Methacholine reactivity in lymphangioleiomyomatosis is inversely related to FEV_1 and VEGF-D

To the Editor:

Lymphangioleiomyomatosis (LAM) is a multisystem disease characterised by cystic lung destruction, leading to respiratory failure, and associated with kidney (*e.g.* angiomyolipomas (AML)) and lymphatic involvement (*e.g.* lymphangioleiomyomas, chylous effusions) [1, 2]. LAM occurs sporadically or in association with tuberous sclerosis complex (TSC), an autosomal-dominant disorder characterised by mutations of the *TSC1* or *TSC2* genes. Lung destruction results from the proliferation of LAM cells, which possess neoplastic properties and are found in LAM lung nodules, in association with fibroblasts, mast cells, lymphocytes and lymphatic endothelial cells [3, 4]. LAM patients may show increases in serum levels of the lymphangiogenic factor, vascular endothelial growth factor-D (VEGF-D), a LAM biomarker used in differential diagnosis of cystic lung diseases and to identify LAM patients likely to respond to sirolimus treatment [5–7].

Cystic lung destruction in LAM is associated with decline in force expiratory volume in 1 s (FEV_1) and/or diffusing capacity of the lung for carbon monoxide (D_{LCO}), reduction in the 6-min walk test and an abnormal cardiopulmonary exercise test [3, 8]. About a third of LAM patients exhibit an intermittent bronchodilator response to β-adrenergic agonists, which is associated with the presence of LAM lung nodules and a more rapid decline in FEV_1 [3, 9]. Although LAM airways showed evidence of airway inflammation [3], histopathology did not correlate with bronchodilation in response to β-adrenergic agonists [8].

Bronchodilator responsiveness is characteristic of patients with asthma, a clinically heterogeneous disease marked by differences in histopathology and responses to therapeutic agents [10]. In some patients with asthma-like symptoms, airway hyper-reactivity may be established by responsiveness to methacholine [11], a non-selective muscarinic receptor agonist that acts on the parasympathetic nervous system and a bronchoprovocative agent that may cause shortness of breath and wheezing in susceptible individuals.

Given the LAM histopathology, we questioned whether the bronchodilator responsiveness seen in some LAM patients occurred in association with methacholine responsiveness, as seen in asthma. If so, we asked whether methacholine responsiveness was preferentially associated with other markers of LAM severity and drug responsiveness, *e.g.* VEGF-D, FEV_1 and bronchodilators.

43 LAM patients participated prospectively in the methacholine study. Diagnosis of LAM was obtained by video-assisted thoracoscopic surgery (11 patients), transbronchial biopsy (one), computed tomography (CT) and AML (20), CT and TSC (seven), and CT and lymphatic involvement (four). Patients were excluded if they had a history of asthma, baseline FEV_1 <1 L, history of cigarette smoking, or treatment of LAM with sirolimus or other mTOR inhibitors. The study was approved by the ethical committee of Ospedale San Giuseppe, Milan. Written, informed consent was obtained from all patients.

Lung volumes, flow rates and single-breath D_{LCO} were measured (Vmax 229; SensorMedics, Yorba Linda, CA, USA), according to American Thoracic Society (ATS) recommendations [12]. Patients abstained from using inhaled bronchodilators and corticosteroids for 12 h prior to testing. A positive response to bronchodilators consisted of an increase in FEV_1 of 12% over baseline and of at least 200 mL. On day 1 of the study, flow rates were measured before and after inhalation of 400 µg of salbutamol sulfate in solution. On the following day (day 2) and at the same time of day, flow rates were measured before and after

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Methacholine bronchoprovocation in LAM patients was used to assess airway reactivity. A negative methacholine challenge was associated with a lack of a response to β-adrenergic agonist, higher levels of FEV_1, and higher levels of VEGF-D https://bit.ly/3aVTFl2

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nebulisation of 500 µg of ipratropium bromide. On day 3, the bronchoprovocation challenge test with methacholine was performed. Patients were excluded if the FEV_1 was <1 L. The test was conducted according to ATS recommendations [12]. Methacholine (Lofarma Metacolina 1% solution), 30 mg was administered with progressively more concentrated solutions *via* nebuliser in 2 min increments; each dose was followed by reassessment of spirometry. If a patient became symptomatic, the test was interrupted. It was continued if FEV_1 did not fall >20% from baseline. The test was stopped and considered positive when an FEV_1 decline >20% from baseline was observed. The test was considered negative when the patient did not respond to the maximum concentrations of methacholine. VEGF-D concentration was determined by a quantitative sandwich enzyme immunoassay technique [5, 6].

14 of the 43 patients exhibited bronchoconstriction in response to bronchoprovocation with methacholine. We initially performed univariate analyses of possible predictors of response to methacholine challenge. p-values were derived using a t-test for continuous variables and Fisher's exact test for binary variables. Then, a multiple logistic regression was used to identify factors associated with the response to the methacholine challenge. Variables with p-value <0.10 in the univariate analyses were considered for inclusion. All tests were two-sided and no p-value adjustments for multiple comparisons were made.

From the univariate analyses, FEV_1 (p=0.076), VEGF-D (p=0.036) and response to salbutamol (p=0.040) were associated with a methacholine response. The response to salbutamol, however, was not associated with FEV_1 or VEGF-D. LAM airways also exhibited bronchodilatation in response to ipratropium but an ipratropium response was not concordant with methacholine responsiveness (p=0.488) (table 1). Also, no factors were found to be significantly associated with the response to ipratropium. Significant predictors of a negative methacholine challenge in the multivariate model were lack of a response to salbutamol (p=0.019), higher levels of FEV_1 (p=0.049), and higher levels of serum VEGF-D (p=0.015). Thus, subjects who did not respond to salbutamol, or had higher levels of FEV_1 or VEGF-D levels, tended to have a negative response to methacholine challenge. Thus, reactivity to methacholine correlated positively with β-adrenergic agonist responsiveness, and inversely with greater FEV_1 and higher serum VEGF-D levels.

As these data suggest, in LAM, like in asthma, airway hypersensitivity can be assessed by methacholine challenge. A subset of LAM patients was sensitive to methacholine. The methacholine-sensitive patients were examined for bronchodilator responsiveness with salbutamol (albuterol), a short-acting β-adrenergic agonist and ipratropium, an anti-cholinergic agonist. The methacholine-responsive patients exhibited a statistically significant response to salbutamol (p=0.04). The same methacholine-responsive LAM patients, however, did not show a concordant bronchodilator response to ipratropium (p=0.488). Thus, there was an apparent dissociation between the methacholine responsiveness and that of the bronchodilators.

The association of serum VEGF-D levels with methacholine sensitivity was unexpected. Serum VEGF-D levels have been shown to be a biomarker for LAM [5, 6], with levels greater than 800 $pg \cdot mL^{-1}$ consistent

TABLE 1 Univariate analyses of possible predictors of response to methacholine challenge

	Methacholine challenge		p-value#
	Negative	Positive	
Subjects n	29	14	
Age years	37.7±2.2	43.0±3.2	0.178
FEV_1 mL per year	2.4±0.1	2.1±0.1	0.076
FEV_1 % pred	84.9±3.5	77.6±3.9	0.214
D_{LCO} $mL \cdot min^{-1} \cdot mmHg^{-1}$	14.8±0.9	13.5±1.0	0.382
D_{LCO} % pred	58.0±3.3	54.2±4.0	0.492
VEGF-D $pg \cdot mL^{-1}$	1396.8±233.6	771.7±167.4	0.036
Salbutamol	26 (90)	6 (43)	0.040
Ipratropium	21 (72)	6 (43)	0.488
Tuberous sclerosis complex	23 (79)	11 (79)	1.000
Angiomyolipoma	16 (55)	7 (50)	1.000
Lymphatic	25 (86)	11 (79)	0.665
Menopause	27 (93)	12 (86)	0.585
Treatment history	28 (97)	13 (93)	1.000

Data are presented as mean±SEM or n (%), unless otherwise indicated. FEV_1: forced expiratory volume in 1 s; D_{LCO}: diffusing capacity of lung for carbon monoxide; VEGF-D: vascular endothelial growth factor D. #: p-values derived using a t-test for continuous variables and Fisher's exact test for binary variables (shown are for values either "no response" or "absence of the condition").

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with a LAM diagnosis, although LAM patients may have VEGF-D levels less than 800 $pg \cdot mL^{-1}$ [13]. In this study, we found that higher serum VEGF-D levels were associated with reduced methacholine sensitivity in LAM patients not treated with sirolimus. Since VEGF-D is a lymphangiogenic growth factor, enhanced lymphangiogenesis may reduce methacholine sensitivity. In the MILES trial [5, 7], higher levels of VEGF-D were associated with an enhanced response to sirolimus treatment and correlated with increased rate of disease progression; in other studies, sirolimus appears to inhibit the lymphatic component [14]. Methacholine bronchoprovocation in patients with LAM was used to assess airway reactivity. A negative methacholine challenge was associated with a lack of response to β-adrenergic agonist, higher levels of FEV_1, and unexpectedly, higher levels of the lymphangiogenic factor, VEGF-D. Our current study suggests that the lymphatic component may be involved in methacholine responsiveness.

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