ADVANCES AND UPDATES IN DIFFUSE CYSTIC LUNG DISEASES

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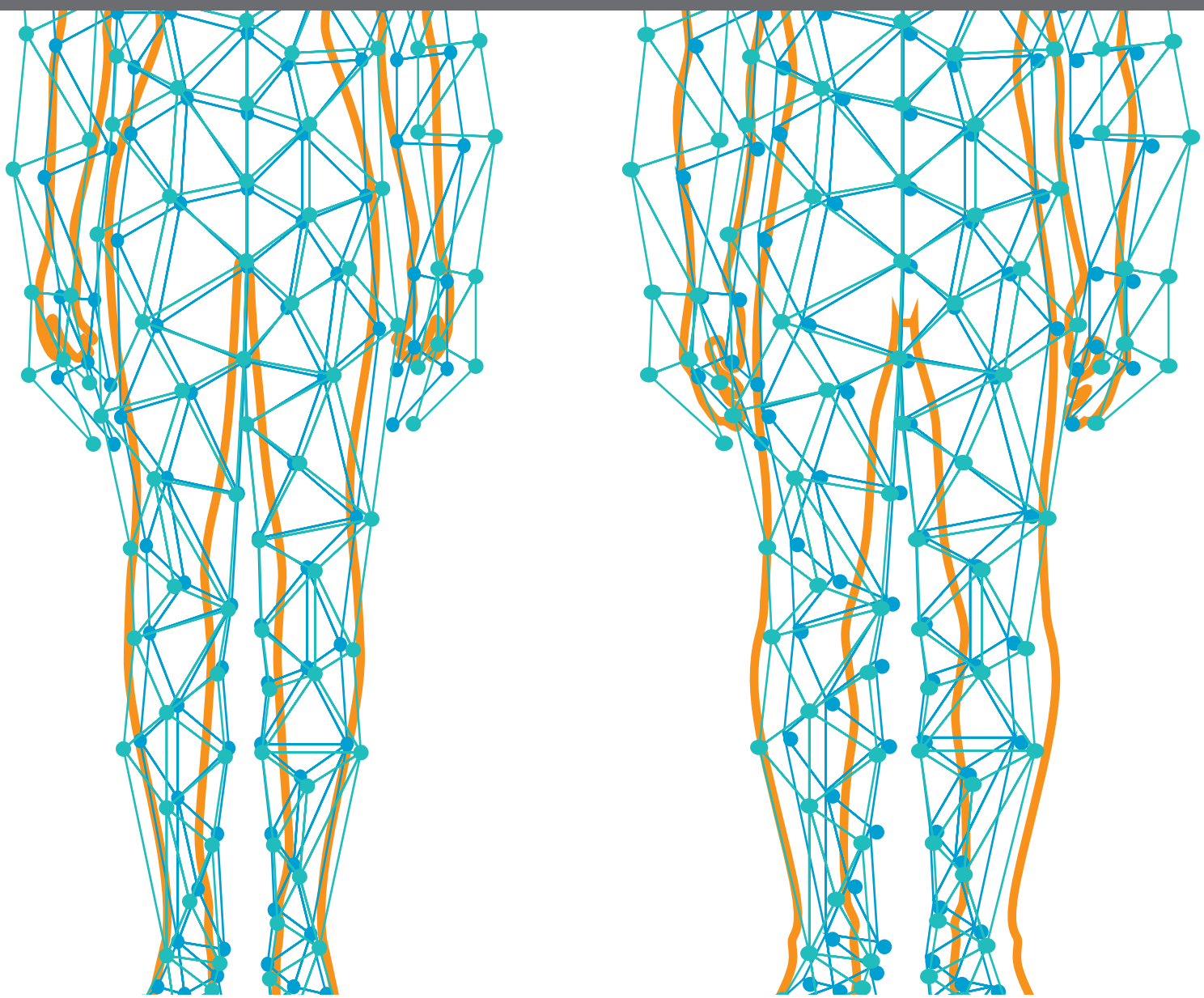

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ADVANCES AND UPDATES IN DIFFUSE CYSTIC LUNG DISEASES

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EDITORIAL
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Editorial: Advances and Updates in Diffuse Cystic Lung Diseases

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Editorial on the Research Topic

Advances and Updates in Diffuse Cystic Lung Diseases

This Frontiers Research Topic aimed to update relevant information, to publish advances and to determine future research directions regarding diffuse cystic lung diseases (DCLDs), such as lymphangioleiomyomatosis (LAM), pulmonary Langerhans cell histiocytosis (PLCH), and Birt-Hogg-Dubé (BHD) syndrome.

Diffuse cystic lung diseases are a variety group of diseases characterized by the presence of multiple cysts in more than one lung lobe, usually bilateral (1). Chest high-resolution computed tomography (HRCT) is the main tool and a multidisciplinary discussion is desirable in the approach to DCLDs. The main etiologies of DCLDs include LAM, PLCH, and other smoking-related diseases, BHD syndrome, lymphocytic intertitial pneumonia, pulmonary amiloidosis, bronchiolitis, metastatic neoplasms, and light-chain deposition disease, etc. (2, 3). New disorders are continuously included in the differential diagnosis of DCLDs, and based on the fact that management and prognosis vary according to the etiology, it is important to try to narrow such differential and, if possible, to confirm the cause, which might require a lung biopsy (1, 4).

Several advances were obtained in the understanding of DCLDs in the last decades, especially in the patogenesis, genetics, and in the diagnostic approach of LAM and PLCH, and, additionally, in the treatment of LAM (5–7). Despite improvements in such issues, there is still no curative treatment for LAM and PLCH (5, 7). Furthermore, the pathophysiology and natural history of several DCLDs are still not fully understood.

Therefore, new studies addressing DCLDs are desired to expand knowledge in the area, and to improve the management of patients. Five studies were published in this Research Topic, two about LAM, two focusing on BHD syndrome and a review about PLCH, highlighting diverse topics.

Previous studies demonstrated that serum matrix metalloproteinases (MMP) −2 and −9 were higher in LAM compared to healthy controls (8, 9). However, doxycycline, a MMP inhibitor, had no effects on pulmonary function decline in a clinical trial in LAM (10). Serum vascular endothelial growth factor (VEGF) -D is a useful biomarker included in the diagnostic guidelines of LAM (5). Previous studies demonstrated that serum VEGF-D was greater in patients with LAM than in healthy controls and in those with other cystic lung diseases (11, 12). Terraneo et al. assessed the role of MMP-2, MMP-7, VEGF-C, and VEGF-D in women with LAM and TSC. The authors found that MMP-2 was greater in LAM compared to controls and TSC patients, and that MMP-7 was higher in TSC-LAM patients. The authors reinforced the importance of VEGF-D in the diagnosis of LAM, and demonstrated that VEGF-C seems to have no role in the diagnosis of LAM and TSC. The authors concluded that MMP-2 and MMP-7 are promising biomarkers in LAM, although further studies with larger populations and with other DCLDs are required to confirm their findings.

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Song et al. provided a comprehensive review of the pathogenesis of LAM and discussed the role of the mTOR signaling pathway in cell growth and proliferation. Although the authors highlighted the role of mTOR inhibitors in attenuating the lung function decline in LAM, they reinforced that such drugs are not a curative therapy (6). Furthermore, other potential therapeutic targets for LAM were presented in this review, such as inhibition of autophagy, inhibition of receptor tyrosine kinases, including VEGF receptors, hormonal blockade, urokinase-type plasminogen activator, blockade of the mTORC2 pathway, and DNA damage checkpoint (13, 14). This review supports the idea that in the future the treatment of LAM will be based on the combination of drugs, acting on different pathogenic pathways.

Zong et al. presented a novel heterozygous variant (c.912delT/p.E305KfsX18) in exon 9 of the folliculin gene, a tumor supressor, in seven patients with BHD syndrome from a Chinese family. All patients presented with bilateral pulmonary cysts on HRCT, three had primary spontaneous pneumothorax (PSP), and none had cutaneous or renal lesions. This case series strengthens the importance of screening for folliculin gene variants in patients with pulmonary cysts and PSP, and of trying to establish a correlation between genotype and phenotypic presentation, although it is not always clear in genetic disorders.

Muller et al. performed a meta-analysis of published epidemiological data using Bayes equation to determine for the first time the prevalence of BHD syndrome and, additionally, information regarding PSP. The main findings of this study were as follows: (1) The prevalence of BHD in the general population was 1.86 (95% confidence interval: 1.16–3.00) per million; (2) The probability of having BHD syndrome among patients with apparent PSP was 9%; (3) The incidence rate of PSP in the general population was 8.69 (95% confidence interval: 6.58–11.46) per 100,000 person-years; (4) The prevalence of PSP was 0.77 (95% confidence interval: 0.52–1.02) per 100,000; (5) The overall probability of PSP in BHD syndrome was 43%. Therefore, this study reinforces that BHD syndrome is rare and has a high probability of developing PSP, and the recommendation to screen for BHD syndrome in patients with PSP.

Diverse topics about PLCH are discussed in this broad narrative review by Radzikowska. The author presented updated issues about PLCH, emphasizing definition, classification, epidemiology, pathogenesis, genetic features, clinical manifestations, radiological and histological presentations, and diagnostic approach. Moreover, potential therapeutic modalities are highlighted in such review, including smoking cessation, corticosteroids, chemotherapy and target drugs. Future perspectives in PLCH include to expand the understanding of the pathogenesis, the identification of biomarkers to support diagnosis, prognosis and therapeutic response, to define efficacy and enhance experience with target drugs, and to identify curative treatments.

In summary, the manuscripts included in this Research Topic highlighted diverse issues and added relevant data to improve knowledge in the area of DCLDs. Although several advances were obtained in the genetic characterization, pathogenesis and diagnostic approach regarding DCLDs in recent years, there is still a need to identify novel targets and therapeutic modalities with curative results, especially in LAM and PLCH.

AUTHOR CONTRIBUTIONS

All authors wrote, reviewed, and approved the final version of the manuscript.

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REVIEW
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Possible Novel Therapeutic Targets in Lymphangioleiomyomatosis Treatment

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Lymphangioleiomyomatosis (LAM) is a rare systemic neoplastic disease that exclusively happens in women. Studies focusing on LAM and tuberous sclerosis complex (TSC) have made great progress in understanding the pathogenesis and searching for treatment. The inactive mutation of TSC1 or TSC2 is found in patients with LAM to activate the crucial mammalian target of rapamycin (mTOR) signaling pathway and result in enhanced cell proliferation and migration. However, it does not explain every step of tumorigenesis in LAM. Because cessation of rapamycin would break the stabilization of lung function or improved quality of life and lead to disease recurrent, continued studies on the pathogenesis of LAM are necessary to identify novel targets and new treatment. Researchers have found several aberrant regulations that affect the mTOR pathway such as its upstream or downstream molecules and compensatory pathways in LAM. Some therapeutic targets have been under study in clinical trials. New methods like genome-wide association studies have located a novel gene related to LAM. Herein, we review the current knowledge regarding pathogenesis and treatment of LAM and summarize novel targets of therapeutic potential recently.

Keywords: lymphangioleiomyomatosis, mTOR, pathogenesis, target, treatment

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INTRODUCTION

Lymphangioleiomyomatosis (LAM) is a rare low-grade neoplasm that predominantly affects young and middle-aged women (1, 2). This disease appears as two forms. S-LAM occurs in 3.35–7.76/million women with an incidence of 0.23–0.31/million women/year (3). As a major feature of the clinical criteria to help diagnose tuberous sclerosis complex (TSC) (4), LAM occurs in 30–80% of women with TSC, which is the inherited form (TSC-LAM). TSC is another variable disease that affects the skin, brain, kidneys, lung, and heart, leading to multiple organ dysfunction. Cystic changes of pulmonary are also observed in 10–12% of males with TSC, but seldomly with symptomatic LAM. The pathogenic mutation in genes TSC1 or TSC2 is the definitive diagnostic criterion for TSC and related to the molecule etiology of LAM. LAM is characterized by progressively diffusive cystic destruction of lungs, which results in clinical symptoms including dyspnea, wheeze, cough, recurrent spontaneous pneumothorax, chylothorax, and a decline in expiratory flow rates (FEV_1) and/or lung diffusion capacity (DLCO) (5). LAM lesions affect both pulmonary parenchyma and interstitium. The formation of the thin-wall cysts in parenchyma is associated with hyperplasia of type II pneumocytes and destructive changes in the elastic fibers and collagen (6). The structural changes may be related to "LAM cells." The smooth muscle–like cells (LAM cells) in small clusters are observed on the edges of lung cysts and along blood

vessels, lymphatics, and bronchioles in interstitium (7). These nodular LAM lesions are composed of more proliferative LAM cells in the center and less proliferative epithelioid cells at the periphery, and contain type II pneumocytes, lymphatic endothelial cells, and mast cells. The infiltration of LAM cells results in airway obstruction, vascular wall thickening, disruption of lymphatic vessels, venous occlusion, and hemorrhage with hemosiderosis (7). Extrapulmonary features consist of renal angiomyolipomas and lymphatic involvement such as lymphangioleiomyomas and chylous effusions (2, 8). Because of the rare nature and unremarkable symptoms as well as the chest radiograph, patients frequently tend to be misdiagnosed with asthma, COPD, idiopathic pulmonary fibrosis, or tuberculosis, and the delayed diagnosis may even come after decades (9). To make a clinical diagnosis of LAM, characteristic high-resolution CT (HRCT) feature—thin-wall cystic air sacs evenly distributed in bilateral lobes, surrounded by normal pulmonary tissues (9)—plus one or more of the following: presence of TSC, angiomyolipomas, chylous effusions, lymphangioleiomyomas, or elevated serum vascular endothelial growth factor-D (VEGF-D) $\geq$800 pg/ml, is required (10). When a definitive diagnosis is required in patients who have characteristic cysts on HRCT, but no additional confirmatory features (i.e., clinical, radiologic, or serologic), a transbronchial lung biopsy before a surgical lung biopsy is recommended (10). The diagnosis can be based on typical morphological appearance of LAM cells and positive staining for smooth muscle cell markers and human melanoma black-45 (HMB-45) by immunohistochemistry (10). The proliferation of LAM cells is commonly thought to be related to the mutational inactivation of gene TSC1 or TSC2 and subsequently abnormal activation of mTOR signaling pathway—the target of rapamycin and its analogs which become the only drugs for LAM treatment currently.

THE CENTRAL ROLE OF THE mTOR SIGNALING PATHWAY IN PATHOGENESIS OF LAM

Loss of heterozygosity for TSC genes, mostly TSC2, is found in somatic tissues either from women with S-LAM or patients with TSC-LAM who also carry germline TSC1 or TSC2 gene mutations (11). The TSC1 encodes hamartin, whereas the TSC2 encodes tuberin (12). Two proteins compose a tumor suppressor complex TSC1/2 which interacts with dozens of proteins. Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7) is one of the interacting proteins, which stably binds to the TSC1/2 heterodimer to form the TSC1-TSC2-TBC1D7 (TSC–TBC) complex (13). The TSC–TBC complex receives growth signals and suppresses the activity of small GTPase Ras homolog enriched in brain (Rheb) through the GAP domain of tuberin (1, 14), resulting in an inhibition effect on mTOR, a highly conserved serine–threonine kinase that plays an important role in the regulation of cell growth and proliferation (**Figure 1**). Generally, activated mTOR phosphorylates ribosomal S6 kinase 1(S6K1), Ras homolog gene family, member A (RhoA), and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), leading to the activation of S6K1 and RhoA, and the inhibition of interaction between 4E-BP1 and eukaryotic initiation factor 4E (elF4E). Then, these downstream molecules result in enhancement of translation, cell growth, and proliferation, as well as cell survival and migration (15) (**Figure 1**). In LAM, inactivation mutation of TSC2 leads to absence of tuberin and loss of suppression to mTOR, ultimately facilitating the cell growth and proliferation.

Upstream signaling, including AKT, ERK1/2, RSK1, MK2, AMPK, GSK3, IKKβ, CDK1, and PLK1, regulates the mTOR pathway mainly through directly phosphorylating complex TSC1/2 (16). Particularly, the phosphoinositide 3-kinases (PI3K)–dependent signaling serves to inhibit the tuberin–hamartin heterodimer through direct protein kinase B (Akt)–dependent phosphorylation of tuberin at Ser-939 and Thr-1462 (the major Akt phosphorylation sites) (17) (**Figure 1**). The PI3K/Akt cascade was activated by insulin receptor substrate 1 (IRS1) when extracellular growth factors bind to receptor tyrosine kinase (RTK) (**Figure 1**). Coincidentally, the downstream target of mTOR, S6K1, repressed IRS1, thus forming a negative feedback loop in PI3K/Akt/mTOR axis, which is a regulatory strategy common to many signaling pathways (18) (**Figure 1**). Moreover, there exists another mechanism in which the PI3K signaling regulates the Akt/mTORC1 axis through activating mTORC2, which in turn modulates the phosphorylation and stability of Akt (19, 20) (**Figure 1**).

RAPAMYCIN IS AN INHIBITOR OF mTORC1 BUT NOT A PERFECT CURE FOR LAM

As the catalytic subunit, mTOR exists in two complexes called mTOR complex 1 (mTORC1), the rapamycin-sensitive complex which controls cell-autonomous growth and responds to diverse extracellular signals including growth factors and nutrients, and mTORC2, which is insensitive to rapamycin and regulates key aspects of cell proliferation, including assembly of the actin cytoskeleton and cellular survival (14, 21). Rapamycin binds to the cytosolic protein FK506-binding protein 12 (FKBP12) which directly binds to mTORC1, resulting in the dissociation of raptor, which is another important component in complex 1, and inhibition of mTORC1 (5). Apart from lung transplantation, sirolimus (a rapamycin analog) is now U.S. FDA approved for the treatment of LAM based on several clinical studies and especially a landmark randomized controlled trial. The study demonstrates that sirolimus stabilizes lung function decline and improves quality of life and functional performance in patients with LAM (22–24). Although the efficacy and safety of sirolimus and everolimus are still being studied (**Table 1**), now, rapamycin is recommended as the first-line treatment option for qualified patients in the LAM guideline document (10).

However, rapamycin is far from a perfect cure for LAM treatment according to serial discoveries. Most importantly, the monotherapy of sirolimus does not eliminate tumors despite the size reduction of solid proliferative lesions. On cessation of rapamycin therapy, the renal tumors regrow and lung function level even decreases to around baseline. Thus, as the only drug for LAM at the moment, rapamycin requires lifelong

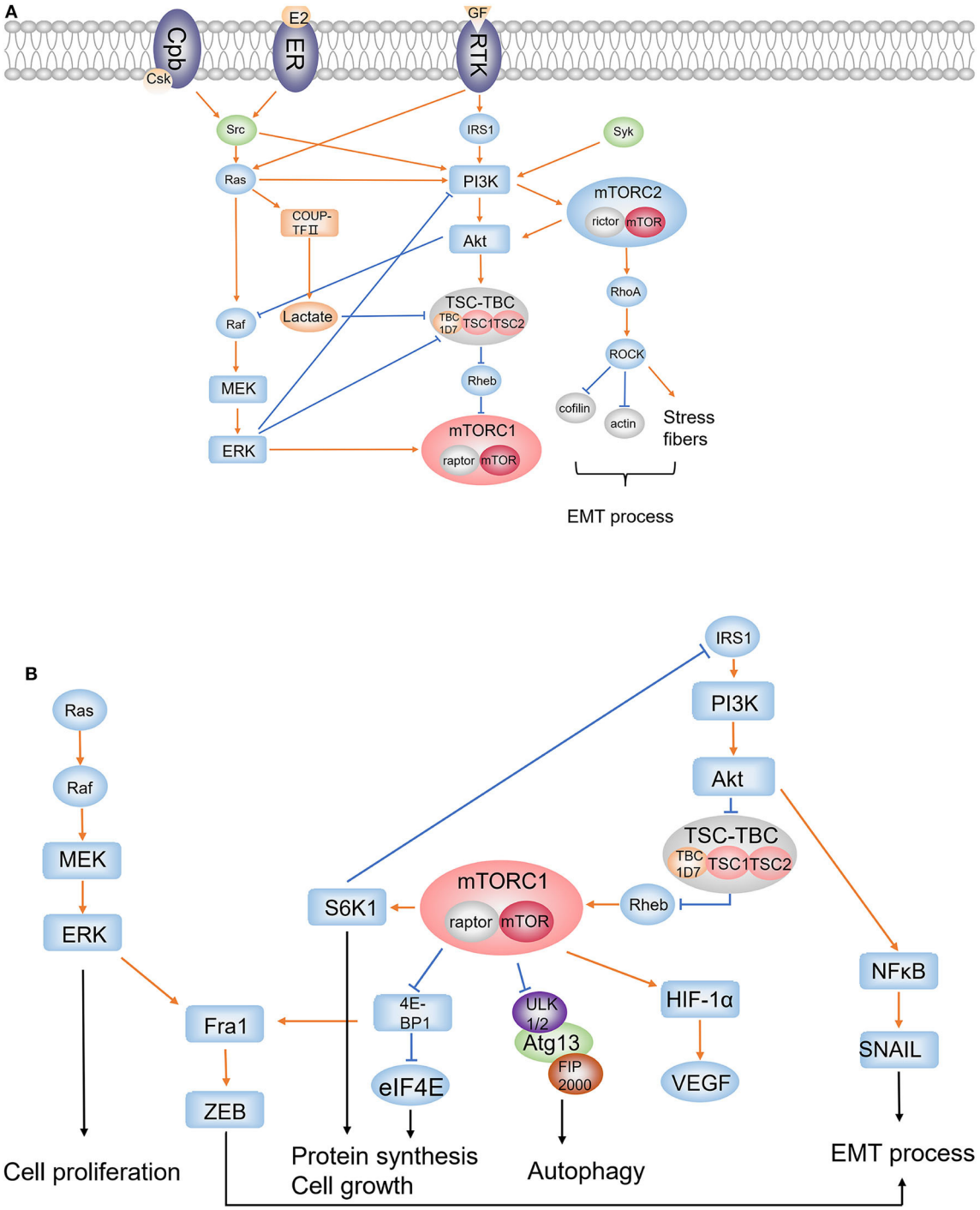

FIGURE 1 | PI3K/Akt/mTORC1 pathway, Ras/Raf/MEK/ERK pathway, mTORC2 signaling, and the EMT process. **(A)** Upstream regulators and accompanying pathways affecting mTORC1; **(B)** downstream effectors regulated by mTORC1. (1) mTORC1 and mTORC2: rictor and mTOR are components of mTORC2, whereas raptor is the binding protein of mTORC1. (2) RTKs: extracellular growth factors bind to RTK and activate IRS1 and Ras. (3) Src: Src is activated by ER or Csk; Src activates PI3K to regulate PI3K/Akt cascade and activates Ras to affect ERK pathway and PI3K/Akt cascade. (4) PI3K/Akt/mTORC1: the PI3K/Akt cascade is activated by IRS1, Src, Ras, and Syk; Akt suppresses TSC1/2 complex, which downregulates mTORC1 through Rheb, to upregulate mTORC1; mTORC1 phosphorylates S6K1 and 4E-BP1 to promote protein synthesis and cell growth, and phosphorylates ULK1 to inhibit autophagy; S6K1 inhibits IRS1 to form a feedback loop in PI3K/Akt/mTORC1 pathway. Activation of mTORC1 results in accumulation of HIF-1α which increases the expression of VEGF. (5) Ras/Raf/MEK/ERK: Ras activates Raf; as a result, activated MEK1/2 upregulates ERK to affect cell proliferation. It is an important compensatory pathway of the PI3K/Akt/mTORC1 pathway. (6) The cross-regulation of Ras/Raf/MEK/ERK and PI3K/Akt/mTORC1: ERK inhibits PI3K and promotes mTORC1 directly or through

(Continued)

FIGURE 1 | repressing TSC1/2 complex. PI3K/Akt/mTORC1 negatively regulates Ras/Raf/MEK/ERK pathway by Akt inhibiting Raf. (7) mTORC2: PI3K activates mTORC2, which in turn activates Akt; RhoA is activated by mTORC2 and activates ROCK to induce the inhibition of cofilin and actin, and the increase of stress fibers; RhoA signaling plays an important role in cell migration and invasiveness through EMT process. (8) EMT: ERK pathway and the constitutive mTORC1 pathway converge on the Fra1–ZEB-1/2 transcriptional network to promote migration and invasion; NFκB activated by Akt induces SNAIL1 stabilization which plays an integral role throughout EMT. (9) NR2F2: activated RAS upregulates COUP-TFII and increases lactate production; overproduction of lactate disrupts the interaction of TSC2 and Rheb to increase mTORC1 activity.

TABLE 1 | Current clinical trials studying potential targets for LAM.

Target	Targeting therapy	Clinical trial	ClinicalTrials.gov identifier	Phase
mTORC1	Sirolimus	Multicenter interventional lymphangioleiomyomatosis (LAM) early disease trial	NCT03150914	III
	Sirolimus and everolimus	Multicenter international durability and safety of sirolimus in LAM trial	NCT02432560	
Autophagy	Resveratrol	Resveratrol and sirolimus in lymphangioleiomyomatosis trial	NCT03253913	II
RTKs	Nintedanib	A pilot study of nintedanib for lymphangioleiomyomatosis (LAM)	NCT03062943	
Src	Saracatinib	Safety and efficacy of saracatinib in subjects with lymphangioleiomyomatosis	NCT02737202	II
RhoA	Simvastatin	The safety of simvastatin (SOS) in patients with pulmonary lymphangioleiomyomatosis (LAM) and with tuberous sclerosis complex (TSC)	NCT02061397	II

use, but it comes with a risk of remarkable adverse effects and possible acquired rapalog resistance (16). Moreover, LAM patients with lymphatic involvement may respond to sirolimus with a greater improvement in lung function than those without lymphatic disease, indicating that not all patients respond to rapamycin therapy in the same way (25, 26). These limitations of rapamycin monotherapy may be explained by its nature which is mainly cytostatic rather than pro-apoptotic and its highly selective specificity to mTORC1, leaving rapamycin-insensitive molecules like mTORC2 continuing to function. In addition, rapamycin promotes autophagy by re-activating RTKs and Akt through a negative feedback loop; not to mention the function of compensatory pathways who also promote cell growth (27, 28). Therefore, novel therapies for LAM and more specific studies about tumorigenesis are in an urge.

AUTOPHAGY

Rapamycin Promoting Autophagy and Cell Survival

The status of mTORC1 as a key inhibitor of autophagy may help explain why rapamycin requires lifelong usage and its limited efficacy. Autophagy is a catabolic process in which damaged proteins and organelles are degraded in lysosomes and recycled to provide metabolic precursors (29). This feature has given autophagy a critical effect in tumorigenesis. Three vital genes, unc51-like autophagy activating kinase (ULK), autophagy-related protein 13 (Atg13), and focal adhesion kinase family interacting protein of 200 kDa (FIP200), were required to initiate autophagy through the product ULK1/2-Atg13-FIP200 complex which is suppressed when mTORC1 phosphorylates ULK1 directly (5) (**Figure 1**). Through this pathway, rapamycin-induced inhibition of mTORC1 might increase the kinase activity of ULK1 to enhance autophagy. In an *in vitro* study, the level of hypoxia-induced autophagy was found lower in $Tsc2^{-/-}p53^{-/-}$ MEFs than in $Tsc2^{+/+}p53^{-/-}$ MEF; when treated with rapamycin, $Tsc2^{-/-}$ $p53^{-/-}$ MEFs showed increased autophagy marker LC3-II and decreased ubiquitin-binding protein p62/Sequestosome-1 (SQSTM1), the autophagy substrate (30). Another study observed fewer renal tumors in $Tsc2^{+/-}$ Beclin $1^{+/-}$ mice than in $Tsc2^{+/-}$ mice and extensive central necrosis of xenograft tumors, indicating that downregulated autophagy level inhibited cell survival in TSC-related tumor (31). One hypothesis is that autophagy is a protective mechanism for survival because it promotes the removal of damaged mitochondria thereby lowering levels of reactive oxygen species (ROS) in a metabolic stressed environment including nutrient deprivation, hormone stimulation, and hypoxia (29, 32). As a result of hyperactive mTORC1, low levels of autophagy in TSC2-null LAM cells limit their survival in the circumstance of bioenergetic stress. In tumor tissues, nutrients, and oxygen tend to be more insufficient in the inner region, which is exactly a natural bioenergetics stress (1). Thus, although it seems contrary, mTOR inhibitors restrain LAM cell growth while very likely benefit cell survival by promoting autophagy. It is obvious that extensive autophagy leads to cell death, so more questions about rapamycin and autophagy need to be answered, but the therapeutic potential of autophagy inhibitors has already shown its attractiveness in LAM treatments.

Therapeutic Potential of Autophagy Inhibitors in the Treatment of LAM

Chloroquine and hydroxychloroquine are known as autophagy inhibitors, mainly used to treat malaria. Their effects in LAM have been revealed in *in vivo* and *in vitro* studies in which chloroquine inhibited TSC2-deficient cell survival and reduced xenograft tumor size to 60% (30). The effect was even more significant when chloroquine is combined with rapamycin than monotherapy of either. Based on these results,

TABLE 2 | Completed clinical trials studying therapeutic targets for LAM.

Target	Targeting therapy	Clinical trial	ClinicalTrials.gov identifier	Completion date
mTORC1	Sirolimus	Rapamycin therapy for patients with tuberous sclerosis complex and sporadic LAM	NCT00457808	March 2006
	Everolimus	A study to determine the effectiveness of escalating doses of RAD001 (everolimus) in patients with lymphangioleiomyomatosis	NCT01059318	June 2012
		RAD001 therapy of angiomyolipomata in patients with TS complex and sporadic LAM	NCT00457964	July 2013
		Long term follow up for RAD001 therapy of angiomyolipomata in patients with tuberous sclerosis (TSC) and sporadic lymphangioleiomyomatosis (LAM)	NCT00792766	September 2013
		Efficacy and safety of RAD001 in patients aged 18 and over with angiomyolipoma associated with either tuberous sclerosis complex (TSC) or sporadic lymphangioleiomyomatosis (LAM)	NCT00790400	November 2015
mTORC1 and autophagy	Sirolimus and hydroxychloroquine	Safety study of sirolimus and hydroxychloroquine in women with lymphangioleiomyomatosis (SAIL)	NCT01687179	August 2015
	Octreotide	Treatment with octreotide in patients with lymphangioleiomyomatosis	NCT00005906	April 2008
MMP	Doxycycline	Doxycycline in lymphangioleiomyomatosis (LAM)	NCT00989742	January 2013
Aromatase	Letrozole	Trial of aromatase inhibition in lymphangioleiomyomatosis (TRAIL)	NCT01353209	September 2014
Src	Saracatinib	The tolerability of saracatinib in subjects with lymphangioleiomyomatosis (LAM) (SLAM-1)	NCT02116712	July 2015
PDGFR	Imatinib mesylate	LAM pilot study with imatinib mesylate	NCT03131999	March 2019

a clinical trial in patients with LAM is conducted to examine the safety, adverse effects, and efficacy of combined use of sirolimus and hydroxychloroquine (33) (**Table 2**). Results of phase I revealed that hydroxychloroquine in combination with sirolimus increased post-bronchodilator FEV_1 (ml) significantly and decreased VEGF-D levels significantly during therapy. The walk distance in the 6-min walk distance test also increased significantly at the end of the treatment phase compared with the screening visit, and no serious adverse effect related to study drugs was reported. Nevertheless, patients with angiomyolipoma did not report any significant change in tumor size from baseline, the same with DLCO levels and St. George's Respiratory Questionnaire scores in all patients. Even the benefits went back to around baseline levels in the observation phase, like the consequence in sirolimus monotherapy. Considering the fact that only 14 patients were enrolled, and several have withdrawn, larger phase II/III trials are needed to further establish the long-term effectiveness of the combination therapy (33).

Another medicine that inhibits autophagy and raises much attention is resveratrol, which may suppress the Akt negative feedback loop to function. It was demonstrated in *in vivo* studies that combination therapy of rapamycin with resveratrol blocked autophagy and induced apoptosis in TSC2-null cells (34). In *in vivo* and *in vitro* studies, the combination therapy prevented rapamycin-induced upregulation of Akt while maintaining inhibition of S6K1 signaling, which means it keeps suppressing the hyperactivation of mTORC1 (35). Moreover, resveratrol is well-tolerated with a low toxicity profile, so it may be worthy of further study. A clinical trial has been set up to study the potential benefit of resveratrol in combination with sirolimus (**Table 1**).

Another Target in Autophagy

When studying the regulation of ULK1 and mTORC1 autophagy pathway, 50-AMP-activated protein kinase (AMPK) is found to be an important player. During energy starvation, AMPK could activate autophagy in three mechanisms: binding to and activating ULK1 through direct phosphorylation, inhibiting mTORC1 directly, or through activating TSC2 to suppress mTORC1 (36) (**Figure 2**). A nuclear protein, Poly (ADP-ribose) (PAR) polymerase (PARP)-1, was found to change the ratio of AMP to ATP which represents energy depletion and could be sensed by AMPK (**Figure 2**). Hyperactivated PARP-1 in response to ROS-induced DNA damage causes a depletion of ATP and activation of AMPK, inhibiting mTOR via TSC1/2 complex, ultimately inducing autophagy (29) (**Figure 2**). Upregulated PARP-1 expression was also found in TSC2-null cells derived from patients with LAM, as well as in TSC2-null xenograft tumors, renal tumors from TSC2 heterozygous mice, human angiomyolipomas, and LAM nodules (37). Studies then found the PARP-1 inhibitors suppress the growth and survival of TSC-null cells from LAM patients but not in a synergistic way with rapamycin *in vivo* (37). This study indicates the potential of targeting PARP1 in LAM therapy through PARP-1 inhibitors. However, although the PAR has been identified for over 50 years, serving as an initial sensor and mediating the early recruitment of DNA brake repair, it is not until recently that the anti-cancer effect of PARP-1 inhibitors was under emerging study, mainly in treating breast cancer (BRCA)–deficient tumors (38). Nevertheless, a recent study reported a contrary effect that the cytotoxicity of PARP inhibitors was dramatically enhanced by mTOR inhibitors in BRCA-proficient triple-negative breast

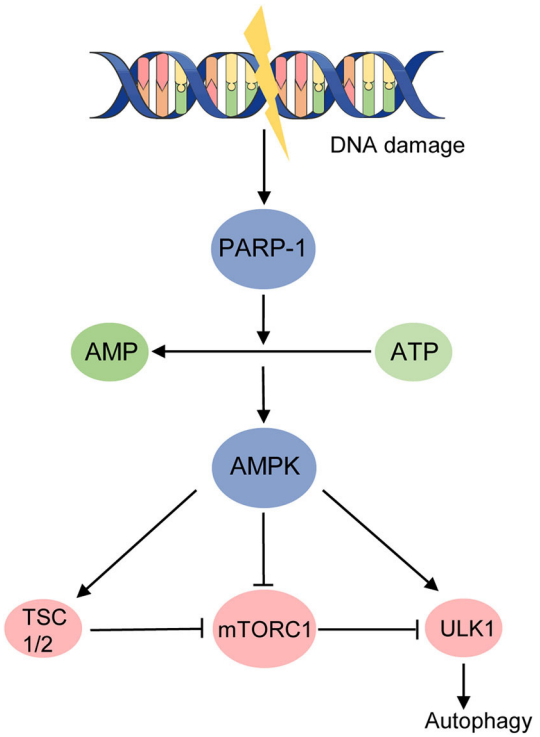

FIGURE 2 | DNA damage activates autophagy in an energy starvation environment. AMPK activates autophagy in three mechanisms during energy starvation: (1) binding to and activating ULK1 through direct phosphorylation; (2) inhibiting mTORC1 directly; (3) activating TSC2 to suppress mTORC1. Hyperactivated PARP-1 induced by DNA damage raises the ratio of AMP to ATP and activates AMPK, ultimately promoting autophagy.

cancers *in vitro* and *in vivo*, at the same time revealing a novel mechanism for mTOR signaling to regulate the homologous recombination process (39). The role of PARP-1 in autophagy and tumorigenesis is worthy to be studied further and PARP1 inhibitors might have potential efficacy in LAM treatment.

RECEPTOR TYROSINE KINASES AND NON-RECEPTOR TYROSINE KINASES

RTKs are a critical group of cell surface receptors located upstream of PI3K/Akt/mTORC1 pathway to receive and transfer growth factors including PDGF, VEGF-C, and VEGF-D, activating several downstream signal pathways to regulate tumor progression. Overexpression of various types of RTKs such as epidermal growth factor receptors (EGFRs), vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), and insulin-like growth factor receptors (IGFRs) is found in different types of cancer including LAM (40–42), indicating the important role of RTKs in LAM. The inhibition of RTKs is proposed to block the mTOR pathway. Studies proved its feasibility when they found that anti-EGFR antibody caused progressive cell death in $TSC2^{-/-}$ and $TSC2^{-}$/meth cells ($TSC2^{-}$/meth cells refer to smooth muscle-like cells from AML cells for these cells are methylated in the TSC2 promoter, and their proliferative, morphological, and biochemical characteristics are very similar to $TSC2^{-/-}$ smooth muscle cells) (43). Subsequently, the *in vivo* study showed that numbers of mice with lung nodules and the average area of the nodules was significantly reduced by Ab treatment and in combination with rapamycin (44). Moreover, angiogenesis and lymphangiogenesis reduction and even lung degeneration reservations were observed in Ab treatment (44). Another typical RTK, VEGFR, expressed on cancer cells, endothelial cells, and other stromal cells is found to be related to tumor lymphangiogenesis, angiogenesis, and growth (45). A study showed that axitinib, a small molecule tyrosine kinase inhibitor that targets VEGFR, attenuated VEGF-D upregulation in serum and lung lining fluid, as well as inhibited Tsc2-null lung lesion growth and abnormal lymphangiogenesis in a mouse model of LAM (42). It suggested that targeting this receptor may be beneficial for the treatment of LAM, especially considering that VEGF-D is a critical serum biomarker for LAM diagnosis and for evaluating the severity of LAM and efficacy of treatment in the presence (46, 47). A well-studied RTK inhibitor is nintedanib that dose-dependently inhibits PDGFR phosphorylation and has been used to treat IPF. Based on these data, a clinical trial assessing the efficacy, safety, and tolerability of nintedanib in the treatment of LAM is being carried on (**Table 1**).

A classic representative of non-RTKs is the Src family, which is also involved in the regulation of extracellular regulated protein kinases (ERK) and PI3K activation (**Figure 1**), modulating the equilibrium between survival and apoptosis, and might be affecting cystic fibrosis (48). The phosphorylation of Src is regulated by C-terminal Src kinase (Csk), a cytoplasmic protein–tyrosine kinase, which possesses a greater affinity for Src when recruited to the membrane by a transmembrane Csk binding protein (Cbp) than the free state (49) (**Figure 1**). A study has found upregulated phosphorylation of Src on Tyr416 in lung tissues of LAM patients compared with normal tissues, and enhanced activation of Src-kinase signaling pathway resulted from autophagy inhibition was observed *in vitro* studies in which Src inhibition of saracatinib reduced the epithelial–mesenchymal transition (EMT) which is promoted by overexpression of Src in TSC-deficient cells (50). EMT is believed to be correlated with infiltrative growth pattern, metastatic potential, and altered cell differentiation of LAM cells. Moreover, saracatinib was proved to attenuate migration and invasion activity of $TSC2^{-/-}$ cells in an *in vitro* study and reduce lung colonization in an *in vivo* study, suggesting that Src inhibition could reduce the metastatic potential for $TSC2^{-/-}$ cells (50). Then, a clinical trial studying the safety and efficacy of saracatinib in patients with lymphangioleiomyomatosis is currently being conducted (**Table 1**).

Spleen tyrosine kinase (Syk) is another non-RTK that has a key role in the immune system but arousing much concern in tumorigenesis study recently (51). It had an aberrant expression in various malignant tumors and contributing to the initiation and metastatic progression (52, 53). Phosphorylation of Syk is involved in the activation of the PI3K/Akt signaling pathway, mainly transducing immune response-associated signaling (54) (**Figure 1**). It is also a regulator of mTOR because its depletion

resulted in a significant reduction of S6K1 phosphorylation in follicular lymphoma cells (55). Hence, the hypothesis that Syk may be involved in LAM pathogenesis is worthy to be taken into consideration. As expected, deregulated expression and activation of Syk was detected in TSC2-deficient cells and LAM lung lesions (56). Furthermore, Syk inhibitor fostamatinib reduced the proliferation of TSC2-deficient cells *in vitro* and suppressed TSC2-null xenograft tumor development *in vivo* (56). This study identified a Syk-dependent signaling inducing VEGF-D expression in peripheral blood mononuclear cells by monocyte chemoattractant protein (MCP)-1, which was elevated via signal transducer and activator of transcription (Stat3) signaling downstream of the mTORC1 signaling (56), demonstrating the strong therapeutic potential of Syk in LAM treatment. Fostamatinib, as a Syk inhibitor, has been approved by the FDA to treat immune thrombocytopenia, but clinical trials studying its efficacy and safety in LAM treatment still wait to be accomplished.

HORMONES AND COMPENSATORY ERK PATHWAY

As is illustrated that LAM happens exclusively in women and is exacerbated during pregnancy, roles of female hormones in the development and progression of LAM call for more studies, and the evidence does suggest the relationship between female hormones and LAM pathogenesis. An immunohistochemistry study found progesterone receptor (PR) and estrogen receptor (ER) immunoreactivity in the smooth muscle component of renal angiomyolipomas (100 and 83%) from 12 LAM patients (57), and prevalence of PR expression over ER was confirmed in a large series of pulmonary LAM cases (58); in *in vitro* studies, ER-α and ER-β expression was observed in cultured angiomyolipoma cells derived from LAM patients (59).

Estrogen has been reported to promote changes in gene expression and can induce the activation of signaling proteins such as Src, Akt, and ERK (60) (**Figure 1**). It was proposed that the estrogen-regulated ERK pathway and the constitutive mTORC1 pathway together promote migration and invasion in LAM patient–derived cells by converging on the late response genes product (Fra1)–zinc finger E-box-binding homeobox (ZEB)-1/2 transcriptional network (61) (**Figure 1**). Studies already observed the stimulation effect of estradiol and tamoxifen on the growth of cultured angiomyolipoma cells (59). It was found that when the tuberin function is lost, the proliferation of Eker rat uterine leiomyoma derived smooth muscle (ELT3) cells promoted by estrogen is associated with the activation of PDGFR and ERK pathway (62). Subsequent studies found that estrogen promoted lung colonization of Tsc2-null ELT3 cells in pulmonary metastasis, which could be blocked by mitogen-activated protein kinase kinase (MEK) inhibitors CI-1040 in *in vivo* studies (63). In addition, a recent study found that estrogen increases membrane translocation of glucose transporters and enhances glucose uptake in a PI3K/Akt-dependent way in mTORC1 hyperactive cells (64). Estrogen promotes glucose metabolism including upregulating the expression of glucose-6-phosphate dehydrogenase (G6PD) and increasing nicotinamide adenine dinucleotide phosphate (NADPH) and ROS production, and thereby promotes cell survival under oxidative stress (65). A randomized controlled trial of letrozole (a non-steroidal, competitive aromatase inhibitor lowering serum estrone and estradiol) for postmenopausal women with LAM showed that the rate of change for serum VEGF-D between placebo group and letrozole group was -0.024 ± 0.009 pg/ml/month ($P = 0.015$), but the rate of change in FEV_1 for all subjects was -3 ± 3 ml/month ($P = 0.4$) (66). Considering the small size (15) of the trial, more evidence is required to prove the effects of hormonal treatment in LAM.

The study on estrogen still revealed the possibility of targeting the ERK pathway as a therapy in LAM treatment. ERK belongs to mitogen-activated protein kinases (MAPKs) family and plays a key role in tumorigenesis including cancer cell proliferation, migration, and invasion through cascade Ras/Raf/MEK/ERK, as a critical compensatory signaling pathway of PI3K/Akt/mTOR (67) (**Figure 1**). In the ERK signaling pathway, Ras, a membrane-associated GTP-binding protein, binds and activates several effector proteins including PI3K and rapidly accelerated fibrosarcoma (Ras) when activated by RTKs (68). ERK1/2 then is activated by MEK1/2 as a result of the regulation of RAF (67) (**Figure 1**). The Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways can cross-regulate each other's activity. For instance, ERK inhibits the recruitment of PI3K when induced by EGF and promotes mTORC1 directly or through repressing TSC1/2 complex; IGF1 stimulation could result in negative regulation of Ras/Raf/MEK/ERK pathway by inducing Akt inhibiting Raf (69) (**Figure 1**). In recent years, it has been reported that inhibition of mTORC1 induced activation of Ras/Raf/MEK/ERK signaling in renal cell carcinoma, prostate cancer, and leiomyosarcomas (70–72). In addition, MEK inhibitor as a melanoma treatment is now attractive for the treatment of non–small cell lung carcinoma (NSCLC) for its anti-cancer activity in patients with NSCLC (73). Considering the compensatory nature of the ERK pathway, whether MEK inhibitor in combination with rapamycin would benefit LAM patients requires to be studied carefully.

The unusual high PR/ER ratio detected by immunohistochemistry in renal angiomyolipomas reveals the possible function of progesterone in LAM (58). Progesterone is well–known to be antiproliferative for uterine leiomyomas, leading to the assumption of its anti-tumor function. Previous studies found that progesterone therapy stabilized chylous pleural effusion and ascites in some LAM patients (74). Nevertheless, retrospective studies showed less decline of lung function level in patients receiving progesterone therapy than in those who were not treated with progesterone but with no significance (75, 76), which seems to suggest that patients with LAM may not benefit from this therapy. Conversely, in *in vitro* studies, progesterone was found to activate ERK1/2 and Akt pathways, as well as enhance the proliferation, migration, and invasiveness of TSC2-deficient cells (77). Therefore, the specific mechanism of how estrogen and progesterone affect LAM development and their therapeutic potential still requires further exploration.

EMT PROCESS IN LAM METASTASIS

The dissemination of tumor cells from the primary site to several new sites in the body is typical in a malignant tumor, and so is it in LAM progression. Genetic confirmation of recipient origins of recurrent LAM lesions within the donor allografts of LAM patients, who had received lung transplantation, validates the metastasis theory for LAM, which is also indicated by multiple systemic manifestations and anatomy evidence (1). Therefore, to illuminate and prevent the metastasis process is particularly important and of increasing interest as a therapeutic avenue in the treatment of LAM.

The EMT has been proposed as a critical mechanism during cancer progression and metastasis because it is believed that the transition from epithelial to mesenchymal states allows a cell to break away from its originating tissue, traveling throughout the body (78). Numerous signaling pathways are involved in EMT induction, including PI3K/Akt pathway in which nuclear factor κB (NFκB) activated by Akt induces SNAIL1 stabilization that plays an integral role throughout EMT, and ZEB proteins which were mentioned earlier to engage the integration of mTORC1 and ERK pathway (78) (**Figure 1**). Among this complex pathway network inducing EMT, a protein family consisting of ezrin, radixin, and moesin (ERM) has come into focus of attention in investigations regarding metastatic cancer.

Dynamic rearrangement of the cytoskeleton is crucial to cell mobility and migration in metastasis, and ezrin, radixin, and moesin are important membrane-cytoskeletal crosslinkers which are suggested to play vital roles in cancer progression, especially ezrin and moesin (79). Studies have found that upregulated expression of moesin was associated with higher histological grade, advanced stage, and poor prognosis in gastric adenocarcinoma, breast carcinoma, and oral squamous cell carcinomas (79–81). Early in 2002, abundant expression of ezrin and moesin was observed in TSC-associated cortical tubers (82). Further studies showed that the role of ERM in tumor metastasis might result from a mTORC2-dependent pathway in which mTORC2 is found to modulate the actin cytoskeleton and promote migration and invasiveness through regulating RhoA activity, as is demonstrated previously (**Figure 1**). More specifically, GTPase-loaded RhoA activates Rho-associated protein kinases (ROCK1 and ROCK2) to promote the formation of stress fibers and the inhibition of cofilin and actin, supporting an amoeboid migration instead of a mesenchymal one, both morphologies being adopted during single-cell migration (78) (**Figure 1**). In the intravasation stage of metastasis, RhoA/ROCK provides cellular contractility to produce major cell deformability, enabling the cell to radically adjust its cytoskeleton to squeeze between intercellular spaces (78). As we expected, moesin is found to be a requirement to activate RhoA signaling in response to initial attachment and spreading (83), highlighting the urge to study the specific mechanism of how ERM, EMT, and mTORC2 integrally affect LAM metastasis.

Urokinase-type plasminogen activator (uPA) is a serine protease that also plays a role in tumor invasive process, at the same time upregulated in LAM lesions and angiomyolipomas.

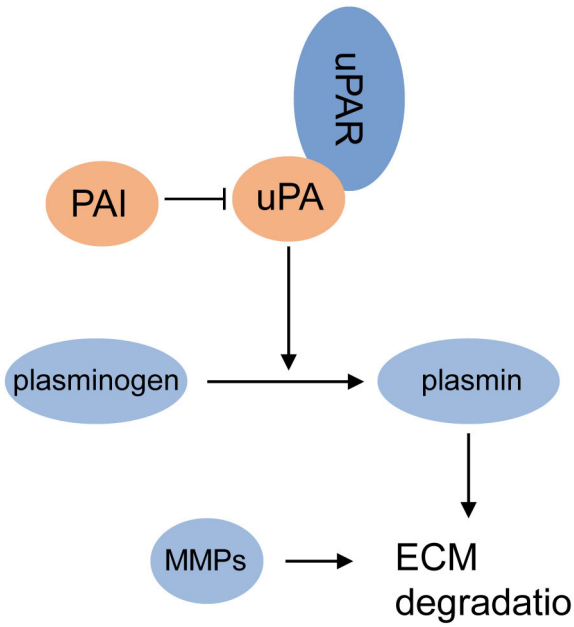

FIGURE 3 | uPA system and MMPs promote ECM degradation. uPA is recruited by uPAR and is negatively regulated by PAI1 or PAI2; overproduction of uPA increases the transformation from plasminogen to plasmin; plasmin promotes the degradation of ECM surrounding the tumor with MMPs, and promotes the initiation of invasion and metastasis.

The uPA system, including uPA, plasminogen activator inhibitor (PAI)-1, PAI-2, and uPA-associated receptor (uPAR), regulates the production and function of uPA to modulate the conversion from plasminogen to plasmin, which promotes the proteolytic cleavage of extracellular matrix (ECM) surrounding the tumor with matrix metalloproteinases (MMPs), facilitating the detachment of tumor cells from an original site and the initiation of invasive and metastasis (84) (**Figure 3**). The abnormal expression of the uPA system has been detected in breast cancer, prostate cancer, ovarian cancer, and lung cancer (84), and was recently reported in TSC-deficient tumors. The study found that overexpression of uPA was a direct consequence of TSC mutation in LAM cells and mTOR inhibitors even increased expression of uPA in cells with compromised TSC function (85). However, inhibition of uPA expression in TSC2-null tumor cells could not only reduce their invasive and mitogenic potentials but also increase their susceptibility to the pro-apoptotic agent simvastatin (85). Together, these studies indicate that uPA might serve as a potential target to prevent the dissemination of tumor cells and the progression of disease in LAM treatment.

NEWLY FOUND POTENTIAL TARGETS

Function of mTORC2

As mentioned previously, mTORC2 is rapamycin insensitive, and the function of mTORC2 in LAM pathogenesis remains unclear, however, which does not mean that mTORC2 contributes little

in tumorigenesis. Further study into the mechanism of the mTORC2 pathway might find novel targets improving the treatment of LAM. Rictor, a GTPase, is another component of mTORC2 besides mTOR (**Figure 1**). siRNA-mediated knockdown of rictor presents thick actin fibers throughout much of the cytoplasm and less prominent actin in HeLa cells, and the pattern of the actin staining is similar to that in cells with reduced mTOR expression, indicating the regulation effect of rictor and mTOR in organization of the actin cytoskeleton (86). Similarly, siRNA rictor microinjected in $TSC^{-/-}$ MEFs is found to attenuate the increased stress fiber formation as well as the enhanced migration and invasiveness of cells, whereas rapamycin has little effect on migration of LAM cells (87). These studies indicated the possibility of targeting mTORC2 as a therapy in combination with rapamycin.

The statin family of pharmacological inhibitors have been proposed as a treatment for LAM and examined in several clinical trials. Atorvastatin and simvastatin separately target farnesylation of Rheb and RhoA activity (88), and showed distinct effects in preclinical *in vivo* studies in which atorvastatin did not reduce tumor growth or improve survival in a Tsc2+/– mouse model with kidney and liver tumors while simvastatin inhibited tumor growth in a model of TSC2-null subcutaneous tumors (14, 89). The effects to preventing TSC2-null tumor growth and inhibiting destruction of the lung tissue were enhanced when simvastatin was in combination with rapamycin (90). The better effect of simvastatin may come from its target, RhoA, through which the mTORC2 could regulate the actin cytoskeleton (**Figure 1**), revealing the unneglected role of mTORC2 in tumor migration and invasiveness. A clinical trial studying the safety of simvastatin in patients with either LAM or TSC who are already being treated with rapamycin was just completed (**Table 1**), and we are looking forward to its outcome.

In addition to affecting cell migration, mTORC2 may also play a part in cell growth in some manner. An immunohistochemical study evaluating the expression of IGFs, IGF-1R, and IGFBPs in the lungs of patients with LAM found that only a proportion of patients have IGF-1 expression in LAM cells while reaction for IGF-2 was detected in almost every patient enrolled (40). The abnormal expression of IGFs in LAM patients may be related to mTORC2. A study reported that increased level of insulin, IGF-1, and IGFBP-3, which is a major IGF1 binding protein that stabilizes IGF-1 in the serum, was observed in adipose-specific Rictor knockout mice (rictor $ad^{-/-}$) that have gained weight due to an increase in the size of non-adipose tissue (91). These results suggested that mTORC2 might affect cell growth through regulating IGF-1, but the specific mechanism is yet to be demonstrated.

DNA Damage Checkpoint

The DNA damage checkpoints trigger cell-cycle arrest, providing sufficient time for the repairment of damaged chromosomes to help prevent the segregation of damaged or mutated chromosomes and promote genomic stability (92). G2/M is the last checkpoint before mitosis which is preferred in cancer cells to repair DNA damage and is regulated by the status of cyclin-dependent kinase 1(CDK1)/cyclin B1 complex (93). DNA damage is detected by related protein kinases and activates cell-cycle checkpoint kinase 1 (CHK1) which then phosphorylates cell division cycle 25 homolog (CDC25) and WEE1, repressing CDC25 and activating WEE1 at the same time. Because WEE1 negatively regulates CDK1/cyclin B1 while CDC25 does conversely, these physiological changes together contribute to the inhibition of CDK1/cyclin B1 complex and trigger the mitosis pause (93) (**Figure 4**). To study the signaling network required for cell-cycle restart, a systems biology approach has recently identified the essential role of mTOR signaling in regulating the recovery from G2/M checkpoint. The study found that transcription of cyclin B1 and polo-like kinase 1 (PLK1) was significantly impaired when DNA gets damaged in mTOR-depleted cells, which provided a suppressive chromatin environment due to decreased KDM4B function (94). As we know, PLK1 has the opposite effect with CHK1 which upregulates CDC25 but downregulates WEE1, helping promote the entry of mitosis (95) (**Figure 4**). This result indicates that in mTOR-activated cells, like LAM cells, cell proliferation seems to be enhanced because of a shorter G2/M checkpoint pause compared with normal cells, leaving insufficient time for DNA repair. In the subsequent study, TSC2-null cells did exhibit accelerated G2/M checkpoint recovery. Furthermore, these cells are sensitive to WEE1 inhibitors MK1775 both in *in vitro* and *in vivo* studies, particularly, more sensitive to rapamycin combined with the WEE1 inhibitor compared with rapamycin alone (94). WEE1 is a key nuclear kinase that is a crucial component to trigger G2/M checkpoint arrest and was reported to be involved in cancer drug resistance. For instance, WEE1 expression was increased after EGFR-TKI resistance in NSCLC and the combination of chemotherapy with WEE1 inhibitor might improve the clinical outcome of NSCLC patients with acquired EGFR-TKI resistance (96). Moreover, a recent study in patients with locally advanced pancreatic cancer demonstrated prolonged overall survival with the addition of the Wee1 kinase inhibitor adavosertib to gemcitabine plus radiotherapy (97). These studies provide a new opportunity for targeting tumor cells with mTORC1 hyperactivation like LAM using WEE1 inhibitors to induce mitotic catastrophe and cell death.

NR2F2 Located by GWAS in S-LAM Patients

Unlike TSC-LAM, the origin of S-LAM is not fully elucidated. The inactive mutations are seen in most LAM lesions but not in the germline of S-LAM patients. Researchers proposed a hypothesis that DNA sequence variants outside of TSC1/TSC2 might be associated with disease risk, and they applied genome-wide association studies (GWASs) to validate the assumption through analysis of over 5.4 million single-nucleotide polymorphisms in 426 S-LAM DNA samples and genotype data of 852 healthy female volunteers obtained from COPDGene project (98). This study finally identified a signal on chromosome 15q26.2 that reached genome-wide

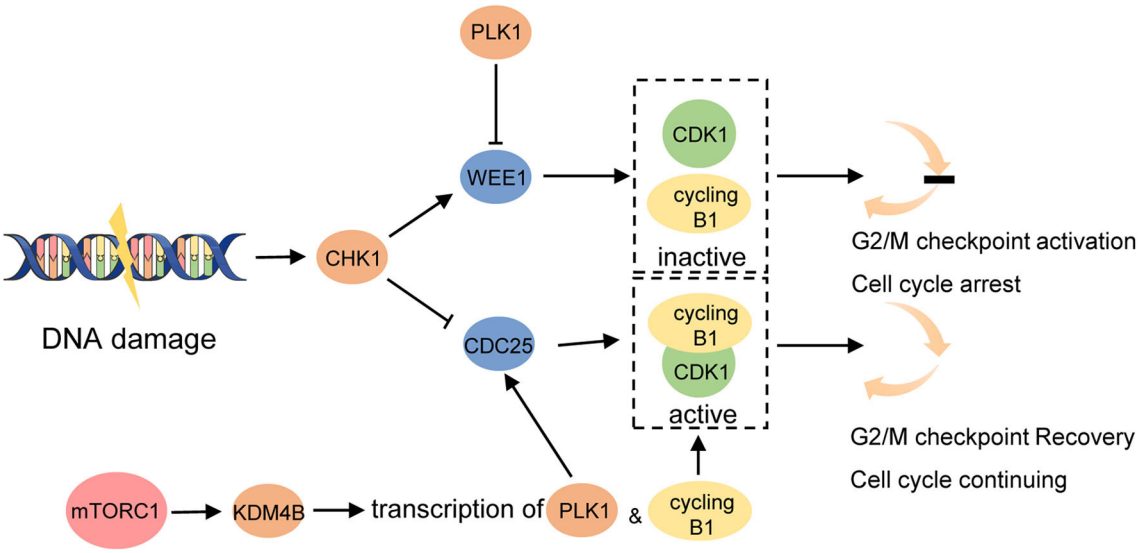

FIGURE 4 | mTORC1 promotes G2/M checkpoint recovery triggered by DNA damage. DNA damage activates CHK1. It then activates WEE1 which maintains cyclin B1 complexed with CDK1 inactivated and prevents cell cycle from entering mitosis. CHK1 inhibits CDC25 at the same time which activates CDK1/cyclin B1 complex and promotes G2/M checkpoint recovery. Hyperactivated mTORC1 increases the function of KDM4B which increases the transcription of cyclin B1 and PLK1. PLK1 activates CDC25 and inhibits WEE1 to promote the cell cycle to continue.

significance adjacent to the gene encoding nuclear receptor subfamily 2, group F, member 2 (NR2F2), which belongs to the steroid receptor superfamily (99). Its expression was higher in LAM and angiomyolipoma by RNA sequencing analysis in comparison with large cancer and normal tissue datasets (98). Studies have suggested that NR2F2, also known as chicken ovalbumin upstream promoter transcriptional factor II (COUP-TFII), serves as a major regulator to control angiogenesis within the tumor microenvironment and tumor lymphangiogenesis through cooperation with VEGF/VEGFR signaling, although the specific details need to be further elucidated (100, 101). VEGF-D serves as a diagnostic biomarker for LAM and possible driver for lymphangiogenesis. In TSC-null cells, inappropriate activation of mTOR results in accumulation of hypoxia-inducible factor-1α (HIF-1α) and increased expression of HIF-responsive genes including VEGF, which could be decreased by rapamycin treatment (102) (**Figure 1**). In addition, a study found that hypoxia significantly downregulated COUP-TFII in early endothelial progenitor cells (103). Further investigation may help reveal the interaction between COUP-TFII, HIF-1α, and VEGF. Previous studies have already shown that the siRNA knockdown of ERα in MCF-7 breast cancer cells decreased COUP-TFII expression while treatment with estradiol increased the expression of COUP-TFII, revealing a positive correlation between COUP-TFII and ERα status (104). A recent study also reported another mechanism of how COUF-TFII might influence LAM pathogenesis, in which lactate production induced by upregulated COUP-TFII in KRAS-activated cells increases mTORC1 activity by disrupting the interaction of TSC2 and Rheb (105) (**Figure 1**). Thus, NR2F2 could be a promising candidate to explain the gene origin of S-LAM and pathogenesis of LAM.

CONCLUSION

Despite the rare nature of this disease, the study into tumorigenesis, migration, and invasiveness of LAM has made tremendous advances in the past 20 years. The activation of mTOR signaling, regulated by inactivation mutation of TSC2, is the key point of LAM pathogenesis and the crucial target of rapamycin therapy, which is a hallmark event in LAM treatment. Nevertheless, rapamycin therapy is not curative and studies about other potential targets have been carried out. Among these hypotheses, autophagy induced by rapamycin may explain its limited efficacy and reveal the probable benefit of autophagy inhibitors. Common methods used in finding and validating new targets usually start from upstream or downstream pathways affecting PI3K/Akt/mTORC1 signaling, like tyrosine kinases, or compensatory pathways independent of mTORC1 signaling, like mTORC2 pathway, modulating actin cytoskeleton, and ERK pathway, related with estrogen signaling. However, they may also coordinate with mTORC1 signaling. Considering the characteristic damage caused by LAM to the lung tissue and pulmonary function, studying the cyst formation mechanism is of significance to prevent the parenchymal destruction of lung and improve the general condition of LAM patients. Another vital process in LAM progression is metastasis in which the EMK process might contribute a lot to the migration and invasiveness of LAM cells. Besides, new methods such as GWAS, which located a new gene NR2F2 involved in LAM, and systems biology approach, which found the correlation of mTOR with DNA damage checkpoint, used in tumor research could help open minds in studying new targets, which, however, need to be further validated. What is more, some of the mechanisms demonstrated in this review, although illustrated from a different angle, present

a potential relation. For example, the little-understood mTORC2 pathway is also found to affect the migration of LAM cells by regulating RhoA signaling, which plays a key role in the EMT process. Therefore, studying the integration of related pathways may help uncover the pathogenesis of this complex disease and find an effective therapy.

AUTHOR CONTRIBUTIONS

LY and MJ contributed to the conception and design of the study. XS and HC organized the database and wrote the article. All authors contributed to article revision, and read, and approved the submitted version.

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Identification of a Novel Pathogenic Folliculin Variant in a Chinese Family With Birt–Hogg–Dubé Syndrome (Hornstein-Knickenberg Syndrome)

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Birt–Hogg–Dubé syndrome (BHDS), which is also called Hornstein-Knickenberg syndrome (HKS), is a hereditary autosomal dominant disorder caused by germline mutations in the folliculin gene (*FLCN*, NM_144997). More pulmonary manifestations (pulmonary cysts and recurrent pneumothoraxes) but fewer skin fibrofolliculomas and renal malignancy are found in Asian BHDS patients compared with other BHDS patients. The atypical manifestation can easily lead to a missed or delayed diagnosis. Here, we report a Chinese family with BHDS that presented with primary spontaneous pneumothorax (PSP) and extensive pulmonary cysts in the absence of skin lesions or renal neoplasms. Next-generation sequencing (NGS) was used to sequence the *FLCN* gene, and Sanger sequencing was carried out on the samples to confirm the presence of these variants. Among the 13 family members, a novel frameshift variant of *FLCN* (c.912delT/p.E305KfsX18) was identified in seven individuals. This variant has not been reported before. Bioinformatics analysis showed that the novel variant might lead to a premature stop codon after 18 amino acid residues in exon 9, and this may affect the expression level of *FLCN*. The identification of this novel frameshift variant of *FLCN* not only further confirms the familial inheritance of BHDS in the proband but also expands the mutational spectrum of the *FLCN* gene in patients with BHDS.

Keywords: pneumothorax, folliculin, Birt–Hogg–Dubé syndrome, pulmonary cysts, Hornstein-Knickenberg syndrome, variant

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INTRODUCTION

Birt–Hogg–Dubé syndrome (BHDS, OMIM#135150), which is also called Hornstein-Knickenberg syndrome (HKS), is a rare autosomal dominant inherited disorder that predisposes individuals to develop benign skin tumors (fibrofolliculomas), renal neoplasms, and pulmonary cysts with a risk of spontaneous pneumothorax (Menko et al., 2009). This disease was first described by Otto P. Hornstein and Monika Knickenberg as a new autosomal dominant trait characterized by "perifollicular fibromatosis cutis," multiple skin tags, and multiple colonic polyps with proneness to cancer in 1975 (Hornstein and Knickenberg, 1975). Two years later, Arthur R. Birt, Georgina R. Hogg, and W. James Dube (Birt and Hogg, 1977) from Canada described similar hereditary skin lesions, fibrofolliculoma, without any extracutaneous cancer proneness.

Then, the disease was named as "BHDS" after the three Canadian doctors. Today, many authors believe that "fibrofolliculoma" is identical with "perifollicular fibroma" (Happle, 2020). Thus, the two diseases are essentially the same. Today, BHDS is more widely accepted, but we should not forget the persons who first discovered the disease.

The most common skin lesions of BHDS are cutaneous fibrofolliculomas occurring on the head, neck, and upper body of greater than 85% of BHDS-affected individuals over 25 years of age (Schmidt and Linehan, 2018). Renal tumors occur frequently in BHDS patients, and the most frequent histological types of neoplasms are hybrid oncocytic/chromophobe tumors or chromophobe renal cell carcinoma (Furuya et al., 2020). More than 80% of BHDS patients have multiple bilateral lung cysts and approximately 22.5–38% patients report a history of single or recurrent spontaneous pneumothorax, which can present as the first symptom (Sattler et al., 2020). The CT examination results showed that the cysts in the lungs were variable with well-defined walls, located mostly in the basal medial regions (58%), less frequently in the basal peripheral regions (27%), and approximately 40% adhere to the pleura. The number of cysts varies from tens to hundreds, and their size also varies greatly, from a few millimeter to 2 cm or more, but most have a diameter smaller than 1 cm (Tobino et al., 2011). Spontaneous pneumothorax may be the only manifestation in BHDS patients, which has been described in many reports (Graham et al., 2005; Painter et al., 2005). It is often misdiagnosed as primary spontaneous pneumothorax (PSP), in particular, in the cases with only isolated lung cysts/pneumothorax presentation (Min et al., 2020). Lack of a comprehensive and profound understanding of BHDS often leads to a misdiagnosis.

The folliculin (*FLCN*) gene is considered to be a tumor suppressor. Localized in the short arm of chromosome 17p11.2, it contains 14 exons, 11 of which encode a 579-amino-acid-long protein called folliculin (Han et al., 2020). Germline mutations of the *FLCN* gene were identified to be responsible for BHDS in 2002 (Nickerson et al., 2002). *FLCN* is expressed in normal skin cells, nephrons, stromal cells, Type I pneumocytes, and the acinar cells of the pancreas and parotid gland. Pathogenic *FLCN* variants may lead to the inactivation of the gene, which destroys the ability of *FLCN* to restrict cell growth and division, resulting in deregulated cell growth and protein synthesis, giving rise to the formation of malignant and benign tumors (Balsamo et al., 2020). To date, more than 280 different types of unique mutations spanning the entire coding region of the *FLCN* gene have been identified, according to the Leiden Open Variation Database.[1] The majority of *FLCN* mutations identified in the germline of BHDS patients are frameshifts (insertion/deletion), nonsense mutations, and splice site mutations (Lim et al., 2010). It has been observed in BHDS patients that *FLCN* mutations in exon 9 are associated with an increased number of lung cysts and exon 9 and 12 mutations are correlated with more episodes of spontaneous pneumothorax (Toro et al., 2007). These genotype-phenotype correlations have only been suggested in some studies.

Here, we reported a large Chinese family in which seven members from three generations developed lung cysts. A genetic study revealed a novel and previously not reported variant in *FLCN* in the seven individuals.

MATERIALS AND METHODS

Patient Characteristics

A 43-year-old female proband from Central China was diagnosed with PSP in the Second Xiangya Hospital of the Central South University. The family histories of her 12 relatives from three generations were collected. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article. All subjects received skin examination by professional dermatologists. Chest CT testing was done to evaluate the pulmonary lesion. And abdominal ultrasound examination was performed to rule out renal involvement. Blood was collected from the probands and their family members.

DNA Extraction

Peripheral blood samples were collected into EDTA anticoagulant tubes and stored at +4°C until DNA isolation was performed within 24 h of collection. Genomic DNA was prepared using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, United States) according to the instructions of the manufacturer. The DNA samples were stored at −20°C until the PCR stage.

Mutation Sequencing

The entire coding regions, including the intronic flanking sequences of FLCN were amplified by PCR. The software IDT[2] was used to design primers for PCR (primer sequences will be provided upon requests). Sequences of PCR products were determined by the ABI 3100 Genetic Analyzer (Thermo Fisher Scientifc, Inc., Waltham, MA, United States). The DNA sequencing reaction of the proband was performed using the next-generation sequencing (NGS) method according to manufacturer's protocols (details are available upon request). The multiple FLCN protein sequences were aligned using the program MUSCLE (version 3.6). The online databases, PolyPhen-2 (polymorphism phenotyping), and MutationTaster programs were used to predict the possible effects of variants on the function of the proteins.

Sanger Sequencing

To validate true positive novel variants identified by NGS, Sanger sequencing was carried out on the samples to confirm the presence or absence of these variants in the proband, and other family members. The sequencing results were later analyzed using Sequencher software (Gene Codes Corporation, MI, United States).

[1]https://www.lovd.nl/

[2]http://www.idtdna.com/site

RESULTS

Germline Variant of the *FLCN* Gene

Sequence analysis of the *FLCN* gene revealed a novel deletion variant (c.912delT/p.E305KfsX18) in exon 9. This variant was then confirmed by Sanger sequencing (**Figure 1**). The c.912delT variant resulted in a frameshift at amino acid position 305 and the introduction of a premature stop codon after 18 amino acid residues (p.E305KfsX18) and was predicted to be disease-causing by MutationTaster.[3] In total, seven of the 13 family members harbored the same variant. The pedigree of the family members included in the study is shown in **Figure 2**. Among the affected individuals, four were female, and three were male. No variant at this site was found in any of the available unaffected family members. The newly identified variant has never been reported in previous studies. According to the dbSNP and Human Gene Mutation Database,[4] this heterozygous variant is novel.

Clinical Characteristics

In our study, all seven patients with the *FLCN* variant showed bilateral multiple pulmonary cysts on CT imaging (**Figure 3**). PSP was detected in three cases, including the proband. Two of the family members underwent conservative treatment due to PSP, while the proband received thoracoscopic bullectomy for a first episode of right-lung pneumothorax. The pathological section of her lung pathology presents a pulmonary cyst. None of the patients exhibited fibrofolliculomas skin lesions or renal involvement. The two family members who suffered from PSP were misdiagnosed with pulmonary cysts due to the atypical manifestations, and they did not receive *FLCN* gene mutation screening. The other four patients without pneumothorax underwent chest CT, and pulmonary cysts were observed for the first time in the present study. The clinical characteristics of most of the living family members are summarized in **Table 1**.

DISCUSSION

In this study, we presented a novel frameshift variant (c.912delT/p.E305KfsX18) in exon 9 of the *FLCN* gene, which has not been previously reported in individuals with BHDS. Frameshift mutation may lead to an early termination of protein synthesis or to nonsense-mediated mRNA decay in which the defective mRNA is prematurely degraded (Ali et al., 2020).

The typical clinical manifestations of BHDS are skin, kidney, and lung involvement. However, patients do not always have all the three characteristic manifestations. Toro et al. (2008) reported that most Caucasians with BHDS (85–90%) have skin lesions as their major complaint, 34% of patients developed kidney tumors, and the incidence of pneumothorax is about one-third. A Japanese study conducted by Furuya et al. (2016) found that recurrent episodes of pneumothorax were more prevalent (73.7%) in BHDS patients and were more informative as diagnostic criteria for BHDS in their Japanese Asian population.

[3]www.mutationtaster.org

[4]http://www.hgmd.cf.ac.uk/ac/index.php

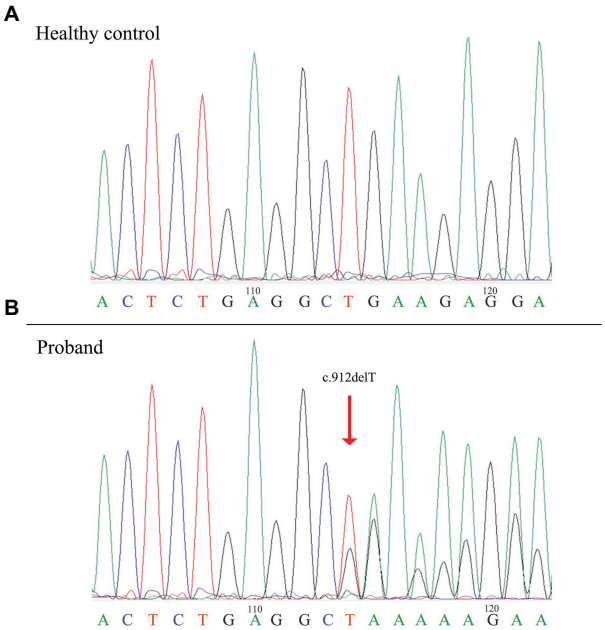

FIGURE 1 | Identification of a novel frameshift mutation of the *FLCN* gene in the patients. Sanger sequencing showing the novel *FLCN* mutation in exon 9 of the proband compared with a healthy control. The red arrow indicates the site of the mutation (c.912delT/p.E305KfsX18). *FLCN*: Folliculin.

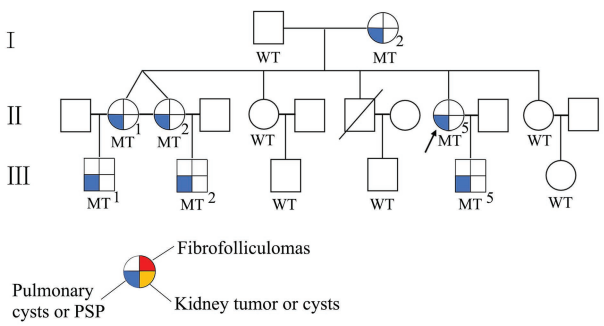

FIGURE 2 | The pedigree of the Chinese family with the *FLCN* mutation. Squares indicate male family members; and circles indicate female members. MT, mutant; WT, wildtype; arrow indicates proband.

In contrast, cutaneous manifestations are not the major complaint in the Japanese population (Furuya et al., 2016). BHDS patients in China also present with more pulmonary manifestations but fewer skin lesions and renal malignancies (Ren et al., 2008; Liu et al., 2017). Selection bias may be responsible for the different frequencies of pulmonary manifestations between Asian and Western BHDS cases (Liu et al., 2019). Most Caucasian patients with BHDS were recruited through referrals from departments of dermatology or urology, while most patients with BHDS in China were diagnosed by respiratory physicians due to the pulmonary manifestations (Liu et al., 2019). Patients with cutaneous fibrofolliculoma or renal neoplasms may not

receive regular chest CT examinations if the patients do not have any marked respiratory symptoms. Apart from renal tumors, several other tumor entities have been reported in association with BHDS, including colon polyps and tumors, breast cancer, lung cancer, thyroid cancer, parathyroid adenoma, lipoma, melanoma, and parotid oncocytoma (Steinlein et al., 2018). Among these tumors, colon cancer has been investigated in more detail. However, the incidence of colon cancer varies greatly among different studies (Zbar et al., 2002; Nahorski et al., 2010; Steinlein et al., 2018). In Asian, there are only sporadic reports about BHDS accompanied by colon polyposis or carcinoma (Kashiwada et al., 2012; Motegi et al., 2018). Whether these tumors are genuine associations or merely coincidences with BHDS has not yet been clinically validated.

In the present study, the proband and her six relatives also exhibited pulmonary involvement without skin or renal lesions, and three out of seven (42.8%) members suffered from PSP. These manifestations are in accordance with the previous studies in China (Ren et al., 2008; Liu et al., 2017). Since renal neoplasms frequently occur in BHDS patients, surveillance by renal imaging annually, such as low dose spiral CT or MRI (Hindman, 2018; Kay and Pedrosa, 2018), should be carried out in this family to identify any kidney tumors as early as possible. Due to the nontypical clinical manifestations, it is difficult to diagnose BHDS in patients without cutaneous lesions or renal pathology. *FLCN* mutation screening is recommended and is a reliable method for the clinical molecular diagnosis of BHDS, especially in patients who do not have skin and renal manifestations (Zheng et al., 2019).

The *FLCN* gene encodes the protein folliculin, a 579-amino-acid-long protein, which was first reported to be responsible for BHDS in 2002 (Nickerson et al., 2002). Since then, over 280 *FLCN* gene mutations have been identified according to the Leiden Open Variation Database. Half of BHDS families exhibited frameshift mutations in coding exons of *FLCN* (Furuya et al., 2016), while splice site, nonsense, missense, and deletion mutations are less prevalent (Kim et al., 2012).

The genotype-phenotype correlations between *FLCN* mutation status and skin, lung, or renal manifestations are still not clear. Some researchers have reported that *FLCN* mutations in exon 9 and 12 are associated with a higher number of pulmonary cysts, a larger cyst diameter, and more episodes of pneumothorax (Toro et al., 2007). A deleted cytosine in exon 11 results in a significantly lower frequency of renal neoplasia compared with the patients with an inserted cytosine at the same location (Schmidt et al., 2005). In the present study, a heterozygous frameshift variant (c.912delT/p.E305KfsX18) was detected in exon 9 of *FLCN* of seven members from three generations of the same family. This novel variant is predicted to cause premature truncation of the *FLCN* protein, leading to functional haploinsufficiency of *FLCN*. Loss of function of this protein can cause alveolar enlargement and cysts formation, consequently leading to pneumothorax (Xing et al., 2017).

The exact mechanism of *FLCN* mutations leading to pulmonary cysts and pneumothorax in BHDS patients has not yet been fully elucidated. The stretch hypothesis has been proposed based on observations of increased cell-cell adhesion in *FLCN*-deficient cells, which may reduce the flexibility of the cell-cell junctions, resulting in stretch-induced lung injury and subsequent airspace enlargement (Medvetz et al., 2012; Kennedy et al., 2016). Animal studies have shown that *FLCN* deletion in SP-C expressing lung epithelial cells leads to alveolar enlargement and impaired lung function by inducing alveolar epithelial cell apoptosis (Goncharova et al., 2014). A recent study (Chu et al., 2020) showed that the deletion of mesenchymal *FLCN* resulted in a reduction of postnatal alveolar formation and destruction of alveolar walls through the suppression of cell proliferation and alveolar myofibroblast differentiation, and the inhibition of extracellular matrix proteins and elastin expression. These results suggested that *FLCN* deficiency may lead to pulmonary cystic lesions and pneumothorax by inducing alveolar hypoplasia. So far, most of the studies have concentrated on the functions of *FLCN* outside the lung, so the exact mechanism of how the *FLCN* gene and involved pathways contribute to lung cyst formation is very limited.

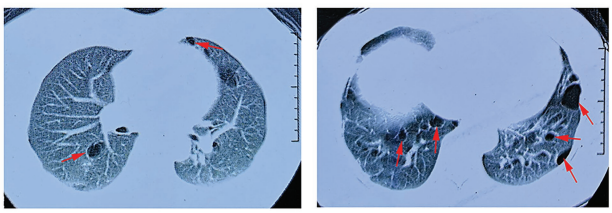

FIGURE 3 | Lung CT scan of the proband. Red arrows indicate multiple pulmonary cysts.

TABLE 1 | Clinical features of family members with folliculin (FLCN) gene mutation.

Patient No.	Sex	Age	BMI (kg/m^2)	Smoking history	Age at first episode of pneumothorax	No. of PSP attack	Treatment	Lung cysts	Skin lesions	Kidney lesions
I-2	F	75	21.2	N	56	1	TD	Bilateral, multiple	N	N
II-1	F	54	23.1	N	N	0	N	Bilateral, multiple	N	N
II-2	F	54	24.5	N	49	1	TD	Bilateral, multiple	N	N
II-5	F	44	22.8	N	43	1	VB + MP	Bilateral, multiple	N	N
III-1	M	29	25.6	Y	N	0	N	Bilateral, multiple	N	N
III-2	M	31	24.9	Y	N	0	N	Bilateral, multiple	N	N
III-5	M	20	23.7	N	N	0	N	Bilateral, multiple	N	N

BMI, body mass index; F, female; M, male; Y, yes; N, no; TD, tube drainage; VB, video-assisted thoracoscopic surgery bullectomy; MP, mechanical pleurodesis.

CONCLUSION

In conclusion, we found a novel heterozygous frameshift variant in exon 9 of *FLCN* (c.912delT/p.E305KfsX18) in a Chinese BHDS family, which might cause lung cysts and PSP. These findings highlight the importance of screening for *FLCN* gene variants in patients with pulmonary cysts and PSP, even in the absence of skin and/or kidney lesions. The identification of this novel variant expands the mutation spectrum of the *FLCN* gene in the Chinese population.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Review Board of The Second Xiangya Hospital of the Central South University. The patients/participants provided their written informed consent obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors contributed to the reviewing of the paper. DZ and XL performed the laboratory work, statistical analyses, and drafted the manuscript. JL and RO collected the clinical data. TG performed NGS analysis. RO supervised the study and helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Update on Pulmonary Langerhans Cell Histiocytosis

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Pulmonary Langerhans cell (LC) histiocytosis (PLCH) has unknown cause and is a rare neoplastic disorder characterized by the infiltration of lungs and various organs by bone marrow-derived Langerhans cells with an accompanying strong inflammatory response. These cells carry somatic mutations of *BRAF* gene and/or *NRAS*, *KRAS*, and *MAP2K1* genes, which cause activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway. PLCH occurs predominantly in young smokers, without gender predominance. Lungs might be involved as an isolated organ or as part of a multiorgan disease. High-resolution computed chest tomography plays an outstanding role in PLCH diagnosis. The typical radiological picture of PLCH is the presence of small intralobular nodules, "tree in bud" opacities, cavitated nodules, and thin- and thick-walled cysts, frequently confluent. Histological examination of the lesion and demonstration of characteristic eosinophilic granulomas with the presence of LCs that display antigen CD1a or CD207 in immunohistochemistry are required for definite diagnosis. Smoking cessation is the most important recommendation for PLCH patients, but treatment of progressive PLCH and multisystem disease is based on chemotherapy. Recently, new targeted therapies have been implemented.

Keywords: pulmonary Langerhans cell histiocytosis, adults, lung, BRAF, MAPK

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INTRODUCTION

Pulmonary Langerhans cell (LC) histiocytosis (PLCH) is a rare neoplastic disorder of unknown etiology, characterized by the infiltration of the lungs and various organs by bone marrow-derived LCs with an accompanying strong inflammatory response (1). These cells carry somatic mutations of the *BRAF* gene and/or *NRAS*, *KRAS*, and *MAP2K1* genes, which cause activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (2–5).

The histiocytic diseases have been reclassified into five categories; LCH is considered a member of group L, along with Erdheim–Chester disease, indeterminate cell histiocytosis, and mixed LCH/Erdheim–Chester disease (1, 6). PLCH occurs predominantly in young smokers and shows no predominance in either sex. The lungs may be involved as isolated organs or as part of a multisystem disease (7). Proliferating LCs form nodular lesions of various sizes, which infiltrate neighboring tissues and damage their structure. In adults, LCH most commonly affects the lungs, bones, skin, and pituitary gland. The involvement of lymphopoietic organs (e.g., lymph nodes, liver, spleen, and bone marrow), alimentary system, and central nervous system (CNS) is rare. In the course of LCH, pulmonary lesions may be isolated, occur after chronic systemic disease, or are an initial sign of disease. The isolated pulmonary form of LCH is observed in approximately 50–70% of patients with PLCH (8–11).

Single-system LCH (SS-LSH) comprises single or multiple involvement of the following single organs or systems:
Bone, with involvement of a single or multiple bones and many foci in many bones;
Skin;
Lymph nodes, excluding lymph nodes that drain the area of histiocyte infiltration or multiple lymph nodes (i.e., more than one lymph node group);
Hypothalamus–hypophysis/CNS;
Isolated pulmonary involvement;
Other, with involvement of oral mucosa, thyroid, thymus, or intestine.
Multisystem LCH (MS-LCH) comprises disease in which two or more organs or systems are affected:
Involvement of critical organs, e.g., hemopoietic system, spleen, liver;
No involvement of critical organs.

The involvement of specific areas and bones [e.g., vertebrae (possible compression spinal fracture and damage to the spinal cord), orbital bones, mastoid process, sphenoid bone, and temporal bones with transition into soft tissue (possible injury to facial nerves and pituitary gland)] is associated with the risk of CNS involvement, which impacts decisions regarding treatment (7, 12).

Previously, the lungs were considered as a "risk organ," but multivariable analysis of cohorts of 420 children with MS-LCH showed that lung involvement was not the significant prognostic variable (13). However, patients with lung lesions have a high risk of developing life-threatening complications (e.g., pneumothoraces, pulmonary infections). Recently, Le Louet et al. (14) presented the assessment of French LCH registry, and it was found that severe lung involvement in children was associated with high mortality. These findings suggested revision of treatment guidelines for this group of patients.

EPIDEMIOLOGY

LCH has an estimated prevalence in adults of 1–2/1,000,000. However, there have been few population-based epidemiological studies regarding this disease. In Japanese hospitalized patients, the prevalence rates of PLCH were reported to be 0.07/100,000 in women and 0.27/100,000 in men. PLCH is presumed to affect approximately 5% of patients undergoing open lung biopsy. The prevalence of PLCH may be underestimated due to the nature of the disease (7, 15).

PLCH affects young people in the third and fourth decades of life, with no sex predominance. Approximately 90 to 95% of patients with PLCH are tobacco smokers (16, 17). Recently, it was noticed that 20–33% of patients smoked both cigarettes and cannabis [(18), personal observations]. The intensity and smoking duration do not influence the development of PLCH. Isolated PLCH in children is extremely rare; the disease is reportedly associated with passive exposure to tobacco smoke in the majority of pediatric patients, and ∼10–30% of children with multisystem LCH exhibit pulmonary lesions (11, 19). Familial occurrence of LCH has been reported, but no genetic predisposition toward LCH has been found (7).

PATHOGENESIS

There have been many investigations regarding the pathogenesis of LCH; the finding that dendritic cells with mutations in MAPK pathway genes contribute to the development of the disease has yielded new insights. Dendritic cells are a heterogeneous group of cells, which mainly serve to process and present antigens to immune cells. Normal LCs are present in the skin, as well as beneath the epithelia of the bronchial tree and other mucosae (3, 6). Toll-like receptors, expressed on pathogen-sensing cells, and factors released from damaged or dying cells, stimulate the activity of normal LCs. Subsequently, the immunological response involves the migration of activated dendritic cells to the nearest lymph nodes and presentation of antigens to naive T lymphocytes. In addition, tolerance to harmless inhaled antigens is mediated by normal LCs (20).

Tobacco smoke plays a major role in the development of PLCH (21). It causes inflammatory cell accumulation in the lungs; these cells include LCs, which release cytokines such as tumor necrosis factor alpha, interleukin 1 beta, granulocyte-macrophage colony-stimulating factor, transforming growth factor beta, and the dendritic cell chemokine (chemokine ligand 20) (22). In addition, bronchial epithelial cells and fibroblasts release granulocyte-macrophage colony-stimulating factor, which is a strong mitogenic factor for LCs.

Moreover, PLCH lesions exhibit elevated expression of osteopontin, a glycoprotein that induces chemotactic activity in macrophages, monocytes, and dendritic cells, including LCs. Activated pathological LCs show strong lymphocyte-stimulating properties and are characterized by elevated expression of CD40, CD80, and CD86. Previous studies have yielded inconsistent results regarding the importance of interleukin-17 released by T lymphocytes in the pathogenesis of histiocytic lesions. This cytokine promotes the development of giant cells and formation of granulomas. Elevated expression of the antiapoptotic protein, Bcl-xL, within histiocytic granulomas maintains the pathogenic process (23).

In addition to inflammation, granuloma formation is accompanied mPAP, mean pulmonary arterial presure; by remodeling of the lung parenchyma. Cystic destruction of the lung is presumably caused by activation of metalloproteinases 2 and 9, produced by dendritic cells, LCs, and monocytes. Moreover, elevated expression of transforming growth factor beta released by granuloma cells causes fibrotic lesions (12).

There have been a number of investigations regarding the pathogenesis of PLCH. Inflammatory cell accumulation and the

Abbreviations: ARAF, A serine/threonine kinase; AKT, serine/threonine kinase; BRAF, B serine/threonine kinase; 2-CDA, 2-chloro-2'-deoxyadenosine; CNS, central nervous system; ERK, extracellular signal-regulated kinase; HRCT, High-resolution computed tomography; LAM, lymphangioleiomyomatosis; LC, Langerhans cell; LCH, Langerhans cell histiocytosis; MAPK, mitogen-activated protein kinase; MS-LCH- multisystem Langerhans cell histiocytosis; mTOR, mechanistic target of rapamycin; P, phosphorylation; PIK, phosphatidylinositol kinase; PIP-1, phosphatidylinositol monophosphate; PIP-2, phosphatidylinositol biphosphate; PLCH, pulmonary Langerhans cell histiocytosis; PTEN, phosphate and tensin homolog; SS-LCH single system Langerhans cell histiocytosis.

presence of LCs with dysfunctional apoptosis, as well as clonal proliferation, have been associated with the onset of PLCH.

Identification of the role of the RAS/MAPK signaling pathway in the pathogenesis of LCH was a key discovery regarding the pathogenetic mechanism of this disorder (24). This pathway transmits proliferative signals from the cell surface *via* the RAS pathway to phosphorylate MAPK and ERK, which transmit signals to the nucleus. The MAPK pathway regulates the activities of many enzymatic proteins and transcription factors. Activating mutations of the BRAF, ARAF, MAP2K1, N/K/HRAS, and PIK3CA genes have been found in patients with PLCH (25–27) (**Figure 1**). The *BRAF* V 600E mutations were found in 50% of LCH pulmonary nodules and MAP2K1 in an additional 20% of cases (26). Moreover, whole-exome sequencing analysis revealed *MAP2K1* mutations in seven of 21 *BRAF*-wild-type LCH biopsy tissue samples, but not in *BRAF*-mutant specimens (5). The activation of ERK was detected in >90% of patients with LCH. In patients with multisystem LCH, mutation of the *BRAF* V 600E gene may occur in myeloid cells; in patients with single-system LCH, mutations may occur in cells originating from peripheral lesions (28). The single-cell analysis showed cellular, transcriptomic, and epigenomic heterogeneity among LCH cells, which ware connected with the development of LCH lesions. LCH cells share enrichment of genes involved in interferon (IFN) signaling and antigen presentation, indicating the inflammatory nature of the disease. In addition, genes involved in cell cycle pathways and DNA repair were presented. It is suggested that interindividual differences in subsets of LCH cells may be connected with clinical presentation of the disease. Validation of the differences in LCH subset composition in bulk RNA sequencing, immunohistochemistry, immunofluorescence imaging in a large cohort of LCH patients might be helpful in patient management (29).

Mutations in genes involved in the MAPK pathway related to precursors of distinct macrophage types (i.e., bone marrow or yolk sac) result in the occurrence of an overlapping syndrome of LCH and Erdheim–Chester disease or chronic myeloid leukemia. Notably, PLCH was recently recognized as a neoplasm with a strong inflammatory component (30).

CLINICAL PRESENTATION

Respiratory Symptoms

Patients with PLCH exhibit dry cough (50–70%), reduced exercise tolerance (40–80%), exertional dyspnea (40–87%), fatigue (50–80%), weight loss (20–30%), chest pain (10–30%), night sweats (10–20%), and fever (10–15%). Approximately 10–30% of affected patients are diagnosed following incidental pneumothorax. Pneumothorax occurs during the course of disease in 30–45% of affected patients (17, 31). Changes are found incidentally on routine chest X-rays without symptoms in 5–25% of affected patients. Dyspnea at rest and characteristics of right-ventricular circulatory failure occur in the late stages of PLCH. More than 10% of patients with PLCH develop pulmonary hypertension; it is not always related to the exacerbation of pulmonary lesions but may be due to the involvement of pulmonary vessels that occurs during the course of the disease (9, 11). Pulmonary hypertension and dynamic hyperinflation are the most important factors that limit exercise capacity in PLCH patients (32). Moderate or severe pulmonary hypertension [mean pulmonary arterial pressure (mPAP) >35 mmHg] was revealed in 92% of the PLCH patients who presented for lung transplantation (33).

Between 25 and 50% of patients without pulmonary symptoms at the time of presentation show symptoms related

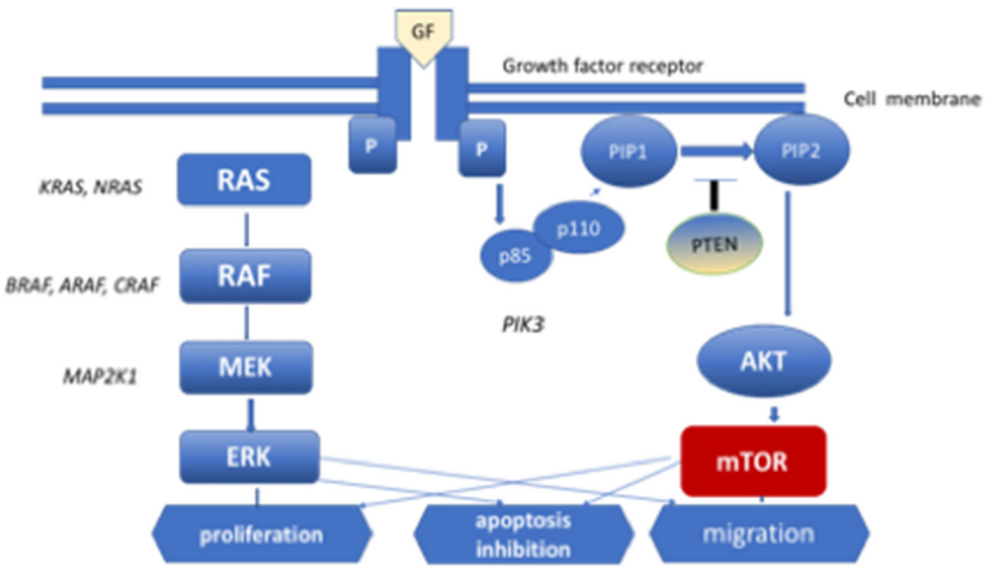

FIGURE 1 | MEK-mitogen activated protein kinase–extracellular signal-regulated kinase (ERK) signaling cascade of the mitogen-activated protein kinase (MAPK) pathway and phosphoinositide 3-kinase (PI3K)–AKT-serine/threonine protein kinase Akt pathway. Diagram of RAS protein activation.

to other organ involvement, such as polyuria–polydipsia (20–30%), bone pain (20–50%), and oral mucosa or skin lesions (∼10%). Initial evaluation of patients with LCH requires whole-body assessment. Symptoms typically arise 6–20 months prior to recognition of the disorder; in some affected patients, the diagnosis is established after many years of observation. Patients with spontaneous pneumothorax as an initial symptom of the disease are reportedly younger, more frequently men, and exhibit greater respiratory impairment compared with those who do not have pneumothorax (17).

Radiological Findings

Chest Radiography

Standard chest radiography has limited value, as multiple lesions may be small. In patients with advanced disease, nodular, reticular, and cystic lesions are visible in the middle and upper lung fields (**Figure 2**). Lung volumes are typically preserved but may be diminished in patients with a history of pneumothorax and pleurodesis. Enlargement of the hilar and mediastinal lymph nodes is present in approximately 10% of affected patients (11).

Computed Tomography

High-resolution computed tomography (HRCT) plays a major role in the diagnosis of PLCH (7, 34). The typical findings are centrilobular nodules (frequently with a "tree-in-bud" appearance), nodules with or without a lacuna, and initially thick-walled cysts of various shapes (these may be isolated or confluent with a "cloverleaf" appearance) (**Figures 3–5**). As the disease progresses, the cysts become larger and thin-walled. Lesions have a characteristic distribution, with predominance in the upper and middle parts of the lungs; the costophrenic angles are spared (>90%). In children, both lungs are symmetrically affected and lesions may be present in the lower lobes (14). In some patients, different degrees of pneumothorax are present. In patients with PLCH, other radiological symptoms of smoking-related diseases are often visible (e.g., emphysematous bullae or ground-glass opacities). Enlarged lymph nodes may be present in approximately 10% of affected patients. Signs of pulmonary hypertension, megalocardia, and enlarged pulmonary trunk are observed in patients with advanced disease (35).

Positron Emission Tomography

Positron emission tomography with ^{18}F-fluorodeoxyglucose has limited value in the assessment of patients with PLCH. Only 20–25% of patients show higher ^{18}F-fluorodeoxyglucose uptake in the lungs, particularly in thick-walled cysts and nodular lesions. Positron emission tomography is more sensitive than CT in terms

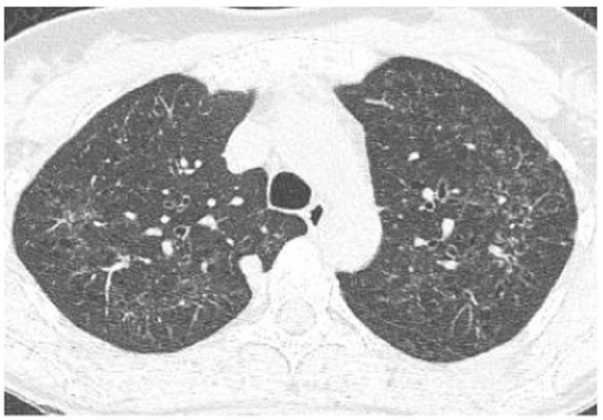

FIGURE 3 | High-resolution computed tomography (HRCT) scan of a pulmonary Langerhans cell histiocytosis (PLCH) patient. Multiple intralobular nodules, nodules with cavitations, and small nodular lesions in both lungs.

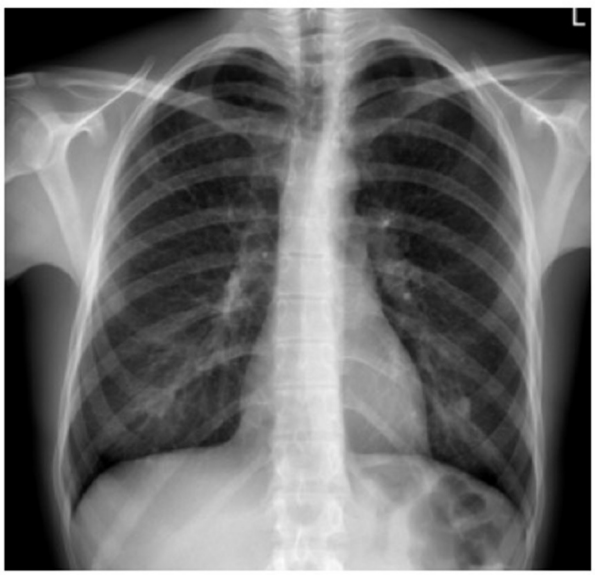

FIGURE 2 | X-ray film of a pulmonary Langerhans cell histiocytosis (PLCH) patient. Multiple reticular and nodular changes in the upper and middle parts of both lungs, with an increase in lung volume.

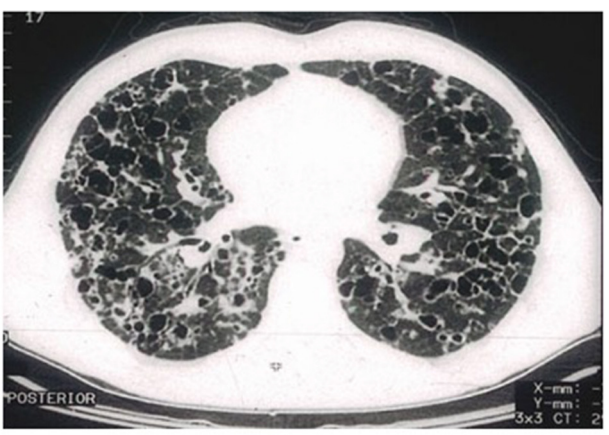

FIGURE 4 | High-resolution computed tomography (HRCT) scan of a pulmonary Langerhans cell histiocytosis (PLCH) patient. Multiple cystic lesions, frequently confluent with bizarre shapes.

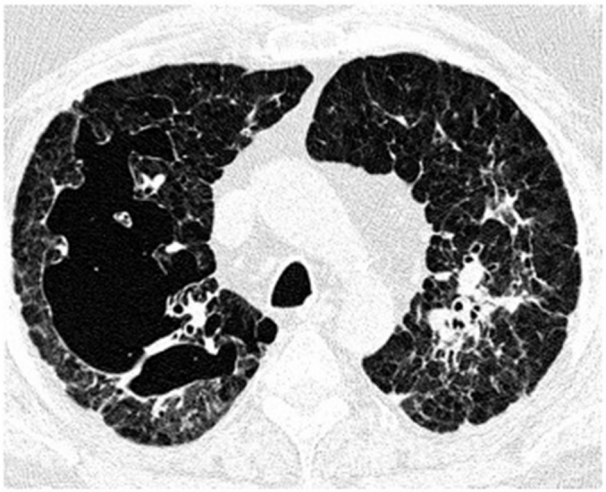

FIGURE 5 | High-resolution computed tomography (HRCT) scan of a pulmonary Langerhans cell histiocytosis (PLCH) patient. Large confluent cystic lesions in both lungs.

of identifying bone lesions, particularly those that are subclinical, as well as those that involve lymph nodes, liver, spleen, or thyroid. Positron emission tomography is reportedly sensitive for the assessment of early response to treatment and disease relapse (36, 37).

Laboratory Tests

The results of laboratory tests are usually unremarkable. However, patients with PLCH often show elevated levels of serum inflammatory markers. Serum hyperosmolarity with lower urine specific gravity and hypoosmolarity are observed in patients with diabetes insipidus. Elevated serum levels of hepatic enzymes and bilirubin are characteristic of liver involvement (7). Fluid phase biopsy is valuable for the assessment of *BRAF* mutations in patients who are potential candidates for targeted therapy.

Pulmonary Function Testing

Patients with PLCH exhibit various patterns of ventilation disorders. Initially, approximately 20% of affected patients have normal values on pulmonary function tests. Obstruction with hyperinflation is the main abnormality and can often be reversed to some degree. Restricted breathing is uncommon and mainly affects individuals with recurrent pneumothorax and pleurodesis. The most common abnormality is reduced diffusing capacity of the lungs for carbon monoxide, which is observed in approximately 70–90% of affected patients. The 6-min walk test is a useful examination; desaturation during exercise is a sensitive marker of lung impairment in patients with less advanced disease, and a reduced walking distance is observed in patients with advanced disease (17, 38, 39).

Bronchoscopy and Bronchoalveolar Lavage

Bronchoscopy is typically performed to exclude other disorders, especially infections. The diagnostic value of transbronchial biopsy is limited and estimated at 10–50% due to focal distribution of lesions, despite the bronchiolocentric nature of the disease (40, 41). Transbronchial biopsy is most useful in patients with nodular lesions but in patients with cystic lesions is associated with a high risk of pneumothorax. A new technique for lung tissue sampling, cryobiopsy, yields a diagnosis in ∼70% of patients with interstitial lung disease. The value of this technic in PLCH is under evaluation because in only a few cases has diagnosis of PLCH been established by cryobiopsy (42, 43). The presence of >5% cells with CD1a expression in bronchoalveolar lavage (BAL) fluid with a typical radiological pattern on chest HRCT is specific for PLCH but is detected in only 0–25% of affected patients (44). Auerswald et al. (45) noticed that all their six patients with histologically proven histiocytosis displayed more than 5% CD1-positive cells, but Torre and Harari (46) were able to establish PLCH in four (25%) out of 16 patients on the basis of BAL assessment. Similarly, only three (38%) out of eight patients with PLCH presented by Baqir et al. (41) and 10 (25%) out of 40 patients presented by Elia et al. (11) in whom BAL was performed displayed the presence of CD1a-positive cells over 5%. In our group of 38 patients with PLCH, only in eight (21%) did BAL assessment have a diagnostic value (47).

Lung Biopsy

Open lung biopsy is the gold standard for a definitive diagnosis of PLCH. In patients with LCH confirmed by histological examination of specimens obtained from other foci (e.g., skin, mucosa, or bones), it is not necessary to confirm pulmonary lesions (7).

Histological Examination

PLCH has a focal, bronchiolocentric localization. Initially, inflammation with eosinophilic granuloma formation occurs near small bronchi and bronchioles, destroying their walls; it exhibits varying degrees of extension into the adjacent lung interstitium. Frequently, macrophages with dark inclusions are observed; these macrophages are characteristic of exposure to inhaled fumes, mainly tobacco smoke. Nodules contain a mixture of inflammatory cells, such as T cells, macrophages, monocytes, and LCs; these LCs are relatively large, with a pale, slightly eosinophilic cytoplasm and a convoluted nucleus that exhibits a longitudinal crease resembling a coffee bean. Electron microscopic examination shows pentalammelar structures (consisting of lectin) in the cytoplasm associated with the cell membrane; these so-called Birbeck granules are characteristic of LCs. These structures can also be identified by immunohistochemistry using antibodies against langerin (i.e., CD207). LCs show CD1a expression, which is necessary for diagnosis (24). The expression of S-100 protein is not specific but may be present. Inflammatory eosinophilic granulomas may be observed, depending on the stage of disease progression. The central part of the nodule has a lacuna, which is presumably a component of the remaining bronchiole lumen or the

effect of cytokine and metalloproteinase-mediated destruction. Subsequently, the inflammation regresses; however, fibrosis develops in the form of stellar scars or confluent cystic cavities in the fibrous ring. At this stage, LCs are absent. In LCH lesions, LCs constitute 1–80% of cells (mean ∼8%) (3). Lung specimens from patients with PLCH may also show signs of other smoking-related diseases, such as bronchiolitis, desquamative interstitial pneumonia, respiratory bronchiolitis with accompanying interstitial lung disease, or emphysema. In patients with advanced disease, the internal walls of both arteries and veins are thickened, resulting in pulmonary hypertension (48).

Patients who qualify for targeted therapy must be tested for mutations in the *BRAF*, *ARAS*, *NRAS*, *KRAS*, and *MAP2K* genes (5). Moreover, as a novel diagnostic technique for monitoring patients undergoing treatment with BRAF inhibitors, analysis of BRAF V600E gene mutations in cell-free DNA in serum has been implemented (49, 50).

DIAGNOSIS

Definite diagnosis of PLCH requires adequate clinical presentation and the identification of LCs in biopsy specimens that exhibit either CD207 (langerin) or CD1a.

Probable diagnosis of PLCH relies on clinical presentation confirmed radiologically (characteristic cysts and nodules mainly observed in upper and middle lung fields on chest CT). Histological verification of all extrapulmonary lesions is recommended (7).

Differential Diagnosis

Disorders that should be taken into account while diagnosing the patients with cystic pulmonary lesions are presented in **Table 1**.

In patients with nodular lesions, diseases such as neoplasms, sarcoidosis, hypersensitivity pneumonitis, and infections are in the scope of differential diagnosis (7).

TABLE 1 | Diseases with a cystic pattern of lung lesions.

- Pulmonary Langerhans cell histiocytosis
- Lymphangioleiomyomatosis (LAM)
  - Sporadic LAM
  - Tuberous sclerosis complex LAM
- Birt–Hogg–Dubé syndrome
- Erdheim–Chester disease
- Lymphatic disorders
  - Lymphocytic interstitial pneumonia
  - Light-chain deposition disease
  - Amyloidosis
  - Hyper-IgE syndrome
- Genetic disorders
  - Ehlers–Danlos syndrome
  - Neurofibromatosis
  - Marfan syndrome
  - Proteus syndrome
- Congenital pulmonary airway malformations
- Infections
  - Respiratory papillomatosis
  - Staphylococcal pneumonia
  - *Pneumocystis jirovecii*
  - Endemic fungal diseases (coccidiomycosis, paragonimiasis)
- Emphysema
- Alpha-1 antitrypsin deficiency
- Post-traumatic pseudocysts
- Pulmonary neoplasms
  - Adenocarcinoma
  - Lymphoma
  - Mesenchymal hamartoma
  - Pleuropulmonary blastoma
- Sarcomas
  - Angio and osteosarcomas
  - Synovial cell sarcoma
  - Leiomyosarcoma
  - Rhabdomyosarcoma
  - Endometrial stromal sarcoma
  - Wilms tumor
  - Ewing sarcoma
- Metastatic tumors
  - Adenocarcinoma of genitourinary and gastrointestinal tract
  - Breast Cancer
  - Cancers of the head and neck

TREATMENT

Treatment of LCH depends on the disease activity and affected organs, including lesions in critical organs and high-risk bones, as well as the degree of damage (**Figure 6**).

Smoking Cessation

Because of the crucial role of tobacco smoke in the development of PLCH, smoking cessation is the most important recommendation for affected patients. In ∼50% of patients with isolated PLCH, smoking cessation leads to partial regression and subsequent stabilization of the disorder without immunosuppressive therapy (16, 51). However, patients require systematic follow-up examinations because reactivation of the disease can occur in the lungs or other organs. Thus far, no biological markers have been found to identify patients in whom smoking cessation would be sufficient and patients in whom the disease is likely to progress despite smoking cessation. Cessation of both cigarette smoking and marijuana smoking is becoming a challenge for both patients and doctors [(52), personal observation].

Glucocorticosteroids

Systemic corticosteroid therapy has been advised for many years; it has been reportedly associated with symptomatic, radiological, and functional improvements. However, there have been insufficient studies to determine the appropriate corticosteroid dose and duration of treatment, as well as comparative studies with respect to smoking cessation. It is

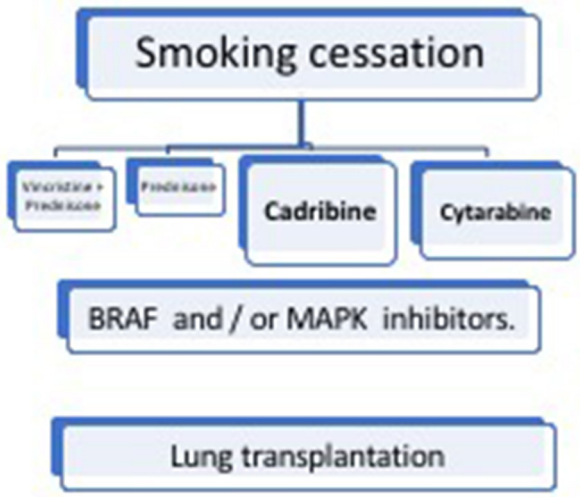

FIGURE 6 | Management of pulmonary Langerhans cell histiocytosis (PLCH).

possible that some effects of therapy are due to smoking cessation, rather than steroid treatment (10). In addition, relapse after treatment is common (∼80%); long-term corticosteroid therapy is associated with many (sometimes severe) adverse effects. Therefore, this treatment is no longer recommended. However, inhaled corticosteroids may be useful for treatment of patients with reversible obstruction (7).

Chemotherapy

Various chemotherapy regimens have been proposed for use in children with LCH, but they have failed to show satisfactory results in adults. The disease has a diverse clinical course in adults, particularly in patients with isolated PLCH, and many drugs are differently tolerated (53–55). Vinblastine, methotrexate, mercaptopurine, and etoposide have shown benefits in some patients with multisystem LCH, but their effects in patients with isolated PLCH are controversial (56). A retrospective study involving a population of 35 adults with LCH (17 patients with pulmonary involvement) treated with vinblastine and steroids showed a response rate of 70%; however, the relapse rate was 40% during the 5-year follow-up period (57). No improvements in ventilation parameters due to vinblastine treatment were observed in any patient. Cantu et al. (58) reported that vinblastine treatment was less effective than cladribine or cytarabine in adult patients with multifocal bone disease (28% had pulmonary lesions). However, the effects of these treatments on lung function parameters were not discussed. Recently, Néel et al. (59) reported an overall response rate of 91% in 22 patients treated with cladribine; however, disease progression occurred in 30% of these patients during the 5-year follow-up period.

Saven and Burian (60) reported that 2-chloro-2′-deoxyadenosine (2-CDA) had beneficial effects in 12 patients (six with lung involvement), affording an overall response rate of 75%. Grobost et al. (61) reported that cladribine (three or four courses) improved pulmonary function parameters in four of five patients with PLCH; however, one of these patients exhibited relapse. Pardanani et al. (62) and Adam et al. (63) in five and seven patients with PLCH who received 2-CDA, respectively reported over than 60% response rate.

Cladribine treatment in adult PLCH patients is a subject of an ongoing phase II clinical trial (NCT01473797, www.ClinicalTrials.gov). The most frequently observed severe adverse event during the course of cladribine treatment is cytopenia, whereas the most severe event associated with vinblastine is neuropathy. For patients with disease progression after cladribine treatment, salvage therapy is cytarabine administered at a dose of 100 mg/m^2 on five consecutive days at 4-week intervals. Another phase II trial (NCT04121819, www.ClinicalTrials.gov) to investigate the efficacy and safety of cytarabine in adult patients with LCH is currently underway. Notably, the preferred chemotherapeutic regimen in patients with brain lesions is cytarabine and cladribine because both drugs cross the blood–brain barrier.

Clofarabine is another drug that is effective as salvage therapy in children and is the subject of another ongoing phase II clinical trial (NCT02425904). In addition, severe lung cystic lesions and chemotherapy, particularly with concomitant corticosteroid treatment, are predisposing factors to opportunistic infections, particularly *Pneumocystis jiroveci* pneumonia. Grobost et al. (61) recommended that sulfamethoxazole/trimethoprim and valaciclovir prophylaxis should be administered during cessation of 2-CDA treatment, as well as for 6 months afterward.

Targeted Therapies

Vemurafenib and Other BRAF Inhibitors

The presence of mutations in genes involved in the MAPK pathways in patients with PLCH implies that targeted therapy may be useful. Vemurafenib, a BRAF kinase inhibitor, is reportedly an effective treatment in patients with LCH (64, 65, 70). However, this drug did not eliminate all LCs; discontinuation of treatment resulted in disease progression. Diamond et al. (67) reported the beneficial effects of vemurafenib in a group of 22 patients with Erdheim–Chester disease and four patients with LCH; these patients exhibited a 2-year progression-free survival rate of 86% and an overall survival rate of 96%. Notably, the patients with LCH showed a particularly good response. Bhatia et al. (68) reported a partial response in patients with refractory Erdheim–Chester disease overlapping with LCH during treatment with another BRAF inhibitor, dabrafenib. Recently, Hazim et al. (66) presented results of treatment with vemurafenib and dabrafenib in six adult patients with LCH. Complete and partial responses were noticed in 30 and 50% of patients, respectively (**Table 2**).

In a study of children with LCH performed in France, the presence of *BRAF* mutations was associated with a weaker response to first-line treatment (vinblastine and steroids) and second-line treatment, as well as a higher proportion of recurrence (65, 71). However, no correlations were found between *BRAF* mutations, clinical presentation, and prognosis in adults (5).

TABLE 2 | Targeted therapies in LCH adult patients.

Study	Trial	Patients	BRAF inhibitor	Time of response assessment	Response
Hyman (70)	Phase 2	13 ECD 1 LCH	Vemurafenib	2 months	ORR-43%
Diamond et al. (67)	Phase 2	22 ECD 4 LCH	Vemurafenib	2–40 months	ORR-61%
Bhatia (68)	Retrospective	7 ECD 4 ECD/LCH	Dabrafenib	2–40 months	ORR-100%
Diamond et al. (69)	Prospective	12 ECD 2 LCH	Cobimetinib	12 months	ORR-89%
Hazim (66)	Retrospective	6 LCH	3 Vemurafenib 3 Dabrafenib	4–27 months	CR-33% PR-50%

CR, complete response; ECD, Erdheim–Chester disease; LCH, Langerhans cell histiocytosis; ORR, overall response rate; PR, partial response.

Mitogen-Activated Protein Kinase Inhibitors

Drugs that inhibit mitogen-activated kinase 1 (MEK1) and 2, both downstream of BRAF, were shown to be beneficial in patients with LCH who exhibited *MAP2K1* deletion. Lorillion et al. (72) reported a good response to trametinib. Cobimetinib, a MEK1 and 2 inhibitor, was the subject of a clinical trial in patients with various histiocytic disorders. All patients showed a response to this treatment; the relapse rate was only 10% at 1 year (69). Studies regarding melanoma cells harboring mutations in genes involved in the MAPK pathway showed that dual inhibition of BRAF and MEK was more effective and less toxic than monotherapy with a BRAF inhibitor. Awada et al. (73) reported beneficial results of this treatment in adult patients with multisystem LCH. Multiple clinical trials involving BRAF and other RAS pathway inhibitors in adults and children with LCH are currently underway (www.ClinicalTrials.gov).

Tyrosine Kinase Inhibitors

Contradictory results have been reported regarding the effects of imatinib treatment. Montella et al. (74) and Janku et al. (75) reported beneficial effects of this drug in a 37-year-old woman with multisystem LCH and in two adult patients with multisystem LCH, respectively. However, the follow-up periods in these patients were both shorter than 2 years. In contrast, Wagner et al. (76) reported treatment failure in two patients treated with imatinib.

Pneumothorax

Pneumothorax, as the first symptom of PLCH, is observed in 10–30% of affected patients. In addition, these patients have a greater probability of pneumothorax recurrence (∼60%). Generally, 30–40% of patients develop this condition during long-term observation. Moreover, this group often includes young men who smoke fewer cigarettes and whose lung function is more affected by the disease. These data support the recommendation that HRCT should be performed in all patients with spontaneous pneumothorax to identify patients with possible PLCH (17, 31, 77).

Pleurodesis should be recommended after the first episode of pneumothorax. However, it was recently reported that the time of the first ipsilateral recurrence and the overall number of pneumothorax recurrences were similar after conservative and thoracic surgical treatments. Pneumothorax recurrences may be linked with active disease (18). Notably, pleurodesis does not constitute a contraindication for lung transplantation (7).

Treatment of Pulmonary Hypertension

The progression of PLCH leads to the destruction of lung parenchyma with elements of fibrosis, as well as the development of pulmonary hypertension. In a small proportion of patients, those with stable ventilation parameters may exhibit pulmonary hypertension.

Oxygen treatment is the main recommendation for this group of patients. Furthermore, drugs that lower pulmonary artery blood pressure (e.g., inhibitors of phosphodiesterase and endothelin receptor, as well as prostacyclin) were shown to have beneficial effects in patients with PLCH who exhibited pulmonary hypertension (78). Also other small series of patients and case reports presented beneficial effects of pulmonary antihypertensive therapies; however, it is not proven as standard treatment and should be delivered in very experienced centers (79, 80).

Lung Transplantation

Lung transplantation is a salvage therapeutic option for patients with features of respiratory failure and those developing pulmonary hypertension. The prognosis of patients undergoing transplantation does not significantly differ from that of patients with other interstitial lung diseases. The 1-year survival rate is 75%; notably, >50% of patients survive for 5 years. Patients with multisystem LCH and lung involvement have a worse prognosis after transplantation, and recurrence of LCH in the transplanted organ has been found in approximately 20% of affected patients (33). Based on 87 transplant patients, Wajda et al. (81) reported outcomes similar to those of prior studies; 1-year, 5-year, and 10-year survival rates were 85, 49, and 22%, respectively. Moreover, the median survival was significantly better for women than for men (mean survival time, 9.3 vs. 3.9 years).

Follow-Up Examination

Patients with PLCH should undergo systematic follow-up examinations, initially performed after 3–4 months with subsequent evaluations at intervals of 3–12 months, depending on disease activity (7). HRCT is not necessary at every follow-up examination because it is not highly sensitive for evaluation of disease activity (34). Similarly, assessments of organs other than the lungs are not required; however, these evaluations should be performed in the event of new symptoms. Pulmonary function tests are important in follow-up assessment of patients with PLCH. Deterioration of exercise tolerance or the presence of new symptoms requires close examination. Participation in tobacco and marijuana cessation programs is highly recommended.

PROGNOSIS

The natural course and prognosis of PLCH are unpredictable and variable. Spontaneous regression and/or stabilization of

the disease after smoking cessation, as well as the presence of addiction to substances other than tobacco in some patients, results in loss to follow-up for many patients; this may influence survival assessment. However, patients with PLCH were reported to exhibit lower mean survival compared to sex- and age-matched members of the general population (7). Vassallo et al. (9) observed a median survival of 12.5 years in adult patients with biopsy-proven PLCH.

Reduced spirometry parameters, low diffusing capacity of the lungs for carbon monoxide, severe cystic lesions detected on HRCT, low oxygen arterial pressure, older age, pulmonary hypertension, and multisystem LCH are negative prognostic factors. Moreover, a higher St. George's Respiratory Questionnaire (SGRQ) score and continued tobacco smoking have negative impacts on survival. Tazi et al. (16) presented an early decline of pulmonary function parameters (over 15%) in 40% of newly diagnosed PLCH patients, but in only 10% of them, progression of pulmonary lesions was noticed.

Recently, severe lung involvement in children was recognized as a factor that substantially influences the course of the disease and survival. Early administration of new targeted LCH therapies and viral infection prophylaxis are suggested (14).

Patients with PLCH also have a higher risk of infections; therefore, influenza and pneumococcal vaccines are recommended.

In addition, the presence of $BRAF^{V600E}$ mutations and MS RO+ LCH in children was recognized as a factor that negatively influences prognosis (71), but in adults with PLCH, the $BRAF^{V600E}$ mutation was not associated with LCH presentation and outcome (5).

LCH promotes the development of other neoplasms originating from the lymphatic and hematopoietic systems, including LCH overlapping with chronic myelogenous leukemia. In a group of 132 adult patients with LCH, Ma et al. (82) found that 32% were diagnosed with additional neoplasms, mostly before [58%] and concurrently (21%) with LCH diagnosis. Lung (18%), breast (18%), and colon (12%) cancers were the most frequently detected additional neoplasms.

Pregnancy and Labor

Pregnancy does not worsen the course of PLCH, but delivery by cesarean section is recommended due to the enhanced likelihood of pneumothorax (83).

Air Travel

Patients with PLCH have a higher risk of pneumothorax during air travel. However, the risk is not high; approximately 1% of patients exhibit this condition, and no severe instances of pneumothorax have been reported thus far (84).

CONCLUSIONS

1. LCH is a rare disorder of unknown etiology caused by clonal proliferation of geno- and phenotypically altered LCs.
2. It usually involves the bones, lungs, skin and pituitary, lymphopoietic organs (lymph nodes, liver, spleen, bone marrow), gastrointestinal tract, thyroid, CNS, and others.
3. PLCH may be as follows: isolated, anticipating even for many years the occurrence of systemic changes, or from the very beginning, the lungs may be one of the sites involved.
4. Tobacco smoke is a crucial causative factor for PLCH. Smoking cessation is the most vital recommendation.
5. Chest HRCT has an outstanding importance in PLCH diagnosis. Pulmonary lesions are the following: centrilobular nodules, nodules with or without a central cavity, initially thick-walled cysts of various shapes that may convolute, forming the so-called clover leaves. The lesions are usually (>90%) sparing the costophrenic angles.
6. The definite diagnosis of PLCH is based on adequate clinical and radiological presentation and the identification of LCs in the examined tissue samples showing in the immunohistochemistry the presence of one of the following antigens: CD207 (langerin), CD1a.
7. Treatment of LCH depends on extension of the disease. Currently, the preferred cytostatic treatment option includes cladribine or cytarabine. Lung transplantation is recommended in patients with isolated PLCH with respiratory insufficiency.
8. PLCH patients have a lower mean survival than individuals of the same sex and age. Negative prognostic factors are age, tobacco smoking, severe obstruction, lowered PaO_2, a higher score in SGRQ, pulmonary hypertension, and multiple organ involvement, particularly risk organs.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Exploring the Role of Matrix Metalloproteinases as Biomarkers in Sporadic Lymphangioleiomyomatosis and Tuberous Sclerosis Complex. A Pilot Study

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Background: Lymphangioleiomyomatosis can develop in a sporadic form (S-LAM) or in women with tuberous sclerosis complex (TSC). The matrix metalloproteinases (MMPs) are extracellular matrix-degrading enzymes potentially involved in cystic lung destruction, and in the process of migration of LAM cells. The aim of the study was to explore the role of MMP-2 and MMP-7, such as vascular endothelial growth factor (VEGF) -C and -D in women with LAM, including patients with minor pulmonary disease (i.e., <10 lung cysts), and TSC with or without LAM.

Methods: We evaluated 50 patients: 13 individuals affected by S-LAM, 20 with TSC-LAM, of whom six with minor pulmonary disease, and 17 with TSC without pulmonary involvement. Sixteen healthy women were used as controls.

Results: MMP-2 resulted higher in LAM compared to healthy volunteers, and TSC patients ($p = 0.040$). MMP-7 was higher in TSC-LAM patient, with even greater values in patients with TSC-LAM minor pulmonary disease, than in S-LAM patients, and in controls ($p = 0.001$). VEGF-D level was lower than 800 pg/mL in all healthy controls and resulted higher in S-LAM and TSC-LAM than in TSC patients and controls ($p < 0.001$). VEGF-C values were not statistically different in the study population ($p = 0.354$). The area under ROC curves (AUCs) of MMP-2, and MMP-7 for predicting LAM diagnosis were of 0.756 ± 0.079 ($p = 0.004$), and 0.828 ± 0.060 ($p < 0.001$), respectively. Considering only

patients with TSC, the AUCs for MMP-2, and MMP-7 in predicting LAM were 0.694 ± 0.088 ($p = 0.044$), and 0.713 ± 0.090 ($p = 0.027$), respectively.

Conclusions: Our data suggest that MMP-2 and MMP-7 could be promising biomarkers for LAM diagnosis.

Keywords: lymphangiomeiomyomatosis, tuberous sclerosis complex, biomarkers, matrix metalloproteinases, vascular endothelial growth factor

INTRODUCTION

Lymphangioleiomyiomatosis (LAM) is a rare progressive cystic lung disease affecting mostly women in the childbearing age (1). LAM, seen either as a sporadic form (S-LAM) or as a manifestation of tuberous sclerosis complex (TSC-LAM), is clinically characterized by progressive dyspnea, recurrent pneumothorax, and chylous pleural effusions (2). The extent of pulmonary cystic involvement in both forms of LAM varies from minor disease, with the identification of few cysts on chest CT scan, to diffuse destruction of the lungs (3). In most cases LAM progresses into respiratory failure (1, 2), but in some patients the disease can remain asymptomatic or even undiagnosed (4).

The cystic modification of the lung parenchyma is thought to be caused by the proliferation of abnormal smooth muscle-like cell called "LAM cells" in the pulmonary interstitial and along the axial lymphatics in the thorax and abdomen. LAM cells exhibit a unique immunophenotype as they express desmin and actin and gp100 and react with HMB-45 antibody (5). The mechanism of cysts development by LAM cells is not completely understood; however different mechanisms seem to contribute to lung destruction (6). LAM cells can express vascular endothelial growth factor (VEGF), a lymphangiogenic protein. Two isoforms of VEGF have been isolated in LAM patients: VEGF-C, which is expressed in lung tissues, and VEGF-D, which was isolated in the serum of patients with LAM (7, 8). VEGF is able to recruit lymphatic endothelial cells, driving the formation of lymphatic vascular channels. This abnormal lymphangiogenesis leads to vascular and airways obstruction and consequent development of air cystic lesions in the pulmonary parenchyma (6). Serum levels of VEGF-D are high in most patients with LAM and—in association with the characteristic cystic images seen on CT scan—could be diagnostic for LAM, thus avoiding invasive testing such as lung biopsy (9, 10). In patients with LAM, VEGF-D serum level correlates with disease severity, namely chylous effusions and/or lymphatic involvement (11, 12), lung function at presentation and rate of disease progression (13) systemic involvement in patients with TSC (14), and pulmonary functional impairment (15, 16). Patients with high VEGF-D serum level are more likely to respond to treatment with sirolimus than patients with low VEGF-D serum level (17).

Immunochemical studies demonstrated an over expression of matrix metalloproteinases (MMPs) and MMPs inducers in lung tissues of patients with LAM, and a tissue paucity of MMPs tissue inhibitor (TIMP)-1 (18). MMPs are zinc-dependent endopeptidases that are active in tissue remodeling by degrading extracellular matrix collagen and elastine (19). Degradation of elastic fibers in areas of smooth muscle proliferation was found in lung biopsy specimens of patients with LAM (20). Hence, MMPs are suggested to be involved in lung tissue destruction and cysts formation in LAM. Odajima et al. demonstrated that serum levels of MMP-9 isoform were higher in LAM patients compared to healthy controls (21), suggesting a possible involvement of MMP-9 in LAM development. However, MMP-9 levels cannot be correlated with LAM severity, evaluated as cystic parenchymal involvement quantified by lung high resolution computed tomography (HRCT) (22). MMP-2 is the most expressed MMP isoform in bronchiolar and vascular smooth muscle cells, in basement membranes of LAM tissue, and in overlying epithelial cells (18). However, some studies suggest that serum MMP-2 levels cannot be correlated with the extent of pulmonary cystic involvement. MMP-7 is one of the most important MMPs in elastin turnover (23). Barnes et al. demonstrated that the invasion of tuberin-null cells might be mediated by MMP-7 (matrilysin), a component of cell invasion, in a TSC model and in LAM tissues (24). However, data about serum level of MMP-7 in patients with LAM are lacking.

Thus, the aim of the present study was to explore the role of MMP-2 and MMP-7 as biomarkers in a cohort of patients with LAM, both S-LAM and TSC-LAM, and in a subgroup of TSC patients with minor cystic parenchymal involvement. An association of such biomarkers, including VEGF-D and -C, with systemic involvement was also explored.

METHODS

Study Design and Population

We performed a cohort study involving adult female outpatients affected by S-LAM and/or TSC followed (1) at the pulmonary clinic (S-LAM, TSC-LAM) and (2) at the Tuberous Sclerosis Center (TSC) of San Paolo Hospital, Milan, Italy, from January 2014 to December 2017.

The diagnoses of both LAM and TSC were based on previously published guidelines (25–27). A multidisciplinary evaluation was performed according to the international guidelines as previously reported (14) in the following patients: (1) individuals with a diagnosis of TSC; (2) patients with diagnosis of LAM who were evaluated in the Pulmonology clinic to rule out a form of TSC-related LAM. Imaging was performed when needed (i.e., abdominal magnetic resonance imaging (MRI) or ultrasound for angiomyolipoma (AML) diagnosis and follow up, MRI of the brain for neurologic involvement). A clinical and radiological

follow-up plan was established on a case-by-case basis and according to the international guidelines (27). Thoracic imaging with lung HRCT was requested during the first pulmonary evaluation and repeated in case of respiratory symptoms. A peripheral blood sample for the evaluation of MMP-2, MMP-7, VEGF-C and VEGF-D was obtained during one of the scheduled pulmonary evaluations in clinic. Clinical and radiological data were collected within 3 months after the serum biomarkers results. All patients were in an observational cohort with Hospital Ethics Committee approval. All patients or relatives, in case of patients with intellectual disability, provided informed consent.

Quantification of Serum VEGF-D, VEGF-C, MMP-2, and MMP-7

Peripheral blood was collected in serum separator tubes, allowed to clot for 30 min at 4°C, centrifuged at 1000 × g for 15 min. Serum was aliquoted and stored at −80°C. Serum VEGF-D, VEGF-C, MMP-2, and MMP-7 were measured using Quantikine Human Immunoassays (R&D Systems; Minneapolis, MN) according to the manufacturers' instruction.

Pulmonary Involvement Assessment

Spirometry, body pletismography and lung diffusion tests (Platinum Elite™ MGC Diagnostic, USA) were performed according to the ATS/ERS guidelines (28, 29). Dyspnea was investigated throughout the Italian version of the modified Medical Resource Council (MRC) scale (30). The six-minute walk test (6MWT) was performed along a flat, straight, 30-m walking course supervised by a well-trained researcher according to the ATS guidelines (31).

Genetic Analyses

Qiamp DNA blood mini DNA kit (Qiagen, Germany) was employed to extract DNA from peripheral lymphocytes (Qiagen, Germany). *TSC1* and *TSC2* exons from genomic DNAs were amplified by means of standard polymerase chain reaction (PCR) using previously described primers (32). Pathogenic variants were detected by denaturing high-performance liquid chromatography (DHPLC) (Transgenomic, Crewe, UK). The products showing variant DHPLC melt profiles were directly sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems), and the results were analyzed using sequence analysis 3.4.1 software (ABI 3130, Applied Biosystem). The sequencing reactions for identified mutations were repeated. Patients that had negative investigations for DHPLC were evaluated with Multiple Ligation-dependent Probe Amplification test for *TSC1* (P124-MRC-Holland) and *TSC2* (P046-MRC-Holland) as previously described (33). Patients in whom genetic analysis was inconclusive were classified as having no mutation identified (NMI) after conventional genetic testing, as Next Generation Sequencing for TSC was not yet available in the laboratory at the time of the study.

Image Analysis

All chest CT examinations (performed using a LightSpeed VCT—GEHealthcare, Milwaukee, WI-−64 slices scanner) were evaluated by a radiologist experienced in LAM, blinded to other researchers. The severity of cystic lung disease was graded according to a visual quantitative grading system (34). The lung involvement was classified as minimal pulmonary disease (Grade 0) if patients showed <10 lung cysts (25). If more than 10 cysts were identified then the extent of disease was graded as "mild" (Grade 1) if less than one third of the lung was involved; "moderate" (Grade 2) if one to two thirds of the lungs resulted involved; and "severe" (Grade 3) if cysts involved more than two third of the lungs. The presence of parenchymal nodules compatible with multifocal micronodular pneumocyte hyperplasia (MMPH) was also evaluated. Lymphatic involvement was considered in the presence of mediastinal lymph node enlargement, and/or pleural effusion attributed to chylothorax and/or lymphangioleiomyomas. Pneumothorax, when detected, was reported, too.

Statistical Analysis

The results are shown as median and interquartile range (IQR), unless otherwise stated. Lilliefors corrected K-S test was performed before the data analysis in order to examine the distribution of the residuals of the parametric tests. For comparisons between patients, the Wilcoxon rank-sum test, Mann-Whitney test, and Kruskal-Wallis test were used, as appropriate. To detect the optimal cut off point at which the sensitivity and specificity of every biomarker were maximized, we developed a receiver operating characteristic (ROC) curve. Since there were no previous works that have analyzed the link between serum MMP-2 and MMP-7 and systemic involvement in LAM or TSC, we divided the patients (both with S-LAM and TSC) into two groups based on the 50th percentile of the distribution of the biomarker. All tests were two-sided, and $p < 0.05$ were considered statistically significant. Statistical tests were performed using the Statistical Package for Social Sciences (version 21.0; SPSS, Chicago, IL) and GraphPad Prism 7 (GraphPad Software, San Diego, California, USA).

RESULTS

Data from 50 patients were available for the analysis: 13 patients (20%) were affected by S-LAM, and 37 (56%) by TSC. Of these, 14 (38%) had TSC-LAM, six TSC-LAM minor pulmonary disease, and 17 TSC without LAM. Sixteen healthy women were used as controls. S-LAM was histologically confirmed in nine patients (by pulmonary biopsy in seven patients, by biopsy of abdominal lymphangioma in one patient and by identification of LAM cells in the chylous effusion in one patient). In four patients with S-LAM the diagnosis was clinically confirmed by the simultaneous presence of characteristic chest HRCT and renal AMLs. For patients with TSC the disease was clinically confirmed on the basis characteristic or compatible chest HRCT. All patients affected with LAM were taking standard inhalation therapy (long acting B_2-agonists and long acting muscarinic agents). None was treated with Sirolimus or Everolimus at the time of biomarkers analysis. Demographic, clinical and genetic characteristics of the study population are reported in **Table 1**. There were no differences between groups in age at LAM

TABLE 1 | Demographic and clinical characteristics of the population in analysis.

	S-LAM $N = 13$	TSC-LAM $N = 14$	TSC-LAM minor pulmonary disease $N = 6$	TSC $N = 17$	HEALTHY $N = 16$	*p*
Age*, yrs, median (IQR)	36 (31–43)	36 (30–50)	34 (24–63)	32 (24–42)	36 (28–49)	0.831
Age at LAM diagnosis, yrs, median (IQR)	35 (29–44)	33 (27–45)	29 (22–61)	–	–	0.930
Smoking history (yes/no/ex), %	9/27/64	14/21/64	17/1/67	6/94/0	0/100/0	0.309
Pulmonary involvement and symptoms						
MMPH, *n* (%)**	–	8 (57)	6 (100)	10 (59)	–	0.068
Dyspnea***, *n* (%)	5 (38)	3 (21)	2 (33)	5 (29)	–	0.637
SpO2 < 90% during 6mWT, *n* (%)	4 (36)	3 (21)	0 (0)	2 (12)	–	0.235
Pneumothorax, *n* (%)	4 (36)	3 (21)	1 (17)	0 (0)	–	0.082
Respiratory failure, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	–	–
Chylotorax, *n* (%)	3 (27)	1 (7)	0 (0)	0 (0)	–	0.063
Lymphocele, *n* (%)	2 (18)	1 (7)	1 (6)	0 (0)	–	0.0592
Mediastinal lymphadenopathy, *n* (%)	1 (9)	2 (17)	1 (8)	1 (16)	–	0.896
Abdominal involvement						
Renal AMLs, *n* (%)	4 (36)	14 (100)	6 (100)	11 (65)	–	**0.001**
AMLs size (> 3 cm), *n* (%)	2 (11)	10 (53)	4 (21)	3 (16)	–	0.368
Hepatic AMLs, *n* (%)	1 (9)	6 (43)	5 (24)	1 (17)	–	0.268
Genotype**						
TSC1, *n* (%)	–	3 (21)	2 (33)	9 (56)	–	0.189
TSC2, *n* (%)	–	7 (50)	4 (67)	5 (31)	–	
NMI, *n* (%)	–	4 (29)	0 (0)	2 (13)	–	
Systemic TSC involvement**						
Renal tumor, *n* (%)	–	1 (8)	0 (0)	4 (24)	–	0.258
Cutaneous involvement, *n* (%)	–	13 (100)	6 (100)	17 (100)	–	>0.999
Epilepsy, *n* (%)	–	3 (23)	4 (67)	12 (71)	–	**0.027**
Cortical tubers, *n* (%)	–	12 (92)	6 (100)	15 (88)	–	0.665
Pulmonary function, median (IQR) or median [min–max]						
FVC (L)	2.81 (2.39–3.49)	3.64 (3.13–3.94)	2.96 [2.88–4.16]	3.53 (3.09–4.00)	–	0.264
FVC (%)	82 (75–97)	99 (90–110)	108 [67–122]	98 (86–109)	–	0.174
FEV1 (L)	2.36 (1.79–2.84)	3.02 (2.52–3.21)	2.57 [2.27–3.57]	3.14 (2.74–3.38)	–	0.063
FEV1 (%)	86 (64–97)	100 (85–110)	98 [69–99]	98 (89–113)	–	0.080
FEV1/FVC (%)	100 (84–106)	100 (92–105)	99 [99–102]	101 (100–103)	–	0.754
DLCO (mL/min/mmHg)	13.48 (10.39–16.31)	20.98 (17.66–23.05)	21.03 [13.71–23.62]	21.87 (19.57–22.90)	–	0.002
DLCO (%)	53 (41–67)	79 (65–81)	70 (60–91)	83 (74–90)	–	0.002
KCO (mL/min/mmHg/L)	3.47 (2.27–4.10)	4.11 (3.55–4.92)	5.07 [3.66–5.55]	4.56 (4.08–5.53)	–	0.015
KCO (%)	66 (44–88)	73–(67–78)	81 [80–99]	78 (76–95)	–	0.043
Radiological involvement§**						
Grade 0, *n* (%)	0 (0)	0 (0)	6 (100)	–	–	<0.001
Grade 1 *n* (%)	2 (22)	10 (77)	0 (0)	–	–	
Grade 2, *n* (%)	4 (44)	1 (8)	0 (0)	–	–	
Grade 3, *n* (%)	3 (33)	2 (15)	0 (0)	–	–	

: referred to age at time of blood sample evaluation; **: percentage are referred to total patients with TSC; *: mMRC>1; §: percentage are referred to patients with radiological analysis available. IQR, interquartile range; MMPH, multifocal micronodular pneumocyte hyperplasia; TSC1/2/NMI, pathogenic variant of TSC1 or TSC2/no mutation identified; AML: angiomyplipoma; 6mWT, six minutes walking test. p < 0.050 in bold.*

diagnosis. One patient with S-LAM, three patients with TSC-LAM, three healthy, controls and one patient with TSC were menopausal, without any difference between groups ($p = 0.429$). All patients with a TSC-LAM minor pulmonary disease showed multifocal micronodular pneumocyte hyperplasia (MMPH) at chest HRCT. Dyspnea was common in patients with LAM, and almost half of the individuals with sporadic LAM reported the symptoms, whereas no patients had respiratory failure (**Table 1**). History of pneumothorax was reported in patients with LAM, including patients with minor pulmonary disease, while patients with S-LAM tended to have more frequently chylothorax than other patients ($p = 0.063$). Individuals with TSC-LAM showed

more frequently renal AMLs than patients with S-LAM ($p = 0.001$). No differences were observed regarding the size of AMLs or ymphatic involvement, (such as lymphocele or mediastinal lymphadenopathy), hepatic AMLs, or *TSC1/TSC2* pathogenic variants between the groups. The analysis of functional data show that patients with S-LAM tended to lower FEV1 and significantly lower DLCO (mL/min/mmHg) than other subjects (**Table 1**).

Distribution of Serum Biomarkers Between Groups

As shown in **Figure 1**, serum MMP-2 was higher in the S-LAM group [median value: 298 ng/ml, range 277–416 ng/ml], and in the TSC-LAM patients (median value: 293 ng/ml, range 248–480 ng/ml) compared to healthy volunteers (median value: 225 ng/ml, range 203–336 ng/ml) and TSC patients (median value: 232 ng/ml, range 213–323 ng/ml, $p = 0.040$ of ANOVA). Serum MMP-7 was higher in TSC-LAM patients (median value: 4.78 ng/ml, range 3.5–5.3 ng/ml) with even greater values in patients with TSC-LAM minimal pulmonary disease (median value: 5.69 ng/ml, range: 4.90–7.43 ng/ml) than in S-LAM patients (median: 3.39 ng/ml, range: 3.16–4.35) and in controls (median: 2.99 ng/ml, range 2.62–3.59 ng/ml, $p = 0.001$ of ANOVA). There was no difference in genotype, lymphatic involvement, abdominal and systemic involvement between patients with MMP2 or MMP7 lower or higher than the 50th percentile except for a higher frequency of cortical tubers in TSC patients with MMP-7 >50th percentile (100 vs. 77%, $p = 0.040$).

Serum VEGF-D level was lower than the diagnostic threshold of 800 pg/mL in all healthy controls and resulted higher in the S-LAM group (median value: 1,456 pg/ml, range 457–3,167 pg/ml) and TSC-LAM group (median value: 1,057 pg/ml, IQR range 574–3,302 pg/ml) than in TSC patients (median value: 396 pg/ml, range 322–646 pg/ml) and controls (median value: 378 pg/ml range 335–444 pg/ml, $p < 0.001$ of ANOVA) (**Figure 1**). Patients with a VEGF-D higher than 800 pg/mL were significantly younger (median age 32 yrs, IQR 26–37 vs. 37 yrs, IQR 30–49; $p = 0.005$); they were diagnosed with LAM at a younger age (median age 32 yrs, IQR 24–36 vs. 44 yrs, IQR 29–56; $p = 0.072$), and had

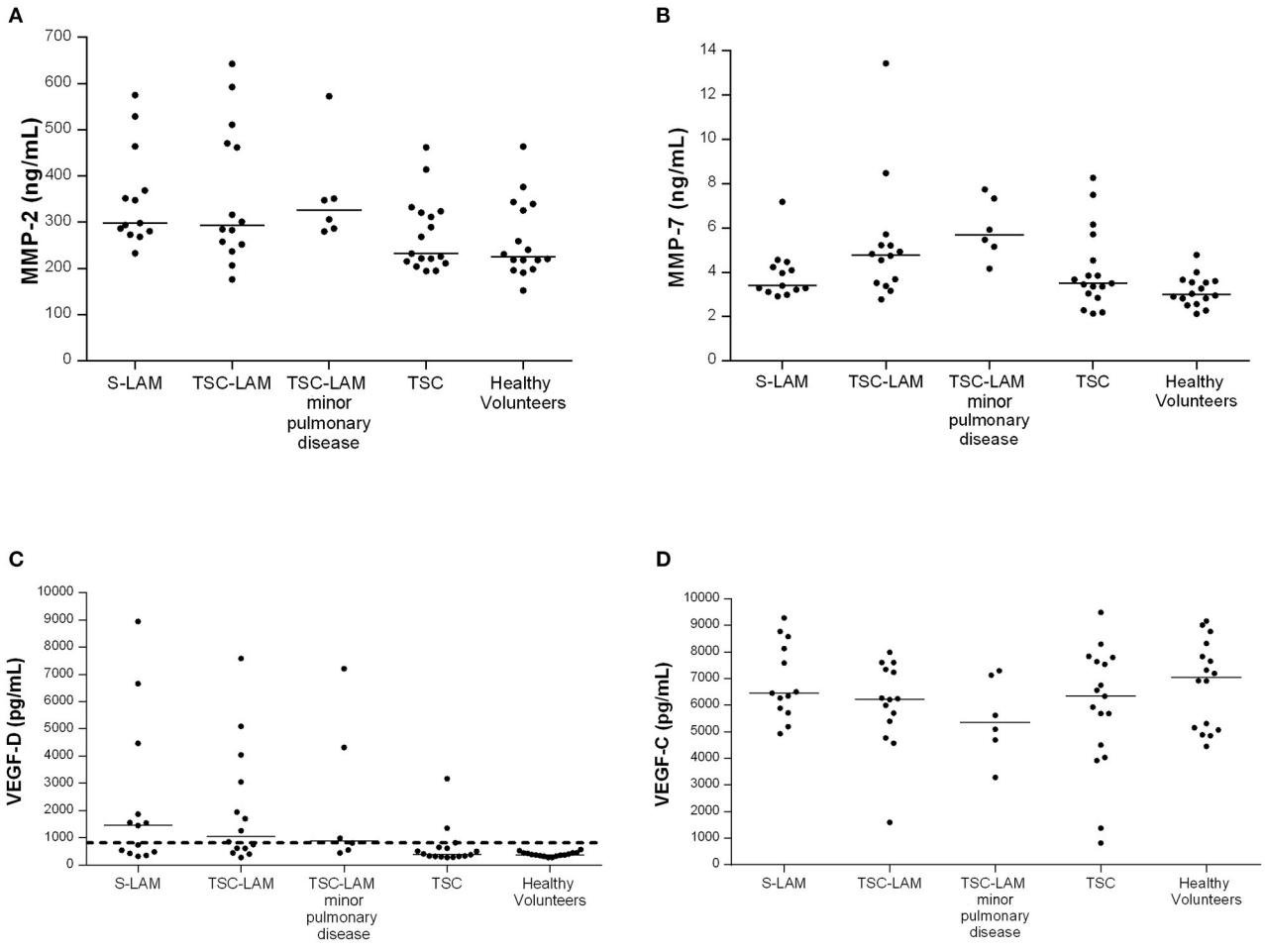

FIGURE 1 | Distribution of MMP-2 **(A)** $p = 0.040$, MMP-7 **(B)** $p = 0.001$, VEGF-D **(C)** $p < 0.001$, VEGF-C **(D)** $p = 0.354$, in the study population (p refers to ANOVA); LAM, lymphangioleiomyomatosis; TSC, tuberous sclerosis complex; minor pulmonary disease: patients with <10 cysts identified at chest CT scan; bar represents the median value, dashed line represents VEGF-D value of 800 pg/mL.

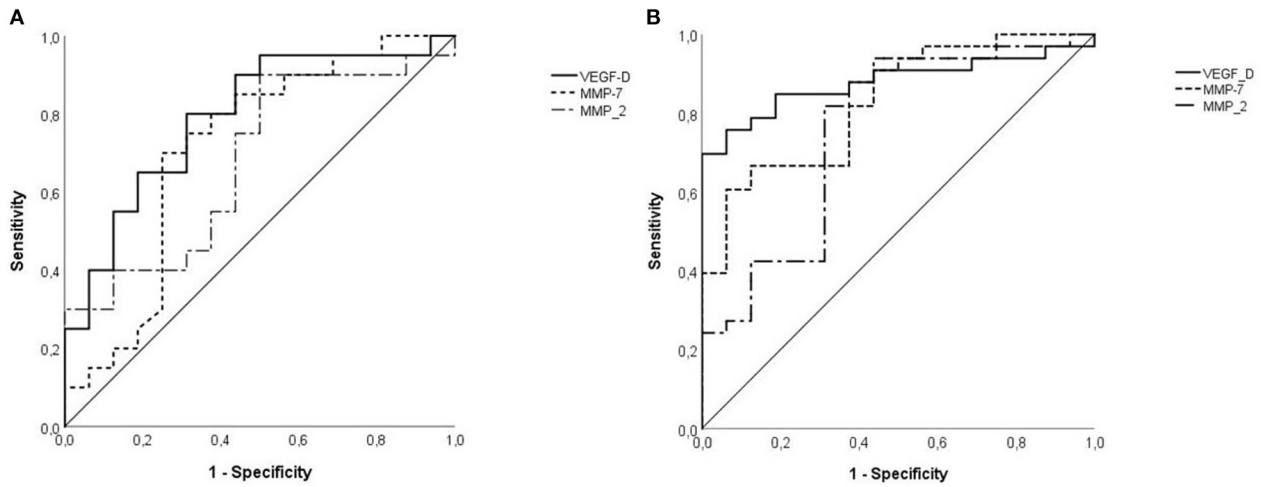

FIGURE 2 | (A) VEGF-D was an effective diagnostic test to predict LAM [area under curve (AUC): 0.879 ± 0.049 (95% CI: 0.782–0.975), $p < 0.001$] continuous line, respect to MMP2 [AUC: 0.756 ± 0.079 (95% CI: 0.601–0.910)], dotted line, and MMP7 [0.828 ± 0.060 (95% CI: 0.710–0.945), $p < 0.001$], punctuate line. **(B)** Specificity of VEGF-D for LAM disease in TSC patients was lower than in previous analysis but remains significant [AUC: 0.791 ± 0.077 (95% CI: 0.640–0.941), $p = 0.003$], continuous line. MMP-2 has lower accuracy than VEGF-D with an AUC of 0.694 ± 0.088 (95% CI: 0.521–0.867), $p = 0.044$, dotted line and similarly MMP-7 has an AUC of 0.713 ± 0.090 (95% CI: 0.538–0.889), $p = 0.027$, punctuate line.

more frequent chylothoraces (19 vs. 0%, $p = 0.034$). They had more frequently pathogenic variants in *TSC2* (71 vs. 24%, $p = 0.004$), larger AMLs (>3 cm 13.87 vs. 6.30% $p = 0.002$), and more frequently retinal hamartomas than patients with low VEGF-D (6.43 vs. 2.10%; $p = 0.042$).

VEGF-C in serum was slightly but not significantly lower in S-LAM patients (median value: 6,453 pg/ml, range 585–8,325 pg/ml), TSC-LAM patients (median value: 6,230 pg/ml, range 5,240–7,411 pg/ml), and TSC patients (median value: 6,338 pg/ml, range 4,271–7,718 pg/ml) than controls (median value 7,058 pg/ml, range 5,093–8,198 pg/ml, $p = 0.354$ of ANOVA).

Diagnostic Yield of Serum Biomarkers for LAM Diagnosis

The area under ROC curves (AUC) exploring the ability of MMP-2 to predict LAM diagnosis was 0.756 ± 0.079 (95% CI: 0.601–0.910, $p = 0.004$). At a cut-off level of 263 pg/ml MMP-2 showed a sensitivity for LAM diagnosis of 81%, and a specificity of 69% (**Figure 2A**). Serum level of MMP2 ≥463 ng/ml showed a specificity for LAM diagnosis of 100%. Only nine patients had MMP-2 serum level higher than 463 ng/ml, with a sensitivity of 24%. MMP-7 resulted a better biomarker for the diagnosis of LAM than MMP-2 with an AUC of 0.828 ± 0.060 (95% CI: 0.710–0.945, $p < 0.001$). The optimal cut off value resulted as 3.27 pg/ml, with a sensitivity and specificity of 67 and 82%, respectively (**Figure 2A**). Serum level of MMP-7 ≥4.8 pg/ml showed a specificity for LAM diagnosis of 100%. Fourteen patients had MMP-7 serum level higher than 4.8 pg/ml, with a sensitivity of 40%.

The cut off value of 800 pg/mL for VEGF-D had sensitivity and specificity for the diagnosis of LAM of 58 and 100%, respectively The ROC AUC for the diagnosis of LAM of VEGF-D was 0.879 ± 0.049 (95% CI: 0.782–0.975, $p < 0.001$, **Figure 2A**).

Diagnostic Yield of Biomarkers for LAM in TSC Patients

Considering all patients with TSC, the AUC for MMP-2 in predicting LAM was 0.694 ± 0.088 (95% CI: 0.521–0.867, $p = 0.044$, **Figure 2B**). With a cut off value of 340 pg/dL, the specificity was 88%, and the sensitivity was 40%. Serum level of MMP-2 ≥461 pg/ml showed a specificity for LAM diagnosis of 100%. Seven patients had MMP-2 serum level higher than 461 pg/ml, with a sensitivity of 30%. The diagnostic yield of MMP-7 for LAM diagnosis was similar to MMP-2, with an AUC of 0.713 ± 0.090 (95% CI: 0.538–0.889, $p = 0.027$, **Figure 2B**). 4.0 pg/mL was the optimal cut off value for LAM with a sensitivity and a specificity of 75 and 71%, respectively. Serum level of MMP-7 ≥8.73 ng/mL showed a sensitivity of 100%. Three patients had serum level of MMP-7 higher than 8.73 ng/mL, with a sensitivity of 10%. In patients with TSC the threshold of 800 pg/mL for VEGF-D had a sensitivity of 55% and a specificity of 82%. The AUC for LAM diagnosis was 0.791 ± 0.077 (95% CI: 0.640–0.941, $p = 0.003$, **Figure 2B**).

DISCUSSION

The most important results of this study are: (1) serum level of MMP-2 is significantly elevated in patients with S-LAM and TSC-LAM compared to patients with TSC and controls, while serum MMP-7 is higher in patients with TSC-LAM than in patients with S-LAM and in controls; (2) patients with minimal pulmonary disease seem to have higher levels of MMP-7 and MMP-2; whether this is due a more active disease and LAM-cell migration needs further studies; (3) high levels of MMP-2 or MMP-7 are not associated with a more severe systemic involvement; (4) high VEGF-D seems to be associated with younger age, *TSC2*

mutational status, and more severe systemic involvement; (5) VEGF-C does not seem to have a role as biomarker in LAM and TSC.

The MMPs and their tissue inhibitors *in vivo* are involved in remodeling the extracellular matrix and basement membranes both in normal and pathologic conditions (35). Overespression of MMPs was correlated with a more aggressive phenotype of cancer (36); the overexpression of MMP-7 is associated with tumor proliferation and a poor prognosis in some pulmonary disorders such as non-small cell lung carcinoma and idiopathic pulmonary fibrosis (36, 37). Some immunohistochemical studied have demonstrated that MMP-2 and their tissue inhibitors are over expressed in the pulmonary tissue of patients with LAM compared to normal bronchial tissue (18, 38). MMPs are highly susceptible to a variety of regulators including hormones, pro-inflammatory cytokines, and growth factors. Although the subtypes of the MMP family are not abundant in the healthy lung, they are produced by inflammatory cells and lungs in response to inflammatory chemokines and free oxygen radicals (39). In cancer, interleukins (ILs) induce neo-angiogenesis and enhance the activity of MMPs, which increases the metastatic activity as demonstrated in hepatocellular carcinoma where IL-8 and IL-17 cause MMP-2 and MMP-9 activation (40). MMPs may likely be the result or the cause of a condition, as demonstrated by the increased expression of MMP-7 in the bronchial epithelial cells of asthmatic patients, and, conversely, the association of MMP-7 with wound repair and low expression in asthmatic patients (41). Natural inhibitors such as tissue inhibitor of TIMPs, protease inhibitor homologs known as TIMP-1, TIMP-2, TIMP-3, and TIMP-4, also regulate MMPs activity (42). The imbalance between MMPs and TIMPs, together with the increased levels of MMPs, seems to cause the cystic destruction of lung LAM parenchyma (43).

Several agents can target the MMPs to inhibit MMPs activation, MMPs enzyme activity, and to suppress MMPs at the transcription/translation levels. Among these we can list natural products such as Neovastat and curcumin, the broad-spectrum antibiotics tetracyclines, bisphosphonates generally used to treat osteoporosis and bone diseases, and histone deacetylase inhibitors (HDACIs) including vorinostat and valproic acid (44–46). Various antiepileptic drugs (AEDs) such as phenytoin (47), valproic acid and lamotrigine (48) have also been demonstrated to exert an inhibitory effect on MMPs. Since epilepsy is a common manifestation of TSC, MMPs expression should be evaluated taking into account whether the patient is taking any AEDs and, in such case, which one. However, in our study, serum MMP-7 level was higher in TSC-LAM patients than in healthy individuals and MMP-2 was increased in S-LAM and TSC-LAM patients compared to TSC patients and controls. Moreover, the low number of patients for each group limits the statistical analysis of the possible antiepileptic drug administration related to MMPs levels.

Lee et al. demonstrated that cells lacking the *TSC1/TSC2* genes overexpressed MMP-2 and that this overexpression was not affected by rapamycin (49). Our data indicate that the MMP-2 serum levels are higher in patients with LAM than in patients without LAM, but we also observed a high overlap between subgroups of the single values. These data are consistent with previous studies on this field. Moses et al. described a patient with LAM in whom urinary levels of some MMPs isoforms (in particular MMP-2 and−9) were elevated and decreased after treatment with doxycycline (an MMP inhibitor) (50). Pimenta et al. described a cohort of 41 patients also treated with doxycycline. Serum and urinary levels of MMP-2 were higher in patients with LAM then in healthy controls and decreased after treatment with the antibiotic; however, the median of MMP-2 in serum was below the detection limit both at baseline and after treatment (50, 51). Chang and colleagues analyzed some serum biomarkers as diagnostic and prognostic tools, and found higher MMP-2 levels in patients with LAM than in controls with a considerable overlap of single values between the two groups (52). Finally, Odajima et al. studied in 2009 the serum level of MMP-2 and MMP-9 in 36 patients with LAM and did not find any significant differences from healthy controls (21). In this study the ROC analysis have furthermore demonstrated that the ability of MMP-2 to predict LAM disease was lower than the ability of VEGF-D in line with the previously cited work by Chang et al. (52). The previous demonstration that MMP-7 and β-catenin are expressed in LAM tissues suggests that tuberin-deficient cells acquire invasive characteristics which may underlie the development of LAM disease (24). Interestingly, the serum MMP-7 levels were high in all the LAM/TSC patients, while in S-LAM and TSC patients MMP-7 show similar levels of healthy subjects. To understand this data further analysis are needed, likely keeping in consideration the significant relationship of high MMP-7 levels with the higher frequency of cortical tubers.

VEGF-D serum levels were analyzed in previous studies that led this biomarker to be included in the recent diagnostic guidelines as a diagnostic tool in the presence of a typical chest CT pattern, thus reducing the need for lung biopsy in patients with suspected LAM (10). In two studies developed by Seyama et al. and Glasgow et al., VEGF-D serum levels were significantly higher in LAM patients that in controls (12, 53). In 2013 Xu et al. found similar results with serum VEGF-D levels significantly increased in the definite LAM group, compared with that of healthy controls (11). Young et al. measured serum level of VEGF-D in patients with LAM, healthy controls and patients with other pulmonary diseases and found that serum levels of VEGF-D were significantly higher in the first group of patients (9). Similarly, Radzikowska et al. showed that VEGF-D could discriminate between LAM and other pulmonary cystic diseases such as pulmonary Langerhans cell histiocytosis and lymphocytic interstitial pneumonia (54). In line with this data, our results demonstrate higher VEGF-D serum levels in LAM patients than in healthy controls. Nevertheless, in our study group more than 40% of patients with a definite diagnosis of LAM showed serum levels of VEGF-D lower than the diagnostic threshold of 800 pg/mL; VEGF-D has a sensitivity of 58% and a specificity of 100% for the diagnosis of LAM. VEGF-D high specificity is confirmed with a low sensitivity. This is consistent with the study by Chang in which 42% of patients with LAM showed VEGF-D serum levels lower than the diagnostic threshold and a sensitivity of 56% and a specificity of 100% (52). On the contrary, Xu et al. found a VEGF-D sensitivity of 96% (11). In a study from Glasgow et al., a statistically significant difference between LAM and healthy

controls for VEGF-D serum level was maintained only for LAM patients with lymphatic involvement (lymphangioleiomyomas and/or lymphadenopathy) but not for those patients with a disease restricted to the lungs (12). In our analysis, however, we did not find any difference in lymphatic involvement in patients with a VEGF-D higher or lower than the diagnostic threshold of 800 pg/mL except for chylothorax, more frequent in patients with a VEGF-D higher than 800 pg/mL. However, it is possible that these results may be related to some differences in the studied population. The differences in the number of patients involved in the studies could in part explain these discrepancies and affect the statistical significance. Furthermore, in our cohort there was a low percentage of lymphatic involvement.

We found a trend to higher VEGF-D serum levels in patients with TSC-LAM compared to patients with TSC and a normal high-resolution CT scan. This is in line with data published by Young et al. in 2008, where VEGF-D levels were much higher in women with TSC and LAM than in women with TSC and normal high-resolution CT scan (9). However, the authors found a very strong difference between the two groups in contrast to our data that show only a trend to statistical significance. This difference may be ascribed to some differences in the studied population as well. In fact, the majority of our patients with TSC-LAM had a mild disease, while we do not have data on the population analyzed in Young's study. Furthermore, TSC is a disease with a very heterogeneous presentation and systemic involvement, and the extent of lymphatic involvement could have influenced the analysis. Our data indicate that patients with VEGF-D serum levels above the diagnostic threshold of 800 pg/ml show more frequently pathogenic variant in *TSC2*. This is consistent with previous studies showing that there is a higher rate of *TSC2* mutations than *TSC1* mutations in patients with TSC-LAM (55–57) and that patients with TSC and pathogenic variants in *TSC1* usually have a milder disease in comparison with patients carrying *TSC2* pathogenic variants (58).

This work has two innovative characteristics: first, we performed deep phenotyping of the whole population analyzing separately and comparing the serum levels of four biomarkers in patients with S-LAM and TSC-LAM and exploring a possible link with clinical and genetic characteristics of the single groups. Secondly, this is the first study that has analyzed serum levels of MMP-7 in relationship to LAM. However, some potential limits to the present study deserve discussion. The main limitation of our study is the limited number of patients included, because of the rarity of the disease and the monocentric nature of the study. We enrolled patients with a definite diagnosis of TSC and LAM and healthy individuals. In clinical practice, the diagnostic challenge is represented by the patients with a cystic lung disease. In keeping with the explorative nature of the study we tried to evaluate the serum biomarkers level in a "pure" population to investigate the data distribution (narrow distribution or very scattered data). Further studies involving other cystic lung diseases as controls (i.e., Birt Hogg Dubè Syndrome, lymphoid interstitial pneumonia, Langherans cells Histiocytosis) and with multiple samplings are needed to strengthen the specificity of the biomarkers for LAM and to evaluate data reproducibility over time and changes in case of treatment or recurrence.

Finally, enrolled patients show a relatively mild disease, in terms of pulmonary function and the clinical and radiological data used to explore a possible link between single biomarkers and systemic involvement were requested for clinical follow up purpose, and some data are therefore missing due to the "retrospective" nature of the analysis.

In conclusion, MMP-2 and especially MMP-7 are promising biomarkers for LAM and validation in longitudinal studies and with a larger patient population is needed. The diagnostic value of VEGF-D for LAM was confirmed in this cohort of Italian patients.

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 studies involving human participants were reviewed and approved by Comitato Etico Interaziendale Milano Area A. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

STe, FD, and EL had full access to all of the data in the study and takes responsibility for the integrity of the data, the accuracy of the data analysis, study concept, and design. STe, SA, GI, LG, GP, PC, and STr: acquisition of data. STe, FD, and GI: analysis and interpretation of data. STe and FD: drafting of the manuscript. OT, AP, SC, and EL: critical revision of the manuscript for important intellectual content. STe and GI: statistical analysis. All authors reviewed and approved the final version of the manuscript.

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Prevalence of Birt-Hogg-Dubé Syndrome Determined Through Epidemiological Data on Spontaneous Pneumothorax and Bayes Theorem

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Background: Birt-Hogg-Dubé syndrome (BHD) is a rare inherited disorder characterized by cutaneous fibrofolliculomas, multiple pulmonary cysts, recurrent spontaneous pneumothorax (SP), and renal tumors. More than 40 years after its description, the prevalence of BHD in the general population remains unknown. This study aimed at determining the prevalence of BHD by applying the Bayes theorem of conditional probability to epidemiological data on SP.

Methods: We performed a meta-analysis of published data on: (1) the probability of having BHD among patients with apparent primary SP (4 studies), (2) the incidence rate of primary SP in the general population (9 studies), and (3) the probability of experiencing a SP in BHD (16 studies). Results were corrected for SP relapses, stratified by gender and year of study publication (before and after 2000), and computed with the Bayes equation.

Results: The probability of having BHD among patients with apparent primary SP was 0.09 (95% confidence interval: 0.07, 0.11) or 9%. It was 0.20 (0.14, 0.27) in women and 0.05 (0.04, 0.07) in men. The incidence rate of primary SP in the general population was 8.69 (6.58, 11.46) per 100,000 person-years (p-y). It was 3.44 (2.36, 4.99) per 100,000 p-y in women and 13.96 (10.72, 18.18) per 100,000 p-y in men, and was about 2 times higher in studies published after 2000 than in those published before 2000. The probability of experiencing at least one SP among patients with BHD was 0.43 (0.31, 0.54) or 43%, without gender difference. By combining these data in the Bayes equation, we found a prevalence of BHD in the general population of 1.86 (1.16, 3.00) per million, with values of 1.86 (1.02, 3.39) per million in men, and 1.88 (0.97, 3.63) per million in women.

Conclusion: The prevalence of BHD in the general population is about 2 cases per million, without difference between genders.

Keywords: Birt-Hogg-Dube syndrome, prevalence, pneumothorax, epidemiology, meta-analysis, Bayes theorem, gender

INTRODUCTION

Birt-Hogg-Dubé syndrome (BHD) is a rare inherited autosomal dominant disorder caused by germline mutations in the tumor suppressor gene *FLCN* encoding the protein folliculin (1). Its clinical expression includes cutaneous fibrofolliculomas, multiple pulmonary cysts, recurrent spontaneous pneumothorax (SP), and renal tumors. However, BHD shows a wide phenotypic variability, and affected subjects can present with any combination of skin, pulmonary, or renal manifestations of varying degrees of severity, even within the same family. Hence, recognition of BHD remains difficult and, more than 40 years after its first description (2), its prevalence in the general population is still unknown.

One characteristic of BHD is the frequent occurrence of SP due to rupture of pulmonary cysts, which affects about half of individuals during their life, and frequently recurs. Despite the rarity of BHD, the occurrence of SP in BHD is so common that 5–10% of apparently primary spontaneous pneumothorax (PSP) in the general population appears in fact due to BHD (3–6).

To determine the prevalence of BHD, we used an indirect approach based on available epidemiological data on SP in the general population and in BHD. For this purpose, we performed meta-analyses of published studies on: (1) the probability of having BHD among patients with apparent PSP, (2) the incidence of PSP in the general population, and (3) the probability of experiencing at least one SP in BHD. Results of these meta-analyses were computed with the Bayes equation, which allows to determine the probability of an event based on prior knowledge of conditions that might be related to this event (7).

METHODS

Literature Search

A literature search was performed in April 2020 in the PubMed electronic database. The search was limited to full-text journal articles in English, French, and German. Articles whose primary or secondary outcome met the searched items were retrieved. All articles were then reviewed to identify other studies of interest in the reference lists.

To assess the incidence of PSP in the general population, a search was performed with the Medical Subject Heading (MeSH) keyword "Pneumothorax/epidemiology." To assess the probability of having BHD among patients with apparent PSP and the probability of experiencing a SP in BHD, a search was performed with the keywords "pneumothorax" and "Birt-Hogg-Dube" combined with the Boolean operator "AND".

Statistics

We expressed the Bayes theorem as follows:

$$P(BHD) = \frac{P\,(BHD|PSP) \cdot P(PSP)}{P(PSP|BHD)}$$

where $P\,(BHD|PSP)$ is the probability of having BHD in individuals experiencing an apparent PSP in the general population, $P(PSP)$ the prevalence of PSP in the general population, and $P(PSP|BHD)$ the probability of experiencing a SP (written "PSP" in the Bayes equation) in individuals with BHD. As the prevalence $P(PSP)$ is not directly measurable, we estimated it using the following prevalence formula (8, 9):

$$P\,(PSP) \cong IR \cdot \overline{D}$$

where IR is the yearly incidence rate of PSP, and $\overline{D}$ is the average duration of a PSP event. This prevalence formula is valid in a steady state setting (i.e., when the total population of affected and unaffected individuals remains constant over time) and provides a good approximation when the prevalence $P(PSP)$ is small. The value of $\overline{D}$ was based on a recently published randomized trial on the treatment of PSP, which showed that the median time of recovery for a PSP treated conservatively was 30 days, whereas it was 16 days with interventional treatment (10).

In each study, data on the number of events E, the number of individuals at risk at the beginning of the follow-up period, and the duration of follow-up were extracted to compute the annual incidence rate IR of PSP (11):

$$IR = \frac{E}{PT}$$

Where PT is the person-time product expressed in person-years (p-y). Given the small number of events in comparison to the number of individuals, PT was simply computed by multiplying the number of individuals by the duration of the follow-up period. The variance of IR was computed based on the Poisson distribution. To build a 95% confidence interval (95% CI), the log-transformation was used and the delta method was applied to compute the variance. In 4 studies (12–15), the number of individuals at risk was not reported, and the population figures were retrieved from census data available online (16–20).

As the 3 components of the Bayes equation were provided by different studies, a separate meta-analysis for each component was conducted. Except for the probability of BHD given PSP, where a fixed-effect analysis was carried out given the very small number of studies, all meta-analyses were carried out using the random-effects model. For the probability $P\,(BHD|PSP)$ of having BHD in individuals experiencing an apparent PSP, the Freeman-Tuckey double arcsine transformation was used to ensure confidence intervals covering the appropriate [0–1] support. As the incidence rate IR of PSP is known to be different across genders, separate analyses were carried out for each group. Also, as all studies on PSP incidence published before year 2000 had much smaller sample sizes than those published after 2000 and had IRs smaller than those published after 2000, a random-effects subgroup meta-analysis was carried out within each gender, with the first group defined by studies published before 2000 and the second by those published after 2000 (21). The same approach was used for the meta-analysis of the prevalences $P(PSP)$, as they were computed based on the IRs. Regarding the meta-analysis of $P(PSP|BHD)$, the Freeman-Tuckey double arcsine transformation was used (22).

Abbreviations: BHD, Birt-Hogg-Dubé syndrome; PT, person-time; SP, spontaneous pneumothorax; PSP, primary spontaneous pneumothorax; P, prevalence; IR, incidence rate.

TABLE 1 | Studies reporting the number of patients with BHD among patients presenting with apparent primary spontaneous pneumothorax.

References	Country	Recruited patients	Number of patients with apparent PSP			Number of patients with BHD		
			Women	Men	Total	Women	Men	Total
Ren, 2008 (3)	China	Admitted for PSP in 2 tertiary hospitals	8	94	102	2	8	10
Johannesma, 2015 (4)	Netherlands	Admitted for PSP in one university hospital	N/A	N/A	40	1	2	3
Ebana, 2018 (5)	Japan	Admitted for PSP needing VATS in one specialized center	119	452	571	32	22	54
Toricelli, 2019 (6)	Italy	Admitted for PSP in one hospital	31*	83*	114	1	5	6

*PSP, primary spontaneous pneumothorax; VATS, video-assisted thoracoscopic surgery; N/A, not available. *Retrieved from author.*

TABLE 2 | Bayes equation's components estimated by random-effects models.

		Women	Men	All
Probability of BHD in apparent PSP (95% CI)		0.20 (0.14, 0.27)*	0.05 (0.04, 0.07)*	0.09 (0.07, 0.11)*
Incidence rate of PSP in the general population, per 100,000 person-years (95% CI)	overall	3.44 (2.36, 4.99)	13.96 (10.72, 18.18)	8.69 (6.58, 11.46)
	<2000	2.63 (1.31, 5.31)	10.73 (7.23, 15.92)	6.71 (4.43, 10.17)
	>2000	4.11 (2.32, 7.29)	16.95 (12.17, 23.61)	10.55 (7.40, 15.04)
Prevalence of PSP in the general population with PSP duration of 16 days, per 100,000 persons (95% CI)	overall	0.17 (0.09, 0.25)	0.65 (0.45, 0.86)	0.41 (0.27, 0.55)
	<2000	0.12 (0.00, 0.25)	0.47 (0.15, 0.79)	0.30 (0.09, 051)
	>2000	0.21 (0.09, 0.32)	0.79 (0.52, 1.07)	0.50 (0.31, 0.68)
Prevalence of PSP in the general population with PSP duration of 30 days, per 100,000 persons (95% CI)	overall	0.32 (0.16, 0.48)	1.22 (0.85, 1.60)	0.77 (0.52, 1.02)
	<2000	0.23 (0.00, 0.48)	0.88 (0.29, 1.48)	0.56 (0.17, 0.95)
	>2000	0.39 (0.18, 0.59)	1.49 (0.97, 2.01)	0.93 (0.59,1.28)
Probability of occurrence of a SP in BHD (95% CI)		0.41 (0.28, 0.54)	0.43 (0.27, 0.59)	0.43 (0.31, 0.54)
Prevalence of BHD in general population with PSP duration of 16 days, per million persons (95% CI)	overall	0.83 (0.42, 1.65)	0.82 (0.44, 1.50)	0.82 (0.50, 1.34)
	<2000	0.61 (0.23, 1.63)	0.59 (0.27, 1.27)	0.59 (0.30, 1.18)
	>2000	1.00 (0.52, 1.93)	0.99 (0.54, 1.81)	0.99 (0.62, 1.60)
Prevalence of BHD in general population with PSP duration of 30 days, per million persons (95% CI)	Overall	1.56 (0.79, 3.09)	1.53 (0.83, 2.81)	1.53 (0.94, 2.51)
	<2000	1.14 (0.42, 3.05)	1.10 (0.51, 2.38)	1.11 (0.56, 2.22)
	>2000	1.88 (0.97, 3.63)	1.86 (1.02, 3.39)	1.86 (1.16, 3.00)

*95% CI, 95% confidence interval; PSP, primary spontaneous pneumothorax; SP, spontaneous pneumothorax; <2000, before year 2000; >2000, after year 2000. *Fixed-effect model.*

Finally, the pooled effect sizes estimated in each strata (defined by gender and publication date <2000 or >2000) were used to compute the prevalence of BHD for each stratum based on Bayes equation. The multivariate delta method was used to compute the variance estimate of the logit transform of $P(BHD)$.

To compute the prevalence $P(PSP)$, the numerator of the incidence rate IR should include relapses to reflect the true prevalence. For 3 of the 9 selected studies, PSP recurrences were already included in the reported numerators and the published data were directly used in the calculation. For the 6 other studies, a correction was applied to the numerators to incorporate a 29% annual recurrence rate, based on the result of a recent meta-analysis (23). The prevalence $P(PSP)$ was computed in each stratum defined by gender, publication date <2000 or >2000, and numerator corrected to include recurrences. Results were expressed as effect size with 95% CI. To assess the robustness of results to modeling assumptions, the analyses were repeated using a fixed-effect approach instead of a random-effects (as in a random-effects approach small studies have more impact on the pooled effect-size estimate).

RESULTS

Probability of BHD in Apparent PSP

The literature search identified 206 articles. Four original articles were retrieved (3–6). No additional article was found after manual review. **Supplementary Figure S1** shows the flow diagram depicting the search strategy. Missing data in one study (6) were completed through correspondence with the first author. Characteristics of the studies are shown in **Table 1**.

The overall probability of having BHD among patients presenting with apparent PSP was 0.09 (0.07, 0.11) or 9%. The prevalence was 0.05 (0.04, 0.07) in men, and 0.20 (0.14, 0.27) in women (**Table 2**). **Figure 1** summarizes the statistical results of

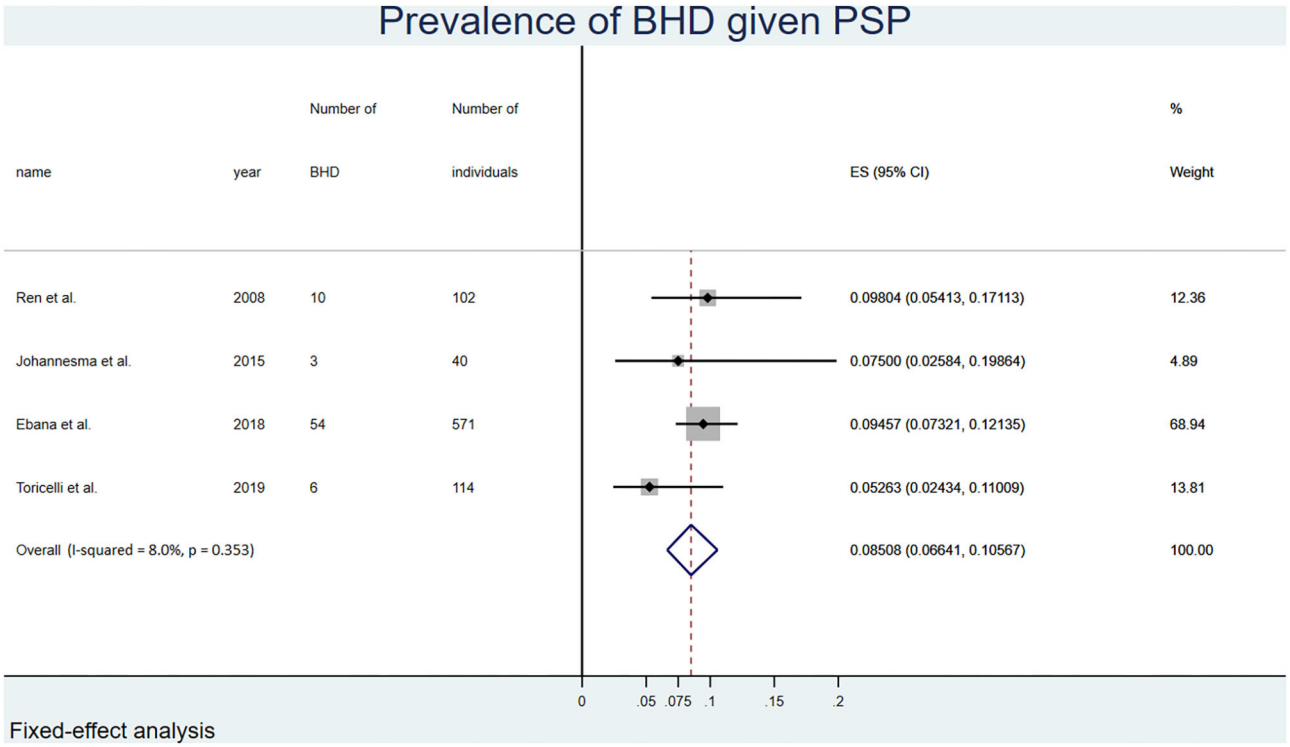

FIGURE 1 | Forest plot of the overall prevalence of BHD in PSP, random-effects model.

the overall meta-analysis. The **Supplementary Figure S2** shows the subgroup analyses by gender.

Incidence of PSP in the General Population

The Pubmed search retrieved 195 articles. Six articles were added after review of the reference lists. A total of 11 original articles reporting PSP incidence in the general population were retrieved. One paper was rejected because the results provided were not based on identified cases but on mathematically inferred cases. Another article was excluded because sample size and gender proportion were not given. Nine original studies were kept for meta-analysis (12–15, 24–28). Their main characteristics are shown in **Table 3**. **Supplementary Figure S3** shows the flow diagram depicting the search strategy.

By meta-analysis, the overall incidence rate of PSP in the general population was 8.69 (6.58, 11.46) per 100,000 p-y. Important differences appeared with gender stratification. The overall incidence rate was 3.44 (2.36, 4.99) per 100,000 p-y in women and 13.96 (10.72, 18.18) per 100,000 p-y in men. With both gender and time period stratification, women had an incidence rate of 2.63 (1.31, 5.31) per 100,000 p-y before 2000 and 4.11 (2.32, 7.29) after 2000. In men, the incidence was 10.73 (7.23, 15.92) per 100,000 p-y before 2000, and 16.95 (12.17, 23.61) per 100,000 p-y after 2000 (**Table 2**). **Figure 2** shows the results of the overall meta-analysis. **Supplementary Figure S4** show the analyses by gender and <2000/>2000 stratification.

Prevalence of PSP in the General Population

With a random-effects model, and a 30 days PSP duration, the overall prevalence of PSP in the general population was 0.77 (0.52, 1.02) per 100,000. In men, it was 0.88 (0.29, 1.48) per 100,000 before 2000, and 1.49 (0.97, 2.01) per 100,000 after 2000. In women, it was 0.23 (0.00, 0.48) per 100,000 before 2000, and 0.39 (0.18, 0.59) per 100,000 after 2000. With a PSP duration of 16 days, the prevalence was about half of these values. Results are detailed in **Table 2. Figure 3** shows the results of the overall meta-analysis. **Supplementary Figure S5** show the analyses by gender and <2000/>2000 stratification.

Using a fixed-effect model, the overall prevalence as well as the prevalence stratified by gender and time period were very similar to those observed with the random-effects analysis. Results are detailed in **Supplementary Table S1**.

Probability of SP in BHD

The search identified 206 articles. Fifteen original articles containing data on SP in BHD were retrieved. One article was excluded because it focused only on SP in BHD after air travel (29). One original article was added after review of the reference lists (30). Another paper published by our group in June 2020 was also added (31). Thus, 16 original studies were kept for meta-analysis (3, 30–44). Their characteristics are shown in **Table 4**. **Supplementary Figure S6** shows the flow diagram depicting the search strategy.

TABLE 3 | Studies reporting the incidence of primary spontaneous pneumothorax in the general population.

References	Country	Observation period	Recruited participants	Person-time			Number of participants with PSP		
				Women	Men	Total	Women	Men	Total
Wynn-Williams, 1957 (24)	England	1947–1956	Admitted for PSP to the General hospital of a county town	*750,000*	*750,000*	*1,500,000*	11	59	70
Hallgrimsson, 1978 (25)	Iceland	1950–1974	Diagnosed with pneumothorax in any primary care setting or hospital in Iceland	*519,500*	*531,500*	*1,051,000*	9	33	42
Melton, 1979 (12)	USA, Minnesota	1950–1974	Diagnosed with pneumothorax in any primary care setting, hospital or at autopsy in the whole county	*923,075*	*844,150*	*1,767,225*	12	65	77
Primrose, 1984 (26)	Scotland	1976–1981	Admitted for pneumothorax to one hospital respiratory unit	*630,000*	*630,000*	*1,260,000*	11	59	70
Bobbio, 2015 (13)	France	2008–2011	Admitted for pneumothorax to any private or public hospital in France	*124,000000*	*132,000000*	*256,000,000*	12,088	38,508	50,596
Schnell, 2017 (15)	Germany	2011–2015	Admitted for PSP to any hospital in Germany AND >10 years old	*218,000,000*	*214,000,000*	*432,000,000*	12,654	40,084	52,738
Huang, 2017 (14)	Taiwan	2001–2013	Admitted for PSP to a hospital in Taiwan AND > 11 and < 40 years old	*151,000,000*	*151,000,000*	*302,000,000*	*2,836*	*16,726*	*19,562*
Hallifax, 2018 (27)	England	2015	Admitted for pneumothorax as first diagnosis to any public hospital AND > 15 years old	*23,000,000*	*22,000,000*	*45,300,000*	564	1,804	2,368
Olesen, 2019 (28)	Denmark	2009–2014	Admitted for a first episode of pneumothorax to hospital AND < 40 years old	*6,818,182*	*7,138,211*	*14,000,000*	150	878	1,028

PSP: primary spontaneous pneumothorax. Population numbers represents the yearly population multiplied by the number of year of the observed period. Values in italics: results were not available in the original paper but were re-calculated from census data.

The overall probability of ever SP among patients with BHD was 0.43 (0.31, 0.54) or 43%. There was no gender difference. **Figure 4** shows the overall results of the meta-analysis. The **Supplementary Figure S7** shows the subgroup analysis by gender.

Prevalence of BHD in the General Population

To determine the prevalence of BHD in the general population, the above components were combined using the Bayes equation. Results are detailed in **Table 2**. We assumed that the highest accuracy would be provided by studies on the incidence of PSP published after 2000, by integrating the occurrence of relapses in the incidence of PSP, and by using a median pneumothorax duration of 30 days reflecting the natural history of the condition for the calculation of PSP prevalence. Using these assumptions, we found a prevalence of BHD in the general population of 1.86 (1.16, 3.00) per million. The prevalence by gender was 1.86 (1.02, 3.39) per million in men, and 1.88 (0.97, 3.63) per million in women. Lower figures were found when integrating studies before and after 2000, and using a median PSP duration of 16 days (**Table 2**).

The same calculations were made with a fixed-effect model (**Supplementary Table S1**). Using studies on the incidence of PSP published after 2000, taking relapses into account, and using a median PSP duration of 30 days, the prevalence of BHD was very similar to that obtained by the random-effects model with values of 1.81 (1.41, 2.31) per million for the whole population, including 2.18 (1.47, 3.23) per million in men, and 1.75 (1.20, 2.56) per million in women. Lower figures were found when

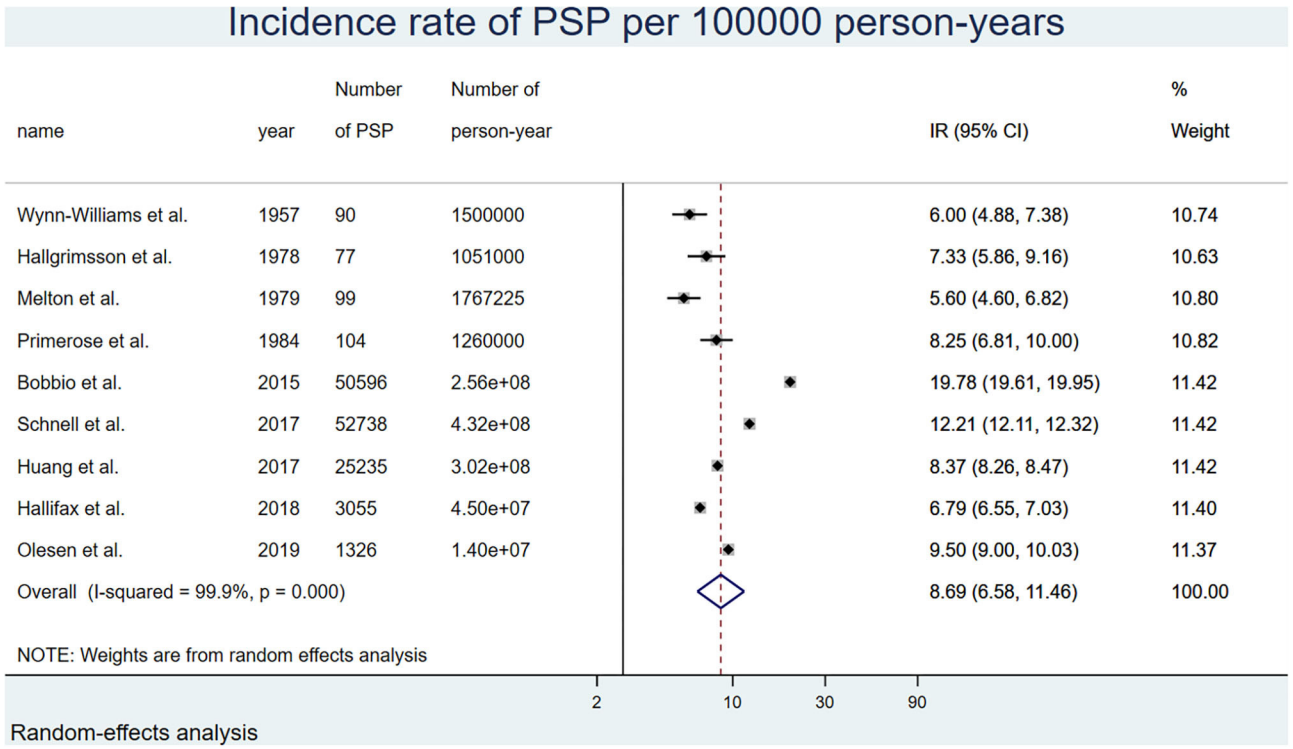

FIGURE 2 | Forest plot of the overall incidence rate of pneumothorax for 100,000 person-years corrected for relapses, random-effects model.

integrating studies before and after 2000, and using a median PSP duration of 16 days (**Supplementary Table S1**).

As sensitivity analysis, we recomputed the prevalence of BHD using the Bayes formula and considering two extreme scenarios: first, a low scenario where the numerator of the formula is minimized [by using the lowest observed values of P(BHD|PSP) and P(PSP)] and the denominator maximized [by using the highest observed value of P(PSP|BHD)], then a high scenario where the numerator of the formula is maximized [by using the highest observed values of P(BHD|PSP) and P(PSP)] and the denominator minimized [by using the lowest observed value of P(PSP|BHD)]. For the low scenario, we found $P(\text{BHD}) = 0.3$ per million individuals, and for the high scenario $P(\text{BHD}) = 14.4$ per million individuals.

In summary, the prevalence of BHD in the general population was about 2 cases per million, and was equally distributed among men and women.

DISCUSSION

In this study, we took advantage of available data on epidemiology of pneumothorax to determine the prevalence of BHD in the general population, using an indirect approach based on Bayes equation. We performed meta-analyses of published studies to assess each component of the Bayes equation. We found a prevalence of BHD in the general population of about 2 cases per million, without differences between men and women. To our knowledge, this is the first study to determine the prevalence of BHD.

Probability of Having BHD in Individuals With Apparent PSP

The first component of the calculation used in this study was the probability of having BHD in individuals presenting with apparent PSP. Only 4 studies addressing this issue were available, with number of PSP ranging from 40 to 571, and number of BHD among these cases ranging from 3 to 54 (3–6). The proportion of BHD among patients with apparent PSP ranged from 5 to 10% in individual studies, with a pooled value of 9%. Although a recruitment bias with enrichment in BHD patients may have occurred in studies performed in tertiary hospitals and a Pneumothorax Research Center (5), this suggests that BHD is not rare among patients presenting with apparent PSP, and should be carefully looked for in this population. One simple measure to screen for BHD is to systematically inquire about a family history of pneumothorax in patients presenting with apparent PSP. Familial pneumothorax accounts for about 10% of apparent PSP (45), with BHD being the most common cause (46). Indeed, 2 studies showed a prevalence of BHD of 64–86% in patients presenting with apparent PSP and a positive family history of pneumothorax (5, 6). It is therefore recommended to systematically look for lung abnormalities and a genetic cause, especially BHD, in any individual presenting with apparent

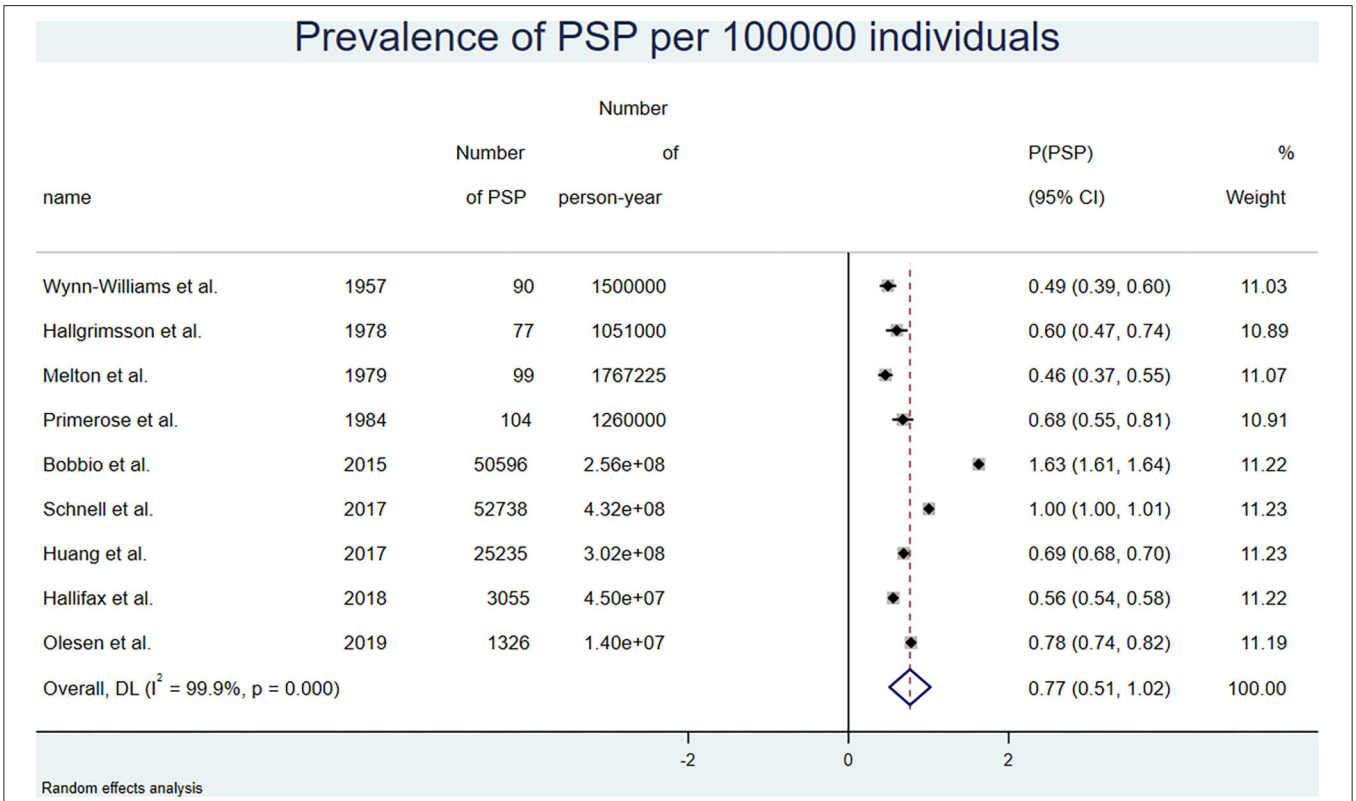

FIGURE 3 | Forest plot of the overall prevalence of pneumothorax for 100,000 person-years corrected for relapses, random-effects model.

PSP, and even more in case of a positive family history of pneumothorax (47, 48).

In the 3 studies with available gender data, we found a meaningful difference between genders in the probability of having BHD among patients presenting with apparent PSP, with a rate of 5% in men and 20% in women. Although this finding has to be interpreted with caution due to limited number of studies and small sample sizes, it suggests that the distribution of causes of apparent PSP differs between men and women. Indeed, the incidence of PSP in the general population is known to be higher in men (49), with smoking being the main other contributing factor. Consequently, true PSP appears more frequent in men, and SP due to BHD accounts for a lower proportion of apparent PSP in this population. In contrast, true PSP is less common in women, and apparent PSP in this population may be due to a greater extent to BHD, and to other diseases specific to women such as lymphangioleiomyomatosis (LAM), and catamenial pneumothorax associated with endometriosis. Indeed, LAM has been estimated to account for 5–30% of apparent PSP in women (50), whereas catamenial pneumothorax is estimated to be its cause in 25–31% (51, 52). Thus, the likelihood of finding an underlying cause is higher in women with apparent PSP as compared to men, and should prompt to carefully look for such a cause in the female population.

Incidence and Prevalence of PSP in the General Population

For the second component of the Bayes equation, the incidence and the prevalence of PSP in the general population had to be determined. Regarding incidence, the 9 studies addressing this issue were performed in 3 different continents (North America, Europe, Asia), and the observation periods covered a large time span between 1947 and 2015. Important differences in PSP incidence were observed between studies performed before 2000 and those performed after 2000, the latter consistently showing a higher incidence both in women and men. As a true increase in incidence over time appears unlikely, we believe that the observed differences are due to more comprehensive case finding and larger sample size in more recent studies. Indeed, the 4 oldest studies, published between 1957 and 1984, were performed at a regional level (county, island, or a region smaller than a country) (12, 24–26). In contrast, the 5 most recent studies, published between 2015 and 2019, were performed at a national level and included much larger samples (13–15, 27, 28). Also, they were based on national registries of hospitalizations or medical care networks, which allowed to retrieve data more precisely and at a larger scale than the smaller studies performed decades ago. We thus considered that the true incidence of PSP was better appraised in recent studies, and chose to use the data of this subgroup for subsequent calculations.

TABLE 4 | Studies reporting the proportion of patients with BHD who experienced at least one episode of spontaneous pneumothorax.

References	Country	Recruited patients	Number of patients with BHD			Number of BHD patients with SP		
			Women	Men	Total	Women	Men	Total
Zbar, 2002 (32)	USA	Diagnosed with BHD	N/A	N/A	111	N/A	N/A	25
Schmidt, 2005 (33)	USA	Diagnosed with BHD and relatives	N/A	N/A	198	N/A	N/A	64
Toro, 2007 (34)	USA	Diagnosed with BHD	97	101	198	28	20	48
Leter, 2008 (36)	Netherlands	Diagnosed with BHD	15	21	36	1	3	4
Ren, 2008 (3)	China	Diagnosed with PSP and relatives	11	12	23	3	11	14
Toro, 2008 (35)	USA	Diagnosed with BHD	52	37	89	N/A	N/A	34
Kluger, 2010 (37)	France	Diagnosed with BHD	10	12	22	2	5	7
Houweling 2011 (38)	Netherlands	Suspected of BHD and relatives	N/A	N/A	115	11	17	28
Tobino, 2012 (39)	Japan	Diagnosed with BHD	14	0	14	11	0	11
Furuya, 2016 (30)	Japan	Suspected of BHD and first-degree relatives	N/A	N/A	312	N/A	N/A	230
Skolnik, 2016 (40)	Canada	One family with members diagnosed with BHD	N/A	N/A	32	N/A	N/A	13
Gupta, 2017 (41)	USA	Diagnosed with BHD	86	18	104	N/A	N/A	79
Geilswick, 2018 (42)	Denmark	BHD diagnosed cohort	57	52	109	26	17	43
Lee, 2019 (43)	R. of Korea	Suspected of BHD	8	4	12	5	3	8
Daccord, 2020 (31)	France	Diagnosed with BHD	46	50	96	30	27	57
Sattler, 2020 (44)	Germany	Diagnosed with BHD and relatives	94	103	197	38	48	86

Three studies included relapses (13, 15, 26), resulting in a higher overall incidence as compared to studies not including this parameter (12, 14, 24, 25, 27, 28). After applying a 29% correction factor to the latter studies, the difference was blunted. We therefore chose to take relapses into account to appraise the true incidence of PSP with the best possible accuracy. This value of 29% was based on a recent meta-analysis on the incidence of PSP (23).

PSP is an acute disease resulting in most cases in complete resolution. Consequently, the prevalence of PSP is usually not a relevant issue clinically, and no data on this parameter were found in the literature. We therefore used data provided by a recent interventional study comparing the outcomes of PSP treated with chest tube vs. observation (10). In this study, the median time to spontaneous resolution of PSP was 30 days, whereas it was 16 days with interventional treatment. Both results were used to calculate the prevalence of PSP. However, we considered that a duration of 30 days reflecting the natural history of the disease was more appropriate to determine a natural phenomenon such as the prevalence of BHD.

Probability of SP in Individuals With BHD

The third component used in the Bayes equation was the probability of having SP in patients with BHD. The meta-analysis included 16 studies with available data on SP. We found a probability of having at least one SP in BHD of 43% (95%CI 0.31–0.54). The reported prevalence rates of SP tended to be higher in pulmonary cohorts (range: 42–76%) (29, 30, 53) than in renal/dermatologic cohorts (range: 23–38%) (32–35, 38), probably due to selection bias. This meta-analysis allowed to appraise more accurately the true probability of SP in BHD independently of its main clinical presentation, and to overcome the selection bias of individual studies. Our analysis confirmed that the occurrence of SP in BHD has no gender predilection, with similar SP rates between men and women of 43 and 41%, respectively.

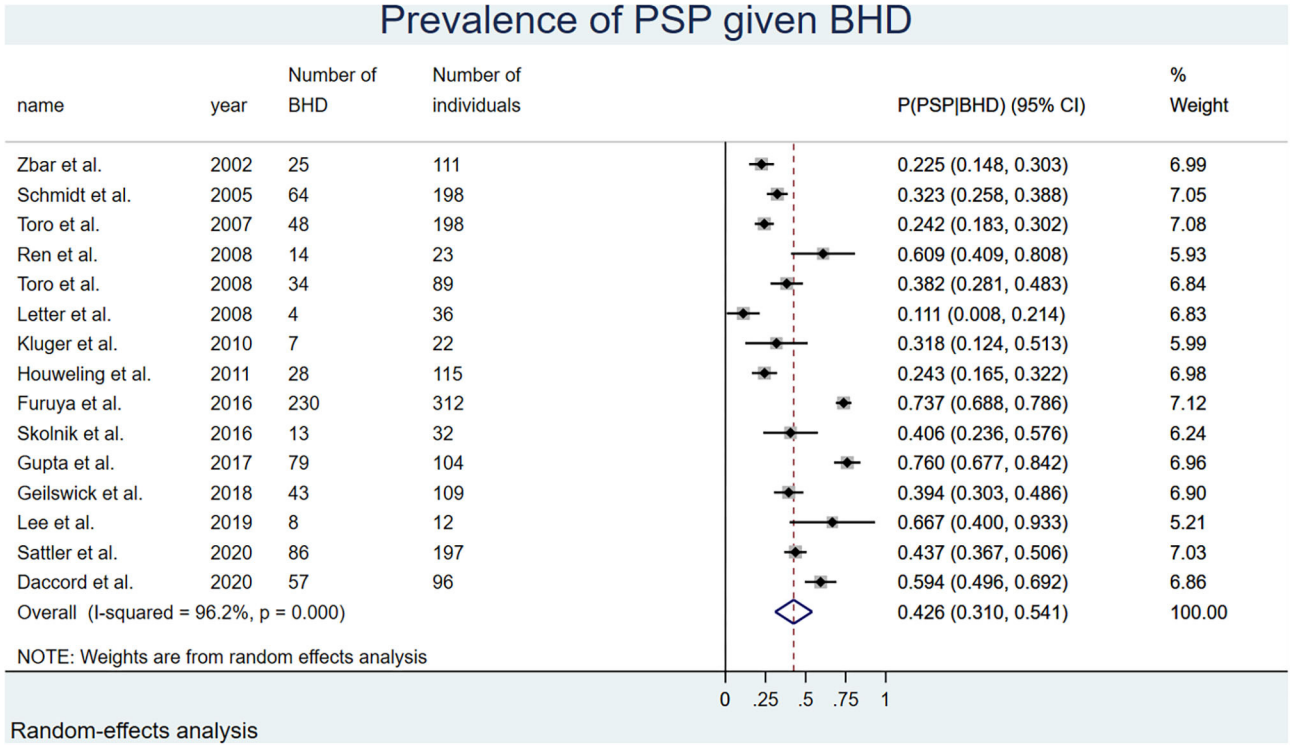

FIGURE 4 | Forest plot of the overall probability of pneumothorax in BHD, random-effects model. Only 15 studies were analyzed, as one study by Tobino et al. (39) included only women.

Prevalence of BHD

The BHD prevalence determined in this study varied moderately according to assumptions made regarding period of publication of studies on PSP incidence, and duration of PSP used to calculate PSP prevalence. The value of about 2 cases per million was the highest among the possible outcomes, but we believe that it is the most reliable. A value of about one case per million was found by pooling all available studies on PSP incidence (both before and after year 2000) and using the lowest value of PSP duration of 16 days. However, all values remained within the same degree of magnitude, thus reinforcing the validity of our observations. We primarily chose to use the random-effects model, but very similar results were observed with the fixed-effect model, demonstrating that our results were independent of the method used. We did not find any previous publication on BHD prevalence for comparison. Very close to our findings, an estimated prevalence of BHD of 1–9 cases per million is mentioned in the Orphanet database, but how this value was determined is not specified (54). Our findings confirm that BHD is a rare disease, with a prevalence similar to or even lower than LAM, which has been estimated to occur in 2.6–7.8 per million women (55–57).

Our study has limitations. The number of studies reporting the probability of BHD in apparent PSP was small, and the number of patients included was also limited. Data about the average age of participants was incomplete in several studies and it was not possible to stratify further the analyses by age. Goodness-of-fit was difficult to assess given the still important heterogeneity even after stratifying and the uselessness of the funnel plot in this context with proportions as outcomes (58). Nevertheless, we believe that stratifying by gender and publication date already accounted for a part of the heterogeneity and provided reasonable effect sizes.

Two components of the Bayes formula exhibited high residual heterogeneity, the prevalence of PSP in the general population and the probability of SP in BHD patients. Unfortunately, most of the heterogeneity could not be explained by the two variables used (before/after year 2000 and gender) to perform the stratification, and no other study variables (such as age) were available to further stratify.

There are several possible explanations for the high residual heterogeneity of the prevalence of PSP in the general population. First, the selected studies have been carried-out in geographic subpopulations that differed importantly regarding the incidence rate of PSP in the general population (as exemplified by the study of Bobbio et al. reporting an IR of 19.78 per 100,000 p-y and that of Melton et al. with an IR of 5.6). This heterogeneity might have resulted from a different exposure to risk factors of PSP such as smoking, air pollution, meteorological conditions, genetic background, or socio-demographic characteristics. Second, different definitions of primary and secondary SP have been used in the various studies, as shown in **Table 3**. Third, criteria used to select the individuals and recruitment settings might also have differed across studies. A fourth source of heterogeneity of the

prevalence of PSP, which we have not investigated, is the possible variability in the duration of a PSP episode across studies.

Regarding heterogeneity of the prevalence of SP in BHD patients, it could be due to different settings of patient enrollment based on the presenting clinical picture (pulmonary vs. cutaneous or renal involvement). Additionally, the risk of SP in BHD may depend on genetic factors having a variable distribution in different subpopulations. For example, genetic variants associated with multiple pneumothorax have been recently identified in European BHD patients (44).

Altogether, our results show that the incidence of PSP in the general population and the probability of SP in BHD are not uniform, and it is likely that there are subpopulations more exposed to the risk of developing these conditions. The end impact of this on the prevalence of BHD in the general population is difficult to apprehend. There might also be subpopulations more exposed to the risk of developing a BHD syndrome due to genetic backgrounds, but it is difficult to answer this question based on our meta-analyses, as a variation in the numerator of the Bayes formula may be compensated by another in the denominator.

In summary, in this first approach of BHD epidemiology, we found a prevalence of BHD of about 2 cases per million, confirming the rarity of this disorder, and the equal distribution between men and women suggested by observational case series. We also believe that the method used in this study provides a new approach to determine the epidemiology of other rare diseases.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

RL and PT: study design. MEM: data collection. PT: statistical analyses. MEM, RL, and PT: data interpretation. MEM, CD, RL, and PT: manuscript writing, revision and final approval of the last version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.631168/full#supplementary-material

Supplementary Figure S1 | Flow diagram depicting the search strategy regarding probability of BHD in apparent PSP.

Supplementary Figure S2 | Forest plot of the prevalence of BHD in apparent PSP, random-effects model, stratified by gender (**A**, in males and **B**, in females).

Supplementary Figure S3 | Flow diagram depicting the search strategy regarding incidence of PSP in the general population.

Supplementary Figure S4 | Forest plot of the incidence rate of PSP for 100,000 person-years with correction for relapses, random-effects model, stratified by gender and period <2000/>2000 (**A**, in males and **B**, in females). Note: due to software features, the values shown in this figure are slightly different from those of the text, as the between-study variance was calculated by the DerSimonian & Laird method, whereas in the text it was computed by restricted maximum likelihood.

Supplementary Figure S5 | Forest plot of the prevalence of PSP for 100,000 person-years with correction for relapses, random-effects model, stratified by gender and period <2000/>2000 (**A**, in males and **B**, in females). Note: due to software features, the values shown in this figure are slightly different from those of the text, as the between-study variance was calculated by the DerSimonian & Laird method, whereas in the text it was computed by restricted maximum likelihood.

Supplementary Figure S6 | Flow diagram depicting the search strategy regarding probability of SP in BHD.

Supplementary Figure S7 | Forest plot of the prevalence of SP in BHD, random-effects model, stratified by gender (**A**, in males and **B**, in females). Due to software features, the values shown in this figure are slightly different from those of the text, as the between-study variance was calculated by the DerSimonian & Laird method, whereas in the text it was computed by restricted maximum likelihood.

Supplementary Table S1 | Bayes equation's components estimated by fixed-effects models.

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