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Flow Cytometric Determination of Circulating Progenitor Cells in Patients With Pulmonary Arterial Hypertension: A Systematic Review

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ABSTRACT

Pulmonary arterial hypertension (PAH) is characterized by progressive narrowing and obliteration of distal, pre-capillary pulmonary vessels. Yet, noninvasive biomarkers that reflect this disease-defining process are lacking. A systematic review of PAH studies that measured circulating progenitor cells (CPCs) or circulating endothelial cells (CECs) in PAH by flow cytometry was performed to understand how future studies, leveraging state-of-the-art single-cell analyses, can advance the field. The study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews. Of the 2422 studies identified, 20 met inclusion criteria. Nineteen studies measured CPCs by flow cytometry, only one study examined CECs. A total of 647 PAH patients were included across all 19 CPC studies. Marker schemes chosen to define CPCs, and the methods of flow cytometry used, varied significantly across studies. Meta-analysis of a subgroup of CPC studies ($n = 8$) similarly identified a significant amount of heterogeneity even amongst studies using the same marker scheme. In conclusion, a systematic review of CPC studies in PAH patients reveals the limitations of the current literature. Future studies should include contemporary risk assessments, disease duration, reporting of comorbid conditions, and serial sampling over time. Furthermore, methods that incorporate best practices for detecting rare cell populations by flow cytometry are essential and should be reported in sufficient detail in future publications. With the emergence of single-cell technologies, future studies of circulating progenitor and endothelial cells in PAH remain relevant and may incorporate several insights from the current review to build upon the existing literature.

Contemporary pulmonary arterial hypertension (PAH) management is guided by risk assessment tools that integrate multiple clinical variables associated with disease severity to estimate a patient's risk of death [1]. Nevertheless, most clinical measures incorporated into current risk assessment tools are largely removed from the characteristic structural abnormalities within the pulmonary circulation that define the disease. For example, many noninvasive parameters [echocardiography, 6-min walk test, New York Heart Association/World Health Organization Functional Classification (NYHA/WHO FC)] and blood biomarkers [brain natriuretic

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peptide (BNP) and N-terminal pro-BNP] currently in clinical use are more closely tied to right ventricular function than lung vascular remodeling. Importantly, the lack of a reliable marker of lung vessel remodeling in PAH limits the assessment of therapies aimed at reversing these vascular lesions [2, 3]. Therefore, identifying biomarkers that more directly reflect the pathological changes in the pulmonary vasculature remains an unmet need [4].

Circulating progenitor cells (CPCs) are a heterogenous group of cells that, in vitro, can differentiate into cells of either hematopoietic or endothelial lineage and to a varying degree, maintain proliferative capacity [5, 6]. Subpopulations of CPCs appear to participate, either directly or indirectly, in vascular homeostasis, including angiogenesis and vasculogenesis [6]. In contrast to CPCs, circulating endothelial cells (CECs) are terminally differentiated cells shed from the endothelial lining of blood vessels into the circulation, lack proliferative capacity [7], and by one established marker scheme, the majority appear to be senescent in healthy volunteers [8]. While detected at low levels in healthy subjects, CEC shedding is increased in diseases characterized by vascular injury and/or endothelial dysfunction [9].

While there has been progress in distinguishing these subpopulations of circulating cells by flow cytometry, current consensus definitions rely on specific ex vivo cell culture techniques as well as in vitro phenotypic and in vivo functional assays [5, 10]. Despite the limitations inherent in assigning specific cell types based on surface protein expression (e.g., CD34, CD133, KDR/VEGFR2, and CD45), flow cytometric determination of these rare circulating cell populations appears to predict important patient outcomes such as the occurrence of major cardiovascular events, death due to cardiovascular disease and all-cause survival [11–14]. Studies of PAH patients have sought to enumerate these rare circulating cells and investigate their role in pathologic lung vessel remodeling [15]. For example, CPCs may be recruited to the lung as part of a reparative response to pulmonary vascular injury [15]. Conversely, mobilization of these cells may drive pathologic vessel remodeling [16, 17]. Nevertheless, the various studies investigating CPCs in PAH patients have been difficult to synthesize given their seemingly discordant results. With the emergence of technologies enabling lineage tracing [18], single-cell analysis of lung endothelial cell diversity [19], and its relevance to PAH [20–23], future studies of CPCs and CECs and their role in vascular biology are primed to generate novel insights. A comprehensive examination of the current literature is an important first step toward this goal and these future investigations. Our systematic review specifically focused on PAH studies that utilized flow cytometry to enumerate circulating cell populations with at least one progenitor or stem cell marker and/or one endothelial cell marker. Importantly, the goal of this review was not to adjudicate specific marker schemes or conclude whether a given study was investigating the "correct" cell population. Instead, a broad, inclusive approach was taken to unbiasedly capture all studies reporting on these rare circulating cell populations in PAH patients.

1 | Methods

This study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) and Meta-Analyses of Observational Studies in Epidemiology (MOOSE) guidelines. The protocol for this systematic review and meta-analysis is registered on PROSPERO (CRD42022362175). The MOOSE reporting checklist (Supporting Information File S2). and PRISMA 2020 checklist are provided in the online Supporting Information File S3.

1.1 | Information Sources

A search of PubMed, Embase and Web of Science from inception to December 15th, 2021 was conducted by a systematic review certified biomedical research librarian using a pre-defined strategy without language restrictions and including preprints and conference abstracts. The search was updated up to and including August 26, 2024. Keywords and MESH terms used for the search strategy are provided in the online Supporting Information File S1. The total number of citations retrieved from all three databases was 2422. After 112 duplicates were removed, the remaining 2310 citations were uploaded into Covidence, a web-based software platform that facilitates data extraction for systematic reviews [24].

1.2 | Selection Criteria and Study Screening

Criteria for inclusion in the systematic review were: (1) diagnosis of PAH based upon published clinical guidelines [1], (2) flow cytometric determination of circulating progenitor (must express at least one progenitor/stem cell marker with or without expression of an endothelial cell marker), and/or endothelial (must express at least one endothelial cell marker) cells from human peripheral blood, and (3) analysis of circulating cell populations relative to a comparator group (e.g., healthy controls) or relative to clinical measure(s) or marker(s) of disease severity. Studies that relied only on magnetic bead capture or in vitro culture of blood-derived circulating cells, as well as studies only reporting on extracellular vesicles, cultured cells, and non-human blood samples, were excluded. Articles lacking original research (e.g., review articles, book chapters, editorials) were also excluded. Titles and abstracts were independently screened against inclusion and exclusion criteria by at least two reviewers (KJ, CJ, MAS, JME). Full texts of papers that passed screening were further reviewed and assessed based on inclusion and exclusion criteria by at least two reviewers (KH, CJ, JME). Disagreements at each stage were referred for arbitration to a third reviewer and resolved by discussion. The screening process was summarized in a PRISMA flow diagram.

1.3 | Data Extraction

Three reviewers (KH, CJ, and JME) independently extracted data from each article meeting eligibility criteria and compared extractions. Pertinent data included patient characteristics (age, sex, PAH etiology, current treatment, clinical assessments),

study characteristics (author, year of publication, study design, number of participants, comparator groups, cell type(s) identified and marker scheme used to define each cell type, and flow cytometric methods), and study results (number or fraction of cells identified in study population, differences between PAH patients and healthy controls or other comparator, and association of circulating cells with clinical assessments).

1.4 | Quality Assessment and Risk of Bias Reporting

The quality of reporting in each study was assessed based on the following criteria: (1) clear hypothesis or statement of research aims (1 point); (2) explanation of subject enrollment and sampling (2 points); (3) description of the flow cytometric methods used for data acquisition and analysis (6 points); (4) statement and (5) discussion of the findings (1 point each). The maximum score was 11 points. In assessing whether each paper met criteria for "subject enrollment and sampling," 1 point was given for stating or referencing PAH diagnostic criteria, and 1 point was given if the authors obtained samples prospectively from consecutive subjects meeting their study criteria. To evaluate the methods described in each study, we adapted a previously published scoring system [25] based on recommended standards for reporting flow cytometry experiments [26]. These recommendations aim to standardize the details of experimental methodology that must be reported in scientific publications to ensure reproducibility. The following elements of flow cytometry were assessed: (1) clear description of the sample type and methods of processing before flow cytometry (1 point); (2) whether or not a gating strategy was reported (1 point); (3) clear description of the results reported as either cell frequency or absolute cell number (1 point); (4) clear reference to the parent population (1 point); (5) description of negative controls (1 point); and (6) mention of viability staining (1 point). Next, the risk of bias was assessed using an adapted version of the critical appraisal tool from the Joanna Briggs Institute Checklist for cohort studies [27], which assesses risk of bias in cohort studies using the following categories: (1) clearly defined inclusion/exclusion criteria, (2) description of study subjects (3) exposure (defined as PAH disease duration for the purposes of our assessment), (4) objective standard criteria for the measurement of PAH, (5) identification of confounding factors, (6) enumeration of strategy to address confounding factors, (7) reliable measurement of outcomes by flow cytometry, and (8) appropriate use of statistical methods. Any disagreement between two reviewers was resolved by iteration until a consensus was reached.

1.5 | Meta-Analysis

Standardized mean differences (SMD) were used to analyze CPCs in PAH patients compared to healthy controls since the units of CPC measurement differed across studies. Studies using consistent marker schemes, regardless of the sample type [e.g., whole blood, isolated peripheral blood mononuclear cells (PBMCs)], were grouped together for meta-analysis. Most studies reported data appropriate for meta-analysis in the original manuscripts. Requests were made to the corresponding authors of three studies to obtain data in the proper format necessary for meta-analysis [28–30]. One author responded but was unable to provide additional data, and another author did not respond despite two attempts. A third study provided data but ultimately was not included because it did not share a marker scheme with any of the other included studies. Studies within each group were combined using a random-effect model, which accounts for heterogeneity across included studies [31]. Heterogeneity among studies was assessed using the Q statistic and I^2 value [32]. All analyses were performed using R (version 4.2.2) [33] packages meta (version 6.2-0) [34]. Two-sided p values ≤ 0.05 were considered significant.

2 | Results

2.1 | Search Results

We identified 2422 studies and assessed 62 full-text manuscripts after removal of 112 duplicates and 2248 irrelevant studies (Figure 1). Forty-four of the full-text studies assessed for eligibility were excluded, leaving 18 studies that met inclusion criteria (Figure 1). Two reviewers (KH and CT) hand searched references of the 62 full-text articles and found two additional articles that met inclusion criteria (Figure 1). Detailed study characteristics and major findings of the 20 studies that met inclusion criteria are summarized in Table 1. Of the 20 studies, 19 measured CPCs by flow cytometry in PAH patients, while only one study specifically examined CECs using flow cytometry [52]. Therefore, the remainder of the systematic review focuses only on the 19 CPC studies.

2.2 | Characteristics of CPC Studies

A total of 647 PAH patients were included across all 19 CPC studies. The median (IQR) number of PAH patients per study was 20 [9–37, 41, 46, 50, 52–54]. Most CPC studies included a heterogenous patient cohort with at least two different PAH subtypes. Idiopathic PAH (263/647, 41%) and PAH due to congenital heart disease (CHD-PAH; 184/647, 28%) made up the two largest subtypes of patients in the 19 studies (Figure 2). Three studies included only pediatric patients with CHD-PAH [46, 53, 54]. Eleven studies compared levels of CPCs in PAH patients to healthy controls [28–30, 35–37, 39–41, 43, 50], 5 studies reported comparisons between PAH subtypes [28, 29, 43, 50, 54], 6 compared PAH patients to patients affected by other cardiopulmonary or systemic diseases [36, 43, 46, 48, 49, 53], and 5 compared levels of CPCs pre- and posttreatment with either pulmonary vasodilators [40, 43, 54] or experimental therapy [47, 51] (Table 1). Lastly, one study compared the percentage of CPCs in PAH patients at baseline and 1 year follow-up [38]. Several studies included more than one comparison. Peripheral venous blood samples were the most common sample type ($n = 16$ studies), but some studies also collected additional sample types, including bone marrow [50], radial artery [36], pulmonary artery [36, 49, 53], and/or pulmonary vein [53].

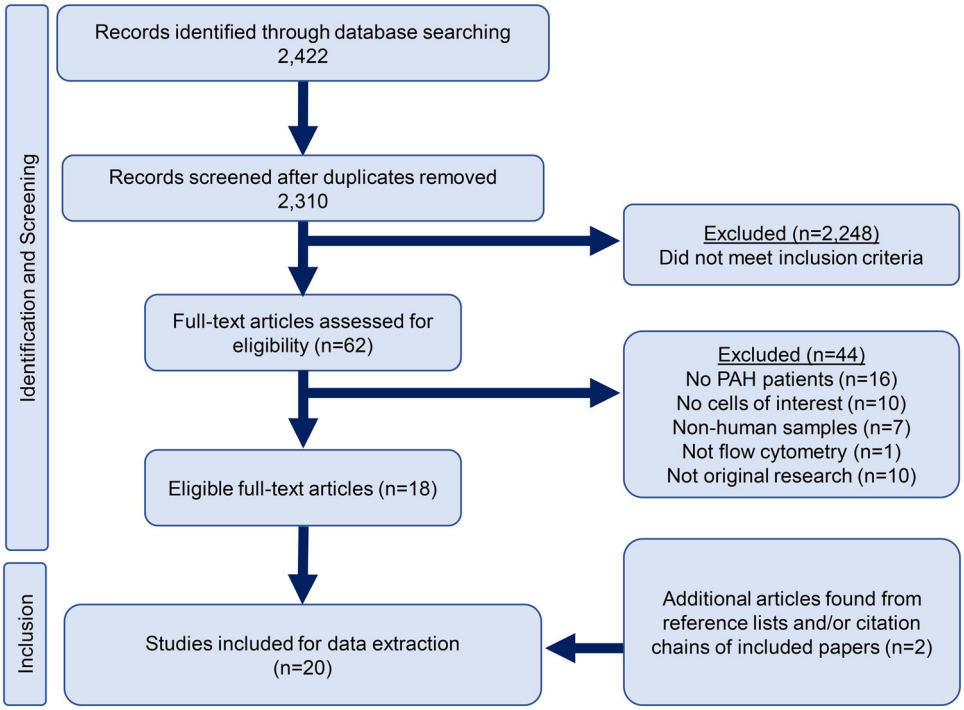

FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram summarizing the process of literature identification, screening, and inclusion.

2.3 | Quality and Risk of Bias Assessments

2.3.1 | Enrollment Criteria

Although nearly all CPC studies provided a clear statement of their research aims, study findings and a discussion of their results, a clear explanation of subject enrollment and sampling was not uniformly reported across CPC studies (Figure 3). Only 8 studies included clear criteria for subject enrollment and patient sampling (Supporting Information Table 1). Most cohort studies were cross-sectional without specific mention of how PAH subjects were selected for enrollment. Corresponding authors from research groups that published multiple CPC studies confirmed that the data reported was independently collected for each study and not included in any of their other CPC manuscripts [37, 38, 40, 43, 47, 49–51, 53, 54]. In most cases, authors were able to confirm that the patient populations were indeed unique, without any overlap between studies [37, 40, 43, 47, 49, 53, 54]. In the remaining studies, the extent of patient overlap, if any, could not be confirmed.

2.3.2 | PAH Patient Clinical Characteristics

The extent of clinical information describing the PAH cohort in each study varied widely (Supporting Information Table 2). Only three studies provided the duration of subjects' symptoms or time from diagnosis to enrollment [28, 41, 48]. Patient level hemodynamics or summary hemodynamic data was included in 15 studies, however several of these studies included only one measure (e.g., mean pulmonary artery pressure or pulmonary vascular resistance) or a limited set of measurements. Whether or not patients were receiving PAH therapy at the time of study enrollment and details of their treatment regimens were included in 14 studies (Supporting Information Table 2). Clinical assessments of functional capacity and disease severity reported across studies included NYHA/WHO FC ($n = 7$ studies), 6-min walk distance (6MWD; $n = 9$ studies), and BNP concentrations ($n = 5$ studies). Currently recommended clinical risk assessments were not reported in any of the 19 studies.

2.3.3 | Strategies to Address Confounding

Approximately a third of the studies acknowledged and attempted to address confounding factors in their study design (Figure 3). For example, Diller et al. conducted subgroup analyses based on sex, class of vasodilator therapy, and, in Eisenmenger patients, coexistence of down syndrome [28]. Garcia-Lucio et al. specifically included treatment naïve patients in an attempt to mitigate confounding due to concurrent PAH therapies [40]. Lastly, five studies included a disease control group [e.g., unrepaired CHD, advanced lung disease, or systemic sclerosis (SSc) without PAH] [36, 43, 46, 48, 53].

2.3.4 | Flow Cytometry Methods

The validity and reliability of CPC study results was based on a composite flow cytometry quality score that evaluated 6 different assay components (see online supplement for further details and Supporting Information Table 3 for individual scores). Twelve studies received a flow cytometry quality score of ≥ 5 (maximum score 6) and thus may be considered more valid and reliable than studies with scores ≤ 4. Nearly all studies provided an adequate description of

TABLE 1 | Study characteristics and major findings.

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
Asosingh 2008[b] [35]	Cross-sectional	Peripheral vein; whole blood	IPAH (13), HPAH (3), Healthy controls (16)	• $CD34^+CD133^+$	• Elevated $CD34^+CD133^+$ cells in PAH vs controls	• $\%CD34^+CD133^+$ cells positively correlated with systolic PAP
Diller 2008 [31]	Cross-sectional	Not reported; PBMCs	IPAH (55), CHD-PAH with Eisenmenger Syndrome (41), Healthy controls (47)	• $CD34^+$ • $CD34^+CD133^+$ • $CD34^+KDR^+$ • $CD34^+CD133^+KDR^+$	• $CD34^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells are reduced in IPAH and CHD-PAH with ES vs controls • Compared to male IPAH patients, male ES patients with Down Syndrome had lower percentages of $CD34^+$, $CD34^+CD133^+$, $CD34^+KDR^+$, and $CD34^+CD133^+KDR^+$ cells	• In CHD-PAH patients with ES, % of progenitor cells was higher in patients with NYHA/WHO FC II symptoms compared with FC III and positively correlated with 6MWD • In IPAH patients, $\%CD34^+KDR^+$ cells did not vary with NYHA/WHO FC or 6MWD but were positively correlated with sildenafil dose and cardiac index
Zhu 2008[c] [36]	Cross-sectional	Peripheral vein; whole blood	IPAH (20), Healthy controls (20)	• $CD133^+KDR^+$	• Decreased $CD133^+KDR^+$ cells in PAH vs controls	• Number of $CD133^+KDR^+$ cells positively correlated with 6MWD and negatively correlated with BNP, mPAP and PVR.
Smadja 2009 [37]	Cross-sectional	Peripheral vein, pulmonary artery, and pulmonary vein; whole blood	CHD with reversible (16) or irreversible (10) PAH, CHD without PAH (5)	• $CD34^+CD133^+$	• Number of $CD34^+CD133^+$ cells is similar regardless of sampling site (peripheral vein, pulmonary artery, or pulmonary vein) • Number of $CD34^+CD133^+$ cells is similar in patients with reversible and irreversible CHD-PAH vs controls	• None explored
Toshner 2009[d] [32]	Cross-sectional	*Giessen:* venous blood (sample location unclear)[d]; PBMCs *Cambridge:* Peripheral vein; whole blood	*Giessen:* IPAH (10), CHD-PAH (2), CTD-PAH (3), PoPH (3), Healthy controls (11) *Cambridge:* IPAH (7), HPAH (4), Healthy controls (7)	• *Giessen:* $CD133^+KDR^+$ • *Cambridge:* $CD34^+CD133^+KDR^+$ $CD34^+CD133^+KDR^-CD34^+$ $CD133^+$	• Compared to healthy controls, the number of $CD133^+KDR^+$ cells was higher in IPAH, and APAH patients (*Giessen*) and the number of $CD34^+CD133^+KDR^+$ cells was higher in IPAH and HPAH patients (*Cambridge*)	• In Giessen cohort, the number of $CD133^+KDR^+$ cells was not correlated with PAP, cardiac index, or PVR

(Continues)

TABLE 1 | (Continued)

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
					• The number of $CD133^+KDR^+$ cells was similar in IPAH and APAH patients (*Giessen*) • The number of $CD34^+CD133^+KDR^+$ cells was similar in IPAH and HPAH patients (*Cambridge*)	
Smadja 2010[e] [38]	Cross-sectional	Peripheral vein and pulmonary artery; whole blood	IPAH (3), CTD-PAH (3), PoPH (2), HIV-PAH (1), CTEPH (9), Patients who underwent RHC for suspected PH but found to have mPAP < 25 mmHg (7)	• $CD34^+CD133^+$ • $CD34^+CD133^+KDR^+$ • $CD34^+CD133^-KDR^+$	• Number of $CD34^+CD133^+$ cells is similar regardless of sampling site (peripheral vein, pulmonary artery) • No significant difference in $CD34^+CD133^+$ cells between PAH, CTEPH and patients with a mPAP < 25 mmHg by RHC	• None explored
Farha 2011 [39]	Cross-sectional	Peripheral vein; whole blood	IPAH (24), HPAH (13), APAH (15), Healthy controls (62), Unaffected family members (9)	• $CD34^+CD133^+$ • $CD34^+CD133^-$ • $CD34^-CD133^+$	• % $CD34^+CD133^+$ were greater in PAH patients versus healthy controls • % $CD34^+CD133^+$ cells were higher in HPAH compared to IPAH and APAH patients, which had similar proportions of $CD34^+CD133^+$ cells • % $CD34^+CD133^+$cells in unaffected family members were similar to their HPAH-affected relatives but higher than healthy controls	• None explored
Montani 2011 [40]	Cross-sectional	Not reported; PBMCs	IPAH (9), Healthy controls (7)	• $CD34^{high}CD133^{high}$ • $CD34^{low}CD133^-$	• % $CD34^{high}CD133^{high}$ cells and % $CD34^{low}CD133^-$ cells were significantly increased in IPAH versus controls	• None explored
Smadja 2011[f] [41]	Prospective	Peripheral vein; whole blood	IPAH (27), CHD with reversible (28) or irreversible PAH (22)	• $CD34^+$	• Number of $CD34^+$ cells was higher in IPAH patients ($n = 9$) compared to those with	• None explored

(Continues)

TABLE 1 | (Continued)

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
					reversible ($n = 28$) and irreversible ($n = 13$) CHD-PAH • Number of $CD34^+$ cells was similar in untreated versus treated PAH patients • Number of CD34+ cells was similar in patients on no therapy, oral therapy alone and dual oral therapy + SQ treprostinil	
Farha 2012 [42]	Prospective	Peripheral vein; whole blood	IPAH (5), HPAH (1), APAH (3)	• $CD34^+CD133^+$	• In PAH patients treated with cromolyn and fexofenadine for 12 weeks, % $CD34^+CD133^+$ cells were significantly lower at 4 and 12 weeks compared to baseline	• None explored
Schiavon 2012 [43]	Cross-sectional	Pulmonary artery and radial artery; whole blood	IPAH (4), IPF with mPAP < 25 mmHg (5) IPF with mPAP >25 mmHg (7), COPD with mPAP < 25 mmHg (3), Healthy controls (lung donors) (6)	• $CD34^+KDR^+$ • $CD34^+CD133^+KDR^+$	• The number of $CD34^+KDR^+$ cells was similar between IPAH, IPF (with and without PH), COPD and healthy controls and there was no difference between pulmonary artery and radial artery samples in any of these groups • No numeric data presented for $CD34^+CD133^+KDR^+$ cells, but state in the discussion that they still found no differences between different lung diseases or between sample locations when comparing triple positive cells	• None explored
Lundgrin 2013 [44]	Prospective	Peripheral vein; whole blood	IPAH (9), HPAH (3), APAH (2)	• $CD34^+CD133^+$	• % $CD34^+CD133^+$ tended to decrease in 8 patients who had	• Positive correlation between % $CD34^+CD133^+$ cells and fasting FDG uptake in the RA and RV

(Continues)

TABLE 1 | (Continued)

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
					baseline and 1 year samples collected	• Negative correlation between the change in % $CD34^+CD133^+$ cells over 1 year and the change in fasting FDG uptake in the RV and LV
Farha 2014[g] [45]	Prospective	Peripheral vein; whole blood	PAH treated with imatinib (8) or placebo (4)	• $CD34^+CD133^+$	• % $CD34^+CD133^+$ cells decreased during 24 months of imatinib therapy ($n = 8$) but did not change in placebo-treated patients.	• None explored
Li 2015 [46]	Cross-sectional	Peripheral vein; whole blood	CHD-PAH (43), CHD without PAH (15), Healthy controls (30)	• $CD31^+CD3^+$	• % $CD31^+CD3^+$ cells are higher in CHD-PAH compared to either CHD without PAH or healthy controls	• Positive correlation between % $CD31^+CD3^+$ cells and systolic PAP as well as mPAP
Shirai 2015 [47]	Cross-sectional	Peripheral vein; PBMCs	SSc-PAH (7), SSc without PAH (63)	• $CD34^+CD133^+KDR^+$	• The number of $CD34^+CD133^+KDR^+$ cells was lower in SSc-PAH compared to SSc patients without PAH	• None explored
Foris 2016 [48]	Cross-sectional	Peripheral vein and artery; PBMCs	IPAH (11), HPAH (1), CTD-PAH (5), CHD-PAH (1), PoPH (2), Healthy controls (20)	• $CD133^+$ • $CD133^+CD34^+$ • $CD133^+CD34^+KDR^+$ • $CD133^+CD45^+$ • $CD133^+CD117^+$ • $CD133^+KDR^+$ • $CD133^+CD31^+$ • $CD133^+CXCR2^+$	• % $CD133^+$ cells were higher in PAH patients vs healthy controls • % $CD133^+CD45^+$ and $CD133^+CD117^+$ amongst all $CD133^+$ cells are higher in PAH patients vs healthy controls • % $CD133^+KDR^+$, $CD133^+CD31^+$ and $CD133^+CXCR2^+$cells were lower in PAH patients vs controls • $CD133^+CD34^+KDR^+$ cells were not detected in either PAH patients or healthy controls	• None explored

(Continues)

TABLE 1 | (Continued)

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
Garcia-Lucio 2017[h] [49]	Prospective	Peripheral vein; PBMCs	IPAH (11), HPAH (3), CTD-PAH (11), HIV-PAH (4), CHD-PAH (2), PoPH (1), Schistosomiasis-PAH (1), Healthy controls (30)	• $CD34^+CD45^{low}$ • $CD34^+CD133^+CD45^{low}$	• Compared to healthy controls, % $CD34^+CD45^{low}$ and $CD34^+CD133^+CD45^{low}$ cells are lower in treatment naïve PAH patients • Compared to pretreatment baseline levels, % $CD34^+CD45^{low}$ and $CD34^+CD133^+CD45^{low}$ cells increased after 6-12 months of PAH therapy ($n = 18$ patients) and reached levels similar to healthy controls	• % $CD34^+CD133^+CD45^{low}$ cells was higher in treatment-naïve patients with a 6MWD above the median value for the group (≥428 m). This analysis was done on the combined cohort of patients with PAH and CTEPH
Sun 2019 [50]	Cross-sectional	Peripheral vein; whole blood	CHD-PAH (62), CHD without PAH (111)	• $CD133^+KDR^+$	• The proportion of CHD-PAH patients (mPAP >25 mmHg) with >1 $CD133^+KDR^+$ cell/μL is lower than the proportion of patients diagnosed with CHD-PAH (mPAP 20–25 mmHg) or CHD without PAH	• After adjusting for age, sex, and BMI, CHD patients with >1 CD133 + KDR+ cell/μL had a lower odds ratio of having a mPAP >25 mmHg
Hashimoto 2020 [33]	Cross-sectional	Location and sample type stained not reported	IPAH (3), SSc-PAH (5), Healthy controls (5)	• $CD34^+$	• % $CD34^+$ cells were higher in PAH patients vs control	• % $CD34^+$ cells positively correlated with PVR
Tura-Ceide 2021 [51]	Cross-sectional & longitudinal	Peripheral vein; PBMCs	IPAH (52), HPAH (9), CTD-PAH (46), HIV-PAH (20), PoPH (17), SSc (44) and HIV (22) patients without PAH, Healthy controls (47)	• $CD34^+CD133^+CD45^{low}$	• % $CD34^+CD133^+CD45^{low}$ cells were lower in PAH patients (all subtypes combined) vs healthy controls • % $CD34^+CD133^+CD45^{low}$ cells were similar between each of the PAH subtypes • % $CD34^+CD133^+CD45^{low}$ cells was increased in SSc-PAH compared to SSc without PAH • % $CD34^+CD133^+CD45^{low}$ cells were similar in HIV patients with and without PAH	• In treatment naïve PAH patients, baseline mPAP, cardiac index, RAP, PVR, 6MWD, BNP, DLCO and NYHA/WHO FC did not correlate with % $CD34^+CD133^+CD45^{low}$ cells

(Continues)

TABLE 1 | (Continued)

Author and year (Text reference)	Study type	Location of sample collection; sample type stained	Subjects (N)	Marker schemes[a]	Major findings	Correlation with clinical parameters
					• In treatment naïve patients ($n = 53$), % $CD34^+CD133^+CD45^{low}$ cells remained unchanged after 3 months of PAH therapy	

Abbreviations: APAH, disease associated pulmonary arterial hypertension; BMI, body mass index; BNP, brain natriuretic peptide; CHD-PAH, congenital heart disease associated PAH; COPD, chronic obstructive pulmonary disease; CTD-PAH, connective tissue disease associated PAH; CTEPH, chronic thromboembolic pulmonary hypertension; DLCO, diffusing capacity of the lungs for carbon monoxide; ES, Eisenmenger Syndrome; FDG, fludeoxyglucose; HIV-PAH, human immunodeficiency virus associated PAH; HPAH, heritable pulmonary arterial hypertension; IPF, idiopathic pulmonary fibrosis; IPAH, idiopathic pulmonary arterial hypertension; LV, left ventricle; mPAP, mean pulmonary artery pressure; NYHA/WHO FC, New York Heart Association/World Health Organization Functional Classification; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PAP, pulmonary artery pressure; PBMCs, peripheral blood mononuclear cells; PVR, pulmonary vascular resistance; PoPH, portopulmonary hypertension; RA, right atrium; RV, right ventricle; RHC, right heart catheterization; SQ, subcutaneous; SSc-PAH, systemic sclerosis associated PAH; RAP, right atrial pressure; 6MWD, 6-min walk distance.

[a]Cell marker schemes that included at least one stem cell marker (e.g., CD34, CD133) or at least one endothelial cell marker (e.g., CD31) are listed for each study.

[b]Patients with PH secondary to OSA ($n = 3$) and sarcoidosis ($n = 1$) included in the original study are not included here.

[c]Analyses included both healthy controls and IPAH patients for the correlations between 6MWD and BNP with the number of $CD133^+KDR^+$ cells.

[d]Patients from the Giessen cohort with chronic thromboembolic PH ($n = 4$) and pulmonary capillary hemangiomatosis ($n = 1$) included in the original study are not included here; regarding sample location, the Methods refer to collection from a central vein, but the results state that peripheral blood samples were measured.

[e]Patients with chronic thromboembolic PH ($n = 9$) included in the original study are not included here.

[f]The methods section states that 24 patients with irreversible CHD-PAH were included,but Table 1 reports 22 patients. In Figures 2 and 3, it is unclear if each data point represents a unique patient or repeated measures over time in some patients. For example, the methods section states that SQ treprostinil was added to 8 patients already on dual oral therapy and samples were collected before treatment, day 2 of treatment, day 5 and monthly thereafter. Repeated measures from these 8 unique patients would explain why the oral bitherapy + SQ treprostinil group in Figure 3a includes 28 data points.

[g]Etiology not specified in the manuscript, but the parent phase 3 clinical trial inclusion criteria stated: "WHO Diagnostic Group I (Dana Point 2008), idiopathic or heritable (familial or sporadic) PAH, PAH associated with collagen vascular disease, PAH present one-year following repair of congenital heart defect (ASD, VSD or PDA), or PAH associated with diet therapies or other drugs."

[h]Patients with chronic thromboembolic PH ($n = 11$) included in the original study are not included here. The manuscript refers to the cell populations of interest as $CD34^+CD45^+$ and $CD34^+CD133^+CD45^+$, but the gating strategy provided in the supplement suggests these cells are $CD34^+CD45^{low}$ and $CD34^+CD133^+CD45^{low}$ similar to Tura-Ceide et al.

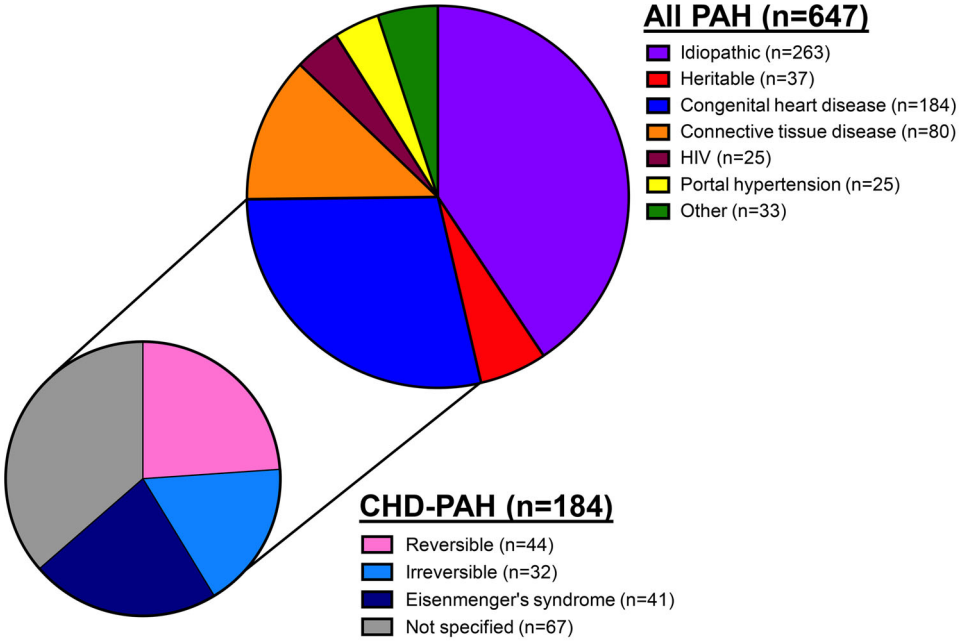

FIGURE 2 | Subtypes of pulmonary arterial hypertension (PAH) represented in the studies of circulating progenitor cells meeting inclusion criteria for systematic review. A total of 647 PAH patients were reported in 19 studies. Congenital heart disease associated PAH (CHD-PAH) was the second largest subgroup of patients ($n = 184$) and was further subdivided based on the designations from the original manuscripts. "Other" subtype, in green, included patients with schistosomiasis-associated PAH ($n = 1$), disease-associated PAH not otherwise specified ($n = 20$) and PAH not otherwise specified ($n = 12$).

Text Reference	Author (Year)	Inclusion criteria clearly defined	Subjects described in detail	Time from PAH diagnosis reported	Standard criteria for PAH diagnosis defined	Confounding factors identified	Strategies to address confounding factors reported	Flow cytometry score	Appropriate statistical analysis used
42	Asosingh (2008)								
31	Diller (2008)								
43	Zhu (2008)								
39	Smadja (2009)								
32	Toshner (2009)								
51	Smadja (2010)								
44	Farha (2011)								
45	Montani (2011)								
40	Smadja (2011)								
52	Farha (2012)								
46	Schiavon (2012)								
54	Lungdrin (2013)								
53	Farha (2014)								
50	Shirai (2015)								
47	Foris (2016)								
48	Garcio-Lucio (2017)								
41	Sun (2019)								
33	Hashimoto (2020)								
49	Tura-Ceide (2021)								

FIGURE 3 | Risk of bias assessment. Each category was scored as either low (green), intermediate (yellow) or high (red) risk of bias using a modified appraisal tool as described in the Methods. The flow cytometry score was used to determine whether flow data were measured in a valid and reliable way. A flow cytometry score of ≥ 5 is green, 4 is yellow; ≤ 3 is red. For the Toshner study, an average flow cytometry score was used (Giessen = 6, Cambridge = 4).

the sample type and processing before flow cytometric analysis. Most studies directly stained whole blood ($n = 11$), while others isolated PBMCs from whole blood before staining ($n = 6$). One study did not clearly indicate whether whole blood or PBMCs were used [30]. Lastly, one study reported results from two institutions where one lab stained whole blood directly and the second isolated PBMCs before staining [29]. Following PBMC isolation, two studies included a subsequent enrichment step using magnetic beads to capture $CD133^+$ or $CD34^+$ cells, respectively [29, 48]. Marker schemes used to define the progenitor cell populations of interest varied across studies, and more than one-third of the studies reported results for two or more marker schemes. The most common marker scheme used to define CPCs across studies was $CD34^+CD133^+$ (Figure 4). Eight studies reported the number of CPCs relative to either blood volume or the number of lymphomonocytic cells, while 11 studies reported the frequency or percentage of CPCs in the parent cell population assayed, typically the total number of PBMCs. The median number of events captured in the 13 studies reporting data was 50,000 with a range as low as 5000 (samples enriched for $CD133^+$ cells before evaluation by flow cytometry) to at least 500,000. Six studies did not specify the total number of events counted. Relevant to any conclusions regarding the functional role of CPCs, only four studies reported using a viability stain as part of their protocol [29, 35, 48, 54]. All studies reported the parent cell reference population, and all but three studies specifically state that isotype control antibodies and/or fluorescence-minus-one samples were included as negative controls. Surprisingly, only 5 studies specifically illustrate their gating strategy as part of the manuscript whereas 7 reference a prior study where the strategy was visually depicted. Nevertheless, a third of the studies did not report nor provide a reference for the gating strategy used to identify CPCs. Importantly, providing a clear illustration of the gating strategy used is among the minimum set of elements recommended when reporting flow cytometry experiments [26].

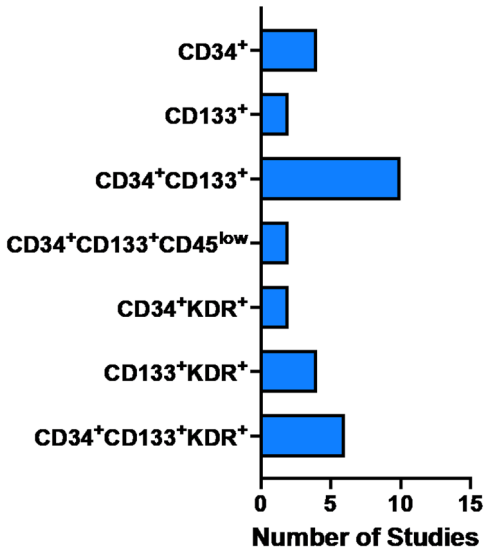

FIGURE 4 | Frequency of common cell surface marker schemes used to define circulating progenitor cells by flow cytometry in PAH studies. Marker schemes reported in at least two studies are plotted on the *y*-axis. The total number of counts is greater than the number of studies included in the systematic review ($N = 19$) because several studies reported results for more than one marker scheme. A complete list of the marker schemes reported in each study is summarized in Table 1.

2.4 | Quantification of CPCs in PAH Patients Compared to Healthy Controls

Among the 11 studies that directly compared the number or percentage of CPCs between PAH patients and healthy controls, 5 studies reported that CPCs were increased in PAH patients [29, 30, 35, 37, 50], 4 studies reported that CPCs were decreased in PAH patients [28, 40, 41, 43], one study did not detect a difference [36] and one study found that the results depended on which subset of $CD133^+$ cells were examined [39]. These seemingly conflicting results could be a result of the different marker schemes used across studies. In an attempt to better synthesize these data sets, studies were grouped together based on shared marker schemes and subjected to meta-analysis. Two studies used CPC marker schemes that precluded grouping them with at least one other study [30, 36]. A third study could not be included in the meta-analysis because the necessary numerical data for comparisons between PAH patients and healthy controls was not reported in the original manuscript, and we did not receive a response to our request for the data. The 8 remaining studies were divided into three groups based on the following marker schemes: (1) $CD34^+CD133^+$; (2) $CD133^+KDR^+$, and (3) $CD34^+CD133^+CD45^{low}$ (Figure 5).

Three studies included patients with diagnoses other than PAH (e.g., PH secondary to OSA, chronic thromboembolic PH and pulmonary capillary hemangiomatosis), but these patients were not included in the meta-analysis [29, 37, 40]. When all 8 studies were pooled together and analyzed using a random effects model, significant heterogeneity ($I^2 = 90\%$, $p < 0.01$; Figure 5) precluded any meaningful conclusion and supports the notion that the three different marker schemes contribute, at least in part, to the variable results across studies. Even when subdivided by marker scheme, heterogeneity was still present in the subgroups of studies that compared $CD34^+CD133^+$ and $CD133^+KDR^+$ cells.

Similar to the analysis across all 8 studies, heterogeneity across the three studies that examined $CD133^+KDR^+$ cells ($I^2 = 91\%$, $p < 0.01$; Figure 5) prohibited a combined analysis [29, 39, 41]. Several important differences between the PAH cohorts and/or the experimental methods used in these studies may explain this degree of heterogeneity. While Zhu et al. directly stained whole blood samples, Foris et al. isolated PBMCs by density gradient centrifugation before staining. Toshner et al. also isolated PBMCs by density gradient centrifugation but then included a subsequent positive enrichment step using magnetic beads to capture $CD133^+$ cells before staining for CD133 and KDR. Although Zhu et al. did not illustrate their gating strategy, they state that $CD133^+KDR^+$ cells were identified in the lymphocyte gate,

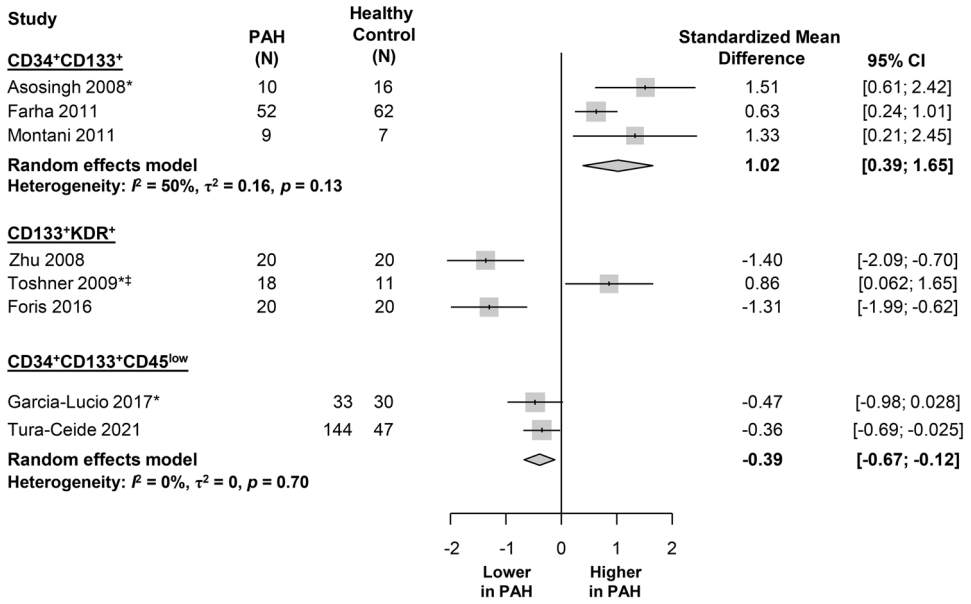

FIGURE 5 | Meta-analysis of studies reporting numerical data for circulating progenitor cells in PAH patients compared to healthy controls. Standardized mean differences between PAH patients and healthy controls from each study were used for meta-analysis. Studies were grouped based on shared marker schemes used for flow cytometry. Significant heterogeneity precluded a combined analysis of all 8 studies ($I^2 = 90\%$, $\tau^2 = 1.09$, $p < 0.01$) as well as the 3 studies that investigated $CD133^+KDR^+$ cells ($I^2 = 91\%$, $\tau^2 = 1.46$, $p < 0.01$). *Patients from these studies with a diagnosis other than PAH [sleep apnea (n = 3), sarcoidosis ($n = 1$), CTEPH ($n = 15$), pulmonary capillary hemangiomatosis ($n = 1$)] were not included in the meta-analysis. ‡Data is from the Giessen cohort reported separately in the original manuscript.

similar to the approach taken by Foris et al. In contrast, Toshner et al. included both lymphocytes and monocytes in their gating strategy. Lastly, Zhu et al. included only IPAH patients, none of whom were on contemporary pulmonary vasodilator therapy. Both Foris et al. and Toshner et al. included IPAH patients and patients with disease-associated PAH (e.g., PAH due to CHD, connective tissue disease, or portal hypertension; APAH), the majority of whom in each study were treated with PAH-specific therapy.

Moderately high heterogeneity ($I^2 = 50\%$, $p = 0.13$) was found between the 3 studies that investigated circulating $CD34^+CD133^+$ cells [35, 37, 50]. While Asosingh et al. and Montani et al. included only IPAH patients, almost a third of the subjects in the study by Farha et al. were diagnosed with APAH. Nevertheless, a meta-analysis of these studies suggests that circulating $CD34^+CD133^+$ cells are increased in PAH patients compared to healthy controls [pooled SMD (95% CI) 1.02 (0.39 to 1.65)].

The most consistent results were observed in the two studies that investigated circulating $CD34^+CD133^+CD45^{low}$ cells ($I^2 = 0\%$, $p = 0.70$) and both studies included patients with a variety of PAH subtypes. The high degree of consistency is not unexpected given that the 2 studies are from the same research group and, therefore, likely followed very similar flow cytometry protocols. In contrast to the three studies that measured $CD34^+CD133^+$ cells, a meta-analysis of these two studies suggests that circulating $CD34^+CD133^+CD45^{low}$ cells are decreased in PAH patients compared to healthy controls [pooled SMD (95% CI) −0.39 (−0.67 to −0.12)]. Notably, the majority of patients in the studies reporting $CD34^+CD133^+CD45^{low}$ cells were diagnosed with APAH rather than IPAH or hereditary PAH (HPAH). Also, in the combined cohort of 177 PAH patients, nearly 50% were treatment naïve, all 33 of the patients in the study by Garcia-Lucio et al. and 53 in the study by Tura Ceide et al. Thus, compared to all other studies in the meta-analysis, including the three studies that measured $CD34^+CD133^+$ cells, the studies reporting $CD34^+CD133^+CD45^{low}$ cells had higher proportions of APAH and treatment naïve patients.

2.5 | Comparison of CPC Levels Across PAH Patient Subtypes

Whether the number or fraction of CPCs differs across different PAH etiologies was examined in five studies (Table 1). Two studies, one in an adult PAH cohort [28] and the other in a pediatric PAH cohort [54], compared CPCs in patients with IPAH to those with CHD-PAH. Diller et al. examined adults with IPAH and CHD-PAH complicated by Eisenmenger syndrome (ES) and stratified the analysis by sex and whether CHD-PAH patients had Down syndrome or not. Significant differences in $CD34^+$, $CD34 + CD133^+$, $CD34^+KDR^+$ or $CD34^+CD133^+KDR^+$ cells were only observed in adult male Down syndrome patients with CHD-PAH and ES compared to adult male IPAH patients. Smadja et al. found that the number of circulating $CD34^+$ cells was similar in pediatric CHD patients following surgical correction whether or not their postoperative hemodynamics normalized (reversible vs. irreversible PAH), and both groups had lower numbers of circulating $CD34^+$cells compared to pediatric IPAH patients. Toshner et al. did not detect differences in circulating $CD34^+CD133^+KDR^+$ cells between IPAH and HPAH patients or differences in circulating $CD133^+KDR^+$ cells in IPAH and

APAH patients. On the other hand, Farha et al. observed a significantly higher proportion of circulating $CD34^+CD133^+$ cells in HPAH compared to IPAH patients, but no difference between IPAH and APAH patients. In the largest study to date, Tura Ceide et al. investigated a diverse cohort of PAH patients but did not observe any significant differences in the proportion of circulating $CD34^+CD133^+CD45^{low}$ cells between patients with IPAH, HPAH, connective tissue disease-associated PAH, SSc-associated PAH, HIV-associated PAH or portopulmonary hypertension.

2.6 | Levels of CPCs in PAH Patients Compared to Patients With Other Cardiopulmonary or Systemic Diseases

Six studies compared the number or fraction of CPCs in patients with PAH to patients with other underlying diseases or disorders (Table 1). Compared to SSc patients without PAH ($n = 63$), Shirai et al. found that SSc patients with PAH ($n = 7$) had lower numbers of circulating $CD34^+CD133^+KDR^+$ cells [48]. In contrast, Tura Ceide et al. detected a higher percentage of circulating $CD34^+CD133^+CD45^{low}$ cells in SSc patients with PAH ($n = 31$) compared to SSc patients without PAH ($n = 44$) [43]. In the later study, SSc patients without PAH exhibited relatively preserved lung function [FVC, FEV1, and DLCO (% predicted): 100.4 ± 15.3, 101.3 ± 12.8 and 73.4 ± 12.2, respectively], and nearly all were WHO/NYHA FC I, and thus the findings may not be generalizable to SSc patients without PAH who exhibit more significant parenchymal lung disease. In the same study, the percentage of circulating $CD34^+CD133^+CD45^{low}$ cells was similar between HIV patients with and without PAH [43]. Lastly, two studies examined levels of CPCs in CHD patients with and without PAH [46, 53]. Smadja et al. found that CHD patients with reversible PAH (mPAP $<$ 25 mmHg 6 months after surgery), irreversible PAH (mPAP $>$ 25 mmHg 6 months after surgery) and repaired CHD without PAH all had similar numbers of circulating $CD34^+CD133^+$ cells. In a large cohort of children with CHD assessed before surgical repair, Sun et al. observed that higher preoperative CPC levels ($>$ 1 $CD133^+KDR^+$ cell/μL) were associated with a lower risk of coexistent PAH (mPAP $>$ 25 mmHg).

2.7 | Correlation Between CPC Levels and Clinical Parameters

Eight studies examined whether the number or fraction of CPCs in PAH patients at a single time point correlated with clinical parameters such as hemodynamics [28–30, 37, 41, 43, 46], WHO/NYHA FC [28, 43], 6MWD [28, 40, 41, 43], and BNP [28, 41, 43]. In general, these studies often reported discordant results for the relationships between CPCs and various clinical parameters (summarized in Table 1). Notably, only two studies specified that CPC measurements were obtained concurrently with the clinical variables to which they were correlated [29, 41]. Zhu et al. reported a significant positive correlation between 6MWD and number of circulating $CD133^+KDR^+$ cells, where $CD133^+KDR^+$ cells were measured on the same day as the 6MWD, however this analysis combined both healthy controls and IPAH patients. Toshner et al. did not observe a significant correlation between $CD133^+KDR^+$ cells measured at the time of right heart catheterization and either mPAP, PVR or cardiac index.

2.8 | Impact of Treatment on CPC Levels

Five studies prospectively investigated the effect of therapy on the number or percentage of CPCs over time (summarized in Table 1) [40, 43, 47, 51, 54]. Levels of CPCs were determined as early as a few days and up to 12 months after treatment initiation. The impact of pulmonary vasodilator therapy on CPC levels was examined in three studies. Two studies specifically evaluated treatment naïve patients at baseline and then again 3–12 months after initiation of PAH therapy. In the first study, the authors observed an increase in the percentage of $CD34^+CD133^+CD45^{low}$ cells 6-12 months after the initiation of PAH treatment ($n = 18$ patients) [40]. Yet, the second and larger of the two studies, reported no change in $CD34^+CD133^+CD45^{low}$ cells following 3 months of PAH therapy ($n = 53$ patients) [43]. Two small, proof-of-concept studies examined the impact of novel therapeutic interventions for PAH on circulating $CD34^+CD133^+$ cells when added to standard background therapy. Compared to baseline levels, treatment with cromolyn and fexofenadine reduced the percentage of $CD34^+CD133^+$ cells at 4 and 12 weeks [51]. Likewise, imatinib treatment for 24 weeks reduced circulating $CD34^+CD133^+$ cells compared to placebo [47]. However, nearly half of the subjects ($n = 3$ in imatinib group, $n = 2$ in placebo group) withdrew before the 24-week endpoint. Lastly, in a retrospective analysis, Diller et al. observed that the percentage of circulating $CD34^+CD133^+$ and $CD34^+KDR^+$ cells in IPAH patients was positively correlated with sildenafil dose. In contrast, the number of circulating $CD34^+CD133^+$ cells was similar in IPAH patients, whether they were receiving an endothelin-1 receptor antagonist or prostacyclin analogue [28]. Interestingly, in studies that reported differences in CPC levels with the addition of new therapy, each observed a relative change towards "normal," even though the direction of change differed between studies.

3 | Discussion

Systematic review of studies quantifying CPCs by flow cytometry in PAH patients highlights the opportunities to build upon the current literature. Less than half of the studies included detailed enrollment strategies and/or clearly stated inclusion and exclusion criteria. Details regarding disease severity, time from diagnosis and/or co-morbid conditions were also frequently absent. The small sample size in many studies commonly necessitated combining patients with diverse PAH etiologies. Further, detailed descriptions of the flow cytometry methods (e.g., gating strategy) were not universally reported. Considering these limitations, the observed discordance between studies that compared CPCs in PAH patients to healthy controls or correlated CPCs with various clinical parameters is not surprising. Meta-analysis of studies that compared CPCs in PAH patients to healthy controls suggests that factors other than the marker scheme contribute to the observed heterogeneity. For instance, comparisons were likely confounded by

differences such as comorbidities, disease duration and severity, sample timing, and evolving treatment paradigms between study cohorts. Despite the noted shortcomings, future studies of circulating progenitor and endothelial cells remain relevant, and similar to studies that have characterized endothelial cell diversity in the lung [19–23], may benefit from current and emerging technologies that enable even deeper single-cell characterization.

The current review focused on flow cytometric studies of CPCs identified in blood samples from PAH patients rather than the application of cell culture methods for isolating and characterizing CPCs ex vivo. Nevertheless, it is important to recognize that consensus definitions of different subsets of CPCs are principally based on in vitro phenotypic characterization of cultured cells and in vivo functional assays [5, 10]. For example, "true" endothelial progenitor cells (EPCs), referred to as endothelial colony-forming cells (ECFCs), late or blood outgrowth ECs, are capable of endothelial differentiation in vitro and de novo vessel formation in vivo. In contrast, there exist a proportion of CPCs that also form colonies when cultured ex vivo, albeit morphologically distinct from ECFCs, and largely exhibit a myeloid/monocytic cell phenotype [5]. These cells, variably referred to as myeloid angiogenic cells, circulating angiogenic cells, early or early outgrowth EPCs, or colony forming unit-ECs, participate indirectly in postnatal vasculogenesis and/or angiogenesis in vivo. Justification for culturing blood cells ex vivo is the fact that these are rare cell populations, and thus expansion facilitates detection [6]. A potential disadvantage is that in vitro culture conditions may artificially produce a cell population and phenotype that does not exist in vivo. Further, these in vitro cell culture techniques may be more difficult to scale when considering biomarker development.

Three-fourths of the cohort studies included in this systematic review and meta-analysis were cross-sectional studies. In comparison with optimally designed cohort studies, several studies did not report a clear enrollment strategy. Reliance of convenience sampling may have increased the risk of sampling error and weakened the accuracy of results [27]. Further, insufficient detail about enrollment including clearly defined inclusion/exclusion criteria poses a high risk of bias and presents a challenge when attempting to make meaningful comparisons across studies. Six studies analyzed circulating cell populations at more than one time point, usually following treatment initiation or a change in treatment from baseline [38, 40, 43, 47, 51, 54]. Follow up was largely limited to one additional time point collected less than 12 months from the initial assessment and was not analyzed with respective to changes in clinical status. More comprehensive sampling over a longer timeframe may uncover insights into whether changes in clinical status are associated with alterations in the number of CPCs or whether CPC levels can predict clinically relevant outcomes such as lung transplantation or death. Similarly, longitudinal studies can now leverage state-of-the-art single-cell transcriptomics and proteomics assays to interrogate CPCs.

Since PAH is a rare disease, many of the studies included in this review combined patients with a wide range of PAH etiologies, yet the overall small size of the cohorts limited the ability to compare sub-groups. Likewise, small sample size may have precluded adjustment for baseline covariates such as comorbid diseases, time from PAH diagnosis, PAH treatments, and/or severity of illness. Even still, these covariates were frequently not reported, and thus it is unclear whether patient cohorts were comparable beyond their diagnosis of PAH. For instance, comorbid conditions such as obesity and diabetes are much more frequent among PAH cohorts than previously recognized [42, 45]. The timing of CPC measurement in any given patient relative to their disease onset may have differed both within a single study and across studies. One possible exception is the study that only included treatment naïve patients [40]. The duration of disease in this cohort of incident patients who have not begun treatment may be more similar. While small sample sizes hindered subgroup analyses by treatment within several studies, the medications available and therapeutic approach to PAH has also evolved substantially over the time period in which these studies were published [1], further contributing to differences between studies. Many studies were also conducted before the widespread use of risk assessment tools, and therefore risk stratification could not be included as a potential covariate. In studies that correlated CPC counts with various clinical variables (e.g., mPAP, PVR, 6MWD), only two studies specified that CPCs were measured on the same day that clinical testing was performed [29, 41]. A long interval between clinical assessment and CPC measurement may obscure any potential correlation, particularly if a patient's clinical status and/or the number of CPCs in circulation is dynamic over time. Likewise, discordant results of correlations between any given clinical variable and the number of CPCs could be due to variations in the time between their measurement among studies. It is notable that the current systematic review identified only one study that examined CECs in PAH patients using flow cytometry [52]. Future studies may consider simultaneous determination of CECs and incorporating a ratio of progenitor cells to CECs in analyses as a means of adjustment and/or an approach to evaluating these circulating cell populations over time in PAH patients.

Regarding recommended best practices for flow cytometry [26], all but two studies reported many of the principal components necessary for proper interpretation. Nevertheless, there was considerable variability in the flow cytometric methods used across studies and the elements assessed here were only the minimum information necessary for interpretation. In addition to minimizing artifacts, the acquisition of a large number of events is necessary to overcome the signal-to-noise challenge of detecting CPCs amongst the more numerous populations of circulating cell [44, 55]. Additional techniques recommended for accurately determining rare, circulating cell populations include serum or Fc blocking before staining, including a nuclear stain, assessing cell viability, gating strategies to minimize artifacts caused by fluidic disturbances, and cell doublet or aggregate exclusion [44, 55].

4 | Summary

In conclusion, an opportunity remains to clarify the role of CPCs in PAH pathobiology and their utility as a biomarker. Based on a systematic review of the current literature, we propose several suggestions for future studies. Several factors

related to study design and reporting are necessary to yield robust results and facilitate comparison across studies, including prospective enrollment with clear inclusion/exclusion criteria, reporting of comorbid conditions, time from diagnosis and risk assessment and incorporating these variables into covariate adjustment models. Clinical assessments such as hemodynamics, exercise capacity, and functional status should ideally coincide with the timing of blood sampling. Application of standardized flow cytometry protocols, including appropriate techniques for rare event analysis, should be reported in sufficient detail. Collaborative, multi-center studies could facilitate enrollment of larger PAH patient cohorts and foster the harmonization of flow cytometry methods and data collection. In addition, reporting patient-level data and metadata would enable future aggregation and meta-analysis. Lastly, longitudinal studies are needed to determine to what extent these rare circulating cell populations change over time and whether changes reflect clinically relevant information about PAH disease progression.

Author Contributions

All authors have reviewed, contributed, and approved this manuscript.

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Conflicts of Interest

Dr Elinoff is the principal investigator of a clinical trial that is partially funded through a Cooperative Research and Development Agreement (CRADA) between the NHLBI and Zymedi, Co. Ltd.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.