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Pulmonary arterial hypertension (PAH) from autopsy study: T-cells, B-cells and mastocytes detection as morphological evidence of immunologically mediated pathogenesis

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ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is characterized by severe vascular remodelling, resulting in increased pulmonary vascular resistance with cardiac hypertrophy and heart failure. However, the diagnosis of PAH is often inaccurate. Many cases of PAH are incorrectly diagnosed or missed, and they are often associated with death. The aim of this study was to verify the morphological and histological criteria of fatal cases of PAH and evaluate the lymphocytic populations associated to lesions with reactive neo-angiogenesis.
Methods: Pulmonary lung sections from 10 cases of sudden unexpected death (SUD) in the absence of previously diagnosed diseases and in an apparent state of well-being, with final histological post autopsy diagnosis of PAH were collected. The pathological findings were compared using ten controls from non-pathological lung from deaths from other causes. The autopsies included 4 males (40%) and 6 females (60%) with an average age of 52.1 ± 10.1 years. Sections stained with hematoxylin and eosin (H&E) were revised for a morphological diagnosis. Subsequently, serial sections were performed and stained with immunohistochemistry for anti-CD20 (B-lymphocytes), anti-CD3 (T-lymphocytes), anti-CD4 (T-helper lumphocytes), anti-CD8 (T-cytotoxic lymphocytes) and anti-CD117/C-Kit (mast cells/MCs) to detect inflammatory infiltrate and different ratios of cell-type. Statistical analysis was conducted using a paired *t*-test looking at 100 cells in 3 different tissue samples representative of vascular lesion and 3 different random normal lung parenchyma fields without lesion (from 10 normal control lungs), to identify specific lymphocyte subpopulations in inflammatory infiltrates.
Results: There was a significant percentage increase of CD20 ($p < 0.001$), CD8 ($p = 0.002$), CD4 ($p < 0.001$), and CD117/C-Kit positive (C-Kit+; $p < 0.001$) cells mainly detected around wall vessels; while increased MCs positivity and C-Kit+ were observed especially in alveolar septa. In addition, reactive angiomatosis was observed.
Conclusions: The inflammatory infiltrate should be included for a correct diagnosis of PAH besides the vascular remodelling. The inflammatory infiltrate seems to be implicated as a main factor in the pathogenesis. This finding is important to rule out secondary pulmonary hypertension, to identify SUDs of unknown causes and to add new elements to the literature that can explain the immunologically related pathogenesis of PAH.

1. Introduction

Pulmonary hypertension (PH) is clinically defined as an at rest mean pulmonary arterial pressure (mPAP) of 25 mm Hg or higher, confirmed by right cardiac catheterization [1]. PH affects approximately 1% of the global population, up to 10% of individuals over the age of 65 and at

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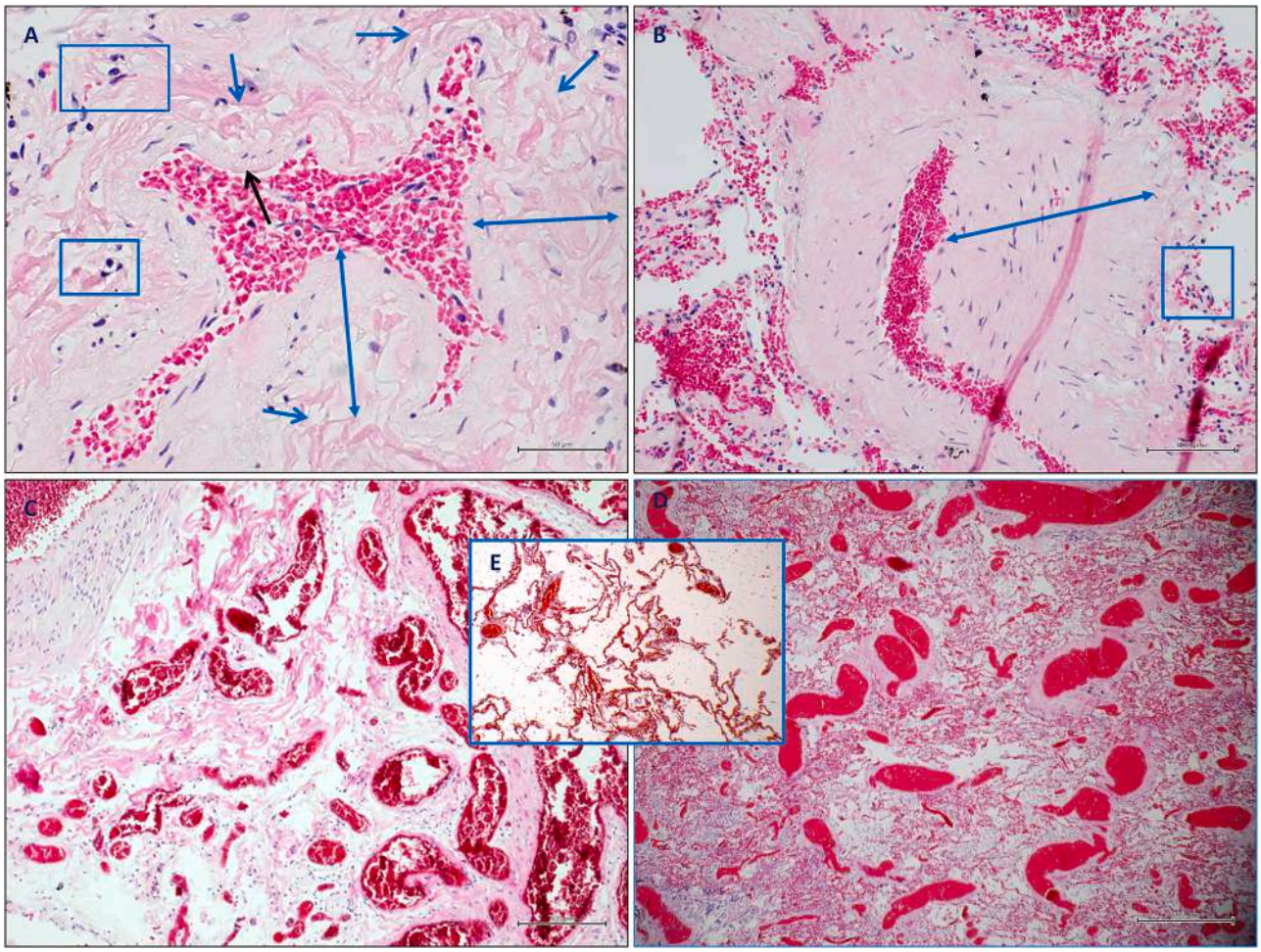

Fig. 1. A-B: a different finding of vascular wall. The plexiform aspect may be more or less evident up to the complete occlusion of the vessel lumen after the rearrangement and hypertrophy of the middle tunic. In A, the blue arrow indicates media disarrangement with fragmentation and homogenization; the double arrow indicates the increase in wall thickness; the blue box indicates the residual inflammatory infiltrate around the vessels; the black arrow points to the vascular endothelium (scale bar on the bottom right 50 μm). In B, the double arrow indicates the complete rearrangement of the vessel wall up to the occlusion of the lumen; the blue box indicates the residual inflammatory infiltrate around the vessels (scale bar on the bottom right 100 μm). In C and in D, different degrees of reactive angiomatosis (scale bar on the bottom right 200 μm in C, 500 μm in D). In E insert, normal histology of the lung (x10 magnification).

least 50% of heart failure (HF) patients. Regardless of the cause, PH leads to a gradual worsening of symptoms and can cause death. The World Health Organization has classified PH into 5 clinical subgroups including pulmonary arterial hypertension (PAH) in the group 1 [2,3].

PAH incidence is low (annual incidence ranges from 2.4 to 7.6 cases per million population) [4], with a prevalence in middle-aged women and a mean survival of about seven years. If not treated, PAH can determine a progressive increase in primary intra-pulmonary resistance and a consequent heart failure up to death [5,6].

Seven subgroups of PAH are identified: idiopathic PAH; hereditary PAH; drug or toxins-induced PAH; PAH associated with congenital heart diseases, infective disease, portal hypertension, etc.; PAH associated calcium channel blocker therapy; PAH with venous/capillary involvement; persistent new-born PAH (mothers receiving selective serotonin reuptake inhibitors) [2,3,7]. It seems, therefore, that the etiopathogenesis of PAH is multifactorial, involving inflammatory/immune-mediated mechanisms, genetic alterations and possibly other mechanisms as well, with increased intrapulmonary vascular resistance, vasoconstriction, remodelling and obstruction/-thrombosis of small vessels and arterioles [8–10].

Data from preclinical and clinical studies on PAH have highlighted the role of inflammation in the development of the disease, which can have severe effects on heart function up to heart failure and death through remodelling of the vascular tree. Few morphological studies have been performed on PAH and most of them use animal models after induction of hypertension and hypoxia [11]. They have still had a significant impact on the definition of etiopathogenesis and above all on the inflammatory / immune-mediated mechanisms involved. However, they need further confirmation and more additional data.

Conventional histology shows thickening of the vessel wall due to hyperplasia and hypertrophy of the intima, media and adventitia, with reduction of the vessel lumen that appears typically plexiform/scalloped and with associated reactive angiomatosis. The thickening of the wall is due to the remodelling following the damage which in the initial stages is evident with fragmentation and degeneration of the middle tunic. This damage is still not noticeably clear. Associated reactive angiomatosis is classically due to increased pulmonary pressures and is characterized by crowded areas of distorted, thickened and congested vessels with an angioma-like arrangement pattern. Recent studies, mainly conducted in animal models, have reported an increase in mast-cells (MCs) around the pulmonary vessels [12–15]. Other studies reported a predominant CD45/LCA+ (leucocyte common antigen) cell type switched from neutrophils to CD3 + T-cells, with increases in CD4 + , CD8 + , additional diversely activated classical myeloid-derived dendritic cells (CD14-HLA-DR+CD11c+CD1a+/-) and non-classical plasmacytoid dendritic cells (pDCs; CD14-CD11c-CD123 +HLA-DR+), together with

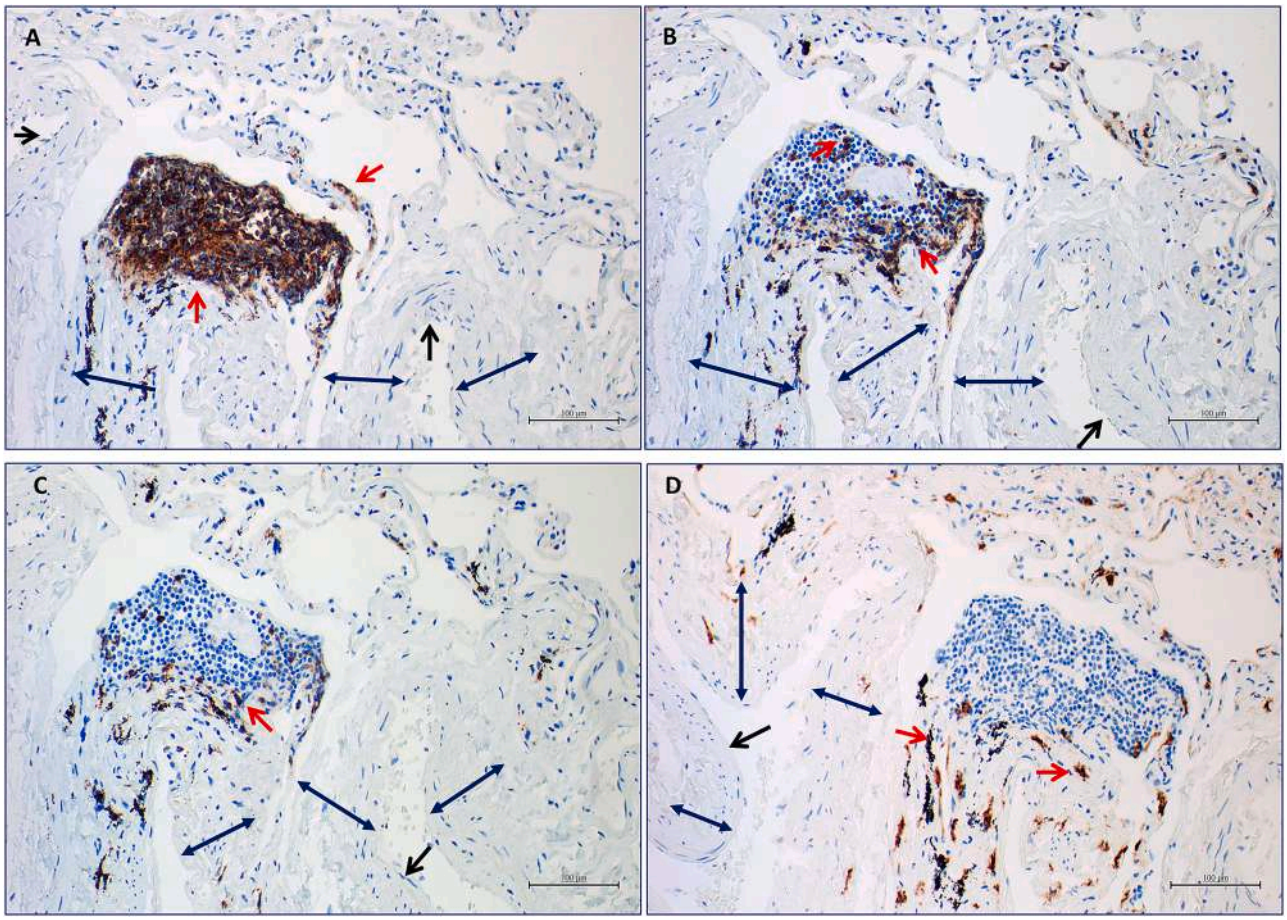

Fig. 2. Immunohistochemistry for B-cells (anti-CD20), T-helper (anti-CD4), T-suppressor (anti-CD8), MCs (anti-CD117). The particular follicular-like pattern of lymphoid cells with prevalent ratio of CD20 + cells (the red arrow indicates CD20 + cells in A), accompanying ratio of CD4 + cells (red arrow in B), and lower share of CD8 + cells (the red arrow indicates CD8 + cells in C). The red arrows indicate CD117 + cells in the periphery of the vessel, especially in the alveolar walls in D. The double blue arrow indicate the parietal thickening, in the different section levels. The most typical image is the section marked (D) in which the distorted lumen of the transversely cut vessel is best evident. For each image, the black arrow in the vascular lumens indicates the endothelium (scale bar for each image on the bottom right 100 μm).

MCs and basophils [15,16].

In this study, a series of sudden unexpected death (SUD) with histological findings of pulmonary hypertension, cardiac hypertrophy and peripheral findings of heart failure was revised with additional immunohistochemistry for inflammatory infiltrate staining and a conclusive diagnosis of PAH.

2. Materials and methods

All autopsies were performed in accordance with the Recommendations on the Harmonization of Forensic Autopsy Rules of the Committee of Ministers of the Council of Europe (1999). Pulmonary sections from 10 PHA were collected. The study group was represented by 4 male (40%) and 6 females (60%), with an average age of 52.1 ± 10.1,. All deceased subjects had no history of previous heart or genetic or other diseases and were in a state of well-being prior to death. Autopsy findings were mostly represented by cyanosis, organ congestion and increase in cardiac diameters with right parietal thickness greater than 0.5 mm and dilation of the right ventricular cavity. We also included as controls 10 normal lungs from subjects died by other causes with evidence of normal lung parenchyma fields without lesions.

Histological evaluation. The original lung samples were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Section (4 μm thick) were stained with hematoxylin and eosin (H&E) for diagnosis of the PAH. The immunohistochemistry was performed to identify lymphocyte sub-populations (ab Cd20 (L26), ab Cd3 (3GV6), ab Cd4 (sp35), ab Cd8 (sp57), ab Cd117 (9.7); ultraView DAB Detection Kit; Banchmark XT Ventana Roche) according to the common protocol [17]. Ten controls of non-pathological lung from deaths from other causes were used. All stained samples were examined under digital and light microscope from two independent researchers. Immunohistochemical evaluation was scored using a three-point scale of range values. For lymphocytes and MCs, we attributed a score of 0 for no increase, scores 1, 2, or 3 for little (<15%), moderate (15–30%), or high (>30%) increase of cell content at high magnification in representative fields.

Statistical analysis: The absolute cell counts were expressed with means and standard error of mean (SEM). Results were analyzed using a paired *t*-test looking at 100 cells in 3 different tissue samples representative of vascular lesion and 3 different random lung control parenchyma fields without lesion for each case.

3. Results

The H&E stain showed in all deaths, thickening of the wall of medium vessels with sometimes the presence of plexiform aspects and remodelling with reduced lumens. In almost all cases, aspects of reactive angiomatosis and areas of alveolar damage were associated [Fig. 1]. In all cases, the highest score of 3 was obtained for B cells, a score of 2 for lymphocytes CD4 + and for CD117 positive MCs, while a score of 1 for lymphocytes CD8 + . The lymphoid infiltrate was organized with a

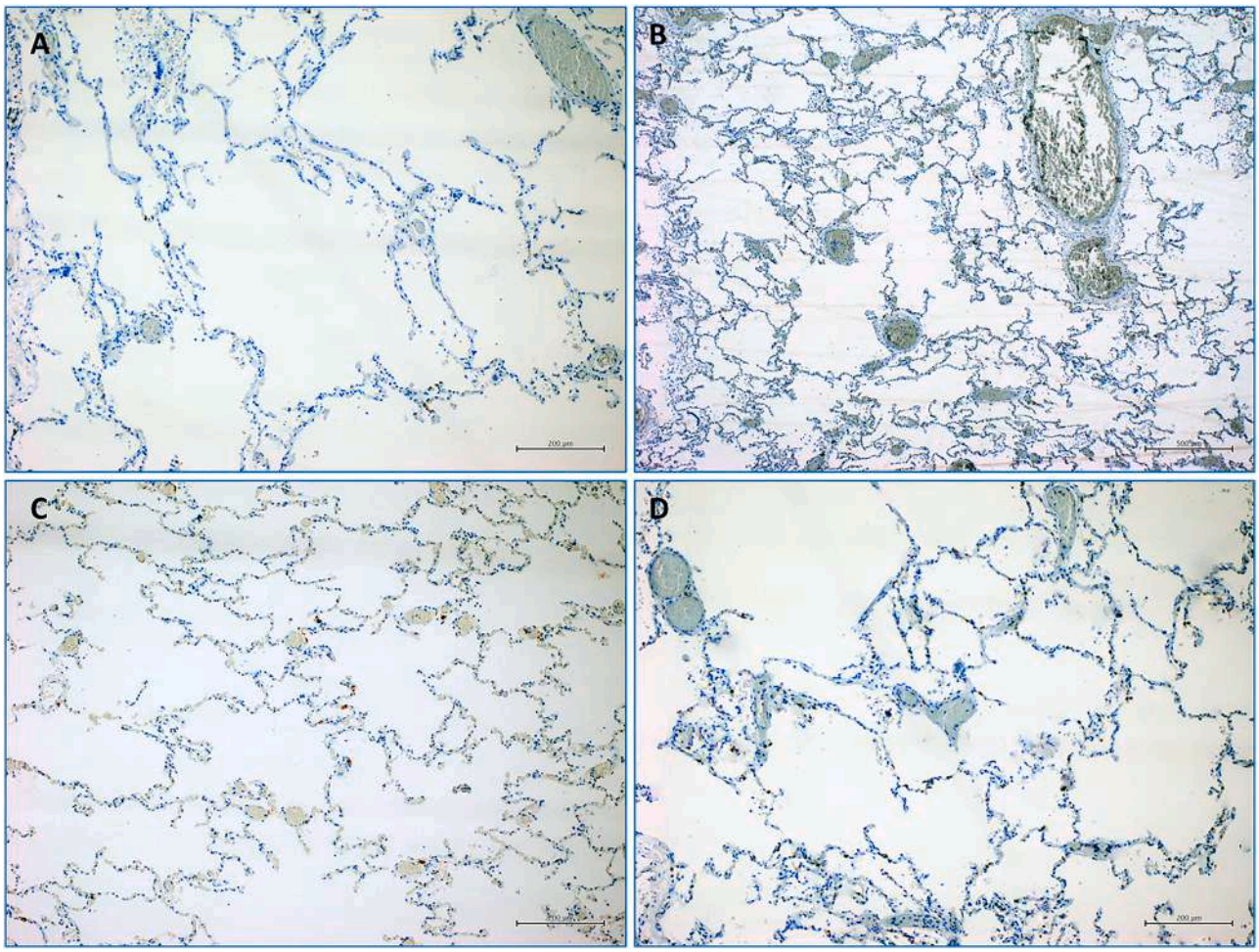

Fig. 3. Negative control for CD20 antibody in **A**, CD4 in **B**, CD8 in **C**, and CD117 in **D** (only rare intersectal inflammatory elements (brown signal of detection)**;** (scale bar on the bottom right 200 μm in A, C, D, and 500 μm in B).

similar follicular pattern around the vessels, whilst the MCs were more evident in the alveolar septa, contrary to the non-pathological lung coming from deceased for other causes [Fig. 2, Fig. 3].

The percentage changes of absolute numbers of lymphocyte subpopulation according to different zones (3 different tissue samples representative of vascular lesion and 3 different random lung parenchyma fields without lesion for each case) are reported in Fig. 4. The amount of percentage (± SEM) of CD20 (79.4 ± 2.7%; $p < 0.001$), CD4 (20.6 ± 1.1%; $p < 0.001$), CD8 (3.6 ± 0.9%; $p = 0.002$), and CD117 (12.5 ± 1.2%; $p < 0.001$) cells grew significantly in all patients ranging between 3.6% in CD8 to 79.4% in CD20.

In addition, histology of heart showed thickened and fragmented muscle fiber cells with anisonucleosis as observed in the ventricular hypertrophy and according with the macroscopic increase in wall thickness observed in association with the dilation of the right ventricular cavity in some cases (Fig. 5).

4. Discussion

In this study, we have examined the immune T-cell and B-cell populations within PAH lungs in SUD. The data were overlapping in all cases with an increase of MCs around the vessels and in the alveolar septa, a prevalent and typical CD20 + lymphocytes (score 3, >30%) with follicular-like pattern, associated minor ratio of CD4 + lymphocytes T helper (score 2, 15–30%), and smaller percentage of CD8 + T cytotoxic/suppressor lymphocytes (score 1, <15%). Furthermore, the remodelling of the vascular wall with thickening of the middle tunic was observed especially when the inflammation was not marked. In addition, aspects of reactive angiomatosis were present in the surrounding areas and sometimes also with accompanying alveolar damage and extravasation. These last two aspects appear to be reported for the first time at the best of our knowledge. The presence of a PAH was supported by the cardiac changes observed such as the right ventricle (RV) hypertrophy with characteristic appearance of thickened and fragmented muscle fiber cells and anisonucleosis. The pulmonary vascular wall with its cellular elements plays a dual role being both the source and the target of inflammatory mediators. The vascular endothelium is probably the initial site of the disease in PAH, being the target of inflammation but being able at the same time to synthesize inflammatory mediators from hypoxic stimulus and ROS [18]. However, the mechanisms are still unclear [19–21]. The most common gene mutations implicated in PAH are found in bone morphogenetic protein type 2 (BMPR2) and correlate with the high expression of the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1); hence with the further recruitment of inflammatory cells into diseased vessels. In support of this, some studies have shown that BMPR2 deficiency promotes an exaggerated inflammatory response in PAH progression [22–24]. Other studies instead evaluated the methylation pattern of the BMPR2 promoter region in patients with PAH and in control subjects by PCR and identified a probable site of methylation in the BMPR2 promoter region compared to control cases [25]. Associations between TBX4 and KCNK3 genes and PAH are also described with more benign course in mutation related TBX4 forms [26].

Likewise, the smooth muscle cells of the vascular walls can secrete

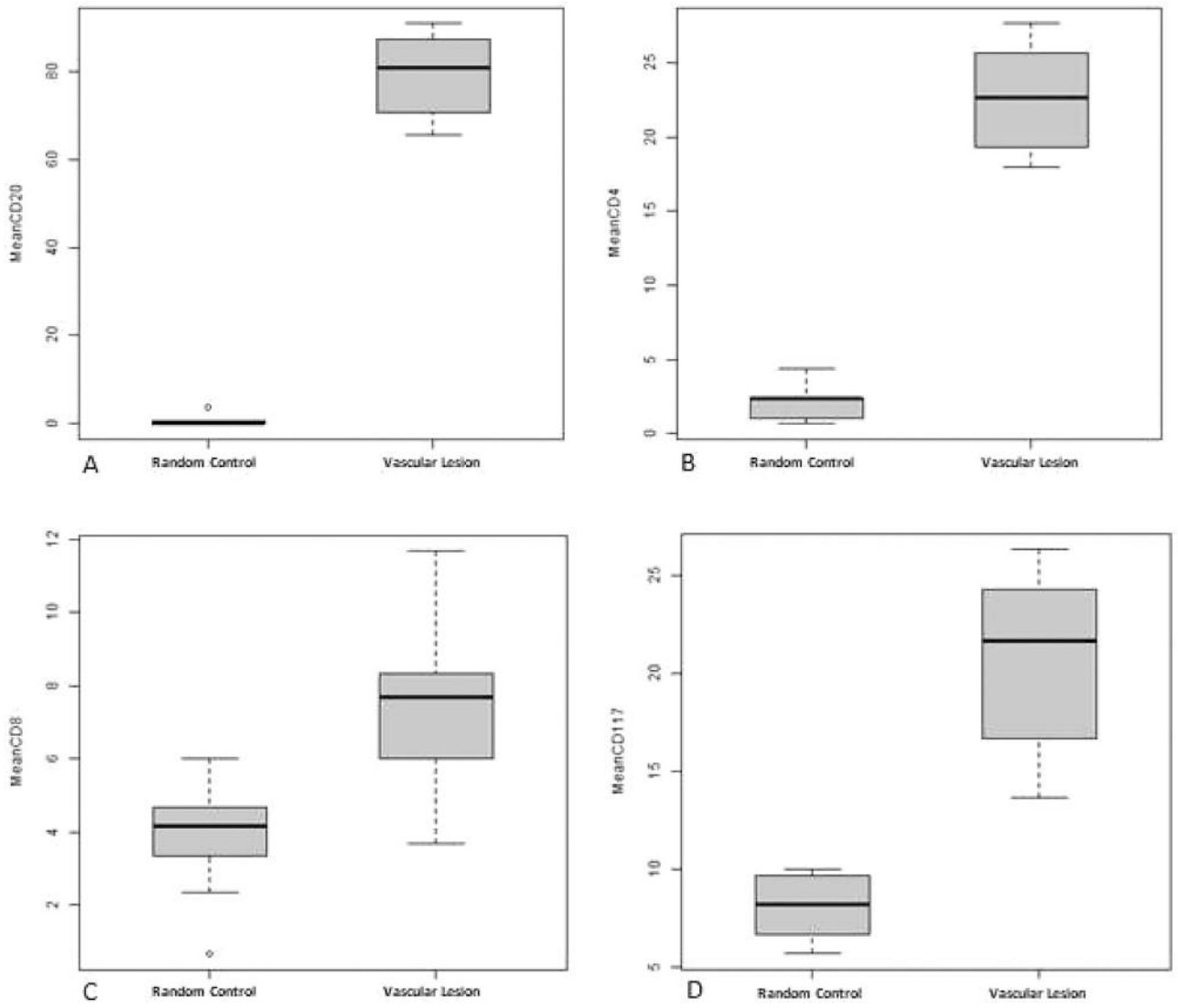

Fig. 4. The changes of absolute numbers of lymphocyte subpopulation according to vascular lesion zones and random lung parenchyma fields without lesion. The numbers are means of 3 different tissue samples. Units are in percentage (%).

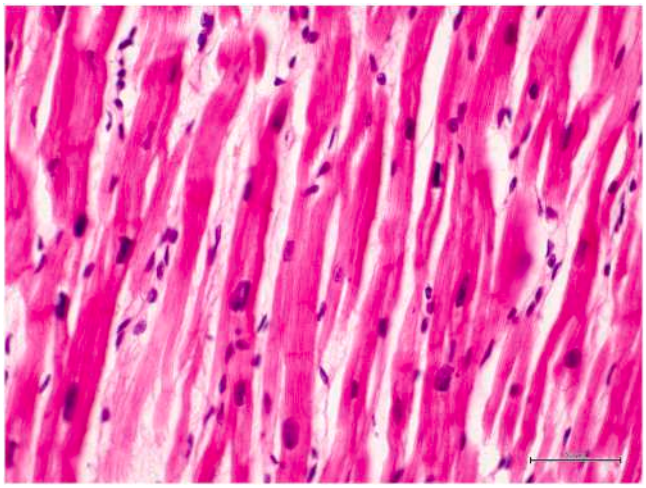

Fig. 5. Right ventricular hypertrophy: the thickening and the fragmentation of myofibers with anisonucleosis (H&E; scale bar on the bottom right 50 μm).

pro-inflammatory cytokines [27], and fibroblasts respond to various stimuli, including hypoxia assuming a pro-inflammatory phenotype, increasing the production of cytokines and adhesion molecules [28], and promoting leukocyte survival with delayed resolution of inflammation [29].

The literature indicates changes in T lymphocytes in PAH from peripheral blood, reporting an increase in CD4 + lymphocytes and a decrease in CD8 + T cells on the contrary to other pathological or physiological conditions. However, these results are not univocal and above all, they denote a variability as happens for neoplasms [30–34].

Lung tissue and bronchoalveolar washing fluid studies report the presence of B and T lymphoid cells as well as in experimental studies with induction of hypertension CD4 + cellular T subpopulations and vascular remodelling with inhibition of PAH progression are reported [35–38]. Similarly, there are experimental and clinical data on B lymphocytes related to PAH severity and pulmonary vascular remodelling [38–41]. On the other hand, increased levels of autoantibodies are commonly detected in autoimmune diseases associated with PAH [42–44]. The presence of perivascular MCs in the lung has also been described with an increase in the percentage of expression in PAH [43–45], but in our case series the MCs are located also in the septal alveolar walls. It has been observed that inhibition of MCs activation or

degranulation with subsequent inhibition of release of mediators such as histamine, serotonin, protease, lipid mediators, cytokines and chemokines is effective for attenuating PAH and pulmonary vascular remodelling [44–46].

In addition, MCs have an important role in modulating the immune response by inducing activation, proliferation and secretion of cytokines, CD4 + T lymphocytes, recruitment of CD8 + T lymphocytes and inhibiting the activity of regulatory T lymphocytes (secretion of histamine and IL-6) [47–52]. These data confirm the rarity of the lesion, the difficult clinical classification of patients and the multifactorial etiopathogenesis. However, the morphological data of this study and a few other studies on lung sampling constitute the only evidence of the prevailing role of the immune-mediated and cell-mediated response, with a prevalent action of CD20 cells and MCS, in addition to damage to the endothelium and of the release of chemical mediators.

Finally, it is important to evaluate other diseases in the differential diagnosis of PAH. Primarily, the secondary pulmonary hypertension from heart disease must be considered. The changes of PAH in the wall thickness of the pulmonary vessels with homogenization and fragmentation of the middle tunic are generally absent in secondary hypertension, while the identification of the cardiac diseases and other pulmonary findings such as cardiogenic edema constitute a valid support to distinguish a secondary hypertension. The identification of pulmonary infections, drugs use, neoplasia and others pulmonary disease, that cause an increase in intrapulmonary resistance with subsequent primitive pulmonary hypertension must be considered in differential diagnosis with PAH. Exuberant interstitial or endoalveolar inflammatory infiltrates, endoalveolar haemorrhages, diffuse alveolar damage, epithelial hyperplasia and numerous other histological morphological aspects suggest a differential diagnosis with other diseases. We must emphasize that the modifications of the vascular wall should not be confused with a degeneration due to fibrinoid necrosis because in this case it must be considered a fibrinoid or necrotizing vasculitis which are typical of both vasculitic processes and infectious diseases with vascular involvement. We must again emphasize that the hyperplasia and remodelling of the medial tunic must be considered together with other modifications, it is present, because in this case secondary and non-primary pulmonary hypertension must be considered [53–58]. In our cases, there were no other organ or pulmonary diseases that could have been responsible for the increase in pulmonary resistance and hypertension.

Finally, the presence of lymphoid infiltrate with typical localization must be considered for small and medium vessels excluding bronchus-associated lymphoid tissue (BALT), which is generally present in the immediate vicinity of the bronchi and more noticeable in infants. In the evaluation of the interstitial inflammatory infiltrate, the possibility of interstitial pneumonia must be considered and excluded, to which other morphological aspects useful for the differential diagnosis are also associated. Based on these small criteria, the evaluation of lymphocyte populations offers the starting point for further progress in the definition of PAH and in its pathogenesis.

5. Conclusions

Many cardiovascular and degenerative diseases can cause SUD compromising the heart's mechanical function, in old and young individuals [53–56]. The vascular wall in PAH is a crucial element in the complex interactions between cells and chemical mediators of inflammation that define their remodelling. The fundamental role of some gene mutations and of the immune system in the pathogenesis is now accepted although the mechanisms are still unclear.

Our morphological data agree with the literature and confirmed the important role of perivascular T-Cells, B-Cells and MCs in the lung. The morphological data represents the phenotypic evidence of pathogenic events up to remodelling of the vascular wall. In the absence of other histological findings and in the absence of heart or primary pulmonary diseases that can induce increased resistance with primary pulmonary hypertension, the possibility of a PAH must always be considered, even in the anamnestic history there are no elements. This data is also especially important for family screening. Finally, we are still far from a therapy that replaces vasodilators, but the identification of the key role of cell types and mediators that take part in the pathogenesis of PAH can certainly represent a valid aid for targeted and effective therapies that can stop or even induce reversible remodelling.

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Ethics approval and consent to participate

This is an autopsy based study not requiring ethical approval.

Consent to publish

Not applicable.

CRediT authorship contribution statement

Gelsomina Mansueto: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Supervision, Project administration. **Mario Di Napoli:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Carlo Pietro Campobasso:** Methodology, Validation, Data curation, Writing – review & editing. **Mark Slevin:** Methodology, Validation, Data curation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

Data and materials are full available.

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