BMPR2 mutation status influences bronchial vascular changes in pulmonary arterial hypertension

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ABSTRACT The impact of bone morphogenetic protein receptor 2 (*BMPR2*) gene mutations on vascular remodelling in pulmonary arterial hypertension (PAH) is unknown. We sought to identify a histological profile of *BMPR2* mutation carriers.

Clinical data and lung histology from 44 PAH patients were subjected to systematic analysis and morphometry.

Bronchial artery hypertrophy/dilatation and bronchial angiogenesis, as well as muscular remodelling of septal veins were significantly increased in PAH lungs carrying *BMPR2* mutations. We found that patients displaying increased bronchial artery remodelling and bronchial microvessel density, irrespective of the mutation status, were more likely to suffer from severe haemoptysis. History of substantial haemoptysis (>50 mL) was significantly more frequent in *BMPR2* mutation carriers. 43.5% of *BMPR2* mutation carriers, as opposed to 9.5% of noncarriers, displayed singular large fibrovascular lesions, which appear to be closely related to the systemic lung vasculature.

Our analysis provides evidence for the involvement of the pulmonary systemic circulation in *BMPR2* mutation-related PAH. We show that *BMPR2* mutation carriers are more prone to haemoptysis and that haemoptysis is closely correlated to bronchial arterial remodelling and angiogenesis; in turn, pronounced changes in the systemic vasculature correlate with increased pulmonary venous remodelling, creating a distinctive profile in PAH patients harbouring a *BMPR2* mutation.

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Introduction

Pulmonary arterial hypertension (PAH; group 1 of the pulmonary hypertension classification) is characterised by precapillary pulmonary hypertension (mean pulmonary arterial pressure (PAP) ⩾25 mmHg) with a normal pulmonary capillary wedge pressure (⩽15 mmHg). Increase of pulmonary vascular resistance (PVR) is paralleled by progressive thickening and occlusion of small pulmonary arteries and arterioles, as well as by fibrous occlusion of pulmonary veins in specific subgroups of PAH, *e.g.* scleroderma-associated PAH [1–3]. The prevalence of PAH is estimated to be 15–50 subjects per million population in Western countries [4, 5]. In 70% of the familial form of PAH a germline mutation of the bone morphogenetic protein receptor type 2 gene (*BMPR2*) is found. In addition, *BMPR2* mutations are identified in 11–40% of sporadic PAH patients, indicating that it is a main predisposing genetic factor for PAH. The penetrance of *BMPR2* mutations ranges from 14% in males and 42% in females [6], suggesting that other factors are probably required to initiate pulmonary vascular remodelling [7].

Studies comparing clinical and haemodynamic data in *BMPR2* mutation carriers and noncarriers have shown that patients harbouring a *BMPR2* mutation are younger at the time of diagnosis, with a more severe haemodynamic compromise (higher mean PAP and PVR) and a lower cardiac index [5, 8–11]. Functional differences in PAH patients carrying a *BMPR2* mutation or not, have been described [12], *e.g.* after acute vasoreactivity testing. In addition, a systematic analysis of pulmonary imaging using computed tomography angiography or magnetic resonance imaging in 129 patients with PAH indicated increased bronchial arterial hypertrophy in *BMPR2* mutation carriers compared with noncarriers [13]. Although clinical and radiological differences between PAH patients with positive and negative *BMPR2* mutation status have been detected, the underlying cause of these observations remains largely unexplained. Therefore, we questioned whether *BMPR2* mutations are linked to specific histological characteristics in the lungs of PAH patients.

Material and methods

Study population

We aimed to study and to compare patients with idiopathic and heritable PAH. Patients from our institution undergoing lung transplantation for PAH between 2005 and 2014 were included in this study. Out of 80 transplanted patients with group 1 PAH, 57 fell into the categories 1.1 (idiopathic PAH) or 1.2 (heritable PAH). 44 had available *BMPR2* mutation status tested at the French pulmonary hypertension reference centre at the time of patient selection and hence were selected for histological analysis [7]. 23 patients were positive for *BMPR2* gene mutation status and constituted the carrier group; mutation details for each patient are summarised in table 1. 21 patients tested negative and constituted the noncarrier group. Clinical and haemodynamic data were retrieved from the registry of the French pulmonary hypertension centre (Hôpital Bicêtre, Le Kremlin-Bicêtre, France). This study was approved by the local ethics committee. All patients underwent genetic counselling.

Histology, immunohistochemistry and morphometry

In all 44 patients, lung explants had been formalin-fixed (10% neutral buffer) and parenchymal samples were collected from all lobes and in random fashion regarding orienting macroscopic structures (*e.g.* bronchioli, vessels and septa) and regarding the section angle, in order to avoid systematic sampling of the same areas and systematic observation from the same angle. For our study, seven haematoxylin and eosin-stained slides from each case were randomly chosen and subjected to histopathological assessment and morphometry. Selected slides were examined by two field-experienced pathologists in a blinded fashion (MRG and PD). The morphologic items examined included patent lumen fraction of pulmonary arteries (arteries 70–300 µm in diameter), density of plexiform lesions, degree of arterial inflammation, density of septal veins displaying muscular hyperplasia, hypertrophy of bronchial arteries and bronchial microvessel density. Further details are described in the online supplementary material.

For assessment of bronchial microvessel density, remodelled vein density and singular millimetric fibrovascular lesions (SiMFis) lesions immunohistochemical stainings were performed: 5-µm sections were dewaxed and rehydrated progressively. Citrate buffer (pH 6.0) was used for antigen retrieval, and endogenous peroxidase was quenched with hydrogen peroxide. Sections were blocked using 5% bovine serum albumin and incubated overnight with monoclonal antibody against PECAM-1 (CD31, as recommended by the manufacturer; Invitrogen, Cergy-Pontoise, France) or actin (smooth muscle actin, as recommended by the manufacturer; Thermo Scientific, Asnières-sur-Seine, France).

Ink-injection procedure

The right lower lobe of one patient from the *BMPR2* mutation carrier group was injected within 5 min following right lung explantation (during bipulmonary transplantation): the inferior lobar pulmonary artery was identified at the lung hilus and explored using a 0.7 mm Jelco intravenous catheter (Smiths Medical International, Ashford, UK). After catheterisation, a 2-mL syringe filled with black ink was

TABLE 1 *BMPR2* mutations identified

Case	Mutated gene	Mutation location	Nucleotide change	Amino acid change	Mutation category
1	*BMPR2*	Exon 110	c.1471C>T	p.Arg491Trp	Missense
2	*BMPR2*	Exon 11	c.1471C>T	p.Arg491Trp	Missense
3	*BMPR2*	Exon 1	c.48G>A	p.Trp16X	Nonsense
4	*BMPR2*	Exon 12	c.2617C>T	p.Arg873X	Nonsense
5	*BMPR2*	Exon 11–13	Deletion of exons 11–13	Deletion of exons 11–13	Large rearrangement
6	*BMPR2*	Exon 3	c.339C>A	p.Tyr113X	Nonsense
7	*BMPR2*	Exon 6	c.631C>T	p.Arg211X	Nonsense
8	*BMPR2*	Exon 12	c.2521_2522dupCA	p.Arg842IlefsX18	Nonsense
9	*BMPR2*	Exon 3	Deletion of exon 3	Deletion of exon 3	Large rearrangement
10	*BMPR2*	Exon 8	c.901T>C	p.Ser301Pro	Missense
11	*BMPR2*	Exon 3	c.418+5G>A	p.Cys84_Ser140del	Splice defect
12	*BMPR2*	Exon 8	c.994C>T	p.Arg332X	Nonsense
13	*BMPR2*	Exon 6	Deletion of exon 6	Deletion of exon 6	Large rearrangement
14	*BMPR2*	Exon 11–13	Deletion of exons 11–13	Deletion of exons 11–13	Large rearrangement
15	*BMPR2*	Exon 7	c.901T>C	p.Ser301Pro	Missense
16	*BMPR2*	Exon 3	c.320C>G	p.Ser107X	Nonsense
17	*BMPR2*	Exon 11	c.1471C>T	p.Arg491Trp	Missense
18	*BMPR2*	Exon 3	c.407_408del	p.Thr136AsnfsX10	Nonsense
19	*BMPR2*	Exon 3	c.280T>G	p.Cys94Gly	Missense
20	*BMPR2*	Exon 10	Deletion of exon 10	Deletion of exon 10	Large rearrangement
21	*BMPR2*	Intron 3	c.418+3A>T	Splice defect	Splice defect
22	*BMPR2*	Exon 7	c.961C>T	p.Arg321X	Nonsense
23	*BMPR2*	Exon 8	c.1001T>G	p.Leu334X	Nonsense

manually injected into the vessel, which was clamped eventually. The lung was then formalin-fixed by airway injection. Dissection was performed after 24 h of fixation.

Haemodynamic parameters

Haemodynamic evaluation by right-heart catheterisation was performed at baseline, as well as 3–6 months before transplantation, according to previously described protocols of the French referral centre [14].

Statistical analysis

The differences between two groups of patients were calculated by using the nonparametric Mann–Whitney U-test. The dotplots present median and interquartile range. Correlations between two histological items were calculated using the Spearman correlation coefficient. All statistical calculations were conducted using Prism 6 software (GraphPad, San Diego, CA, USA).

Results

Clinical and haemodynamic characteristics of transplanted PAH patients

Details of demographics, New York Heart Association (NYHA) functional class and specific PAH treatment at the time of lung transplantation are summarised for each patient in table 2. Mean±SD age at PAH diagnosis was 28.5±11.5 years and age at lung transplantation was 35.2±11.7 years. At time of lung transplantation, patients were mainly in NYHA functional class III/IV (n=41; 93%). 29 patients received triple therapy with prostacyclin, endothelin receptor antagonists (ERA), and type 5 phosphodiesterase inhibitors (PDE5-i), eight patients received dual therapy with prostacyclin and ERA, two patients received dual therapy with ERA and PDE5-i, 2 patients received dual therapy with prostacyclin and PDE5-i, one patient reveived a monotherapy with prostacyclin and one patient received a monotherapy with PDE5-i. Moreover, one patient had not been treated with specific PAH medication before transplantation. *BMPR2* mutation carriers and noncarriers were of similar age at PAH diagnosis, as well as at lung transplantation, and presented with same haemodynamic characteristics, except for a higher PVR at diagnosis in *BMPR2* mutation carriers (table 3). Moreover, *BMPR2* mutation carriers experienced a history of severe haemoptysis (>50 mL) more frequently than noncarriers (43% *versus* 14%, respectively, p=0.034).

PAH patients carrying a BMPR2 *mutation show a similar degree of pulmonary arterial remodelling compared to the noncarrier group*

We found no difference in patency of distal pulmonary arteries of the muscular type in *BMPR2* mutation carriers and noncarriers (median 6.3% *versus* 6.9% of patent lumen area) (figure 1). In addition, no

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TABLE 2 Details of demographic characteristics, New York Heart Association (NYHA) functional class and specific pulmonary arterial hypertension (PAH) treatment at the time of lung transplantation

Patient	Sex	Disease span years	Age at diagnosis years	Age at transplantation years	NYHA functional class	Specific PAH therapy	*BMPR2* mutation
1	M	2	46	48	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
2	M	3	38	41	III–IV	*i.v.* prostacylin, ERA, PDE5-i	Yes
3	M	3	52	55	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
4	F	7	27	34	III	*s.c.* prostacyclin, ERA, PDE5-i	Yes
5	F	8	38	46	III – IV	*i.v.* prostacylin, ERA, PDE5-i	Yes
6	F	10	8	18	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
7	F	9	35	44	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
8	F	9	21	30	III	*i.v.* prostacylin, ERA	Yes
9	M	1	17	18	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
10	M	17	21	38	III	Other prostanoid, ERA, PDE5-i	Yes
11	F	12	40	52	III	*s.c.* prostacyclin, ERA, PDE5-i	Yes
12	F	3	36	39	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
13	F	4	22	26	IV	No specific PAH therapy	Yes
14	F	4	34	38	IV	*s.c.* prostacyclin, PDE5-i	Yes
15	M	6	29	35	IV	*i.v.* prostacylin, ERA, PDE5-i	Yes
16	F	3	28	31	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
17	F	2	30	32	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
18	M	7	10	17	II	ERA, PDE5-i	Yes
19	M	12	32	44	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
20	F	11	26	37	III	*i.v.* prostacylin, ERA, PDE5-i	Yes
21	F	13	3	16	III	*i.v.* prostacylin, ERA	Yes
22	M	6	48	54	III	*i.v.* prostacylin, ERA	Yes
23	F	4	16	20	III	*i.v.* prostacylin, ERA	Yes
24	F	5	36	41	IV	*i.v.* prostacylin, ERA	No
25	F	2	29	31	IV	*i.v.* prostacylin, ERA, PDE5-i	No
26	M	3	31	34	II	PDE5-i	No
27	F	16	23	39	III	*i.v.* prostacylin, ERA, PDE5-i	No
28	M	2	24	26	III	*i.v.* prostacylin, ERA, PDE5-i	No
29	F	4	27	31	III	*i.v.* prostacylin, ERA, PDE5-i	No
30	F	13	35	48	III	*i.v.* prostacylin, PDE5-i	No
31	F	15	26	41	III	*i.v.* prostacylin, ERA, PDE5-i	No
32	M	4	42	46	IV	*i.v.* prostacylin, ERA, PDE5-i	No
33	M	5	12	17	II	ERA, PDE5-i	No
34	M	11	11	22	III	*i.v.* prostacyclin, ERA, PDE5-i	No
35	M	0.5	22	22	III	*i.v.* prostacylin, ERA, PDE5-i	No
36	M	7	43	50	III	*i.v.* prostacylin, ERA	No
37	F	5	33	38	IV	*i.v.* prostacylin, ERA, PDE5-i	No
38	F	6	19	25	III	*i.v.* prostacylin, ERA	No
39	F	4	15	19	III	*i.v.* prostacylin, ERA, PDE5-i	No
40	F	5	32	37	III	*i.v.* prostacylin, ERA, PDE5-i	No
41	F	5	39	44	III	*i.v.* prostacylin, ERA, PDE5-i	No
42	F	16	31	47	III	*i.v.* prostacylin, ERA, PDE5-i	No
43	F	7	50	57	III	*i.v.* prostacylin, ERA	No
44	F	3	19	21	IV	*i.v.* prostacylin	No

M: male; *i.v.*: intravenous; ERA: endothelin receptor antagonists; PDE5-i: phosphodiesterase-type 5 inhibitor; F: female; *s.c.*: subcutaneous.

difference was observed in the average density of plexiform lesions in our two groups (0.69 *versus* 0.51 lesions·cm^{-2} in *BMPR2* carriers *versus* noncarriers, respectively) (figure 2a–d). Importantly, we observed SiMFis of unparalleled morphology in 12 (27.3%) out of 44 patients: the lesions were easily detectable at lowest magnification (objective ×2.5) (figure 2e–i), ranging from 1 to 2 mm in their largest diameter. They displayed multiple large vascular channels with broad, fibrous or hyalin vessel walls and narrowed lumen. Other than that, these vascular conglomerations were surrounded by collagen-rich connective tissue (orange colour in haematoxylin–eosin–saffron (HES) staining), fortifying the fibrous aspect of the lesion (figure 2e–g). Frequently, SiMFis were topographically close to a bronchiole. Immunohistochemical analysis showed a large conglomerate of CD31-positive vessels with an important muscular, actin-positive component (figure 2g–j). There was no glomeruloid pattern with predominant

TABLE 3 Mean values of demographic and haemodynamic data from transplanted *BMPR2* mutation carriers and noncarriers

	BMPR2 mutation carriers	Noncarriers	p-value
Subjects	23	21	
Age at diagnosis years	28.6±12.7	28.5±10.4	NS
Age at transplant years	35.3±12.0	35.0±11.5	NS
Sex F/M	14/9	14/7	NS
History of haemoptysis >50 mL %	43	14	0.034
mPAP at diagnosis mmHg	63±13.4	64±19.5	NS
mPAP at last evaluation mmHg	58±13.8	64±22.8	NS
PVR at diagnosis Wood units	17.3±5.6	15.3±6.2	0.05
PVR at last evaluation Wood units	13.2±6.3	16.0±5.2	NS
PCWP mmHg	10.5±4.4	7.4±2.6	NS
Cardiac index at diagnosis L·min^{-1}·m^{-2}	1.97±0.6	2.36±0.8	NS
Cardiac index at last evaluation L·min^{-1}·m^{-2}	2.74±0.5	2.74±0.9	NS
***D*LCO % predicted**	65.1±10.7	66.2±10.5	NS

Data are presented as n or mean±SD, unless otherwise stated. NS: nonsignificant; F: female; M: male; mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; PCWP: pulmonary capillary wedge pressure; *D*LCO: diffusing capacity of the lung for carbon monoxide.

endothelial cell proliferation, as can be observed in plexiform lesions. We injected black dye into a lobar pulmonary artery at the lung hilus, right after lung explantation in one of the *BMPR2* mutation carriers from the cohort: after analysis of HES-stained paraffin-slides, we were able to retrieve an injected SiMFis. The arterially delivered dye could be traced towards adjacent bronchial vessels, as well as towards a nearby septal pulmonary vein, highlighting a functional, or at least patent conncetion of these three vascular compartments (figure 3).

When sorting for mutation status, we found that 10 (43.5%) out of 23 *BMPR2* carriers and two (9.5%) out of 21 noncarriers were harbouring SiMFis.

PAH patients carrying a BMPR2 *mutation show a similar degree of perivascular inflammation as the noncarrier group*

Since the degree of perivascular inflammation has been demonstrated to correlate with disease severity, and in particular with pulmonary vascular wall remodelling and thickness, we next studied pulmonary artery inflammation score as previously described [15]. Consistent with the similar degree of pulmonary arterial remodelling, we did not find discrepancies between the *BMPR2* mutation carriers and noncarriers and no correlation between inflammation and any morphometric parameter was observed (data not shown).

Increase of bronchial arterial hypertrophy/dilatation and bronchial microvessel density in PAH patients carrying a BMPR2 *mutation*

Since the bronchial and pulmonary circulations are connected through bronchopulmonary anastomoses on the precapillary, the capillary and the postcapillary level, which become functional during obstructive

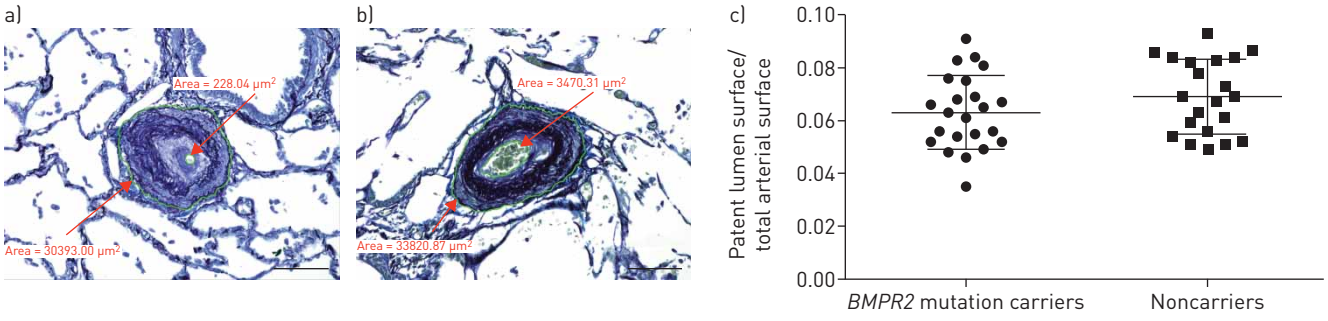

FIGURE 1 Histology and morphometry of pulmonary arteries of the muscular type from patients with pulmonary arterial hypertension (Weigert's elastic staining). Arterial obstruction through intimal and medial thickening in a) a patient harbouring a *BMPR2* mutation and b) a noncarrier. The patent lumen area and the total area of the arteries are measured; in this example, patent lumen fraction is <1% (a), and <10% (b). c) Degree of patent lumen fraction in *BMPR2* mutation carriers (n=23) and in noncarriers (n=21): no significant difference is observed between the two groups (p=0.105). Data are presented as median (interquartile range). Scale bars=100 µm.

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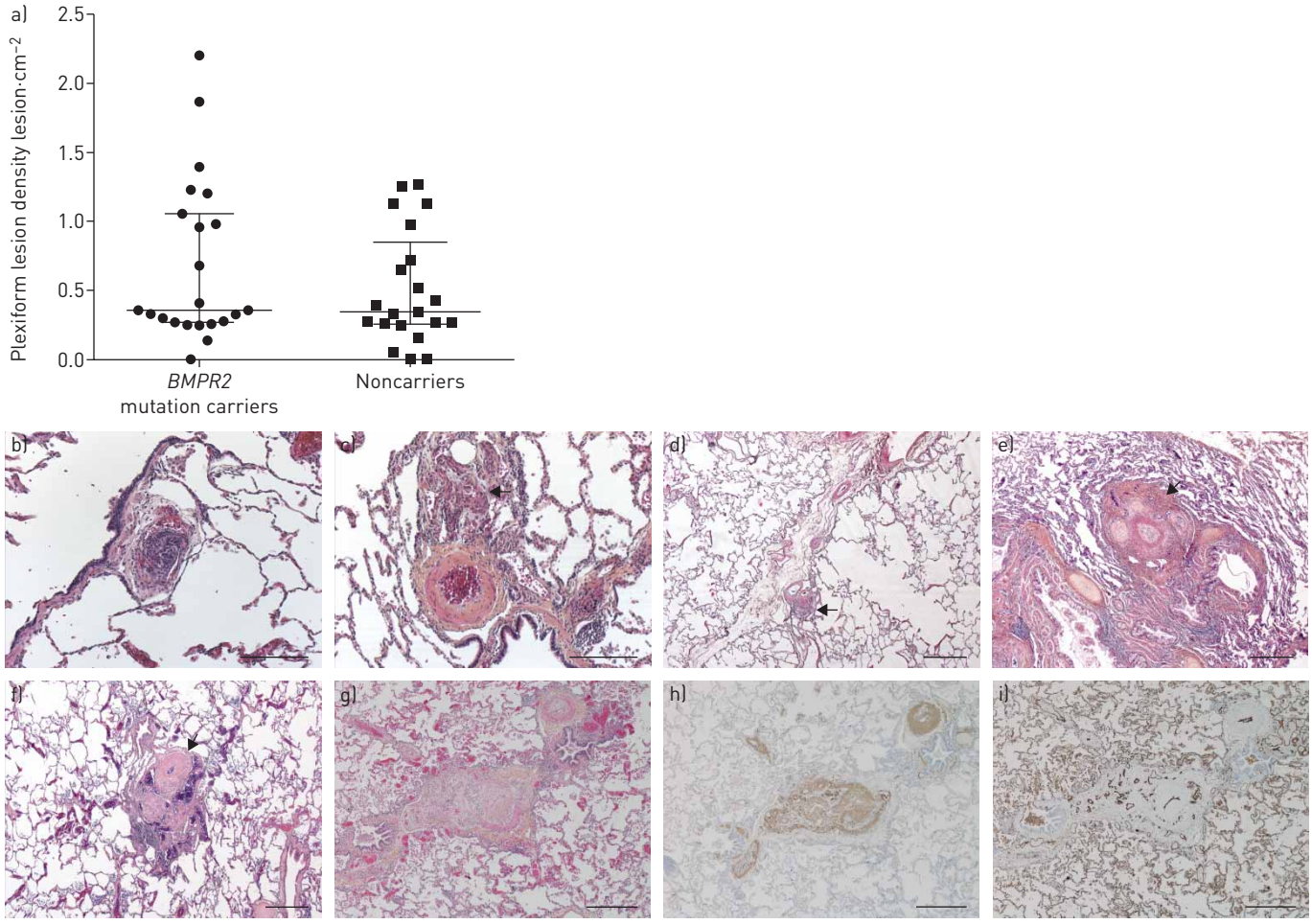

FIGURE 2 Morphometry and histology of plexiform and singular millimetric fibrovascular lesions (SiMFis) in lungs from patients with pulmonary arterial hypertension. a) Plexiform lesion density in *BMPR2* mutation carriers and in noncarriers: no significant difference is observed between the two groups (p=0.488); data are presented as median (interquartile range). b–d) Plexiform lesions close to pulmonary arteries in *BMPR2* mutation (b) carriers and (c) noncarriers; d) exceptional periseptal location of a plexiform lesion in a mutation carrier. e–g) Haematoxylin–eosin–saffron stainings showing SiMFis: large fibrous lesions comprising vascular channels with a narrow lumen and dilated vessels, usually found close to small-sized bronchi; all taken from mutation carriers; collagen is highlighted by orange colour from saffron. h, i) Serial sections of (g) stained by immunohistochemistry using antibodies against (h) actin and (i) CD31, in order to highlight smooth muscle/myofibroblast components and endothelial cells, respectively. SiMFis do not show a classical glomeruloid pattern with predominant/important endothelial cell proliferation as seen in plexiform lesions, but rather a large, collagen-rich conglomerate of vessels with a prominent muscular component. Scale bars=200 μm (b, c) and 500 μm (d–i).

events, we compared bronchial artery remodelling in our patients (when central sections of segmental bronchi were available): 20 out of 23 PAH patients carrying a *BMPR2* mutation and 20 out of 21 PAH patients without identified *BMPR2* mutation were analysed. A greater degree of bronchial arterial surface per bronchial surface was observed in *BMPR2* mutation carriers (p=0.0019) (figure 4). In addition, computer-based semiautomated quantitative assessement on anti-CD31 immunostained slides from 20 *BMPR2* mutation carriers and from 18 noncarriers revealed that bronchial microvessel density was increased in bronchial walls of PAH patients carrying a *BMPR2* mutation compared to the noncarrier group (p=0.0002) (figure 5). In 44 patients with PAH, we found 13 patients with recent history of haemoptysis >50 mL, of which 10 harboured a *BMPR2* mutation. We also found that patients with episodes of haemoptysis >50 mL showed higher degree of bronchial artery hypertrophy/dilatation (p=0.0006) and bronchial microvessel density (p=0.001) irrespective of their *BMPR2* status (figure 6).

When comparing morphological parameters, we revealed a close association of bronchial artery hypertrophy/dilatation or degree of bronchial microvessel density and presence of SiMFis (figure 7a and b). Patients with SiMFis displayed significantly increased bronchial artery hypertrophy as compared with patients lacking SiMFis (p=0.012); the same association was found for SiMFis and bronchial microvessel density (p=0.003). In contrast, no association was found between plexiform lesion density and hypertrophy of bronchial arteries or bronchial microvessel density (data not shown).

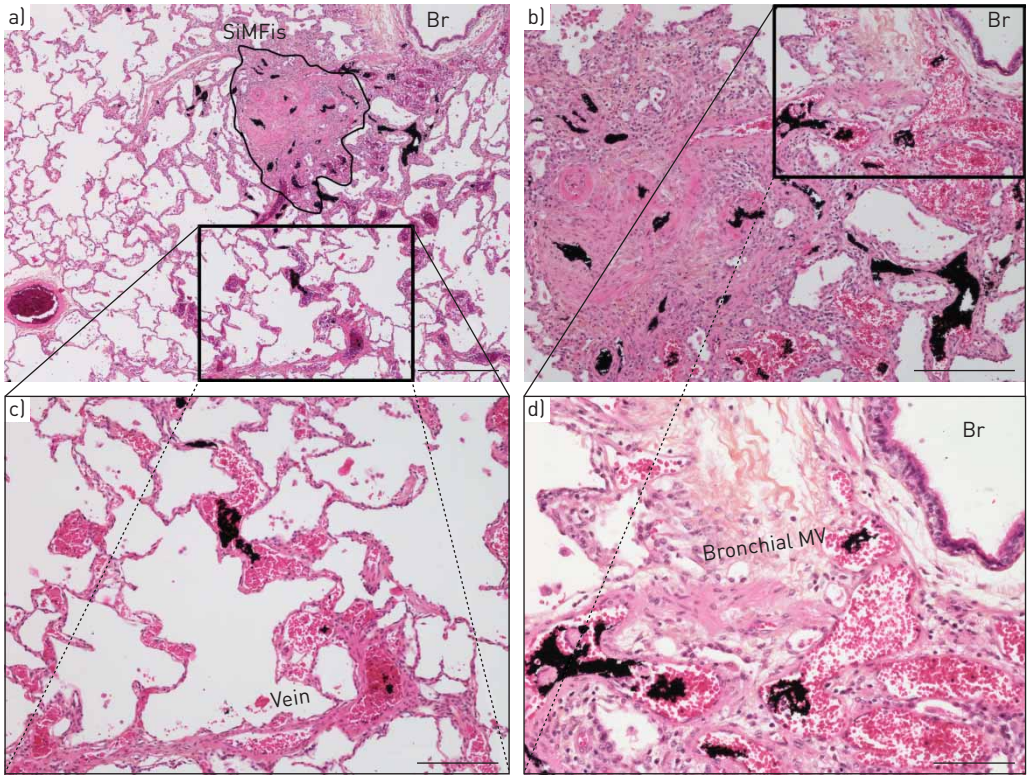

FIGURE 3 Ink-injected pulmonary lobe of a patient harbouring a *BMPR2* mutation. a) Overview comprising a singular millimetric fibrovascular lesions (SiMFis): black dye is present within the numerous vascular lumina of the lesion; b) and d) magnification of the lesion core: the intraluminal black dye can be traced up to subepithelial, thin-walled vessels of the adjacent bronchioles (Br), topographically and anatomically corresponding to bronchial venules. c) magnification of a lower area from (a) (box): to the opposite side of the bronchiole, the intraluminal black dye can be traced from the SiMFis to a septal pulmonary vein. MV: microvasculature. Scale bars=500 µm (a), 250 µm (b), 200 µm (c) and 100 µm (d).

When correlating haemodynamic parameters, such as mean PAP, PVR or cardiac index with bronchial artery hypertrophy/dilatation or degree of bronchial microvessel density no significant association was found. A comparison of haemodynamic parameters in patients with and without SiMFis lesions did not show any significant differences (data not shown).

With regard to possible predominant mutation types in patients with increased bronchial vascular remodelling or presence of SiMFis, no specific pattern was found: missense and nonsense mutations were both present (table 1).

Muscular remodelling of pulmonary veins is more extensive in PAH patients carrying a BMPR2 *mutation*

We determined the density of veins with smooth muscle hyperplasia per interlobular septum in all 44 PAH patients (figure 8a and b) and in seven control patients (data not shown). We found a substantial increase in their number in patients with PAH carrying a *BMPR2* mutation as compared with noncarriers ($p=0.008$) (figure 8c). Importantly, we observed that an increase of venous muscular remodelling closely correlated with bronchial microvessel density ($r=0.82$; $p<0.0001$) and bronchial artery hypertrophy ($r=0.60$, $p<0.0001$), independently of *BMPR2* status (figure 8d and e). In many patients presenting with smooth muscle hyperplasia of pulmonary veins we also observed irregular, collagen-rich thickening of the intima, corresponding to intimal fibrosis; however, this histological feature was very heterogeneously distributed and difficult to quantify. We therefore limited our assessment to the more homogeneous hyperplasia of the venous media.

Discussion

Our study is the first to focus on histopathological characteristics of heritable PAH patients harbouring a *BMPR2* mutation, compared with noncarriers suffering from idiopathic PAH. We found a substantial increase of bronchial artery hypertrophy/dilatation and bronchial microvessel density in *BMPR2* mutation carriers, which was closely related to pulmonary venous remodelling. Our results

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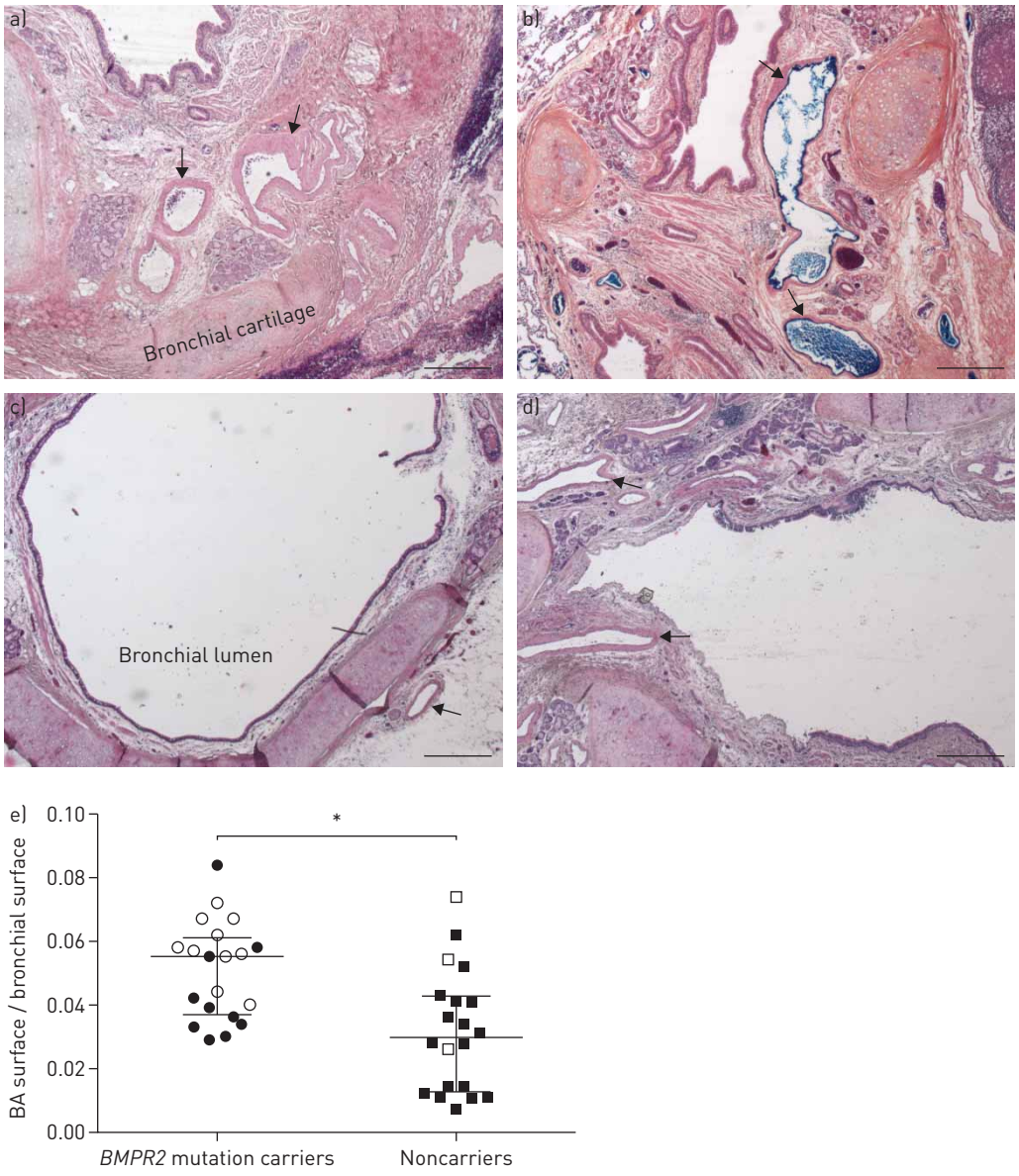

FIGURE 4 Histology and morphometric results of bronchial vasculature from patients with pulmonary arterial hypertension. a, b) dilated and thickened bronchial arteries (arrows) in *BMPR2* mutation carriers; b) hilar bronchial arteries of one lung transplant specimen were injected with blue dye; c) bronchial artery in a lung transplant specimen from a noncarrier (arrow); d) dilated and moderately thickened bronchial arteries in lungs from a noncarrier (arrows); e) bronchial artery remodelling in *BMPR2* mutation carriers and in noncarriers. A higher degree of bronchial artery remodelling is observed in *BMPR2* mutation carriers (p=0.0019); white circles and white squares indicate patients with positive history of haemoptysis. Data are presented as median (interquartile range). *: p<0.05.

demonstrate that haemoptysis is more frequently encountered in PAH patients carrying *BMPR2* mutation. In addition, we newly identify unusual fibrovascular lesions, which are overrepresented in *BMPR2* mutation carriers.

Typical histological traits characterising PAH include pulmonary arterial wall thickening, plexiform lesions, perivascular arterial inflammation and, to some extent, pulmonary vein remodelling. These classic features have been studied extensively in PAH irrespective of genetic mutation status [15–21]. In the current study, no difference was found in the degree of pulmonary arterial patency or of perivascular inflammatory cell infiltration between PAH patients, whether or not they carried a *BMPR2* mutation. Studies indicate a potential role for BMPR2 in the promotion of inflammation, a phenomenon that may explain the predisposition of *BMPR2* carriers to develop PAH [22–25]. The tissue sections we studied were from patients with end-stage PAH receiving drug therapy; hence we cannot exclude that the degree of

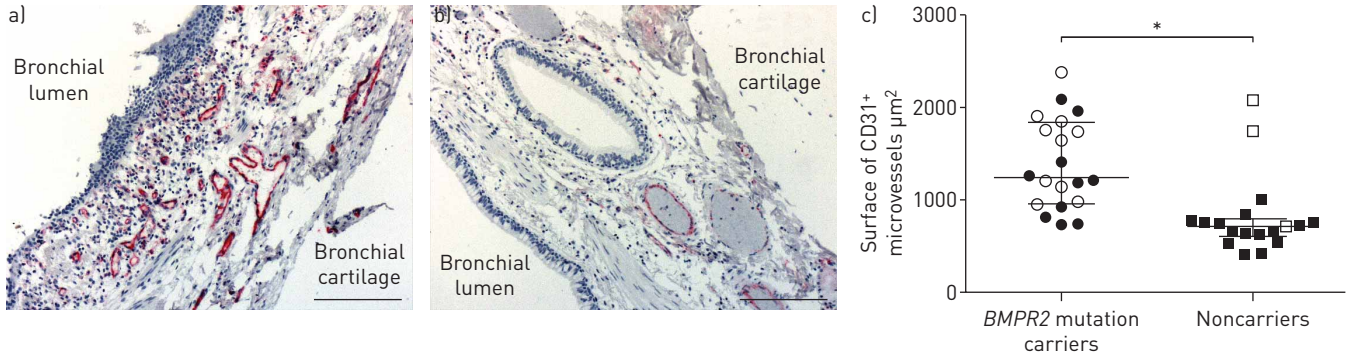

FIGURE 5 Histology and morphometry of bronchial microvasculature from patients with pulmonary arterial hypertension; a) CD31 immunostaining (red) in a *BMPR2* mutation carrier showing increased bronchial microvessel density; b) bronchial microvessel density is less pronounced in lungs from a noncarrier patient. Scale bars=200 µm. c) Bronchial microvessel density in *BMPR2* mutation carriers and noncarriers; a higher density of microvessels is observed in the bronchial wall of *BMPR2* mutation carriers (p=0.0002); white circles and white squares indicate patients with positive history of haemoptysis. Data are presented as median (interquartile range). *: p<0.05.

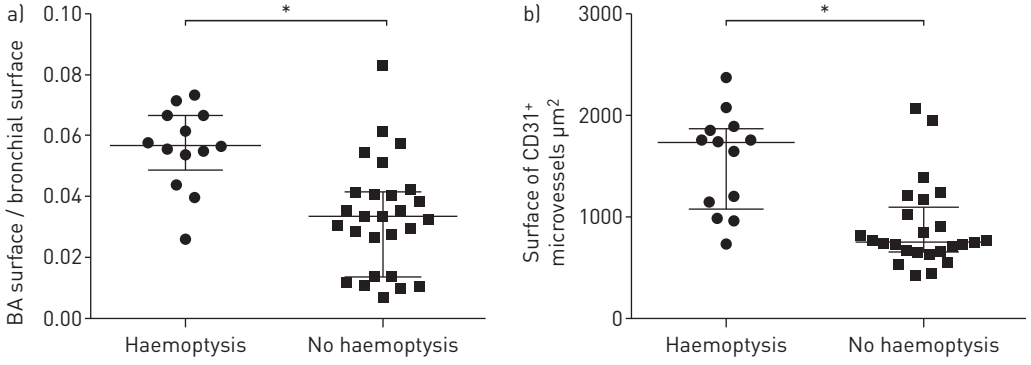

FIGURE 6 Association of bronchial vascular remodelling and history of haemoptysis in all patients. a) Patients with haemoptysis display a significantly higher degree of bronchial artery hypertrophy/dilatation (p=0.0006); b) patients with hemoptsysis display a significantly higher degree of bronchial microvessel density (p=0.001). Data are presented as median (interquartile range). *: p<0.05.

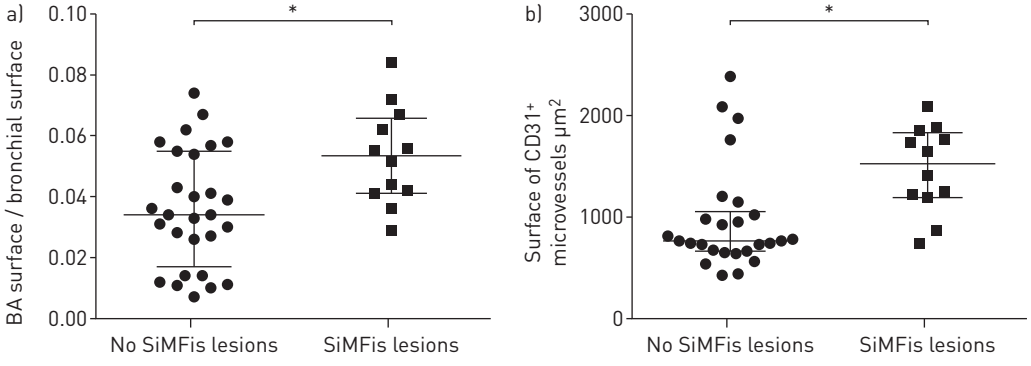

FIGURE 7 Degree of bronchial artery remodelling and microvessel density in patients with and without singular millimetric fibrovascular lesions (SiMFis). a) Patients with SiMFis display significantly increased bronchial artery hypertrophy/dilatation compared with patients lacking SiMFis (p=0.012); b) patients with SiMFis display significantly increased bronchial microvessel density compared with patients lacking SiMFis (p=0.003). Data are presented as median (interquartile range). *: p<0.05.

pulmonary arterial patency or of perivascular inflammatory cell infiltration is different in early stages of pulmonary vascular remodelling in PAH.

Although their pathophysiological significance and origin are far from being elucidated, classically, plexiform lesions have been considered as markers of PAH progression and severity [16, 26]. We

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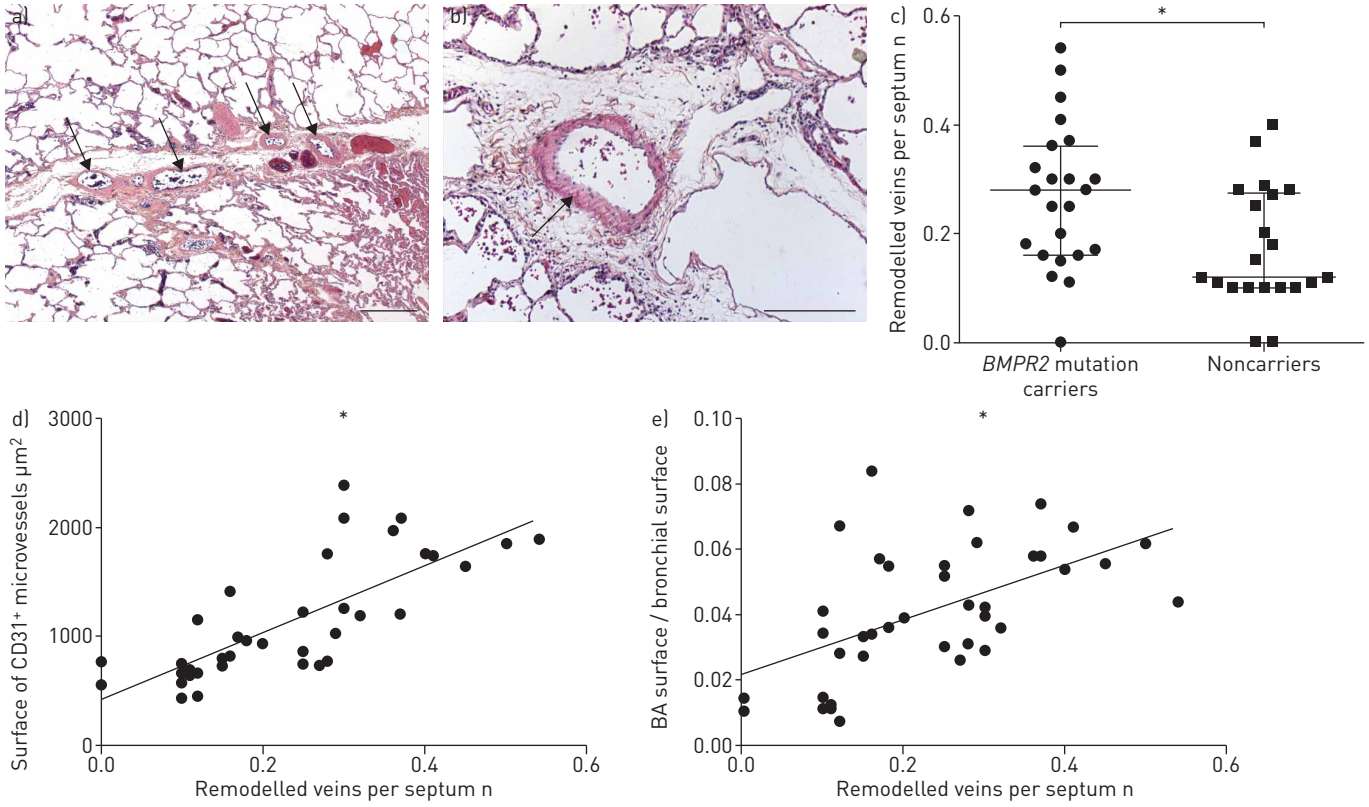

FIGURE 8 Muscular remodelling of septal veins in pulmonary arterial hypertension patients. a) Muscular hyperplasia of a septal vein in a *BMPR2* mutation carrier (arrows); scale bar=500 µm. b) Septal vein in a noncarrier (arrow); scale bar=200 µm. c) Muscular remodelling of septal veins in *BMPR2* mutation carriers and in noncarriers: a significantly higher degree of septal vein remodelling in *BMPR2* mutation carriers is observed (p=0.008). Data are presented as median (interquartile range). d) Correlation between bronchial microvessel density and number of remodelled veins in all patients: a significant positive correlation is observed (r=0.82; p<0.0001); e) correlation between the number of remodelled veins and bronchial artery remodelling in all patients: a significant positive correlation is observed (r=0.60; p<0.0001). *: p<0.05.

found that the density of plexiform lesions is comparable in lung samples from *BMPR2* mutation carriers and noncarriers.

Importantly, we observed SiMFis of unusual morphology more frequently in *BMPR2* mutation carriers *versus* noncarriers. The acronym SiMFis comprises the most distinguishing characteristics of the lesion: "singular", meaning rare and of unparalleled morphology; we never found more than one lesion per slide, and sometimes only two or three per seven analysed slides; "millimetric", meaning that these lesions ranged between 1 and 2 mm in diameter, while classic pulmonary arterial lesions in PAH concern arteries <500 µm in diameter, and even more frequently arteries that measure ⩽250 µm; and "fibrovascular", because these lesions were extremely rich in collagen and displayed more than one vascular channel. Note that SiMFis did not show a classic glomeruloid pattern with predominant endothelial cell proliferation as seen in plexiform lesions, but rather a large conglomerate of hypertrophic vessels. The collagen-rich, scar-like appearance could of course indicate that the lesion is a product/relic of earlier arterial lesions. However, we feel that there is more to it: performing an ink injection experiment in a freshly explanted lung from one of our patients allowed us to highlight a patent connection between SiMFis, bronchial/systemic vessels and pulmonary septal veins (figure 3). We compared morphometry and found that patients displaying SiMFis had an increased amount of bronchial microvessels and showed increased hypertrophy of larger bronchial arteries. Thus, SiMFis could be directly related to hypertrophy and/or angiogenesis (increase in number of bronchial microvessels) of vasa vasorum/bronchial arteries in the vicinity of the diseased artery.

We do not necessarily think that SiMFis have a more important impact on disease evolution than other arterial lesions, but we suspect them to be an extreme form of connection/anastomoses between pulmonary arteries, bronchial vessels and pulmonary veins. They appear to be typical of heritable PAH. Another pulmonary hypertension group we have meticulously studied and that has a most prominent systemic vascular hypertrophy is chronic thromboembolic pulmonary hypertension (CTEPH): no SiMFis lesions were found in 17 patients with this condition [27]. However, we believe that the link between

bronchial artery hypertrophy and venous muscular hyperplasia might be similar in both conditions: 1) primary occlusion of a pulmonary artery; 2) resulting hypertrophy of the bronchial vasculature and the vasa vasorum (and in some areas evolution into SiMFis), *e.g.* through hypoxia; 3) draining of the increased systemic bloodflow into pulmonary veins; and 4) resulting muscular hyperplasia of pulmonary veins.

Bronchial artery remodelling and extent of bronchial microvessel density in terms of histology have never been systematically explored in lungs from PAH patients, even though an increase of hypertrophic bronchial arteries has been recently reported in *BMPR2* mutation carriers with PAH. To *et al.* [13] reviewed 129 cases of PAH and showed that haemoptysis in PAH was associated with bronchial artery hypertrophy on computed tomography angiograms, and that the latter was more frequently observed in *BMPR2* mutation carriers. In our cohort of 44 PAH patients having undergone lung transplantation, positive history of haemoptysis was more frequent in *BMPR2* mutation carriers, compared to noncarriers. This direct and significant association between mutation status and history of haemoptysis may be more pronounced in our study than in the above-mentioned article, due to the inevitable selection bias of transplantation and hence disease progression and/or severity. Importantly, we found a marked increase in bronchial artery hypertrophy/dilatation and bronchial microvessel density in PAH patients carrying a *BMPR2* mutation: bronchial arteries were enlarged and displayed important wall thickening. Automated immunohistochemistry-based quantification of systemic microvessels within the bronchial walls revealed a significant increase of the latter in the *BMPR2* mutation carriers, paralleling the remodelling of larger systemic arteries. Multiplication of microvessels within the bronchial wall has been described for inflammatory and ischaemic diseases of the lungs such as asthma, pulmonary fibrosis and chronic thromboembolic pulmonary hypertension, respectively [27–31]. Furthermore, human studies and animal models of these conditions have shown that an increase in bronchial microvessels is due to a mediator-triggered angiogenetic process involving predominantly chemokines with a CXC-motif and growth factors such as vascular endothelial growth factor (VEGF) [30, 32–34]. Our findings suggest that in PAH patients harbouring *BMPR2* mutations bronchial angiogenesis is more prevalent compared to PAH patients lacking these mutations. In this context, it is important to emphasise that functional BMPR2, when binding to its ligand bone morphogenetic protein-9 supresses VEGF expression, an established stimulating growth factor in bronchial angiogenesis [35]. Of note, a direct relationship between the degree of bronchial vessel remodelling and disease severity was not observed in our study: no correlation was found between bronchial arterial remodelling/bronchial microvessel density and haemodynamic parameters, such as mean PAP, PVR or cardiac index (data not shown). Moreover, a comparison of these haemodynamic parameters in patients displaying or lacking SiMFis showed no difference (data not shown). Hence, a shortcut between remodelling of the systemic lung vasculature and severity of disease appears improbable. In contrast, patients with episodes of haemoptysis from our cohort showed a higher degree of bronchial artery remodelling and angiogenesis irrespective of their *BMPR2* status.

Our group has shown that bronchial artery hypertrophy and bronchopulmonary shunting were associated with pulmonary venous changes, including intimal fibrosis of septal veins and venular remodelling [27]. Interestingly, these post-capillary changes were also found in *BMPR2* mutation carriers more frequently than in noncarriers. The concordance of morphological changes in the bronchial and in the pulmonary venous vasculature appears to be related, independent of a specific disease entity: we have recently shown in patients with CTEPH that remodelled pulmonary veins are functionally connected to bronchial vessels through anastomoses [27]. The quantitatively unprecedented functional connection to systemic vessels could lead to a proliferative reaction of the low-pressure accustomed pulmonary veins, which could explain increased smooth muscle hypertrophy (and intimal fibrosis). Our results suggest that rapid disease progression and increase of complications that can be seen in *BMPR2*-mutated PAH patients is maybe at least in part due to a substantial hypertrophy of the systemic lung vasculature. This relationship is illustrated in figure 9.

The percentage of *BMPR2* mutation carriers in our patient cohort does not reflect the generally reported frequency of this mutation in idiopathic and heritable PAH [10, 36]: for this study, we initially identified 80 transplanted patients with PAH group 1 of the Nice classification. 57 fell into categories 1.1 (idiopathic PAH) or 1.2 (heritable PAH) and 44 had available *BMPR2* mutation status at the time of patient selection, and hence were selected for evaluation. Of these 44 transplanted patients 23 (52.3%) were *BMPR2* mutation carriers and 21 (47.7%) were noncarriers. To the best of our knowledge, systematic *BMPR2* mutation screening is not performed in a majority of lung transplantation centres. In contrast, since January 2003, the French referral centre for pulmonary hypertension has been offering genetic counselling and *BMPR2* mutation screening (point mutations and large rearrangements) to all patients with PAH considered to be idiopathic, patients with a family history of PAH, patients exposed to anorexigen drugs and patients with pulmonary hypertension associated with other rare diseases [37]. Thus, the higher percentage of *BMPR2* mutation carriers in our cohort of transplanted patients, compared with published

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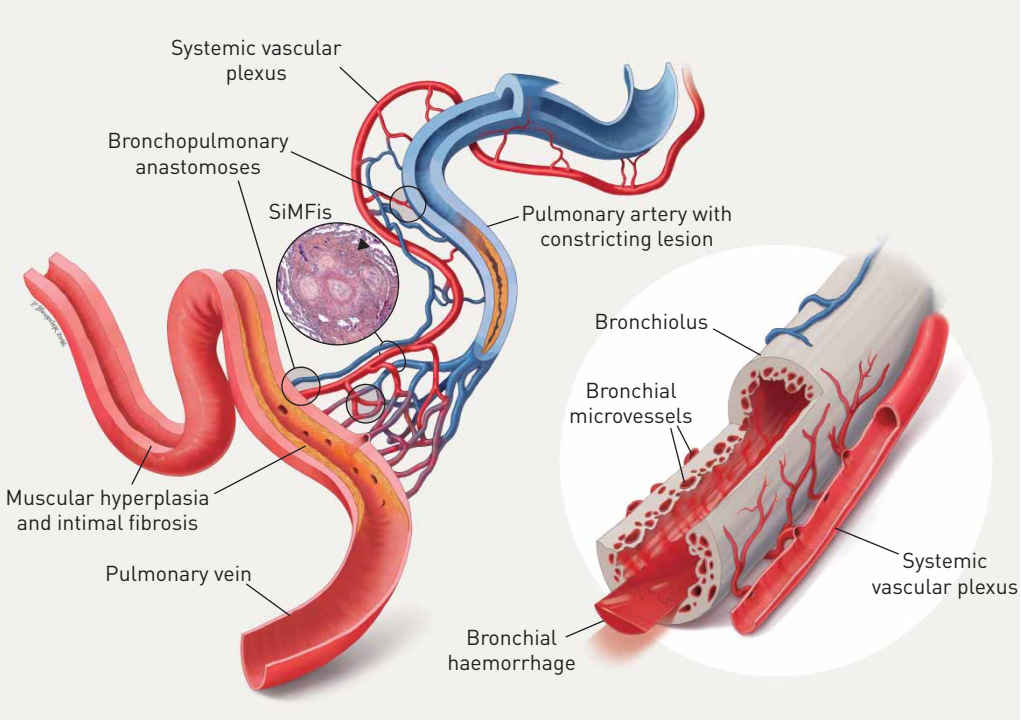

FIGURE 9 Impact of hypertrophic systemic vasculature in pulmonary arterial hypertension (PAH): an explanatory approach. The pulmonary artery (top centre, blue) and the adjacent bronchiole (right, grey) are covered by a hypertrophic systemic vascular plexus, comprising systemic arterial (red) and venous (blue) vessels and microvessels. The systemic plexus entertains several anastomoses with the pulmonary artery, the capillary bed and the pulmonary vein (bottom left, red): these bronchopulmonary anastomoses appear to bypass a constricting PAH-lesion, represented by medial thickening and intimal fibrosis (centre). In our view, the observed singular millimetric fibrovascular lesions (SiMFis) (photo inset) corresponds to a transversal section through a dense association of a (remodelled) pulmonary artery and several hypertrophic systemic vessels. Eventually, the increased systemic blood flow into arterioles, capillaries and the pulmonary vein leads to structural changes of the latter: muscular hyperplasia and focal intimal fibrosis within the pulmonary vein are observed. The hypertrophic systemic vasculature has a parallel impact on the adjacent airway (right): an increase in size and number of bronchial vessels and subepithelial microvessels leads to intrabronchial haemorrhage and eventually haemoptysis.

PAH registries presumably reflects our routine systematic mutation screening beyond familial cases. Of note, *BMPR2* mutation carriers usually present at a younger age and display more severe haemodynamic compromise at the time of PAH diagnosis [10, 11, 38], thus increasing the likelihood of undergoing transplant surgery. In addition, some patients within the transplanted cohort studied presented with "moderate" cardiopulmonary limitations. Interestingly, such patients were transplanted because of severe PAH complications, including haemoptysis in three out of six patients that had been transplanted with a last record of NYHA functional class II. Such patients with severe haemoptysis benefit from a priority listing, which guarantees shorter waiting time.

In conclusion, our work provides new insights into the characteristics of pulmonary vasculopathy in heritable *versus* idiopathic PAH. The results of our study on 44 PAH patients with or without *BMPR2* gene mutations do not reveal categorical differences between these two groups: we did not identify a pathognomonic standalone feature that would have permitted a definite diagnosis of heritable PAH and excluded every other cause of pulmonary hypertension. Nonetheless, we show that *BMPR2* mutation carriers are more prone to haemoptysis and that haemoptysis is closely correlated to bronchial arterial remodelling and angiogenesis; in turn, pronounced changes in the systemic vasculature correlate with increased pulmonary venous remodelling, hereby creating a distinctive profile in PAH patients harbouring a *BMPR2* mutation.

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