COLUMBIA UNIVERSITY MEDICAL CENTER

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**Evaluation of lymphedema severity and treatment response to propranolol using novel imaging technologies**
(Abbrievated title: Evaluation of lymphedema severity and treatment response)

**Participation Limited to Columbia University**

**Center:** *Columbia University Medical Center*

**Principle Investigator:** *June K. Wu, MD*
*Division of Plastic Surgery*
*161 Fort Washington Ave Suite 511*
*Phone: 212-342-3704*
*Fax: 212-305-9626*
*Email: jw92@cumc.columbia.edu*

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## STUDY COMMITTEE

CO-INVESTIGATOR
Dympna Gallagher, PhD
Obesity Research Center
21 Audubon Ave
New York, NY 10032
Phone: 212-523-4196
Fax:
e-mail: dg108@cumc.columbia.edu

CO-INVESTIGATOR
Jacques Merab, MD
Cardiology
161 Fort Washington Ave, 6th Fl
New York, NY 10032
Phone: 212-305-7934
Fax:
e-mail: jpm6@cumc.columbia.edu

CO-INVESTIGATOR
Thomas Starc, MD
Pediatric Cardiology
3959 Broadway, 2N
New York, NY 10032
Phone: 212-305-4432
Fax:
e-mail: tjs1@cumc.columbia.edu

CO-INVESTIGATOR
Angela Kadenhe-Chiweshi, MD
Pediatric Surgery
3959 Broadway, 2N-215
New York, NY 10032
Phone: 212-342-8586
Fax:
e-mail: avk2102@cumc.columbia.edu

CO-INVESTIGATOR
,MD
Pathology
Address
Address
Phone:
Fax:
e-mail:

RESEARCH NURSE, TBA
, RN or NP
Discipline
Address
Address
Phone:
Fax:
e-mail:

STUDY COORDINATOR/DATA MANAGER
TBA
Dept
Address
Address
Phone:
Fax:
e-mail:

STUDY STATISTICIAN
Codruta Chiuzan,Ph.D.
Biostatistics
Mailman School of Public Health
722 W 168th St, 6th Fl
Phone:
Fax:
e-mail: cc3780@cumc.columbia.edu

**Study Exemption from IND Requirements per 21 CFR 312.2(b) is pending**

**ABSTRACT**

Lymphatic malformations (LMs) arise from abnormal development of lymphatic vasculature. Associated morbidity can be severe, including bleeding, sepsis, airway and visual axis obstruction (cervicofacial LMs), respiratory compromise (thoracic LMs), ascites and malabsorption (peritoneal/pelvic LMs), and limitations on mobility (extremity LMs). Severe symptoms can be life-threatening. Treatment options are often palliative and recurrence is common [1, 2]. Recently, results in our laboratory demonstrated that propranolol, a pan beta-adrenergic receptor (βAR) antagonist, had cytotoxic and anti-proliferative effects against cells isolated from LM tissues. Preliminary results from treating symptomatic LM patients at our institution with propranolol at a dose range from 0.7-1mg/kg/day demonstrated a 70% positive response rate, with patients reporting improvement in their symptoms.

Despite severe morbidities and even mortality, there are no effective, objective, repeatable diagnostic tools to evaluate lymphedema severity, and there is no FDA-approved medical treatment for this devastating condition. Thus, we hypothesize that quantitative magnetic resonance (QMR) can accurately and quickly evaluate lymphedema severity, and propranolol will be effective in improving lymphedema, using QMR as a treatment response evaluator.

QMR has been validated in obesity research but has never been validated for lymphedema. It has the ability to quantify total body water, extracellular and intracellular water. Lymphedema results when there is excess, pathological accumulation of fluid in the interstitial spaces (extracellular water). Therefore, QMR has the potential to sensitively and accurately quantify the severity of extracellular water accumulation, thus severity of lymphedema.

Propranolol has been used for different indications for many years. Most recently, propranolol gained FDA approval for use in infants with hemangiomas, a related vascular anomaly (Hemangeol ®) (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.DrugDetails, accessed May 23, 2014). FDA-approved dose range for treating hemangiomas in infants (>5 weeks old, >2kg) ranged from 1-3mg/kg/day in divided doses. Propranolol has also been used to treat pediatric supraventricular tachycardia at doses up to 4mg/kg/day[3, 4]. However, βAR antagonists are not without potential adverse effects, including hypotension, bradycardia, hypoglycemia, bronchospasms, and sleep disturbances[5].

The dose range of 0.7-1mg/kg/day was chosen for our LM patients as it was the low end of dose range for infants treated with propranolol for problematic hemangiomas. At this dose, no significant hemodynamic adverse effects were noted in our LM patients. However, when patients stopped propranolol or their dose fell below 0.7mg/kg/day, they suffered rebound worsening of their symptoms. Moreover, inflammatory events such as infections temporarily overcame the effects of 0.7-1mg/kg/day of propranolol. Thus, it is unknown whether we have achieved maximum propranolol efficacy at our current dose range. We propose to escalate propranolol dosages up to 3mg/kg/day in this study, still well below the dose ranges currently used in clinical settings.

In this clinical trial, we will examine whether QMR can quickly and accurately quantify lymphedema severity, as well as evaluating whether we have optimized propranolol usage for treatment of LM patients. The primary endpoints for this study are to: 1) validate QMR as a tool for evaluation of lymphedema, and 2) ascertain whether LM patients can tolerate higher doses of propranolol, as measured by known propranolol adverse effects and patient-reported symptoms. A secondary endpoint will address whether patient-reported LM symptoms and quality of life are improved with higher doses of propranolol; objective findings such as LM size on physical examination and imaging studies will be analyzed as well. In addition, LM tissue biopsies will acquired from patients before and after propranolol treatment for further analyses of disease progression.

## EXPERIMENTAL DESIGN SCHEMA

The dose escalation for each patient will be as follows (**Table 1**):

**Table 1. Propranolol intra-patient dose escalation schedule and assessments**

| | Propranolol | Assessments | | |
|---|---|---|---|---|
| | Dose Schedule* | Subjective | Objective | Other |
| Week 1 (initiation) | 0.5 mg/kg/day, divided bid | Enterance Interview Quality of Life Questionnaire | Physical Exam, Imaging | Blood Draw, Tissue Biopsy |
| Week 2 | 1 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | |
| Week 4 | 2 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | Blood Draw |
| Week 6 | 3 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | |
| Week 8 | 3 mg/kg/day, divided bid[a,b] | Exit Interview, Quality of Life Questionnaire | Physical Exam, Imaging | Blood Draw, Tissue Biopsy |

*Weight will be rounded to the nearest 10-kg increment
[a]Dose escalation in the absence of clinical contraindications.
[b]In the presence of clinical contra-indications, the patients will remain on their current level of dosage, or decreased to the previous level, or have their medications held.
A maximum of 8-weeks of therapy may be administered.

## 1.0 GOALS AND OBJECTIVES (SCIENTIFIC AIMS)

### 1.1 Primary Aims

1.1.1 To assess whether QMR can accurately and quantitatively assess lymphedema severity. QMR results will be compared to validated, gold standard quantitation of total body, extracellular and intracellular water distribution using deuterium ("heavy water") and sodium bromide (NaBr) and whole body MRI.

1.1.2 To assess tolerability, and define and describe the toxicities of propranolol, administered two times daily in patients with lymphedema both by

1.1.2a subjective patient-reported symptoms (energy levels, fatigue, dizziness, respiratory issues/wheezing, activities of daily living) by validated questionnaires (SF-36, LYMPH—ICF, LYMPH—ICF--LL)

1.1.2b objective measures (heart rate, blood pressure) by physical exam

### 1.2 Secondary Aims

1.2.1 to assess disease-specific lymphedema patients' response to propranolol

1.2.1a to assess *subjective* patient-reported lymphedema-related symptoms

i) Validated questionnaires: LYMPH—ICF, LYMPH—ICF--LL

1.2.1b to assess *objective* measures

i) Physical examination: Weight, BMI, limb girth discrepancy
ii) Imaging evaluation: MRI
iii) Blood and tissue analysis: circulating and tissue biomarkers
iv) Genetic analysis: correlation of somatic mutations to propranolol response

**Table 2. Primary and secondary endpoints**

| | Primary Endpoint (Aim 1) | Secondary Endpoint (Aim 2) |
|---|---|---|
| Subjective Assessment | Questionnaires QOL | Questionnaires |
| | Short Form 36<br>Lymph-ICF<br>Lymph-ICF-LL | Symptoms (Drainage, pain)<br>Activity levels<br>Clothing size change |
| Objective Assessment | Clinical and radiological Exam | Clinical and radiological Exam |
| | Blood pressure<br>Heart rate<br>Blood sugar<br>Whole Body MRI<br>QMR<br>Deuterium and NaBr | Weight, BMI<br>Limb girth discrepancy<br>CTCAE Grading for Lymphedema<br>Imaging: QMR, whole body MRI<br>Blood and Tissues<br>Circulation biomarkers<br>IHC lymphatic vessel phenotype<br>PiK3CA mutational analysis<br>Next Gen sequencing analysis |

## 2.0 BACKGROUND

## 2.1 Introduction/Rationale for Development

Primary lymphedema is a vascular malformation of the lymphatic system, or a lymphatic malformation (LM)[6]. In lymphedema, regional edema develops, most commonly in the lower extremities, although it can affect any location[7, 8]. Primary lymphedema is classified based on the age of onset: congenital, childhood (praecox), and adulthood (tarda)[9]. Primary lymphedema with early onset (excluding tarda) is considered rare and estimated to affect 1.2/100,000 people under the age of 20[9], while another study reported a 1/6000 incidence for primary lymphedema presenting under the age of 36[10]. Since adult-onset of primary lymphedema is defined to occur after 35, these data possibly underestimate the true incidence.

Lymphatics function to maintain tissue fluid homeostasis, immune surveillance and lipid uptake from the intestines. Defective lymphatic development can cause both mass effects and physiologic derangements. Over time, the abnormal lymphatic drainage leads to chronic, progressive swelling of tissues, increased adipose deposition and fibrosis[8], interfering with ambulation and use of the affected extremity. Such abnormal lymphatic tissue also harbors pools of lymph, which do not drain or circulate normally, and are consequently static and prone to severe infections. Cutaneous involvement can lead to weeping of lymph or blood-tinged lymph from skin lesions. Psychological trauma can be experienced by these patients, who may struggle with the social isolation consequent to physical differences and repeated illness[7-9]. Collectively, co-morbidities of lymphedema include pain, swelling, lymph leakage, hyperkeratosis, interference with function, cellulitis and sepsis and produce a severe lifelong burden of disease associated with significant morbidities.

Because LM pathobiology is poorly understood, existing treatments are largely empirical, and often ineffective. Fuerthermore, there are no objective diagnostic toolds. Treatment options are limited and ranges from lymphatic massage, compression garments, diuretics[11, 12], to surgery[13], only with partial success. Residual tissue after incomplete resection is prone to recur when infectious or inflammatory stimuli occur, and recurrence is frequent. Without effective diagnostic tools, any response to treatment can not be documented or quantified. *Patients with primary lymphedema are frequently therapeutic orphans and new approaches to treatment are desperately needed.* Interest in identifying medical treatments has been great, although limited by the lack of knowledge of the basic pathophysiology of these lesions.

Recently our laboratory has identified two cell types in LMs: lymphatic malformation progenitor cells (LMPCs) and lymphatic malformation endothelial cells (LMECs) that are potential therapeutic targets as they can both recapitulate the LM phenotype in our LM mouse model[14]. In a screen of different drugs using LMPCs, we identified propranolol, a non-selective pan-beta adrenergic receptor (βAR) inhibitor, as a candidate for lymphedema/LM treatment. We find low levels of β2AR expression in normal lymphatic endothelium, and high levels of β1AR/β2AR expression in LMPC/ECs of LM tissues. *In vitro*, propranolol suppresses LMPC/EC proliferation, and in our LM mouse model, propranolol decreases the abnormal lymphatic vessel dilation that is associated with lymphatic vessel dysfunction. *These data suggest that propranolol normalizes LM vessel phenotype, possibly by decreasing LM cell proliferation*.

In addition to our laboratory studies, there are prior anecdotal reports of propranolol efficacy in children with lymphatic anomalies[15-18]. More recently, it has become the therapy of choice for infants and children with complicated hemangiomas[19], and FDA approval for Hemangeol (March 2014) as a treatment for infantile hemangiomas heralded the first time any medications has obtained FDA approval in treatment of a vascular anomaly. Based on our pre-clinical data, published anecdotal reports of efficacy

against lymphatic anomalies, FDA approval for treatment of a vascular anomaly, and the lack of effective treatments led us to offer propranolol to families of severely affected patients.

In a retrospective study, we have found that propranolol was effective in 5 out of 6 lymphatic malformations patients (2 out of 2 patients with lymphedema) with a dose of 0.75mg/kg/day-1mg/kg/day (Wu, et al, *unpublished)*. LM patient symptoms improvement included reduced edema, infections, and need for transfusions without any obvious adverse events (unpublished data). However, we are at the lower end of propranolol dosing, when compared to other vascular anomalies[19, 20]. In fact, in one lymphedema patient, symptoms reappeared when the daily propranolol dose was reduced in half. In the other lymphedema patient, the girth of his lower extremities increased and lymphedema recurred to pre-treatment levels after propranolol discontinuation. Thus, we find that propranolol in the dose window of 0.75mg/kg/day-1mg/kg/day improves lymphedema symptoms, but is not curative. It is possible that increasing the propranolol dose will further improve the lymphedema and minimize morbidities associated with this condition. In pediatric patients, 4mg/kg/day of propranolol can be safely used for the treatment of arrhythmias such as supraventricular tachycardia[3, 4]. Our goal of the trail is to optimized propranolol dosing to treat our lymphedema patients.

The initial positive experience with this therapy, and its lack of apparent toxicity, suggest that systematic evaluation of the effect of propranolol in patients with lymphedema may provide a useful addition to the therapeutic armamentarium.

In the United States, most lymphedema patients are monitored for response to therapy with clinical examinations. Therefore, establishment of standardized, validated tools to stage severity of lymphedema, as well as treatment response, is urgently needed as well to test the efficacy of any proposed treatment method. In addition to the currently used weight, BMI, and clinical examinations, we have proposed to collaborate with Dr. Dympna Gallagher, as expert is using MRIs to quantify adiposity and edema in patients[21-23] to monitor treatment response in our study patients (details on assessment below). Therefore, as an endpoint in the second aim of our study, we will use imaging with MRIs to evaluate treatment response. In addition to imaging studies, we also propose to study circulating biomarkers and genetic profiling of lymphedema tissues and correlate with treatment response.

## 2.2 **Preclinical Studies**

### 2.2.1 Anti Lymphatic Malformation Activity

Our laboratory has used a cytotoxicity assay to screen for candidate molecules for use against LMPCs and LMECs. Our results demonstrated that propranolol was cytotoxic against both LMPCs and LMECs with an LD50 300-500μm. At lower concentrations (50-100μm), propranolol had anti-proliferative effects on both LMPCs and LMECs, and in LMPCs upregulated transcript expression of lymphatic endothelial differentiation genes (Prox1, podoplanin, VEGFR-2, VEGFR-3, and LYVE1) in a dose-dependent manner (range 10-100μm). When LMPCs were injected into nude mice, the development of dilated lymphatic channels was disrupted in mice that were treated with propranolol (6mg/kg/day) when compared to control mice. In mice the improved lymphatic vessel phenotype correlated with reduced infiltration of inflammatory macrophages. Thus, both *in vitro* and *in vivo* pre-clinical data demonstrated propranolol was efficacious against LM formation and LM physiology.

### 2.2.2 Animal Toxicology (if applicable)

Preliminary results in our laboratory treating immunosuppressed mice in our

murine LM model demonstrated that these mice tolerated up to 40mg/kg/day of propranolol by echocardiography. Based on general clinical observations, these mice continued to behave normally, without signs of weight loss, lethargy, or mortalities. Propranolol has been used in other murine studies with no reported toxicities or intolerance reported[24, 25]. We have found that 6mg/kg/day, a dose far below the 40mg/kg/day, in a mouse model was sufficient to improve LM vessel abnormalities. Using equivalent surface area dosage conversion factors, 6mg/kg/day in a mouse correlated to 3.3-4.8mg/kg/day, higher than the 0.7-1mg/kg/day we used to treat our patients (https://ncifrederick.cancer.gov/lasp/acuc/frederick/Media/Documents/ACUC42.pdf, accessed Nov 7, 2015)[26, 27].

2.2.3 Preclinical Pharmacokinetic Studies

Preliminary *in vitro* studies performed in our laboratory has shown that propranolol had anti-proliferative effects in "low-dose" range (in the nanomolar range: $10^{-9}$ to $10^{-7}$M). However, as propranolol dosage is increased, it exhibits cytotoxic effects on cells (in the micromolar range: $10^{-6}$ to $10^{-4}$M). The anti-proliferative effects were also accompanied by a does-dependent decrease in cAMP activity. The cytoxic dose range coincided with increased MAPK activation (ERK 1/2). Our results are consistent with other reports that propranolol had partial agonist effects with MAPK activation [Azzi, Baker, Galandrin].

The dose range of propranolol used to treat infantile hemangiomas ranged from 1-3mg/kg/day. This correlates to plasma concentration of 21-448ng/mL (private communication, Dr. Christine Cazeau) corresponding to $10^{-6}$ to $10^{-5}$M dose range. In adults given 0.5-1mg/kg/day, an average of 50ng/mL was achieved[28, 29]. This correlates to approximately $10^{-6}$M range. We propose to study patient responses at 2- to 3-mg/kg/day, corresponding to $10^{-6}$ to $10^{-5}$M, within the $10^{-6}$ to $10^{-4}$ anti-proliferative dose range.

## 2.3 Pediatric Studies

2.3.1 Prior Experience in Children

Recent reviews of the activity of propranolol in children with vascular anomalies report apparent efficacy in infantile hemangioma[19, 20, 30], and anecdotal experience with lymphatic malformations of the tongue and other anatomic locations[15-18]. The biologic basis for these effects is not known, but is hypothesized to relate to role of beta-adrenergic receptors in regulation of angiogenic factor expression[31-35]. Our pilot biologic studies of cells derived from lymphatic malformations suggest that beta-adrenergic signaling functions as survival pathway in both lymphatic progenitor and lymphatic endothelial cells.

2.3.2 Risk Assessment

2.3.2.1 Hypotension and Bradycardia:

Studies have shown that the peak effect of oral propranolol on blood pressure and heart rate occurs 1 to 3 hours after it is taken[28, 29]. As such, current recommendations are that patients should have HR and BP done at baseline and also hourly for the first 2 hours after a dose increase, and at least once after the target dose is achieved. As dose response is generally most significant after the first dose, once normal vitals are obtained with the first dose there is no recommendation that vitals should be re-checked at the same dose level, unless the patient is very young, has cardiovascular issues or co-morbidities, or if clinical complaints arise.

Pediatric patients must have vitals within the normal range for their age group, and adults with normal established vitals, prior to starting a dose. Dose escalations will take

place as given in the table above. Patient must have no evidence of clinical contraindications with acceptable HR and BP parameters prior to dose escalation occurring. If individuals are found to have a fall in their blood pressure or heart rate outside of normal range, they will have dose de-escalation to the prior tolerated dose level. If they are able to tolerate this lower dose level with normal parameters and no clinical symptomatology they will have a re-challenge of the dose escalation after 2 weeks. If on re-escalation they once again have fall in HR and blood pressure, they will again undergo dose de-escalation to the prior tolerated dose level for 2 weeks and re-evaluated again until the end of the 8-week study. If the patients are already at the lowest doses, they will be given a 2-week rest and re-evaluated after 2 weeks. If HR and BP then fall within normal parameters they will be reintroduced to the study at the lowest study dose of propranolol. If patient is once again found to have fall in HR/BP below normal parameters for age upon an attempt to re-start propranolol, they will be removed from the study. That is, all patients who do not tolerate a dose increase for the 2$^{nd}$ time will be held at the last tolerated dose until the end of the study. If a patient cannot tolerate the lowest doses twice, they will be removed from the study.

2.3.2.3 Hypoglycemia:

Propranolol has been reported to cause hypoglycemia in infants treated with propranolol. While these data is not established in old patients, all study patients will be encouraged to keep a regular food schedule and snack if they feel fatigue or dizzy.

If patients become ill for other reasons during the study period (eg URIs, gastroenteritis) during the study period, they are encouraged to contact us. Their propranolol dose will be held until they are able to tolerate POs.

2.3.2.4 Bronchospasm:

Patients will be monitored for respiratory symptoms such as coughing, wheezing, or shortness of breath.

2.3.2 Pharmacology/Pharmacokinetics/Correlative Biological Studies

Propranolol is well absorbed after oral administration. It is lipophilic and subject to significant first-pass hepatic metabolism so that only approximately 25% of propranolol reaches the systemic circulation. Peak blood levels occur at about 1-4 hours after an oral dose. Multiple metabolic pathways are involved in propranolol elimination (including those in the cytochrome P450 system).

## 2.4 Overview of Proposed Lymphedema Study

After patients have been registered, screened, and consented (Eligibility Screening Worksheet, Registration Form Consent form, HIPAA authorization form, Appendix 1), they will be entered into the study. Propranolol as a single agent will be administered orally, equally divided, two times a day (BID). Patients may receive up to 8-weeks of therapy in the absence of progressive disease or unacceptable toxicity. Treatment response will be assessed by patient self-reported symptoms and questionnaires (Appendix 2), physical exam, and radiograpically by MRI.

The starting dose of propranolol will be 0.5 mg/kg/day, which is the current institutional dose for initial treatment for cardiovascular and hemangioma indications in children. Patients will be weighed and their weight will be rounded to the closest 10kg (eg 50-54kg = 50kg, 55-59 = 60kg).

**Table 1**

**Table 1. Propranolol intra-patient dose escalation schedule and assessments**

| | Propranolol | Assessments | | |
|---|---|---|---|---|
| | Dose Schedule* | Subjective | Objective | Other |
| Week 1 (initiation) | 0.5 mg/kg/day, divided bid | Enterance Interview Quality of Life Questionnaire | Physical Exam, Imaging | Blood Draw, Tissue Biopsy |
| Week 2 | 1 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | |
| Week 4 | 2 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | Blood Draw |
| Week 6 | 3 mg/kg/day, divided bid[a,b] | Quality of Life Questionnaire | Physical Exam | |
| Week 8 | 3 mg/kg/day, divided bid[a,b] | Exit Interview, Quality of Life Questionnaire | Physical Exam, Imaging | Blood Draw, Tissue Biopsy |

*Weight rounded to the nearest 10-kg increment

[a]Dose escalation in the absence of clinical contraindications.

[b]In the presence of clinical contra-indications, the patients will remain on their current level of dosage, or decreased to the previous level, or have their medications held.

A maximum of 8-weeks of therapy may be administered

Studies have shown that the peak effect of oral propranolol on blood pressure and heart rate occurs 1 to 3 hours after it is taken. As such, current recommendations are that patients should have HR and BP done at baseline and also hourly for the first 2 hours after a dose increase, and at least once after the target dose is achieved. As dose response is generally most significant after the first dose, once normal vitals are obtained with the first dose there is no recommendation that vitals should be re-checked at the same dose level, unless the patient is very young, has cardiovascular issues or co-morbidities, or if clinical complaints arise.

Patients must have vitals within the normal range for their age group prior to starting in the study. After the first dose (0.5mg/kg/day), they will be re-evaluated. If their heart rate (HR) and blood pressure (BP) are within normal limits (WNL), they will continue on this dose for the next week until the next evaluation. If there is a fall below normal range (>15%) of HR and BP, they will rest off medications for 2 weeks and re-challenged. If they tolerate the dose, they will continue on the study. If they cannot tolerate the lowest dose for the 2nd time, they will be removed from the study, as they cannot tolerate the lowest dose.

At the 2nd-week re-evaluation, they will be escalated to the new dose (1mg/kg/day) if the baselines HR and BP are WNL. Patients will have HR and BP noted after first new dose (1mg/kg/day) of propranolol to ensure that the propranolol is tolerated. If the HR and BP are WNL, they will continue on this new dose and re-evaluated in 2 weeks for the 4th-week evaluation. If they have a drop in HR and BP to below normal range, they will be de-escalated and remain on the prior dose (0.5mg/kg/day) until the next visit (week 4).

At the 4th and 6th-week evaluation, their baseline vitals will be evaluated and their propranolol escalated to the next dose (if vitals are WNL) and have their HR and BP assessed after first new dose. Propranolol will stay increased or held at old dose depending on HR and BP after the first new dose as described above. If on re-evaluation in 2 weeks a patient continues to have below normal HR and blood pressure on re-challenge of the escalated dose, they will not be escalated and remain at the last tolerated dose for the remainder of the 8 week period with no further re-challenges. For patients with below normal HR and BP and needed their doses held, they will be reported as AEs.

At each study visit, patients will be asked to complete validated questionnaire to assess for tolerance to propranolol treatment, subjective changes in lymphedema symptoms, and self-reported function and activities of daily living (SF-36, LYMPH—ICF, and LYMPH—ICF—LL) (See attached documents, Appendix 2). Physical examination will include weight and BMI, vitals (HR, BP), and measurement of girth of normal and affected extremity.

MRIs and blood tests will be performed at study entry and study endpoint to assess for changes in lymphedema tissue composition[21-23] in response to propranolol.

Blood draws will be performed at study entry, at the midpoint, and study endpoint to assess for changes in circulating biomarkers in response to propranolol treatment.

Correlative biomarkers assessed will include markers of progenitor, endothelial, lymphatic, macrophage, and adipocyte cells, and expression of beta-adrenergic receptors (Table 3).

Patients will also receive punch biopsies at study entry and at study endpoint to assess for expression of biomarkers. Genetic studies will be performed from tissues obtained at study entry to evaluate for presence of PIK3CA mutation[36, 37] and correlated to response to propranolol. Genomic data generated will be deposited into an NIH database per NIH regulations (de-identified).

In the context of this study, we will perform immunohistochemical analyses on biopsied tissues to assess alterations in lymphatic vessel/channel formation, and changes in inflammation (Table 3). We have selected markers that we have previously shown to be altered by propranolol either in isolated LMPCs in culture or in the mouse model. Expression of the beta-adrenergic receptors will be assessed, as propranolol is a pan-beta-adrenergic receptor antagonist. To date, no tissue nor circulating molecular markers have been identified to correlate with LM patient improvement. Identification of such markers could provide an important benefit to affected patients. Morphological changes in the lymphatic vasculature will also be quantified using ImageJ software. Student's T-test will be employed to determine significance, with $p < 0.05$ considered significant.

**Table 3. Markers to be probed and assessed in blood and tissues**

| | |
|---|---|
| Progenitor cell markers | CD133, CD45, podoplanin, CD90, NG2 |
| Lymphatic endothelial cell markers | VEGFR-2, VEGFR-3, podoplanin, Prox1, LYVE1 |
| Beta-adrenergic receptors | Beta-1, beta-2, beta-3 adrenergic receptors |
| Macrophage markers | CD68, iNOS, F4/80 |

These analyses have been optimized and validated using discarded de-identified involved and uninvolved LM specimens. Fixed frozen tissue will be prepared and 5-micron sections generated. Standard immunohistochemistry will be performed with commercially available antibodies and H&E staining performed. Results will be captured by microscopy and digital camera. Results will be quantified using ImageJ software and *Student's t-test* to determine significance, with a $p < 0.05$ considered significant. We have already isolated similar specimens from > 10 patients and have obtained consistent results using these methodologies.

Analysis of circulating biomarkers will also be done. Identification of pertinent circulating biomarkers of a positive response will aid in future studies and minimize the need for more invasive procedures. We will use FACS to determine if LMPCs double positive for podoplanin and CD90 are in circulation. Expression of circulating VEGFR-2 and VEGFR-3 will also be assessed as it has been reported that propranolol reduced circulating VEGFR-3 levels in a patient with a generalized lymphatic anomaly[18].

## 3.0 SCREENING AND STUDY ENROLLMENT PROCEDURES

### 3.1 General Guidelines

Eligible patients will be registered on-study at Columbia University by the Study Coordinator. The required forms (Eligibility Screening Worksheet and Study Registration Form) can be found in Appendix 1.

**Following registration, patients should have baseline evaluations (physical examination, EKG, MRI, blood draw and tissue biopsy—see Table 8) before beginning protocol treatment within 14 days.** Issues that would cause treatment delays should be discussed with the Principal Investigator. If a patient does not receive protocol

therapy following registration, the patient's registration on the study may be canceled. The Study Coordinator should be notified of cancellations as soon as possible.

### 3.2 Patient Registration

To register a patient, the following documents should be completed by the research nurse, PI, or co-Investigator and faxed [212-305-9626] or e-mailed [jw92@cumc.columbia.edu] to the Study Coordinator/Data Manager:

- Copy of required laboratory and radiology tests
- Signed patient consent form
- HIPAA authorization form
- *Other appropriate forms (*e.g.*, Eligibility Screening Worksheet, Registration Form)*

To complete the registration process, the Study Coordinator will:

- Assign a patient study number.
- Register the patient on the study.

## 4.0 PATIENT ELIGIBILITY

Baseline evaluations are to be conducted within 2 weeks (14 days) prior to start of protocol therapy. In the event that the patient's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next dose escalation of propranolol.

**Important note: The eligibility criteria listed below are interpreted literally and cannot be waived. All clinical and laboratory data required for determining eligibility of a patient enrolled on this trial must be available in the patient's medical or research record which will serve as the source document for verification at the time of audit.**

### 4.1 Inclusion Criteria

4.1.1 Age: Patients must be ≥ 12 years of age at the time of study enrollment.

4.12a Diagnosis: Patients must have a clinically, pathologically, and/or radiographically diagnosed (lymphscintogram or MRI) lymphedema affecting an extremity without prior surgical intervention or major trauma at that extremity.

4.1.3 Disease Status: Patients must have either measurable or evaluable disease (see Sections 12.2 and 12.3 for definitions).

4.1.4 Therapeutic Options: Patient's current disease state must be one for which there is no known curative therapy or therapy proven to prolong survival with an acceptable quality of life.

4.1.5 Performance Level: Karnofsky ≥ 50% for patients > 16 years of age and Lansky ≥ 50 for patients ≥ 12 to ≤ 16 years of age. Patients who are unable to walk because of paralysis, but who are up in a wheelchair, will be considered ambulatory for the purpose of assessing the performance score.

4.1.6 Prior Therapy

Prior therapy may have included surgery or interventional radiology procedures,

but must have occurred > 4 weeks prior to baseline imaging studies and study entry. Prior therapy may have included other investigational drugs (e.g. sirolimus, sildenafil), since there are no standard medical therapies for lymphedema, but must have concluded > 4 weeks prior to baseline imaging studies.

4.1.7 Organ Function Requirements
These are required for enrolled patients with LMs.

4.1.7.1 Adequate Bone Marrow and Renal Function Defined as:

**Table 4. Organ Function Criteria**

| | |
|---|---|
| Hemoglobin | ≥ 8 mg/dl |
| Creatinine | Within normal limits (<1.2) |
| | OR |
| Creatinine Clearance | ≥ 60 mL/min/1.73 $m^2$ for patients with creatinine levels above institutional normal |

4.1.7.4 Adequate Cardiac Function Defined as:
- Baseline heart rate and blood pressure on physical exam within age-defined normal ranges*
- Normal cardiac rhythm by electrocardiogram
- Normal intracardiac anatomy, pericardial space, and function by echocardiogram (if indicated by screening cardiologist)

*See Appendix 7.

4.1.7.5 Adequate Pulmonary Function Defined as:
- No evidence of dyspnea at rest, no exercise intolerance, and a pulse oximetry > 94% if there is clinical indication for determination.

4.1.8 Pregnancy: A negative pregnancy test must be obtained in girls who are post-menarchal. Males or females of reproductive potential may not participate unless they have agreed to use an effective contraceptive method.

4.1.9 Informed Consent: All patients and/or their parents or legal guardians must sign a written informed consent. Assent, when appropriate, will be obtained according to institutional guidelines.

## 4.2 Exclusion Criteria

4.2.1 Patients with secondary lymphedema.

4.2.2 Patients who have had surgery or interventional radiology treatment within 4 weeks prior to baseline imaging studies and/or study entry, or those who have not recovered from these interventions.

4.2.3 Patients who are receiving any other investigational agents.

4.2.4 Patients with known contraindications to propranolol therapy, or a history of allergic, hypersensitive, or adverse reaction to propranolol.

4.2.5 History of allergic reactions attributed to compounds of similar chemical or biologic composition to propranolol.

4.2.6 Patients requiring treatment with drugs known to interact with propranolol including those with other cardiovascular drugs (e.g. antiarrhythmics, calcium channel blockers); migraine drugs; theophylline; neuroleptic, anti-ulcer, and lipid-lowering drugs; and warfarin.

4.2.7 Patients must be able to swallow capsules or liquid.

4.2.8 Patients who in the opinion of the investigator may not be able to comply with the safety monitoring requirements of the study are not eligible.

4.2.9 Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection; asthma, reactive airway disease, or bronchospasm requiring medication; ulcers or nonhealing wounds of the lymphatic malformation; symptomatic congestive heart failure, pericardial effusion, cardiac arrhythmia; or psychiatric illness/social situations that would limit compliance with study requirements.

4.2.10 Other contraindications including pheochromocytoma, bradycardia, first degree heart block, decompensated heart failure.

## 5.0 TREATMENT PROGRAM

### 5.1 Overview of Treatment Plan

Patients will be provided with propranolol prescriptions. Dosing is weight-based (corrected to the closest 10kg increment) and will be prescribed by the PI after medical evaluation by the study cardiologist.

The starting dose of propranolol will be 0.5 mg/kg/day, which is the current institutional dose for initial treatment for cardiovascular and hemangioma indications in children. Dose escalation to 1 mg/kg/day will take place at the 2-week evaluation in the absence of clinical contraindications. If patient tolerates 1 mg/kg/day dose, they will be escalated to 2 mg/kg/day at the 4-week evaluation. In the absence of clinical contraindications, dose escalation will be increased to 3mg/kg/day at week 6 and continued for the duration of the study. A maximum of 8-weeks of therapy may be administered.

### 5.2 Dose Escalation Schema

5.2.1 Inter-Patient Escalation

There is no inter-patient escalation allowed on this study.

5.2.2 Intra-Patient Escalation

The starting dose will be 1 mg/kg/day (BID) with dose escalation up to 3 mg/kg/day (BID) in the absence of clinical contra-indications. There will be no escalations beyond 3 mg/kg/day (Table 1).

At the return visit, patients must have no evidence of clinical contraindications with normal HR and BP parameters prior to dose escalation occurring. They will be re-evaluated 2 hours after the first escalated dose. If individuals have normal heart rate and blood pressure and report no clinical symptoms (dizziness, increased fatigue), they will be given the new escalated dose and re-evaluated 2 hours after the new dose. If their HR and BP are within normal limits (WNL), they will be sent home on this new escalated dose and will return in another 2 weeks for evaluation. If individuals are found to have a fall in their blood pressure or heart rate below the normal value at 2 hours after the new dose, they will not be escalated and will be sent home at the current dose. They will return in 2 weeks for evaluation for dose escalation.

If individuals are found to have a fall in their blood pressure or heart rate below the normal value at the return visit *and* clinically asymptomatic, they will held at the current tolerated dose level (no escalation). If they are able to tolerate this lower dose level with normal parameters and no clinical symptomatology they will return for evaluation of dose escalation at the next visit. If their parameters are WNL, they will be escalated and evaluated. If they do not tolerate dose escalation, they will be held at the current tolerated dose of propranolol until the end of the study without further attempts to escalate. Patients who are not able have their dose escalation at 2 consecutive visits will have no more dose escalation challenges and stay for the remainder of the study at the current tolerated dose.

Individuals found to have a fall in HR and BP below normal parameters at initiation (0.5mg/kg/day, week 1) will be removed from the study for 1 week. At week 2 they will be re-evaluated. If HR and BP are within normal parameters they will be reintroduced to the study. If patient is once again found to have fall in HR/BP below normal parameters upon an attempt to re-initiate (failed the lowest intiation dose of 0.5mg/kg/day twice), they will be removed from the study.

### 5.3 Grading of Adverse Events

Adverse events (toxicities) will be graded according to the NIH's Common Terminology Criteria for Adverse Events v4.0 (CTCAE):
Grade 1 Mild AE
Grade 2 Moderate AE
Grade 3 Severe AE
Grade 4 Life-threatening or disabling AE
Grade 5 Death related to AE
Any life-threatening toxicity (Grades 4 and 5) should be reported immediately (within 24 hours) to the PI and to the Data Safety Monitoring Board.

## 6.0 DOSE MODIFICATIONS FOR ADVERSE EVENTS

**The Study Chair must be notified of any dosage modification.**

### 6.1 Dose Modifications for Potential Propranolol Specific Side Effects

#### 6.1.1 Hypotension and Bradycardia

**Baseline blood pressure (BP)** is defined as the blood pressure obtained at the examination used for study enrollment. This baseline BP should be obtained as follows: 1) Obtain 3 serial blood pressures from the same extremity with the patient in the same position that are separated by at least 5 minutes. Avoid using the lower extremity if possible. BP will not be obtained from the affected limb. 2) Average the systolic blood pressure from the 2nd and 3rd measurements. 3) Average the diastolic blood pressure from the 2nd and 3rd measurements. 4) The baseline BP is the average of the systolic over the average of the diastolic measurements.

Heart rate will be measured by palpation of the radial pulse serially from the same extremity for 15 secs, separated by at least 5 minutes. In the case of extremity LMs, pulse rate will not be obtained from the affected limb. 2) Average the pulse rate from 2nd and 3rd measurements.

6.1.1.1 Patient must have no evidence of clinical contraindications with normal HR and BP parameters prior to dose escalation occurring. If individuals are found to have a fall in their blood pressure or heart rate to below normal limits, but asymptomatic:

a. patient will be not have dose escalation
b. patient will be evaluated for dose escalation at the next 2-week cycle.
c. if HR and BP fall below normal ranges and patient is symptomatic, propranolol will be held for 2 weeks, and re-introduced at the next visit at the lowest tolerated dose.
d. if patient is at the lowest dose already (0.5mg/kg/day), they will be pulled from the study and re-evaluated at the next visit.
c. Patients receiving 0.5mg/kg/day who experience HR or BP decrease below the normal range at 4 weeks must be taken off protocol therapy.

Table 5. Side effects and propranolol dosing guidelines

| **Hypotension and Bradycardia** | Propanolol Dosing Guidelines |
|---|---|
| Within normative range | No change in dose |
| Below normal range, asymptomatic | Cardiology evaluation, EKG, echocardiogram; if cardiac status is acceptable, patient will not have dose escalation and remain at current dose level with subsequent re-evaluation at 2 weeks |
| Below normal range, symptomatic | Discontinue propranolol (off protocol therapy. Re-evaluate after 2 weeks. If HR and BP then fall within normal parameters they will be reintroduced to the study with dose de-escalation to the prior tolerated dose of propanolol. If HR/BP below normative range at 0.5mg/kgday, patient will be removed from study |

| **Hypoglycemia** | **Management/Next Dose for** propranolol |
|---|---|
| Blood glucose >80 mg/dl | No change in dose |
| Blood glucose <80, >60, asymptomatic | Clinical evaluation, review feeding schedule, repeat test after more |

| **Hypoglycemia** | **Management/Next Dose for** propranolol |
|---|---|
| | frequent/supplemental feeds |
| Blood glucose <80, symptomatic | Discontinue propranolol (off protocol therapy) |

| **Bronchospasm** | **Management/Next Dose for** propranolol |
|---|---|
| Transient/self-limited episode | Clinical evaluation, may continue at same dose |
| Associated with URI | Hold propranolol till URI resolves; if >7 days, off protocol |
| Not associated with URI, persistent or requiring new medical treatment | Discontinue propranolol (off protocol therapy) |

## 7.0 SUPPORTIVE CARE AND OTHER CONCOMITANT THERAPY

### 7.1 Concurrent Anti Lymphatic Malformation Therapy

Concurrent lymphatic malformation therapy, including surgery, immunotherapy, or biologic therapy may NOT be administered to patients receiving study drug >4 weeks prior to study initiation and during the study period. If these treatments are administered the patient will be removed from protocol therapy.

### 7.2 Investigational Agents

No other investigational agents may be given while the patient is on study.

### 7.3 Supportive Care

Patients with lymphedema should continue to receive appropriate supportive care, including compression garments, massage, and protective skin care dressings/preparations (emollients or barrier agents that protect involved skin).

### 7.4 Concomitant Medications

Patients who require treatment with oral antibiotics for minor soft tissue infections may continue to receive these during the study. Antibiotics prescribed to be determined by infectious disease specialist, based on the patient's particular past history of infections and potential involved flora. If the patient is determined to need hospital admission for IV antibiotics, the patient will be removed from the study.

## 8.0 EVALUATIONS/MATERIAL AND DATA TO BE ACCESSIONED

### 8.1 Required Clinical, Laboratory and Disease Evaluation

Baseline evaluations are to be conducted within 2 weeks prior to start of protocol therapy. Scans and x-rays must be done ≤4 weeks prior to the start of therapy. In the event that the patient's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next dose escalation of propranolol.

**Table 6. Study schedule and evaluations**

| STUDIES TO BE OBTAINED | Pre-Study | Wk 1 | Wk 2 | Wk 3 | Wk 4 | Wk 5 | Wk 6 | Wk 7 | Wk 8 |
|---|---|---|---|---|---|---|---|---|---|
| Informed consent | x | | | | | | | | |
| Demographics | x | | | | | | | | |
| Medical history | x | | | | | | | | |
| Questionnaires | x | x | x | | x | | x | | x |
| Physical exam | x | x | x | | x | | x | | x |
| LM measurements | x | x | x | | x | | x | | x |
| Vital signs | x | x | x | | x | | x | | x |
| Height | x | x | x | | x | | x | | x |
| Weight | x | x | x | | x | | x | | x |
| CBC w/diff, platelets | x | | | | x | | | | x |
| Cr | x | | | | x | | | | x |
| Blood glucose | x | x[a] | x[a] | | x[a] | | x[a] | | x[a] |
| EKG | x | x[a] | x[a] | | x[a] | | x[a] | | x[a] |
| Echocardiogram[a] | x | | | | | | | | |
| serum β-HCG for females of childbearing potential | x | | | | | | | | |
| Adverse event evaluation | x | x | x | x | x | x | x | x | x |
| Skin Biopsies | x | | | | | | | | x |
| CXR[a] | x | | | | | | | | |
| MRI | x | | | | | | | | x |
| QMR | x | | | | | | | | x |
| Body water content evaluation | x | | | | | | | | x |

[a]If clinically indicated after initial evaluation

## 8.2 Correlative Studies

### 8.2.1 Description of Studies

Correlative biology studies will include markers for progenitor, endothelial, lymphatic, macrophage, and adipocyte identity, and expression of beta-adrenergic receptors.

### 8.2.2 Sampling Schedule

In patients with skin lesions on the affected extremity, skin biopsy samples will be collected at study entry and at the two-month evaluation point (Week 8).

### 8.2.3 Sample Labeling

Each biopsy specimen must be labeled with the patient's study registration number and the date and time the sample was taken. Data should be recorded on the Correlative Study Form (Appendix 1).

## 8.3 Radiology Review

MRIs to be reviewed for comparison and analysis by Dr. Dympna Gallagher.

## 8.4 Statistical Analysis

Descriptive statistics will be used to summarize patient characteristics, toxicity data, response data and biomarker expression. Toxicity rates at each dose tested will be presented with 95% exact confidence intervals. Responses will be evaluated among patients treated at the highest tolerable dose. Continuous outcomes will be summarized using mean ± standard deviation or median (interquartile range) for non-normal data. Comparisons between groups and response changes from baseline will be evaluated using two-sided t-tests or the non-parametric equivalent Wilcoxon Rank-Sum test (for non-normal data). Association between PIK3CA mutational status and patient response will be assessed using chi-square/Fisher exact test. All analyses will be performed using a type I error of 0.05. Biostatistics will be done using Irving Institute for Clinical and Translational Research statistical services at Columbia University Medical Center.

# 9.0 AGENT INFORMATION

## 9.1 Drug Name

Propranolol (Inderal, Inderal LA, Innopran XL)

### 9.1.1 Structure

Propranolol hydrochloride (2-Propanol, 1-[(1-methylethyl)amino]-3-(1-naphthalenyloxy)-, hydrochloride is a synthetic, nonselective antagonist of beta-adrenergic receptors. It has been used extensively in pediatrics for more than 40 years, principally for cardiac indications (e.g. supraventricular tachycardias) but more recently for other indications. In particular, broad experience with propranolol for the treatment of pediatric hemangiomas has recently emerged, after the initial serendipitous observation of its efficacy in this condition[30].

9.1.2. Supplied by: Commercially available

9.1.3 Formulation
Commercially available.

9.1.4 Storage
Patients will obtain propranolol from commercial pharmacy.

9.1.5 Solution Preparation
N/A.

9.1.6 Administration
Propranolol will be administered orally, either as a tablet or as a suspension, in equal divided doses two times a day.

For children (12-18 years of age): Parents will be informed of the signs of hypoglycemia, hypotension, and bradycardia.

9.1.7 Toxicities
A list of those adverse events encountered in treatment of infantile hemangioma is included below with estimates of overall frequency provided in the recent review by Drolet et al.[19]. Because infantile hemangiomas are vascular anomalies of childhood, like lymphatic malformations, we believe these may best represent adverse events that would affect the patients in our study.

Table 7. Known toxicities of propranolol

| **Known Toxicities** | **Frequency** |
|---|---|
| Sleep disturbance | 3.7% |
| Asymptomatic hypotension | 2.8% |
| Somnolence | 2.2% |
| Bronchopulmonary symptoms (wheezing, breathing disturbance) | 1.9% |
| Cool or mottled extremities | 1.7% |
| Asymptomatic bradycardia | 0.9% |
| Hypoglycemia | 0.9% |
| Diarrhea | 0.8% |
| Gastroesophageal reflux | 0.7% |

Detailed pharmacologic data is available on the FDA website: http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/016418s080,016762s017,017683s008lbl.pdf.

## 10.0 CRITERIA FOR REMOVAL FROM PROTOCOL THERAPY AND OFF STUDY CRITERIA

10.1 **Criteria for Removal from Protocol Therapy**

a) Intercurrent illness that prevents further administration of treatment.
b) Adverse Events requiring removal from study (See Section 6.0).
c) Refusal of further protocol therapy by patient/parent/guardian

d) Non-compliance that in the opinion of the investigator does not allow for ongoing participation.
e) Completion of 2 months of therapy.
f) Physician determines it is not in the patient's best interest.

Patients who are off protocol therapy are to be followed until they meet the criteria for Off Study (see below). Ongoing adverse events, or adverse events that emerge after the patient is removed from protocol therapy, but within 30 days of the last dose of investigational agent, must be followed until resolution or stabilization of the adverse event. Follow-up data will be required unless consent is withdrawn.

10.2 **Off Study Criteria**

a) Completion of 10 months of follow-up after the last dose of the investigational agent.
b) Death
c) Lost to follow-up
d) Withdrawal of consent for any further data submission
e) Enrollment onto another anti-lymphatic malformation study

## 11.0 STATISTICAL AND ETHICAL CONSIDERATIONS

11.1 **Sample Size and Study Duration**

**Sample Size:** Based on our current clinical volume, we anticipate a total enrollment of 15-20 (this is just an example to show a range) patients over a period of 24 months.

**Accrual Rate:** We anticipate that 8-10 patients per year will be accrued to the study.

**Accrual Targets:** LMs affect the general population without known differences in genders or ethnicities. Therefore, we expect accrual to reflect the makeup of the population in the region(s) surrounding our institution.

11.2 **Study Design/ Endpoints (Table 2)**

11.2.1 Study design: Each patient will serve as his/her own control, for comparison of study questionnaires (subjective) and with changes in the clinical measurements and imaging measurements of the anatomic area affected by lymphatic malformation compared before and after treatment (objective). In our experience, children exhibiting responses to propranolol displayed these within 2 weeks of beginning propranolol treatment, with estimated reduction in the overall volume of the affected area exceeding 20%.

11.2.2 Primary endpoints: The primary endpoint will be the tolerability of escalating dosage of propranolol both by subjective reporting (survey instrument) as well as objective measurements of vital signs. Survey instruments include a general quality of life (QOL) and function survey (SF-36), as well as validated survey instruments for patients with lymphedema (LYMPH—ICF, LYMPH—LCF—LL)[38, 39].

11.2.3 Secondary endpoints: The secondary endpoints of the study will be both subjective and objective evaluation of the lymphedema after propranolol treatment, as measured by both survey instrument and objective measurements (clinical exam and radiological studies), as well as biological assays from tissues obtained from biopsies.

11.2.3.1 We will determine whether higher doses of propranolol are well tolerated in this patient population. Secondarily, we will measure determine whether higher doses correlate with improvement in lymphedema symptoms and signs, and healing or improvement of skin lesions, if present (reduced lymphatic leakage, reduced bleeding). Skin lesions will be photographed at clinical visits, and daily lymphatic fluid losses estimated by weight measurements.

Using MRI and body composition analysis, we will determine whether propranolol decreases lymphedema, alters edema water content and location (intracellular vs. interstitial), or alters adiposity of affected extremity.

We will study the biologic activity of oral propranolol against lymphedema, by examining the expression of progenitor cell markers, lymphatic endothelial cell markers, beta-adrenergic receptor expression, macrophage markers and detection of adipocyte content of biopsy specimens (see table below). Results will be compared to ultrasound and magnetic imaging data.

## 11.3 **Analysis of Secondary Endpoints**

### 11.3.1 Biomarkers

In the context of this study, we plan to perform immunohistochemical studies to assess alterations in lymphatic vessel/channel formation, adipocyte differentiation, and changes in inflammation. We have selected markers that we have previously shown to be altered by propranolol either in isolated LMPCs in culture or in the mouse model (*unpublished*). Expression of the beta-adrenergic receptors will be assessed as propranolol is a pan-beta-adrenergic receptor antagonist. To date, no molecular markers have been identified to correlate with LM patient improvement. Identification of such markers could provide an important benefit to affected patients. Morphological changes in the lymphatic vasculature will also be quantified using ImageJ software. Student's T-test will be employed to determine significance, with $p < 0.05$ considered significant.

**Table 3. Markers to be probed and assessed in blood and tissues**

| | |
|---|---|
| Progenitor cell markers | CD133, CD45, podoplanin, CD90, NG2 |
| Lymphatic endothelial cell markers | VEGFR-2, VEGFR-3, podoplanin, Prox1, LYVE1 |
| Beta-adrenergic receptors | Beta-1, beta-2, beta-3 adrenergic receptors |
| Macrophage markers | CD68, iNOS, F4/80 |

The analyses we plan to perform on the specimens have been validated by our laboratory using discarded de-identified specimens[14]. Fixed frozen tissue will be prepared and 5-micron sections generated. Standard immunohistochemistry will be performed with commercially available antibodies and H&E staining performed. Results will be captured by microscopy and digital camera. Results will be quantified using ImageJ software and *Student's t-test* to determine significance, with a $p < 0.05$ considered significant. We have already isolated similar specimens from > 10 patients and have obtained consistent results using these methodologies.

We have comprehensive data on *in vitro* and *in vivo* analysis of 12 cells lines from patients with LMs. We have demonstrated consistently that LMPCs have unique characteristics by FACS analysis and gene expression profile analysis. They express mesenchymal stem cell markers CD90 and NG2, and can be differentiated into various terminal cell types including adipocytes, mural and endothelial cells. When implanted into immunocompromised mice, these LMPCs formed cystic structures that phenocopy lymphatic malformations. These features are reproducible between LMPC cell lines, and demonstrate consistent changes in gene and protein expression after treatment with propranolol.

We are expecting to recruit 20 patients into this study. Our assays are sufficiently sensitive to detect 5-10% differences in proliferation rate and gene and protein expression changes.

11.3.2 Imaging Studies

Lymphedema MRI characteristics before and after propranolol therapy will be assessed and related to clinical outcome, as applicable. Specifically, whole body MRI will be used to evaluate body composition and changes in regional adipose tissue depots in the affected limb[21-23].

Furthermore, changes in body composition (water and fat content) in response to propranolol treatment will be evaluated in this study. To evaluate water content, the standard deuterium dilution technique will be used. Fasting patients will have blood draw before drinking stable deuterium isotopes and sodium bromide and after isotope equilibration (about 3-4 hours). Total body water, extra-cellular and intracellular water content can be calculated[22, 40]. Furthermore, a quantitative magnetic resonance (QMR) system will be used to evaluate body (fat and water) content in response to propranolol treatment[21]. Quantification and analysis of results will be performed by Dr. Gallagher.

11.3.3 Patient-Reported Outcomes; Symptom Assessment etc.

Patients' self-reported tolerance to propranolol will be assessed using validated quality of life (QOL) health surveys (SF-36, Lymph-ICF, Lymph-ICF-LL) SF-36 is an accepted general health status survey[38, 39]. Lymph-ICF and Lymph-ICF-LL are validated, disease-specific survey instruments for patients with lymphedema that evaluate physical and mental activity, impairments in function and activity limitations related to lymphedema.

11.3.4 Genetic analysis

Primary lymphedema has been shown to be associated with genetic mutations. There is a mutation in flt4 (vegfr3) gene in Milroy's[8]. More recently, somatic mutations have been identified in another subset of patients with lymphatic malformations[36, 37]. Patient blood and tissues will also be sequenced using Next-Gen exome sequencing analyses to probe for de novo genetic mutations and screening for mutations in genes known to be associated with lymphedema (vegfr3, pik3ca) and correlated with treatment response to propranolol. Columbia University has core facilities available for these analyses. As these tests are performed under experimental and not clinical indications, individual results will not be shared with study subjects. However, once the studies are published, the published aggregate results can be made available to study subjects.

### 11.4 Ethical Considerations

Patients with LMs of the extremities may experience a range of complications, including pain, bleeding, infections, and functional limitation. These can result in a severe lifelong burden of disease, exacerbated by the social difficulties imposed by physical differences and school absence caused by acute illness. No validated medical therapies currently exist. Surgical and interventional treatments can improve and palliate disease, but are rarely curative. The identification of effective treatments for these patients is therefore urgent.

The recent development in our lab of model LMs *in vivo* and *in vitro* has provided a rational basis for identifying potential new therapeutic agents[14]. Our *in vitro* and *in vivo* studies are consistent with recent reports of propranolol efficacy in small groups of patients[15-18], and with our initial clinical experience. The systematic study of propranolol, which has a favorable risk profile in patients and a biologic basis for efficacy in pediatric patients with lymphatic malformations, would thus appear ethically justified.

### 11.5 Reporting and Exclusions

All of the patients who met the eligibility criteria (except those who received no propranolol) will be included in the main analysis of the response. All conclusions will be based on all eligible patients.

Sub-analyses may then be performed on the basis of a subset of patients, excluding those for whom major protocol deviations have been identified. However, these sub-analyses will not serve as the basis for drawing conclusions concerning treatment efficacy, and the reasons for excluding patients from the analysis will be clearly reported.

### 11.6 Inclusion of Children, Women and Minorities

Children (≥ 12 years) and adults of both gender and of all races and ethnic groups are eligible for this trial.

## 12.0 EVALUATION CRITERIA

### 12.1 Adverse Events

Any patient who receives the study drug will be evaluable for Adverse Effects. This study will utilize the NIH Common Terminology Criteria for Adverse Events v4.0 (CTCAE) for evaluating adverse events.

### 12.2 Disease Parameters

Measurable disease: Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as > 50 mm by clinical exam and MRI. All measurements must be recorded in millimeters (or decimal fractions of centimeters).

### 12.3 Methods for Evaluation of Measurable Disease

All measurements should be taken and recorded in metric notation using a ruler, measuring tape, or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment.

The same method of assessment and the same technique should be used to characterize

each identified and reported lesion at baseline and during follow-up.

Clinical lesions**:** The circumference of the affected (vs. unaffected) extremity will be documented in the patient's clinical chart along pre-determined landmarks), as this is a standard part of care for patients with extremity LM. Landmarks include: midway point between ASIS (anterior superior iliac spine) to the patella for the thigh, and midway point between patella to the medial malleolus for lower extremities. The midway point between the axillary crease to medial epicondyle (upper arm) and midway point between medial epicondyle to hamate bone of the wrist (forearm) for upper extremities.

MRI and QMR have excellent contrast, spatial, and temporal resolution. The same scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. While CT scans may give even improved spatial resolution, MRI has the advantage of no ionizing radiation, gives superior soft tissue resolution, and allows for multi-planar imaging.

12.4 **Response**

Patients should be re-evaluated for response by clinical examination every 2 weeks. Baseline MRI will be obtained, and an MRI repeated at 8 weeks. Response will be evaluated in this study using quality of life survey instruments, physical examination and radiologic studies (MRI at baseline and 8 weeks).

12.4.1 Definitions

Subjective Response: Patients who complete at least the questionnaires at baseline (study entry) and at the last study visit will be included for subjective evaluation.

Evaluable for Objective Response: by physical examination and radiological studies as outlined above.

## 13.0 ADVERSE EVENT REPORTING REQUIREMENTS

Adverse events (AEs) will be graded according to the NIH's Common Terminology Criteria for Adverse Events v4.0 (CTCAE) (Section 5.3):

Grade 1 Mild AE
Grade 2 Moderate AE
Grade 3 Severe AE
Grade 4 Life-threatening or disabling AE
Grade 5 Death related to AE

Any life-threatening (grades 4 and 5) adverse events (AEs) should be reported immediately (within 24 hours) to the PI and Data Safety Monitoring Board. All other AEs (grades 1-3) will be reported to the PI, the IRB, and the Data Safety Monitoring Board as soon as possible, but not later than five (5) workdays from first awareness of the problem. Significant AEs include, but are not limited, to:

a. any death, excluding death due to the progression of the disease, and
b. the combination of the following 3 conditions:
   i. Unexpected: any adverse experience that is not identified in nature, severity, or frequency in the consent form, and is not due to the progression of a disease process.
   ii. Serious: any event that is fatal or life threatening, is permanently or significantly

disabling, requires inpatient hospitalization or prolongation of hospitalization, or any medical event that requires treatment to prevent one of the medical outcomes listed above.
iii. Related/possibly related: as determined by the research team.

The event will be formally reported on the Significant Adverse Event Report Form (Medwatch 3500 Form, Appendix 1). All events that do not meet the criteria of significant or trends as listed in numbers 1 and 2 above, should be recorded in the summary form.

## 14.0 RECORDS, REPORTING, AND DATA AND SAFETY MONITORING PLAN

### 14.1 Data Safety and Monitoring

The frequency and severity of all toxicities and/or adverse effects will be tabulated on an ongoing basis and summarized for review under the Data Safety and Monitoring Plan for this study (DSMP) as per the guidelines for the Data and Safety Monitoring Committee of Columbia University. Data for this study will be reviewed and monitored by the PI, as this study is deemed minimal or no more than minor increased over minimal risk. All serious and/or unexpected events will be communicated to the Study Chair and reviewed within 2 working days for consideration of notification, amendment, or immediate study suspension.

### 14.2 Data and Safety Monitoring Plan

14.2.1 Monitoring mechanisms

It is the responsibility of the Principal Investigator to oversee the safety of the study at his/her site. This safety monitoring will include careful assessment and appropriate reporting of adverse events, as well as the construction and implementation of a site data and safety-monitoring plan. Medical monitoring will include a regular assessment of the number and type of serious adverse events.

Data and Safety Monitoring Board

The PI will oversee the conduct of this trial with an *ad hoc* Data and Safety Monitoring Board. The DSMB will review the progress of the trial following the enrollment of subjects and to ensure that patient safety is not being compromised. We have formed an ad hoc DMSB comprising of a vascular surgeon (Dr. Alan Bevenisty), a cardiologist (Dr Yi-ming Yang), and a pediatric hematologist with expertise in vascular malformations (Dr. Francine Blei).

Reporting of Adverse Events

All STEAEs should be recorded on a MedWatch 3500 Form and faxed to:

Columbia University/June K. Wu, M.D.
Fax: (212) 305-9626

AND

Columbia University Data and Safety Monitoring Committee of Columbia University
622 West 168th Street, PH-18 Room 200
New York, NY 10032
Telephone: (212) 305-8615
Fax: (212) 342-3036

MedWatch 3500 Reporting Guidelines:

In addition to completing appropriate patient demographic and suspect medication information, the report should include the following information within the Event Description (section 5) of the MedWatch 3500 form:

-Treatment regimen (dosing frequency, combination therapy)
-Protocol description (and number, if assigned)
-Description of event, severity, treatment, and outcome, if known
-Supportive laboratory results and diagnostics
-Investigator's assessment of the relationship of the adverse event to each investigational product and suspect medication

Follow-up information:

Additional information may be added to a previously submitted report by any of the following methods:

-Adding to the original MedWatch 3500 report and submitting it as follow-up
-Adding supplemental summary information and submitting it as follow-up with the original MedWatch 3500 form
-Summarizing new information and faxing it with a cover letter including subject identifiers (i.e. D.O.B. initial, subject number), protocol description and number, if assigned, suspect drug, brief adverse event description, and notation that additional or follow-up information is being submitted (The subject identifiers are important so that the new information is added to the correct initial report)

Assessing Causality:

Investigators are required to assess whether there is a reasonable possibility that propranolol caused or contributed to an adverse event. The following general guidance may be used.

*Yes:* if the temporal relationship of the clinical event to propranolol administration makes a causal relationship possible, and other drugs, therapeutic interventions or underlying conditions do not provide a sufficient explanation for the observed event.

*No:* if the temporal relationship of the clinical event to propranolol administration makes a causal relationship unlikely, or other drugs, therapeutic interventions or underlying conditions provide a sufficient explanation for the observed event.
For **Investigator Sponsored IND Exempt Studies**, there are some reporting requirements for the FDA in accordance with the guidance set forth in 21 CFR 314.80.

*Postmarketing 15-Day "Alert Report"*:
The Sponsor-Investigator is required to notify the FDA of any fatal or life-threatening adverse event that is **unexpected and assessed by the investigator to be possibly related to the use of propranolol**. An unexpected adverse event is one that is not already described in the Investigator Brochure. Such reports are to be submitted to the FDA (2 copies) at the following address: Central Document Room, 12229 Wilkins Avenue, Rockville, MD 20852.

For questions related to safety reporting, contact:

Columbia University/June K. Wu, M.D.
Fax: (212) 305-9626

AND

Columbia University Data and Safety Monitoring Committee of Columbia University
622 West 168th Street, PH-18 Room 200
New York, NY 10032
Telephone: (212) 305-8615
Fax: (212) 342-3036

Adverse Event Reporting Period
The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. However, any serious treatment emergent adverse events must be reported within 24 hours.

Post-study adverse event
All unresolved adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator should instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study. The investigator should notify the study sponsor of any death or adverse event occurring at any time after a subject has discontinued or terminated study participation that may reasonably be related to this study.

14.2.2 Frequency of monitoring

Patients will be monitored and assessed during their study visits (weeks 1, 2, 4, 6, 8). In addition, patients will be encouraged to contact the PI and/or study coordination if they have any issues, concerns, unexpected symptoms to be assessed.

14.2.3 Stop or change rules

Patients will be removed from study if they cannot tolerate the intiation dose of propranolol. Patients who failed 2 challenges to escalate to 1mg/kg/day (lowest study dose) will be pulled from the study. Patients who failed 2 challenges to escalate to subsequent doses will be kept at the highest tolerated dose without any further attempts to escalate through the study.

14.2.4 Plans for interim analyses and/or futility analyses

This study only lasts 8 weeks and no interim analyses nor futility analyses are planned.

14.2.5 Information to be monitored

a. Information to be monitored include assessment of data quality, timeliness and patient recruitment and retention, accrual and retention consistent with plans for diversity and generalizability.

b. A review of outcome and adverse event data to determine whether there is any change to the risk/benefit ratio of the study, if a stop rule has been invoked, or a study endpoint has been reached whether the study should continue as originally deisgned, be changed, or be stopped.

c. An assessment of external factors or relevant information (eg. developments in the literature, reults of related studies, etc) that may have an impact on the safety of pariticipants or on the ethics of the

research study.

14.2.6 Communication

The PI will be available to the study coordinator, study patients, and the DSMB by person, phone, or email (study coordinator and DSMB) for any concerns. Since this is an IND exempt study, the only communication with the FDA is for adverse event reporting (Postmarketing 15-day “Alert Report”)

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