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All authors declare no conflicts of interest and no competing interests.

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Take home message: Airway hyperreactivity increases with sirolimus treatment in LAM patients with moderate disease, despite LAM disease stabilization. A possible explanation for the increase in airway hyperreactivity is stimulation of the ryanodine receptor by sirolimus.
To the Editor:

Lymphangioleiomyomatosis (LAM) is a multisystem disease affecting primarily women, characterized in the lung by proliferation of LAM cells, abnormal smooth muscle-like cells with dysfunctional tuberous sclerosis complex genes. This dysfunction results in activation of mechanistic target of rapamycin (mTOR), leading to LAM cell proliferation. Sirolimus (rapamycin) is the only FDA-approved treatment for pulmonary LAM, resulting in decreased LAM cell growth/size and stabilized lung function [1].

LAM is characterized by lung destruction, with cysts lined by nodules containing LAM cells and immune cells. Regions of the basement membrane are disrupted, with loss of lung parenchyma and gas exchange impairment [1]. LAM patients may exhibit partially reversible airflow obstruction [2], and patients with more LAM nodules have higher frequencies of bronchodilator response (BDR), suggesting that airflow obstruction results from an increase in airways resistance due to the proliferation of LAM cells surrounding the airways [2]. Patients with advanced stage LAM had bronchi showing LAM cell infiltration [3]. A greater frequency of BDR was a clinical marker of worse lung function and a greater rate of decline in FEV\textsubscript{1} and DL\textsubscript{CO} in LAM patients without sirolimus treatment [2].

Airway hyperresponsiveness may stem from increased airway smooth muscle (ASM) (i.e., LAM cell growth and proliferation)[2], an increase in contractile mediators (i.e., mast cell products [4]), and/or increases in intracellular calcium that enhance airway smooth muscle contractility (i.e., ryanodine receptor activation) amongst other factors [5, 6]. Since sirolimus stabilizes LAM disease, we predicted that BDR frequency in patients receiving sirolimus would decrease as compared to BDR frequency of patients pretreatment, and thus be indicative of treatment success.

To test this idea, we examined the BDR of 133 patients (105 with both before and during sirolimus treatment visits, 28 with visits only during treatment), with 1528 visits. Patients were diagnosed based on clinical, radiological, physiological, and pathological criteria [7, 8], with written informed consent from the National Institutes of Health Institutional Review Board protocols 95-H-0186 and 96-H-0100. BDR was defined as an albuterol-induced increase of FEV\textsubscript{1} of $\geq 12\%$ over baseline. As 48.0\% of visits on sirolimus had a baseline FEV\textsubscript{1} of less than
1.5L, this definition of BDR using a percentage-based difference over baseline as opposed to a volume-based cut-off is warranted, as clinically relevant BDR may be missed in patients with low baseline FEV$_1$ who cannot produce large changes in volume [9].

Unexpectedly, a BDR was seen at 34.9% (310/887) of visits during treatment as compared to 25% (160/641) of pretreatment visits (P<0.001). To determine if this result was due to the association of worse lung function and BDR [2], we defined severe pulmonary disease as having percent-predicted FEV$_1$ or DL$_{CO}$ $\leq$40, with 14.4% of pretreatment and 36.4% of during treatment visits falling into this category. Regardless of sirolimus treatment, the frequency of BDR was increased in those with severe disease as compared to those with normal/mild/moderate disease (P<0.001) (Table 1). However, in those with normal/mild/moderate disease, there was an increase in BDR frequency during sirolimus treatment (27.9%) as compared to pretreatment (21.4%) (P=0.013) that was not seen in those with severe disease (46.7% during treatment versus 45.7% pretreatment). This increase in BDR frequency in those patients with normal/mild/moderate disease during treatment is opposite to our prediction that sirolimus treatment would decrease BDR frequency.

To control for the inherent variability in BDR, we examined BDR in patients over multiple visits, grouping them as never having a BDR, having a BDR $<$50% of the time, or having a BDR $\geq$50% of the time, followed for at least 5 visits. As determined previously, patients without sirolimus treatment were more likely to have BDR when they had worse lung function and also showed a trend to faster decline in pulmonary function as compared to those without BDR [2]. For patients during sirolimus treatment, there was a trend toward worse pulmonary function values in those patients with BDR. Interestingly, patients during sirolimus treatment who showed a BDR more than 50% of the time had significantly slower rates of decline of pulmonary function than those with BDR less than 50% of the time, suggesting that those with more stable disease show more frequent BDR during sirolimus treatment than those with declining disease (Table 1).

When we examine BDR over time, we find that pretreatment, the longer time since the first visit, the higher the probability of BDR (P=0.002), suggesting that BDR frequency increases as disease worsens, as expected. Surprisingly, during treatment, increasing the time on sirolimus decreases the probability of BDR (P<0.001), despite more severe disease. This may be due to the
inability of the destroyed lung to support an increase in FEV$_1$ of 12% upon beta-agonist stimulation. Examination of transplanted lungs by *ex-vivo* computed tomography indicated that LAM lungs have at least a three-fold reduction in airway number, with collapse of airways due to cysts and filling of airways with exudate [10].

The increase in BDR frequency in patients with normal/mild/moderate disease during sirolimus treatment as compared to the BDR frequency in the same patients before treatment is unexpected as treatment would be expected to slow LAM disease, relieving airway obstruction [11]. A possible explanation may be activation of the ryanodine receptor [12], a channel responsible for changes in calcium concentrations in the cell. It is stabilized in a closed position by FKBP12. Sirolimus binds to FKBP12 and removes it from the ryanodine receptor, resulting in its activation. Stimulation of the ryanodine receptor in normal cells by sirolimus may explain some side effects seen with sirolimus treatment [1], since the ryanodine receptor is linked to airway hyperreactivity, lymphedema, and hypertension [12-14], as airway smooth muscle, lymphatic, and endothelial cells express ryanodine receptors. We immunostained LAM lung tissue from three patients with polyclonal antibodies to FKBP12 and ryanodine receptor type 2 (RyR2) using an antigen retrieval procedure and peroxidase/DAB method (data not shown). Proliferative LAM cells, epithelial cells from bronchioles, and alveoli were immunoreactive with anti-FKBP12 antibodies. RyR2 was present in alveoli, LAM lung nodules, bronchioles, and endothelial cells. Interestingly, RyR2 was detected in LAM cells, in addition to its expected presence in normal cells. The effect of ryanodine receptor activation in LAM cells warrants further investigation.

In patients before treatment with sirolimus, mTOR would be active and the FKBP12/ryanodine receptor complex would be stabilized in a closed state, suggesting that airway hyperreactivity is due to LAM cell hypertrophy/hyperplasia or other factors, such as mast cell recruitment [4]. When patients are treated with sirolimus, FKBP12/sirolimus/mTOR would be inactive and the ryanodine receptor would be in an open state, suggesting that airway hyperreactivity is due to increases in intracellular free Ca$^{2+}$ in normal airway cells. Thus, in LAM, the clinical marker of a BDR during sirolimus treatment may not be indicative of worsening disease, but may be indicative of adverse events due to ryanodine receptor activation in non-targeted normal cells. Ryanodine receptor activation by sirolimus explains airway
hyperreactivity seen with treatment via effects on normal cells, while also allowing for LAM
disease stabilization by sirolimus. Patients with BDRs may be treated with steroids; in the case of
those with sirolimus treatment, perhaps therapy targeting the ryanodine receptor may be a better
choice.

Acknowledgements

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National Heart, Lung, and Blood Institute.
Table 1: Frequency of BDR seen at visits either pretreatment or during sirolimus treatment for patients with normal/mild/moderate lung disease or severe lung disease, with severe disease defined as either percent-predicted FEV$_1$ or DL$_{CO}$ ≤40, and BDR frequency, average percent-predicted pulmonary function values, and rates of change in percent-predicted FEV$_1$ or DL$_{CO}$ for patients with at least 5 visits before and during treatment. Repeated measurements of PFTs were analyzed using mixed-effects models. Analysis of each variable (FEV$_1$, DL$_{CO}$), was adjusted for its initial value, time of visit, and sirolimus treatment. Data analysis was performed with SAS 9.3.

<table>
<thead>
<tr>
<th>BDR$^a$</th>
<th>pretreatment</th>
<th>during sirolimus treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal/mild/moderate lung disease</td>
<td>severe lung disease</td>
</tr>
<tr>
<td>number of visits (%)</td>
<td>number of visits (%)</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>431 (78.6)</td>
<td>50 (54.3)</td>
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<tr>
<td>yes</td>
<td>118 (21.4)</td>
<td>42 (45.7)$^b$</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>BDR</th>
<th>pretreatment</th>
<th>during sirolimus treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of patients$^d$</td>
<td>%</td>
</tr>
<tr>
<td>never</td>
<td>15</td>
<td>33.3</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>19</td>
<td>42.2</td>
</tr>
<tr>
<td>≥50%</td>
<td>11</td>
<td>24.4</td>
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</table>

<table>
<thead>
<tr>
<th>BDR</th>
<th>FEV$_1$</th>
<th>DL$_{CO}$</th>
<th>FEV$_1$</th>
<th>DL$_{CO}$</th>
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<tbody>
<tr>
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<td>84.4</td>
<td>75.8</td>
<td>56.5</td>
<td>46.9</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>70.7$^e$</td>
<td>64.0$^e$</td>
<td>55.2</td>
<td>47.2</td>
</tr>
<tr>
<td>≥50%</td>
<td>72.4$^e$</td>
<td>67.5</td>
<td>51.9</td>
<td>43.5</td>
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</table>

Average percent-predicted

<table>
<thead>
<tr>
<th>Rate of change (percent-predicted/year)</th>
<th>BDR</th>
<th>FEV$_1$</th>
<th>DL$_{CO}$</th>
<th>FEV$_1$</th>
<th>DL$_{CO}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>never</td>
<td>-2.01</td>
<td>-1.94</td>
<td>-0.55</td>
<td>-0.71</td>
<td></td>
</tr>
<tr>
<td>&lt;50%</td>
<td>-2.37</td>
<td>-2.45</td>
<td>-0.98</td>
<td>-1.33</td>
<td></td>
</tr>
<tr>
<td>≥50%</td>
<td>-2.16</td>
<td>-2.41</td>
<td>0.07$^f$</td>
<td>-0.50$^f$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Flow rates were measured before and after nebulization with 2.5mg albuterol. An increase in FEV$_1$ of ≥12% over baseline was considered a positive response.

$^b$BDR frequency increases with severe disease regardless of sirolimus treatment (P<0.001 for both pretreatment normal/mild/moderate disease versus severe lung disease and during treatment normal/mild/moderate disease versus severe lung disease).

$^c$Patients with normal/mild/moderate lung disease show an increase in BDR frequency with sirolimus treatment (P=0.013).
133 patients: 132 female, 1 male; 10 Asian, 3 African-American, 1 Hawaiian/Pacific Islander, 4 multiple race (2 Hispanic/Latino), 5 unknown race (4 Hispanic/Latino), 110 Caucasian (6 Hispanic/Latino); 110 sporadic LAM, 22 LAM/TSC, 1 LAM with questionable TSC status; mean age at start of study: 42.0±0.8 years; mean age at start of sirolimus treatment: 46.8±0.9 years; mean percent-predicted FEV1 and DLCO at the first pretreatment visit: 79.0±2.3 and 70.3±2.2, respectively; mean percent-predicted FEV1 and DLCO at the start of sirolimus treatment: 67.7±2.0 and 59.5±1.9, respectively.

Average percent-predicted pulmonary function test (PFT) value is significantly different (p<0.005) as compared to that in the “never” category.

Rate of change is significantly different comparing that of <50% to ≥50% (p<0.001).
References

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FKBP12 and RyR2 are present in LAM lung nodules and bronchioles. Images are representative of results from 3 patients. **Top** panels show the tissue architecture of tissue sections from explanted lungs of patients with LAM (H&E stained). **Middle** panels show proliferative LAM cells immunoreactive with anti-FKBP12 polyclonal antibodies (Invitrogen). Inset is the negative control for antibodies using a non-specific rabbit antibody. Epithelial cells from bronchioles are also immunoreactive to anti-FKBP12 antibodies. Alveoli present a reactivity to anti-FKBP12 as well. **Bottom** panels, RyR2 is present in LAM lung nodules, bronchioles, and endothelial cells. Alveoli reacted to the anti-RyR2 polyclonal antibodies (Invitrogen). Bar in all the panels represents 100 μm. Tissue sections were stained using an antigen retrieval procedure and peroxidase/DAB method.