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TGF beta and IL13 in schistosomiasis mansoni associated pulmonary arterial hypertension; a descriptive study with comparative groups

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Abstract

Background: It is suggested that interleukin (IL)-13 and transforming growth factor (TGF)-beta play a role in the pulmonary vascular changes found in animal models of schistosomiasis. The aim of this study was to assess and compare the serum levels of total TGF-beta and IL-13 of patients with schistosomiasis with pulmonary arterial hypertension (PAH) and patients with schistosomiasis without PAH.

Methods: 34 patients from the schistosomiasis outpatient clinic of the Hospital das Clinicas, Recife, Pernambuco, Brazil, without PAH assessed by echocardiography and 34 patients from the Reference Centre of Pulmonary Hypertension of Pronto Socorro Cardiológico de Pernambuco, Recife, Brazil with PAH, confirmed by right heart catheterization, were enrolled on the study. Both groups presented with schistosomal periportal fibrosis after abdominal ultrasound. Serum levels of TGF-beta1 and IL-13 were determined by ELISA. Student t test to independent samples, Mann-Whitney test to nonparametric variables, Pearson correlation test for correlation analyses and Fisher Chi-squared test to compare categorical analyses were used.

Results: The median value of TGF-beta1 was significantly higher in patients with PAH (22496.9 pg/ml, interquartile range [IR] 15936.7 – 32087.8) than in patients without PAH (13629.9 pg/ml, IR: 10192.2- 22193.8) (p = 0.006). There was no difference in the median value of IL-13 in the group with Sch-PAH compared to patients without Sch-PAH (p > 0.05).

Conclusion: Our results suggest that TGF-beta possibly plays a role in the pathogenesis of schistosomiasis-associated PAH.
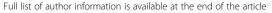
Keywords: Schistosomiasis, Pulmonary hypertension, Transforming growth factor-beta, Interleukin 13

Background

Pulmonary arterial hypertension (PAH) is defined hemodynamically as an increase in mean pulmonary arterial pressure (PAP) ≥ 25 mmHg at rest and pulmonary artery occlusion pressure ≤ 15 mmHg, characterized by the remodelling of the pulmonary vasculature that causes progressive dyspnea and can lead to right-sided heart failure and premature death [1]. It may be caused by several conditions including idiopathic PAH, connective tissue disease, HIV infection, schistosomiasis and others [2]. Around 240 million people are infected with some species of *Schistosoma* throughout the world, mainly *S. japonicum*, *S. haematobium* and *S. mansoni*, the latter being responsible for almost all cases of schistosomiasis-associated pulmonary arterial hypertension (Sch-PAH) [3-5]. Sch-PAH represents 30.8% of all causes of PAH in endemic areas of *S. mansoni*, and is considered the leading cause of PAH in these areas [6]. Hospital-based studies in Brazil, where *S. mansoni* is endemic, have reported pulmonary hypertension in approximately 10% of patients with schistosomal periportal fibrosis, using echocardiographic assessment [7,8], but only 4.7% of patients with the hepatosplenic form of the disease presented PAH confirmed by right heart catheterization (RHC) [8].

The main lesion caused by *S. mansoni* is due to an intense immune reaction against the parasite eggs trapped

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in small branches of the intrahepatic portal vein, resulting in chronic granulomas that stimulate the development of fibrosis in the portal spaces [5,9]. Granuloma formation is a predominantly T helper (Th) cell-dependent process, with an early Th1 proinflammatory response (IL-1, IL-12, interferon gamma, transforming growth factor beta [TGF – beta] and tumour necrosis factor-alfa), in which a switch occurs following egg production to a predominant Th2 immune response (IL-4, IL-5, IL-10 and IL-13) that plays a central role in the modulation of granuloma immune response [10-14].

Depending on the intensity of the fibrotic reaction around the portal veins, almost 5-10% of chronically infected individuals will suffer blockage of the hepatic blood flow and secondary portal hypertension [15]. The downstream effect is when the naturally occurring portal-systemic collateral vessels open to decompress the portal system [16,17], which allows embolization of eggs to the lungs. A secondary granulomatous reaction is triggered in the pulmonary vascular bed and gives rise to obliterative arteritis and vascular remodeling, which in some individuals results in PAH [18]. This is the main mechanism which is generally believed to be related to the development of Sch-PAH. However, a recent study failed to demonstrate a significant amount of parasite-derived antigens within the lungs of individuals who had died of Sch-PAH [19], but the histologic and vascular changes encountered were similar to those described in idiopathic PAH [20]. This suggests that embolization of eggs to the lungs is not the sole mechanism responsible for establishing PAH [19].

It is possible that genetic determinants are involved in the development of the disease. Around 80% of patients with familial PAH and 25% with idiopathic PAH present mutations in the gene encoding the bone morphogenetic protein receptor type II (BMPR-II), a member of the TGF-beta superfamily [21]. This leads to a change in the signaling of the TGF-beta superfamily. There is strong evidence to suggest that the TGF-beta system, by stimulating the proliferation of pulmonary arterioles, induces vasculogenesis [22,23].

Studies have suggested that IL-13, an important mediator of granulomatous and vascular responses in the schistosomotic infection, acts in conjunction with the TGF-beta system, and its increased activity has been found in animals with Sch-PAH [24-26]. Moreover, TGF-beta signaling is higher in mice exposed to *Schistosoma* and patients who have died of Sch-PAH than in control samples. Besides this, the increase in TGF-beta after *Schistosoma* exposure is dependent on IL-4 and IL-13 in the animal model [27].

The aim of this study was to assess the serum concentrations of TGF-beta1 and IL-13 in patients with Sch-PAH and compare them with patients with schistosomiasis without PAH.

Methods

Study population, participants, ethical approval and exclusion criteria

This was a descriptive study with comparative groups. Patients with schistosomal periportal fibrosis and PAH confirmed by RHC (mean PAP ≥ 25 mmHg and pulmonary capillary wedge pressure ≤ 15 mmHg) were recruited from the pulmonary hypertension referral center at the Pronto Socorro Cardiológico de Pernambuco, Recife, Brazil. The comparative group consisted of patients with schistosomal periportal fibrosis without pulmonary hypertension assessed by Transthoracic Doppler echocardiography and matched by sex and age, from the schistosomiasis outpatient clinic at the Hospital das Clínicas, Universidade Federal de Pernambuco, Recife, Brazil.

Patients were selected from July 2010 to December 2012, and the study was approved by the Research Ethics Committee of the Universidade Federal de Pernambuco (registration number at SISNEP Fr-326154/CAAE-0096. 0.172.000-10/REGISTRATION CEP/CCS/UFPE 099/10). All patients received an explanation concerning the scope of the study, and signed an Informed Consent Form. This study was conducted according to the Helsinki Declaration.

Exclusion criteria were: age ≤ 18 years of age; a history of alcohol abuse (alcohol intake in excess of 210 g/week for males and 140 g/week for females over the previous 5 years) [28]; evidence of non-schistosomal liver disease by ultrasound or clinical examination; hepatitis B or C viruses and/or HIV infection; evidence of congenital heart diseases (i.e. atrial septal defects); left-sided heart disease; moderate or severe lung disease (forced vital capacity [FVC] and/or forced expired volume in one second [FEV1] and/or FEV1/VFC ≤ 60% of predicted); and/or diagnosis of connective tissue disease.

Diagnosis of PAH and schistosomiasis

All patients were previously submitted to abdominal ultrasound in order to define the presence of schistosomal periportal fibrosis, which was classified according to Niamey criteria [29] as: C-peripheral, D-central, E-advanced and F-very advanced (Siemens Acuson X150® with3,5 mHz convex transducers). The groups with and without PAH were compared in relation to each pattern of fibrosis.

The included patients with periportal fibrosis presented with the hepatointestinal (spleen longitudinal diameter < 120 mm) and hepatosplenic forms of schistosomiasis (spleen longitudinal diameter of ≥120 mm). The diameters of the porta and splenic veins were considered enlarged if they exceeded 12 mm and 9 mm, respectively. The presence of collateral vessels was also reported.

A Doppler echocardiography was performed on the comparative group to exclude PAH, using standard procedures [30] (Philips EnVisor C® system with 2.5 and 3.5

mHz mHz transducers). The following parameters were considered in order to exclude PAH: tricuspid regurgitation velocity ≤ 2.8 m/s, pulmonary artery systolic pressure ≤ 36 mmHg and no additional echocardiographic variables suggestive of pulmonary hypertension (increased velocity of pulmonary valve regurgitation, short acceleration time of right ventricular ejection into pulmonary artery, increased dimensions of right heart chambers, abnormal shapes and function of the interventricular septum, increased right ventricular wall thickness, and dilated pulmonary artery) [1].

The functional classification of patients with PAH was determined according to the modified World Health Organization classification from the New York Heart Association [31]. Those patients with PAH were previously submitted to RHC. Access to the venous and femoral arteries was obtained using the Seldinger technique. Catheterization was performed in the right and left chambers of the aorta, and manometry was conducted on the left ventricle, pulmonary artery, right ventricle and right atrium using a 06-French pigtail catheter and a 07-French wedge catheter for the occluded capillary wedge pressure. The output and resistance calculations were performed using the Fick method [32].

Sample collection and assay

Venous blood samples were collected under aseptic conditions, using vacuum tubes (BD, Becton Dickinson, UK) without anticoagulants. Samples were centrifuged for 5 min at 2000 × g and the serum was stored in 0.5 ml aliquots at −80°C until assayed. The levels of TGF-beta1 and IL-13 were assessed using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc, Minneapolis, MN). To activate latent TGF-beta1 to the immunoreactive form, 1 N HCl was used for acid activation and 1.2 N NaOH/0.5 M HEPES for neutralization. After activation, the samples (serum) were diluted and the concentration was measured according to the ELISA kit. The presence of cytokines was revealed and the results were expressed as pg/ml in accordance to standard curves. The assay sensitivity to TGF-beta1 was 15.4 pg/ml and to IL-13 57.4 pg/ml.

Statistical analysis

The data was incorporated into the database Epi Info 3.02 and analysed using STATA. Results were presented as median and percentiles (interquartile range). Comparisons for quantitative variables were conducted using the Student t-test for independent samples, the Mann–Whitney test for nonparametric variables, and the Pearson correlation test for correlation analyses. The Fisher Chi-squared test was used to compare the categorical variables: sex, clinical form of schistosomiasis and periportal fibrosis pattern between the patients with and without PAH. A p value <0.05 was considered as statistically significant.

Results

Thirty four patients with Sch-PAH and periportal fibrosis and 34 patients with periportal fibrosis without PAH diagnosed by Doppler echocardiography and matched by sex and age, concluded the study. One female with FEV1/VFC ≤ 60% and one male who was HbsAg antigen positive were excluded from the control group. Two women with PAH were also excluded: one presented with a previous diagnosis of cutaneous scleroderma and the other with atrial septal defect. The clinical and hemodynamic variables of the patients with Sch- PAH are shown in Table 1. Twenty seven (79.4%) patients were taking medication for PAH at the time of inclusion: 25 were on phosphodiesterase inhibitors, one on bosentan and one on calcium channel blockers (Table 1). There was no difference between groups regarding age, sex, the periportal fibrosis pattern and portal vein diameter (Table 2). The longitudinal diameter of the spleen was wider in the control group, without PAH. All patients in the control group and 30 patients with Sch-PAH presented the hepatosplenic form of schistosomiasis and only four patients in the Sch-PAH group presented the hepatointestinal form of the disease (Table 2).

Median serum levels of immunoreactive TGF-1 beta were significantly higher in patients with Sch-PAH compared to patients without PAH (22496.9 [IR: 15936.7 − 32087.8] pg/ml versus 13629.9 [IR: 10192.2 − 22193.8] pg/ml) (p = 0.006). There was no difference in the median value of IL-13 in the group with Sch-PAH (152.3 [IR: 103.7 - 177.8]) pg/ml, compared to patients without PAH (131.8 [IR: 100.2 - 162.5]) pg/ml (p = 0.177) (Table 2).

Discussion

The present study has demonstrated significantly higher serum levels of immunoreactive TGF-beta1 in patients with Sch-PAH compared with patients with schistosomiasis without PAH. TGF-beta 1 is one isoform of TGF-beta that causes enhanced cell proliferation in smooth muscle

Table 1 Clinical and hemodynamic characteristics of patients with schistosomiasis-associated pulmonary arterial hypertension

Variables	Patients	Characteristics
Functional class I/II/III/IV/N	34	9/10/7/8
Drugs to PAH/N (%)	34	27 (79.4%)
Mean PAP mmHg median (P25; P75)	34	55.5 (46; 62.7)
RAP mmHg median (P_{25}; P_{75})	29	13 (9; 16)
LVEDP mmHg median (P_{25}; P_{75})	32	14 (11.7; 15)
CI l/min/m² median (P_{25}; P_{75})	25	2.68 (1.97; 3.67)
PVR Dyn/sec/cm median (P_{25}; P_{75})	30	989.5 (644.5; 1417.5)

Recife, 2012.
CI – cardiac index; LVEDP-left ventricular end-diastolic blood pressure; PAP- pulmonary artery pressure; N - sample size; PAH – pulmonary arterial hypertension; PVR- pulmonary vascular resistance; RAP- right arterial pressure.

Table 2 Comparison of patient characteristics according to the condition of schistosomiasis-associated pulmonary arterial hypertension

Groups	General	With Sch-PAH	Without Sch-PAH	p-value
Samples	68	34	34	-
Age (mean ± sd)	49.3 ± 12.9	49.8 ± 13.2	49.8 ± 12.8	0.759[a]
Sex: Male	24 (35.3%)	11 (32.3%)	13 (38.2%)	0.612
Clinical form				
Hepatosplenic	64 (94.1%)	30 (88.2%)	34 (100%)	0.039[c]
Hepatointestinal	04 (5.9%)	04 (17.8%)	00 (−)	
Periportal fibrosis				
D[x]	24 (35.3%)	13 (38.2%)	11 (32.3%)	
E	37 (54.4%)	18 (52.9%)	19 (55.9%)	0.674[c]
F	07 (10.3%)	03 (8.9%)	04 (11.8%)	0.685
TGF-β1 pg/ml (median − P_{25}; P_{75})	19442.2 (11070.4; 30097.2)	22496.9 (15936.7; 32087.8)	13629.9 (10192.2; 22193.8)	0.006[b]
IL-13 pg/ml (median − P_{25}; P_{75})	152.3 (100.2; 165.0)	152.3 (103.7; 177.8)	131.8 (100.2; 162.5)	0.177[b]
Portal vein diameter cm	1.15 ± 0.33	1.09 ± 0.37	1.23 ± 0.25	0.085[a]
Spleen diameter cm	14.5 ± 3.3	12.7 ± 2.17	16.4 ± 3.3	0.000[a]

Recife, 2012.
[a]Student's t-test; [b]Mann–Whitney nonparametric test; [c] Fisher X^2 test; [x]Reference value, D- central fibrosis; E- advanced fibrosis; F- very advanced fibrosis; TGF-β1 – transforming growth factor-beta 1; IL-13- interleukin-13.

cells in the pulmonary arteries of patients with idiopathic PAH and has a growth-inhibitory effect on normal cells [33]. A previous study has already demonstrated elevated levels of TGF-beta1 and other growth factors in serum samples of 46 patients with PAH with other diverse etiologies compared to 20 controls, and suggested that these substances may contribute to vascular remodeling in PAH [34].

The link between TGF-beta and the pathogenesis of Sch-PAH has been studied in animal models. The altered signaling of the TGF-beta system was demonstrated in experimental mouse models infected with *Schistosoma* and in lung specimens obtained from autopsies of individuals who had died of Sch-PAH through increased Smad2/3 activity found in the affected vessels [19]. Smads are proteins that can be phosphorylated after the attachment of TGF-beta in cell receptors, and which are able to move to the nucleus of the cells, altering essential functions [22]. The TGF-beta system is a probable pathway to direct the proliferation of pulmonary arterioles in PAH, leading to vasculogenesis, as well as intimal hyperplasia and growth of the media layer [21-23]. It is believed that in the case of PAH linked with BMPR-II mutation, the main mechanism is the imbalance secondary to the loss of BMPR-II function, associated with the increased activation of the TGF-beta superfamily of receptors [22].

Studies using the inhibition of TGF-beta in experimental models of PAH have confirmed the role of this growth factor in the pathogenesis of PAH and have indicated its possible use in the treatment of this disease [35-37]. PAH was induced in rats by monocrotaline injection, and the later treatment with an antibody against TGF-beta-ligand decreased the pulmonary artery systolic pressure and right ventricular hypertrophy, increased exercise capacity and reduced pulmonary vascular remodeling as evidenced by decreased vessel-wall thickness and number of pre-capillary arterioles. This effect occurred with bosentan, the endothelin receptor blocker. TGF-beta regulates endothelin-1 synthesis, which is a possible mediator of TGF-beta actions [38]. More recently, Graham et al. [27] demonstrated that a mouse model of Sch-PAH submitted to pharmacological blockage of the TGF-beta ligand and receptor and that mice lacking Smad3 were significantly protected from pulmonary vascular remodeling and PAH. This blockage also led to a decrease in IL-4 and IL-13 concentrations [27]. A significant increase was encountered in the levels of TGF-beta1 mRNA as measured by RNA sequencing in *S. mansoni*-exposed mice compared with unexposed mice, and there was no change in the TGF-beta2 and TGF-beta3 expressions with *S. mansoni* exposure. A more extensive expression was observed of phosphor-Smad2/3 in the pulmonary vascular intimal, medial, and adventitial regions in experimental Sch-PH lungs compared with control mice. These authors also demonstrated an increased expression of Smad2/3 within the pulmonary vascular media compartment in subjects who had died of Sch-PAH compared with control human lungs [27]. Accordingly, the present study also measured the TGF-beta1 isoform, and our results were similar, in that they supported a role played by this growth factor in the pathogenesis of Sch-PAH.

There was no difference in the median value of IL-13 in the group with Sch-PAH, compared to patients without PAH, contrary to that observed in animal models. Crosby et al. [26], using mice experimentally infected with Schistosoma, discovered that the grade of pulmonary vascular remodeling correlated with the egg burden in the lungs and with plasma Th1 and Th2 cytokines. Furthermore, IL-13 stimulated the migration of mouse pulmonary artery smooth muscle cells in transwell assays. There was a peak of cytokines IL-10, IL-13, IL-6 and IL-4, 17 weeks after infection [26]. In this animal model, transcutaneous infection of the animals with cercariae before the intravenousinjection of Schistosoma eggs some weeks later was required for PAH development, suggesting that a potent inflammatory response in the lungs due to prior sensitization is necessary to elicit PAH, more than the mere embolization of eggs in the lungs [25,26].

IL-13 signaling is mediated by a complex receptor system [39]. Some studies suggest that it is the balance between levels of IL-13Rα1 versus IL-13Rα2 that regulates the IL-13 mediated response. Graham et al. [25] demonstrated that vascular remodeling was reduced in mice with the loss of IL-13Rα1 receptors, which leads to the loss of IL-13 function, in a non-significant manner. On the other hand, mice without IL-13Rα2, and with an IL-13 gain-of-function, presented thicker intimal layers in the pulmonary vessels as well as higher right ventricular pressure. The increased IL-13 signaling through the loss of IL-13Rα2 receptors was able to elicit PAH in this model. Other authors have already reported that the imbalance in IL-13 receptors is the key feature for changing IL-13 signaling in other animal models of PAH and in patients with idiopathic PAH, more than changes in circulating levels of this cytokine [40,41]. Maybe, for this reason, the present study failed to demonstrate increased serum levels of IL-13 in patients with Sch-PAH. More recently, it was demonstrated that increased levels of IL-4 and IL-13 mRNA and proteins seen after exposure to *S. mansoni*, tended toward suppression after blockage of the TGF-β signaling pathway. These data suggest that IL-4/ IL-13 and TGF-β act mutually [27].

Although parasite eggs and *S. mansoni*-soluble egg antigens were found in the lungs of experimentally infected mice and in specimens of human intestine, there was no significant amount of parasite-derived antigens within the lungs of individuals who had died of Sch-PAH, despite the presence of pulmonary vascular remodeling with plexiform lesions and arterial medial thickening [19]. This suggests that after an initial acute inflammatory process, which may be triggered when the schistosomula passes through the lungs in genetically predisposed individuals, vascular remodeling is established and can progress or persist regardless of the presence of the antigen. Maybe this process starts and progresses with the release of cytokines and growth factors by the granulomas produced in other sites, such as the liver.

Crosby et al. [42] demonstrated that treatment with praziquantel was effective in eradicating adult worms of *S. mansoni*, prevented PAH development and reversed pulmonary vascular remodeling in infected mice. Contrary to what occurs with liver fibrosis, which may reverse or reduce with parasite treatment; this does not seem to occur with the lung pathology of humans [19,43,44]. For all these reasons it is important to clarify the immune mechanisms involved in the pathogenesis of Sch-PAH, in order to discover more effective therapy targets, acting directly on the proliferative component of PAH.

Study limitations
The sample size in the present study was probably small, but if we consider that this study enrolled individuals with PAH of one single etiology, it was an important sample of patients. TGF-beta1 and IL-13 were chosen according to the new evidence encountered in studies with animal models of Sch-PAH. This was a preliminary study, therefore new studies using larger samples and evaluating other growth factors and cytokines need to be performed to confirm our results and bring new insights into the immunopathogenesis of this devastating disease.

Conclusions
This study has demonstrated significantly increased serum levels of TGF-beta1 in patients with Sch-PAH compared with patients with schistosomiasis without PAH, suggesting that this growth factor may contribute to vascular remodeling in this disease. However, this study was not able to detect a significantly elevated level of IL-13 in patients with Sch-PAH, maybe because either a larger sample size is necessary or an alteration in the IL-13 receptors is responsible for the augmented IL-13 signaling found in animal models.

Authors' contributions
RCSF, SMLM, ALCD, APB contributed to the conception and design of this study, the acquisition of patients data, analysis and interpretation of data and were involved in drafting the manuscript. SMLM carried out the imunnoassays. The other authors (CAMS, LACL, CAP, IMF, ABM, MOA) contributed to the acquisition of data and clinical specimens. All authors have given approval for the final version of the manuscript to be published.

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