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Targets of anti-endothelial cell antibodies in pulmonary hypertension and scleroderma

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ABSTRACT: Anti-endothelial cell antibodies (AECAs) have been identified in patients with systemic sclerosis (SSc) with and without pulmonary arterial hypertension (PAH) and in patients with idiopathic pulmonary arterial hypertension (iPAH). However, their target antigens remain poorly identified.

Sera from 24 patients with SSc without PAH, 20 patients with SSc with PAH, 30 with iPAH and 12 healthy controls were collected. Target antigens were identified by two-dimensional electrophoresis and immunoblotting in protein extracts of human umbilical vein endothelial cells. Targeted antigens were identified by mass spectrometry.

Serum immunoglobulin G from patients with SSc with or without PAH and patients with iPAH specifically recognised 110, 82 and 37 protein spots, respectively. Among others, target antigens of AECAs included lamin A/C, tubulin β-chain and vinculin. One-dimension immunoblotting experiments confirmed the identification of lamin A/C and tubulin β-chain.

In conclusion, our results confirm the presence of AECA in patients with systemic sclerosis with and without pulmonary arterial hypertension and in those with idiopathic pulmonary arterial hypertension, and provide evidence for the identification of target antigens of these autoantibodies including lamin A/C and tubulin β-chain.

KEYWORDS: Autoantibodies, autoantigens, endothelial cells, pulmonary arterial hypertension, systemic sclerosis

Systemic sclerosis (SSc) is a connective tissue disorder characterised by microvascular damage and excessive fibrosis of the skin and various internal organs. Although incompletely understood, the pathogenesis of SSc involves a dysregulation of endothelial cells (ECs), fibroblast dysfunction and inflammation, with altered leukocyte responses resulting in collagen overproduction [1]. Although the link between autoimmunity and tissue fibrosis is still unclear, autoantibodies to cellular components are detected in most patients with SSc [2]. Three of these autoantibodies are disease-specific and mutually exclusive: anti-centromere antibodies [3] detected in the limited cutaneous form of SSc, anti-topoisomerase 1 antibodies detected in the diffuse form of SSc [4] and anti-RNA polymerase III antibodies associated with renal crisis [5]. Other autoantibodies identified in SSc patients are directed against the nucleus and cytoplasm, membrane structures, extracellular matrix components, fibroblasts and ECs [2].

Anti-endothelial cell antibodies (AECAs) are detected in 44–84% of patients with SSc [6, 7] and are associated with an increased incidence of vascular manifestations [7], such as severe digital ischaemia and pulmonary arterial hypertension (PAH). PAH is characterised by a progressive increase in pulmonary vascular resistance, eventually leading to right-heart failure and premature death [8]. PAH can be idiopathic (iPAH) in the absence of other conditions or associated with diseases such as SSc. Thus, PAH develops in ~8–12% of patients with SSc and is responsible for high mortality [9]. PAH has a multifactorial pathophysiology. The remodelling of the pulmonary vessel wall is a hallmark of severe PAH. It is marked by enhanced proliferation and survival of ECs and the formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina [10].

We and others previously identified AECAs in the serum of patients with iPAH or with SSc

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without PAH and with SSc associated with PAH (SSc-PAH) [11, 12]. AECAs can activate ECs and induce apoptosis in SSc patients, but the pathogenic role of AECAs in patients with iPAH has not been documented. Moreover, the target antigens of AECAs in patients with SSc-PAH and iPAH remains to be identified.

We aimed to identify the target antigens of immunoglobulin (Ig)G AECAs in the sera from patients with SSc with or without PAH and in patients with iPAH by two-dimensional electrophoresis and immunoblotting with protein extracts of human umbilical vein ECs (HUVECs) and mass spectrometry (MS).

PATIENTS AND METHODS

Ig sources

Serum samples were collected from 24 patients with SSc without PAH, 20 with SSc-PAH, and 30 with iPAH. PAH was confirmed by right-heart catheterisation. In all patients with PAH, mean pulmonary artery pressure at rest was >25 mmHg. Serum samples from patients were tested in pools of three within the same disease group. By convention, iPAH patients excluded patients with familial or dexfenfluramine-associated PAH or any other associated condition. Patients with SSc fulfilled the American Rheumatism Association criteria [13] and/or the LeRoy and Medsger [14] criteria. Clinical and biological characteristics of patients are depicted in online supplementary table S1. 12 healthy blood donors (healthy controls; HCs) were recruited as controls (six males; mean±SD age 39.5±7.8 yrs, range 33–61 yrs). HCs did not differ significantly from patients in age or sex. HCs had no detectable disease, no remarkable medical history and did not take any medication at the time of blood sampling. All patients and HCs were included in the Hypertension Artérielle Pulmonaire (HTAP)-Ig study (investigation and clinical research contract 2005, no. CIRC #05066; Assistance Publique-Hôpitaux de Paris) and gave their written, informed consent to participate according to the ethics committee of Cochin Hospital, Paris, France.

EC culture, protein extraction, indirect immunofluorescence and one-dimensional immunoblotting

Culture, protein extraction and indirect immunofluorescence procedures with HUVECs, pulmonary (p) and dermal (d) human microvascular endothelial cells (HMVECs) are detailed in the online supplementary material.

Two-dimesional electrophoresis, electro-transfer and two-dimensional immunoblotting

HUVECs were stored at -80°C in 1 mM phenylmethyl sulphonyl fluoride and protease inhibitors (Complete Mini; Roche Diagnostics, Meylan, France). Briefly, cells were suspended at 1×10^6 cells·mL^{-1} in a sample solution extraction kit (Kit 3; Bio-Rad Laboratories, Hercules, CA, USA). Cell samples were sonicated and the supernatant was collected after ultracentrifugation (Optima L90-K ultracentrifuge; Beckman Coulter, Fullerton, CA, USA) at 150,000×g for 25 min at 4°C. Protein quantification was carried out using the Lowry method. The supernatant was aliquoted and stored at -80°C.

We used pH 3–10 and on acrylamide gradient of 7–18% in all experiments, which allowed for studying a wide range of antigens of 10–250 kDa [15, 16]. The instrumentation and related reagents were from Bio-Rad, unless otherwise indicated. Proteins underwent isoelectrofocusing with 17-cm immobilised pH gradient (IPG) strips on the Protean IEF Cell System, essentially as described by Görg *et al*. [17] and reported by Servettaz *et al*. [16]. For preparation of two-dimensional gels, 100 μg of HUVEC protein extract was loaded onto IPG strips.

Prior to the second dimension of electrophoresis, the strips were equilibrated as described, then transferred to a 7–18% polyacrylamide gradient gel [16]. The equilibrated IPG gels were sealed on top of the polyacrylamide gels with use of 1% agarose containing bromophenol blue, and running buffer (24.8 mM Tris, 192 mM glycine and 0.1% sodium dodecyl sulfate) was added. Gels were run initially at 40 V (constant) for 1 h and then at 80 V for 1 h and, finally, at 120 V for 21 h 15 min.

The gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by semidry transfer (Bio-Rad) at 320 mA for 1 h 30 min. After being blocked with

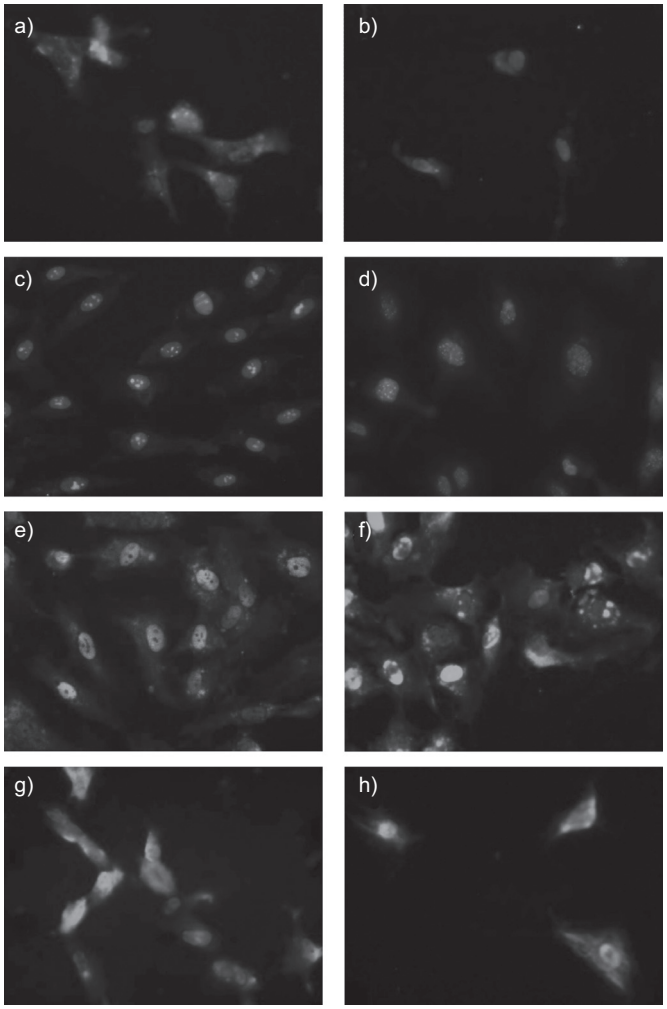

FIGURE 1. Indirect immunofluorescence on unpermeabilised human umbilical vein endothelial cells serum immunoglobulin (Ig)G from two representative individuals from each group: a, b) healthy controls, c, d) patients with systemic sclerosis without pulmonary arterial hypertension (PAH), e, f) systemic sclerosis-associated PAH and g, h) idiopathic PAH. Sera were tested at a 1:200 dilution. Secondary antibody: goat anti-human IgG antibody conjugated with fluorescein isothiocyanate nuclear staining: 4′,6-diamidino-2-phenylindole (DAPI). Magnification ×63.

TABLE 1 Proteins specifically recognised by serum immunoglobulin G from patients with systemic sclerosis without pulmonary artery hypertension, identified by mass spectometry (MS)

Protein ID on gel	Protein	SwissProt accession number	Theoretical/estimated molecular weight kDa	Theoretical/estimated pI	MS			
					Number of unique identified peptides#	Total ion score	Best ion score	Sequence coverage %
290	Splicing factor, proline- and glutamine-rich	SFPQ_HUMAN	76/100	9.5/7.5	5/14	146	54	28
342	Endoplasmin	ENPL_HUMAN	92/99	4.8/5.5	9/22	128	23	32
367	HSP 90-β	HS90B_HUMAN	83/93	5/6.2	8/19	182	57	33
389	Mitochondrial inner membrane protein	IMMT_HUMAN	84/9.1	6.1/6.5	4/5	53	24	7
438	Caldesmon	CALD1_HUMAN	93/83	5.6/6.6	2/9	58	44	13
483	NADH-ubiquinone oxidoreductase 75-kDa subunit, mitochondrial	NDUS1_HUMAN	79/75	5.9/6.1	6/21	86	35	41
526	Haematopoietic lineage cell-specific protein	HCLS1_HUMAN	54/75	4.7/5.9	5/9	91	38	22
594	Lamin-B1	LMNB1_HUMAN	66/74	5.1/5.6	6/19	86	33	40
634	Lamin-A/C	LMNA_HUMAN	74/71	6.6/7.1	11/31	415	88	46
691	Ras GTPase-activating protein-binding protein 1	G3BP1_HUMAN	52/66	5.4/6.2	5/9	227	77	26
704	Catalase	CATA_HUMAN	60/65	6.9/7.4	6/12	88	24	32
785	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	SCOT_HUMAN	56/58	7.1/6.6	7/9	175	65	30
787	Protein disulfide-isomerase A3	PDIA3_HUMAN	57/58	6/6.5	9/14	304	67	37
820	T-complex protein 1 subunit β	TCPB_HUMAN	57/55	6/6.6	11/12	421	92	38
831	Cytosol aminopeptidase	AMPL_HUMAN	56/55	8/6.8	7/12	194	55	29
899	RuvB-like 2	RUVB2_HUMAN	51/50	5.5/5.9	4/13	86	41	36
991	Mitochondrial-processing peptidase subunit β	MPPB_HUMAN	54/50	6.4/6.4	5/14	87	41	35
1101	Heterogeneous nuclear ribonucleo-protein A/B	ROAA_HUMAN	36/43	8.2/8.4	2/3	62	36	8
1105	Serpin B9	SPB9_HUMAN	42/43	5.6/6.2	6/12	182	64	31
1113	Reticulocalbin-3	RCN3_HUMAN	37/43	4.7/5.1	4/9	147	60	49
1116	Poly(rC)-binding protein 1	PCBP1_HUMAN	37/43	6.7/6.9	2/11	42	32	48
1117	Mitochondrial import receptor subunit TOM40 homolog	TOM40_HUMAN	38/43	6.8/7.7	5/10	157	45	37
1205	Eukaryotic translation initiation factor 3 subunit I	EIF3I_HUMAN	36/38	5.4/6	8/14	335	76	52
1246	60S acidic ribosomal protein P0	RLA0_HUMAN	34/37	5.7/6.2	6/9	213	74	41
1248	60S acidic ribosomal protein P0	RLA0_HUMAN	34/37	5.7/6	4/8	121	47	33
1328	Inorganic pyrophosphatase	IPYR_HUMAN	33/34	5.5/6	9/11	359	82	47
1488	Phosphoglycerate mutase 1	PGAM1_HUMAN	29/28	6.7/7.2	6/7	99	41	42
1495	Endoplasmic reticulum protein ERp29	ERP29_HUMAN	29/27	6.8/6.2	5/8	141	48	38
2098	Septin-11	SEP11_HUMAN	49/52	6.4/7.1	7/11	157	51	27
2106	Eukaryotic translation initiation factor 4B	IF4B_HUMAN	69/82	5.6/6.1	2/4	50	27	9

pI: isoelectric point; HSP: heat shock protein; NADPH: reduced nicotinamide adenosine dinucleotide; GTP: guanosine triphosphate; ERp29: 29-kDa endoplasmic reticulum protein 29; CoA; coenzyme A; TOM40: translocase of outer membrane. #: number of unique identified peptides with MSMS and MS+MSMS searches.

PBS–0.2% Tween for 90 min, membranes were incubated overnight at 4°C with pools of sera from three patients or the pool from 12 HCs.

IgG immunoreactivities were revealed as described [16]. Specific reactivities were determined by densitometrically scanning the membranes (densitometer GS-800; Bio-Rad) by use of Quantity One software (Bio-Rad). The membranes were then stained with colloidal gold (Protogold; British Biocell International, Cardiff, UK) and subjected to a second densitometric analysis to record labelled protein spots for each gel.

Gel staining

Analytical gels were stained with ammoniacal silver nitrate [18].

Image analysis of gels and two-dimensional blots

Images of gels and membranes were acquired by use of a densitometer (GS-800; Bio-Rad) and analysed by use of ImageMaster 2-D Platinum 5 (Amersham Biosciences, Amersham, UK) as described previously [16]. In order to maintain an objective detection of reactivities, protein spots were preselected by the analysis software using standard values of smoothing, saliency and minimal area. Each spot was confirmed by two experienced scientists (H. Dib and A. Regent). Thus, artefacts were eliminated and a threshold of intensity was determined in order to discriminate false positives. However, a visual and manual analysis was necessary to seek minor reactivities not detected by the software in order to avoid false-negative reactivities.

Only protein spots recognised by at least four different pools of three sera of patients in a given group were selected for in-gel excision and MS identification. Protein spots that were recognised by more than one group, including the group of HCs, were not selected for identification.

Protein identification by MS

Statistical analyses were used only for protein identification by mass spectrometry. The protein identification technique is detailed in the online supplementary material.

RESULTS

Detection of AECAs by indirect immunofluorescence

Using indirect immunofluorescence on unpermeabilised HUVECs, we have observed that AECAs from patients with PAH with or without SSc, as well as HCs, bind to the cell membranes (fig. 1). The intensity of immunofluorescence was more important in the case of patients with SSc-PAH and iPAH than in the case of patients with SSc without PAH. We have also performed indirect immunofluorescence studies using unpermeabilised p and d HMVECs (online supplementary figs S1 and S2). Interestingly, comparing reactivities of individuals in each group with HUVECs and HMVEC-p and HMVEC-d, we noted a more intense immunofluorescence for all of the groups tested with HMVEC-d, particularly in the case of patients with SSc without PAH.

Two-dimensional immunoblotting of IgG reactivities from HC sera directed against HUVEC proteins

The proteome of HUVECs contained 826 different protein spots with different isoeletric points (pI) ranging from 3 to 10 masses and from 10 to 250 kDa. Among these, a mean±SD of 595±145 spots were successfully transferred onto PVDF membranes (data not shown). IgG from the sera pool of 12 HCs recognised 97 protein spots (data not shown).

Two-dimensional immunoblotting of IgG reactivities from sera from SSc patients without PAH directed against HUVEC protein extracts

IgG from the seven pools of sera from SSc patients without PAH recognised a mean±SD of 230±100 protein spots corresponding to 424 different protein spots. Most of these 424 spots were recognised in only one or two sera pool(s) from patients and/or HCs. 110 out of these 424 spots were specifically recognised by serum IgG from patients with SSc without PAH. 30 spots of interest were identified, corresponding to 29 different proteins (table 1). Localisation of identified spots in the analytical gel is presented in figure 2. We found lamin A/C among the identified proteins (fig. 3).

Two-dimensional immunoblotting of IgG reactivities from sera from patients with SSc-PAH

IgG from the eight pools of sera from SSc-PAH patients recognised a mean of 202±73 protein spots corresponding to 363 different protein spots. Most of these 363 spots were

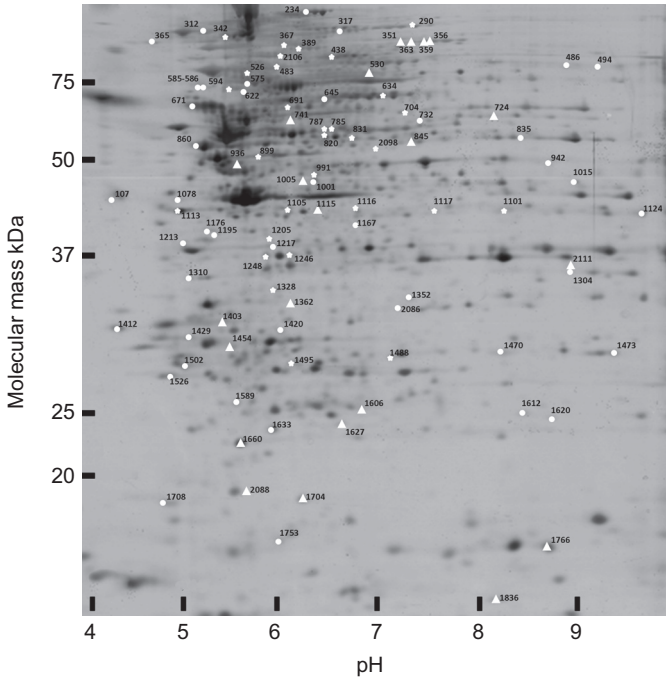

FIGURE 2. Localisation of the protein spots specifically recognised by serum immunoglobulin (Ig)G from patients. Two-dimensional silver-stained protein spots of total protein extracted from human umbilical vein endothelial cells. First dimension (*x*-axis): pH range 4–10; second dimension (*y*-axis): acrylamide gradient varying from 7 to 18%, allowing discrimination of 880 protein spots with a molecular mass range from 150 to 15 kDa. 30 protein spots were specifically recognised by serum IgG from patients with systemic sclerosis without pulmonary artery hypertension (stars), 41 protein spots specifically recognised by serum IgG from patients with systemic sclerosis with pulmonary artery hypertension (circle) and 21 protein spots specifically recognised by serum IgG from patients with idiopathic pulmonary artery hypertension (triangle). See tables 1–3 for the names of these proteins. All these protein spots were identified by mass spectrometry.

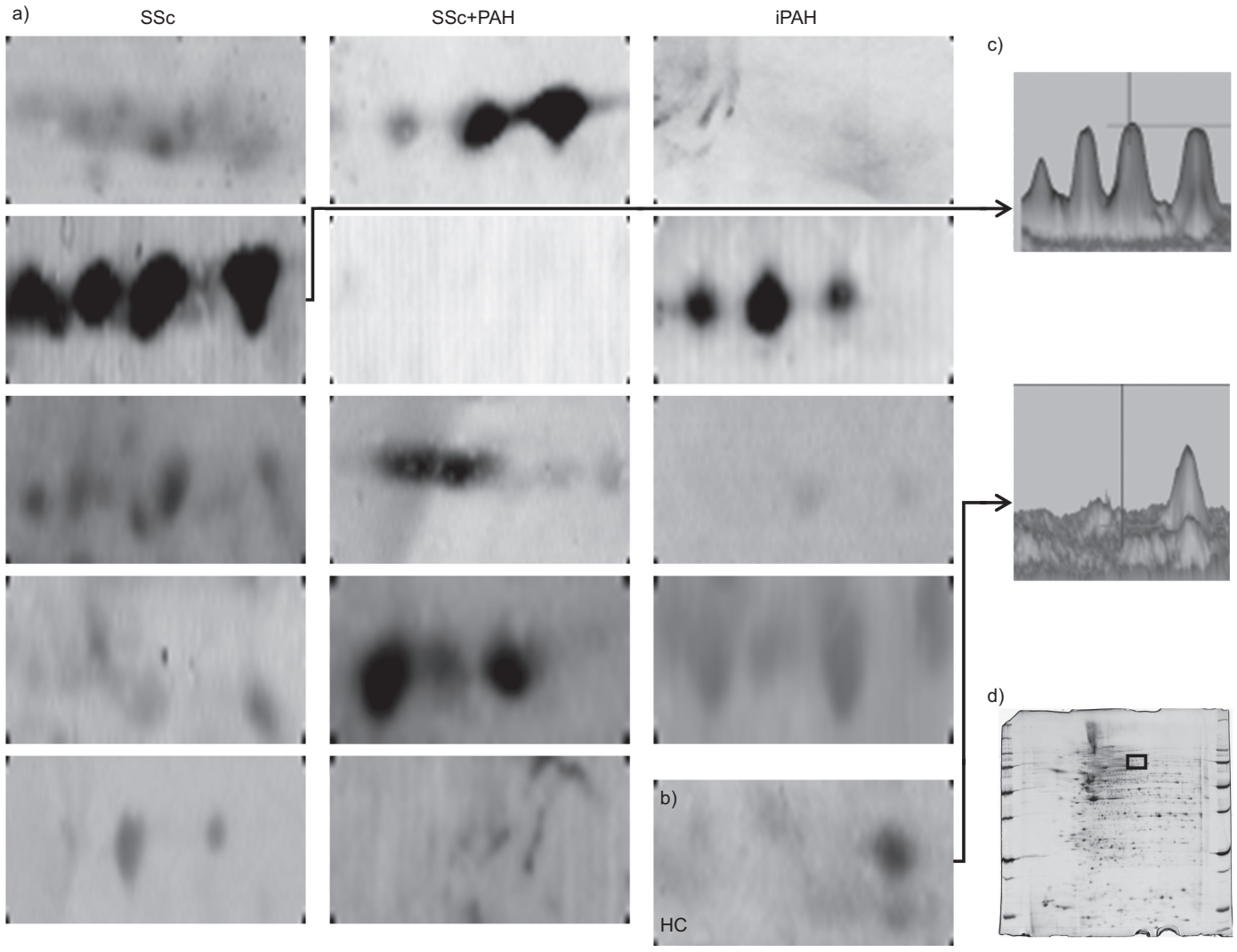

FIGURE 3. Two-dimensional immunoblots of immunoglobulin (Ig)G reactivities with lamin A/C in pools of sera from patients and healthy controls. a) IgG reactivities directed towards lamin A/C of a human umbilical vein endothelial cells (HUVECs) protein extract in five different serum pools from three systemic sclerosis (SSc) patients (left column), five serum pools from three SSc-pulmonary artery hypertension (PAH) patients (middle column) and four serum pools from three idiopathic PAH (iPAH) patients each (right column). b) IgG reactivities directed towards lamin A/C in one pool from 12 healthy controls (HC). c) Three-dimensional representation of IgG reactivity peaks towards lamin A/C in one representative sera pool from three patients (upper panel) and in the pool from the 12 healthy blood donors (lower panel). d) Two-dimensional silver-stained gel of HUVECs protein extract. The area delineated by a rectangle corresponds to the region of membranes magnified in panel a) (pH 6.8–7.5; 65–75 kDa).

recognised by only one or two sera pool(s) from patients with SSc-PAH and/or HCs. Among these 363 spots, 82 were specifically recognised by serum IgG from SSc-PAH patients. 43 protein spots of interest were identified corresponding to 39 different proteins (table 2). Localisation of identified spots in the analytical gel is depicted in figure 2. We found tubulin β chain (fig. 4) and vinculin among the identified proteins.

Two-dimensional immunoblotting of IgG reactivities from sera from patients with iPAH

IgG from the 10 pools of sera from iPAH patients recognised a mean$\pm$SD of 111$\pm$41 protein spots corresponding to 295 different protein spots. Most of these 295 spots were recognised by only one or two sera pool(s) from patients with iPAH and/or HCs. Among these 295 spots, 37 were specifically recognised by sera from iPAH patients. 22 protein spots of interest were identified corresponding to 22 different proteins (table 3). Localisation of identified spots in the analytical gel is depicted in figure 2. We found profilin 1 among the identified proteins.

Characterisation of target antigens of AECA

One-dimensional immunoblotting experiments were performed with HUVECs, HMVEC-p and HMVEC-d protein extracts with polyclonal antibodies raised against human recombinant lamin A/C, tubulin β-chain and vinculin. Anti-lamin A/C antibodies bound to the same 70- and 60-kDa proteins that were recognised by serum IgG form patients with SSc with or without PAH on HUVEC protein extract (fig. 5). Anti-tubulin antibodies bound to the same 50- and 40-kDa proteins bands that were recognised by serum IgG from patients with SSc-PAH (fig. 5). Similar results were obtained with HMVEC-p and HMVEC-d protein extracts (data not

TABLE 2 Proteins specifically recognised by serum immunoglobulin G from patients with systemic sclerosis and pulmonary artery hypertension, identified by mass spectrometry (MS)

Protein ID on gel	Protein	SwissProt accession number	Theoretical/estimated molecular weight kDa	Theoretical/estimated pI	MS			
					Number of unique identified peptides[#]	Total ion score	Best ion score	Sequence coverage %
234	Vinculin	VINC_HUMAN	124/114	5.5/6.4	15/23	739	108	28
312	Endoplasmin	ENPL_HUMAN	92/100	4.8/5.3	11/22	311	58	32
317	Splicing factor, proline- and glutamine-rich	SFPQ_HUMAN	76/100	9.5/6.7	4/7	95	50	13
365	Protein NOXP20	NXP20_HUMAN	61/96	4.6/4.8	4/12	92	57	34
486	TATA-binding protein-associated factor 2N	RBP56_HUMAN	62/76	8.0/9.0	2/4	66	39	9
494	TATA-binding protein-associated factor 2N	RBP56_HUMAN	62/76	8/9.3	2/5	57	44	15
575	Heat shock cognate 71-kDa protein	HSP7C_HUMAN	71/75	5.4/5.7	9/10	308	77	21
585	Heat shock cognate 71-kDa protein	HSP7C_HUMAN	71/74	5.4/5.3	2/3	50	29	6
586	Heat shock cognate 71-kDa protein	HSP7C_HUMAN	71/74	5.4/5.3	6/7	210	49	16
622	Heat shock cognate 71-kDa protein	HSP7C_HUMAN	71/72	5.4/5.7	5/13	88	37	24
645	Lamin-A/C	LMNA_HUMAN	74/70	6.6/6.5	9/26	223	49	38
671	Cytoskeleton-associated protein 4	CKAP4_HUMAN	66/67	5.6/5.2	3/6	80	32	12
732	Lamin-A/C	LMNA_HUMAN	74/62	6.6/7.5	3/18	88	39	25
835	ATP synthase subunit α, mitochondrial precursor	ATPA_HUMAN	60/55	9.2/8.5	4/7	109	41	15
860	Tubulin β chain	TBB5_HUMAN	50/53	4.8/5.3	9/16	379	88	48
942	NADPH:adrenodoxin oxidoreductase, mitochondrial precursor	ADRO_HUMAN	54/50	8.7/8.8	7/13	122	28	30
1001	Heterogeneous nuclear ribonucleoprotein D0	HNRPD_HUMAN	38/49	7.6/6.5	2/2	79	51	6
1015	Phosphoglycerate kinase 1	PGK1_HUMAN	45/49	8.3/9.1	10/16	437	71	46
1077	Calumenin precursor	CALU_HUMAN	37/45	4.5/4.4	6/10	148	32	41
1078	40S ribosomal protein SA	RSSA_HUMAN	33/45	4.8/5	8/10	433	125	51
1124	Fructose-bisphosphate aldolase A	ALDOA_HUMAN	39/42	8.3/9.8	7/12	273	90	46
1167	28S ribosomal protein S22, mitochondrial	RT22_HUMAN	41/40	7.7/6.9	5/7	73	28	20
1176	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRPC_HUMAN	34/39	5/5.4	3/4	118	63	18
1195	Protein SET	SET_HUMAN	33/39	4.2/5.5	3/7	64	36	27
1213	60-kDa heat shock protein, mitochondrial	CH60_HUMAN	61/37	5.7/5.1	2/8	49	33	20
1217	*N*(G),*N*(G)-dimethylarginine dimethylamino-hydrolase 1	DDAH1_HUMAN	31/37	5.5/6	5/9	212	72	36
1304	Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN	37/36	9.0/9.0	7/8	320	83	30
1310	Elongation factor 1-delta	EF1D_HUMAN	31/36	4.9/5.2	4/7	53	21	35
1352	Voltage-dependent anion-selective channel protein 2	VDAC2_HUMAN	32/33	7.5/7.4	3/7	90	42	37
1412	Complement component 1 Q subcomponent-binding protein, mitochondrial precursor	C1QBP_HUMAN	31/30	4.7/4.5	6/7	471	113	48
1420	*N*(G),*N*(G)-dimethylarginine dimethylamino-hydrolase 2	DDAH2_HUMAN	30/30	5.7/6.1	4/7	84	48	35
1429	Lysosomal protective protein precursor	PPGB_HUMAN	54/29	6.2/5.1	2/2	55	29	5
1470	Eukaryotic translation initiation factor 4H	IF4H_HUMAN	27/28	6.7/8.3	3/3	176	87	14
1473	Electron transfer flavoprotein subunit β	ETFB_HUMAN	28/28	8.2/9.5	5/8	120	49	40
1502	14-3-3 protein β/α	1433B_HUMAN	28/27	4.8/5.1	3/5	191	103	28
1526	Proteasome subunit α type-5	PSA5_HUMAN	26/26	4.7/5	6/8	160	70	41
1589	Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1_HUMAN	25/25	5.3/5.6	5/6	219	72	38
1612	Flavin reductase	BLVRB_HUMAN	22/25	7.1/8.6	4/8	82	24	65
1620	Peroxiredoxin-1	PRDX1_HUMAN	22/25	8.3/8.8	6/7	202	60	37
1633	Peroxiredoxin-2	PRDX2_HUMAN	22/23	5.7/6	6/10	367	95	50
1708	Myosin regulatory light chain 2, nonsarcomeric	MLRM_HUMAN	20/19	4.7/4.9	6/7	377	102	52
1753	Actin-related protein 2/3 complex subunit 5	ARPC5_HUMAN	16/17	5.5/6.1	6/6	426	102	52
2086	S-formylglutathione hydrolase	ESTD_HUMAN	31/32	6.5/7.3	4/9	150	73	52

pI: isoelectric point; ATP: adenosine triphosphate; NADPH: reduced nicotinamide adenosine dinucleotide. [#]: number of unique identified peptides with MS/MS and MS+MS/MS searches.

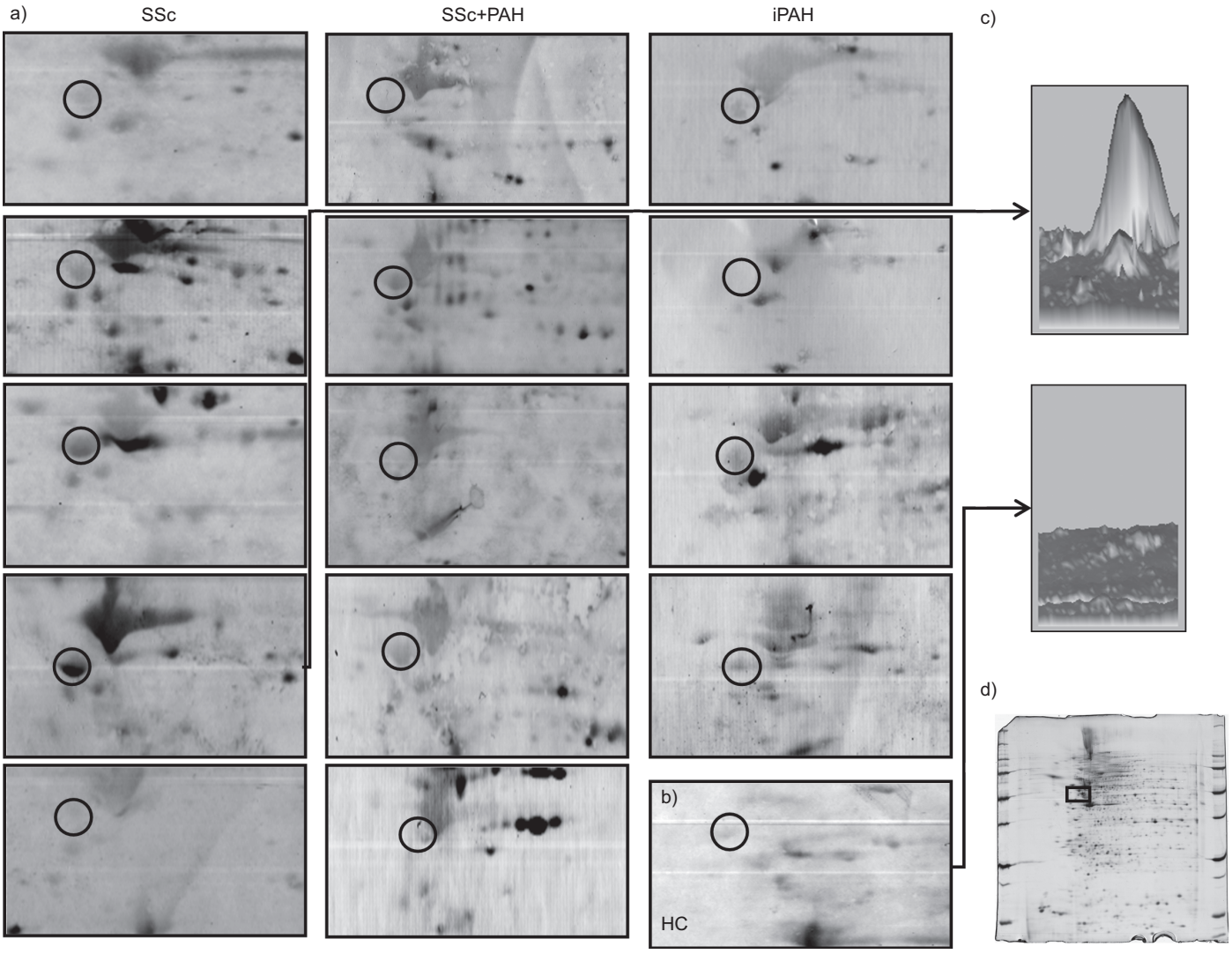

FIGURE 4. Two-dimensional immunoblots of immunoglobulin (Ig)G reactivities with tubulin in pools of sera from patients and healthy controls. a) IgG reactivities directed towards tubulin (circle) of a human umbilical vein endothelial cell (HUVEC) protein extract in five different serum pools from three systemic sclerosis (SSc) patients (left column), five serum pools from three SSc pulmonary artery hypertension (PAH) patients (middle column) and four serum pools from three idiopathic PAH (iPAH) patients (right column). b) IgG reactivities directed towards tubulin (circle) in one pool from 12 healthy controls (HC). c) Three-dimensional representation of IgG reactivity peaks towards tubulin in one representative sera pool from three patients (upper panel) and in the pool from the 12 healthy blood donors (lower panel). d) Two-dimensional silver-stained gel of HUVEC protein extract. The area delineated by a rectangle corresponds to the region of membranes magnified in panel a) (pH 4.5–5.5; 50–60 kDa).

shown). Unfortunately, we failed to identify similarities between reactivities observed in individually tested patients with SSc-PAH and antibodies specific for vinculin.

DISCUSSION

We have previously reported that IgG from patients with SSc bound to DNA topoisomerase 1 [19] and centromeric protein B [20] in protein extracts of HUVECs. To our knowledge, target antigens of AECAs had never been identified before in patients with iPAH. In the present work, using a proteomic approach, we have identified target antigens of AECAs in patients with SSc with or without PAH and in patients with iPAH. Among others, target antigens of AECAs included lamin A/C, tubulin β-chain and vinculin. Additional one-dimensional immunoblotting experiments confirmed the identification of lamin A/C and tubulin β-chain.

Lamins A/C are major constituents of the inner nuclear membrane. Mutations of the *LMNA* gene have been identified in Hutchinson–Gilford progeria syndrome [21], which represents a major differential diagnosis of juvenile SSc. Mutant lamin A (progerin), which accumulates within the nuclei of human vascular cells, may be directly responsible for vascular involvement in progeria [22]. Anti-lamin antibodies were found in sera from patients with linear morphea [23]. In our present work, lamin A/C was identified in 15 different spots, although the different pools of sera recognised each spot with a variable intensity.

β-tubulins, along with α-tubulin, are the major components of microtubules. β-tubulin isotype 5 has been reported as a target antigen of perinuclear anti-neutrophil cytoplasmic antibodies. Furthermore, tubulin autoantibodies are detected in several

TABLE 3 Proteins specifically recognised by serum immunoglobulin G from patients with idiopathic pulmonary artery hypertension, identified by mass spectrometry (MS)

Protein ID on gel	Protein	SwissProt accession number	Theoretical/estimated molecular weight kDa	Theoretical/ estimated pI	MS			
					Number of unique identified peptides[#]	Total ion score	Best ion score	Sequence coverage %
351	Far upstream element-binding protein 2	FUBP2_HUMAN	73/98	6.8/7.3	6/6	230	66	13
356	Aconitate hydratase, mitochondrial	ACON_HUMAN	85/96	7.4/7.7	8/17	243	42	25
359	Aconitate hydratase, mitochondrial precursor	ACON_HUMAN	85/96	7.4/7.6	10/18	385	56	31
363	Far upstream element-binding protein 2	FUBP2_HUMAN	73/96	6.8/7.4	11/16	401	64	29
530	GMP synthase (glutamine-hydrolysing)	GUAA_HUMAN	77/75	6.4/7	4/6	73	26	10
724	Far upstream element-binding protein 1	FUBP1_HUMAN	68/63	7.2/8.2	8/14	385	107	24
741	Protein disulfide-isomerase A3 precursor	PDIA3_HUMAN	57/62	6/6.2	3/6	31	16	14
845	Fascin	FSCN1_HUMAN	54/54	6.8/7.4	9/14	318	81	37
936	Thioredoxin domain-containing protein 5	TXND5_HUMAN	48/50	5.6/5.7	11/15	566	116	43
1005	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate	ODO2_HUMAN	49/49	9.1/6.3	3/4	66	34	11
1115	DnaJ homolog subfamily B member 11 precursor	DJB11_HUMAN	40/43	5.8/6.5	7/8	316	116	34
1362	Annexin A4	ANXA4_HUMAN	36/33	5.8/6.2	6/11	195	70	41
1403	EF-hand domain-containing protein D2	EFHD2_HUMAN	27/31	5.2/5.5	6/9	213	60	37
1454	Proteasome subunit alpha type-3	PSA3_HUMAN	28/29	5.2/5.6	5/6	241	84	25
1606	Acyl-protein thioesterase 1	LYPA1_HUMAN	25/25	6.3/6.9	3/3	138	54	13
1627	GTP-binding protein SAR1a	SAR1A_HUMAN	22/24	6.2/6.7	3/3	93	54	15
1660	ATP synthase subunit d, mitochondrial	ATP5H_HUMAN	18/21	5.2/5.7	8/10	596	112	65
1704	Nucleoside diphosphate kinase A	NDKA_HUMAN	17/19	5.8/6.3	7/9	399	101	70
1766	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	18/17	7.7/8.8	6/9	443	141	46
1836	Profilin-1	PROF1_HUMAN	15/15	8.4/8.3	5/9	284	78	72
2088	ATP synthase subunit d, mitochondrial	ATP5H_HUMAN	18/22	5.2/5.9	2/2	107	78	15
2111	Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN	37/37	9.0/9.0	8/9	374	77	33

pI: isoelectric point; GMP: guanosine monophosphate; GTP: guanosine triphosphate; ATP: adenosine triphosphate. [#]: number of unique identified peptides with MS/MS and MS+MS/MS searches.

FIGURE 5. Immunoglobulin (Ig)G from patients bind to lamin A/C and/or tubulin. Western blotting of serum IgG from two representative individuals from each group: healthy controls (HCs), patients with systemic sclerosis (SSc) without pulmonary artery hypertension (PAH), patients with SSc-PAH and patients with idiopathic PAH (iPAH) tested at 1:200 with protein extracts of human umbilical vein endothelial cell. Rabbit anti-lamin A/C and rabbit anti-tubulin β-chain antibodies were tested at 1:200 on the same protein extract.

clinical neurological diseases, such as chronic inflammatory demyelinating polyneuropathy [24], diabetic neuropathy and systemic lupus erythematosus [25].

In SSc, many studies have suggested that AECAs could be linked to vascular injury and could reflect EC damage [26]. Whether AECAs play a role in these processes remains controversial and further work is needed. Only one study reported a correlation between the detection of AECAs in patients with SSc and the development of severe digital ischaemia and PAH [27]. As our study is descriptive and did not aim to establish a correlation between autoantibody reactivity and disease evolution, further studies are needed to document whether AECAs might help to characterise a subgroup of SSc patients at higher risk of developing vascular complications and whether AECAs might, therefore, be considered as a predictor of outcome in SSc patients. In iPAH, where disorganised proliferation and apoptosis of EC are observed [28] and lead to the formation of plexiform lesions, it would be important to investigate the role of AECAs.

Proteins identified as targets of AECAs are ubiquitous and play key roles in different cell types as they are implicated in cell morphology, metabolism and protein folding, Interestingly, we have previously reported the presence of anti-fibroblast antibodies in patients with SSc-PAH and iPAH [29] and identified that their target antigens also comprised ubiquitous proteins. Interestingly, calumenin, which is also identified in the present work as a target of AECAs in patients with SSc-PAH, is one of the targets of antifibroblast antibodies identified in patients with SSc-PAH [30].

Our work has several limitations. Thus, although the combined use of two-dimensonal electrophoresis and immunoblotting

offers an interesting approach to identify target antigens of autoantibodies [15, 16, 30], it does not allow targeting of membrane antigens. As expected, none of the identified antigens was located at the cell surface, because protein extraction for two-dimensional electrophoresis does not allow for identifying membrane proteins. However, using indirect immunofluorescent studies, we provide evidence that AECAs from patients and HCs bind to the EC surface. Moreover, we have used HUVECs protein extracts to identify target antigens of AECAs. EC represent a heterogeneous group of cells and there are differences between HUVECs and, for instance, HMVEC-p and HMVEC-d. We have performed indirect immunofluorescence experiments using HMVEC-p and HMVEC-d and observed a more intense immunofluorescence for patients with SSc without PAH with HMVEC-d than with HUVECs. Thus, additional experiments using HMVEC protein extracts might help to identify additional target antigens of AECAs in patients with SSc with or without PAH and iPAH patients. In addition, the total number of protein spots stained in the reference gel of the HUVEC protein extract was <1,000, which is less than the total number of proteins contained in these cells. Finally, our pools of sera were from three patients each because, in previous work, this number was sufficiently low to allow for detection of strong reactivity that would be present in the serum of only a single individual [30]. However, we cannot rule out that a low-intensity reactivity specific to a given individual could not be detected by this pooling approach.

In conclusion, our results confirm the presence of AECAs in patients with SSc with and without PAH and in those with iPAH and provide evidence for the identification of target antigens of these autoantibodies. Functional analysis will be necessary to demonstrate their potential role. The usefulness of the identified targets of AECAs for PAH or SSc screening, diagnosis or follow-up needs further confirmation by extensive laboratory screening with large groups of patients and HCs.

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STATEMENT OF INTEREST

Statements of interest for M.C. Tamby, A. Berezné, L. Guillevin, G. Simonneau, M. Humbert and L. Mouthon, and for the study itself can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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