Zieliński J. Pulmonary haemodynamics at rest and during exercise in patients with sarcoidosis. *Respiration.* (1984) 46:26–32. doi: 10.1159/000194667
89. Baughman RP, Engel PJ, Taylor L, Lower EE. Survival in sarcoidosis-associated pulmonary hypertension: the importance of hemodynamic evaluation. *Chest.* (2010) 138:1078–85. doi: 10.1378/chest.09-2002
90. Bourbonnais JM, Samavati L. Clinical predictors of pulmonary hypertension in sarcoidosis. *Eur Respir J.* (2008) 32:296–302. doi: 10.1183/09031936.00175907
91. Handa T, Nagai S, Miki S, Fushimi Y, Ohta K, Mishima M, et al. Incidence of pulmonary hypertension and its clinical relevance in patients with sarcoidosis. *Chest*. (2006) 129:1246–52. doi: 10.1378/chest.129.5.1246
92. Rosen Y, Moon S, Huang CT, Gourin A, Lyons HA. Granulomatous pulmonary angiitis in sarcoidosis. *Arch Pathol Lab Med.* (1977) 101:170–4.
93. Smith LJ, Lawrence JB, Katzenstein AA. Vascular sarcoidosis: a rare cause of pulmonary hypertension. *Am J Med Sci.* (1983) 285:38–44. doi: 10.1097/00000441-198301000-00004
94. Takemura T, Matsui Y, Saiki S, Mikami R. Pulmonary vascular involvement in sarcoidosis: a report of 40 autopsy cases. *Hum Pathol.* (1992) 23:1216–23. doi: 10.1016/0046-8177(92)90288-E
95. Hoffstein V, Ranganathan N, Mullen JB. Sarcoidosis simulating pulmonary veno-occlusive disease. *Am Rev Respir Dis.* (1986) 134:809–11.
96. Jones RM, Dawson A, Jenkins GH, Nicholson AG, Hansell DM, Harrison NK. Sarcoidosis-related pulmonary veno-occlusive disease presenting with recurrent haemoptysis. *Eur Respir J.* (2009) 34:517–20. doi: 10.1183/09031936.00044609
97. Labyk A, Wretowski D, Zybińska-Oksiutowicz S, Furdyna A, Ciesielska K, Piotrowska-Kownacka D, et al. Balloon pulmonary angioplasty - efficient therapy of chronic thromboembolic pulmonary hypertension in the patient with advanced sarcoidosis - a case report. *BMC Pulm Med.* (2018) 18:139. doi: 10.1186/s12890-018-0695-4
98. Ungprasert P, Crowson CS, Matteson EL. Association of sarcoidosis with increased risk of VTE: a population-based study, 1976 to 2013. *Chest.* (2017) 151:425–30. doi: 10.1016/j.chest.2016.09.009
99. Birnie D, Ha AC, Gula LJ, Chakrabarti S, Beanlands RS, Nery P. Cardiac Sarcoidosis. *Clin Chest Med.* (2015) 36:657–8. doi: 10.1016/j.ccm.2015.08.008
100. Birnie DH, Sauer WH, Bogun F, Cooper JM, Culver DA, Duvernoy CS, et al. HRS expert consensus statement on the diagnosis and management of arrhythmias associated with cardiac sarcoidosis. *Heart Rhythm.* (2014) 11:1305–23. doi: 10.1016/j.hrthm.2014.03.043
101. Boucly A, Cottin V, Nunes H, Jaïs X, Tazi A, Prévôt G, et al. Management and long-term outcomes of sarcoidosis-associated pulmonary hypertension. *Eur Respir J*. (2017) 50:1700465. doi: 10.1183/13993003.00465-2017
102. Preston IR, Klinger JR, Landzberg MJ, Houtchens J, Nelson D, Hill NS. Vasoresponsiveness of sarcoidosis-associated pulmonary hypertension al. *Chest.* (2001) 120:866–72. doi: 10.1378/chest.120.3.866
103. Culver DA. Treatment of pulmonary hypertension in sarcoidosis. *Proc Am Thorac Soc.* (2005) 2:A862.
104. Fisher KA, Serlin DM, Wilson KC, Walter RE, Berman JS, Farber HW. Sarcoidosis-associated pulmonary hypertension: outcome with long-term epoprostenol treatment. *Chest.* (2006) 130:1481–8. doi: 10.1378/chest.130.5.1481
105. Milman N, Burton CM, Iversen M, Videbaek R, Jensen CV, Carlsen J. Pulmonary hypertension in end-stage pulmonary sarcoidosis: therapeutic effect of sildenafil? *J Heart Lung Transplant.* (2008) 27:329–34. doi: 10.1016/j.healun.2007.11.576
106. Barnett CF, Bonura EJ, Nathan SD, Ahmad S, Shlobin OA, Osei K, et al. Treatment of sarcoidosis-associated pulmonary hypertension. a two-center experience. *Chest.* (2009) 135:1455–61. doi: 10.1378/chest.08-1881
107. Baughman RP, Judson MA, Lower EE, Highland K, Kwon S, Craft N, et al. Inhaled iloprost for sarcoidosis associated pulmonary hypertension. *Sarcoidosis Vasc Diffuse Lung Dis.* (2009) 26:110–20.
108. Judson MA, Highland KB, Kwon S, Donohue JF, Aris R, Craft N, et al. Ambrisentan for sarcoidosis associated pulmonary hypertension. *Sarcoidosis Vasc Diffuse Lung Dis.* (2011) 28:139–45. doi: 10.1164/ajrccm-conference.2010.181.1_MeetingAbstracts.A2368
109. Dobarro D, Schreiber BE, Handler C, Beynon H, Denton CP, Coghlan JG. Clinical characteristics, haemodynamics and treatment of pulmonary hypertension in sarcoidosis in a single centre, and meta-analysis of the published data. *Am J Cardiol.* (2013) 111:278–85. doi: 10.1016/j.amjcard.2012.09.031
110. Baughman RP, Culver DA, Cordova FC, Padilla M, Gibson KF, Lower EE, et al. Bosentan for sarcoidosis-associated pulmonary hypertension: a double-blind placebo controlled randomized trial. *Chest.* (2014) 145:810–7. doi: 10.1378/chest.13-1766
111. Keir GJ, Walsh SL, Gatzoulis MA, Marino PS, Dimopoulos K, Alonso R, et al. Treatment of sarcoidosis-associated pulmonary hypertension: a single centre retrospective experience using targeted therapies. *Sarcoidosis Vasc Diffuse Lung Dis.* (2014) 31:82–90.
112. Bonham CA, Oldham JM, Gomberg-Maitland M, Vij R. Prostacyclin and oral vasodilator therapy in sarcoidosis-associated pulmonary hypertension: a retrospective case series. *Chest.* (2015) 148:1055–62. doi: 10.1378/chest.14-2546
113. Ford HJ, Baughman RP, Aris R, Engel P, Donohue JF. Tadalafil therapy for sarcoidosis-associated pulmonary hypertension. *Pulm Circ.* (2016) 6:557–62. doi: 10.1086/688775
114. Condado JF, Babaliaros V, Henry TS, Kaebnick B, Kim D, Staton GW Jr. Pulmonary stenting for the treatment of sarcoid induced pulmonary vascular stenosis. *Sarcoidosis Vasc Diffuse Lung Dis*. (2016) 33:281–7.

115. Shang W, Li Y, Ren Y, Li W, Wei HL, Dong J. Prevalence of pulmonary hypertension in patients with chronic kidney disease without dialysis: a meta-analysis. *Int Urol Nephrol.* (2018) 50:1497–504. doi: 10.1007/s11255-018-1853-6
116. Tang M, Batty JA, Lin C, Fan X, Chan KE, Kalim S. Pulmonary hypertension, mortality, and cardiovascular disease in CKD and ESRD patients: a systematic review and meta-analysis. *Am J Kidney Dis.* (2018) 72:75–83. doi: 10.1053/j.ajkd.2017.11.018
117. Zhang Q, Wang L, Zeng H, Lv Y, Huang Y. Epidemiology and risk factors in CKD patients with pulmonary hypertension: a retrospective study. *BMC Nephrol.* (2018) 19:1–8. doi: 10.1186/s12882-018-0866-9
118. O'Leary JM, Assad TR, Xu M, Birdwell KA, Farber-Eger E, Wells QS, et al. Pulmonary hypertension in patients with chronic kidney disease: invasive hemodynamic etiology and outcomes. *Pulm Circ.* (2017) 7:674–83 doi: 10.1177/2045893217716108
119. Böger RH. Asymmetric dimethylarginine (ADMA) and cardiovascular disease: insights from prospective clinical trials. *Vasc Med.* (2005) 10(Suppl. 1):S19–25 doi: 10.1177/1358836X0501000104
120. Nakhoul F, Yigla M, Gilman R, Reisner SA, Abassi Z. The pathogenesis of pulmonary hypertension in haemodialysis patients via arterio-venous access. *Nephrol Dial Transplant.* (2005) 20:1686–92. doi: 10.1093/ndt/gfh840
121. Nickel NP, O'Leary JM, Brittain EL, Fessel JP, Zamanian RT, West JD, et al. Kidney dysfunction in patients with pulmonary arterial hypertension. *Pulm Circ.* (2017) 7:38–54 doi: 10.1086/690018
122. Yu TM, Chen YH, Hsu JY, Sun CS, Chuang YW, Chen CH, et al. Systemic inflammation is associated with pulmonary hypertension in patients undergoing haemodialysis. *Nephrol Dial Transplant.* (2009) 24:1946–51. doi: 10.1093/ndt/gfn751
123. Rol N, Kurakula KB, Happé C, Bogaard HJ, Goumans MJ. TGF-β and BMPR2 signaling in PAH: Two black sheep in one family. *Int J Mol Sci.* (2018) 19:1–17. doi: 10.3390/ijms19092585
124. Chen L, Yang T, Lu DW, Zhao H, Feng YL, Chen H, et al. Central role of dysregulation of TGF-β/Smad in CKD progression and potential targets of its treatment. *Biomed Pharmacother.* (2018) 101:670–81. doi: 10.1016/j.biopha.2018.02.090
125. Alkhouli M, Sandhu P, Boobes K, Hatahet K, Raza F, Boobes Y. Cardiac complications of arteriovenous fistulas in patients with end-stage renal disease. *Nefrologia.* (2015) 35:234–45. doi: 10.1016/j.nefroe.2015.07.005
126. Basile C, Lomonte C, Vernaglione L, Casucci F, Antonelli M, Losurdo N. The relationship between the flow of arteriovenous fistula and cardiac output in haemodialysis patients. *Nephrol Dial Transplant.* (2008) 23:282–7. doi: 10.1093/ndt/gfm549
127. ESRD NCC (2019). Available from: https://esrdncc.org/en/fistula-first-catheter-last/ (accessed June 13, 2019).
128. Yigla M, Banderski R, Azzam ZS, Reisner SA, Nakhoul F. Arterio-venous access in end-stage renal disease patients and pulmonary hypertension. *Ther Adv Respir Dis.* (2008) 2:49–53. doi: 10.1177/1753465808089456
129. Rhodes CJ, Wharton J, Howard L, Gibbs JS, Vonk-Noordegraaf A, Wilkins MR. Iron deficiency in pulmonary arterial hypertension: a potential therapeutic target. *Eur Respir J.* (2011) 38:1453–60. doi: 10.1183/09031936.00037711
130. Ganz T. Iron balance and the role of hepcidin in chronic kidney disease. *Semin Nephrol.* (2016) 36:87–93. doi: 10.1016/j.semnephrol.2016.02.001
131. Navaneethan SD, Roy J, Tao K, Brecklin CS, Chen J, Deo R, et al. Chronic renal insufficiency cohort investigators. prevalence, predictors, and outcomes of pulmonary hypertension in CKD. *J Am Soc Nephrol.* (2016) 27:877–86. doi: 10.1681/ASN.2014111111
132. Abdelwhab S, Elshinnawy S. Pulmonary hypertension in chronic renal failure patients. *Am J Nephrol.* (2008) 28:990–7. doi: 10.1159/000146076
133. Mahiout A, Jörres A, Hiss R, Meinhold H, Kessel M. Effects of blood-dialyser interaction on prostaglandins in uraemic patients and in healthy man. *Nephrol Dial Transplant.* (1987) 2:546–50.
134. Kiykim AA, Horoz M, Ozcan T, Yildiz I, Sari S, Genctoy G. Pulmonary hypertension in hemodialysis patients without arteriovenous fistula: the effect of dialyzer composition. *Ren Fail.* (2010) 32:1148–52. doi: 10.3109/0886022X.2010.516854
135. Barak M, Nakhoul F, Katz Y. Pathophysiology and clinical implications of microbubbles during hemodialysis. *Semin Dial.* (2008) 21:232–8. doi: 10.1111/j.1525-139X.2008.00424.x
136. Perkett EA, Brigham KL, Meyrick B. Continuous air embolization into sheep causes sustained pulmonary hypertension and increased pulmonary vasoreactivity. *Am J Pathol.* (1988) 132:444-54.
137. Sakaguchi Y, Shoji T, Kawabata H, Niihata K, Suzuki A, Kaneko T, et al. High prevalence of obstructive sleep apnea and its association with renal function among nondialysis chronic kidney disease patients in Japan: a cross-sectional study. *Clin J Am Soc Nephrol.* (2011) 6:995–1000. doi: 10.2215/CJN.08670910
138. Zoccali C, Mallamaci F, Tripepi G. Nocturnal hypoxemia predicts incident cardiovascular complications in dialysis patients. *J Am Soc Nephrol.* (2002) 13:729–33.
139. Barceló A, de la Peña M, Ayllón O, Vega-Agapito MV, Piérola J, Pérez G, et al. Increased plasma levels of asymmetric dimethylarginine and soluble CD40 ligand in patients with sleep apnea. *Respiration.* (2009) 77:85–90. doi: 10.1159/000165630
140. Conger JD, Hammond WS, Alfrey AC, Contiguglia SR, Stanford RE, Huffer WE. Pulmonary calcification in chronic dialysis patients. clinical and pathologic studies. *Ann Intern Med.* (1975) 83:330–6. doi: 10.7326/0003-4819-83-3-330
141. Amin M, Fawzy A, Hamid MA, Elhendy A. Pulmonary hypertension in patients with chronic renal failure: role of parathyroid hormone and pulmonary artery calcifications. *Chest.* (2003) 124:2093–7. doi: 10.1378/chest.124.6.2093
142. Pabst S, Hammerstingl C, Hundt F, Gerhardt T, Grohé C, Nickenig G, et al. Pulmonary hypertension in patients with chronic kidney disease on dialysis and without dialysis: results of the PEPPER-study. *PLoS ONE.* (2012) 7:e35310. doi: 10.1371/journal.pone.0035310
143. Yigla M, Nakhoul F, Sabag A, Tov N, Gorevich B, Abassi Z, et al. Pulmonary hypertension in patients with end-stage renal disease. *Chest.* (2003) 123:1577–82. doi: 10.1378/chest.123.5.1577
144. Vaes RHD, Wouda R, Van Loon M, Van Hoek F, Tordoir JH, Scheltinga MR. Effectiveness of surgical banding for high flow in brachial artery-based hemodialysis vascular access. *J Vasc Surg.* (2015) 61:762–6. doi: 10.1016/j.jvs.2014.09.034
145. Rao NN, Stokes MB, Rajwani A, Ullah S, Williams K, King D, et al. Effects of arteriovenous fistula ligation on cardiac structure and function in kidney transplant recipients. *Circulation.* (2019) 139:2809–18 doi: 10.1161/CIRCULATIONAHA.118.038505
146. Frey R, Becker C, Saleh S, Unger S, van der Mey D, Mück W. Clinical pharmacokinetic and pharmacodynamic profile of riociguat. *Clin Pharmacokinet.* (2018) 57:647–661. doi: 10.1007/s40262-017-0604-7
147. Montani D, Chaumais MC, Savale L, Natali D, Price LC, Jaïs X, et al. Phosphodiesterase type 5 inhibitors in pulmonary arterial hypertension. *Adv Ther.* (2009) 26:813–25. doi: 10.1007/s12325-009-0064-z
148. Lentine KL, Villines TC, Axelrod D, Kaviratne S, Weir MR, Costa SP. Evaluation and management of pulmonary hypertension in kidney transplant candidates and recipients: Concepts and controversies. *Transplantation.* (2017) 101:166–81 doi: 10.1097/TP.0000000000001043
149. Foderaro AE, Baird GL, Bazargan-Lari A, Morrissey PE, Gohh RY, Poppas A, et al. Echocardiographic pulmonary hypertension predicts post-transplantation renal allograft failure. *Transplant Proc.* (2017) 49:1256–61. doi: 10.1016/j.transproceed.2017.01.085
150. Issa N, Krowka MJ, Griffin MD, Hickson LJ, Stegall MD, Cosio FG. Pulmonary hypertension is associated with reduced patient survival after kidney transplantation. *Transplantation.* (2008) 86:1384–8 doi: 10.1097/TP.0b013e318188d640
151. Wolfe JD, Hickey GW, Althouse AD, Sharbaugh MS, Kliner DE, Mathier MA, et al. Pulmonary vascular resistance determines mortality in end-stage renal disease patients with pulmonary hypertension. *Clin Transplant.* (2018) 32. doi: 10.1111/ctr.13270
152. Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am J Respir Crit Care Med.* (2005) 172:530–51. doi: 10.1164/rccm.200410-1384SO
153. Vassallo R, Ryu JH, Schroeder DR, Decker PA, Limper AH. Clinical outcomes of pulmonary Langerhans'-cell histiocytosis in adults. *N Engl J Med.* (2002) 346:484–90. doi: 10.1056/NEJMoa012087

154. Casolaro MA, Bernaudin JF, Saltini C, Ferrans VJ, Crystal RG. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am Rev Respir Dis.* (1988) 137:406–11. doi: 10.1164/ajrccm/137.2.406
155. Vassallo R, Walters PR, Lamont J, Kottom TJ, Yi ES, Limper AH. Cigarette smoke promotes dendritic cell accumulation in COPD; a Lung Tissue Research Consortium study. *Respir Res.* (2010) 11:45. doi: 10.1186/1465–9921-11-45
156. Suri HS, Yi ES, Nowakowski GS, Vassallo R. Pulmonary langerhans cell histiocytosis. *Orphanet J Rare Dis.* (2012) 7:16. doi: 10.1186/1750–1172-7-16
157. Prasse A, Stahl M, Schulz G, Kayser G, Wang L, Ask K, et al. Essential role of osteopontin in smoking-related interstitial lung diseases. *Am J Pathol.* (2009) 174:1683–91. doi: 10.2353/ajpath.2009.080689
158. Tazi A, Bonay M, Bergeron A, Grandsaigne M, Hance AJ, Soler P. Role of granulocyte-macrophage colony stimulating factor (GM-CSF) in the pathogenesis of adult pulmonary histiocytosis X. *Thorax.* (1996) 51:611–4. doi: 10.1136/thx.51.6.611
159. Travis WD, Borok Z, Roum JH, Zhang J, Feuerstein I, Ferrans VJ, et al. Pulmonary langerhans cell granulomatosis (histiocytosis X). a clinicopathologic study of 48 cases. *Am J Surg Pathol.* (1993) 17:971–86. doi: 10.1097/00000478-199310000-00002
160. Fartoukh M, Humbert M, Capron F, Maître S, Parent F, Le Gall C, et al. Severe pulmonary hypertension in histiocytosis X. *Am J Respir Crit Care Med.* (2000) 161:216–23. doi: 10.1164/ajrccm.161.1.9807024
161. Harari S, Brenot F, Barberis M, Simmoneau G. Advanced pulmonary histiocytosis X is associated with severe pulmonary hypertension. *Chest.* (1997) 111:1142–4. doi: 10.1378/chest.111.4.1142-a
162. Wajda N, Zhu Z, Jandarov R, Dilling DF, Gupta N. Clinical outcomes and survival following lung transplantation in patients with pulmonary Langerhans cell histiocytosis. *Respirology.* (2020) 25:644–50. doi: 10.1111/resp.13671
163. Chaowalit N, Pellikka PA, Decker PA, Aubry MC, Krowka MJ, Ryu JH, et al. Echocardiographic and clinical characteristics of pulmonary hypertension complicating pulmonary Langerhans cell histiocytosis. *Mayo Clin Proc.* (2004) 79:1269–75. doi: 10.4065/79.10.1269
164. Dauriat G, Mal H, Thabut G, Mornex JF, Bertocchi M, Tronc F, et al. Lung transplantation for pulmonary langerhans' cell histiocytosis: a multicenter analysis. *Transplantation.* (2006) 81:746–50. doi: 10.1097/01.tp.0000200304.64613.af
165. Bois MC, May AM, Vassallo R, Jenkins SM, Yi ES, Roden AC. Morphometric study of pulmonary arterial changes in pulmonary langerhans cell histiocytosis. *Arch Pathol Lab Med.* (2018) 142:929–37. doi: 10.5858/arpa.2017-0463-OA
166. Heiden GI, Sobral JB, Freitas CSG, Pereira de Albuquerque AL, Salge JM, Kairalla RA, et al. Mechanisms of exercise limitation and prevalence of pulmonary hypertension in pulmonary langerhans cell histiocytosis. *Chest.* (2020) 158:2440–8. doi: 10.1016/j.chest.2020.05.609
167. Le Pavec J, Lorillon G, Jaïs X, Tcherakian C, Feuillet S, Dorfmüller P, et al. Pulmonary Langerhans cell histiocytosis-associated pulmonary hypertension: clinical characteristics and impact of pulmonary arterial hypertension therapies. *Chest.* (2012) 142:1150–7. doi: 10.1378/chest.11-2490
168. Hamada K, Teramoto S, Narita N, Yamada E, Teramoto K, Kobzik L. Pulmonary veno-occlusive disease in pulmonary Langerhans' cell granulomatosis. *Eur Respir J.* (2000) 15:421–3. doi: 10.1034/j.1399-3003.2000.15b33.x
169. Tazi A, de Margerie C, Naccache JM, Fry S, Dominique S, Jouneau S, et al. The natural history of adult pulmonary Langerhans cell histiocytosis: a prospective multicentre study. *Orphanet J Rare Dis.* (2015) 10:30. doi: 10.1186/s13023-015-0249-2
170. Mogulkoc N, Veral A, Bishop PW, Bayindir U, Pickering CA, Egan JJ. Pulmonary Langerhans' cell histiocytosis: radiologic resolution following smoking cessation. *Chest.* (1999) 115:1452–5. doi: 10.1378/chest.115.5.1452
171. Wolters PJ, Elicker BM. Subacute onset of pulmonary langerhans cell histiocytosis with resolution after smoking cessation. *Am J Respir Crit Care Med.* (2014) 190:e64. doi: 10.1164/rccm.201405-0974IM
172. Elia D, Torre O, Cassandro R, Caminati A, Harari S. Pulmonary Langerhans cell histiocytosis: a comprehensive analysis of 40 patients and literature review. *Eur J Intern Med.* (2015) 26:351–6. doi: 10.1016/j.ejim.2015.04.001
173. Kinoshita Y, Watanabe K, Sakamoto A, Hidaka K. Pulmonary Langerhans cell histiocytosis-associated pulmonary hypertension showing a drastic improvement following smoking cessation. *Intern Med.* (2016) 55:491–5. doi: 10.2169/internalmedicine.55.5152
174. Delobbe A, Durieu J, Duhamel A, Wallaert B. Determinants of survival in pulmonary Langerhans' cell granulomatosis (histiocytosis X). Groupe d'Etude en Pathologie Interstitielle de la Société de Pathologie Thoracique du Nord. *Eur Respir J.* (1996) 9:2002–6. doi: 10.1183/09031936.96.09102002
175. Schönfeld N, Frank W, Wenig S, Uhrmeister P, Allica E, Preussler H, et al. Clinical and radiologic features, lung function and therapeutic results in pulmonary histiocytosis X. *Respiration.* (1993) 60:38–44. doi: 10.1159/000196171
176. Grobost V, Khouatra C, Lazor R, Cordier JF, Cottin V. Effectiveness of cladribine therapy in patients with pulmonary Langerhans cell histiocytosis. *Orphanet J Rare Dis.* (2014) 9:191. doi: 10.1186/s13023-014-0191-8
177. Lorillon G, Bergeron A, Detourmignies L, Jouneau S, Wallaert B, Frija J, et al. Cladribine is effective against cystic pulmonary Langerhans cell histiocytosis. *Am J Respir Crit Care Med.* (2012) 186:930–2. doi: 10.1164/ajrccm.186.9.930
178. Tazi A, Lorillon G, Haroche J, Neel A, Dominique S, Aouba A, et al. Vinblastine chemotherapy in adult patients with langerhans cell histiocytosis: a multicenter retrospective study. *Orphanet J Rare Dis.* (2017) 12:95. doi: 10.1186/s13023-017-0651-z
179. Alves Júnior SF, Zanetti G, Alves de Melo AS, Souza AS Jr, Souza LS, de Souza Portes Meirelles G, et al. Neurofibromatosis type 1: state-of-the-art review with emphasis on pulmonary involvement. *Respir Med.* (2019) 149:9–15. doi: 10.1016/j.rmed.2019.01.002
180. Oikonomou A, Vadikolias K, Birbilis T, Bouros D, Prassopoulos P. HRCT findings in the lungs of non-smokers with neurofibromatosis. *Eur J Radiol.* (2011) 80:e520–3. doi: 10.1016/j.ejrad.2010.11.033
181. Zamora AC, Collard HR, Wolters PJ, Webb WR, King TE. Neurofibromatosis-associated lung disease: a case series and literature review. *Eur Respir J.* (2007) 29:210–4. doi: 10.1183/09031936.06.00044006
182. Lie JT. Vasculopathies of neurofibromatosis Type 1 (von Recklinghausen Disease). *Cardiovasc Pathol.* (1998) 7:97–108. doi: 10.1016/S1054-8807(97)00081-1
183. Oderich GS, Sullivan TM, Bower TC, Gloviczki P, Miller DV, Babovic-Vuksanovic D, et al. Vascular abnormalities in patients with neurofibromatosis syndrome type I: clinical spectrum, management, and results. *J Vasc Surg.* (2007) 46:475–84. doi: 10.1016/j.jvs.2007.03.055
184. Jutant EM, Girerd B, Jaïs X, Savale L, O'Connell C, Perros F, et al. Pulmonary hypertension associated with neurofibromatosis type 1. *Eur Respir Rev.* (2018) 27:180053. doi: 10.1183/16000617.0053-2018
185. Rasmussen SA, Yang Q, Friedman JM. Mortality in neurofibromatosis 1: an analysis using U.S. death certificates. *Am J Hum Genet.* (2001) 68:1110–8. doi: 10.1086/320121
186. Zöller M, Rembeck B, Akesson HO, Angervall L. Life expectancy, mortality and prognostic factors in neurofibromatosis type 1. a twelve-year follow-up of an epidemiological study in Göteborg, Sweden. *Acta Derm Venereol.* (1995) 75:136–40.
187. Jutant EM, Jaïs X, Girerd B, Savale L, Ghigna MR, Perros F, et al. Phenotype and outcomes of pulmonary hypertension associated with neurofibromatosis Type 1. *Am J Respir Crit Care Med.* (2020) 202:843–52. doi: 10.1164/rccm.202001-0105OC
188. Montani D, Coulet F, Girerd B, Eyries M, Bergot E, Mal H, et al. Pulmonary hypertension in patients with neurofibromatosis type I. *Medicine.* (2011) 90:201–11. doi: 10.1097/MD.0b013e31821be2b7
189. Aoki Y, Kodama M, Mezaki T, Ogawa R, Sato M, Okabe M, et al. von Recklinghausen disease complicated by pulmonary hypertension. *Chest.* (2001) 119:1606–8. doi: 10.1378/chest.119.5.1606
190. Chaddha U, Puscas I, Prosper A, Ganesh S, Yaghmour B. A 63-year-old woman with neurofibromatosis Type 1 and pulmonary hypertension with worsening hypoxemia. *Chest.* (2017) 152:e89–93. doi: 10.1016/j.chest.2017.05.014
191. Merlo CA, Studer SM, Conte JV, Yang SC, Sonnett J, Orens JB. The course of neurofibromatosis type 1 on immunosuppression after lung

transplantation: report of 2 cases. *J Heart Lung Transplant.* (2004) 23:774–6. doi: 10.1016/S1053-2498(03)00265-1

192. Tamura Y, Ono T, Sano M, Fukuda K, Kataoka M, Satoh T. Favorable effect of sorafenib in a patient with neurofibromatosis-associated pulmonary hypertension. *Am J Respir Crit Care Med.* (2012) 186:291–2. doi: 10.1164/ajrccm.186.3.291
193. Baris HN, Cohen IJ, Mistry PK. Gaucher disease: the metabolic defect, pathophysiology, phenotypes and natural history. *Pediatr Endocrinol Rev.* (2014) 12(Suppl. 1):72–81.
194. Weinreb NJ, Charrow J, Andersson HC, Kaplan P, Kolodny EH, Mistry P, et al. Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: a report from the Gaucher Registry. *Am J Med.* (2002) 113:112–9. doi: 10.1016/S0002-9343(02)01150-6
195. Charrow J, Scott CR. Long-term treatment outcomes in Gaucher disease. *Am J Hematol.* (2015) 90(Suppl. 1):S19–24. doi: 10.1002/ajh.24056
196. Jmoudiak M, Futerman AH. Gaucher disease: pathological mechanisms and modern management. *Br J Haematol.* (2005) 129:178–88. doi: 10.1111/j.1365-2141.2004.05351.x
197. van Dussen L, Biegstraaten M, Dijkgraaf MG, Hollak CE. Modelling Gaucher disease progression: long-term enzyme replacement therapy reduces the incidence of splenectomy and bone complications. *Orphanet J Rare Dis.* (2014) 9:112. doi: 10.1186/s13023-014-0112-x
198. Mistry PK, Sirrs S, Chan A, Pritzker MR, Duffy TP, Grace ME, et al. Pulmonary hypertension in type 1 Gaucher's disease: genetic and epigenetic determinants of phenotype and response to therapy. *Mol Genet Metab.* (2002) 77:91–8. doi: 10.1016/S1096-7192(02)00122-1
199. Lo SM, Liu J, Chen F, Pastores GM, Knowles J, Boxer M, et al. Pulmonary vascular disease in Gaucher disease: clinical spectrum, determinants of phenotype and long-term outcomes of therapy. *J Inherit Metab Dis.* (2011) 34:643–50. doi: 10.1007/s10545-011-9313-9
200. Elstein D, Klutstein MW, Lahad A, Abrahamov A, Hadas-Halpern I, Zimran A. Echocardiographic assessment of pulmonary hypertension in Gaucher's disease. *Lancet.* (1998) 351:1544–6. doi: 10.1016/S0140-6736(98)10194-0
201. Harats D, Pauzner R, Elstein D, Many A, Klutstein MW, Kramer MR, et al. Pulmonary hypertension in two patients with type I Gaucher disease while on alglucerase therapy. *Acta Haematol.* (1997) 98:47–50. doi: 10.1159/000203562
202. Dawson A, Elias DJ, Rubenson D, Bartz SH, Garver PR, Kay AC, et al. Pulmonary hypertension developing after alglucerase therapy in two patients with type 1 Gaucher disease complicated by the hepatopulmonary syndrome. *Ann Intern Med.* (1996) 125:901–4. doi: 10.7326/0003-4819-125-11-199612010-00005
203. de Boer GM, van Dussen L, van den Toorn LM, den Bakker MA, Hoek RA, Hesselink DA, et al. Lung transplantation in Gaucher disease: a learning lesson in trying to avoid both scylla and charybdis. *Chest.* (2016) 149:e1–5. doi: 10.1016/j.chest.2015.09.011
204. den Bakker MA, Grünberg K, Boonstra A, van Hal PT, Hollak CE. Pulmonary arterial hypertension with plexogenic arteriopathy in enzyme-substituted Gaucher disease. *Histopathology.* (2012) 61:324–6. doi: 10.1111/j.1365-2559.2012.04261.x
205. Goobie GC, Sirrs SM, Yee J, English JC, Bergeron C, Nador R, et al. Lessons from lung transplantation: cause for redefining the pathophysiology of pulmonary hypertension in gaucher disease. *Respir Med Case Rep.* (2019) 28:100893. doi: 10.1016/j.rmcr.2019.100893
206. Roberts WC, Fredrickson DS. Gaucher's disease of the lung causing severe pulmonary hypertension with associated acute recurrent pericarditis. *Circulation.* (1967) 35:783–9. doi: 10.1161/01.CIR.35.4.783
207. Ross DJ, Spira S, Buchbinder NA. Gaucher cells in pulmonary-capillary blood in association with pulmonary hypertension. *N Engl J Med.* (1997) 336:379–81. doi: 10.1056/NEJM199701303360516
208. Fernandes CJ, Jardim C, Carvalho LA, Farias AQ, Filho MT, Souza R. Clinical response to sildenafil in pulmonary hypertension associated with Gaucher disease. *J Inherit Metab Dis.* (2005) 28:603–5. doi: 10.1007/s10545-005-0603-y
209. Al-Naamani N, Roberts KE, Hill NS, Preston IR. Imatinib as rescue therapy in a patient with pulmonary hypertension associated with Gaucher disease. *Chest.* (2014) 146:e81–3. doi: 10.1378/chest.13-2795

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 30 April 2021
doi: 10.3389/fmed.2021.625763

Novel *TNIP2* and *TRAF2* Variants Are Implicated in the Pathogenesis of Pulmonary Arterial Hypertension

Shaun Pienkos[1†], Natalia Gallego[2,3†], David F. Condon[1†], Alejandro Cruz-Utrilla[4,5], Nuria Ochoa[4,5], Julián Nevado[2,3,6], Pedro Arias[2,3,6], Stuti Agarwal[1], Hiral Patel[1], Ananya Chakraborty[1], Pablo Lapunzina[2,3,6], Pilar Escribano[4,5], Jair Tenorio-Castaño[2,3,6†] and Vinicio A. de Jesús Pérez[1*†] on behalf of Spanish PAH Consortium[2,3]*

[1] Division of Pulmonary and Critical Care Medicine and Department of Medicine, Stanford University, Stanford, CA, United States, [2] Medical and Molecular Genetics Institute (INGEMM), IdiPaz, Hospital Universitario La Paz, Madrid, Spain, [3] CIBERER, Centro de Investigación en Red de Enfermedades Raras, Instituto de Salud Carlos III, Madrid, Spain, [4] Pulmonary Hypertension Unit, Department of Cardiology, Hospital Universitario Doce de Octubre, Madrid, Spain, [5] Centro de Investigación Biomedica en Red en Enfermedades Cardiovasculares, Instituto de Salud Carlos III (CIBERCV), Madrid, Spain, [6] Intellectual Disability, TeleHealth, Autism and Congenital Anomalies (ITHACA), European Reference Network on Rare Congenital Malformations and Rare Intellectual Disability, Brussels, Belgium

OPEN ACCESS

Edited by:
Silvia Ulrich,
University Hospital Zürich, Switzerland

Reviewed by:
Vasile Foris,
Medical University of Graz, Austria
Gabriele Grunig,
New York University, United States

***Correspondence:**
Vinicio A. de Jesús Pérez
vdejesus@stanford.edu
Jair Tenorio-Castaño
jaira.tenorio@salud.madrid.org

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Pulmonary Medicine, a section of the journal Frontiers in Medicine

Received: 03 November 2020
Accepted: 23 March 2021
Published: 30 April 2021

Citation:
Pienkos S, Gallego N, Condon DF, Cruz-Utrilla A, Ochoa N, Nevado J, Arias P, Agarwal S, Patel H, Chakraborty A, Lapunzina P, Escribano P, Tenorio-Castaño J and de Jesús Pérez VA (2021) Novel TNIP2 and TRAF2 Variants Are Implicated in the Pathogenesis of Pulmonary Arterial Hypertension. Front. Med. 8:625763. doi: 10.3389/fmed.2021.625763

Background: Pulmonary arterial hypertension (PAH) is a rare disease characterized by pulmonary vascular remodeling and right heart failure. Specific genetic variants increase the incidence of PAH in carriers with a family history of PAH, those who suffer from certain medical conditions, and even those with no apparent risk factors. Inflammation and immune dysregulation are related to vascular remodeling in PAH, but whether genetic susceptibility modifies the PAH immune response is unclear. *TNIP2* and *TRAF2* encode for immunomodulatory proteins that regulate NF-κB activation, a transcription factor complex associated with inflammation and vascular remodeling in PAH.

Methods: Two unrelated families with PAH cases underwent whole-exome sequencing (WES). A custom pipeline for variant prioritization was carried out to obtain candidate variants. To determine the impact of TNIP2 and TRAF2 in cell proliferation, we performed an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay on healthy lung pericytes transfected with siRNA specific for each gene. To measure the effect of loss of TNIP2 and TRAF2 on NF-kappa-beta (NF-κB) activity, we measured levels of Phospho-p65-NF-κB in siRNA-transfected pericytes using western immunoblotting.

Results: We discovered a novel missense variant in the *TNIP2* gene in two affected individuals from the same family. The two patients had a complex form of PAH with interatrial communication and scleroderma. In the second family, WES of the proband with PAH and primary biliary cirrhosis revealed a *de novo* protein-truncating variant in the *TRAF2*. The knockdown of TNIP2 and TRAF2 increased NF-κB activity in healthy lung pericytes, which correlated with a significant increase in proliferation over 24 h.

Conclusions: We have identified two rare novel variants in *TNIP2* and *TRAF2* using WES. We speculate that loss of function in these genes promotes pulmonary vascular

remodeling by allowing overactivation of the NF-κB signaling activity. Our findings support a role for WES in helping identify novel genetic variants associated with dysfunctional immune response in PAH.

Keywords: pulmonary arterial hypertension, NF-κB, inflammation, massive paralleled sequencing, *TNIP2*, *TRAF2*

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare disorder associated with progressive elevation of pulmonary pressures that, if untreated, leads to right heart failure and death (1, 2). The major pathological features of PAH are obliterative vasculopathy and progressive loss of distal pulmonary microvessels that are unresponsive to available therapies (3, 4). While the cause of PAH remains incompletely understood, pathogenic variants in the bone morphogenetic protein receptor 2 *(BMPR2)* have been associated with both familial (60%) and sporadic PAH (20–25%) (5, 6). Studies have now shown that BMPR2 is responsible for ensuring appropriate vascular repair in response to injury and loss of function variants are associated with impaired angiogenesis, as well as endothelial and smooth muscle cell proliferation within the vessel wall (7–9). Given the low penetrance of *BMPR2* variants, it is likely that additional genetic modifiers are required for the development of PAH. Besides genetic predisposition, immune dysregulation and inflammation have been established as risk factors for PAH development (10, 11). Recent work in this area has led to identifying immune subtypes within PAH, which may explain phenotypic differences among patients (12). Understanding the interaction between genetics and immunity could provide an avenue for revealing the basis of PAH susceptibility and might offer opportunities for novel therapeutics that could change the natural history of this disease.

Whole-exome sequencing (WES) is a valuable tool for discovering novel pathogenic variants in the protein-coding regions and the genome's intron-exon boundaries. The use of WES to analyze probands and family members (i.e., trios and quartets) has led to the identification of novel disease genes associated with multiple genetic disorders, including PAH (13–16). In this study, we employed WES to detect novel pathogenic variants in a patient trio and a quartet that had previously tested negative for known PAH pathogenic variants using our customized parallel sequencing PAH gene platform (17). We report the discovery of potentially pathogenic variants in *TNIP2* and *TRAF2*, two genes associated with inflammation and immunity whose role in PAH has not been previously documented. Through the use of patient-derived cells and *in vitro* gene knockdown studies, we show that TNIP2 and TRAF2 act by suppressing the activation of NF-κB, a transcription complex that controls the expression of inflammatory cytokines and genes associated with cell proliferation in PAH (18–21). We conclude that *TNIP2* and *TRAF2* pathogenic variants could increase PAH susceptibility through their capacity to alter cellular immune responses and drive abnormal cellular proliferation in the pulmonary vasculature.

MATERIALS AND METHODS

Patient Studies

All patients involved in this study gave their informed consent to participate. Samples were obtained from the Spanish PAH registries (REHAP and REHIPED), and the ethical committee approved the project of scientific research of the Hospital Universitario La Paz, Madrid (PI-1210, PI-3996). PAH was confirmed in all patients by right heart catheterization (RHC) following the recommendations of the 6th World Symposium and the European Society of Cardiology (1, 2, 22). Screening of family members was carried out by performing a family pedigree of all individual index cases of PAH. In those with a clinical suspicion of heritable PAH, evaluation of medical history, physical examination, laboratory tests, 12-lead electrocardiogram, and transthoracic echocardiography of all family members was done to select possible affected members and RHC candidates.

Whole Exome Sequencing

The workflow used for WES analysis is summarized in **Supplementary Table 1**. Library preparation was carried by Agilent SureSelect^{TM} (v. 6.0), all exons kit followed by sequencing in a NovaSeq Sequencer (Illumina, USA). The exomes were analyzed by Saphetor Varsome software (23) and VarSeq (Golden Helix, USA) to prioritize variants in the probands with segregation patterns consistent with potential disease pathogenesis. Copy number variant (CNV) analysis was carried out through VarSeq to detect large rearrangements (deletions, insertions, and Indels of more than 1 Kb). Segregation analysis and validation of the variants detected in the WES was performed by classical Sanger sequencing. Screening for pathogenic variants in well-known PAH genes was carried out through a custom gene panel described previously (17).

Variant prioritization was carried out by a custom algorithm (see **Supplementary Table 1**). The first step was to filter out variants not meeting the standard quality requirements for depth, genotype quality, and variant allele frequency, followed by segregation analysis based on the variant's suggested pattern of inheritance. All variants with a population frequency above 1% were filtered out, and the pathogenicity of the remaining variants was assessed using several bioinformatic tools included in the dbNSFP database (24) plus the computation of the CADD score, which predict the deleteriousness of variants throughout the human genome (25). The variant analysis was prioritized according to predicted pathogenicity. Predicted impact of variants on candidate gene function and role in PAH pathogenesis was carried out using *in silico* analysis via STRING and literature review, respectively.

Pericyte Isolation and Culture

Pericytes were obtained from human donor lung tissue samples and lineage was confirmed by FACS, and western immunoblotting as previously described (26–28). 3G5 (ATCC CRL-1814) hybridoma cells were cultured in 1X DMEM with 10% FBS. Culture medium was collected and concentrated by centrifugal filter Amicon Ultra-15 (Millipore). 3G5 IgM was isolated using the IgM Purification Kit (Pierce), and concentration was determined using the Lowry Method. The day before the extraction, 40 μl of rat anti-mouse IgM magnetic beads (Invitrogen 11039D) were incubated with 10 μg of 3G5 IgM overnight. The next day, fresh lung tissue was washed with 1X HBSS twice and digested in a 10 ml 1X HBSS solution containing 10 mg Collagenase (Sigma-Aldrich, USA), 1 mg dispase, and 75 μg DNase for 15–30 min at 37 degrees on an orbital shaker at vigorous speed (150 rpm). An equivalent amount of ice-cold pericyte medium (ScienCell, USA) containing 2% FBS was added immediately after digestion. The suspension was passed through a 100 μm mesh filter (BD Falcon, USA) to remove debris and undigested fibrous tissue and washed once with 1X PBS. The cell pellet was resuspended in 1 ml 1X PBS containing 0.2% FBS and 40 μl of antibody-coated magnetic beads and gently rotated in 4 degrees for 45 min. Cells were used between Passage 8–10.

Western Immunoblotting

Cells were washed three times with ice-cold 1x PBS, and lysates were prepared by adding lysis buffer (1X radio-immunoprecipitation assay buffer, 1 mMol/L phenyl-methylsulfonyl fluoride, and 1x phosphatase inhibitor) and vortexed for 10 s before centrifugation. Supernatants were transferred to fresh microcentrifuge tubes and stored at −20°C. The protein concentration was determined by Pierce BCA assay (Thermo Scientific Product #23227, Rockford, Illinois). Equal amounts of protein were loaded onto each lane of a 4–12% Bis-Tris gel (Life Technologies) and subjected to SDS-PAGE electrophoresis under reducing conditions. After blotting, polyvinylidene difluoride (PVDF) membranes were blocked for 1 h in blocking buffer (5% milk powder in 0.1% PBS/Tween 20) and incubated with primary antibodies overnight at 4°C, followed the next day by incubation with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using the ECL kit (Thermo Fisher).

RNA Interference

To achieve gene knockdown, 2 μmol/L silencing RNA (siRNA) of ABIN2 (encoded by TNIP2) (L-014328-01, Dharmacon, Lafayette, CO), TRAF2 (L-005198-00, Dharmacon, Lafayette, CO), or non-targeting siRNA control transfected into healthy pulmonary pericytes. Transfection was performed using a Nucleofector II (Program U-025; Lonza) with the Basic Smooth Muscle Cell Nucleofection kit (Lonza). All experiments were performed 48 h after electroporation.

MTS Proliferation Assay

Cells were seeded in eight replicates on collagen-coated 96-well plates (5.0×10^3 cells per well) (Nunc, Rochester, NY) and cultured in complete medium overnight at 37°C and 5% CO_2. The next day, reduced serum media and MTS reagent were added to each well for 1 h in accordance with the manufacturer's instructions. MTS absorbance was measured at 490 nm.

Statistical Analysis

The number of samples studied per experiment is indicated in the figure legends. Values from multiple experiments are expressed as mean +/− SEM. Statistical significance was determined using the Mann-Whitney test. A value of $P < 0.05$ was considered significant.

RESULTS

Discovery of the *TNIP2* Variant

The pedigree of the first family is shown in **Figure 1A**. The index case was a 63-year-old female with a history of Ostium Secundum atrial septal defect (ASD), which was surgically corrected in 1994, and severe restrictive lung disease caused by thoracic deformities and diaphragmatic paralysis. The patient was diagnosed with PAH when she presented with exertional dyspnea and chest pain. Despite treatment with sildenafil and ambrisentan, the patient died 4 years after diagnosis from progressive right heart failure. Her 45-year-old daughter was diagnosed in 2015 with PAH associated with limited scleroderma and remains clinically stable with triple therapy of tadalafil, bosentan, and intravenous epoprostenol. The clinical characteristics of the two affected patients are summarized in **Table 1**. The remaining family members were screened and demonstrated no signs and symptoms of PAH to date. Given the familial predisposition, we conducted a genetic screen of the patients and family members using an in-house custom panel (HAPv2.1) (17), which was negative for mutations in 21 genes associated with PAH. As a next step, we conducted WES on the two patients and four unaffected family members, followed by segregation analysis (**Figure 1A**).

Analysis of WES data confirmed the absence of mutations in PAH associated genes. However, we discovered that both PAH patients were carriers of a novel missense variant in TNFAIP3 Interacting Protein 2 (*TNIP2*). This gene encodes for the protein ABIN2, which acts as an inhibitor of the NF-κB signaling pathway (29). The variant is predicted to change an adenine to a cytosine base at position 697 (NM_024309.4:c.697A>C), which results in a switch from serine to glycine at position 233 [p.(Ser233Gly)] of the peptide chain, located between the two ubiquitin-binding domains of the protein. This novel *TNIP2* variant was predicted to be damaging based on the estimates obtained via dbSNFP and would likely result in loss of protein function (**Table 2**). Additionally, this variant is absent from several control population databases (gnomAD exomes, gnomAD genomes, Kaviar, Beacon, Bravo, ESP, 1000G phase III). Interestingly, one of the unaffected family members was also found to be a carrier of the same *TNIP2* variant and is currently undergoing clinical surveillance in our clinic.

Discovery of the *TRAF2* Variant

The pedigree of the second family is shown in **Figure 1B**. The index case was a 27-year-old female that presented to the clinic in 2015 complaining of dizziness and was subsequently diagnosed with idiopathic PAH. Following initiation of tadalafil and ambrisentan, the patient developed elevated liver function

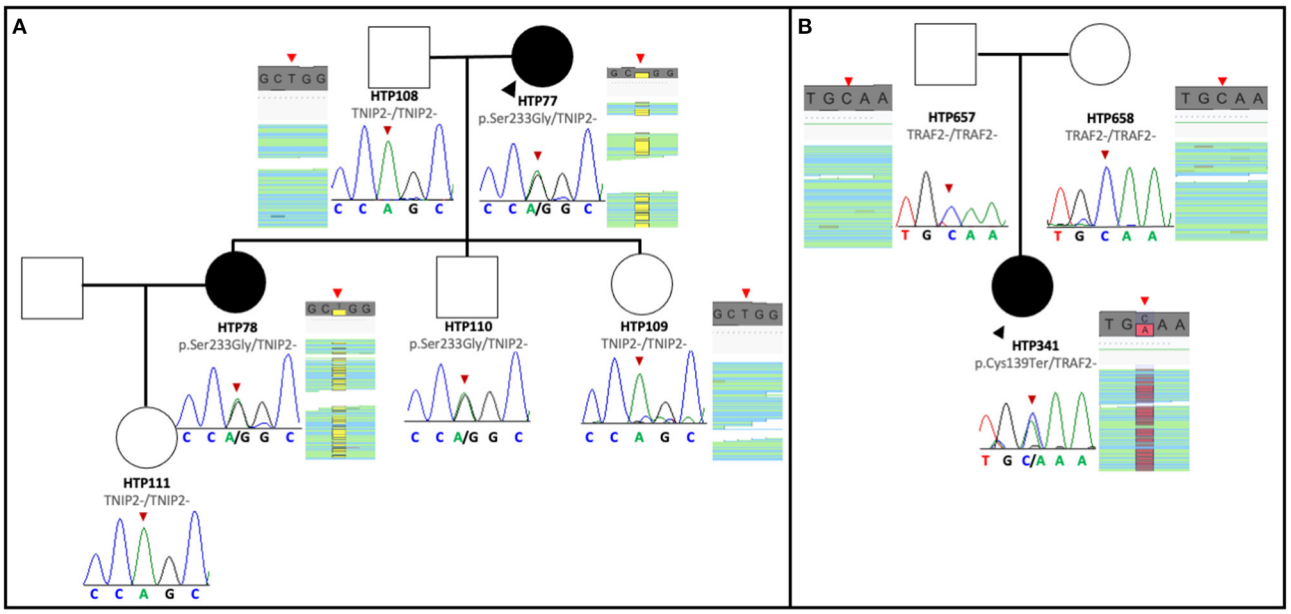


FIGURE 1 | Pedigree of the families with *TNIP2* and *TRAF2* variants. **(A)** The *TNIP2* variant was found in two affected family members and an unaffected sibling of the proband. Sanger sequencing segregation in all available members is presented, with BAM capture from the IGV software showing the vertical read coverage at the specific position (red arrows). **(B)** Pedigree of the family with the *TRAF2* variant. The nonsense variant was detected only in the proband and confirmed with Sanger sequencing of the variant. BAM capture from the IGV software showing the vertical read coverage at the specific position (red arrows).

enzymes, which prompted ambrisentan cessation. During the workup for elevation in liver enzymes, the patient was diagnosed with primary biliary cirrhosis, and the PAH subtype was revised to portopulmonary hypertension. Intravenous epoprostenol was temporarily initiated to stabilize the patient, and treatment was later switched to triple oral therapy with tadalafil, macitentan, and selexipag (**Table 1**). At the time of writing, the patient remains clinically stable.

As part of the clinical evaluation, we applied the custom gene panel HAPv.2.1 to screen the patient and her unaffected parents but found no evidence of pathogenic variants. However, WES analysis detected a novel pathogenic variant in the Tumor Necrosis Factor Receptor-Associated Factor (*TRAF2*). This gene encodes for a protein involved in the TNF-dependent activation of NF-κB (30). The variant is predicted to change cysteine to adenosine in position 417 (NM_021138.4:c.417C>A), introducing a premature stop codon and resulting in a truncated TRAF2 transcript. Analysis of the parents' exomes showed that the variant is *de novo* in the proband. This variant is also absent from the control population databases (gnomAD exomes, gnomAD genomes, Kaviar, Beacon, Bravo, ESP, 1000G phase III), and the majority of the pathogenic *in silico* tools from the dbSNFP suggested a damaging effect for this variant (**Table 2**).

TNIP2 and TRAF2 Interact With Pathways Associated With PAH

To date, no studies have shown evidence of *TNIP2* and *TRAF2* involvement in the pathogenesis of PAH. To determine whether these genes could potentially be interacting with components of know PAH-associated pathways, we carried out an *in silico* protein-protein screen using STRING (**Figure 2A**). This tool illustrates protein-protein interactions based on function and previously described associations (31). Our STRING analysis showed that TNIP2 and TRAF2 indirectly interacted with the transforming growth factor-β (TGF-β) family genes via CAV1, a known risk gene for hereditary PAH (13). Notably, BMPR2, the gene most commonly implicated in hereditary and idiopathic PAH, is part of the TGF-β family identified within the STRING cluster. As expected, we found strong associations between TNIP2 and TRAF2 with proteins involved in the NF-κB pathway (**Figures 2B,C**). Of the proteins associated with familial PAH, only CAV1 directly interacts with TRAF2 based on *in vitro* studies demonstrating that these proteins form a cytoplasmic protein-protein complex that modulates NF-κB activation in response to TNF (32, 33).

Loss of TNIP2 and TRAF2 Result in Increased Pericyte Proliferation and Activation of NF-κB

TNIP2 and TRAF2 are part of a gene network responsible for regulating the NF-κB pathway's signaling activity. The NF-κB proteins exist in the cytoplasm in an inactive state and can be rapidly activated in response to harmful stimuli such as oxidative stress, bacterial lipopolysaccharides, environmental toxins, and inflammation. Once activated, the NF-κB protein complex translocates to the nucleus and binds to target genes involved in cell proliferation, survival, and the production of cytokines that regulate innate and adaptive immune

TABLE 1 | Clinical features of patients.

Patient ID	HTP 77	HTP 78	HTP 341
Etiology	Group 1: PAH + ASD	Group 1: PAH + scleroderma	Group 1: PAH + primary biliary cirrhosis
Date of birth	22/09/1946	15/10/1975	24/08/1988
Age at diagnosis (years)	63	27	27
Current age (years)	Dead with 67 years	45	32
WHO functional class	III	I	I
Last known PAH therapies	Sildenafil, ambrisentan	Bosentan, tadalafil, epoprostenol	Selexipag, macitentan, tadalafil
6-min walk test (meters)	Not performed	595	454
CPET (date) RER Maximum V02—mL/Kg/min (% of predicted value) VE/VCO2 slope	Not performed	May of 2018 1.41 11.0 (33) 33	March of 2018 1.15 17.0 (53) 38
Mean pulmonary artery pressure (mmHg)	42	51	55
Pulmonary capillary wedge pressure (mmHg)	14	6	8
Pulmonary vascular resistance (Wood units)	6.0	9.1	17.0
Cardiac output (lpm)	4.6 lpm	4.6 lpm	2.7 lpm
Cardiac index (lpm/m^2)	3.0	3.1	1.8
Mean arterial pressure or blood pressure (mmHg)	96	83	74
Echocardiographic abnormalities	Repaired Ostium Secundum ASD. Mild dilatation and hypertrophy of the RV and mild RV systolic dysfunction. Mild RA enlargement.	Eccentricity index of 1.7. RV hypertrophy and dilatation. RV systolic dysfunction. Moderate RA enlargement.	Eccentricity index of 1.1. RV hypertrophy and dilatation. Subtle RV systolic dysfunction. Mild RA enlargement.
Chest computed tomography abnormalities	Thoracic deformities and diaphragmatic paralysis	Bilateral ground glass opacities related with PH.	No abnormalities noted in chest CT Arterio-venous hepatic fistulae
Forced expiratory volume in 1 s (mL) and predicted value by age and sex (%)	660 (43%)	2,790 (86%)	2,00 (89%)
Forced vital capacity (mL) and predicted value by age and sex (%)	1,060 (56%)	3,240 (61%)	3,185 (91%)
Diffusing capacity of the lungs for carbon monoxide (DLCO)	Not available	45%	56%
Partial pressure of oxygen (PaO_2)	75 mmHg	57 mmHg	55 mmHg
V/Q scintigraphy and/or CT pulmonary angiography	Global matched perfusion and ventilation defects	Normal V/Q scan. Main pulmonary artery enlargement. RA and RV enlargement	Matched perfusion and ventilation defects in the upper right lung segment in V/Q scan.

Summary of the most relevant clinical characteristics of the patients in which a genetic variant was detected. ASD, atrial septal defect; CPET, cardiopulmonary exercise test; CT, Computed tomography; PH, pulmonary hypertension; RA, right atrium; RER, respiratory exchange ratio; RV, right ventricle; V/Q, Ventilation-Perfusion scan; WHO, World Health Organization.

TABLE 2 | Candidate genes and variant *in silico* analysis.

	Variant information (hg19)				Pathogenicity		Population frequencies
Gene	**cDNA position**	**Protein position**	**Effect**	**Zygosity**	**dbSNFP**	**CADD**	**AF**
TNIP2	NM_024309.3:c.697A>G	NP_077285.3:p.Ser233Gly	Missense	Heterozygous	12/19	25.8	0
TRAF2	NM_021138.3:c.417C>A	NP_066961.2:p.Cys139Ter	Non-sense	Heterozygous	5/9	35	0

*Variants were annotated with the human reference genome hg19. Pathogenicity in silico prediction was obtain from the aggregation database dbSNFP and CADD (**Table 1** for more info). AF, Alleled frequency was estimated from several population pseudo-control databases: gnomAD genomes (v2.1.1), gnomAD exomes (v2.1.1), Kaviar (version 160204-Public), Beacon (v2.0), 1000G, Phase III and Bravo.*

responses (34). Inappropriate NF-κB activation is associated with a wide range of diseases, including cancer, autoimmune conditions such as scleroderma, and PAH (35). Given that TNIP2 and TRAF2 serve to regulate NF-κB activity, we hypothesized that the loss of these proteins would lead to NF-κB overactivation.

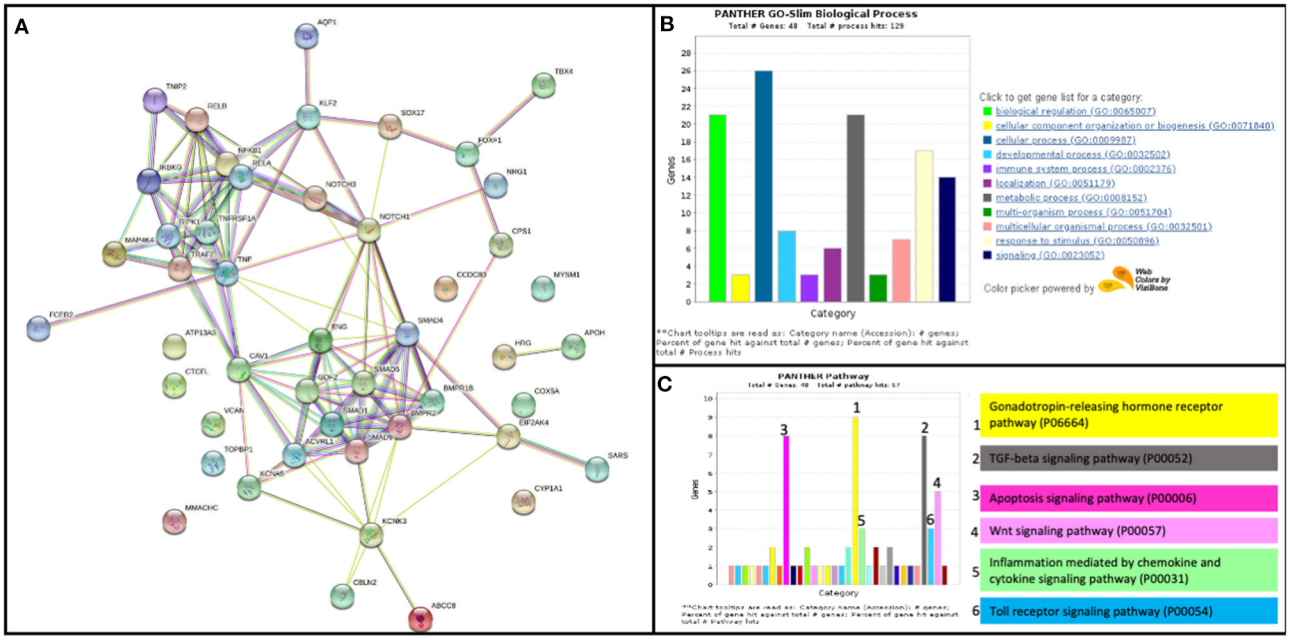


FIGURE 2 | STRING analysis of TRAF2 and TNIP2 related to previously associated genes to PAH. **(A)** Protein network showing the possible interactions between all genes associated with PAH. This graph includes genes previously associated with PAH and candidate genes described in case reports/series. The strength of evidence for the relation between TRAF2 and CAV1 comes from published experimental/biochemical data and association in curated databases (see Methods). **(B,C)** Gene enrichment analysis, including biological processes **(B)** and interaction pathways **(C)** in which the STRING analysis genes are involved. Several genes are involved in the immune system process and participate in inflammation mediated by chemokine and cytokine signaling.

To evaluate the biological consequences of deleterious variants affecting *TNIP2* and *TRAF2*, gene silencing via siRNA transfection in human-derived pulmonary pericytes was performed. We chose to study pericytes because these cells have been shown to contribute to progressive small vessel loss and muscularization through hyperproliferation and inflammatory cytokine production (26, 27, 36). A query of our recently published pericyte RNA-seq analysis confirmed that both TNIP2 and TRAF2 are expressed in healthy and PAH pericytes, although no significant differences in expression were observed (26). Transfection of siTNIP2 and siTRAF2 resulted in >50% reduction in corresponding protein levels (**Figure 3A**). To determine whether TNIP2 and TRAF2 knockdown led to inappropriate activation of NF-κB, we used western immunoblotting to probe whole cell lysates for phospho-p65-NF-κB, the active form of p-65-NF-κB that becomes activated to trigger transcription of NF-κB target genes (34, 35). Stimulation with lipopolysaccharide (LPS, a known activator of NF-κB) triggered an increase in phospho-p65-NF-κB and was used as a positive control (37). Compared to baseline controls and cells transfected with a non-specific siRNA (siCtl), both siTNIP2 and siTRAF2 pericytes demonstrated a significantly increased phospho-p65-NF-κB of similar magnitude as that observed with LPS stimulation (**Figure 3B**). To assess the effect of TNIP2 and TRAF2 knockdown on pericyte proliferation, we used an MTS assay to quantify cell numbers under different experimental conditions over 24 h. Compared to the controls, both siTNIP2 and siTRAF2 pericytes demonstrated significantly increased cell proliferation (**Figure 3C**). Interestingly, the degree of proliferation observed in both siTNIP2 and siTRAF2 pericytes was similar to that of healthy pericytes stimulated with LPS.

DISCUSSION

In this study, we utilized a WES custom gene prioritization method optimized to identify potentially pathogenic variants in PAH patients and family members to elucidate possible genetic mechanisms influencing disease development. This approach enabled the discovery of novel variants in *TRAF2* and *TNIP2*, two genes involved in regulating the NF-κB pathway. We demonstrated that loss of TNIP2 and TRAF2 results in inappropriate NF-κB activation and increased proliferation of healthy lung pericytes through the use of siRNA-based knockdown. A graphical abstract is included in **Figure 4**. To our knowledge, ours is the first report to document a potential link between TNIP2/TRAF2 loss of function and PAH in humans.

Since the description of *BMPR2*, the increasing prevalence of genetic screening has shown that pathogenic variants in this gene are present in ∼60% of hereditary PAH cases and 20-25% of idiopathic cases. *BMPR2*, as well as other genes (i.e., *KDR, SOX17, TBX4*), may also be involved in the development of associated forms of PAH (APAH) (38–41). Multiple groups have

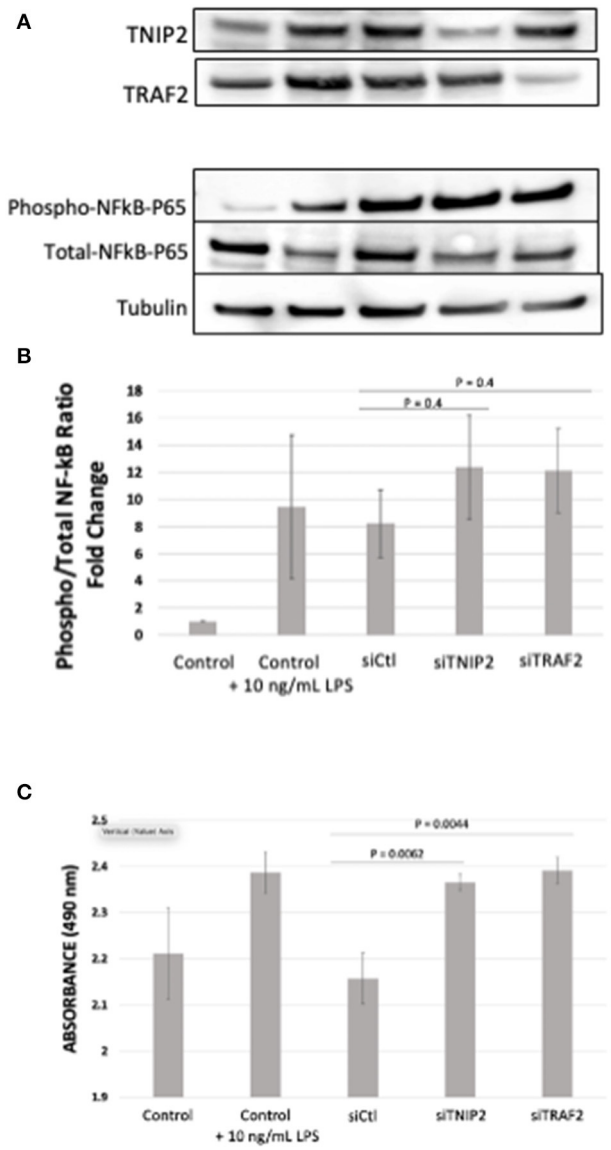


FIGURE 3 | Phospho- to Total-p65-NF-κB determination and MTS Assay in lung pericytes transfected with siTNIP2 and siTRAF2. **(A,B)** Representative western immunoblots demonstrating knockdown efficiency of TNIP2 and TRAF2 in healthy lung pericytes transfected with the corresponding siRNA **(A)**, and the fold change of phosphorylated to total p65-NF-κB **(B)**. **(C)** MTS assay of healthy lung pericytes transfected with siControl (siCtl), siTNIP2, and siTRAF2 under different experimental conditions. The data in the graphs are the average of three independent experiments.

applied custom panels, whole-exome sequencing (WES), or even whole-genome sequencing to elucidate these mechanisms (42), which include variation in gene regulatory elements in addition to the protein-coding sequences themselves (43). Whole-genome sequencing in more extensive and more inclusive cohorts will be vital for uncovering intronic and regulatory genetic sequences that may impact PAH development in patients with unrevealing results upon initial gene panel screening. In many pulmonary hypertension centers, however, genetic analysis is not frequently offered in patients with PAH associated with other medical conditions (e.g., connective tissue diseases, congenital heart diseases), limiting the data available in this population. This practice will be essential to address future clinical guidelines to deepen our understanding of genetic variation in APAH.

As part of the clinical evaluation, our group routinely performs genetic screening for patients diagnosed with idiopathic, heritable, and associated PAH forms with suspected genetic components. We have developed a custom NGS panel of genes that is updated yearly to reflect published risk genes in PAH and recently reported our experience applying it in 300 patients, some of whom suffered from APAH (17). In all patients with inconclusive results on the gene panel, we move to WES, preferably including parents and other proband relatives, to screen for new risk genes. In recent years, multiple publications have demonstrated the role of genetic variants in the development of many PH subtypes, some of which were previously not known to have a genetic cause. For example, variants in the endoglin gene are linked with PAH associated with connective tissue disease (44), polymorphisms in estrogen-related genes may impact the risk of portopulmonary hypertension (45), and *SOX17* have been implicated in PAH associated with congenital heart disease (41). Also, variants in *CAV1, TRPM8*, and *BNP* have been linked with PH secondary to chronic obstructive pulmonary disease (46–48). These findings underscore the importance of expanding our genetic sequencing application to PH populations not traditionally thought to have a genetic predisposition.

In the present study, our WES data suggest an association between *TNIP2* and *TRAF2* variants with the development of APAH. By analyzing exome data of individuals affected with PAH compared to healthy family members, we focused on genes that correlate with phenotypic differences. After our prioritization analysis, we selected the variants in *TNIP2* and *TRAF2* based on the criteria described in **Supplementary Table 1**. These two variants were absent in control population databases, and the majority of the *in silico* pathogenic tools suggest a damaging effect for these variants. Both variants are highly conserved through evolution, highlighting these residues' importance in the affected protein's function. Importantly, these two genes have plausible mechanistic significance as regulators of the NF-κB pathway, previously associated with PAH (49–53). As is the case with the discovery of all novel variants, consideration of pseudocontrol human population gene frequency, conservation in orthologs through evolution, pathogenicity prediction, and biological context was necessary to establish how *TNIP2* and *TRAF2* were involved in PAH.

The family genetic analysis showed that the variant in *TNIP2* is present in both affected individuals and one unaffected member. We suspect two possible explanations for this observation. First, the variant may have incomplete penetrance or cause variable age of disease onset, allowing some carriers to be unaffected for some or all of their lives. Secondly, it may be necessary for an additional disease process to trigger the development of PAH in those who carry this variant. The high-impact nonsense *TRAF2* variant identified in a separate family was only seen in the affected proband, suggesting a *de novo* event

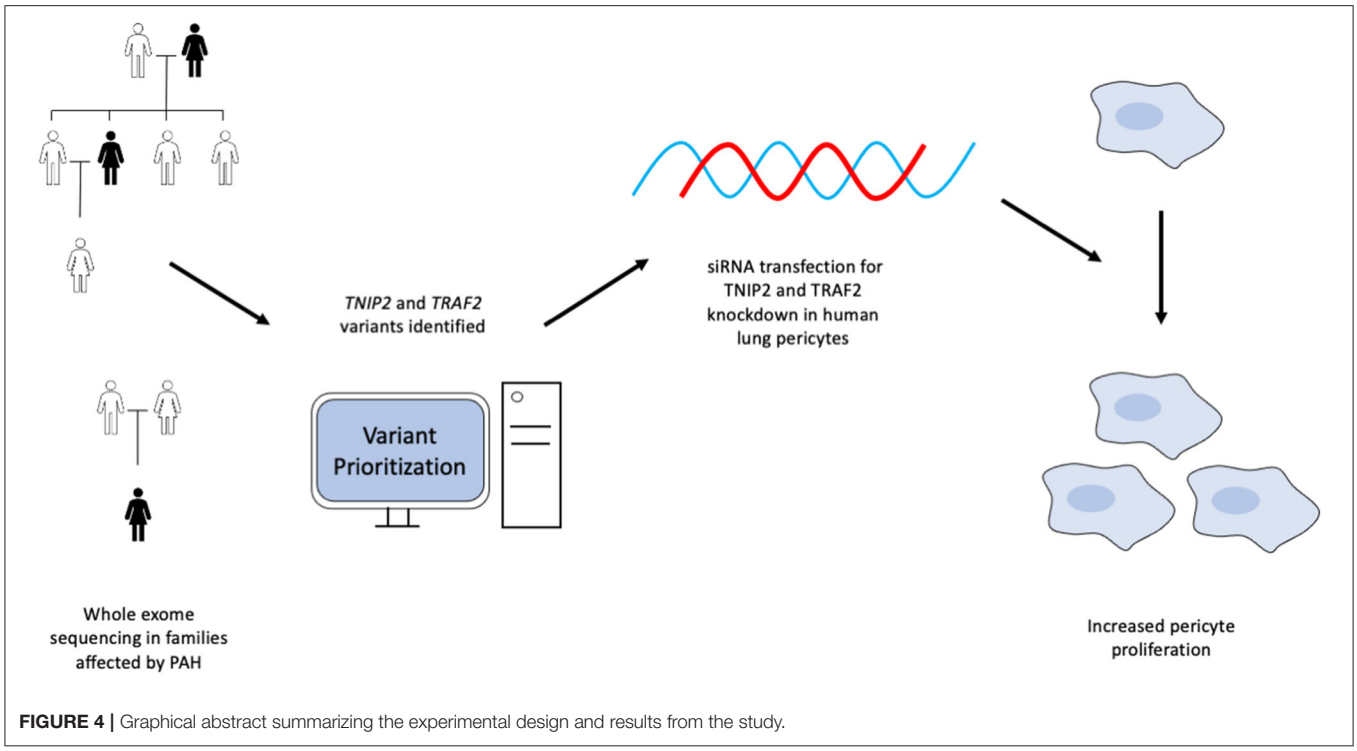


FIGURE 4 | Graphical abstract summarizing the experimental design and results from the study.

or germinal mosaicism of the parents. Because there was no previous evidence linking pathogenic variants in *TNIP2* and *TRAF2* with PAH, we performed *in silico* analysis to search for relationships between these genes and others implicated in PAH pathogenesis. A study of the protein network by STRING showed that TRAF2 interacts with *CAV1*, a gene linked to hereditary PAH (54). In addition to STRING, there are numerous methods to identify critical gene-gene interactions and visualize pathways affected by genes of interest. Available tools include GSEA from the Broad Institute for gene list analysis and EnrichmentMap to visualize relationships between distinct pathways (55). We believe that investigators should implement these algorithms as part of ongoing genetic studies in PAH.

Inflammation has garnered increasing interest in PAH in recent years (56). Immune phenotypes characterized by differing signatures of inflammatory cytokines offer prognostic and potentially therapeutic significance (12). Antiinflammatory agents such as anakinra and tocilizumab are currently being investigated in PAH clinical trials (57, 58). NF-κB is known to regulate the expression of a wide array of inflammatory genes, and both human and animal studies indicate that NF-κB activity is increased in PAH. Core processes in PAH pathophysiology, such as aberrant pulmonary artery wall cell proliferation, apoptosis, and arterial obliteration, are attenuated with NF-κB inhibition (50). The *TNIP2* gene codes for ABIN2 (A20 binding inhibitor of NF-κB activation-2), a cytosolic protein that interacts with several membrane receptors (CD40, TNF receptor 1, and toll-like receptor 4) to suppress downstream activation of the NF-κB pathway (29, 59–61). TRAF2 belongs to a family of adaptor proteins associated with TNF receptors 1 and 2 to modulate TNF stimulation response in immune cells. Prior work supports TRAF2 as an essential mediator of cell survival and apoptosis through its capacity to activate or inhibit NF-κB, depending on the cell type (62, 63). Also, TRAF2 is involved in a membrane complex that can recruit caspases to promote apoptosis independent of the NF-κB pathway (64). Lastly, some authors have noted common hyperproliferative mechanisms driving PAH pathogenesis and cancer development (65), and pathogenic variants in TRAF proteins have been previously described in cancer (66). Specifically, *TRAF2* has suggested roles as an oncogene in epithelial cancer, osteotropic breast cancer and colon cancer (67–69), mainly through the NF-kB pathway. As evidenced by our *in vitro* studies, decreased expression of TNIP2 and TRAF2 can increase NF-κB activation in human pericytes. More importantly, pericytes demonstrated a change in proliferative activity under these conditions. These data support our hypothesis that pathogenic variants in *TNIP2* and *TRAF2* result in dysregulated NF-κB activation and could drive abnormal cell proliferation in PAH, as illustrated by the response of lung pericytes transfected with the corresponding siRNAs (**Figure 3**). However, a manifestation of clinically detectable PAH may require the presence of predisposing conditions (a "second-hit"), such as primary biliary cirrhosis or scleroderma, as seen in our subjects.

Our study has several limitations. First, whole-exome data was only available for a limited number of probands and their relatives, therefore limiting our power to detect novel variants potentially associated with PAH. Secondly, the technology used for our genetic analysis variants does not allow identification of variants outside of exons or exon-intron boundaries, which may hold essential regulatory elements. Additionally, our cell studies used pericytes from human donors, not from the subjects

analyzed in our study. These donors do not have predisposing inflammatory or cardiac conditions, which we believe in having been essential contributors to PAH development in the probands we analyzed. However, our data support the ongoing investigation of NF-κB as a potential therapeutic target in PAH and show that our genomics-to-bench approach can be useful for expanding our view of PAH genetics.

CONCLUSION

Identifying genetic biomarkers related to PAH holds the potential for a tremendous impact on patient care and the development of personalized treatments. We suggest that WES be considered for patients with associated forms of PAH that do not have causative variants identified with targeted gene panels, especially in cases with family history supportive of inherited disease. Genetic sequencing of family members with and without the disease, when feasible, will provide additional power to link genotype, phenotype, and endotype in PAH. Understanding the genetic underpinnings that predispose individuals to immune dysregulation in the pulmonary vascular wall will be paramount for advancing targeted therapies for this deadly disease. Variants in *TRAF2* and *TNIP2* may contribute to hyperproliferation and increased NF-κB activation in some individuals with PAH, however further studies are needed to describe the possible relationship of these genes with aberrant immune activity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, accession no: PRJNA702898 (ACCESSION PRIVATE).

ETHICS STATEMENT

All patients involved in this study gave their informed consent to participate. Samples were obtained from the Spanish PAH registries (REHAP and REHIPED), and the Ethical Committee approved the project of scientific research of the Hospital Universitario La Paz, Madrid (PI-1210, PI-3996).

AUTHOR CONTRIBUTIONS

JT and VDJP designed and supervised the work. NG, PA, and JT performed all genetic studied and NGS analysis. SP, DC, SA, HP, and AC performed all the functional and *in vitro* studies. ACU, NO, PE, and PL reviewed all the clinical features of the patients. JN performed the microarray analysis. JT, VDJP, SP, NG, and DC wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

VDJP was supported by NIH NHLBI R01HL134776 and R01 HL139664. This project was also supported by FEDER ISCIII Grant: PI18/01233, and by an unrestricted grant from the Spanish Foundation Against PAH (FCHP) (https://www.fchp.es/). AC holds a research-training contract Rio Hortega (CM20/00164) from the Spanish Ministry of Science and Innovation (Instituto de Salud Carlos III), Grant from FEDER (Federación Española de Enfermedades Raras; https://enfermedades-raras.org/).

ACKNOWLEDGMENTS

We would like to thank the National registries of patients with PAH, REHAP, and the REHIPED.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.625763/full#supplementary-material

REFERENCES

1. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, et al. 2015 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension. *Rev Espanola de Cardiol.* (2016) 69:177. doi: 10.1016/j.recesp.2016.01.002
2. Condon DF, Nickel NP, Anderson R, Mirza S, de Jesus Perez VA. The 6th world symposium on pulmonary hypertension: what's old is new. *F1000Res.* (2019) 8:F1000 Faculty Rev-888. doi: 10.12688/f1000research.18811.1
3. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Investig.* (2012) 122:4306–13. doi: 10.1172/JCI60658
4. de Jesus Perez VA, Yuan K, Orcholski ME, Sawada H, Zhao M, Li CG, et al. Loss of adenomatous poliposis coli-alpha3 integrin interaction promotes endothelial apoptosis in mice and humans. *Circul Res.* (2012) 111:1551–64. doi: 10.1161/CIRCRESAHA.112.267849
5. Evans JD, Girerd B, Montani D, Wang XJ, Galie N, Austin ED, et al. BMPR2 mutations and survival in pulmonary arterial hypertension: an individual participant data meta-analysis. *Lancet Respir Med.* (2016) 4:129–37. doi: 10.1016/S2213-2600(15)00544-5
6. Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, et al. Genetics and genomics of pulmonary arterial hypertension. *J Am College Cardiol.* (2009) 54:S32–42. doi: 10.1016/j.jacc.2009.04.015
7. Cai J, Pardali E, Sanchez-Duffhues G, ten Dijke P. BMP signaling in vascular diseases. *FEBS Lett.* (2012) 586:1993–2002. doi: 10.1016/j.febslet.2012.04.030
8. Dyer LA, Pi X, Patterson C. The role of BMPs in endothelial cell function and dysfunction. *Trends Endocrinol Metabol TEM.* (2014) 25:472–80. doi: 10.1016/j.tem.2014.05.003
9. Tada Y, Majka S, Carr M, Harral J, Crona D, Kuriyama T, et al. Molecular effects of loss of BMPR2 signaling in smooth muscle in a transgenic mouse model of PAH. *Am J Physiol Lung Cell Mol Physiol.* (2007) 292:L1556–63. doi: 10.1152/ajplung.00305.2006
10. NMorrell W, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, et al. Genetics and genomics of pulmonary arterial hypertension. *Eur Respir J.* (2018) 53:1801899. doi: 10.1183/13993003.01899-2018
11. Zhu N, Pauciulo MW, Welch CL, Lutz KA, Coleman AW, Gonzaga-Jauregui C, et al. Novel risk genes and mechanisms implicated by exome sequencing of 2572 individuals with pulmonary arterial hypertension. *Genome Med.* (2019) 11:69. doi: 10.1186/s13073-019-0685-z

12. Sweatt AJ, Hedlin HK, Balasubramanian V, Hsi A, Blum LK, Robinson WH, et al. Discovery of distinct immune phenotypes using machine learning in pulmonary arterial hypertension. *Circulat Res.* (2019) 124:904–19. doi: 10.1161/CIRCRESAHA.118.313911
13. Austin ED, Ma L, LeDuc C, Berman Rosenzweig E, Borczuk A, Phillips JA, et al. Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. *Circ Cardiovasc Genet.* (2012) 5:336–43. doi: 10.1161/CIRCGENETICS.111.961888
14. de Jesus Perez VA, Yuan K, Lyuksyutova MA, Dewey F, Orcholski ME, Shuffle EM, et al. Whole-exome sequencing reveals TopBP1 as a novel gene in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med.* (2014) 189:1260–72. doi: 10.1164/rccm.201310-1749OC
15. Eyries M, Montani D, Girerd B, Perret C, Leroy A, Lonjou C, et al. EIF2AK4 mutations cause pulmonary veno-occlusive disease, a recessive form of pulmonary hypertension. *Nat Genet.* (2014) 46:65–9. doi: 10.1038/ng.2844
16. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson SK, Soubrier F, et al. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med.* (2013) 369:351–61. doi: 10.1056/NEJMoa1211097
17. Castano JAT, Hernandez-Gonzalez I, Gallego N, Perez-Olivares C, Ochoa Parra N, Arias P, et al. Customized massive parallel sequencing panel for diagnosis of pulmonary arterial hypertension. *Genes.* (2020) 11:1158. doi: 10.3390/genes11101158
18. Fan J, Fan X, Guang H, Shan X, Tian Q, Zhang F, et al. Upregulation of miR-335-3p by NF-kappaB transcriptional regulation contributes to the induction of pulmonary arterial hypertension via APJ during hypoxia. *Int J Biol Sci.* (2020) 16:515–28. doi: 10.7150/ijbs.34517
19. Li Y, Yang L, Dong L, Yang ZW, Zhang J, Zhang SL, et al. Crosstalk between the Akt/mTORC1 and NF-kappaB signaling pathways promotes hypoxia-induced pulmonary hypertension by increasing DPP4 expression in PASMCs. *Acta Pharmacol Sin.* (2019) 40:1322–33. doi: 10.1038/s41401-019-0272-2
20. Liu WY, Wang L, Lai YF. Hepcidin protects pulmonary artery hypertension in rats by activating NF-kappaB/TNF-alpha pathway. *Eur Rev Med Pharmacol Sci.* (2019) 23:7573–81. doi: 10.26355/eurrev_201909_18878
21. Woods M, Wood EG, Bardswell SC, Bishop-Bailey D, Barker S, Wort SJ, et al. Warner, role for nuclear factor-kappaB and signal transducer and activator of transcription 1/interferon regulatory factor-1 in cytokine-induced endothelin-1 release in human vascular smooth muscle cells. *Mol Pharmacol.* (2003) 64:923–31. doi: 10.1124/mol.64.4.923
22. Thomas CA, Anderson RJ, Condon DF, de Jesus Perez VA. Diagnosis and management of pulmonary hypertension in the modern era: insights from the 6th world symposium. *Pulm Ther.* (2020) 6:9–22. doi: 10.1007/s41030-019-00105-5
23. Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, et al. VarSome: the human genomic variant search engine. *Bioinformatics.* (2019) 35:1978–80. doi: 10.1093/bioinformatics/bty897
24. Liu X, Wu C, Li C, Boerwinkle E. dbNSFP v3.0: a one-stop database of functional predictions and annotations for human nonsynonymous and splice-site SNVs. *Hum Mutat.* (2016) 37:235–41. doi: 10.1002/humu.22932
25. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res.* (2019) 47:D886–94. doi: 10.1093/nar/gky1016
26. Yuan K, Liu Y, Zhang Y, Nathan A, Tian W, Yu J, et al. Mural cell SDF1 signaling is associated with the pathogenesis of pulmonary arterial hypertension. *Am J Respir Cell Mol Biol.* (2020) 62:747–59. doi: 10.1165/rcmb.2019-0401OC
27. Yuan K, Orcholski ME, Panaroni C, Shuffle EM, Huang NF, Jiang X, et al. Activation of the Wnt/planar cell polarity pathway is required for pericyte recruitment during pulmonary angiogenesis. *Am J Pathol.* (2015) 185:69–84. doi: 10.1016/j.ajpath.2014.09.013
28. Zehendner CM, Valasarajan C, Werner A, Boeckel JN, Bischoff FC, John D, et al. Long noncoding RNA TYKRIL plays a role in pulmonary hypertension via the p53-mediated regulation of PDGFRbeta. *Am J Respir Crit Care Med.* (2020) 202:1445–57. doi: 10.1164/rccm.201910-2041OC
29. Banks CA, Boanca G, Lee ZT, Eubanks CG, Hattem GL, Peak A, et al. TNIP2 is a hub protein in the NF-kappaB network with both protein and RNA mediated interactions. *Mol Cell Proteomics.* (2016) 15:3435–49. doi: 10.1074/mcp.M116.060509
30. Pomerantz JL, Baltimore D. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. *EMBO J.* (1999) 18:6694–704. doi: 10.1093/emboj/18.23.6694
31. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* (2019) 47:D607–13. doi: 10.1093/nar/gky1131
32. Feng X, Gaeta ML, Madge LA, Yang JH, Bradley JR, Pober JS. Caveolin-1 associates with TRAF2 to form a complex that is recruited to tumor necrosis factor receptors. *J Biol Chem.* (2001) 276:8341–9. doi: 10.1074/jbc.M007116200
33. Legler DF, Micheau O, Doucey MA, Tschopp J, Bron C. Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. *Immunity.* (2003) 18:655–64. doi: 10.1016/S1074-7613(03)00092-X
34. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther.* (2017) 2:17023. doi: 10.1038/sigtrans.2017.23
35. Karin M. NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol.* (2009) 1:a000141. doi: 10.1101/cshperspect.a000141
36. Ricard N, Tu L, Le Hiress M, Huertas A, Phan C, Thuillet R, et al. Increased pericyte coverage mediated by endothelial-derived fibroblast growth factor-2 and interleukin-6 is a source of smooth muscle-like cells in pulmonary hypertension. *Circulation.* (2014) 129:1586–97. doi: 10.1161/CIRCULATIONAHA.113.007469
37. Guijarro-Munoz I, Compte M, Alvarez-Cienfuegos A, Alvarez-Vallina L, Sanz L. Lipopolysaccharide activates Toll-like receptor 4 (TLR4)-mediated NF-kappaB signaling pathway and proinflammatory response in human pericytes. *J Biol Chem.* (2014) 289:2457–68. doi: 10.1074/jbc.M113.521161
38. Roberts KE, McElroy JJ, Wong WP, Yen E, Widlitz A, Barst RJ, et al. BMPR2 mutations in pulmonary arterial hypertension with congenital heart disease. *Eur Respir J.* (2004) 24:371–4. doi: 10.1183/09031936.04.00018604
39. Liu D, Liu QQ, Guan LH, Jiang X, Zhou DX, Beghetti M, et al. BMPR2 mutation is a potential predisposing genetic risk factor for congenital heart disease associated pulmonary vascular disease. *Int J Cardiol.* (2016) 211:132–6. doi: 10.1016/j.ijcard.2016.02.150
40. Eyries M, Montani D, Girerd B, Favrolt N, Riou M, Faivre L, et al. Familial pulmonary arterial hypertension by KDR heterozygous loss of function. *Eur Respir J.* (2020) 55:1902165. doi: 10.1183/13993003.02165-2019
41. Zhu N, Welch CL, Wang J, Allen PM, Gonzaga-Jauregui C, Ma L, et al. Rare variants in SOX17 are associated with pulmonary arterial hypertension with congenital heart disease. *Genome Med.* (2018) 10:56. doi: 10.1186/s13073-018-0566-x
42. Graf S, Haimel M, Bleda M, Hadinnapola C, Southgate L, Li W, et al. Morrell, identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat Commun.* (2018) 9:1416. doi: 10.1038/s41467-018-03672-4
43. Rhodes CJ, Batai K, Bleda M, Haimel M, Southgate L, Germain M, et al. Genetic determinants of risk in pulmonary arterial hypertension: international genome-wide association studies and meta-analysis. *Lancet Respir Med.* (2019) 7:227–38. doi: 10.1016/S2213-2600(18)30409-0
44. Wipff J, Kahan A, Hachulla E, Sibilia J, Cabane J, Meyer O, et al. Association between an endoglin gene polymorphism and systemic sclerosis-related pulmonary arterial hypertension. *Rheumatology.* (2007) 46:622–5. doi: 10.1093/rheumatology/kel378
45. Al-Naamani N, Krowka MJ, Forde KA, Krok KL, Feng R, Heresi GA, et al. Pulmonary vascular complications of liver disease study, estrogen signaling and portopulmonary hypertension: the pulmonary vascular complications of liver disease study (PVCLD2). *Hepatology.* (2020) 73:726–37. doi: 10.1002/hep.31314
46. Xiong M, Wang J, Guo M, Zhou Q, Lu W. TRPM8 genetic variations associated with COPD risk in the Chinese Han population. *Int J Chron Obstruct Pulmon Dis.* (2016) 11:2563–71. doi: 10.2147/COPD.S109026
47. Li M, Cai W, Chen Y, Dong L. The CAV1 gene 3' Untranslated region single nucleotide polymorphisms are associated with the risk of pulmonary hypertension in Chinese Han chronic obstructive pulmonary patients. *Genet Test Mol Biomarkers.* (2019) 23:634–43. doi: 10.1089/gtmb.2019.0053
48. Jin G, Chen Z, Zhang J, Song J, Shi J, Zhou B. Association of brain natriuretic peptide gene polymorphisms with chronic obstructive pulmonary disease

complicated with pulmonary hypertension and its mechanism. *Biosci Rep.* (2018) 38:BSR20180905. doi: 10.1042/BSR20180905
49. Hosokawa S, Haraguchi G, Sasaki A, Arai H, Muto S, Itai A, et al. Pathophysiological roles of nuclear factor kappaB (NF-kB) in pulmonary arterial hypertension: effects of synthetic selective NF-kB inhibitor IMD-0354. *Cardiovasc Res.* (2013) 99:35–43. doi: 10.1093/cvr/cvt105
50. Farkas D, Alhussaini AA, Kraskauskas D, Kraskauskiene V, Cool CD, Nicolls MR, et al. Nuclear factor kappaB inhibition reduces lung vascular lumen obliteration in severe pulmonary hypertension in rats. *Am J Respir Cell Mol Biol.* (2014) 51:413–25. doi: 10.1165/rcmb.2013-0355OC
51. Price LC, Caramori G, Perros F, Meng C, Gambaryan N, Dorfmuller P, et al. Nuclear factor κ-B is activated in the pulmonary vessels of patients with end-stage idiopathic pulmonary arterial hypertension. *PLoS ONE.* (2013) 10:e75415. doi: 10.1371/journal.pone.0075415
52. Sawada H, Mitani Y, Maruyama J, Jiang BH, Ikeyama Y, Dida FA, et al. A nuclear factor-kappaB inhibitor pyrrolidine dithiocarbamate ameliorates pulmonary hypertension in rats. *Chest.* (2007) 4:1265–74. doi: 10.1378/chest.06-2243
53. Pang Y, Liang MT, Gong Y, Yang Y, Bu PL, Zhang M, et al. HGF reduces disease severity and inflammation by attenuating the NF-κB signaling in a rat model of pulmonary artery hypertension. *Inflammation.* (2018) 3:924–31. doi: 10.1007/s10753-018-0747-1
54. Han B, Copeland CA, Kawano Y, Rosenzweig EB, Austin ED, Shahmirzadi L, et al. Characterization of a caveolin-1 mutation associated with both pulmonary arterial hypertension and congenital generalized lipodystrophy. *Traffic.* (2016) 17:1297–312. doi: 10.1111/tra.12452
55. Grimes T, Potter SS, Datta S. Integrating gene regulatory pathways into differential network analysis of gene expression data. *Sci Rep.* (2019) 9:5479. doi: 10.1038/s41598-019-41918-3
56. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circulat Res.* (2014) 115:165–75. doi: 10.1161/CIRCRESAHA.113.301141
57. Trankle CR, Canada JM, Kadariya D, Markley R, De Chazal HM, Pinson J, et al. IL-1 blockade reduces inflammation in pulmonary arterial hypertension and right ventricular failure: a single-arm, open-label, phase IB/II pilot study. *Am J Respir Crit Care Med.* (2019) 199:381–4. doi: 10.1164/rccm.201809-1631LE
58. Hernandez-Sanchez J, Harlow L, Church C, Gaine S, Knightbridge E, Bunclark K, et al. Clinical trial protocol for TRANSFORM-UK: a therapeutic open-label study of tocilizumab in the treatment of pulmonary arterial hypertension. *Pulm Circ.* (2018) 8:2045893217735820. doi: 10.1177/2045893217735820
59. Besson B, Sonthonnax F, Duchateau M, Ben Khalifa Y, Larrous F, Eun H, et al. Regulation of NF-kappaB by the p105-ABIN2-TPL2 complex and RelAp43 during rabies virus infection. *PLoS Pathog.* (2017) 13:e1006697. doi: 10.1371/journal.ppat.1006697
60. Nanda SK, Nagamori T, Windheim M, Amu S, Aviello G, Patterson-Kane J, et al. ABIN2 function is required to suppress DSS-induced colitis by a Tpl2-independent mechanism. *J Immunol.* (2018) 201:3373–82. doi: 10.4049/jimmunol.1700614
61. Papoutsopoulou S, Symons A, Tharmalingham T, Belich MP, Kaiser F, Kioussis D, et al. ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses. *Nat Immunol.* (2006) 7:606–15. doi: 10.1038/ni1334
62. Etemadi N, Chopin M, Anderton H, Tanzer MC, Rickard JA, Abeysekera W, et al. TRAF2 regulates TNF and NF-kappaB signalling to suppress apoptosis and skin inflammation independently of Sphingosine kinase 1. *Elife.* (2015) 4:e10592. doi: 10.7554/eLife.10592.018
63. Grech AP, Amesbury M, Chan T, Gardam S, Basten A, Brink R. TRAF2 differentially regulates the canonical and noncanonical pathways of NF-kappaB activation in mature B cells. *Immunity.* (2004) 21:629–42. doi: 10.1016/j.immuni.2004.09.011
64. Van Quickelberghe E, De Sutter D, van Loo G, Eyckerman S, Gevaert K. A protein-protein interaction map of the TNF-induced NF-kappaB signal transduction pathway. *Sci Data.* (2018) 5:180289. doi: 10.1038/sdata.2018.289
65. Boucherat O, Vitry G, Trinh I, Paulin R, Provencher S, Bonnet S. The cancer theory of pulmonary arterial hypertension. *Pulm Circ.* (2017) 2:285–99. doi: 10.1177/2045893217701438
66. Zhu S, Jin J, Gokhale S, Lu AM, Shan H, Feng J, et al. Genetic alterations of TRAF proteins in human cancers. *Front Immunol.* (2018) 9:2111. doi: 10.3389/fimmu.2018.02111
67. Shen RR, Zhou AY, Kim E, O'Connell JT, Hagerstrand D, Beroukhim R, et al. TRAF2 is an NF-κB-activating oncogene in epithelial cancers. *Oncogene.* (2015) 2:209–16. doi: 10.1038/onc.2013.543
68. Peramuhendige P, Marino S, Bishop RT, de Ridder D, Khogeer A, Baldini I, et al. TRAF2 in osteotropic breast cancer cells enhances skeletal tumour growth and promotes osteolysis. *Sci Rep.* (2018) 1:39. doi: 10.1038/s41598-017-18327-5
69. Peng C, Zhu F, Wen W, Yao K, Li S, Zykova T, et al. Tumor necrosis factor receptor-associated factor family protein 2 is a key mediator of the epidermal growth factor-induced ribosomal S6 kinase 2/cAMP-responsive element-binding protein/Fos protein signaling pathway. *J Biol Chem.* (2012) 287:25881–92. doi: 10.1074/jbc.M112.359521

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership