

Doxycycline use in patients with lymphangioleiomyomatosis: biomarkers and pulmonary function response^{*,**}

Doxiciclina em pacientes com linfangioleiomiomatose:
biomarcadores e resposta funcional pulmonar

Suzana Pinheiro Pimenta, Bruno Guedes Baldi,
Ronaldo Adib Kairalla, Carlos Roberto Ribeiro Carvalho

Abstract

Objective: To assess blockade of matrix metalloproteinase (MMP)-2 and MMP-9, as well as the variation in FEV₁, in patients with lymphangioleiomyomatosis (LAM) treated with doxycycline (a known MMP inhibitor) for 12 months. **Methods:** An open-label, single-arm, interventional clinical trial in which LAM patients received doxycycline (100 mg/day) for 12 months. Patients underwent full pulmonary function testing, a six-minute walk test, and quality of life assessment, as well as blood and urine sampling for quantification of MMP-2, MMP-9, and VEGF-D levels—at baseline, as well as at 6 and 12 months after the initiation of doxycycline. **Results:** Thirty-one LAM patients received doxycycline for 12 months. Although there was effective blockade of urinary MMP-9 and serum MMP-2 after treatment, there were no significant differences between pre- and post-doxycycline serum levels of MMP-9 and VEGF-D. On the basis of their response to doxycycline (as determined by the variation in FEV₁), the patients were divided into two groups: the doxycycline-responder (doxy-R) group (n = 13); and the doxycycline-nonresponder (doxy-NR) group (n = 18). The patients with mild spirometric abnormalities responded better to doxycycline. The most common side effects were mild epigastric pain, nausea, and diarrhea. **Conclusions:** In patients with LAM, doxycycline treatment results in effective MMP blockade, as well as in improved lung function and quality of life in those with less severe disease. However, these benefits do not seem to be related to the MMP blockade, raising the hypothesis that there is a different mechanism of action.

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Keywords: Lymphangioleiomyomatosis; Doxycycline; Matrix metalloproteinases; Respiratory function tests.

Resumo

Objetivo: Avaliar o bloqueio da metaloproteinase da matriz (MMP)-2 e da MMP-9 e a variação do VEF₁ em pacientes com linfangioleiomiomatose (LAM) após o uso de doxiciclina, um conhecido inibidor de MMP, durante 12 meses. **Métodos:** Ensaio clínico aberto de braço único no qual as pacientes com diagnóstico de LAM receberam doxiciclina (100 mg/dia) durante 12 meses. Elas foram submetidas a prova de função pulmonar completa, teste de caminhada de seis minutos, avaliação da qualidade de vida e coleta de amostras séricas e urinárias para dosagem de MMP-2, MMP-9 e VEGF-D antes do início do tratamento com doxiciclina e após 6 e 12 meses de tratamento. **Resultados:** Trinta e uma pacientes com LAM receberam doxiciclina durante 12 meses. Embora tenha havido um bloqueio efetivo da MMP-9 urinária e da MMP-2 sérica após o tratamento, os níveis séricos de MMP-9 e VEGF-D permaneceram estáveis. Com base na resposta à doxiciclina (determinada pela variação do VEF₁), as pacientes foram divididas em dois grupos: respondedoras (doxi-R; n = 13) e não respondedoras (doxi-NR; n = 18). As pacientes com alterações espirométricas leves apresentaram melhor resposta à doxiciclina. Os efeitos colaterais mais comuns foram epigastralgia, náusea e diarreia, todos de leve intensidade. **Conclusões:** Em pacientes com LAM, o tratamento com doxiciclina resulta em um bloqueio eficaz das MMP, além de melhorar a função pulmonar e a qualidade de vida daqueles com doença menos grave. No entanto, esses benefícios não parecem estar relacionados ao bloqueio das MMP, o que sugere um mecanismo de ação diferente.

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Descritores: Linfangioleiomiomatose; Doxiciclina; Metaloproteinases da matriz;
Testes de função respiratória.

* Study carried out in the Department of Pulmonology, *Instituto do Coração* – InCor, Heart Institute – University of São Paulo School of Medicine *Hospital das Clínicas*, São Paulo, Brazil.

Correspondence to: Carlos R. R. Carvalho. Rua Sampaio Viana, 509, ap. 21, CEP 04004-001, São Paulo, SP, Brasil.

Tel. 55 11 3887-4607. Fax: 55 11 3885-7036. E-mail: crccarvalho@uol.com.br

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Introduction

Lymphangiomyomatosis (LAM) is a rare disease that affects women of childbearing age. It can occur sporadically or in association with tuberous sclerosis complex (TSC), being characterized by lung cysts.⁽¹⁾ The most common clinical manifestations of LAM are dyspnea and pneumothorax; other, less common, manifestations include hemoptysis, cough, and chylothorax.⁽²⁻⁶⁾ In patients with LAM, the most common abnormal pulmonary function test results are airflow obstruction and decreased DLCO.⁽⁷⁾

There is currently no curative treatment for LAM. Hormonal blockade is the most widely used treatment. However, there is a lack of evidence in this field. A retrospective study involving patients with LAM showed that the rates of decline in DLCO were significantly higher in those who were treated with progesterone.⁽⁸⁾ The results of small studies involving the use of gonadotropin-releasing hormone agonists have been controversial.^(9,10)

A randomized clinical trial showed that sirolimus, an inhibitor of the mammalian target of rapamycin pathway, can be useful in treating patients with moderately severe LAM-related lung disease.⁽¹¹⁾

Recently, there has been interest in the possible role of matrix metalloproteinases (MMPs) in the pathogenesis of cystic lung destruction in LAM, including the possibility that MMPs represent a therapeutic target.⁽¹²⁾ It is known that MMPs are functional components of the extracellular matrix. They degrade a variety of matrix substrates and play an important role in lung remodeling and lymphangiogenesis.^(13,14) In addition, MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs), which are indispensable in the regulation of MMPs. An imbalance between MMPs and their inhibitors has been implicated in the pathogenesis of lung diseases such as asthma, COPD, and Langerhans cell histiocytosis.^(15,16) Protease imbalance has been described in LAM lesions.⁽¹⁴⁾ A study examining lung biopsy samples from patients with LAM found elastic fiber degradation in areas of smooth muscle cell proliferation.⁽¹⁷⁾ Likewise, immunohistochemical analysis of lung biopsy samples from patients with LAM revealed increased immunoreactivity of MMP-2 and MMP-9, although not of TIMP-1 and TIMP-2, in the LAM cells when compared with that of those in normal lung cells.⁽¹⁸⁾ One

case report showed an improvement in pulmonary function test results and a reduction in urinary MMP levels in an LAM patient who had severely impaired lung function and was treated with doxycycline, an MMP inhibitor.⁽¹⁹⁾ In a previous open-label trial, our group found a significant decrease in serum levels of MMP-2 in a cohort of LAM patients treated with doxycycline for 6 months.⁽²⁰⁾

The objective of the present study was to assess serum and urinary MMP-2 and MMP-9 levels in LAM patients treated with doxycycline for 12 months, as well as to assess the variation in FEV₁ in those patients.

Methods

This was an open-label, single-arm, interventional clinical trial. All LAM patients receiving outpatient treatment at the University of São Paulo School of Medicine *Hospital das Clínicas* Outpatient Clinic for Interstitial Diseases, located in the city of São Paulo, Brazil, were invited to participate in the study. The diagnosis of LAM was made in accordance with the European Respiratory Society guidelines.^(21,22) The study was approved by the local research ethics committee, and all participants gave written informed consent. One LAM patient had undergone lung transplantation and therefore was not enrolled in the trial.

At baseline, the patients underwent full pulmonary function testing, which was followed by a six-minute walk test (6MWT) and health-related quality of life assessment. Urine and blood samples were collected in order to determine the levels of MMP-2, MMP-9, and VEGF-D. After this initial evaluation, the patients were started on doxycycline at a dose of 100 mg/day, which was maintained for at least 12 months. All of the abovementioned tests were repeated at 6 and 12 months after treatment initiation.

The primary endpoints were MMP-2 and MMP-9 blockade, defined as decreased MMP-2 and MMP-9 levels, and the variation in FEV₁ after treatment with doxycycline for 12 months. The secondary endpoints were the variation in FVC, the variation in RV, and the variation in DLCO. We also evaluated the variation in the six-minute walk distance (6MWD), the variation in the minimum SpO₂ (as assessed during the 6MWT), and the variation in serum VEGF-D levels.

Urinary and serum MMP-2 and MMP-9 levels, as well as serum VEGF-D levels, were measured

by ELISA (R&D System, Inc., Minneapolis, MN, USA), in accordance with the manufacturer instructions. The procedure is described in detail in our previous study⁽²⁰⁾ and in the appendix to this article (available online at http://www.jornaldepneumologia.com.br/english/artigo_detalhes.asp?id=1975). The detection limit of the assay was 15.6 pg/mL for MMP-9, 156.2 pg/mL for MMP-2, and 15.6 pg/mL for VEGF-D. Urine and blood samples were also collected from 10 age-matched healthy female nonsmokers.

All patients underwent spirometry, determination of lung volumes, and determination of DLCO with a whole-body plethysmograph (Elite D MedGraphics; Medical Graphics Co., Saint Paul, MN, USA). Pulmonary function testing was performed in accordance with the recommended guidelines for pulmonary function testing in Brazilian adults. The reference values for spirometry, lung volumes, and DLCO were those established for the Brazilian population.⁽²³⁻²⁵⁾

A symptom-limited 6MWT was performed in accordance with the recommended standards.⁽²⁶⁾ A pulse oximeter (WristOx[®] 3100; Nonin Medical, Inc., Plymouth, MN, USA) was used in order to record SpO₂ on room air every 2 s. The data obtained during the 6MWT were analyzed with the nVISION[®] software (Nonin Medical, Inc.) and exported to a Microsoft Excel spreadsheet. The parameters analyzed were the minimum SpO₂ maintained for at least 10 s and the 6MWD, which was expressed in meters and in percentage of the predicted values for the Brazilian population.⁽²⁷⁾

The Medical Outcomes Study 36-item Short-form Health Survey (SF-36) was used in order to assess quality of life, having previously been validated for use in the Brazilian population.^(28,29)

The statistical analysis was performed with the Student's t-test or the Wilcoxon signed rank test for parametric variables and the Mann-Whitney test for nonparametric variables. Fisher's exact test was used for categorical variables. The ROC curve was used in order to identify the FEV₁/FVC ratio that was most accurate in predicting the response to doxycycline treatment.

The data were analyzed with the Statistical Package for the Social Sciences, version 15.0 (SPSS Inc., Chicago, IL, USA), and differences were considered significant if $p < 0.05$.

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Results

Between 2006 and 2009, 41 patients with LAM were enrolled to receive doxycycline (100 mg/day). Of those 41 patients, 10 were lost to follow-up: 5 withdrew from the trial; 2 had worsening of the symptoms; and 3 had drug-related adverse events.

A total of 31 patients completed 12 months of doxycycline treatment (Appendix, section 2, Table A1). Mean age was 43 ± 8 years. In 29 patients, the diagnosis of LAM was based on histopathological features, whereas, in 2, the diagnosis was based on typical clinical and radiological features. The median time from the onset of symptoms to the diagnosis of LAM was 9 months (range, 6-24 months).

In the 31 patients, baseline pulmonary function was characterized by mild obstruction and slightly reduced DLCO (Table 1). After 12 months of doxycycline treatment, there was a significant mean decrease in FEV₁ (70 mL) but no significant variation in DLCO. The results of the 6MWT showed that there was an insignificant (25-m) increase in the mean 6MWD and a reduction in the minimum SpO₂. There was an improvement in some of the SF-36 physical and mental domain scores (Table 1).

As shown in Figure 1A, there was a significant reduction in urinary MMP-9 levels (from 10,487 pg/mL at baseline to 4,061 pg/mL after 12 months of treatment with doxycycline; $p < 0.001$). Serum MMP-2 was also blocked. However, there were no significant differences between pre- and post-treatment serum MMP-9 levels (Figure 1B), and urinary MMP-2 levels were undetectable. All MMP-related data are shown in Table 1.

Blood and urine samples were collected from 10 healthy women (controls), whose mean age was 40 ± 4 years ($p = 0.29$ vs. the mean age of the LAM patients).

Median baseline serum and urinary MMP-9 levels were significantly higher in the LAM patients than in the healthy controls (933 vs. 89.6 ng/mL; $p < 0.0001$, and 10,487 vs. 200 pg/mL; $p < 0.0001$, respectively). Baseline serum and urinary MMP-2 levels were undetectable in the control group, with no significant differences between the controls and the LAM patients in terms of those levels.

Median baseline serum VEGF-D levels were significantly lower in the controls than in the LAM patients (185 vs. 821 pg/mL), in whom

Table 1 – Pulmonary function test results, six-minute walk test results, biomarkers, and Medical Outcomes Study 36-item Short-form Health Survey scores at baseline and after 12 months of treatment with doxycycline in 31 patients with lymphangioleiomyomatosis.^a

Variable	Result		p
	Baseline	After treatment	
	(n = 31)	(n = 31)	
Pulmonary function test			
FVC, L	3.17 ± 0.60	3.19 ± 0.60	0.792
FVC, % of predicted	92 ± 14	93 ± 14	0.751
FEV ₁ , L	2.24 ± 0.70	2.17 ± 0.70	0.034
FEV ₁ , % of predicted	79 ± 23	77 ± 25	0.042
FEV ₁ /FVC ratio	0.70 ± 0.20	0.67 ± 0.20	0.003
TLC, L	5.03 ± 0.80	5.09 ± 0.70	0.408
TLC, % of predicted	103 ± 14	105 ± 14	0.393
RV, L	1.82 ± 0.60	1.91 ± 0.70	0.263
RV, % of predicted	130 ± 47	137 ± 52	0.282
RV/TLC ratio	0.36 ± 0.08	0.37 ± 0.10	0.295
DLCO, mL/min/mmHg	17.0 ± 6.8	16.76 ± 6.0	0.713
DLCO, % of predicted	65 ± 25	64 ± 22	0.745
Six-minute walk test^b			
Distance, m	490 ± 109	515 ± 90	0.132
Distance, % of predicted	90 ± 20	95 ± 17	0.113
Baseline SpO ₂ , % ^c	96 (95-98)	96 (94-97)	0.282
Minimum SpO ₂ , % ^c	94 (87-95)	93 (84-95)	0.020
Biomarkers^c			
Serum MMP-9, ng/mL	933 (730-1,202)	1,076 (809-1,367)	0.140
Urinary MMP-9, pg/mL	10,487 (4,565-20,963)	4,061 (712-9,985)	< 0.001
Serum MMP-2, pg/mL	0 (0-833)	0 (0-179)	0.005
VEGF-D, pg/mL	821 (407-2,113)	913 (313-2,262)	0.590
SF-36 domains^c			
Physical functioning	70 (60-85)	70 (50-88)	0.465
Role-physical	50 (50-50)	75 (25-100)	0.054
Bodily pain	78 (56-95)	70 (58-90)	1.000
General health	75 (48-80)	75 (58-80)	0.717
Vitality	65 (53-80)	65 (55-80)	0.611
Role-emotional	100 (33-100)	100 (67-100)	0.147
Mental health	62 (52-84)	80 (58-84)	0.006
Social functioning	88 (56-94)	88 (63-100)	0.165

Minimum SpO₂: minimum SpO₂ sustained for 10 s; MMP: matrix metalloproteinase; and SF-36: Medical Outcomes Study 36-item Short-form Health Survey. ^aValues expressed as mean ± SD, except where otherwise indicated. ^bOne patient did not perform the six-minute walk test after the 12-month treatment. ^cValues expressed as median (interquartile range).

serum VEGF-D levels remained stable over the course of doxycycline treatment (Figure 1C).

Although all 31 patients showed a decrease in mean FEV₁ after doxycycline treatment, a subset of those patients showed an increase in or a stabilization of FEV₁. The 31 patients were divided into two groups on the basis of the variation in FEV₁ (from baseline to doxycycline treatment month 12): the doxycycline-responder (doxy-R) group, which comprised 13 patients with increased or stable FEV₁; and the doxycycline-

nonresponder (doxy-NR) group, which comprised 18 patients with decreased FEV₁ (Figure 2A).

The functional parameters, SF-36 scores, MMP levels, and VEGF-D levels in the doxy-R and doxy-NR groups are shown in Tables 2 and 3. The patients in the doxy-NR group showed an obstructive pattern, with air trapping, whereas those in the doxy-R group did not. The median variation in FEV₁ (in mL) from baseline to treatment month 12 was calculated. There was a median increase of 70 mL (range, 30-110 mL) in the

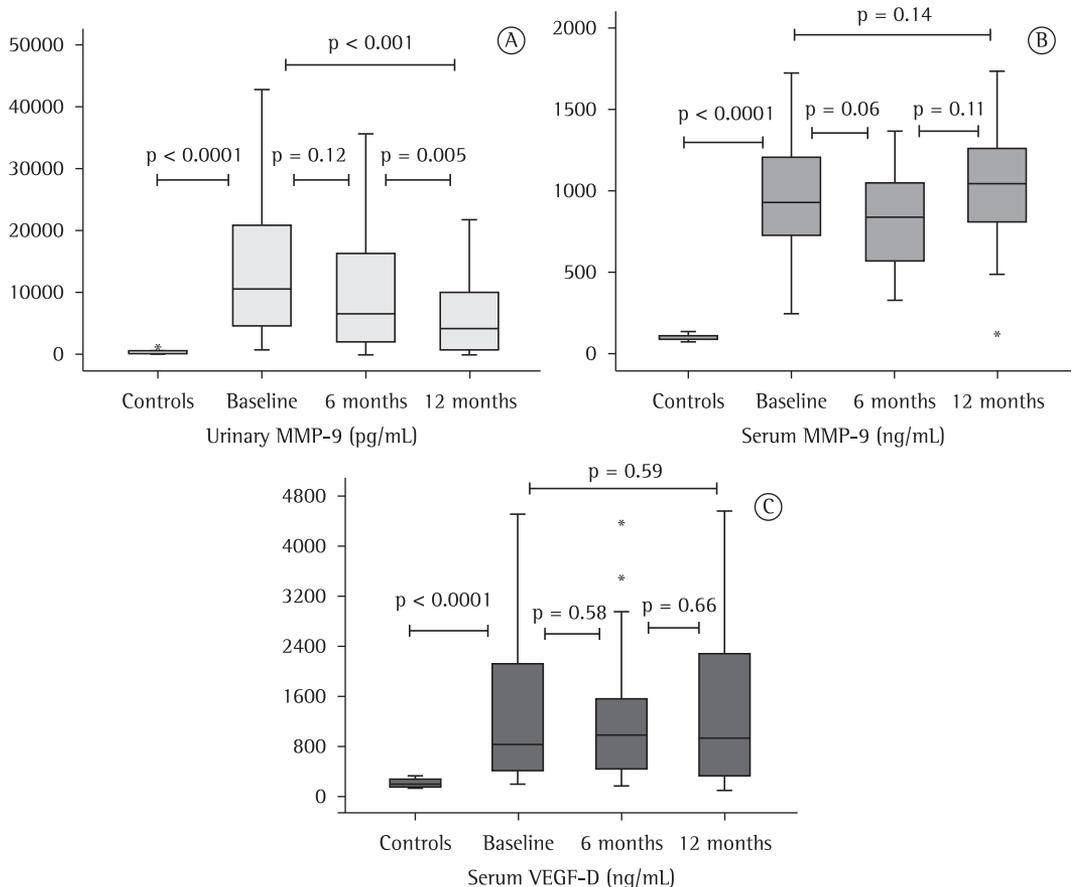


Figure 1 - Urinary matrix metalloproteinase (MMP)-9 levels (in A), serum MMP-9 levels (in B), and serum VEGF-D levels (in C) in 31 lymphangioleiomyomatosis patients treated with doxycycline for 12 months in comparison with those in 10 healthy controls.

doxy-R group and a median decrease of -140 mL (range, -260 to -110) in the doxy-NR group. The median variation in FVC in both groups followed the same trend as that of the variation in FEV₁, with significant differences between the two groups ($p = 0.005$; Figure 2B). Functional impairment was greater in the doxy-NR group, as were MMP and VEGF-D levels (Tables 2 and 3).

After treatment with doxycycline, the patients in the doxy-R group showed an improvement in the SF-36 scores, the social functioning and role-physical domain scores being significantly higher. The patients in the doxy-NR group showed an improvement, although not significant, in the physical and mental domain scores. The SF-36 scores are shown in Tables 2 and 3.

In order to evaluate functional markers of response to doxycycline, we compared the doxy-R and doxy-NR groups before treatment initiation and found that the patients with less

severe disease (lower TLC and milder obstruction) responded better to doxycycline (Appendix, section 2, Table A2). The ROC curve analysis showed that the FEV₁/FVC ratio that was most accurate in predicting the response to treatment with doxycycline was 0.71 (area under the curve = 0.690; 95% CI: 0.50-0.88; $p = 0.07$; Appendix, section 2, Figure A1, panel A).

Of the 31 patients enrolled in the trial, 22 had previously received some type of hormonal blockade therapy. However, there was no significant difference between the doxy-R and doxy-NR groups in terms of the prevalence of previous hormonal blockade therapy ($p = 0.696$; Appendix, section 2, Table A3).

Twenty patients continued to receive doxycycline for a median time of 18 months after the end of the study period. Regarding the variation in FEV₁ after the end of the study, the patients in the doxy-R group showed a slight

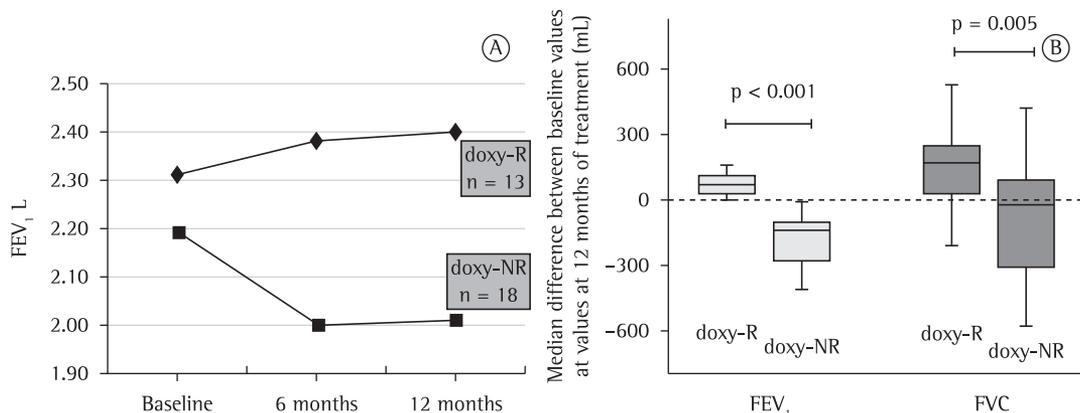


Figure 2 – In A, variation in FEV₁ over the course of 12 months of treatment with doxycycline in the lymphangioleiomyomatosis patients who responded to the drug—the doxycycline-responder (doxy-R) group—and in those who did not—the doxycycline-nonresponder (doxy-NR) group. In B, median difference between FEV₁ at baseline and FEV₁ at 12 months of doxycycline treatment and between FVC at baseline and FVC at 12 months of doxycycline treatment in the doxy-R and doxy-NR groups. The doxy-R group comprised the lymphangioleiomyomatosis patients who responded to treatment with doxycycline, as evidenced by increased or stable FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline. The doxy-NR group comprised the lymphangioleiomyomatosis patients who did not respond to treatment with doxycycline, as evidenced by decreased FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline.

reduction in FEV₁, whereas those in the doxy-NR group showed a sizeable reduction, the variation in FEV₁ after the end of the study having followed the same trend as that of the variation in FEV₁ during the study period (Appendix, section 2, Figure A1, panel B).

The most common adverse events were epigastric pain (45%), nausea (19%), diarrhea (16%), and itching (6%). They were mild and self-limiting, specific treatment being rarely required. Of the 41 patients enrolled in the trial, 3 (7%) had adverse events requiring doxycycline discontinuation. Of those 3 patients, 1 had acute colitis, 1 had chronic diarrhea, and 1 had hemorrhoids.

Discussion

To the best of our knowledge, the present study is the first to demonstrate the effects of doxycycline, a known MMP inhibitor, on serum MMP levels, urinary MMP levels, and serum VEGF-D levels, as well as on pulmonary function test results and quality of life, in a cohort of LAM patients. The main findings of the present study include effective urinary MMP-9 and serum MMP-2 blockade induced by a 12-month treatment with doxycycline, without a significant influence on VEGF-D levels. We believe that treatment with doxycycline can be beneficial for patients with less

severe functional impairment and that doxycycline is safe for patients with LAM.

One of the factors likely involved in cystic lung destruction in LAM is MMP overexpression. It is known that MMPs are enzymes that are capable of degrading collagen and elastin in the extracellular matrix of lung tissue.⁽¹²⁾ Chang et al. demonstrated the in vitro effect of doxycycline on TSC2-null cell adhesion, as well as on MMP-2 and MMP-9 expression, revealing a reduction in LAM cell proliferation, although high doses of doxycycline were required and there was no significant blockade of MMP expression.⁽³⁰⁾ They also suggested that the antiproliferative effects of doxycycline are unlikely to be directly due to MMP inhibition, given that cell proliferation was not inhibited by ilomastat, a potent MMP inhibitor.⁽³⁰⁾ However, Moir et al. demonstrated that doxycycline significantly reduced MMP-2 levels in LAM-derived cells and in TSC2-null mouse embryonic fibroblasts.⁽³¹⁾

Doxycycline has been described as an MMP blocker in previous studies of other diseases, being able to suppress cerebral MMP-9 expression and angiogenesis in a mouse model and to reduce MMP-2 and MMP-9 levels to prevent thoracic aortic aneurysms.^(32,33)

In the present study, doxycycline was found to induce effective MMP blockade, a finding

Table 2 – Pre- and post-doxycycline functional parameters, matrix metalloproteinase levels, VEGF-D levels, and Medical Outcomes Study 36-item Short-form Health Survey scores in the 13 lymphangioliomyomatosis patients allocated to the doxycycline-responder group^a on the basis of their response to doxycycline (as determined by the variation in FEV₁).^b

Variable	Doxy-R group		p
	Pre-doxycycline	Post-doxycycline	
Pulmonary function test			
FVC, L	3.0 ± 0.3	3.2 ± 0.3	0.023
FVC, % of predicted	90 ± 13	95 ± 12	0.026
FEV ₁ , L	2.31 ± 0.40	2.40 ± 0.40	0.003
FEV ₁ , % of predicted	84 ± 14	86 ± 14	0.002
FEV ₁ /FVC ratio	0.77 ± 0.10	0.76 ± 0.10	0.173
TLC, L	4.6 ± 0.5	4.8 ± 0.6	0.310
TLC, % of predicted	98 ± 13	101 ± 15	0.327
RV, L	1.6 ± 0.5	1.6 ± 0.6	0.956
RV, % of predicted	117 ± 43	118 ± 47	0.940
RV/TLC ratio	0.34 ± 0.08	0.33 ± 0.08	0.640
DLCO, mL/min/mmHg	18.5 ± 6.0	18.0 ± 5.0	0.514
DLCO, % of predicted	72 ± 20	70 ± 18	0.448
Six-minute walk test			
Distance, m	515 ± 66	523 ± 52	0.633
Minimum SpO ₂ , % ^c	94 (93-95)	94 (91-95)	0.500
Biomarkers^c			
Serum MMP-9, ng/mL	837 (716-1,033)	1,103 (773-1,237)	0.328
Urinary MMP-9, pg/mL	8,905 (5,607-21,829)	6,735 (674-8,983)	0.050
Serum MMP-2, pg/mL	0 (0-182)	0 (0-40)	0.144
VEGF-D, pg/mL	413 (277-2,113)	328 (257-2,008)	0.374
SF-36 domains^c			
Physical functioning	75 (65-85)	80 (75-95)	0.281
Role-physical	50 (50-50)	100 (50-100)	0.020
Bodily pain	57.5 (55-90)	70 (55-77.5)	1.000
General health	80 (45-90)	75 (70-85)	0.412
Vitality	70 (45-90)	70 (70-85)	0.428
Role-emotional	33 (33-100)	100 (33-100)	0.158
Mental health	68 (40-84)	76 (52-88)	0.051
Social functioning	75.0 (50.0-88.5)	87.5 (62.5-100)	0.040

Doxy-R group: doxycycline-responder group; minimum SpO₂: minimum SpO₂ sustained for 10 s; MMP: matrix metalloproteinase; and SF-36: Medical Outcomes Study 36-item Short-form Health Survey. ^aGroup of patients who responded to treatment with doxycycline, as evidenced by increased or stable FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline. ^bValues expressed as mean ± SD, except where otherwise indicated. ^cValues expressed as median (interquartile range).

that corroborates our previous findings in a slightly larger cohort of patients.⁽²⁰⁾ However, there was no association between MMP blockade and functional improvement. We also found a reduction in MMP levels in the doxy-R and doxy-NR groups, a finding that is suggestive of an MMP-independent mechanism of action for doxycycline. However, we tested only MMP-2 and MMP-9 and did not explore the possibility of involvement of other MMPs, such as MMP-1

and MMP-8, or the inhibition of TIMP activity, as previously described.^(34,35)

Serum VEGF-D levels remained stable after doxycycline treatment (Figure 1A). Although vascular permeability and angiogenesis inhibition^(36,37) have been reported to be induced by doxycycline, the pathway seems to be MMP-independent. Although median baseline VEGF-D levels were found to be higher in the patients in the doxy-NR group than in those in

Table 3 – Pre- and post-doxycycline functional parameters, matrix metalloproteinase levels, VEGF-D levels, and Medical Outcomes Study 36-item Short-form Health Survey scores in the 18 lymphangiomyomatosis patients allocated to the doxycycline-nonresponder group^a on the basis of their lack of response to doxycycline (as determined by the variation in FEV₁).^b

Variable	Doxy-NR group		p
	Pre-doxycycline	Post-doxycycline	
Pulmonary function test			
FVC, L	3.2 ± 0.7	3.2 ± 0.7	0.114
FVC, % of predicted	94 ± 15	91 ± 16	0.151
FEV ₁ , L	2.2 ± 0.9	2 ± 0.9	< 0.001
FEV ₁ , % of predicted	75 ± 28	70 ± 28	< 0.001
FEV ₁ /FVC ratio	0.65 ± 0.2	0.61 ± 0.2	0.009
TLC, L	5.3 ± 0.8	5.3 ± 0.7	0.922
TLC, % of predicted	107 ± 13	107 ± 12	0.844
RV, L	2 ± 0.6	2.1 ± 0.7	0.129
RV, % of predicted	140 ± 49	150 ± 54	0.155
RV/TLC ratio	0.37 ± 0.08	0.4 ± 0.11	0.032
DLCO, mL/min/mmHg	15.9 ± 7.5	15.8 ± 6.6	0.967
DLCO, % of predicted	60 ± 27	60 ± 24	0.948
Six-minute walk test			
Distance, m	472 ± 130	509 ± 112	0.119
Minimum SpO ₂ , % ^c	92.5 (83-95)	92 (78-95)	0.087
Biomarkers^c			
Serum MMP-9, ng/mL	1,011 (748-1,272)	1,042 (809-1,307)	0.756
Urinary MMP-9, pg/mL	10,487 (4,526-15,653)	3,970 (765-9,985)	0.001
Serum MMP-2, pg/mL	188 (0-905)	0 (0-214)	0.017
VEGF-D, pg/mL	995 (535-2,000)	1,124 (810-2,262)	0.211
SF-36 domains^c			
Physical functioning	70 (51-84)	62.5 (46-81)	0.084
Role-physical	50 (50-50)	50 (25-100)	0.668
Bodily pain	79.0 (60.0-97.5)	67.5 (57.5-90.0)	0.821
General health	70 (51-80)	67.5 (50-80)	0.905
Vitality	63 (55-79)	65 (55-80)	0.954
Role-emotional	100 (33-100)	100 (67-100)	0.426
Mental health	66 (57-83)	80 (63-84)	0.053
Social functioning	87.5 (75.0-97.0)	87.5 (62.5-97.0)	1.000

Doxy-NR group: doxycycline-nonresponder group; minimum SpO₂: minimum SpO₂ sustained for 10 s; MMP: matrix metalloproteinase; and SF-36: Medical Outcomes Study 36-item Short-form Health Survey. ^aGroup of patients who did not respond to treatment with doxycycline, as evidenced by decreased FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline. ^bValues expressed as mean ± SD, except where otherwise indicated. ^cValues expressed as median (interquartile range).

the doxy-R group (995 ng/mL vs. 413 ng/mL), the difference was not significant ($p = 0.325$). These findings are concordant with those of previous studies that found no association between VEGF-D levels and lung function impairment.⁽³⁸⁻⁴⁰⁾

After 12 months of treatment with doxycycline, we found a mean decrease of 70 mL in FEV₁ and a mean decrease of 0.24 mL/min/mmHg in DLCO. In comparison with the annual rates of decline in FEV₁ reported by Taveira et al.⁽⁸⁾

and Johnson & Tattersfield,⁽⁴¹⁾ i.e., 75 mL and 118 mL, respectively, the rate of decline in FEV₁ in the present study was slightly lower, although we found that doxycycline had no impact on pulmonary function decline. The rate of DLCO decline in our patients was also lower than were those reported in the two studies cited above (0.69 and 0.90 mL/min/mmHg, respectively). In the present study, the patients in the doxy-R group showed no decline in FEV₁, having shown

a slight but significant increase in FVC and FEV₁ and stable SpO₂ during the 6MWT. However, the doxy-NR group showed a decrease in FEV₁ and in the FEV₁/FVC ratio, as well as showing an increase in the RV/TLC ratio. By comparing the doxy-R and doxy-NR groups (Appendix, section 2, Table A2), we found that doxycycline can be beneficial for patients with mild spirometric abnormalities. This finding was supported by the ROC curve analysis, which showed that the FEV₁/FVC ratio that was most accurate in predicting the response to doxycycline was 0.71. In addition, because the prevalence of hormonal blockade was similar between the doxy-R and doxy-NR groups, we hypothesized that hormonal therapy has no influence on the response to doxycycline.

Twenty patients continued to receive doxycycline after the end of the 12-month study period. An evaluation performed after the end of the study period revealed differences between the two groups in terms of the rate of FEV₁ decline. This finding suggests that doxycycline is more effective in slowing functional impairment in patients with LAM that is less severe (Appendix, section 2, Figure A1, panel B).

The LAM patients investigated in the present study underwent health-related quality of life assessment before and after treatment with doxycycline, an assessment that was not performed in previous studies. We found an improvement in some of the physical and mental domain scores after treatment with doxycycline, a finding that was more prominent in the patients in the doxy-R group.

Doxycycline was found to be safe and well-tolerated. The most common side effects were those affecting the gastrointestinal tract and were self-limiting.

Our study has some limitations. Because the trial was not placebo-controlled, the fact that we included patients with less severe forms of LAM can be considered a bias, which could account for the beneficial effect of doxycycline in the doxy-R group patients, because those patients had less functional impairment at baseline. Another potential limitation is the fact that more than 10% of the patients enrolled in the trial were lost to follow-up. In addition, we did not assess MMPs other than MMP-2 and MMP-9, and we did not assess TIMPs. Furthermore, we tested doxycycline at only one dose level.

In summary, the present study showed that, in patients with LAM, doxycycline treatment resulted in effective MMP blockade. Doxycycline treatment also resulted in improved or stable lung function and in improved quality of life, particularly in the LAM patients with mild spirometric abnormalities. However, these benefits do not seem to be related to the blockade of MMPs, which raises the hypothesis of a different mechanism of action. Because the lack of a placebo arm limits the analysis of the clinical benefits of doxycycline, randomized placebo-controlled studies are needed in order to establish the actual role of doxycycline in the treatment of patients with LAM.

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About the authors

Suzana Pinheiro Pimenta

Pulmonologist. A. C. Camargo Hospital, São Paulo, Brazil.

Bruno Guedes Baldi

Attending Physician. Pulmonary Division, Heart Institute (InCor), University of Sao Paulo Medical School, São Paulo, Brazil.

Ronaldo Adib Kairalla

Assistant Professor. Pulmonary Division, Heart Institute (InCor), University of Sao Paulo Medical School, São Paulo, Brazil.

Carlos Roberto Ribeiro Carvalho

Tenured Associate Professor. Pulmonary Division, Heart Institute (InCor), University of Sao Paulo Medical School, São Paulo, Brazil.

APPENDIX

Section 1 - Additional methodological details

Urinary matrix metalloproteinase (MMP)-2 levels, serum MMP-9 levels, and serum VEGF-D levels were measured by ELISA (R&D System, Inc., Minneapolis, MN, USA), according to the specific antibody binding.

Samples were centrifuged at 1,500 rpm for 10 min and stored at -80°C . The MMP levels were determined by optical density comparison in an ELISA reader (Power Wave; Bio-Tek Instruments Inc., Winooski, VT, USA) with a 450-nm filter. The detection limit of the assay was 15.6 pg/mL for MMP-9, 156.2 pg/mL for MMP-2, and 15.6 pg/mL for VEGF-D.

Section 2 - Additional results

Table A1 - Baseline characteristics of the 31 lymphangioleiomyomatosis patients who completed the study, as well as pulmonary function test results and biomarkers in those patients.^a

Variable	Result
Age, years	43 \pm 8
Tuberous sclerosis ^b	4 (13)
Biopsy-proven diagnosis ^b	29 (94)
Hormonal blockade ^b	
GnRH analogue	14 (45)
Oophorectomy	4 (13)
Menopause	4 (13)
Pulmonary function test	
FVC, L	3.2 \pm 0.6
FVC, % of predicted	92 \pm 1.4
FEV ₁ , L	2.2 \pm 0.7
FEV ₁ , % of predicted	79 \pm 23
FEV ₁ /FVC ratio	0.7 \pm 0.2
TLC, L	5.0 \pm 0.8
TLC, % of predicted	103 \pm 14
RV, L	1.8 \pm 0.6
RV, % of predicted	130 \pm 47
RV/TLC ratio	0.36 \pm 0.08
DLCO, mL/min/mmHg	17.0 \pm 6.8
DLCO, % of predicted	65 \pm 25
Six-minute walk test	
Distance, m	490 \pm 109
Distance, % of predicted	90 \pm 20
Baseline SpO ₂ , % ^c	96 (95-98)
Minimum SpO ₂ , % ^c	94 (87-95)
Biomarkers ^{c,d}	
Serum MMP-9, ng/mL	933 (730-1,202)
Urinary MMP-9, pg/mL	10,487 (4,565-20,963)
Serum MMP-2, pg/mL	0 (0-833)
VEGF-D, pg/mL	821 (407-2,113)

GnRH: gonadotropin-releasing hormone; minimum SpO₂: minimum SpO₂ sustained for 10 s; and MMP: matrix metalloproteinase. ^aValues expressed as mean \pm SD, except where otherwise indicated. ^bValues expressed as n (%). ^cValues expressed as median (interquartile range). ^dTwo patients provided no blood or urine samples.

Table A2 – Comparison between the lymphangioliomyomatosis patients in the doxycycline-responder group and those in the doxycycline-nonresponder group in terms of baseline markers of functional response to doxycycline.^a

Variable	Group		p
	doxy-R	doxy-NR	
	(n = 13)	(n = 18)	
FVC, L	3 ± 0.3	3.2 ± 0.7	0.120
FVC, % of predicted	90 ± 13	94 ± 15	0.520
FEV ₁ /FVC ratio	0.77 ± 0.10	0.65 ± 0.20	0.031
TLC, L	4.6 ± 0.5	5.3 ± 0.8	0.006
TLC, % of predicted	98 ± 13	107 ± 13	0.720
RV, L	1.6 ± 0.5	2.0 ± 0.6	0.087
RV, % of predicted	117 ± 43	140 ± 49	0.201
RV/TLC ratio	0.34 ± 0.08	0.37 ± 0.08	0.382
DLCO, mL/min/mmHg	18.5 ± 6.0	15.9 ± 7.5	0.297
DLCO, % of predicted	72 ± 20	60 ± 27	0.198

Doxy-R: doxycycline-responder; and doxy-NR: doxycycline-nonresponder. ^aValues expressed as mean ± SD.

Table A3 – Comparison between the lymphangioliomyomatosis patients in the doxycycline-responder group and those in the doxycycline-nonresponder group in terms of the prevalence of hormonal blockade therapy before the treatment with doxycycline.^a

Hormonal blockade	Groups				Total		p*
	doxy-R		doxy-NR		n	%	
	n	%	n	%			
No	3	33	6	67	9	29	0.696
Yes	10	45	12	55	22	71	
GnRH analogue	6		8				
Oophorectomy	2		2				
Menopause	2		2				

Doxy-R: doxycycline-responder; doxy-NR: doxycycline-nonresponder; and GnRH: gonadotropin-releasing hormone.

^aValues expressed as n (%). *Fisher's exact test.

Table A4 – Doxycycline-related adverse events during the study period.^a

Adverse event	Baseline	Post-treatment
	(n = 41)	(n = 31)
Nausea	10 (24)	6 (19)
Epigastric pain	20 (49)	14 (45)
Diarrhea	8 (20)	5 (16)
Vomiting	1(2)	0 (0)
Itching	2 (5)	2 (6)
Chest pain	1 (2)	1 (3)
Photosensitivity reaction	1 (2)	1 (3)
Maculopapular rash	2 (5)	2 (6)

^aValues expressed as n (%).

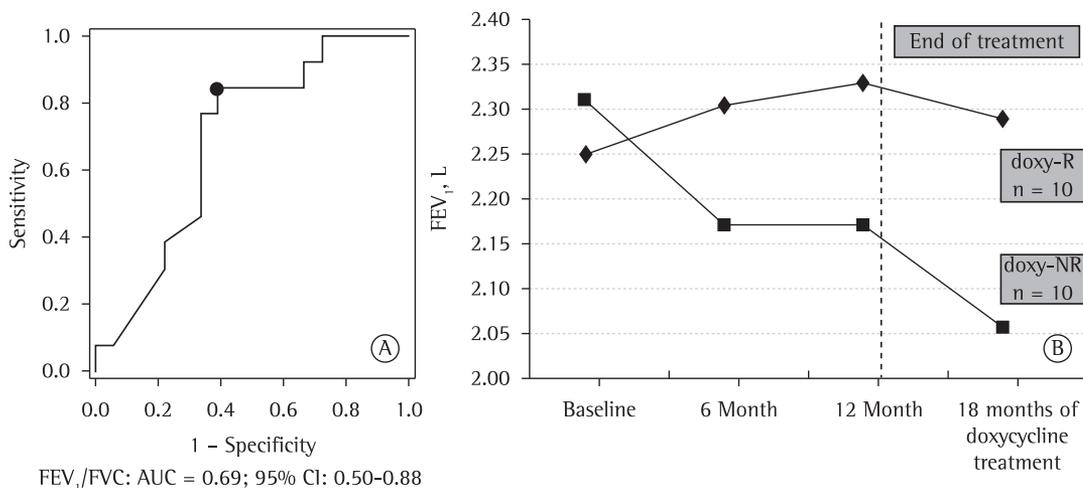


Figure A1 - ROC curve and FEV₁ follow-up. In A, ROC curve for predicting the response to doxycycline treatment. The solid dot indicates the cut-off point for the FEV₁/FVC ratio that was most accurate in predicting the response to treatment (0.71). In B, FEV₁ follow-up of 20 lymphangioleiomyomatosis patients who continued to use doxycycline for a median time of 18 months after the end of the 12-month study period. The patients in the doxycycline-responder (doxy-R) group showed a slight reduction in FEV₁ (from 2.33 L to 2.29 L), whereas those in the doxycycline-nonresponder (doxy-NR) group showed a sizeable decline in FEV₁ (from 2.17 L to 2.06 L). AUC: area under the curve. The doxy-R group comprised the lymphangioleiomyomatosis patients who responded to treatment with doxycycline, as evidenced by increased or stable FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline. The doxy-NR group comprised the lymphangioleiomyomatosis patients who did not respond to treatment with doxycycline, as evidenced by decreased FEV₁ at doxycycline treatment month 12 in comparison with FEV₁ at baseline.