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Corso di Dottorato in Medicina Clinica e Sperimentale

WOMEN WITH LYMPANGIOLEIOMYOMATOSIS: FROM RESPIRATORY FUNCTION TO SERUM BIOMARKERS ANALYSIS. PHENOTYPING OF A RARE DISEASE.

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SUMMARY

FENOTIPIZZAZIONE DI PAZIENTI CON LAM SPORADICA E SCLEROSI TUBEROSA: DALL'ANALISI FUNZIONALE ALLA VALUTAZIONE BIOCHIMICA

Riassunto

La linfangioleiomiomatosi (LAM) è una malattia rara sistemica che determina lo sviluppo di cisti aeree multiple all'interno del parenchima polmonare, di linfangioleiomiomi sia toracici che addominali e angiomiolipomi renali. Può svilupparsi in una forma sporadica (S-LAM) o come parte del Complesso Sclerosi Tuberosa (LAM-TSC), una malattia genetica autosomica dominante che causa la formazione in pressochè tutti gli organi corporei di neoplasie amartomatose. Solo da qualche anno è disponibile una terapia in grado di ralletare la progressione della LAM.

Il programma di dottorato della Dr.ssa Silvia Terraneo è stato concepito con lo scopo di espandere la conoscenza su aspetti ancora poco noti della LAM sia dal punto di vista clinico (diagnosi e follow up) che dal punto di vista della patogenesi e che sono riportati nella presente tesi.

Come primo approccio è stata effettuata una valutazione delle caratteristiche cliniche e genetiche delle pazienti affette da TSC con e senza coinvolgimento polmonare seguite presso il centro per la diagnosi e la cura della Sclerosi Tuberosa dell'Ospedale San Paolo. E'stata indagata una possibile associazione tra LAM e altre caratteristiche della TSC; è stata inoltre condotta un analisi sul possibile ruolo diagnostico degli esami di funzionalità polmonare, nell' ottica di limitare l'esposizione delle pazienti alle radiazioni ionizzanti della TC del torace. Il risultato dello studio ha dimostrato come la prevalenza della LAM nelle pazienti con TSC aumenti con l'età. A causa della disabilità mentale presente nella TSC, la funzionalità polmonare è stata eseguibile solo in una parte del campione analizzato e non si è mostrata utile nella diagnosi della malattia, il cui gold standard diagnostico si è confermato essere la TC torace.

Nel contesto della valutazione clinica delle pazienti con LAM-TSC del centro, è stato descritto il caso di una paziente con TSC LAM che durate il follow up ha sviluppato altre 2 malattie rare: sindrome da overlap epatite autoimmune/cirrosi biliare primitiva e sarcoidosi; è stato speculato un possibile ruolo del pathway mTOR e MAPK, ben conosciuto per TSC e LAM, nello sviluppo di altre malattie, quali ad esempio la sarcoidosi.

Nell'ultima parte del percorso di dottorato sono stati analizzati i dati derivanti dal dosaggio su siero di 4 biomarcatori (VEGF-D, VEGF-C, MMP-2 e MMP-7) nelle pazienti con LAM, sia S-LAM che TSC-LAM. Lo studio presentato in dettaglio nel testo, conferma il valore diagnostico del VEGF-D e mostra una possibile utilità del MMP-2 e MMP-7 come biomarcatori di malattia.

WOMEN WITH LYMPANGIOLEIOMYOMATOSIS: FROM RESPIRATORY FUNCTION TO SERUM BIOMARKERS ANALYSIS. PHENOTYPING OF A RARE DISEASE.

Abstract

Lymphangioleiomyiomatosis (LAM) is a rare progressive cystic lung disease that affects almost exclusively women. LAM can occur sporadically, or can be associated with tuberous sclerosis complex (TSC); a rare disorder with multiorgan involvement effecting the brain, kidneys, heart, liver, skin and eyes and is associated with intellectual disability, epilepsy and autism spectrum disorders.
Dr.Terraneo PhD project was developed with the aim to expand clinical knowledge about diagnosis and follow up as well as to analyze pathogenic aspect of the development of the disease.

As a first step of the PhD project, the association between LAM and other features of TSC (e.g. demography, extrapulmonary manifestations, genetic mutations..) was investigated as well as the role of pulmonary function tests (PFTs) for LAM diagnosis. Our results demonstrate that age, but not PFTs, is independently associated with LAM development in patients with TSC. PFTs, even if indicated to assess impairment in lung function, result feasible in a limited number of patients due to cognitive impairment, and are not significantly useful for LAM diagnosis in women with TSC.
Successively, the case of a patients with coexistence of three rare diseases (autoimmune hepatitis/primary biliary cirrhosis overlap syndrome, lymphangioleiomyomatosis/tuberous sclerosis complex (LAM-TSC), and sarcoidosis) was described. We speculated that the dysregulation of the pathway involving mTOR and MAPK and their interaction might play a role in the pathogenesis of diseases other than TSC, including sarcoidosis.
In the last part of PhD project, the serum levels of VEGF-D, VEGF-C, MMP-2 and MMP-7 were assessed in a cohort of patients affected with S-LAM and TSC with and without LAM.
Our results showed that VEGF-D, MMP-2 and MMP7 were higher in patients with LAM than in patients without. VEGF-D was confirmed as the biomarkers with the highest accuracy for LAM diagnosis. MMP-2 and MMP-7 could be a promising biomarker of LAM.

CHAPTER I: USEFULNESS OF PULMONARY FUNCTION IN THE EVALUATION AND FOLLOW UP OF PATIENTS WITH LYMPHANGIOLEIOMYOMATOSIS.

WOMEN WITH TSC: RELATIONSHIP BETWEEN CLINICAL, LUNG FUNCTION AND RADIOLOGICAL FEATURES IN A GENOTYPED POPULATION INVESTIGATED FOR LYMPHANGIOLEIOMYOMATOSIS

ABSTRACT

The advent of pharmacological therapies for lymphangioleiomyomatosis (LAM) has made early diagnosis important in women with tuberous sclerosis complex (TSC), although the lifelong cumulative radiation exposure caused by chest computer tomography (CT) should not be underestimated. We retrospectively investigated, in a cohort of TSC outpatients of San Paolo Hospital (Milan, Italy) 1) the role of pulmonary function tests (PFTs) for LAM diagnosis, 2) the association between LAM and other features of TSC (e.g. demography, extrapulmonary manifestations, genetic mutations, etc.), and 3) the characteristics of patients with multifocal micronodular pneumocyte hyperplasia (MMPH). Eighty-six women underwent chest CT scan; pulmonary involvement was found in 66 patients (77%; 49% LAM with or without MMPH, and 28% MMPH alone). LAM patients were older, with a higher rate of pneumothorax, presented more frequently with renal and hepatic angiomyolipomas, and tended to have a TSC2 mutation profile. PFTs, assessed in 64% of women unaffected by cognitive impairments, revealed a lower lung diffusion capacity in LAM patients. In multivariate analysis, age , but not PFTs, resulted independently associated with LAM diagnosis. Patients with MMPH alone did not show specific clinical, functional or genetic features. A mild respiratory impairment was most common in LAM-TSC patients: In conclusions, PFTs, even if indicated to assess impairment in lung function, are feasible in a limited number of patients, and are not significantly useful for LAM diagnosis in women with TSC.

INTRODUCTION

Lymphangioleiomyiomatosis (LAM) is a rare progressive cystic lung disease that affects almost exclusively women [1]. LAM can occur sporadically, or can be associated with tuberous sclerosis complex (TSC); a rare disorder with multiorgan involvement effecting the brain, kidneys, heart, liver, skin and eyes and is associated with intellectual disability, epilepsy and autism spectrum disorder [2]. In either form, LAM results from mutations affecting the function of TSC1 or TSC2 genes [3], encoding for hamartin and tuberin, respectively. Such proteins inhibit the mammalian target of the rapamycin (mTOR) signaling pathway, a major regulator of cell size and proliferation [4]. Moreover, TSC patients may develop multifocal micronodular pneumocyte hyperplasia (MMPH), a distinct micronodular epithelial proliferative lesion of the lung, with or without the coexistence of LAM [5]. MMPH is caused by the growth of proliferating epithelial cells into the alveolar walls which is not simply just pneumocyte hyperplasia [5]. Lung function abnormalities in LAM patients include the reduction of both forced expiratory volume in one second (FEV1) and lung diffusion for carbon monoxide (DLCO), which clinically corresponds to a reduction in breathing ability, and hypoxemia when performing physical activity and even at rest [6, 7].

A consensus statement issued by the European Respiratory Society in 2012 defined the diagnostic criteria for LAM [1]. In patients with definite or probable TSC, LAM can be diagnosed on the basis of a characteristic pulmonary high-resolution computed tomography (HRCT) pattern with the presence of more than 10 thin-walled, round and well-defined air-filled cysts with preserved or increased lung volume, and no other significant pulmonary involvement (with the exception of possible features of MMPH) present [1]. In the same document, HRCT scanning is recommended for women with TSC at ages between 18 and 30 years [1]. Previous studies run on women affected by TSC found a LAM prevalence ranging between 26 and 49% [8-13], with an increase of prevalence correlated to age that may reach 81% in subjects aged 40 years or older [10].

Sirolimus and its derivate everolimus are immunosuppressive drugs that affect mTOR function. Both have been demonstrated to be somewhat effective in the treatment of LAM [14-17]. With the advent of such therapies, early diagnosis of LAM has become crucial. However, since the prevalence of clinically significant LAM in TSC patients is low [18-22] and LAM-TSC is a milder disease compared to sporadic LAM [6, 22], the lifelong cumulative radiation exposure risk of serial CT should be taken into account. Cudzilo CJ et al. proposed an age-based approach using limited CT scanning methods in order to facilitate screening and limit radiation exposure [10].

In our study, the evaluation of a possible association between pulmonary and extrapulmonary localization of TSC-related abnormalities was investigated with the objective to assess whether specific extrapulmonary manifestations typical of TSC, or other features of the disease may increase the risk of LAM. The aims were: 1) evaluation of the prevalence of LAM in a large TSC Italian population and usefulness of lung function tests for screening purposes; 2) assessment of the association between LAM-TSC and other features of the disease such as demographic characteristics of patients, the presence of extrapulmonary involvement and the identification of the mutation of gene TSC1 or TSC2; and 3) characterization of patients affected by MMPH alone.

METHODS

Study design and population

This is a cohort retrospective study involving outpatients affected by TSC, regularly seen at the Tuberous Sclerosis Center of San Paolo Hospital, Milan, Italy, from 2000 to 2014. The diagnosis of TSC was established using international criteria [23]. In our TSC center every systemic manifestation of TSC is evaluated at least yearly by a specialist experienced in TSC diagnosis and management (neurologist, pulmonologist, nephrologist, dermatologist, ophthalmologist, radiologist, and cardiologist) according with international guidelines [23-26]. Pulmonary evaluation with high-resolution lung CT (HRCT) was performed in women [1]: 1) at the age of 18 years for the patients

diagnosed with TSC in pediatric age; 2) at the moment of TSC diagnosis in adult patients or during the first evaluation in our center; 3) in case of respiratory symptoms. The analyzed data (demographic, clinical, genetic, pulmonary function tests, and extrapulmonary manifestations) refer to the year of chest CT.

Pulmonary involvement

Spirometry, body pletismography and lung diffusion tests (Platinum Elite™ MGC Diagnostic, USA) were performed according to ATS/ERS guidelines [27, 28]. We defined an alteration of pulmonary function test as 1) FEV1/FVC < lower level of normality, 2) reduction of lung diffusion for carbonic monoxide (DLCO) and/or 3) DLCO/alveolar volume (DLCO/VA) < 80% of predicted value using ECCS predicted values [29]. Dyspnea was investigated throughout the Italian version of the modified Medical Resource Council (MRC) scale consisting in five statements regarding perceived breathlessness [30]. The six-minute walk test (6MWT) was performed along a flat, straight, 30 meters walking course supervised by a well-trained researcher according to ATS guidelines [31].

Chest CT scans

As previously described, in accordance with the ERS document, the presence of LAM in patients with definite or probable TSC was confirmed in the presence of characteristic lung high-resolution CT patterns [1]. Chest CT examinations were performed without contrast media administration either on a 4-slice multidetector CT (Light Speed QX/i; General Electric Medical System, Milwaukee, WI) between January 2000 and August 2008 or, due to the scan system replacement, with a 64-slice multidetector CT (LightSpeed VCT, General Electric Healthcare, Milwaukee, WI) between September 2008 and December 2014. For both scanners, parameters comprised the following: tube voltage, 100-140 kVp; tube current, 120-400 mAs (with automatic tube current modulation for the 64-slice scanner); gantry rotation time, 0.5 s; reconstruction thickness, 1.25 mm; reconstruction increment, 1.25 mm; acquisition kernel, standard. A beam pitch of 1.5 was

used for the 4-slice CT scanner and a pitch of 1 for the 64-slice one. Images were acquired during inspiration and the scan length extended from the lung apices to the adrenal glands.

Genetic analysis

Qiamp DNA blood mini DNA kit (Qiagen, Germany) was employed to extract DNA from peripheral lymphocytes (Qiagen, Germany). *TSC1* and *TSC2* exons from the genomic DNAs were amplified by means of standard polymerase chain reaction (PCR) and previously described primers [32]. Mutations were detected by submitting the PCR products to denaturing high-performance liquid chromatography (DHPLC) (Transgenomic, Crewe, UK). The products showing variant DHPLC melt profiles were directly sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems), and the results were analyzed using sequence analysis 3.4.1 software (ABI 3130, Applied Biosystem). The sequencing reactions for identified mutations were repeated. Patients that had negative investigations for DHPLC were evaluated with Multiple Ligation-dependent Probe Amplification test for TSC1 (P124-MRC-Holland) and TSC2 (P046-MRC-Holland). Patients in whom genetic analysis was inconclusive, were classified as having no mutation identified (NMI).

Neurological manifestations

Neurological manifestations (cortical tubers, subependymal nodules (SEN) and subependymal giant cell astrocytoma (SEGA)) were evaluated by the use of CT and brain magnetic resonance imaging (MRI). Epilepsy and neurodevelopmental psychiatric/cognitive symptoms were also evaluated. Frequency, age at onset, and characteristics of epilepsy, intellectual disability, sleep disorders and anti-epileptic therapy were reported. Intellectual disability was divided into five grades according to intelligence quotient (IQ): (1) normal IQ with IQ > 85; (2) borderline intellectual functioning (BIF) with IQ from 84 to 71;(3) mild intellectual disability (ID) with IQ from 70 to 55;(4) moderate ID with IQ from 54 to 40, and (5) severe ID with IQ < 40 [33]. As part of their clinical

management, patients were evaluated through a psychiatric interview in order to assess possible Axis I and II disorders [33].

Abdominal, dermatological and cardiac manifestations

All patients were evaluated at least once with an abdomen CT or MRI [34], and followed-up with ultrasonography (US) in the majority of cases. Abdominal manifestations of TSC include renal angiomyolipomas, renal cysts and renal cell carcinoma and hepatic angiomyolipomas; the data included in our database refer to the closest CT or MRI available, obtained before or after the chest CT-scan. Skin lesions were also clinically evaluated. TSC manifestations include facial angiofibromas, forehead plaques, hypomelanotic macules, shagreen patches and ungual fibroma. Cardiac involvement (rhabdomyoma, electrocardiographic abnormalities) was investigated by electrocardiography and echocardiography.

Statistics

The results are shown as mean±standard deviation (SD), unless otherwise stated. Lilliefors corrected K-S test was performed before the data analysis in order to examine the distribution of the residuals of the parametric tests. For comparisons between patients, unpaired Student's t test analysis (test for equal variances was performed), Wilcoxon Mann-Whitney test, or Fisher's exact test were used, as appropriate. Variables that resulted in p values < 0.15 were used in a multivariate logistic regression model to predict factors that were associated with TSC-LAM diagnosis. The odds ratios (OR) and their 95% confidence intervals were also derived. All tests were two-sided, and $p < 0.05$ were considered statistically significant. Statistical tests were performed using the Statistical Package for Social Sciences (version 21.0; SPSS, Chicago, IL).

Ethical considerations

The local ethical committee (Comitato Etico Interaziendale Milano Area A) approved the study. All patients recruited were required to give their signed consent for the collection and analysis of clinical data. Patients with cognitive impairment had consent signed for them by appropriate next of kin.

RESULTS

Analysis of the Population and relationship between age and prevalence of LAM in TSC

Among the 200 patients (80 males, 120 females; mean age 29 years, range 1-71) followed up for TSC at San Paolo Hospital (Milan, Italy) during the period of analysis, 142 were older than 18 years of age (and therefore considered "adult patients"). Ninety-two adult women were evaluated; of them 86 (93%) had chest CT scans (Fig 1). Eighty-two of those scans (95%) were done for screening purposes (requested at the time of the first clinical evaluation); two patients underwent HRTC for pneumothorax, and two others for chylothorax (in these subjects the pulmonary involvement preceded the diagnosis of TSC) during hospitalization. Chest CT scan allowed the following identifications: 66 (77%) adult women had pulmonary involvement with LAM in 42 cases (49%), MMPH in 24 cases (28%), and both LAM and MMPH in 19 cases (22%).

Demographic and clinical features of the population are shown in Table 1. The mean age at first CT evaluation was significantly higher (> 9 years difference) for patients with LAM compared to those without LAM ($p< 0.001$). LAM prevalence significantly increased across age quartiles ($p=0.005$) in the overall population (Fig 2A); TSC2 mutation was found in 50% of the cases. Such percentage changed when the presence of LAM was considered, with a statistically borderline higher prevalence of TSC2 mutation in LAM patients (60 *vs.* 38%, $p=0.070$). In the overall population, the most common mutation was "*de novo*" (61%), with the same number of "familial" and "dubious" mutations; in terms of mutations, no significant differences were found in patients with and without LAM ($p=0.282$).

Pulmonary involvement, symptoms and respiratory function

Respiratory function tests were successfully carried out in 55 patients (64%), due to the high prevalence of intellectual disability and/or behavioral problems (Table 2); namely, 14 patients were not co-operative, in 7 cases spirometry was not acceptable due to glottis closure, 5 had variable effort with early termination of forced expiration and 5 patients were not able to perform reproducible tests. LAM patients showed lower DLCO and DLCO/VA (both referred to predicted values); the difference in terms of obstruction (i.e. FEV1/FVC ratio under lower limit of normality, LLN) between LAM and TSC-LAM resulted in borderline statistical significance (p=0.080). Impairment in lung function tests is more common in TSC-LAM patients than in TSC patients without LAM (p=0.055). As shown in Fig 2B, patients with an altered lung function showed a higher percentage of LAM with age. Five TSC-LAM patients had a history of recurrent pneumothorax. Patients with or without LAM did not differ in dyspnoea and oxygen desaturation during 6MWT. We also evaluated the usefulness of pulmonary function tests in the subgroup of 16 women with "respiratory impairment" (i.e. dyspnoea, pneumothorax, or chylothorax). The prevalence of LAM in this subgroup did not differ significantly from asymptomatic patients (26 *vs.* 11%, p=0.068), such as the results of pulmonary function tests, with the only exception of a borderline reduction of DLCO (i.e. <80%) in patients with symptoms (38 vs. 14%, p=0.050).

Extrathoracic involvement and MMPH patients

Renal (multiple and bilateral), and hepatic angiomyolipomas were significantly more frequent in patients with LAM, compared to those without LAM (p=0.011, and p=0.002, respectively). In addition, women with LAM less frequently had a history of epilepsy than patients without LAM (p=0.076) (Table 1).

Twenty-five patients with MMPH alone (i.e. without LAM) have been compared with 24 patients with no pulmonary involvement. Patients having MMPH alone did not differ from the other TSC

patients without any lung manifestation in terms of clinical features, neuropsychiatric symptoms, genetic characteristics, and lung function tests (Table 3).

Predictors of TSC-LAM

Pulmonary function test alterations alone yielded a sensitivity of 45% and specificity of 70% for LAM diagnosis by using CT scan as gold standard, with a positive and negative predicted value of 59% and 57% respectively. A multivariate model was used to estimate the odds for LAM diagnosis. The only element found in the univariate analysis that was independently associated with LAM diagnosis in our TSC group was the age at first CT evaluation, with a higher risk of LAM in older women (Table 4). Our analysis failed to demonstrate alterations of PFT as independently associated with LAM in TSC patients (p=0.245).

DISCUSSION

The main findings of this study, conducted on a large cohort of Italian TSC patients, are the following: 1) LAM and MMPH have a prevalence of 49% and 28% respectively; 2) on average, women with LAM are older, develop renal and hepatic angiomyolipomas more frequently, show a higher rate of pneumothoraces, and have more mutations on the TSC2 gene; 3) Impairment in lung function tests, feasible in patients not affected by major cognitive deficit (64%), is more common in LAM patients; 4) older age is independently associated with LAM whereas multivariate analysis failed to demonstrate pulmonary function test alterations as an independent risk factor for LAM diagnosis; 5) patients with MMPH alone do not show a specific clinical, functional or genetic profile.

To the best of our knowledge, this is the first study that describes the clinical characteristics of a large Italian population of patients with LAM associated with TSC and that investigates the possible role of respiratory function test in detecting pulmonary involvement.

Our data, in line with previous studies [8-13], indicate that in TSC patients there is an age-related LAM prevalence, a higher frequency of TSC2 mutations (statistically borderline in our study) [11, 35, 36], and more frequent occurrence of renal [10, 11, 22] and hepatic angiomyolipomas [37]. The crucial importance of "time" in the manifestation of lung lesions is probably due to the pathogenesis of LAM-TSC, which is consistent with the Knudson "two-hit" tumor suppressor gene mechanism [38]. Moreover, in line with previous data, our results support the evidence that patients with TSC1 mutations have, on average, milder disease in comparison with patients with TSC2 mutations [13, 39].

We encountered two major difficulties in the use of pulmonary function tests in TSC patients: firstly, 36% of TSC women who underwent chest CT failed to correctly perform spirometry and other pulmonary function tests due to TSC-related intellectual disability. This could potentially be aided by the use of other techniques that require a lower level of co-operation, such as using forced oscillation technique (FOT), a simple, noninvasive method which requires minimal patient technical ability, currently used in both children and adults. The second problem was the low sensitivity (<50%)

of PFTs for LAM screening in TSC women. This may be due to numerous issues, e.g. TSC patients usually present with a mild form of LAM, LAM is the initial presenting symptom of TSC only occasionally, and the decline in lung function is typically very gradual in patients with LAM-TSC, with only a minority of patients becoming symptomatic during follow-up [40-42]. However, an interesting study conducted by Taveira-DaSilva AM et al. showed that some young LAM-TSC patients (mean age 26±3 years) can rapidly progress from minimal to severe lung disease [22]. The real question is whether it is important to diagnose LAM in asymptomatic patients, which on average present with very mild lung disease. Screening for LAM in TSC patients should take into consideration potential benefits and risks. Potential benefits of earlier LAM diagnosis include the possibility to inform women about the risk of a pneumothorax, pregnancy, the use of contraceptives, and lifestyle choices, such as scuba diving or smoking, as well as the opportunity to start mTOR inhibitor therapy, even if this choice is currently limited to patients with lung function declining rapidly or respiratory symptoms, chylous pleural effusion or ascites [43]. On the other hand, the risk of carcinogenic ionizing radiation exposure has to be taken into account, as well as the possible anxiety due the diagnosis, and lifestyle limitations (e.g. the risk of pneumothorax and air travel) not supported by a strong level of evidence (e.g. the risk of a life-threatening pneumothorax associated with air travel is minor) [44].

Our study confirms that, so far, HRCT is the only available tool for LAM screening in TSC patients. Multivariate analysis failed to identify a parameter that is independently associated with the presence of LAM, with the only exception of age. To conclude, the need of LAM diagnosis by chest HRCT scan in asymptomatic TSC women with normal lung function should be weighed in each individual circumstance with consideration of the pros and cons.

The significant percentage of TSC patients showing MMPH (50%) correlates halfway between previous reports [11] and a recent work by Muzykewicz et al. [45] that found a nodular lesion prevalence of 57% in TSC patients. We did not find a significant correlation between the presence of MMPH and LAM since the rate of MMPH was the same in patients with and without

LAM (p=0.518). Moreover, even if not confirmed by statistically significant results, MMPH alone seems to be more common in older patients (5 years of difference compared to TSC patients without any lung involvement, p=0.074). However, the presence of MMPH itself does not affect pulmonary function, with pulmonary function tests on average normal in those patients and comparable with TSC women without evidence of lung disease. Thus, the evidence of MMPH at HRCT should be considered as clinically negligible, with the exception, in our experience, of occasional atypical radiological findings which require follow-up in order to exclude other diagnosis (e.g. in situ adenocarcinoma).

A number of potential limits of the present study deserve discussion. First, due to the retrospective nature of our work, we did not determine any biomarker, such as vascular endothelial growth factor-D (VEGF-D) at time of first HRTC, which demonstrated a potential clinical utility in reaching a diagnosis of LAM [46]. The correlation between higher values of VEGF-D and a reduced lung function has been found in some but not all studies [46-50]. However, no study has specifically investigated the potential role of the combination of pulmonary function tests and biomarkers for the diagnosis of LAM, reason why this approach is worth of investigation. Secondly, the understanding of TSC and LAM has significantly changed in the period of our data collection. Even if this is not a limit for pulmonary function tests, since spirometry and DLCO are unchanged for decades, we cannot rule out a possible bias for changes in the evaluation of extrapulmonary manifestation of the disease in the 14 years of data collection. Third, as suggested by many international documents, CT-scan evaluation was limited to women, since the presence of cysts in men is anecdotal. The reason being we cannot rule out some LAM-like lesions that are present in men with TSC, as previously reported [51]. We also did not analyze the data of abdominal lymphangioleiomyomas or of lymphadenopathy since scarcity in the number of patients. Finally the, power of our multivariate analysis is low, due to the limited number of patients with all the parameters available for analysis.

CONCLUSIONS

Impairment in lung function tests is common in LAM, but pulmonary function testing, needed to evaluate the level of lung impairment, does not prove to be a useful tool for detecting LAM in TSC women in clinical practice. Using more sensitive tests which require a lower level of co-operation could assist, if available. However, the weak correlation between lung function impairment, "anatomical" lung cysts and symptoms limits the utility of lung function testing for LAM in patients with TSC. The use of low dose CT methods are suggested to limit the lifelong cumulative radiation exposure.

FIGURES

FIGURE 1.

Population in analysis. Age is shown as mean ± standard deviation and is referred to first evaluation in the center. LAM=lymphangioleiomyomatosis; TSC= tuberous sclerosis complex; *percentage referred to all adult TSC females patients in which lung scan was available for evaluation.

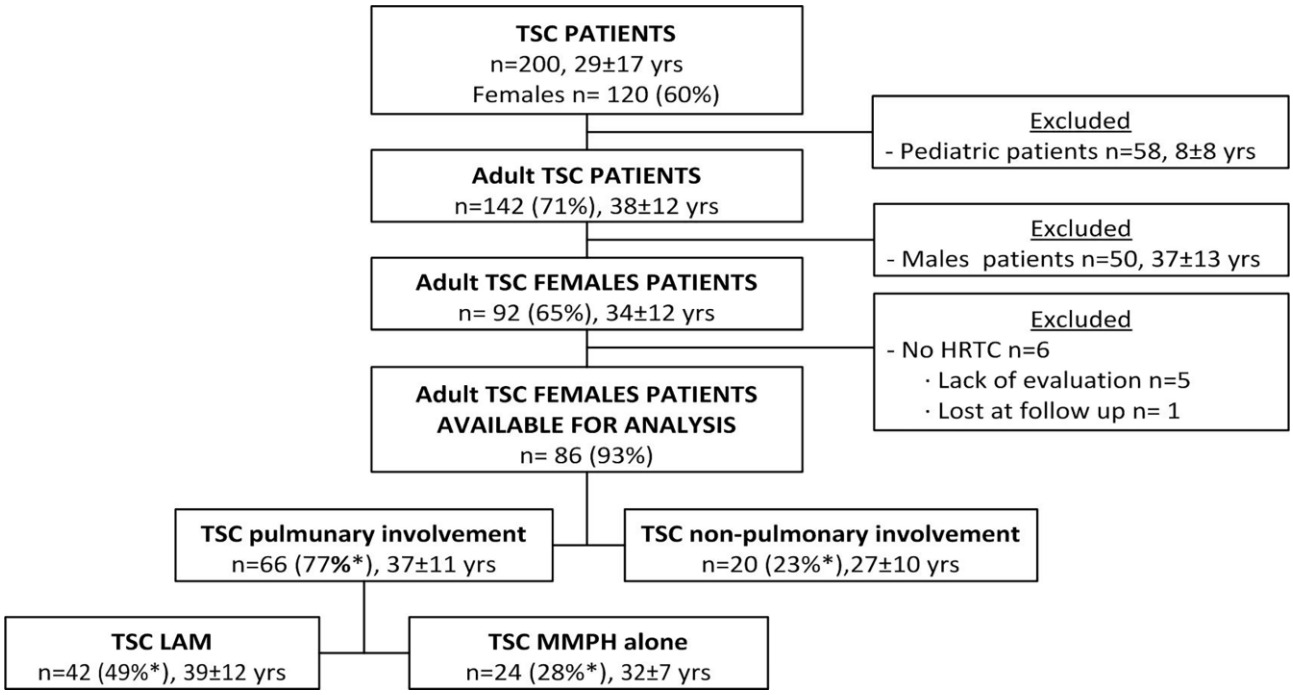
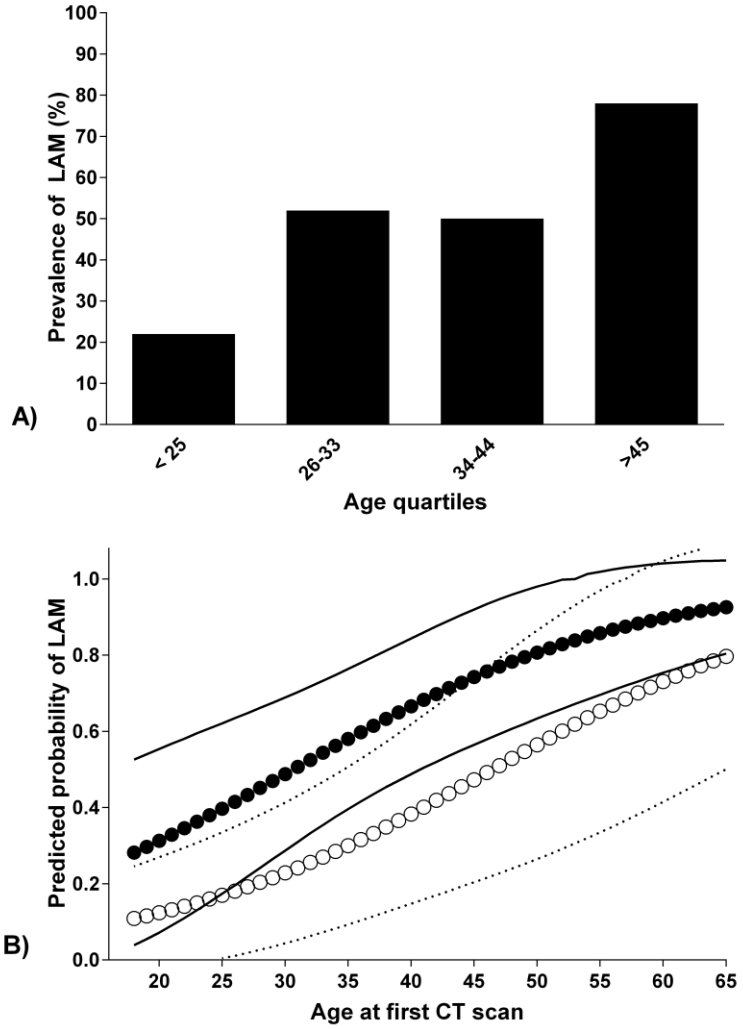


FIGURE 2.

AGE -DEPENDENT RISK OF LAM. **(A)** on age quartiles in the overall population (*p=0.004*) **(B)** predicted probability of LAM in relationship to age and 95% CI in patients with and without altered pulmonary function tests. Points along the central logistic curve are individual predicted probabilities. Black points refer to patients with normal pulmonary function tests (PFT), white points refer to patients with altered PFT. The corresponding 95% CI for each point appears on the outer logistic curves. The dotted lines refer to 95% CI of predicted probability for patients with altered PFT while the continuous line refers to IC in patients with normal PFT



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TABLES

Table 1. Demographic and clinical characteristics of enrolled patients according with LAM.

	ALL PATIENTS	**LAM-TSC**	**TSC**	***P* value**
Number (%)	86	42 (49)	44 (51)	
Age at first CT evaluation, yrs	34 ± 12	39 ± 12	30 ± 9	**<0.001**
Genotype[a]				
TSC1, n (%)	33 (40)	11 (28)	22 (52)	
TSC2, n (%)	40 (49)	24 (60)	16 (38)	**0.070**
No mutation identified (NMI), n (%)	9 (11)	5 (12)	4 (10)	
Abdominal manifestation				
Renal angiomyolipomas, n (%)	63 (75)	36 (88)	27 (62)	**0.011**
n <3/n≥ 3, n (%)	9 (25)/ 27 (75)	3 (11)/ 23 (89)	6 (60)/ 4 (40)	**0.006**
bilateral angiomyolipomas, n (%)	32 (80)	23 (88)	9 (64)	0.102
Renal cysts, n (%)	30 (40)	16 (44)	14 (37)	0.636
Hepatic angiomyolipoma, n (%)	29 (35)	22 (54)	7 (17)	**0.002**
Skin lesions, n (%)	84 (99)	41 (100)	43 (97)	>0.999
Hypomelanotic macules, n (%)	51 (91)	26 (93)	25 (89)	>0.999
Facial angiofibromas, n (%)	58 (97)	30 (100)	28 (90)	0.492
Fiorhead plaque, n (%)	26 (81)	10 (77)	16 (84)	0.666
Shagreen patches, n (%)	13 (41)	7 (50)	6 (33)	0.473
Ungual fibromas, n (%)	30 (77)	18 (95)	12 (60)	**0.020**
Neurological manifestation				
Epilepsy, n (%)	54 (64)	22 (54)	32 (72)	0.076
Cortical tubers, n (%)	78 (92)	37 (88)	41 (95)	0.265
Subependymal nodules, n (%)	58 (76)	29 (78)	29 (74)	0.790

SEGA, n (%)	13 (17)	8 (22)	5 (13)	0.591
Sleep disorders, n (%)	58 (89)	26 (87)	32 (91)	0.695
Intellectual disability, n (%)	35 (44)	19 (47)	16 (40)	0.652
Borderline, n (%)	5 (6)	4 (10)	1 (2)	0.586
Level 1, n (%)	10 (12)	5 (12)	5 (12)	
Level 2, n (%)	8 (10)	3 (7)	5 (12)	
Level 3, n (%)	12 (15)	7 (17)	5 (12)	
Ocular manifestation				
Fundus oculi abnormalities, n (%)	32 (73)	15 (68)	17 (77)	0.736
Retinic amartomas, n (%)	6 (46)	2 (29)	4 (67)	0.286
Cardiac manifestation				
Cardiac rhabdomyomas, n (%)	12 (17)	4 (11)	8 (22)	0.343

Results are shown as mean± standard deviation unless otherwise stated. SEGA: subependymal giant cell astrocytoma; IQR: interquartile range; SD: standard deviation. p < 0.050 in bold. [a]Results of genetic analysis were available for 82 TSC patients.

Table 2. Lung function, pulmonary manifestations and symptoms of patients according with LAM.

		ALL PATIENTS	LAM-TSC	TSC	P value
Lung function[a]					
FEV1 (% pred), median (IQR)		95 (85-106)	95 (85-106)	94 (86-108)	0.736
FVC (% pred)		99 ± 17	100 ± 19	97 ± 15	0.607
FEV1/FVC ratio, median (IQR)		101 (96-104)	99 (94-103)	102 (97-104)	0.166
FEV1/FVC ratio < LLN, n (%)		6 (11)	5 (21)	1 (3)	0.080
DLCO (% pred)		81 ± 18	74 ± 22	86 ± 14	**0.029**
VA (% pred)		96 ± 15	93 ±16	98 ± 14	0.229
DLCO/VA (% pred)		81 ± 18	74 ± 20	87 ± 15	**0.007**
DLCO/VA < 80% pred, n (%)		25 (46)	16 (64)	9 (31)	**0.028**
VR (% pred)		131 ± 59	140 ± 65	127 ± 56	0.502
TGV (% pred)		116 ±34	123 ± 38	112 ± 31	0.358
Alteration of PFT, n (%)		32 (58)	19 (73)	13 (44)	**0.055**
Lung involvement[b]					
Smoke history (current/ex), n (%)		8 (9)/ 1 (1)	6 (14)/ 1 (3)	2 (4)/ 0 (0)	0.166
Pneumothorax, n (%)		5 (6)	5 (12)	0 (0)	**0.024**
Chylothorax		3 (3)	3 (7)	0 (0)	0.112
MMPH, n (%)		43 (50)	19 (45)	24 (55)	0.518
Dyspnea, n (%)		14 (17)	9 (23)	5 (11)	0.241
	mMRC =1, n (%)	2 (14)	1 (11)	1 (20)	0.486
	mMRC >1, n (%)	12 (86)	8 (89)	4 (80)	
SpO2 < 90% during 6mWT, n (%)		11 (14)	4 (10)	7 (18)	0.518

Results are shown as mean± standard deviation unless otherwise stated. PFT: pulmonary function test (alteration: FEV1/FVC < LLN, and/or DLCO < 80%, and/or DLCO/VA < 80%); IQR: interquartile range; FEV1: forced expiratory volume in one second; FVC: forced expiratory volume; LLN: lower limit of normality; DLCO: diffusion capacity for CO; VA: alveolar volume; TLC: total lung capacity; RV: residual volume; TGV: thoracic gas volume; MMPH: multifocal

micronodular pneumocyte hyperplasia; mMRC: Modified Medical Research Council Dyspnea Scale, %pred: % of predicted value; p < 0.050 in bold. [a]Data and percentage referred to 55 patients who performed lung function tests; [b]Data and percentage referred to 86 patients with CT scan.

Table 3. Demographic, pulmonary, clinical characteristic and genetic analysis of TSC patients according with MMPH.

	MMPH-TSC	**TSC**	***P value***
Number	24	20	
Age at first CT evaluation, (yrs)	32 ± 7	27 ± 10	0.074
Dyspnea, n (%)	4 (83)	4 (17)	0.362
Smoke history (current or past), n (%)	1 (4)	1 (5)	>0.999
SO2 < 90% during 6mWT, n (%)	6 (29)	1 (5)	0.095
Respiratory function			
FEV1 (% pred)	96 ± 13	98 ± 8	0.550
FEV1/FVC < LLN, n (%)	1 (5)	0 (0)	>0.999
FVC (% pred)	98 ± 16	97 ±13	0.806
DLCO (% pred)	84 ± 13	90 ±15	0.297
VA (% pred)	100 ±15	96 ±14	0.562
DLCO/VA (% pred)	86 ±13	89 ± 17	0.697
RV (% pred)	125 ±63	128 ±31	0.881
Genotype			
TSC1, n (%)	14 (58)	8 (44)	0.355
TSC2, n (%)	9 (37)	7 (39)	
No mutation identified (NMI), n (%)	1 (4)	3 (17)	
Abdominal manifestations			
Renal angiomyolipomas, n (%)	12 (50)	15 (79)	0.064
Hepatic angiomyolipoma, n (%)	3 (14)	4 (21)	0.760

Skin involvement[a], n (%)	24 (100)	19 (95)	0.455
Neurological manifestations			
Epilepsy, n (%)	18 (75)	14 (70)	0.746
Brain tubers, n (%)	23 (100)	18 (90)	0.210
Intellectual disability, n (%)	7 (33)	9 (47)	0.520
Ocular manifestations			
Retinic hamartoma, n (%)	4 (100)	0 (0)	0.067
Cardiac hamartoma, n (%)	3 (15)	5 (31)	0.422

Results are shown as mean± standard deviation unless otherwise stated. IQR: interquartile range; FEV1: forced expiratory volume in one second; FVC: forced expiratory volume; LLN: lower limit of normality; DLCO: diffusion capacity for CO; VA: alveolar volume; TLC: total lung capacity; RV: residual volume; TGV: thoracic gas volume; yrs: years; %pred: % of predicted value. [a]*Any skin manifestation of TSC.*

Table 4. Multivariate Analysis and ODDS RATIO for LAM risk in overall population.

	UNIVARIATE			MULTIVARIATE		
Variable	**OR**	**95% CI**	**p value**	**OR**	**95% CI**	**p value**
Haepatic AML	4.26	1.39-13.09	0.011	-	-	-
Renal AML	2.26	1.02-5.00	0.430	-	-	-
Altered PFT	3.34	1.07-10.38	0.037	-	-	-
TSC1/TSC2	1.52	0.79-2.95	0.207	-	-	-
Age	1.08	1.03-1.13	0.001	1.083	1.014-1.156	**0.018**

AML: angiomyolipoma; PFT: pulmonary function test; TSC1/2/NMI: mutation of TSC1 TSC2 genes/ no mutation identified; Age: referred to age at first CT evaluation; p < 0.050 in bold.

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CHAPTER II: LYMPHANGIOLEIOMYOMATOSIS, MULTIFOCAL MICRONODULAR PNEUMOCYTE HYPERPLASIA, AND SARCOIDOSIS: MORE PATHOLOGICAL FINDINGS IN THE SAME CHEST CT, OR A SINGLE PATHOLOGICAL PATHWAY?

ABSTRACT

Autoimmune hepatitis/primary biliary cirrhosis overlap syndrome, lymphangioleiomyomatosis/tuberous sclerosis complex (LAM-TSC), and sarcoidosis are three rare diseases. Here we present, to the best of our knowledge, the first description of a patient with the coexistence of these three diseases.

A 47-year-old woman affected by LAM-TSC and primary biliary cirrosis/autoimmune hepatitis overlap syndrome. During her follow up a high resolution chest CT scan (HRTC) confirmed the presence of both multiple cysts and micronodular opacities consistent with multifocal micronodular pneumocytes hyperlasia (MMPH), and revealed multiple hilar-mediastinal symmetrical lymphadenopathies suggestive of sarcoidosis. Simultaneously, subcutaneous nodules appeared on her forearm bilaterally. Cutaneous biopsy showed granulomatous dermatitis with sarcoid-like granulomas. A diagnosis of stage I pulmonary sarcoidosis was made. No treatment for sarcoidosis was initiated since the patient had neither systemic involvement, nor respiratory impairment.

The presence of more than one rare disease should challenge the concept of a potential common underlying mechanism, since the a priori probability of the concomitant presence of different conditions with different pathogenic mechanisms - especially if rare diseases - is low.

We speculate that the dysregulation of the pathway involving mTOR and MAPK and their interaction might play a role in the pathogenesis of other diseases, including sarcoidosis.

BACKGROUND

Tuberous sclerosis complex (TSC) is a rare genetic disorder, characterized by predominantly benign tumours developing potentially in all organ systems. Pulmonary involvement consists of Lymphangioleiomyomatosis (LAM) and Multifocal Micronodular Pneumocyte Hyperplasia (MMPH), which cause cystic and nodular diseases, respectively. Pneumothorax and chylothorax are common clinical presentations of LAM, whereas MMPH is usually asymptomatic. Here we describe a female with TSC and LAM with new pulmonary findings.

CASE PRESENTATION

A 47-year-old woman, affected by TSC with a mutation identified in the *TSC1* gene [c.682C>T (p.Arg228*)], was referred to the TSC Clinic of San Paolo Hospital (Milan, Italy). Family history was noticeable for Addison's disease and brain glioblastoma (in her mother) and idiopatic pulmonary fibrosis (in her father). Her daughter was affected by TSC. The patient suffered from primary biliary cirrosis/autoimmune hepatitis overlap syndrome. She was treated with ursodeoxycholic acid (15 mg/kg/day).

She received a diagnosis of LAM by chest CT scan, which showed bilateral lung cysts randomly distributed thoughout the lungs. Chest CT scan revealed also the presence of sclerotic bone lesions. Pulmonary function tests were normal, such as the 6-minutes walking test. Vascular endothelial growth factor (VEGF)-D, a lymphangiogenic growth factor proposed as a biomarker for LAM diagnosis and severity, was 582 pg/mL (normal limits 153-642 pg/mL). Dermatological examination showed hypomelanotic macules, facial angiofibromas, xantelasma palpebrarum, periungual fibromas and erythematous plaque on the left knee. She had a renal angiomyolipoma of 4.2 cm in the left kidney. Brain magnetic resonance (MR) showed the presence of cortical tubers. There was no ocular or heart involvement.

One year later, the patient was adressed to our clinic. A new high resolution chest CT scan (HRTC) was performed, and confirmed the presence of both multiple cysts and micronodular

opacities, consistent with MMPH. Multiple hilar-mediastinal lymphadenopathies were also identified. Of note, hilar lymphadenopathies were symmetrically enlarged (Figure 1). Simultaneously, subcutaneous nodules appeared on the patient's forearm bilaterally (Figure 1E), prompting a biopsy that resulted in the hystopathologic diagnosis of non-necrotizing granulomas, with mono- and multinucleate epithelioid cells, some of them with asteroid bodies surrounded by a sparse lymphocytic infiltrate, suggestive of granulomatous dermatitis with sarcoid-like granulomas. Diseases other than sarcoidosis were ruled out by second line evaluations on histology samples, such as Ziehl-Neelsen, periodic acid-Schiff (PAS), and Giemsa staining techniques. Immunologic studies showed positive antimitochondrial antibody and antinuclear antibody (titre 1:1,280, speckled pattern). IgG, IgM and IgA levels resulted within normal range. HIV test was negative. Other causes of granulomatous disease such as drug-induced hypersensitivity, pneumoconiosis, pulmonary histiocytic disorders, diseases associated with vascular inflammation were ruled out through clinical history, examination and the results of instrumental tests.

Thus, a diagnosis of sarcoidosis was considered. In order to evaluate the extent of the disease, laboratory tests were performed and showed normal serum angiotensin conversion enzyme (ACE) and serum and urinary calcium levels. Abdominal ultrasonography showed an enlarged liver with irregular edges and nodular heterogeneous echotexture, with mild steatosis and multiple renal cysts without evidence of nephrolithiasis. Bilateral x-ray of the hands did not show alterations in bone structure. The ophthalmologic examination, including funduscopic evaluation showed no signs of uveitis and confirmed the absence of retinal hamartomas and achromic patches related to TSC. EKG and echocardiography showed no abnormalities. Pulmonary function tests, including spirometry and lung volumes were normal; a mild reduction in diffusing capacity for carbon monoxide was detected. The six-minute walk test was normal. A diagnosis of stage I pulmonary sarcoidosis was made.. No treatment for sarcoidosis was initiated since the patient had neither systemic involvement, nor respiratory impairment. A treatment with Sirolimus was not performed due to the limited pulmonary cystic involvement and the lack of respiratory symptoms, and a clinical follow-up was carried on.

DISCUSSION AND CONCLUSIONS

This case report represents the first description of the coexistence of three rare disorders: autoimmune hepatitis/primary biliary cirrhosis overlap syndrome, TSC/LAM, and sarcoidosis. The presence of more than one rare disease should challenge the concept of a potential common underlying mechanism.

Lymphadenopathy, both thoracic and abdominal, has been described as another possible feature of LAM. For instance, a recent study on 138 patients with LAM, both sporadic and associated with TSC, found a prevalence of 9.4% for mediastinum and pulmonary hilum lymphatic lesions [23]. However, the presence of granulomatous skin lesions has not yet been described in LAM associated with TSC. Thus, the results of the skin biopsy together with the presence of bilateral hilar lymphadenopathy lead to the diagnosis of sarcoidosis.

With respect to sarcoidosis, the importance of host susceptibility and gene-environment interaction is widely accepted [24]. Although sarcoidosis does not meet the criteria for autoimmune disease, it can coexist with a wide range of autoimmune disorders, including primary biliary cirrhosis, which is characterized by either hepatic granuloma formation of cutaneous granulomatous inflammation, connective tissue diseases (e.g. systemic sclerosis and Sjogren's syndrome), Addison's disease, and thyroiditis [25]. It is noteworthy that the diagnosis of sarcoidosis in case of granulomatous skin lesions is made by exclusion criteria. Granulomatous lesions have been described in case of granulomatous-lymphocytic interstitial lung disease (GLILD) associated with common variable immunodeficiency (CVID), drug toxicity, or infections such as tuberculosis or fungal infections. All the aforementioned diagnoses were ruled out in our patient. As previously stated, the association between granulomatous lesions and autoimmune hepatitis/PBC, such as other immune-mediated and chronic inflammatory disease, has been previously described [26].

TSC is an autosomal-dominant disease caused by heterozygous loss-of-function mutations in the *TSC1* (chromosome 9q34) or *TSC2* (chromosome 16p13) tumour suppressor genes coding for

hamartin and tuberin, respectively. Tuberin and hamartin, together with TBC1D7, form a complex that functions as a negative regulator of mammalian target of rapamycin (mTOR) through the inhibition of Rheb. Inactivation of TSC1 or TSC2 results in overactivation of mTOR leading to abnormal cell growth, proliferation, metabolism, and angiogenesis. A common molecular mechanism for LAM/TSC and sarcoidosis is not known. However, a strong immunoreactivity for cathepsin-k was demonstrated in spindle and epithelioid-shaped cells of lung LAM and in granulomas of sarcoidosis cases [27]. Since it is known that modulation of cathepsin-k may occur through the mTOR pathway, it is possible to speculate that the mTOR pathway might play a role in sarcoidosis [28]. Several evidences demonstrated an integration of signaling of the mTOR pathway and mitogen-activated protein kinases (MAPKs)-Erk activation. For instance, vascular endothelial growth factors (VEGF)-C, a lymphangiogenic growth factor present at high levels in serum and urine of patients with LAM, induces phosphorylation of Akt, mTOR, S6K, S6 and MAPK-Erk. Uncontrolled inflammation and chronic inflammatory diseases may be caused by the persistent activation of MAPKs, which can occur in sarcoidosis [29]. This finding suggests an explanation for the persistent production of several inflammatory cytokines, such as TNF-α and IL-12, in sarcoidosis. Interestingly, Linke et al. found that activation of mTORC1 due to TSC2 deficiency causes granulomatous disease, including sarcoidosis, in both mice and humans. mTORC1 inhibition resolves granulomas in TSC2-deficient mice, suggesting that treatment with mTOR inhibitors, used to stop the progression of benign tumors and LAM in TSC, might improve the signs of sarcoidosis as well [30]. However, the reason why not all patients with TSC develop sarcoidosis is still unknown.

Although at present it is not possible to demonstrate a common mechanism underlying LAM/TSC, sarcoidosis, primary biliary cirrhosis and autoimmune hepatitis, and their coexistence could well occur by chance, we might speculate that the dysregulation of the pathway involving mTOR and MAPK and their interaction may play a role in the alteration of the diseases. Further reports are needed to demonstrate our hypothesis.

FIGURE 1

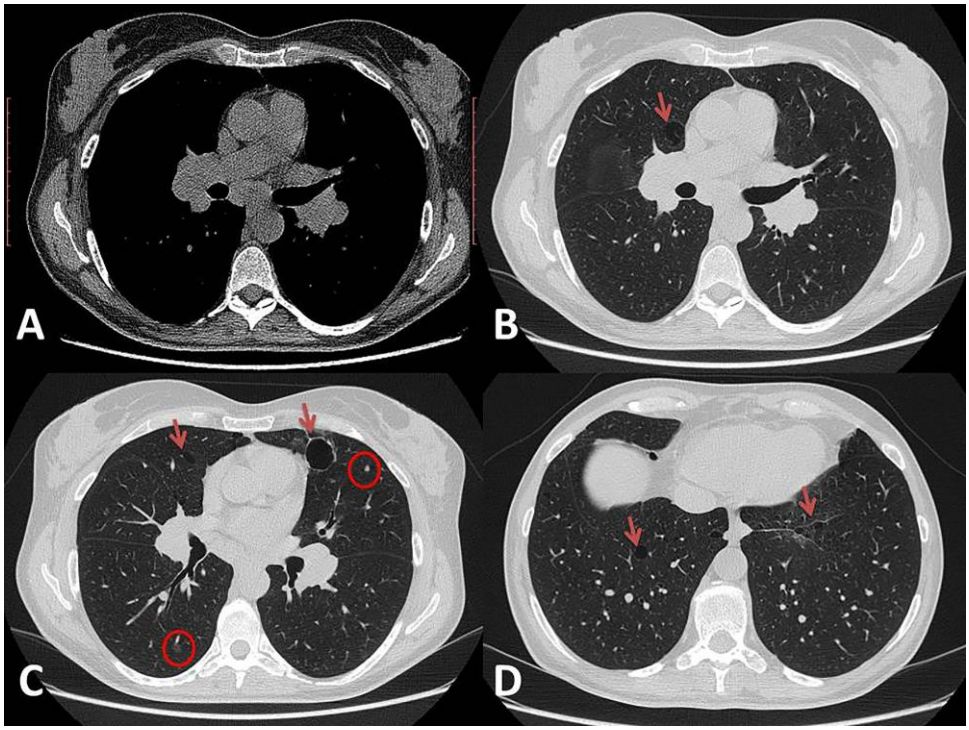

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Figure 1: (A) The mediastinal window HRCT image shows symmetrical hilar and subcarinal enlarged lymphnodes. (B-D) HRCT images show scattered lung cysts (arrows) consistent with lymphangioleiomyomatosis and either solid or subsolid micronodules (circles). (E) Multiple subcutaneous painless, hard papules and nodules covered by eritematous skin on the left forearm

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CHAPTER III: EVALUATION OF SERUM BIOMARKERS IN S-LAM AND TSC

VASCULAR ENDOTHELIAL GROWTH FACTORS AND MATRIX METALLOPROTEINASES SERUM LEVELS FOR LAM DIAGNOSIS IN PATIENTS WITH SPORADIC LAM AND TUBEROUS SCLEROSIS COMPLEX

ABSTRACT

Lymphangioleiomyiomatosis (LAM) is a rare cystic disease affecting primarily young women. It could develop in a sporadic form (S-LAM) and in women with tuberous sclerosis complex (TSC). Serum level of vascular endothelial growth factor D (VEGF-D) higher than 800 pg/mL in presence of typical air cystic changes in pulmonary parenchyma evaluated with the high resolution chest CT scan is diagnostic. Matrix metalloproteinases (MMPs) are extracellular matrix-degrading enzymes that might have a role in cystic lung destruction and in the process of migration of LAM cells.

We assessed serum levels of VEGF-D, VEGF-C, MMP-2 and MMP-7 in a cohort of Italian patients affected with S-LAM and TSC with and without LAM with the aim to explore their role as biomarkers of LAM.

Serum level of VEGF-D and C, MMP-2 and -7 were quantified by ELISA assays for 52 adult women with S-LAM and TSC and for 16 controls. ROC curves were built to explore diagnostic potential of single biomarkers. VEGF-D, MMP-2 and MMP7 were higher in patients with LAM than in patients without; there was no difference in VEGF-C levels between groups. All healthy controls had VEGF-D level less than 800 pg/mL ROC curves analysis confirmed the VEGF-D as the biomarkers with the high accuracy for LAM diagnosis and showed that MMP-2 and MMP-7 could be a promising biomarker of LAM. Patients with VEFG-D higher than diagnostic threshold of 800 pg/mL show more frequently chilothorax and mutation in TSC2 gene than patients with lower VEGF-D levels

while there was no difference regarding MMP-2 and MMP-7 in systemic involvement, except for a higher frequency of cortical tubers in patients with high MMP-7.

INTRODUCTION

Lymphangioleiomyiomatosis (LAM) is a rare progressive disease affecting mostly women in childbearing age[1]. The disease is characterized by abnormal smooth muscle-like cell (LAM cells) proliferation in the pulmonary interstitial and along the axial lymphatics in thorax and abdomen, which could lead to vascular, and airways obstruction and consequent air cystic development[2]. The disease is clinically characterised by progressive dyspnoea, recurrent pneumothorax, and chylous pleural effusions and, in most cases it could lead to respiratory failure[1]. LAM could develop in both a sporadic form (S-LAM) involving lungs, lymphatics and kidney and in patients affected by tuberous sclerosis complex (TSC), a rare tumor-suppressor syndrome associated with hamartomas in multiple organs, seizures and cognitive impairment (TSC-LAM)[3].

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor produced by malignant cells. The "D" and "C" isoform of VEGF, the ligands for the lymphatic-growth factor receptor VEGFR-2 and VEGFR-3/Flt-4, induce formation of lymphatics and promote the spread of tumour cells to lymph nodes. Serum VEGF-D levels are increased in most LAM patients and in presence of characteristic cystic changes at chest CT scan, a serum level ≥ 800 pg/dL is considered diagnostic, thus avoiding invasive assessment such as pulmonary biopsy[4]. The VEGF-D level was associated to disease severity evaluated as presence of chylous effusions and/or lymphatic involvement[5,6] however a strong correlation between VEGF-D and the extent of the parenchymal involvement evaluated with chest CT scan has been observed but not completely demonstrated[5,7]. Furthermore VEGF-D is not indicative of disease activity[8]. LAM cells are immune reactive for VEGF-C, but previous studies demonstrated that the serum level in LAM patients as compared with age and gender matched controls does not differ[9].

Matrix metalloproteinases (MMPs) are extracellular matrix-degrading, zinc-dependent enzymes active in lung tissue remodelling and repair that have caught attention in the pathogenesis of cystic lung destruction and in the process of migration of LAM cells[10]. MMPs are components of ECM that

degrades matrix substrates such as elastin and collagen in the lung parenchyma and their impairment has been implicated in the pathogenesis of several lung diseases including COPD, asthma, idiopathic pulmonary fibrosis and Langerhans cell istiocytosis [11,12]. Immunochemical studies showed that MMP-2 and MMP-9 are expressed in LAM cells[13,14] but serum levels of the former and not of the latter were significantly higher in patients with LAM patients compared to healthy controls[14]. However, the role of MMPs in LAM is controversial and other studies suggest that serum MMP-9 and MMP-2 levels cannot be correlated with extent of pulmonary cystic involvement[7]. LAM is a slowly progressive neoplasm that targets the lungs and presents pathogenic mechanisms similar to cancer, i.e. metastasis via blood and lymphatic circulation, infiltration and invasion. In a TSC model and in LAM tissues the invasion of tuberin-null cells might be mediated by MMP-7, a component of cell invasion[15].

The aim of the present study was to explore the role as biomarkers of MMP-2 and MMP-7 in a cohort of patients with S-LAM and with TSC with or without LAM, or TSC-LAM minimal disease, and confirm diagnostic value of VEGF-D in LAM.

METHODS

Study design and population

This was a cohort study involving adult women affected by S-LAM and TSC evaluated at the Tuberous Sclerosis Centre of San Paolo Hospital, Milan, Italy, from 2014 to 2017. LAM is diagnosed according to ERS criteria and TSC is diagnosed according to clinical and genetics criteria[16,17,18]. During the first visit every systemic manifestation of TSC was evaluated by specialists experienced in TSC diagnosis and management (neurologist, pulmonologist, nephrologist, dermatologist, ophthalmologist, radiologist, and cardiologist) according with international guidelines as previously reported[1918] and the follow up is established, if needed. All patients with a suspicious of LAM or a definite diagnosis of LAM[16] coming primarily from pulmonary clinic were also evaluated in the TSC clinic to find out a possible form of TSC-LAM. Clinical, radiological and genetic data collected in the year of the biomarkers analysis were analysed and compared to biomarkers serum levels results. All patients were in an observational cohort with Hospital Ethics Committee approval. All patients or relatives, in case of patients with intellectual disability, provided informed consent.

Quantification of serum VEGF-D, VEGF-C, MMP-2 and MMP-7

Blood was collected in serum separator tubes, allowed to clot for 30 min at 4°C, centrifuged at 1000 x g for 15 min. Serum was aliquot and stored at -80°C. Serum VEGF-D, VEGF-C, MMP-2 and MMP-7 were measured using Quantikine Human Immunoassays (R&D Systems; Minneapolis, MN) according to the manufacturers' instruction. Measurements were performed in duplicates.

Pulmonary, radiological, systemic involvement and genetics

Every systemic sign of TSC was investigated and evaluated as reported elsewhere [19]. For patients with TSC, thoracic high-resolution lung CT (HRCT) was performed in women [1]: 1) at the age of 18 years for the patients diagnosed with TSC in pediatric age; 2) at the moment of TSC

diagnosis in adult patients or during the first evaluation in our centre; 3) in case of respiratory symptoms. Spirometry, body pletismography and lung diffusion tests (***Platinum EliteTM MGC Diagnostic, USA***) were performed according to ATS/ERS guidelines[20,21]. The six- minute walk test (6MWT) was performed along a flat, straight, 30 meters walking course supervised by a well-trained researcher according to ATS guidelines[22]. Neurological manifestations were evaluated by the use of CT and brain magnetic resonance imaging (MRI). Epilepsy and neurodevelopmental psychiatric/cognitive symptoms were also evaluated. For abdominal manifestations, patients were evaluated at least once with an abdomen CT or MRI, and followed-up with ultrasonography (US) in the majority of cases. TSC related skin lesions were also clinically evaluated. Cardiac involvement was investigated by electrocardiography and echocardiography. Extensive explanation of genetic analysis was previously reported[19]. Patients were classified as having mutation in TSC1, TSC2 gene and when genetic analysis was inconclusive, they were classified as having no mutation identified (NMI).

Interpretation of radiology

All chest CT scan performed in the year of serum analysis were re-evaluated by a radiologist experienced in LAM, blinded to other researchers. The severity of cystic lung disease was graded according to a visual quantitative grading system[23]. The radiologic involvement was classified as "minimal" (Grade 0) if patients showed less than 10 lung cysts. If more than 10 cysts were identified then the extent of cysts was graded as "mild" disease (grade 1) if less than one third of the lung was involved, "moderate" (grade 2) if one to two thirds of the lungs resulted involved and "severe" (grade 3) if cysts involved more than two third of the lungs. Lymphatic involvement was considered if there was the presence of: lymph node enlargement, pleural effusion attributed to cylothorax or lymphangioleiomyomas.

Statistical analysis

The results are shown as median and interquartile range (IQR), unless otherwise stated. Lilliefors corrected K-S test was performed before the data analysis in order to examine the distribution of the residuals of the parametric tests. For comparisons between patients, the Wilcoxon rank-sum test, Mann-Whitney test and Kruskal-Wallis test were used, as appropriate. The ROC curve was used to choose the optimal cut off point at which the sensivity and specificity of every biomarker were maximized.

All tests were two-sided, and $p < 0.05$ were considered statistically significant. Statistical tests were performed using the Statistical Package for Social Sciences (version 21.0; SPSS, Chicago, IL) and GraphPad Prism 7 (GraphPad Software, San Diego, California, USA).

RESULTS

Characteristics of study subjects

Data from sixty-eight adult women with a median age of 32 years (median, IQR: 29-46) were considered (Figure 1). Two patients with TSC were excluded from the study because of the lack of thoracic imaging (chest CT scan was requested during the first pulmonology evaluation but the patients were lost at follow up). Data from 66 patients were available for the analysis; the analysis of serum from 16 healthy female volunteers was also performed (**Figure 1**). 13 patients (19%) were affected by S-LAM, 37 (54%) by TSC, and of these 14 (38%) had TSC-LAM. Six patients with TSC had a chest CT showing less than 10 parenchymal cysts and they were classified as having a TSC-LAM "minimal disease"[16]. In patients with S-LAM the diagnosis was istologically confirmed in 9 patients (by pulmonary biopsy in 7 patients, by biopsy of abdominal lymphangioma in 1 patient and by identification of LAM cells in the chylous effusion in 1 patient). In 3 patients with S-LAM the diagnosis was confirmed by the presence of a chest CT scan compatible in association with evidence of renal angiomyolipoma[24]. There was no difference in the median age and in the age at LAM diagnosis between groups. Six patients currently smoke and 36 have a smoking history. All patients affected with LAM, both S-LAM and TSC-LAM were taking standard therapy (long acting B2 agonists and long acting muscarinic agents). None was treated with sirolimus or everolimus at the time of biomarkers analysis.

The demographic, clinical and genetic characteristics of the study population are reported in **Table 1**. The median values of VEGF-D in whole population was 515 pg/dL (interquartile range, IQR,: 364 pg/dL -1407 pg/dL), the median values of VEGF-C was 6342 pg/dL (IQR: 5142 pg/dL -7613 pg/dL), the median value of MMP-2 was 285 ng/dL (IQR: 221 ng/dL -348 ng/dL), the median value of MMP-7 was 3,63 ng/dL (IQR: 3,03 ng/dL -4,84 ng/dL).

Distribution of serum biomarkers between groups

Figure 2 shows the distribution of the biomarkers between subjects. Serum VEGF-D was higher in S-LAM (median value: 1456pg/ml range 457-3167pg/ml) and TSC-LAM (median value: 1057pg/ml range 574-3302pg/ml) than in TSC patients (median value: 396 pg/ml, range 322-646pg/ml) and controls (median value: 378 pg/ml range 335-444pg/ml), ($p<0.001$) (**Figure 2**). All healthy controls had a VEGF-D lower than 800 pg/mL. Among patients with TSC, 1 patient showed borderline serum VEGF-D level while 2 patients had very high level even without any identified cysts at chest CT scan. Three patients between the 6 classified as having "minimal LAM-TSC" had serum VEGF-D higher than 800 pg/mL. When considering comparison between each subgroup, VEGF-D remains significantly higher in S-LAM, TSC-LAM and minimal LAM TSC compared in healthy controls ($p=0.014$, $p=0.005$, $p=0.034$) but not in TSC compared to healthy controls ($p>0.999$); **Figure 2**. VEGF-D did not differ between TSC and LAM-TSC ($p=0.055$) and between TSC and minimal LAM TSC ($p=0.170$). Serum VEGF- C did not show any significant difference between the groups. As previously reported[8], VEGF-C in serum was slightly lower in S-LAM patients (median value: 6453 pg/ml, range 585-8325 pg/ml), TSC-LAM patients (median value: 6230 pg/ml, range 5240-7411 pg/ml), and TSC patients (median value: 6338 pg/ml, range 4271-7718 pg/ml) than controls (median value 7058 pg/ml, range 5093-8198 pg/ml) (**Figure 2)**.

Considering all LAM patients (both S-LAM and LAM-TSC) compared with subjects without LAM (controls and TSC patients) serum MMP-2 was higher in the former [median value: 301 ng/ml (276-463 ng/ml) vs 231ng/ml (213-324 ng/ml), $p=0.001$] but with a high overlap between the values (data not showed).

Serum MMP-2 levels were higher in S-LAM (median value: 298 ng/ml, range -416 ng/ml)], and TSC-LAM patients (median value: 293 ng/ml (248-480 ng/ml)] compared to healthy volunteers [median value: 225 ng/ml (203-336 ng/ml)] and TSC patients [median value: 232 ng/ml (213-323 ng/ml), $p=0.020$] (**Figure 2**). When considering pairwise analysis, MMP-2 did not differ in S-LAM, TSC-LAM and minimal LAM TSC compared to healthy subjects ($p=0.101$, $p=0.475$, $p=0.271$ respectively)

nor between TSC and LAM-TSC (p=0.977) or between TSC and minimal LAM TSC (p=0.05); **Figure 2**. Serum MMP-7 was higher in patients with LAM (both S-LAM and TSC-LAM) than in patients with TSC and in healthy subjects [median value: 4,5 ng/ml (3,3-5,3 ng/ml) vs 3,4 ng/ml (2,8-3,8 ng/ml), p=0.002]. A high overlap between the values is observed. MMP-7 serum levels were higher in TSC-LAM patient [median value: 4,78 ng/ml (3,5-5,3 ng/ml)] with even greater values in patients with TSC-LAM minimal disease [median value: 5,69 ng/ml (4,90-7,43 ng/ml)] than in S-LAM patients [median value: 3,39 ng/ml (3,16-4,35)] and in controls [median value: 2,99 ng/ml (2,62-3,59 ng/ml); p=0.001] (**Figure 2**). When considering pairwise analysis, MMP-7 is significantly higher in LAM-TSC and minimal TSC-LAM than in healthy subjects (p=0.008 and 0.001 respectively) but did not differ between S-LAM and healthy subjects (p>0.99) and between S-LAM and TSC-LAM (p>0.99); **Figure 2.**

Diagnostic yield of serum biomarkers

When considering patients with LAM (both S-LAM and TSC-LAM and with TSC-LAM with minimal disease) and healthy subjects, with the cut off value of 800 pg/mL the VEGF-D sensitivity and specificity for the diagnosis of LAM in our sample was 58% and 100% respectively with a negative likehood ratio of 0.42, a positive predicted value of 100% and a negative predicted value of 53% and an accuracy of 71%. ROC analysis confirmed VEGF-D as an effective diagnostic test to predict LAM [area under curve (AUC): 0.879±0.049 (95% CI:0.782-0.975), p<0.001] (**Figure 3**). The ability of MMP-2 for predicting LAM disease was lower than the ability of VEGF-D with an AUC of 0.756±0.079 (95% CI:0.601-0.910), p=0.004 for LAM. With a cut off value of 263,18 pg/ml, the sensitivity for LAM was high but the specificity remains low (81% and 69% respectively) (**Figure 3**). ROC analysis showed that MMP-7 was a better biomarker for diagnosis of LAM than MMP-2 with an area under curve of 0.828±0.060(95% CI:0.710-0.945), p<0.001, for LAM. The optimal cut off value for LAM resulted as 3,27 pg/ml; the sensitivity was 67% and specificity 82% (**Figure 3**).

Diagnostic yield of biomarkers for LAM in TSC patients

Considering all patients with TSC, the specificity of the cut off value of 800 pg/dL for LAM was 82% and the sensitivity remaining quite low (55%). The area under ROC curve for LAM diagnosis was 0.791±0.077(95% CI:0.640-0.941), p=0.003 (**Figure 3**). The diagnostic yield of MMP-2 in predicting LAM disease in patients with TSC was lower than the ability of VEGF-D. In fact the area under curve was 0.694±0.088 (95% CI:0.521-0.867), p=0.044; considering a cut off value of 339.8 pg/dL the specificity of MMP-2 was high but the sensitivity remains low (88% and 40% respectively). In this subgroup of our cohort the diagnostic yield of MMP-7 for LAM diagnosis was similar to MMP-2 with a area under curve of 0.713±0.090 (95% CI:0.538-0.889), p=0.027. The optimal cut off value for LAM resulted 4.0 pg/mL with a sensitivity of 75% and a specificity of 71%.

Role of biomarkers for TSC genetic, systemic and LAM radiological involvement

Patients with a VEGF-D higher than 800 pg/mL were significantly younger; they were diagnosed with LAM at a younger age and had more frequent chylothorax (p=0.034) than patients with a VEGF-D serum level lower than 800pg/ml (**Table 2**). The VEGF-D level above 800 pg/ml was related to higher MMP-2 and MMP-7 values (p<0.001 and p=0.001, respectively, data not shown), and to a high frequence of mutation in TSC2 gene. In patients with higher VEGF-D the renal angiomyolipomas were bigger (p=0.002) and the retinic hamartomas were more frequent (**Table 2**) but there was no significant differences in the respiratory function tests (**Table 2b**).

Since there are no previous work that have analysed a link between MMP-2 and MMP-7 and systemic involvement, we divided the patients in two groups based on serum level higher or lower of the 50°percentile of the distribution of the biomarker in the whole population in study. Systemic involvement and functional results did not differ between patients with MMP-2 and MMP-7 higher or lower than 50 percentile (**Table 3; 3a and Table 4; 4a**) except for a higher frequency of cortical tubers in patients with high MMP-7. Based on revised HRTC scan data, 6 patients have a “minimal” disease, 12 patients had “mild” disease, 5 patients had “moderate” disease and 5 patients had “severe” disease. There were no differences in radiological involvement according to serum VEGF-D (**Table**

2). A more severe radiological involvement was seen in patients with MMP-7 lower than 50° percentile compared to patients with MMP-7 serum level higher than 50° percentile (**Table 4**).

DISCUSSION

The most important results of our work are:

1) VEGF-D is confirmed as biomarker of LAM with a high specificity; the specificity decreases in patients with TSC. Patients with LAM-TSC tended to show higher levels of VEGF-D than patients with TSC without pulmonary involvement.

2) MMP-2 and MMP-7 serum levels differ patients with and without LAM but a high overlap of the single values between groups is observed; the diagnostic yield of this two biomarkers for LAM is lower than the diagnostic yield of VEGF-D.

3) Higher level of VEGF-D seems to be related to a higher frequency of chilothorax, ocular involvement and to the presence of the mutation in TSC-2 gene.

VEGF-D serum level were analysed in a couple of previous studies that brought this biomarker to be inserted in the recent diagnostic guidelines as a diagnostic tool in presence of a typical chest CT scan framework, thus reducing the need for lung biopsy in patients with suspected LAM[4]. In two studies developed by Seyama K et al and Glasgow CG et al respectively in 2006 and 2009, VEGF-D serum levels were significantly higher in LAM patients that in controls[9,6]. In 2013 Xu et al found similar results with serum VEGF-D level significantly increased in definite LAM group, compared with that of healthy controls[25]. Successively, Young et al measured serum level of VEGF-D in patients with LAM, healthy controls and patients with other pulmonary diseases and found a serum level of VEGF D significantly higher in the first group of patients. Similarly Radzikowska et al showed that VEGF-D could discriminate between LAM and other pulmonary cystic diseases such as pulmonary Langerhans cell histiocytosis and lymphocytic interstitial pneumonia[26]. Our data showed higher VEGF-D serum levels in LAM patients than in healthy controls, in line with this data. Nevertheless in our study group, more than 40% of patients with a definite diagnosis of LAM showed serum levels of VEGF-D lower than the diagnostic threshold of 800 pg/mL; VEGF-D has a sensitivity of 58% and a specificity of 100% for the diagnosis of LAM. VEGF-D high specificity is confirmed

with a low sensitivity. This is in line with the study by Chang in which 42% of patients with LAM showed VEGF-D serum level lower than the diagnostic threshold and a sensitivity of 56% and a specificity of 100%[27]. On the contrary, Xu et al find a VEGF-D sensitivity of 96%[5]. In a study from Glasgow CG at al, a statistically significant difference between LAM and healthy control for VEGF-D serum level was maintained only for LAM patients with lymphatic involvement (lymphangioleiomyomas and/or lymphadenopathy) and not for those patients with a disease restricted to the lung[6]. In our analysis however we do not find any difference in lymphatic involvement in patients with a VEGF-D higher or lower the diagnostic threshold of 800 pg/mL except for chylothorax, that could be linked to lymphatic involvement, more frequent in patients with a VEGF-D higher than 800 pg/mL. However it is possible that these results could be related to some differences in the studied population. The differences in the number of patients involved in the studies could in part explain these differences and affect the statistical significance. Furthermore in our cohort there was a low percentage of lymphatic involvement.

We find a trend to higher VEGF-D serum levels in patients with TSC-LAM respect to patients with TSC and a normal high-resolution CT scan. This is in line with data published by Young L et al in 2008. In that study in fact VEGF-D levels were much higher in women with the tuberous sclerosis complex and LAM than in women with the TSC and normal high-resolution CT scan. However authors find a very strong difference between two groups in contrast to our data that shows only a trend to statistical significance. This difference may be ascribed to some differences in the studied population as well. In fact the majority of our patients with LAM has a mild disease while we do not have data on the population analysed in Young's study. Furthermore TSC is a disease with a very heterogeneous presentation and systemic involvement and the extent of lymphatic involvement could have influenced the analysis.

The MMPs and their tissue inhibitors (TIMPs) in vivo are involved in remodelling the extracellular matrix and basement membranes both in normal and pathologic conditions. There are not previous data about the level of isoform MMP-7 in human, while the isoform MMP-2 has been deeply

investigated and some works are available in scientific literature with quite contrasting results. Some immunohistochemical studied have demonstrated in fact that the expression of MMP-2 and their tissue inhibitors is over expressed in pulmonary tissue from patients with LAM compared to normal bronchial tissue[13,28]. Lee et al demonstrated that cells lacking TSC1/TSC2 genes, over expressed MMP-2 and that this overexpression was not affected by rapamycin (a inhibitor of the activation of T cells and B cells by reducing their sensitivity to interleukin-2 (IL-2) through mTOR inhibition)[27]. From these observations a possible role of matrix metalloproteinases in the development of parenchymal air cysts in LAM was proposed. Our data indicated that the MMP-2 serum levels are higher in patients with LAM than in patients without LAM but we also observed a high overlap between subgroups of the single values. These data are consistent with the previous studies on this field. Moses et al described a case of a patient with LAM in which urinary levels of some isoform of MMPs (in particular MMP-2 and -9) were elevated and decrease after a treatment with doxycycline (an MMP inhibitor) [29]; Pinheiro Pimenta et al described a group of 41 patients also treated with doxycycline. Serum and urinary levels of MMP-2 were higher in patients with LAM then in healthy controls and decreased after treatment with the antibiotic; however the median of MMP-2 in serum was below the detection limit both at baseline and after treatment [29 30]. Chang and colleague analysed some serum biomarkers as diagnostic and prognostic tools and found higher MMP-2 levels in patients with LAM than in controls with a considerable overlap of single values between the two groups. Finally Odajama et al studied in 2009 serum level of MMP-2 and MMP-9 in 36 patients with LAM and did not find any significant differences from healthy controls[27]. ROC analysis have demonstrated that, in line with the previously cited work by Chang et al, the ability of MMP-2 for predicting LAM disease was lower than the ability of VEGF-D.

Our data indicated that patients with VEGF-D serum level above the diagnostic threshold of 800 pg/ml show more frequent mutation in TSC 2 gene. This is consistent with previous studies that have indicate that in patients with TSC- LAM there is a higher rate of mutation in TSC2 gene respect to

TSC1[31,32,33] and that that patients with TSC and a mutation in TSC1 gene have a milder disease in comparison with patients showing a mutation of TSC2 gene[34].

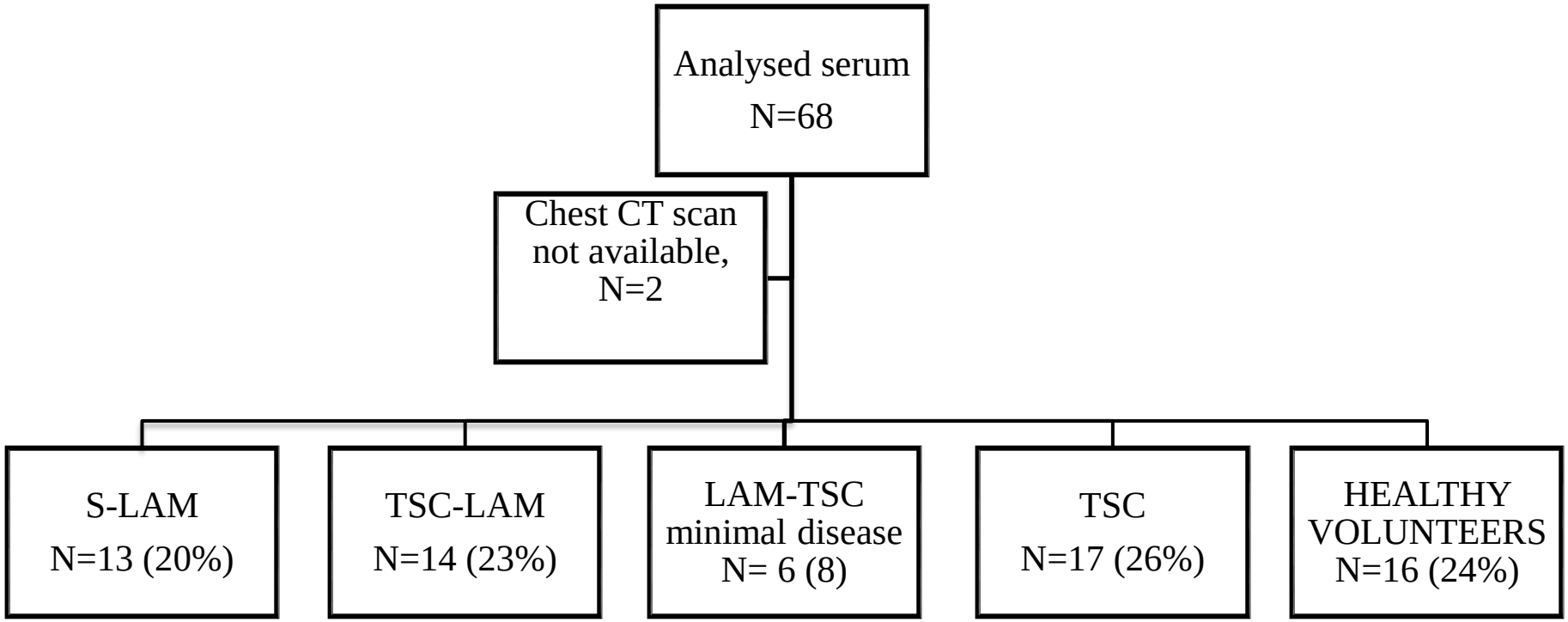
This work has two innovative characteristics: firstly we made deep phenotyping of the whole population analysing separately and comparing the serum level of four biomarkers in patients with S-LAM and TSC-LAM and exploring a possible link with clinical and genetic characteristic of the single groups. Secondly this is the first work that have analysed serum level of MMP-7 in relationship to LAM. A number of potential limits of the present study deserve also discussion. First, our data are about a single centre cohort of patients. Secondly, our patients show a relatively mild disease, in terms of pulmonary function. Third, the clinical and radiological data used to explore a possible link between single biomarkers and systemic involvement were requested for clinical follow up purpose and due to the "retrospective" characteristic of the analysis some data are missing.

CONCLUSIONS

The diagnostic value of VEGF-D for LAM was confirmed in this cohort of Italian patients. VEGF-D specificity decreases considering TSC patients, probably due to lymphatic involvement linked to TSC. MMP-2 and especially MMP 7 are promising biomarkers for LAM but validation in longitudinal studies and with a larger patient population is needed.

FIGURES

Figure 1. Population in analysis



LAM: lymphangioleiomyomatosis, TSC: tuberous sclerosis complex, minimal disease: patients with less than 10 cysts identified at chest CT scan

Figure 2 Distribution of VEGF-D (A) ***p<0.001***, VEGF-C (B) ***p=0.354***, MMP-2 (C) ***p=0.040*** and MMP-7 (D) ***p=0.001*** in the 4 subgroups of patients and in healthy volunteers

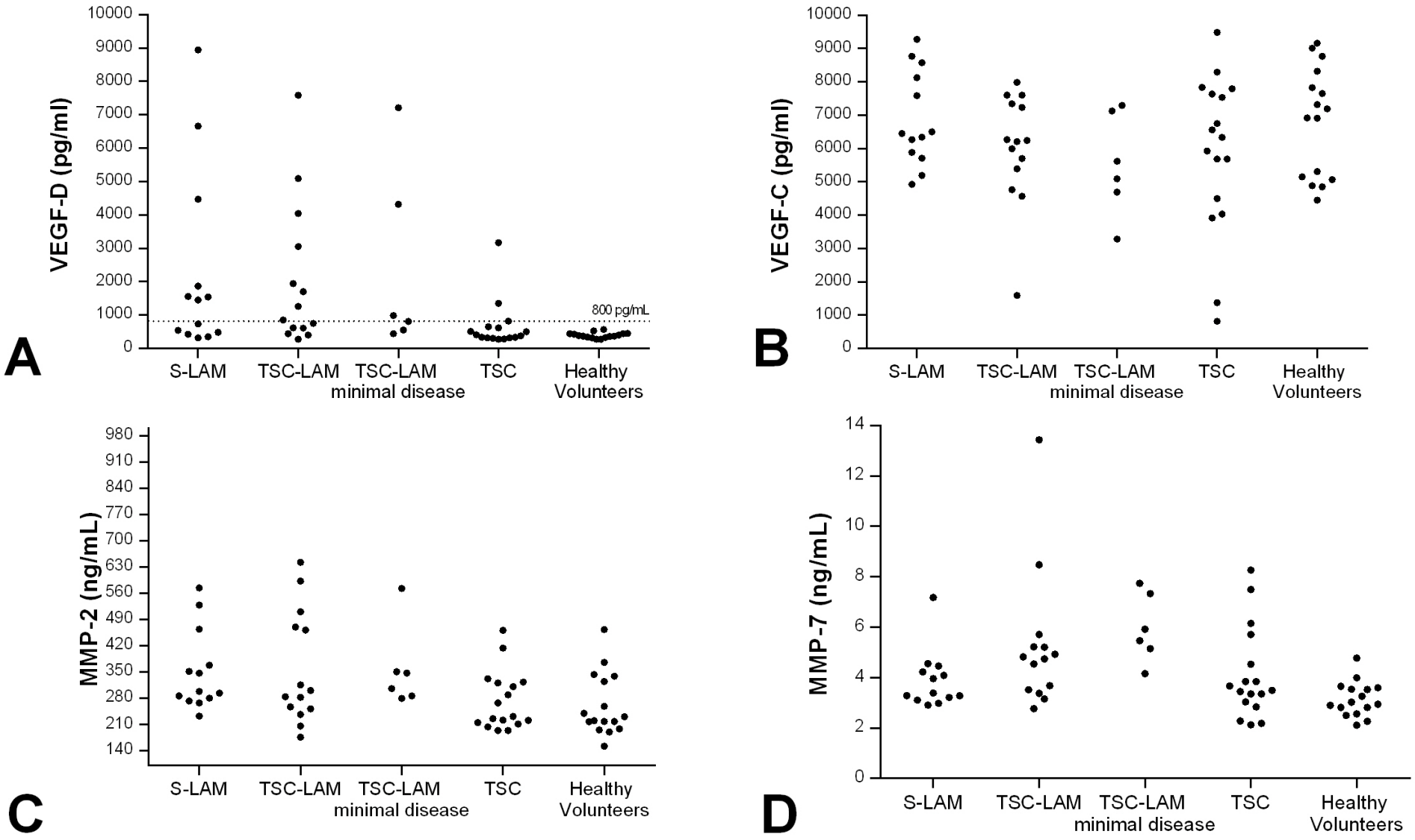


Figure 3 Model representing the diagnostic ability of serum VEGF-D, MMP-2 and MMP-7 for predicting LAM disease (A) in a sample of patients with LAM (both S-LAM and TSC-LAM) and healthy volunteers and (B) in a sample of subject with TSC with and without LAM. A larger AUC indicates higher accuracy for diagnosis of LAM.

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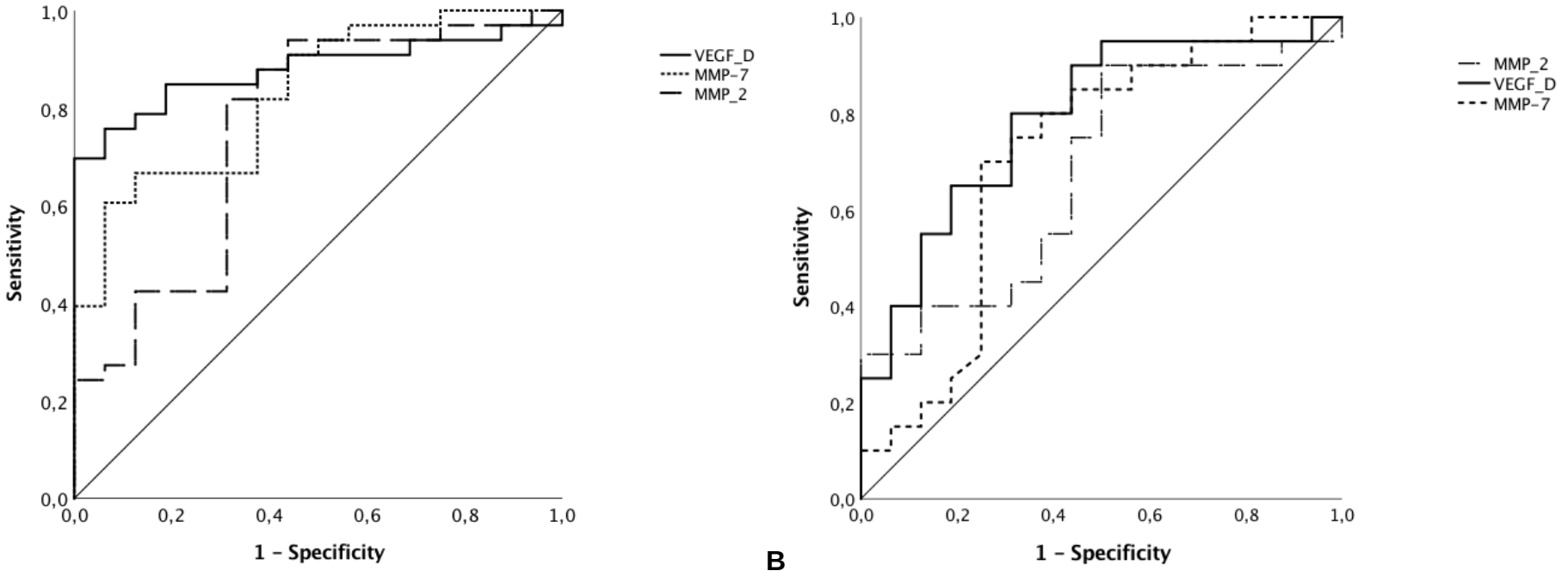

(A) VEGF-D was an effective diagnostic test to predict LAM [area under curve (AUC): 0.879±0.049 (95% CI:0.782-0.975), p<0.001] continuous line, respect to MMP2 [AUC: 0.756±0.079 (95% CI:0.601-0.910)], dotted line, and MMP7 [0.828±0.060(95% CI:0.710-0.945), p<0.001], punctuate line. (B) Specificity of VEGF-D for LAM disease in TSC patients was lower than in previous analysis but remains significant [AUC: 0.791±0.077(95% CI:0.640-0.941), p=0.003], continuous line. MMP-2 showed lower accuracy respect to VEGF-D with a AUC of 0.694±0.088 (95% CI:0.521-0.867), p=0.044, dotted line and similarly MMP-7 showed a AUC of 0.713±0.090 (95% CI:0.538-0.889), p=0.027, punctuate line

TABLES

Table 1. Characteristics of the population in analysis

	S-LAM N=13	TSC-LAM N=14	TSC-LAM minimal disease N= 6	TSC N=17	HEALTHY N=16	p
Age, yrs, median (IQR)	36 (31-43)	36 (30-50)	34 (24-63)	32 (24-42)	36 (28-49)	0.831
Age at LAM diagnosis, yrs, median (IQR)	35 (29-44)	33 (27-45)	29 (22-61)	-	-	0.930
Smoke (yes/no/ex), n (%)	1(9) / 3(27)/ 7(64)	2(14) /3(21) /9(64)	1(17) /1(1)/4 (67)	1 (6)/ 16 (94) / 0(0)		
Pulmonary involvement and symptoms						
MMPH, n (%)	-	8 (57)	6 (100)	10 (59)	-	
Dyspnea, n (%)	5 (46)	3 (21)	2 (33)	5 (29)	-	
spO2 < 90% during 6mWT, n (%)	4 (36)	3 (21)	0 (0)	2 (12)	-	
Respiratory failure, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	-	
Pneumothorax, n (%)	4 (36)	3 (21)	1 (17)	0 (0)	-	
Chylothorax, n (%)	3 (27)	1 (7)	0 (0)	0 (0)	-	
Lymphocele, n (%)	2 (18)	1 (8)	0 (0)	1 (6)	-	
Mediastinal lymph node enlargement, n(%)	1 (9)	2 (17)	1 (17)	1 (6)	-	
Lymphatic involvement*, n (%)	4 (33)	4 (29)	1 (17)	1 (6)	-	0.093
Abdominal involvement						
Renal angiomyolipomas, n (%)	4 (36)	14 (100)	6 (100)	11 (65)	-	

n<3/n≥3, n (%)	3(75)/1(25)	1 (7)/13 (93)	1 (17)/5(83)	6 (50)/6(50)		
<3 cm/≥3 cm, n (%)	2(50)/2(50)	4(29)/10(71)	3(50)/3(50)	7(64)/4(36)	-	
Multiple renal cysts, n (%)	1 (9)	8 (57)	3 (50)	6 (35)	-	
Hepatic angiomyolipomas, n (%)	1 (9)	6 (43)	1 (17)	5 (29)	-	
Genotype						
TSC1, n (%)	-	3 (21)	2 (33)	9 (56)	-	0.189
TSC2, n (%)	-	7 (50)	4 (67)	5 (31)	-	
NMI, n (%)	-	4 (29)	0 (0)	2 (13)	-	
Systemic TSC involvement						
Renal tumour, n (%)	-	1 (8)	0 (0)	4 (24)	-	
Renal failure, n (%)	0 (0)	3 (25)	0 (0)	2 (12)	-	
Cutaneous involvement, n (%)	-	13 (100)	6 (100)	17 (100)	-	
Epilepsy, n (%)	-	3 (23)	4 (67)	12 (71)	-	
Cortical tubers, n (%)	-	12 (92)	6 (100)	15 (88)	-	
Subependymal giant cell astrocytoma, n (%)		1 (8)	0 (0)	1 (6)	-	

*: at least one from chylothorax, lymphocele, mediastinal lymph node enlargement[62]

Table 2. Systemic involvement in patients with TSC and S-LAM according to serum VEGF-D

	VEGF-D < 800 pg/mL	VEGF-D ≥ 800 pg/mL	*p*
Age at evaluation*****, yrs median (IQR)	37(30-49)	32 (26-37)	**0.005**
Age at LAM diagnosis, yrs median (IQR)	44 (29-56)	32 (24-36)	0.072
LAM, n (%)	14 (33)	19 (86)	**<0.005**
MMPH, n(%)******	13 (57)	10 (67)	0.736
Dyspnoea, n(%)	11 (42)	4 (19)	0.121
Pneumothorax, n(%)	3 (12)	5 (24)	0.470
Chylothorax, n(%)	0 (0)	4 (19)	**0.034**
Lymphocele, n (%)	1 (4)	3 (15)	0.209
Mediastinal lymph node enlargement, n(%)	3 (14)	2 (11)	>0.999
Lymphatic involvement*******, n (%)	3 (12)	7 (32)	0.086
spO2 < 90% during 6mWT, n (%)	5 (20)	4 (19)	>0.999
Genotype**			
TSC1, n (%)	13 (62)	1 (7)	**0.004**
TSC2, n (%)	5 (24)	10 (71)	
NMI, n (%)	3 (14)	3 (21)	
Renal AML, n (%),	20 (77)	15 (71)	0.744
n<3/n≥3, n (%)	8(40)/12(60)	2(13)/13(87)	0.134
<3 cm/≥3 cm, n (%)	14(70)/6(30)	2 (13)/13 (87)	**0.002**
Multiple renal cysts, n (%)	12 (46)	6 (29)	0.245
Hepatic angiomyolipomas, n (%)	7 (27)	6 (29)	>0.999
Cutaneous involvement******, n (%)	20 (100)	15 (100)	-
Cortical tubers******, n (%)	17 (85)	15 (100)	0.244
Epilepsy******, n (%)	9 (45)	9 (60)	0.500
Arytmia, n(%)******	1 (5)	1 (7)	>0.990
Cardiac rhabdomyoma******, n (%)	3 (15)	4 (31)	0.393
Fundus oculi abnormalities*****, n (%)	2 (10)	6 (43)	**0.042**

*age: referred to age at time of blood sample evaluation; IQR: interquartile range; ****:** Percentage are referred to total patients with TSC; ***: at least one from chylothorax, lymphocele, mediastinal lymph node enlargement. MMPH: multifocal micronodular pneumocyte hyperplasia; TSC1/2/NMI: mutation of TSC1 TSC2 genes/ no mutation identified; AML: angiomyplipoma

Table 2b Functional and radiologic involvement ** in patients with S-LAM and TSC-LAMaccording to serum VEGF-D

	VEGF-D < 800 pg/mL	VEGF-D ≥ 800 pg/mL	*p*
FEV1, % pred	98 (85-112)	95 (82-101)	0.343
FVC, % pred	99 (84-110)	94 (78-102)	0.300
FEV1/FVC, % pred	101 (99-103)	100 (96-106)	0.581
DLCO, % pred	78 (65-84)	73 (53-82)	0.297
KCO, % pred	77 (68-80)	73 (59-94)	0.929
VA, % pred	101 (84-110)	95 (84-117)	0.705
VR, % pred	86 (65-143)	95 (66-120)	0.888
TLC, % pred	99 (84-113)	102 (87-112)	0.937
RV/TLC, % pred	120 (101-128)	135 (103-173)	
TGV, % pred	84 (70-119)	94 (76-125)	0.599
Radiological severity**			
Minimal disease, n(%)	2 (15)	4 (27)	0.738
Grade I, n (%)	7 (54)	5 (33)	
Grade II, n (%)	2 (15)	3 (20)	
Grade III, n (%)	2 (15)	3 (20)	

**% Data and percentage referred to 28 patients with revised CT scan. FEV1: forced expiratory volume in one second; FVC: forced expiratory volume; DLCO: diffusion capacity for CO; VA: alveolar volume; TLC: total lung capacity; RV: residual volume; TGV: thoracic gas volume; %pred: % of predicted value. p < 0.050 in bold.

Table 3. Systemic involvement in patients with TSC and S-LAM according to serum MMP-2

	MMP-2 < 50° percentile	MMP2- ≥ 50° percentile	*p*
Age at LAM diagnosis, yrs, median (IQR)	44 (24-47)	32 (29-38)	0.582
Age at evaluation*****, yrs, median (IQR)	37 (30-49)	32 (27-41)	0.201
LAM, n (%)	12 (36)	21 (63)	**0.048**
MMPH, n (%)******	12 (63)	12 (60)	0.550
Dyspnoea, n (%)	8 (38)	7 (26)	0.277
Pneumothorax, n (%)	4 (19)	4 (15)	0.495
Chylothorax, n(%)	0 (0)	4 (15)	0.121
Lymphocele, n (%)	1 (5)	3 (12)	0.622
Mediastinal lymph node enlargement, n(%)	2 (11)	3 (13)	>0.999
Lymphatic involvement*******, n (%)	2 (10)	8 (29)	0.155
spO2 < 90% during 6mWT, n (%)	3 (15)	6 (22)	0.407
Genotype**			
TSC1, n (%)	9 (50)	5 (28)	0.131
TSC2, n (%)	5 (28)	11 (61)	
NMI, n (%)	4 (22)	2 (11)	
Renal AML******, n (%),	16 (76)	19 (70)	0.750
n<3/n≥3, n (%)	6 (35)/ 11 (65)	5 (26)/ 14 (74)	0.412
<3 cm/≥3 cm, n (%)	9 (56)/ 7 (44)	7 (37)/ 12 (63)	0.210
Bilateral renal AML******, n (%)	10 (48)	16 (62)	0.225
Hepatic AML*****, n (%)	8 (38)	5 (19)	0.192
Cutaneous involvement******, n (%)	-	17 (100)	-
Cortical tubers******, n (%)	14 (82)	19 (100)	0.095
Epilepsy******, n (%)	7 (41)	12 (63)	0.316
Arytmia, n (%)******	1 (6)	1 (6)	>0.999
Cardiac rhabdomyoma******, n (%)	2 (12)	5 (29)	0.398
Fundus oculi abnormalities******, n (%)	3 (18)	5 (28)	0.691

*age: referred to age at time of blood sample evaluation; IQR: interquartile range; ****:** Percentage are referred to total patients with TSC; ***: at least one from chylothorax, lymphocele, mediastinal lymph node enlargement. MMPH: multifocal micronodular pneumocyte hyperplasia; TSC1/2/NMI: mutation of TSC1 TSC2 genes/ no mutation identified; AML: angiomyplipoma

Table 3b Functional and radiologic* involvement in patients with LAM (both S-LAM and TSC-LAM) according to serum MMP2

	MMP2 < 50° percentile	MMP2 ≥ 50° percentile	*P*
FEV1, % pred	98 (85-115)	96 (82-99)	0.516
FVC, % pred	99 (85-113)	95 (81-103)	0.759
FEV1/FVC, % pred	101 (99-103)	100 (97-106)	0.288
DLCO, % pred	79 (66-84)	71 (53-82)	0.383
KCO, % pred	77 (68-91)	77 (52-85)	0.318
VA, % pred	102 (78-120)	95 (84-109)	0.720
VR, % pred	74 (52-108)	116 (76-144)	0.051
TLC, % pred	90 (82-110)	106 (89-113)	0.026
RV/TLC, % pred	118 (99-124)	136 (104-179)	
TGV, % pred	75 (66-112)	99 (87-127)	0.058
Radiological severity			
Minimal disease, n (%)	1 (9)	5 (29)	**0.040**
Grade I, n (%)	8 (73)	4 (24)	
Grade II, n (%)	2 (18)	3 (18)	
Grade III, n (%)	0 (0)	5 (29)	

*% Data and percentage referred to 28 patients with revised CT scan. FEV1: forced expiratory volume in one second; FVC: forced expiratory volume; DLCO: diffusion capacity for CO; VA: alveolar volume; TLC: total lung capacity; RV: residual volume; TGV: thoracic gas volume; %pred: % of predicted value. $p < 0.050$ in bold.

Table 4 systemic involvements according to serum MMP-7

	MMP-7 < 50° percentile	MMP-7 ≥ 50° percentile	*P*
Age at evalutation, median (IQR) *****	34 (29-45)	37 (28-49)	0.287
Age at LAM diagnosis, median (IQR)	34 (29-44)	32 (24-52)	0.880
LAM, n (%)	11 (33)	22 (67)	**0.013**
MMPH, n (%)******	5 (36)	19 (76)	**0.019**
Pneumothorax, n(%)	4 (21)	4 (14)	0.695
Dyspnoea, n(%)	6 (32)	9 (31)	>0.999
Chylothorax, n(%)	0 (0)	4 (14)	0.142
Lymphocele, n (%)	1 (5)	3 (11)	0.632
Mediastinal lymph node enlargement, n(%)	2 (13)	3 (12)	>0.999
Lymphatic involvement*******, n (%)	2 (11)	8 (27)	0.278
spO2 < 90% during 6mWT, n (%)	4 (21)	5 (18)	>0.999
Genotype**			
TSC1, n (%)	7 (54)	7 (30)	0.154
TSC2, n (%)	3 (23)	13 (57)	
NMI, n (%)	3 (23)	3 (13)	
Renal angiomyolipomas, n (%),	14 (74)	21 (72)	>0.999
n<3/n≥3, n (%)	6 (43)/8 (57)	5 (23)/17 (77)	0.273
<3 cm/≥3 cm, n (%)	8 (57)/6 (43)	8 (38)/13(62)	0.317
Bilateral renal angiomyolipomas, n (%)	9 (47)	17 (61)	0.390
Hepatic angiomyolipomas, n (%)	6 (32)	7 (24)	0.741
Cutaneous involvement******, n (%)	13 (100)	23 (100)	-
Cortical tubers******, n (%)	10 (77)	23 (100)	**0.040**
Epilepsy******, n (%)	5 (39)	14 (61)	0.299
Aritmia, n(%)*	1 (9)	1 (4)	>0.999
Cardiac rhabdomyoma******, n (%)	2 (15)	5 (24)	0.682
Fundus Oculi abnormalities******, n (%)	3 (23)	5 (23)	>0.999
Multiple retinal hamartomas*****, n (%)	1 (8)	5 (23)	0.377

*age: referred to age at time of blood sample evaluation; IQR: interquartile range; ****:** Percentage are referred to total patients with TSC; ***: at least one from chylothorax, lymphocele, mediastinal lymph node enlargement. MMPH: multifocal micronodular pneumocyte hyperplasia; TSC1/2/NMI: mutation of TSC1 TSC2 genes/ no mutation identified; AML: angiomyplipoma

Table 4b Functional and radiologic involvement* in patients with LAM (both S-LAM and TSC-LAM) according to serum MMP7

	MMP-7 < 50° percentile	MMP-7 ≥ 50° percentile	*P*
FEV1, % pred	98 (84-113)	95 (84-102)	0.314
FVC, % pred	98 (82-112)	95 (83-107)	0.282
FEV1/FVC, % pred	100 (94-103)	101 (99-104)	0.173
DLCO, % pred	81 (64-86)	70 (58-80)	**0.002**
KCO, % pred	77 (67-94)	76 (66-85)	0.705
VA, % pred	104 (96-122)	94 (82-110)	0.105
VR, % pred	89 (72-137)	87 (58-130)	0.863
TLC, % pred	99 (86-115)	100 (87-112)	0.863
RV/TLC, % pred	122 (105-139)	127 (94-152)	
TGV, % pred	81 (73-121)	91 (69-122)	0.463
Radiological severity			
Grade 0, n (%)	0 (0)	6 (32)	**0.033**
Grade I, n (%)	6 (67)	6 (32)	
Grade II, n (%)	0 (0)	5 (26)	
Grade III, n (%)	3 (33)	2 (11)	

*% Data and percentage referred to 28 patients with revised CT scan. FEV1: forced expiratory volume in one second; FVC: forced expiratory volume; DLCO: diffusion capacity for CO; VA: alveolar volume; TLC: total lung capacity; RV: residual volume; TGV: thoracic gas volume; %pred: % of predicted value. p < 0.050 in bold.

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