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Thrombotic risk factors in pulmonary hypertension

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Thrombotic risk factors in pulmonary hypertension. M. Wolf, C. Boyer-Neumann, F. Parent, V. Eschwege, H. Jaillet, D. Meyer, G. Simonneau. ©ERS Journals Ltd 2000.

ABSTRACT: Thrombotic lesions are consistently observed in chronic thromboembolic pulmonary hypertension (CTEPH) and frequently found in primary pulmonary hypertension (PPH). It remains unknown, however, whether thrombosis is related to defects of the antithrombotic pathway or to previous vascular injury. This study therefore analysed the frequency of both hereditary and acquired thrombotic risk factors in CTEPH and PPH.

One hundred and forty-seven consecutive patients with CTEPH investigated in the author′s institution were compared to 99 consecutive patients with PPH. In 116 CTEPH patients and 83 PPH patients, phospholipid-dependent antibodies (antiphospholipid antibodies and lupus anticoagulant) were analysed by both immunological and clotting assays. In patients enrolled since 1994 (46 CTEPH and 64 PPH), hereditary thrombotic risk factors were also determined. Antithrombin, protein C and protein S activities were measured by functional assays. Mutations of factor V and factor II were identified by polymerase chain reaction.

The prevalence of hereditary thrombotic risk factors was not increased in patients with either PPH or CTEPH. In contrast, a high frequency of phospholipid-dependent antibodies was observed in PPH (10%) and more notably in CTEPH (20%). Moreover, in PPH, antibodies were present only in low titre whereas in CTEPH, half of the patients with antiphospholipid antibodies had high titres. In addition, in CTEPH all but one of the patients with lupus anticoagulant also had antiphospholipid antibodies.

The most striking finding of this study was the high prevalence of phospholipid-dependent antibodies but their clinical relevance appears to be different in primary pulmonary hypertension and chronic thromboembolic pulmonary hypertension. In primary pulmonary hypertension, these antibodies in low titre probably reflect endothelial dysfunction. In contrast, in chronic thromboembolic pulmonary hypertension the presence of antibodies in high titre associated with lupus anticoagulant, underlines the role of thrombosis in the pathogenesis of this condition.

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Pulmonary hypertension (PH) is a serious condition with significant morbidity and mortality. A large spectrum of diseases are included under this rubric, roughly divided into primary pulmonary hypertension (PPH) and secondary pulmonary hypertension. The definition of PPH is based on the exclusion of secondary causes of PH viz hypoxic lung disease, congenital heart disease, chronic thromboembolic disease, and associated conditions such as collagen vascular diseases, human immunodeficiency virus (HIV) infection, portal hypertension and exogenous substances [1]. Although the pathogenesis of the increased vascular resistance in PPH remains unknown, vasoconstriction, vascular remodelling and thrombosis may all contribute. In fact clinical data and histological features of PH suggest that thrombosis may be important in the pathophysiological state of PH, especially in PPH and chronic thromboembolic pulmonary hypertension (CTEPH). Several recent studies of large series of patients with PPH have shown that primary pulmonary arteriopathy includes a spectrum of histopathological lesions ranging from classic plexogenic arteriopathy to microthrombotic, nonplexogenic forms [1]. On the other hand, CTEPH is an aberrant (and infrequent) outcome of acute pulmonary embolism, defined by the persistance of unresolved thrombi; for reasons still unclear, the emboli in CTEPH patients do not resolve completely and become organized in fibrotic masses that obstruct and narrow major pulmonary arteries leading to an increase in pulmonary vascular resistance [2]. However CTEPH and PPH are distinct entities, as supported by different patterns on lung scan and pulmonary angiography. In all CTEPH patients, at least one segmental or larger perfusion defect is observed on lung perfusion scan, "mismatched" by a normal ventilation scan. Pulmonary angiography confirmed the diagnosis showing changes characteristic of chronic embolism, including webs, bands, pouches and other irregularities, as well as "cut-off" and narrowed vessels [2]. In PPH patients, the lung scan is usually normal or of "low probability" [1]; in the few cases of PPH with thrombotic pulmonary arteriopathy and an intermediate lung scan, pulmonary angiography does not show the pattern of CTEPH. These thrombotic lesions might be due to genetic risk factors for thrombosis, including deficiencies of antithrombin, protein C (PC), protein S (PS) and the two mutations recently

identified on the genes for factors V and II: replacement of the arginine 506 by a glutamine on the factor V (FV) gene (FV Leiden) [3] and mutation 20210 G/A in the 3′ untranslated region of the prothrombin (factor II) gene [4]. To date, these thrombotic factors have been poorly documented in PPH and CTEPH: MOSER *et al.* [5] found low frequencies of antithrombin, PC and PS deficiencies in patients with CTEPH and LANG *et al.* [6] reported a normal prevalence of FV Leiden in a small number of patients with CTEPH.

On the other hand, the presence of vasculitic lesions in PPH, together with the clinical association of PPH with several immunological disorders, suggests the involvement of an autoimmune process in this disease. In that regard, antiphospholipid antibodies have been observed in PPH but they also occur in CTEPH [7, 8]. These antibodies which are directed against negatively charged phospholipids of cells membranes represent the most frequent form of acquired thrombophilia. However, in some cases they are an epiphenomenon and result from cell activation or vascular injury [9].

Recently, in a large study of patients with acute venous thromboembolism (VTE), anticardiolipin antibodies were reported to increase the risk of recurrence of VTE and might therefore lead to the development of CTEPH [10]. Hence, there are several arguments supporting the role of thrombosis in pulmonary hypertension and especially in CTEPH. It remains unknown, however, whether thrombosis is related to inherited or acquired defects of the antithrombotic pathway or results from previous vascular injuries. To address this issue, the authors designed a prospective study of the prevalence of phospholipid-dependent antibodies including antiphospholipid antibodies (APA) measured by enzyme linked immunosorbent assay (ELISA) and lupus anticoagulant (LA) in CTEPH and PPH patients. The authors also determined the frequency of hereditary thrombotic risk factors, antithrombin, PC, PS deficiencies and the presence of FV and factor II (FII) mutations in patients with CTEPH compared to PPH.

Materials and methods

Patients

Among all PH patients referred for initial investigation to the authors′ institution, a French reference centre for PH, two groups of consecutive patients were studied: 147 consecutive patients with CTEPH investigated between 1984–1996 were compared to 99 consecutive patients with PPH investigated between 1994–1996. In all patients, the diagnosis of PH was confirmed by the presence of a mean pulmonary arterial pressure exceeding 25 mmHg at rest during right heart catheterization, with a normal pulmonary wedge pressure (<12 mmHg). Each patient was investigated according to the recommendations of the consensus conference on PPH [1] in order to establish the subgroup of PH: a detailed history was obtained and physical examination, pulmonary function testing, perfusion lung scan, computed tomography (CT) scan and pulmonary angiography in case of abnormal lung scan, serological studies were performed to exclude collagen vascular diseases and HIV infection. In group 1, the diagnosis of CTEPH was based on the presence of at least one segmental perfusion defect on the lung scan confirmed in all CTEPH patients by chronic thromboembolic patterns on pulmonary angiography as described by MOSER *et al.* [5], *i.e.* webs, bands, pouches and other irregularities as well as "cut-off" in main, lobar, or segmental pulmonary arteries. In group 2, the diagnosis of PPH was made when other causes of PH were ruled out according to the criteria defined by the American study group for PH [1]. In all PPH patients, collagen vascular diseases were excluded; however the presence of autoantibodies alone without systemic manifestations was not a criterion for exclusion of PPH.

Blood samples

Blood was drawn from patients into evacuated tubes containing 0.11 M sodium citrate. Following two centrifugations at $2{,}400 \times g$ for 20 min at 15°C, plasma samples were frozen at -30°C and thawed at 37°C just before use.

Blood from patients and controls was also collected in ethylene diamine tetracetic acid (EDTA) for deoxyribonucleic acid (DNA) studies and maintained at 4°C until DNA extraction. There were 100 healthy control subjects, 68 females, 32 males, mean age 41 yrs.

Phospholipid-dependent antibodies. The phospholipid-dependent antibodies include both APA measured by ELISA and LA detected by clotting assays.

Antiphospholipid antibodies. APA were measured by ELISA using a commercial kit, Asserachrom APA (Diagnostica Stago, Asnières, France). Briefly, plates coated with a mixture of cardiolipin, phosphatidic acid and phosphatidylserine and saturated with goat serum as a source of β_2-glycoprotein (β_2GPI), were incubated with 1:100 diluted plasma in duplicate. After washing, the antihuman immunoglobulin (Ig) (IgG, IgA, IgM) peroxidase conjugate was added and incubated for 1 h. The plates were then washed, the peroxidase substrate (ortho-phenylene-diamine with hydrogen-peroxide: OPD/H_2O_2) added and the absorbance measured at 492 nm.

A calibration curve was established using serial dilutions of reference plasma titrated against the first preparation of the standard of HARRIS *et al.* [11]. Results are expressed in total phospholipid units (UPL·mL^{-1}). The threshold level for positivity, defined as mean±3 SD of the values obtained in 100 healthy control subjects, was 8 total UPL·mL^{-1}. The titre was considered to be low for values ranging 8–15 UPL·mL^{-1}, medium between 15–40 UPL·mL^{-1} and high for levels >40 UPL·mL^{-1}. Plasma samples showing >8 total UPL·mL^{-1} were further isotyped with specific monovalent anti-IgG- or anti-IgM-peroxidase conjugates. Results were then expressed in GPL·mL^{-1} for IgG antiphospholipid antibodies and MPL·mL^{-1} for IgM antiphospholipid antibodies. Values >5 GPL·mL^{-1} or MPL·mL^{-1} were considered positive.

Lupus anticoagulant. The detection of LA was performed with an activated partial thromboplastin time (APTT) assay (PTT LA; Diagnostica Stago) and an integrated assay including hexagonal phase phospholipid-neutralization procedure (Staclot LA; Diagnostica Stago) [12, 13]. A shortening of the baseline APTT of a least 7 s was considered as positive.

Hereditary thrombotic risk factors. Hereditary thrombotic risk factors were investigated only in patients enrolled since 1994, representing 64 patients with PPH and 46 patients with CTEPH.

Coagulation inhibitors. Activities of antithrombin (AT), protein C (PC) and protein S (PS) were measured by functional assays as previously described [14]. AT activity was tested in all patients, whereas PC and PS could only be determined in patients not treated by vitamin-K antagonists.

Factor V and Factor II mutations. Genomic DNA was isolated from blood leukocytes as described by MILLER *et al.* [15]. The Arg 506 to Glutamine mutation of FV and the 20210 G to A mutation of FII were identified by polymerase chain reaction (PCR) amplification as previously described [4, 16].

Statistical analysis

Fisher's exact test and Student's t-test were used to compare data (Statview Software, Abacus concepts, Berkeley, CA, USA).

Results

Characteristics of the patients

From 1984–1996, 147 consecutive patients with CTEPH were investigated in the authors' institution and compared to 99 consecutive patients with PPH. In 116 CTEPH patients and 83 PPH patients, the phospholipid-dependent antibodies (APA and LA) were analysed by both immunological and clotting assays. In patients enrolled since 1994 (46 CTEPH and 64 PPH), hereditary thrombotic risk factors were also determined (Antithrombin, PC, PS activities and mutations of FV and FII).

The clinical characteristics and haemodynamic features of the 83 patients with PPH and 116 patients with CTEPH enrolled into the study are listed in table 1. These data were not statistically different from those of the entire population of 147 patients with CTEPH and 99 patients with PPH.

Table 1. – Clinical and haemodynamic characteristics of patients

	PPH	CTEPH
n	83	116
Age yrs	46±15	54±14
M/F ratio	0.47	1.04
mPAP mmHg	63±13	51±16*
CI $L \cdot min^{-1} \cdot m^{-2}$	2.3±0.6	2.5±0.7**
PVR $dyne \cdot s^{-1} \cdot cm^{-5}$	1408±558	1008±95*

Data are presented as mean±SD. PPH: primary pulmonary hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; M: male; F: female; mPAP: mean pulmonary arterial pressure; CI: cardiac index; PVR: pulmonary vascular resistance; *: $p<0.001$ between PPH and CTEPH; **: $p<0.01$ between PPH and CTEPH. 1 $dyne \cdot s^{-1} \cdot cm^{-5} = 1/(80 \times 7.5)$ $kPa \cdot L^{-1} \cdot min^{-1}$.

All patients, either PPH or CTEPH patients, had normal or near normal pulmonary function tests. All patients with CTEPH had patterns of chronic thromboembolism on pulmonary angiography, as described by MOSER *et al.* [5]. As previously reported [5], only 70 (60%) of the 116 patients with CTEPH had a previous history of venous thromboembolism; 21 (18%) other patients had previous unexplained symptoms compatible with undiagnosed pulmonary embolism; 10% of patients had a family history of venous thromboembolism. Each PPH patient fulfilled the diagnostic criteria for PPH; in all of them, secondary causes were excluded on the results of history, physical examination, chest radiography, pulmonary function testing, perfusion lung scan and pulmonary angiography in the case of an abnormal lung scan.

All patients had severe pulmonary arterial hypertension, but PH was significantly more severe in patients with PPH than in those with CTEPH.

Phospholipid-dependent antibodies

In PPH, a similar and high prevalence was found for both APA and LA (table 2). In CTEPH, the prevalence of both antibodies was twice as high as in PPH (close to 20%), although the difference between CTEPH and PPH only reached significance for APA ($p<0.005$). The titres of antiphospholipid antibodies in patients with positive APA are summarized in figure 1. Among the eight PPH patients with a positive APA, the antibody titre was low in five, medium in three and high in only one patient. By contrast, in CTEPH, half of the 25 positive patients had high titres, ranging 40–100 $UPL \cdot mL^{-1}$. Hence, the prevalence of high titre antibodies is markedly different in PPH and CTEPH (1% and 10% respectively). Isotyping of the antibody was performed in the 33 patients with PPH or CTEPH with a positive APA: 22 patients had IgG antibodies alone, seven the association of IgG and IgM, and four IgM alone. Similar distributions were seen in CTEPH and PPH and no relationship was observed between the isotype and titre.

The distribution of positive phospholipid-dependent tests, LA and APA, was different in both groups. Most of the patients with PPH were positive in only one test, either APA or LA. Only two of the eight patients with LA were positive for APA. Conversely, in CTEPH, all except one of

Table 2. – Frequencies of antiphospholipid antibodies and lupus anticoagulant

	PPH	CTEPH	p-value
n	83	116	
APA	8/83	25/116	<0.005
	9.5*	21.5*	
n	73	79	
LA	9/73	16/79	0.07
	12*	20*	

PPH: primary pulmonary hypertension; CTEPH: chronic thromboembolic pulmonary hypertension; APA: antiphospolipid antibodies; LA: lupus anticoagulant. Comparison between PPH and CTEPH groups was performed using a Fisher's exact test. *: percentage. The method used to evaluate LA only became available in 1990. Therefore patients enrolled in 1984–1990 were only investigated for APA.

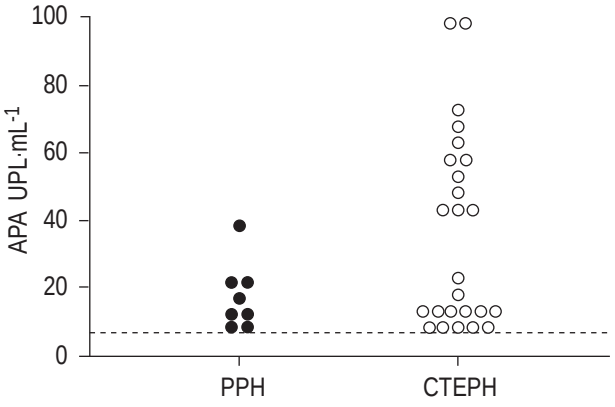

Fig. 1. – Titres of antiphospholipid antibodies (APA) measured by enzyme linked immunosorbent assay (ELISA) in the eight patients (out of 83) with primary pulmonary hypertension (PPH) and the 25 patients (out of 116) with chronic thromboembolic pulmonary hypertension (CTEPH) who had positive APA (>8 total UPL·mL^{-1}). The dashed horizontal line indicates the threshold level for positivity (8 total UPL·mL^{-1}).

the 16 patients with LA also had positive APA. There was no significant association between haemodynamic severity and the presence of phospholipid-dependent antibodies, either LA or APA.

Hereditary thrombotic risk factors

The frequency of antithrombin, PC, PS, deficiencies and FV and FII mutations are shown in table 3. In PPH, there was no deficiency of antithrombin, PC or PS, and both the FV and FII mutations were found in only one patient out of ~60. In CTEPH, the frequency of antithrombin and PS deficiencies and FII mutation was also low and similar to that of control subjects. The prevalences of FV mutation and PC deficiency were slightly higher but not statistically different from those found in PPH or in control subjects. None of the patients had combined defects of these hereditary thrombotic risk factors. One patient with CTEPH had a familial dysfibrinogenemia with hypofibrinolysis related to an abnormal interaction of fibrinogen with tissue plasminogen activator.

Table 3. – Frequencies of inherited thrombotic risk factor abnormalities in patients and control subjects

	AT	PC^{+}	PS^{+}	FV	FII
PPH	0/64	0/26	0/26^{+}	1/64	1/61
	0*	0*	0*	1.5*	1.6*
CTEPH	0/46	1/46	0/46	3/46	1/40
	0*	2*	0*	6.5*	2.5*
Control	0/100	1/100	0/100	3/100	2/100
	0*	1*	0*	3*	2*

AT: antithrombin; PC: protein C; PS: protein S; FV and FII: factor V and factor II mutations, respectively; PPH: primary pulmonary hypertension; CTEPH: chronic thromboembolic pulmonary hypertension. The prevalences are not statistically different between PPH and CTEPH, nor between control subjects and PPH or CTEPH for the five risk factors. *: percentage; $^{+}$: cannot be examined in patients on oral anticoagulants; $^{\#}$: F11 mutation only identified in 1996, therefore not all patients could be evaluated for the mutation.

Discussion

This is the first prospective study of the prevalence of thrombotic risk factors in a large series of patients with PPH or CTEPH. As new hereditary risk factors have only recently been identified they could only be tested in 46 of the 116 CTEPH patients and in 64 of the 83 PPH patients. In PPH, the prevalence of all hereditary risk factors was very low, whereas, in CTEPH, it was slightly higher but not significantly different from that of PPH patients or control subjects. The number of patients tested in this study does not allow the total exclusion of the role of inherited thrombotic risk factors in PPH and CTEPH. However, the results are in agreement with two other studies on CTEPH: Moser *et al.* [5] found a prevalence of only 1% of antithrombin, PC and PS defects and Lang *et al.* [6] reported the same frequency of FV mutation as in the current study. This low prevalence of hereditary thrombotic risk factors in CTEPH is unexpected, since most of these patients had experienced thrombosis. However, thrombosis is a multigenic disorder and to date, such hereditary risk factors have been identified in only 30% of patients with a familial history of venous thromboembolism. These data in CTEPH suggest that there must be additional risk factors that are still unknown. Alternatively, CTEPH could result from a local cellular abnormality promoting *in situ* thrombosis.

In contrast, there was a very high prevalence, close to 20%, of phospholipid-dependent antibodies including both LA and APA in CTEPH. This prevalence is much higher than usually reported in patients with acute VTE where estimates have ranged from 0.4 [17] to 14% for LA [18] and from 5 [19] to 14% for APA [18]. Moreover, in the current study, half of the patients with a positive APA had high titres (>40 UPL·mL^{-1}). Previous studies of APA in CTEPH have included too few patients to draw definitive conclusions. Only Auger *et al.* [8] studied a large series of consecutive patients with CTEPH and found a prevalence of LA close to 10%. This is less than that found in the present study, perhaps due to variable sensitivity of the assays used.

During the last decade, the clinical significance of phospholipid-dependent antibodies has been widely debated. It now seems well established that the presence of LA increases the risk of thrombosis [19–21]. Conversely, the clinical relevance of APA antibodies remains controversial as they have been identified in patients with no history of thrombosis [22], although Finazzi *et al.* [23] showed that high titres are strongly associated with thrombosis. Two other studies reported that the presence of high titre antibodies is associated with an increased risk of recurrent thrombosis [9, 24]. Thus, in CTEPH the presence of LA together with high titre antibodies may increase the thrombotic tendency.

In PPH the current results were strikingly different, with a prevalence of phospholipid-dependent antibodies half of that observed in CTEPH and with only low titres of APA. This finding makes the involvement of these antibodies in the pathogenicity of PPH questionable as they have also been reported to be associated with cell injury and/or autoimmune disease. In that regard, other autoantibodies such as antinuclear antibodies [25] and recently antibodies to tissue plasminogen activator have also been identified in PPH [26]. The presence of phospholipid-dependent

antibodies in PPH probably reflects either an autoimmune process and/or endothelial dysfunction. This hypothesis is supported by the finding of abnormalities of the fibrinolytic system such as increased levels of tissue plasminogen activator and/or plasminogen activator inhibitor [27].

In conclusion, the results provide new evidence that the pathophysiology of primary pulmonary hypertension and chronic thromboembolic pulmonary hypertension is different. In primary pulmonary hypertension, among thrombotic risk factors, only low titres of phospholipid-dependent antibodies were present and probably reflect endothelial dysfunction. Conversely, in chronic thromboembolic pulmonary hypertension, the high prevalence of high titre phospholipid-dependent antibodies and lupus anticoagulant makes their implication in the pathophysiological mechanism of this disease more likely.

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