Protocol Title: A Pilot Clinical Trial of COX-2 inhibition in LAM and TSC

Version Date: 3 Apr 2017

NCT #: 02484664

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A Pilot Clinical Trial of COX-2 inhibition in LAM and TSC

Abbreviated Protocol Title: COLA Trial

Identifying words: LAM, COX-2, Celecoxib, Circulating LAM cells

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Duration of study: 2 years

Number and type of subjects: 12 adult female subjects diagnosed with lymphangioleiomyomatosis

Ionizing radiation: No

Project uses IND/IDE: IND/IDE Exempt- Exemption letter attached

Multi-Institutional Project: Yes

Participating Institutions: Brigham and Women's Hospital, Boston, MA; Division of Intramural Research National Heart, Lung, and Blood Institute, Bethesda, MD.

I- Background and significance

A- Overview

Lymphangioleiomyomatosis (LAM) is a progressive pulmonary disease which affects almost exclusively women. LAM is characterized pathologically by proliferation of abnormal smooth muscle cells in nodules in the lung parenchyma, which typically have mutations in *TSC2*, and by cystic changes within the lung parenchyma (McCormack and Henske 2010, Taveira-DaSilva et al. 2010, Henske and McCormack 2012). Cystic lung changes are seen by chest CT scans in up to 80% of women with TSC of age > 40 years (Cudzilo et al. 2013). LAM is also commonly diagnosed in women who do not have clinical features of TSC or germ line mutations in *TSC1* or *TSC2*, and is called sporadic LAM. Inactivating mutations of both alleles of either *TSC1* or *TSC2* have been found in LAM cells from both TSC-LAM and sporadic LAM patients (Carsillo et al. 2000, Badri et al. 2013). Approximately 60% of women with the sporadic form of LAM also have renal angiomyolipoma (McCormack and Henske 2010). The presence of the same *TSC2* mutation in LAM cells and renal angiomyolipoma cells from women with sporadic LAM, but not in normal tissues, indicates that they are part of the same clonal neoplasm (Carsillo et al. 2000). This has led to the model that LAM is a systemic disease with neoplastic cells that spread to the lungs via a metastatic mechanism (Crino et al. 2006, Henske and McCormack 2012). 63% of TSC LAM patients defined radiographically developed pulmonary symptoms, and 12.5% died from LAM in a recent large series (Cudzilo et al. 2013). Thus, LAM is a major contributor to morbidity and mortality in the TSC population.

The protein products of *TSC1* and *TSC2*, hamartin and tuberin, respectively, along with the TBC1D7 protein form a protein complex called the TSC complex that has GTPase activity toward the small GTPase Rheb (Ras homologue enriched in brain) (Dibble and Manning 2013). Loss of tuberin or hamartin inactivates the TSC protein complex, and leads to constitutive high levels of Rheb-GTP, which leads to hyperactivation of the mammalian target of Rapamycin complex 1 (mTORC1) (Kwiatkowski and Manning 2005, Dibble and Manning 2013). mTORC1 is recognized as a master regulator of cell growth, with many anabolic effects that expand cell mass and all the critical building blocks required for cell growth, including NADPH, glycolytic intermediates, lipids, nucleotides, and ribosomes (Duvel et al. 2010, Dibble and Manning 2013). In the randomized controlled MILES (Multicenter International LAM Efficacy of Sirolimus) trial, the mTORC1 inhibitor rapamycin stabilized lung function and improved symptoms in LAM patients (McCormack et al. 2011). However, lung function in treated subjects declined when rapamycin was discontinued. Furthermore, renal angiomyolipomas that had responded to sirolimus also re-grew when the drug was discontinued (Bissler et al. 2008). Thus, other approaches to LAM treatment and/or control are urgently needed.

Although regulation of mTORC1 activity is recognized as the major canonical function of the TSC protein complex, multiple other effects occur in cells lacking the TSC complex, which appear to be independent of mTORC1 (Henske and McCormack 2012). We have recently demonstrated that loss of TSC2 increases COX-2 and PTGIS levels, and prostaglandin biosynthesis in a rapamycin-insensitive manner, but dependent on mTORC2 and EGFR (Li et al, 2014). In addition, celecoxib, a selective COX-2 inhibitor, reduced renal tumor incidence in *Tsc2*$^{+/-}$ mice, and aspirin suppressed tumor progression in a xenograft tumor model utilizing LAM patient-derived angiomyolipoma cells (Li et al. 2014). This recently published study motivates the current Pilot Clinical Trial proposal of COX-2 inhibition as a novel therapeutic strategy for LAM.

b- In vitro and preclinical evidence

Preliminary studies

Our recent publication provides the rationale for this clinical trial (Li et al. 2014).

Loss of TSC2 upregulates both COX-2 and prostacyclin synthase expression and prostaglandin production, independent of mTORC1 (Li et al. 2014)

Prostaglandins are products of prostaglandin-endoperoxide synthases (PTGSs) 1 and 2, more commonly known as COX-1 and COX-2. COX-1 and COX-2 convert arachidonic acid released from membrane phospholipids into PGH2. Prostaglandin I2 (prostacyclin) synthase (PTGIS) then produces prostacyclin (PGI2) from PGH2. We had previously noted that both COX-2 (*PTGS2*) and *PTGIS* expression were significantly increased, by 2- and 40-fold, respectively, in TSC2-deficient angiomyolipoma (101) cells relative to TSC2-addback cells, through microarray analysis (Lee et al. 2010). To validate these findings, we performed real-time RT-PCR analysis. TSC2-deficient cells exhibited a 101-fold increase of PTGS2, and a 15-fold increase of PTGIS in comparison to addback TSC2-expressing cells ($P < 0.001$).

Effects of COX inhibitors on COX expression, PG levels, and proliferation (Li et al. 2014)

To assess the effects of COX inhibition on TSC2 deficient cells, we treated the angiomyolipoma (101) cells with Sulindac (COX-1 inhibitor), NS398 (COX-2 inhibitor), or aspirin (an irreversible COX-1 and COX-2 inhibitor that acetylates COX-2, resulting in production of 15-epi-LXA4) for 24 h. NS398 and aspirin reduced COX-2 and COX-1 levels without affecting phosphorylation of p44/42–MAPK or S6. Aspirin significantly decreased PGE2 levels and furthermore, reduced proliferation of the 101 cells in vitro (Li et al. 2014)

Effects of COX inhibitors in Tsc mouse models (Li et al. 2014)

We then assessed the effects of COX inhibition on TSC2 null tumor development in vivo. First, we used the COX-2–specific inhibitor Celecoxib to treat *Tsc2*$^{+/-}$ mice, which spontaneously develop renal cystadenomas (Onda et al. 1999). Celecoxib treatment (0.1% wt/wt in chow) for 4 months, beginning at 1 m of age, reduced the volume of renal cystadenomas in *Tsc2*$^{+/-}$ mice by 50% at age 5m relative to vehicle control ($p = 0.0002$). Then, we assessed the effect of aspirin in a xenograft tumor model using Tsc2-deficient ELT3 cells, modified to express luciferase. Aspirin treatment for 3 weeks decreased bioluminescent signal as well as tumor size. Aspirin-treated tumors also had reduced expression of COX-2 and c-Myc, and had increased levels of cleaved-caspase-3 and cleaved-PARP. Aspirin-treated mice bearing ELT3 xenograft tumors had markedly reduced urinary levels of PGE2, consistent with strong inhibition of each of COX-1 and COX-2, as expected. Together, these data indicate that COX inhibition reduces tumor growth in two different in vivo Tsc models (Li et al. 2014).

Evidence for COX-2 activation in vivo in LAM (Li et al. 2014)

We then examined COX-2 expression directly in LAM lung samples. LAM lungs were found to express higher levels of COX-2 in comparison with control lungs by immunohistochemistry. COX-2 expression was seen in cells also expressing smooth muscle actin and phospho-S6 in LAM nodules, commonly called LAM cells (Li et al. 2014).

To gain some insight into the functional effects of COX-2 expression in LAM, 15-epi-lipoxin A4 (LXA4), a product of aspirin-acetylated COX-2, was measured in exhaled breath condensate (EBC) from three LAM subjects. LXA4 was detected in the LAM EBCs and levels were increased following transient aspirin treatment. We also hypothesized that LAM cell activity might increase serum PG metabolites in LAM patients. The mean serum PGE2 level of LAM patients (27.8 pg/ml) was higher than that of healthy women (19.6 pg/ml; $P = 0.0021$). In addition, the mean serum 6-keto-$PGF_1\square$ level of LAM patients (192 pg/ml) was also higher than the mean of healthy women (82.6 pg/ml; $P = 0.0006$). These results suggest that COX-2 activity is high in LAM cells in vivo (Li et al. 2014).

Use of next-generation sequencing (NGS) for *TSC2* mutation detection

We have employed NGS for sensitive detection of *TSC1/TSC2* mutations in multiple settings. This includes on microdissected LAM tissue, in collaboration with Lucia Schuger (Badri et al. 2013), analysis of cultured skin tumor cells, in collaboration with Tom Darling (Tyburczy et al. 2014), and on a large number of TSC patients with no mutation identified (data not shown). In brief we either prepare large amplicons from *TSC1*/*TSC2*, or perform hybrid capture of those genomic regions using Agilent probe sets, to create NGS libraries highly enriched for the *TSC1/TSC2* regions. Following sequencing on the Illumina 2500 machine, we use a set of standard pipeline tools to align sequences to the human genome. We use our own specialized scripts written in python and Matlab to detect all sequence variants present at allele frequency > 1%, and then review all calls using IGV. By this methodology we have identified several sequence variants that are present at 1-2% frequency, and validated them by secondary methods (Badri et al. 2013, Tyburczy et al. 2014).

Current clinical use and toxicities of celecoxib: implications for the COLA trial

Celecoxib is a COX-2 selective non-steroidal anti-inflammatory drug (NSAID). It is used to treat osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and several other inflammatory conditions. It has been in clinical use in the USA for 16 years. Celecoxib is generally well tolerated, with mild to moderate upper GI complaints being the most common adverse event (McCormack 2011). Although celecoxib has been associated with an increased risk of cardiovascular events, including myocardial infarction (MI) and stroke, this has been inconsistent in meta-analyses (McCormack 2011). Nonetheless, due to this potential risk, LAM patients with a history of MI or stroke will not be entered on this trial. Celecoxib and other NSAIDs may increase risk of serious gastrointestinal (GI) ulceration, bleeding, and perforation. To minimize this risk, LAM subjects on this protocol will not take any other NSAID, and omeprazole will be permitted for use concurrently for any LAM subjects on this trial. In addition, LAM patients with a prior history of ulcer disease or GI bleeding will be excluded from this protocol. Celecoxib and other non-steroidal anti-inflammatory drugs (NSAIDs) are known to reversibly affect renal function. For this reason, subjects with impairment of renal function will be excluded from the trial, and renal function will be monitored among subjects on this trial.

c- Rationale and hypothesis

As shown above, we have demonstrated that COX-2 and prostacyclin synthase expression is markedly increased in TSC2 deficient cells, and have also shown that prostaglandin metabolites are altered in angiomyolipoma cells. We have shown that treatment with a COX-2 inhibitor changes the PG metabolite profile of the angiomyolipoma cells, and inhibits their growth in vitro. Furthermore we have shown that COX-2 inhibition reduces growth of TSC2 deficient cells in vivo in two Tsc mouse models. Thus, **we hypothesize that COX-2 inhibition may be effective for TSC and LAM patients in reducing LAM and angiomyolipoma cell growth, and in stabilizing lung function.**

We further hypothesize that COX-2 inhibition will decrease the number of circulating *TSC2*-mutant LAM cells. Previous studies from the Moss laboratory at NIH have shown that circulating LAM cells can be isolated by a combination of density gradient separation and FACS separation using antibodies to CD45 and CD235a (Crooks et al. 2004, Cai et al. 2014). We will use this same methodology followed by NGS to identify mutations in *TSC2* in patients enrolled on this study. We will then use similar methods to quantify the number of circulating LAM cells in the subjects enrolled on this trial, developing a novel biomarker of LAM. Since this method works on a blood sample, it is non-invasive.

II- Specific Aims

The main objective of **COLA: A Pilot Clinical Trial of COX-2 inhibition LAM and TSC** is to test the safety and tolerability of celecoxib in subjects with LAM, and to test in a pilot manner its potential therapeutic effectiveness. If successful, this pilot study will enable future phase II/III studies formally evaluating efficacy of this therapy for LAM. A second objective of this study is to develop a novel biomarker of LAM, the number of circulating LAM cells per ml of blood.

Specific Aims

Aim 1: To investigate whether, in LAM patients, celecoxib is safe and well tolerated, and has evidence of clinical benefit. LAM patients diagnosed by consensus criteria (Johnson et al. 2010) and with mild to moderate disease will be treated with celecoxib at 200mg PO QD. Assessments will include history and physical examination, chemistries, complete blood count, renal function assessment, and health status questionnaire during 6 months of treatment. To assess benefit, we will measure pulmonary function, angiomyolipoma size, VEGF-D levels, and quality of life using the St. George's Respiratory Questionnaire, before and after therapy with celecoxib. Twelve LAM patients at the two sites (BWH and NIH) will be enrolled on this study.
Aim 2: To investigate the potential value of novel biomarkers of LAM, quantitative measurement of the number of *TSC2* mutant LAM cells per ml of blood and/or identification of TSC2 mutations in plasma cell-free DNA, to assess disease severity. We will develop a next gen sequencing (NGS) assay on FACS sorted circulating LAM cells and/or plasma cell-free DNA to quantitatively determine the number of *TSC2* mutant LAM cells per ml of blood and/or the amount of TSC2 mutant DNA molecules per ml of plasma.

III- Subject selection

a- Inclusion and exclusion criteria

We will perform a two-center phase I trial of celecoxib (COX-2 inhibitor) administered at 200mg by mouth daily for 6 months. The two centers are BWH and the National Institutes of Health Clinical Center. Up to 12 adult women with LAM will be recruited at both sites, and up to 8 subjects at BWH (between 4-8 at each site). The study will use the following eligibility criteria.

Inclusion criteria

a. Female of age 18 to 69
b. Ability to give informed consent
c. Definite diagnosis of LAM
Typical cystic change on CT scan of the chest plus one of the following i) biopsy or cytology of any tissue demonstrating LAM, ii) angiomyolipoma, chylothorax, clinical or genetic diagnosis of tuberous sclerosis, iii) serum VEGF-D > 800pg/ml
d. post-bronchodilator forced expiratory volume in one second ≥ 70% of predicted and DLCO ≥ 70% predicted during baseline visit.
e. Women of childbearing potential must agree to use two forms of barrier contraception after screening visit, for the duration of study participation and for 30 days after last dose.

Exclusion criteria

a. History of intolerance to non-steroidal anti-inflammatory drugs (NSAIDs)
b. History of current regular use (daily most days of the week) of NSAIDs
c. History of use of rapamycin or everolimus
d. Uncontrolled intercurrent illness
e. Pregnant, breast feeding or planning to become pregnant in the next 2 years
f. Significant hematological (platelet count <100.000/µl or hepatic abnormalities (Liver function tests >2 times normal).
g. Use of an investigational drug within 30 days of study start
h. Inability to attend scheduled clinic visits
i. Inability to give informed consent
j. Inability to perform spirometry
k. Creatinine > 1.0 mg/dl or eGFR < 60 ml/min
l. Pneumothorax within past 8 weeks
m. History of malignancy in the last 2 years other than basal cell skin cancer
n. Use of estrogen containing medication within 30 days of enrolment
o. Currently taking doxycycline, metformin, lupron or simvastatin

p. Unable to undergo MRI
q. History of seizure within the last year
r. History of hepatitis or known active hepatitis B or C, or HIV positive serology
s. Angiomyolipoma of diameter $\geq$ 4 cm
t. History of vascular disease, including myocardial infarction or stroke
u. History of ulcers or GI bleeding
v. Allergy to sulfonamides, unless subject has previously used Celocoxib without any adverse reactions.
w. Age older than 70

b- Source of subjects and recruitment methods

Participants will be referred by physicians who care for LAM patients at participating institutions. Care will be taken to allow potential subjects to feel free from tension and subtle coercion. The investigators will advise potential subjects that a study is being done, and briefly describe the study to ascertain interest prior to identifying themselves as being involved. Investigators will emphasize that participation is voluntary and that not participating will not jeopardize future care.
The study investigators will also contact providers outside of these institutions who may be caring for patients with LAM to notify them of the clinical trial design, inclusion and exclusion criteria. This contact will be in the form of a letter to referring physicians. Such physicians will be provided with contact information of key personnel in the trial in order to refer potential subjects for participation.
Research staff will seek to enroll any potentially qualified adults regardless of age, ethnic, religious or racial background, and economic status.
Subjects will be recruited from other past studies. We will also post an advertisement on the Brigham and Women's website and on clinicaltrials.gov as well as use flyers to advertise the study in the clinics and events for LAM patients.

IV- Subject enrollment

The primary specialist or heath care provider, usually a physician, who is known to the potential subject and has first- hand knowledge of the patient's medical history must (1) give approval for his/her patient to be contacted for research purposes (2) initially introduce the study to the patient and (3) obtain the patient's permission to be contacted by study staff. A nurse or study coordinator will review the informed consent with the subject to avoid the possibility of coercion or bias since subjects will be recruited from Investigator's own practice. Subjects will be reminded that refusal to participate in this trial will not change the quality of care that she will receive at BWH. Study participation is voluntary and the subject will be advised that she can withdraw at any time.

The subject will be given the opportunity to take the consent home to consider participation. The subject will be asked to read the informed consent form prior to the physician or study coordinator asking the subject any questions concerning their health. Written, informed consent will be obtained after one of the investigators has reviewed the protocol verbally in detail with the subject. The physician will inform the subject that she has the alternative not to participate in the study. Subjects will receive a signed copy of the consent form for their records. After all questions are answered, and only after the consent form is signed by the patient and one of the MDs listed on the application will study screening commence. Subjects unable to give consent will not be approached.

Participation in other trials

Subjects will be allowed to participate in other research studies, as long as they do not involve the use of an investigational therapy.

Inclusion of Women and Minorities in this study
Adult women (>18 years of age) without any restrictions on age, race or ethnic group are eligible to participate in this trial. We will make every attempt to reach women of all ethnic groups, by advertising broadly through patient groups, LAM foundation and the internet.

V- Study procedures

Prior to enrolling in the study subjects must undergo the following tests to determine eligibility and baseline characteristics.

Screening and baseline testing		Timing prior to start of therapy
Informed consent	To be obtained before entry	
Medical history and physical examination	Including weight, height, SaO2, vital signs (heart rate, blood pressure, temperature)	Within 28 days
Hematology	Complete blood count and differential (CBCD)	Within 28 days
Serum chemistries	Must include alkaline phosphatase, AST, ALT, bilirubin, serum creatinine, calicium and magnesium	Within 28 days
Urine test	Including pregnancy test	Within 28 days
Electrocardiogram (ECG)		Within 28 days
Pulmonary function test	Spirometry, Post Bronchodilator spirometry, TLC, DLCO	Within 28 days
Abdominal MRI		Within 3 months

Study Visit Summary

Study Procedures
All study procedures will be performed in accordance with accepted standards of practice. Relevant changes in clinical status and medication use, symptom history and adverse events will be reviewed at each study visit and in between study visit by a phone call placed from the research team to subjects enrolled in the study.

Blood samples (chemistry, CBC) and biomarkers (VEGF-D, circulating TSC2 mutant cells) will be obtained via blood draws at indicated study visits. In addition, urine pregnancy testing will be performed at each study visit during the course of drug administration. Pulmonary function testing will be performed in accordance with ATS guidelines (Anonymous 1991) and according the schedule outlined in Table 1. Abdominal MRI will be performed at enrollment –if not done in the past 3 months-, and if angiomyolipoma or any other kidney abnormalities are detected, follow-up MRI will be performed at the discretion of the study team as indicated in Table 1. The St. Georges Respiratory Questionnaire (SGRQ) will also be administered and scored in accordance with published guidelines (Barr et al. 2000). Information on hospitalization and side effects will be obtained via direct subject interviews and medical record review.
Table 1: study visits and testing

Week

	Baseline	8	16	24
Visit number	1	2	3	4/early termination
Informed consent	X			
History and physical	X	X	X	X
Concurrent Medication Reconciliation	X	X	X	X
Adverse Event Review	X	X	X	X
Drug Administration Record	X	X	X	X
Liver, renal, glucose, cholesterol	X	X	X	X
EKG	X			
Urine pregnancy	X	X	X	X
CBC diff	X	X	X	X
MRI abdomen	X			X
Full PFT	X			X
St George's questionnaire	X			X
Stored serum and plasma	X	X	X	X
Exhaled breath condensate	X			X
measurement of TSC2 mutant cells	X			X

Study Windows

The screening to first dose period will be 28 days. All other study visit windows will be +/- one week.

Study visit procedures

Screening Visit (Visit 1)

The Screening Visit will take about 5 hours.

The following testing will be performed at the screening visit:

- Medical history
- Full physical exam
- Medication reconciliation
- Blood collection as outlined in table 1
- Urine pregnancy testing
- EKG
- MRI of the abdomen if not done in the last 3 months
- St. George's Respiratory Questionnaire (SGRQ)
- Spirometry, Post Bronchodilator spirometry, body plethysmography and diffusing capacity for carbon monoxide (DLCO)
- Stored blood for all exploratory endpoints
- Collection of exhaled breath condensates

Subjects who meet the inclusion criteria will be enrolled in the study and will start taking their medications

Visit 2

Visit 2 will take place 8 weeks after a subject starts taking study medication and will require about one hour.

The following testing will be obtained at this visit:

- Interval history and adverse event review
- Physical exam
- Blood collection as outlined in table 1
- Urine pregnancy testing

Visit 3
Visit 3 will take place 16 weeks after a subject starts taking study medication and will require about one hour. The following testing will be obtained at this visit:

- Interval history and adverse event review
- Physical exam
- Blood collection as outlined in table 1
- Urine pregnancy testing

Visit 4
Visit 4 will take place at 24 weeks after the start of therapy and after this visit the subject will stop taking the medication. This visit will require about 5 hours. The following testing will be obtained at this visit:

- Medical history
- Full physical examination
- Medication reconciliation
- Blood collection as outlined in table 1
- Urine pregnancy testing
- MRI of the abdomen if angiomyolipoma or any other kidney abnormalities are present on screening MRI
- St. George's Respiratory Questionnaire (SGRQ)
- Spirometry, Post Bronchodilator spirometry, body plethysmography and diffusing capacity for carbon monoxide (DLCO)
- Collection of exhaled breath condensates

Early termination study visit
If a subject decides to stop taking part in the study for any reason, she will make a final study visit. The final study visit will take about 6 hours. The following testing will be obtained at this visit:

- Medical history
- Full physical examination
- Medication reconciliation
- Blood collection as outlined in table 1
- Urine pregnancy testing
- MRI of the abdomen if angiomyolipoma are present on screening MRI
- St. George's Respiratory Questionnaire (SGRQ)
- Spirometry, Post Bronchodilator spirometry, body plethysmography and diffusing capacity for carbon monoxide (DLCO)
- Collect any unused study drug and drug diaries
- Collection of exhaled breath condensate

Subject study participation may be discontinued by the PI if:

- At the PI's discretion in the best interest of the subject
- Subject can't make study visits
- The sponsor decides to stop the study
- The subject has intolerable side effects even at the lower dose of Celecoxib
- Subject becomes pregnant
- Subject does not follow study requirements

Labs performing evaluations and special precautions

The clinical laboratory evaluations will be performed in CLIA approved laboratories at BWH and the NIH Clinical Center.

Investigational analyses of VEGF-D and circulating LAM cells will be conducted in research laboratories at BWH and NIH.

i- VEGF-D analysis will be performed in Dr. Kwiatkowski's laboratory at BWH

ii- Next generation sequencing (NGS) assay to detect TSC2 mutant LAM cells in FACS sorted circulating LAM cells and/or TSC2 mutations in plasma cell-free DNA will be performed at BWH in Dr. Kwiatkowski's laboratory

Transfer of specimens between sites will be necessary to complete the investigational analyses. Samples will be shipped overnight on dry ice Monday to Thursday to keep specimens frozen. Transferring site will keep a log of all specimens transferred. The receiving site will keep a log of when samples are received and analyzed.

Primary outcome measurements

We will perform a two-center phase I trial of celecoxib (COX-2 inhibitor) administered at 200mg by mouth daily for 6 months. A study investigator will obtain informed consent after detailed review of the consent form with potential study participants. Consenting subjects will be screened at the initial visit based on predefined inclusion/exclusion criteria. Subjects must have mild to moderate LAM (defined as definite LAM not requiring rapalog therapy), have no history of chronic non-steroidal anti-inflammatory drug use, have no previous history of treatment with rapamycin or everolimus, and have no angiomyolipoma of diameter > 4 cm. Enrolled subjects will receive celecoxib at an initial dose of 200 mg orally daily, with a single dose adjustment to 100mg PO QD permitted if needed.

Any subject who receives at least one dose of celecoxib will be included in the analysis for side effects. Dose limiting toxicity will be graded according to the National Cancer Institute's CTCAE v3.0 (http://ctep.cancer.gov/protocoldevelopment/electronic_applications /docs/ctcaev3.pdf). During the 6 months of the trial, there will be 3 study related visits. Clinical and laboratory evaluation is described below.

Since this dose of celecoxib (200mg PO QD) is routinely well tolerated in the clinical setting in patients with arthritis and other inflammatory disorders, we expect that it will be well-tolerated in LAM patients. However, we will carefully monitor GI tolerance and renal function, since celecoxib can lead to reversible renal impairment.

We will perform assessments of clinical, functional and subject self-reported outcomes to assess potential benefit of celecoxib. Since lung function decline is a hallmark of LAM, we will perform spirometry, which will be interpreted in accordance with standard guidelines (Anonymous 1991). We will utilize the St. George's Respiratory Questionnaire (SGRQ) (Barr et al. 2000), as an assessment of the impact of treatment on dyspnea levels. Finally, we will record the incidence of hospitalization, complications from the disease, and other medical problems occurring during the study period

Stopping rules

The study will be monitored for the occurrence of a specified set of treatment related serious adverse events (TRSAE). The following two types of TRSAEs will be considered for early stopping of the study:

- Death considered to be probably or definitely related to Celecoxib

- Any grade IV toxicity considered to be probably or definitely related to Celecoxib

Subjects will be followed for 24 weeks while on therapy. During the 24 weeks of the trial, there will 4 study visits (screening visit and 3 visits while on therapy). Clinical and laboratory evaluations will be performed as outlined in Table 1.

Secondary outcome measurements

We will be performing several correlative studies as part of this protocol.

a. VEGF-D levels. VEGF-D is well-known as a biomarker of LAM, and disease extent correlates with VEGF-D levels. Therefore we will measure VEGF-D prior to treatment and at serial time points during treatment. As this is a pilot study, formal statistical analysis is not planned.
b. Circulating LAM cells. Circulating LAM cells may be isolated from peripheral blood of subjects on this trial, using density gradient and FACS methods. TSC2 mutations will be identified in these cells, if possible, and then the prevalence of LAM cells will be measured serially over time in subjects on this study. If no TSC2 mutation is identified, then mutations in other genes in this pathway (RHEB, MTOR) will be sought. However, we will not scan the entire genome in these analyses, but rather focus on just a few genes. We will test the number of circulating LAM cells over time to help assess whether the treatment, celecoxib, reduces the number of LAM cells.
c. Plasma biomarker analyses. Prostaglandin metabolites (PGE2 and 6-keto-PGF1-alpha), matrix metalloproteinases MMP2 and MMP9, cathepsin K, and a cytokine array will be measured on serial plasma samples (weeks 0, 8, 16, 24) from subjects on this trial. Marked changes in these metabolites are expected when subjects are on celecoxib, and this will serve as a potential biomarker of drug effect and compliance.
d. Exhaled breath condensates (EBCs). EBCs will be obtained from subjects on this trial at the time of pulmonary function testing (week 0, 24). Subjects will be required to withhold food and carbonated beverages for one hour prior to EBC collection. An Rtube (Respiratory Research, Austin, TX) will be used to collect EBCs by tidal breathing through the mouth for 10 minutes without a nose clip. The metal sleeve surrounding the collection chamber will be chilled to -20°C for at least one hour prior to EBC collection. The EBC will then be stored at -80°C under N2 gas to prevent non-enzymatic oxidation of the parent fatty acids and lipid mediators. Samples will be analyzed for prostaglandin metabolites (PGE2 and 6-keto-PGF1-alpha).
e. Plasma cell-free DNA (cfDNA) will be isolated from each subject on this trial at each time point a sample is drawn. The plasma will be separated, and cfDNA isolated. The cfDNA will be subject to targeted next generation sequencing to search for mutations in TSC2.

VI- <u>Biostatistical analysis</u>

b- Data variables

We will collect information about the safety of Celecoxib in women with LAM and TSC. In particular, we will collect data on the incidence of adverse events and severe adverse events. In addition, we will collect information about lung function at the screening visist and at the end of the study period. If subject has a renal angiomyolipoma at the screening visit, we will collect information about angiomyolipoma size at the end of the 6 months of therapy.

c- Statistical Methods

Sample Size Considerations

Common adverse event rate with Celecoxib provided in the package insert are between 0.2% and 6.0%. The below table gives the probability of seeing at least one case of common adverse event associated with Celecoxib, using different event rates (Table 2).

Table 2. Probability of observing at least one case of an adverse event

	Adverse event rate		
Sample size	6%	2.5%	0.2%
12	52%	26%	2.3%

With the proposed sample size of 12 subjects will allow us to detect at least one case of common adverse event with probability of 2.3% to 52%. Rates of side effects related to celecoxib in the LAM population are unknown.

For lung function parameters (FEV1, FVC, DLCO) and angiomyolipoma size (when applicable), the difference between baseline and 24 weeks of therapy will be assessed using the Wilcoxon signed-rank test, with a two sided alpha at 0.05.
For the exploratory endpoints, difference between baseline and 24 weeks of therapy will be assessed using parametric and non-parametric tests as indicated. Two-sided alpha of 0.05 will be used to determine statistical significance.

VII- Risks and Discomforts

a- Risks of Celecoxib

Cardiovascular Risk

Celecoxib may cause an increased risk of serious cardiovascular thrombotic events, myocardial infarction and stroke which can be fatal. All NSAIDs may have similar risk. This risk may increase with duration of use. Patients with cardiovascular disease or risk factors for cardiovascular disease may be at greater risk. The cumulative risk is 2.5% over 3 years at the Celecoxib dose that we are using in this study.

Hypertension

Celecoxib can lead to new onset hypertension or worsening of exisiting hypertension. The rate of hypertension in one trial was 2.4%

Gastrointestinal Risk

Celecoxib cause an increased risk of serious gastrointestinal adverse events including bleeding, ulceration, and perforation of the stomach or intestines, which can be fatal. These events can occur at any time during use and without warning symptoms. Elderly patients are at greater risk for serious gastrointestinal events. The incidence after 9 months of therapy of complicated and symptomatic ulcers was 0.78% in one study, but the risk was 1.4% in patients older than the age of 65. Concomitant use of aspirin increased the risk to 3.06%.

Hepatic Risk

The incidence of borderline elevation (greater than 1.2 times and less than 3 times the upper limit of normal) of liver enzymes was 6% in one trial. The incidence of significant elevation in liver enzymes was 0.2%

Renal Risk

Renal toxicity can be seen especially in patients with pre-existing impairment of kidney function, heart failure or liver disease taking diuretics. No percentage on the occurrence of renal toxicity with Celecoxib is provided in the package insert.

Skin reactions

Celecoxib is a sulfonamide and can cause serious skin adverse events such as exfoliative dermatitis, Stevens-Johnson syndrome and toxic epidermal necrolysis which can be fatal. These serious adverse events can occur without warning and in patients without prior history of sulfa allergy.

Pregnancy
Use of NSAID late in pregnancy can lead to early closure of the ductus arteriosus.

b- Risk of procedures

Risk of Lung Function Tests:
- Cough
- Lightheadedness
- Discomfort due to technique
- Shortness of breath
- Fatigue

Lung function tests will be performed by a qualified respiratory therapist and will be done according to ATS guidelines

Risk of questionnaires:
- Fatigue
- Distress related to questions

Risk of blood draws:
- Bruising
- Pain
- Infection
- Lightheadedness and/or fainting

Specimens will be collected by a qualified member of the research team at the time of study visit and drawn according to institutional standards.

Risk of MRI:
There have been no ill effects reported from exposure to the magnetism or radio waves used in the MRI. A known risk of MRI is that magnet could attract certain types of metal. Metal within the subject's body is evaluated (this includes certain dyes found in tattoos, pacemakers, implanted defibrillator, metallic particles in the eye, non-fixed metallic particles, vascular clips in the head, prosthetic heart valves, etc) in order to acquire the image data.

Risk of exhaled breath condensate:
It is possible that subject may become lighheaded while breathing into the tube to collect breath condensate.

Genetic Risks
There is a potential psychosocial risk to subjects related to learning genetic information. However, we will not be looking across the entire genome in these subjects, and will be focusing on genetic changes in *TSC2*, the known genetic cause of LAM. In addition, our findings will be somatic mutations, which are very common in neoplasms in general. Last, the information is safeguarded and not provided to anyone outside of our laboratory in a manner that identifiers may be used to identify the specific subject.

c- <u>Celecoxib drug interactions:</u>
- Celecoxib metabolosm is predemoninantly mediated via cytochrome P450 (CYP)2C9 in the liver. Co-administration of celecoxib with drugs that are known to inhibit CYP2C9 should be done with caution.
- Warfarin

- Lithium
- Aspirin
- Angiotensin converting enzyme inhibitors and angiotensin II antagonists.
- Fluconazole
- Furosemide
- Concomittant NSAID use
- Methotrexate

Celecoxib formulation, instruction and storage

Celecoxib 200mg capsules are white, with reverse printed on gold band with markings of 7767 on the cap and 200 on the body. Celecoxib should be stored at 25°C (77°F); excursions permitted to 15-30°C (59-86°F). Celecoxib will be taken once daily with meals.

Dose interruption, modification or discontinuation due to toxicities related to study drug

Single dose adjustement to 100mg daily is allowed if subjects develop adverse events related to study drug. If symptoms persist at the reduced dose of Celecoxib then subject is withdrawn from the study.

Protection against general subject risks

Highly trained physician and nursing personnel familiar with clinical care and research protocols will conduct the study procedures. Experienced personnel following established guidelines will perform venipuncture. To maintain confidentiality, all laboratory specimens, evaluation forms, and reports will be identified only by a coded number. A computer will generate the coded number at random and only the study investigators will have access to the codes. All records will be kept in a locked, password protected computer. The case report forms for the protocols will be maintained on line under the supervision of the investigator. Subjects will be provided with contact information for trial investigators, who will be accessible to subjects at all times through the phone, paging or email.

All testing will be reviewed with the potential subjects during the consent process.

Celecoxib is a category C drug before 30 weeks of gestation and Category D afterwards out of concern of early closure of the ductus arteriosus. Since LAM is a disease that affects women in their childbearing years, this population cannot be excluded from the trial. Urine pregnancy testing will be performed at each study visit. Women will be required to use two forms of contraception during and for at least 8 weeks after the last dose of Celecoxib.

To monitor for liver, kidney impairements laboratory analysis will be perforemd at each visit. Subjects will be instructed to contact study staff if they develop fluid retention, elevated blood pressure, chest pain or any symptoms.

The Principle Investigator will be responsible for monitoring the safety of subjects who have entered this study and for providing appropriate medical
Care. The investigators will be responsible for alerting the sponsor to any event that seems unusual, even if the event may be considered an unanticipated benefit to the subject. By exercising appropriate health-care options, the investigator remains responsible for managing AEs that are serious or that cause the subject to discontinue before completing the study. Frequency of follow-up beyond that mandated in the protocol is left to the discretion of the investigator.

Confidentiality and HIPAA considerations

All subjects must provide written informed consent and signed HIPAA authorization prior to the performance of any screening or main study procedures. Subject confidentiality will be protected throughout the study and no subject-identifying information will be released to anyone outside the project. Confidentiality will be secured through several mechanisms. Each subject will be assigned an anonymous study ID, which be used on all study forms. Any study forms and paper records containing personal identifier information (e.g., address, phone number) will be kept secured and locked at each clinical site. No personal identifiers will be placed on biological samples and other documents forwarded to central labs and reading centers.
Access to all subject data and information at the clinical sites, including biological samples, will be restricted to authorized personnel. Only authorized personnel at the coordinating center will have access to study data files. Authorized personnel will be assigned user logon IDs, passwords and appropriate access privileges to study data. Study subjects will be identified only by their initials and subject ID number. No personal identifiers such as name, address, social security number will be entered into the study database. Any subject-specific data reported to any study committees will only be identified by subject ID number.
Finally, subjects will not be identified by name in any reports or publications, nor will the data be presented in such a way that the identity of individual subjects can be inferred. Analysis files created for further study by the scientific community will have no subject identifiers.

Protection against subject risks related to the study medication

Subjects will be provided with written, detailed instruction for calling the study coordinator or physician, including but not limited to infections requiring antibiotics, planeed or emergent procedures, or addition of drugs known to interact with Celecoxib. The risk associated with Celecoxib will be explained to the subject in detail. A plan for dose interruption and reduction is included in the experiemntal plan. Subjects may withdraw from the trial at any time, or be withdrawn by the site PI for safety reasons at his/her discretion. The Data and Safety Monitor will review routine adverse events and trial results every six months, and serious adverse events within 72 hours. Dr. Kwiatkowski will review urgent, persistent or life-threatening adverse events at all times during the trial. An on-call clinician at each site will be avaialbel for questions and consultation.

Due to the experimental use of celecoxib in LAM and TSC the trial has been designed with a focus on protecting subjects against risk from the medication including:

1- Small sample size consistent with the exploratory nature of the trial.
2- Specific exclusion criteria to prevent enrollment of subjects with significant co-morbidities that might place them at excess risk (see exclusion criteria)
3- Specific defined dose and holding criteria related to study adverse events.

VIII- Potential benefits

Participation in the proposed research will not guarantee direct benefit to participants, although it remains possible that a previously undiagnosed clinical abnormality will be brought to the clinician's attention. Information obtained from this research will provide information about the relationship between LAM treatment and patient outcomes. Sporadic LAM and Tuberous sclerosis affect more than 1.5 million people worldwide. Lung manifestations of TSC, including LAM are its most life threatening manifestations. Participants may also benefit from the effects of the drug in the trial, as well as frequent follow-up and monitoring. Information learned from this research may benefit women with LAM in the future. Understanding the interplay between mTOR dysregulation and COX 2 inhibition may have implications for patients suffering from other diseases as well.

IX-Data management

Data management overview

This study will use REDCap for electronic data capture. REDCap is a secure, web-based data management tool. Data will be entered into REDCap directly from the information collected on the source documents. All staff will be trained on REDCap. Traning modules can be accessed via the website https://redcap.partners.org/redcap Data entry in to Redcap should be done within 5 business days from the time the information is collected.

Data Querries and Corrections
Data will be routinely reviewed for any missing data or discrepancies. Data discrepancies that are identified will be resolved within 5 business days. Each site must correct or update the database to resolve the data discrepancies.

Investigator Electronic Signature
The investigator or designee indicates their review of the data accuracy on all eCRFs pages for a subject with their e-signature within the REDCap system.

Maintaining the Clinical Center Subject File
Keep the following documents in the clinical center subject study file for each subject entered in the trial:

Copy of Confidential Subject Information page
Original signed consent form
Copies of all submitted source documents

Confidentiality Data Security Procedures and HIPAA considerations

All subjects must provide written informed consent and signed HIPAA authorization prior to the performance of any screening or main study procedures. Subject confidentiality will be protected throughout the study and no subject-identifying information will be released to anyone outside the project. Confidentiality will be secured through several mechanisms. Each subject will be assigned an anonymous study ID, which be used on all study forms. Any study forms and paper records containing personal identifier information (e.g., address, phone number) will be kept secured and locked at each clinical site. No personal identifiers will be placed on biological samples and other documents forwarded to central labs and reading centers.
Access to all subject data and information at the clinical sites, including biological samples, will be restricted to authorized personnel. Only authorized personnel at the coordinating center will have access to study data files. Authorized personnel will be assigned user logon IDs, passwords and appropriate access privileges to study data. Study subjects will be identified only by their initials and subject ID number. No personal identifiers such as name, address, social security number will be entered into the study database. Any subject-specific data reported to any study committees will only be identified by subject ID number.
Finally, subjects will not be identified by name in any reports or publications, nor will the data be presented in such a way that the identity of individual subjects can be inferred. Analysis files created for further study by the scientific community will have no subject identifiers.

IX- Monitoring and Quality Assurance

a- Safety Monitoring

We will institute an independent Data Safety Monitoring Board to review procedures and adverse events that occur during the clinical study. Board members will not be involved in the active recruitment and daily routine of the study. The principal investigator will present study progress on a routine basis to the board members. Board members will review new cases and decide whether the study goals should be altered or the study should be stopped.

b- Monitoring of Subjects and Criteria for Withdrawal of Subjects

Subjects will be monitored throughout the clinical trial for new symptoms or complaints.

c- Outcome Monitoring

The clinical outcome of our participants will be continuously monitored by the study staff and the Data Safety Monitoring Board.

d- Adverse event reporting guidelines

Definitions

Adverse event means any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated wit the subject's participation in the research, whether or not considered related to the subject's participation in the research (modified from the definition of adverse events in the 1996 International Conference on Harmonization E-6 Guidelines for Good Clinical Practice).

Serious adverse event means any event temporally associated with the subject's participation in the research that meets any of the following criteria:

- Results in death
- Is life threatening (places the subject at immediate risk of death from the event as it occurred)
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in a persistent or significant disability/incapacity
- Results in a congenital anomaly/birth defect or
- Any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the outcomes listed above.

Important medical events that may not result in death, be life-threatening, or require inpatient hospitalization may be considered an SAE when, based on appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent on of the outcomes listed above.

An Unexpected Adverse Event is an adverse reaction, the nature or severity of which is not consistent with the applicable product information per the Investigator Brochure.
Life-threatening means that the subject was, in the view of the investigator, at immediate risk of death from the AE as it occurred. It does not include an AE that, had it occurred in a more severe form might have caused death.
Persistent or significant disability/incapacity means that the event resulted in permanent or significant and substantial disruption of the subject's ability to carry out normal life functions.
Associated with the use of the drug means there is reasonable possibility that the AE may have been caused by the drug.

Adverse event reporting

All AEs (serious and non-serious) will be recorded from start of study treatment (enrollment) through final study visit on the AE case report form.

All adverse events will be reported to the Partners Human Research Committee (PHRC) in accordance with the accepted guidelines. A 24-hour/7-day telephone number will be provided for contacting the licensed physician investigator for this purpose.

The research staff will monitor all subjects during their study involvement for evidence of any adverse events. Local study site personnel will report all serious adverse events (SAEs) to a designated medical monitor who will collect initial reports and oversee medical record retrieval for determination of final diagnosis.

Serious Adverse Events Reporting
All deaths and all SAEs require reporting form the start of treatment through 28 days after discontinuation of the study. The site is requried to call the coordinating center to inform them of the SAE within the 24 hours of knowledge of the event. The site should also submit an SAE form via fax to the coordinating center and enter the SAE information onto the CRF within 24hours of knowledge of the event.

An updated full written report will be filed as additional information becomes available within 10 working days (14 calendar days). The report will include a complete description of the event, use of all concomitant medications, and the local investigator's assessment of causality of the SAE. Non-serious adverse events will be reported at routine visits, documented on a separate adverse event form, and transmitted to the central coordinating center. All serious and unexpected adverse events will be reported to the overall study PI's.

Expedited Event Reporting
Adverse events that meet the definition of serious, study drug-related and unexpected (per the Investigator's Drug Brochure) qualify for expedited reporting to the regulatory authorities. The DSMB will perform a medical review of all deaths and study drug-related serious adverse events and evaluate for "unexpectedness".
The coordinating center will prepare Med Watch repots for those events identified as serious, study drug-related and unexpected as determined by the DSMB. The coordinating center will submit all unexpected study drug related SAEs as per 21 CRF 32.

Safety events requiring expedite reporting will be sent to the DSMB chair, with forwarding by the chair to the full board as needed, and DOD program office within 1-2 business days. DSMB chair will respond to the overall principal investigator and the coordinating center within 48 hours. Reporting framework and timelines are to be documented in Data and Safety Monitoring Plan after consultation with board members.

Coding for Adverse Events
All reported adverse events will be classified using the Common Terminology Criteria for Adverse Events (CTCAE), version 4.

X- References

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