CLINICAL PROTOCOL

A Proof of Concept Study to Determine the Effects of NOx and Conjugated Linoleic Acid on Asthmatics

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Version 11.0
December 11, 2018

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ABSTRACT

Obesity is an asthma comorbidity associated with increased severity, poor control, reduced steroid responsiveness and greater exacerbation and healthcare utilization rates. These associations are not explained by having a greater degree of Th-2 inflammation. Rather, the obese asthma phenotype defined in several cluster studies, has paradoxically reduced levels of Th-2 biomarkers, including sputum eosinophils and exhaled nitric oxide (NO). Our previous research has shown that the inverse relation between increased body mass index (BMI) and reduced exhaled NO, may be explained by a metabolic imbalance characterized by lower L-arginine and greater asymmetric di-methyl arginine (ADMA) levels. Having a low L-arginine/ADMA ratio has been shown to inhibit and uncouple all isoforms of nitric oxidase synthase (NOS), thereby reducing NO bioavailability and promoting oxidative stress through enhanced superoxide production. In obese asthmatics, this imbalance not only correlates with exhaled NO, but also with lower FEV1% and poorer asthma-related quality of life. Yet the effect of obesity in asthma is unlikely to be solely dependent on a single mechanism. Other factors, such as increased Th1 and Th-17-mediated inflammation have been shown to occur in human and animal models. Given all of these potential avenues, it is imperative that an intervention is sufficiently pleiotropic that can, in addition to restoring airway NO levels, also reduce other obesity-related non-Th2 mechanism of inflammation. We hypothesize that treatment with conjugated linolenic acid (CLA) + nitrate and nitrite (together known as NOx), will restore NO airway bioavailability, reduce oxidative stress and improve airway inflammation in obese asthmatics. To test this hypothesis, we propose a phase II pilot study in which obese asthmatics, will be treated orally with CLA+NOx for 8 weeks, in an open label study design to assess pre to post-intervention changes in airway and systemic biomarkers, and to determine the effects on lung function and bronchial hyperresponsiveness. Participants will undergo a pre and post intervention bronchoscopy. The results obtained from this project will be greatly informative to our understanding of the obese – asthma pathophysiology and for the development of clinical trials to determine the potential benefit of this intervention in improving health outcomes.

1 SPECIFIC AIMS

Obesity is an asthma comorbidity associated with increased exacerbation rates and greater healthcare utilization by mechanisms that are not explained by increases in Type 2 (Th2)-related biomarkers of airway inflammation. However, alterations in airway nitric oxide (NO) metabolism constitute a potential mechanistic pathway for obesity-mediated increases in asthma severity (1). We have shown a correlation between increasing BMI and lower L-arginine to asymmetric di-methyl arginine (ADMA) ratios in asthmatics (2). This imbalance leads to inhibition and uncoupling of airway epithelial inducible nitric oxide synthase (iNOS), resulting in lower exhaled NO (eNO) levels and greater airway oxidative stress, which could impair normal airway function and reduce the response to inhaled steroids (ICS). In fact, lower L-arginine/ADMA ratios is strongly associated with more frequent respiratory symptoms, decreased quality of life, and reduced pre-bronchodilator FEV_1 (FEV1%) among asthmatic adults (2). A potential link between obesity and this NO metabolic derangement may be explained by the metabolic syndrome (MS), which occurs in over 60% of obese persons. MS has been associated with increased systemic ADMA levels and is a well-known pathway to endothelial dysfunction (3). MS, independently of obesity, has been associated with increased risk for incident asthma, poor symptom control, and a steeper decline in lung function (4-7).

However, obesity could also adversely affect asthma via other inflammatory and oxidative mechanisms beyond NOS uncoupling, such as greater Th1 polarization, and increased pro-inflammatory airway leptin and cytokine levels (8, 9). It is plausible that other signaling processes can be modulated, or be susceptible to modulation, in parallel with reduced airway NO, to worsen asthma severity. Therefore, more pleiotropic pharmacologic strategies that can impact multiple inflammatory and metabolic pathways may be useful for improving the health of obese asthmatics. To do this, we propose a novel approach, which includes oral nitrite + nitrate (NO_2^- + NO_3^-) termed oxides of nitrogen (NO_x) + conjugated linoleic acid (CLA) supplementation. CLA is an 18 carbon, 2 double bond-containing unsaturated fatty acid (18:2, n-6) and has been shown to reduce bronchial hyperresponsiveness in overweight or obese asthmatics (10). CLA is readily nitrated (addition of an NO_2 group to a double bond) by the nitrite/nitric oxide derived species, nitrogen dioxide, producing a fatty acid nitro-alkene product with broad anti-inflammatory effects, including partial peroxisome proliferator-activated receptor gamma (PPARγ) agonist activity, blocking TH17-related pathways, nuclear factor-κB (NF-κB) inhibition and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation (11-14). In addition to the anti-

inflammatory effects of CLA, NOx supplementation can be beneficial to obese asthmatics by increasing NO airway bioavailability (15).

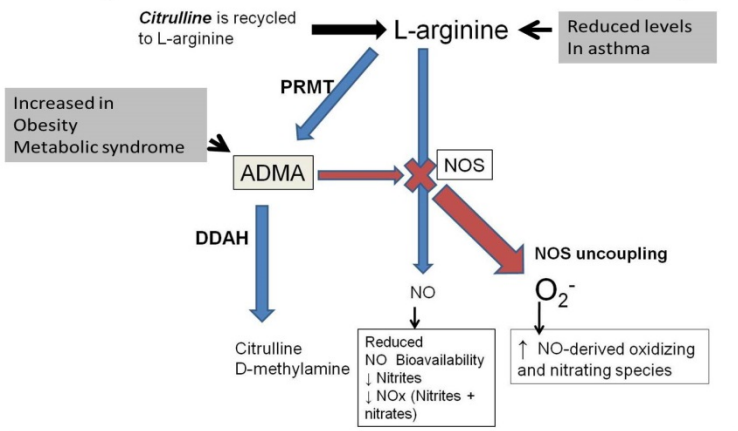
We hypothesize that in obese asthmatics treatment with NO_x + CLA is well tolerated, safe and will increase eNO while reducing airway oxidative stress. Allied with this, we will define whether supplementing with this bioactive mediator modifies the airway microbiome, and reduces airway inflammation.

Specific Aim 1. To determine whether treatment with NO_x + CLA will increase exhaled NO and reduce airway oxidative stress in adult obese asthmatics. To do this, participants will receive 8 weeks of open label treatment with NOx (Sodium nitrate 1000 mg + Sodium nitrite 20 mg/day) + 3,000 mg/day of CLA.

Specific Aim 2. To determine whether treatment with ***NO_x + CLA*** can influence airway NO bioavailability, as well as oxidative, metabolic and inflammatory pathways. Participants will undergo pre and post-treatment bronchoscopies to obtain airway epithelial cells, bronchoalveolar lavage fluid (BALF) and endobronchial biopsies. Peripheral blood and urine will also be collected at each of these bronchoscopies. To do this, we will compare the following pre and post intervention endpoints: a) total CLA levels, nitrite/nitrate in urine and plasma, and NO2-cLA levels in BALF, plasma, and urine; b) NFkB and Nrf2 pathways in peripheral blood monocyte cells (PBMC) and alveolar macrophages, c) leptin/adiponectin in BAL and plasma, d) plasma ADMA e) airway microbiome in BAL f) airway xanthine oxidase (XO) activity from endobronchial biopsies (as an airway oxidative stress source), g) TH17: BAL fluid/cells: IL-17 levels, mRNA, flow cytometry, h) metabolomics study to look at where ^{15}N is incorporated other than NO2-cLA; to do this, participants will take same dose of nitrate and nitrite ^{15}N heavy isotopes, the day prior to the second bronchoscopy.

2 BACKGROUND AND RATIONALE

Figure 1. L-arginine, NOS and ADMA metabolism



PRMT: protein arginine N-methyltransferase, DDAH: Dimethylarginine dimethylaminohydrolase
NOS: Nitric oxide synthase, ADMA: Asymmetric dimethyl arginine

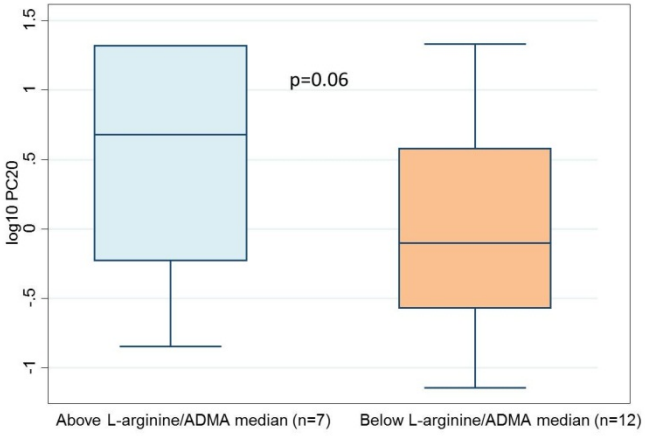
Obesity is one of the most significant comorbidities associated with poor asthma control, high exacerbation rates and increased frequency of healthcare utilization. Considering that in the United States roughly

30% of the population is obese, understanding how it adversely affects asthma is a significant research and public health priority. Increasing body mass index (BMI) inflicts a mechanical load to the respiratory system which predictably increases the frequency and severity of respiratory complaints, particularly shortness of breath, in obese asthmatics (16). In addition, obesity may cause or be the result of dysfunction beyond the mechanical load with impacts of the pathobiology of asthma and/or response to treatment. In different cluster analyses of asthma populations, obese patients with asthma have been identified as a "clinical phenotype" characterized by late onset asthma, reduced atopy, and disproportionate symptoms resulting in high rates of healthcare and medication use. These patients have relatively well preserved lung function and low levels of traditional Th-2 cytokine related airway inflammation, including sputum eosinophils and exhaled nitric oxide (eNO) (17-19). **Obese, late onset asthmatics are a unique phenotype**. Among obese subjects, eNO is relatively lower among those with a greater BMI and later asthma onset vs. those with earlier, childhood onset (20). This observation suggests that the interaction of obesity with late onset age of asthma is what determines lower eNO levels in the obese asthma phenotype. This concept is further supported by the fact that the inverse association between increasing BMI and eNO is only significant among those with late onset asthma (> 12 years) (2). Although higher levels of eNO are thought to be part of the pathophysiology of asthma, a causal role in asthma has not been established. In fact, inhibition of NOS may not benefit humans whereas NO inhalation has been reported to prevent bronchial hyperresponsiveness (21-24). NO contributes to airway physiology by playing a role in airway smooth muscle and vascular relaxation. Reductions in eNO in obese patients with asthma, may be indicative of a detrimental derangement of airway NO metabolism (25).

In obese asthmatics, L-arginine/ADMA metabolism is a potential mechanism to explain disease severity. A critical factor determining NO bioavailability is the balance between L-arginine, the substrate for NOS, and asymmetric di-methyl arginine (ADMA), which is a methylated product of L-arginine and an endogenous inhibitor of all NOS enzymes (26) (**See Figure 1**). A reduced L-arginine/ADMA ratio would therefore result in lower NO production by NOS. This balance, as we have previously shown (27), is inversely associated with increasing BMI categories in late onset asthmatics. Importantly, increased levels of ADMA also enhance the formation of superoxide, as a result of ADMA-mediated NOS uncoupling (28). It is well defined that L-arginine is reduced in asthma. This phenomenon coupled with increased levels of ADMA is associated with obesity and/or the metabolic syndrome and may explain why obese

asthmatics have lower plasma L-arginine/ADMA ratios (29). Both, plasma L-arginine/ADMA ratio and sputum ADMA, are strongly correlated (inversely for ADMA) with eNO (30). In addition, individuals with late onset asthma and reduced plasma L-arginine/ADMA ratios have more respiratory complaints, lower asthma quality of life, less allergic inflammation and lower FEV_1 (2). These associations are significant even after adjusting for asthma severity, FEV_1 and medication use. Further, among obese late onset asthmatics, those with an L-arginine/ADMA levels below the median have greater bronchial methacholine responsiveness, when compared to those that have higher ratios, as shown in **Figure 2**.

Figure 2. Log PC20 among obese – late onset asthmatics by L-arginine/ADMA ratios above and below median distribution



Experimental studies in stimulated airway epithelial cells have shown that ADMA reduces nitrite production while increasing superoxide levels in a dose dependent manner. In murine OVA models, pre-treatment with ADMA increases airway resistance in the absence of traditional biomarkers of allergic airway inflammation (28) (31). Together, these data suggest that the L-arginine/ADMA balance, through reduced airway NO bioavailability and enhanced oxidative stress, is a causal pathway driving the increased symptomatology in the “obese late-onset asthma phenotype” described in previous cluster studies.

Obese asthmatics respond differently to inhaled steroids. Compared to leaner asthmatics, obese asthmatics report more continuous respiratory symptoms, experience a higher rate of nocturnal respiratory symptoms and are more likely to have experienced asthma exacerbations, which required emergency room evaluation or hospitalization within the preceding 12 months (1). In the longitudinal study of difficult asthma (TENOR), patients who gained 5 lb (2.27 kg) during a 12-month interval between baseline and follow-up reported worse asthma control (adjusted odds ratio [OR]: 1.22; 95% 1.01-1.49; p = 0.04) and quality of life (0.18; 95% CI: 0.30 to 0.06; p = 0.003), as well as a greater number of corticosteroid bursts (OR: 1.31; CI: 1.04 - 1.66; p=0.02) than patients who maintained their baseline weight or lost 5 lb (25). While there may be many reasons why obese asthma patients may be more difficult to control, emerging data suggest that obese asthmatics also respond less well to current standard asthma

medications. In a retrospective analysis of a clinical trial comparing ICS with montelukast investigators found that obese asthmatics have reduced responses to ICS therapy, when compared to leaner counterparts (26). Increasing BMI was inversely associated with achieving adequate asthma control days while adjusting for potential confounders. Similarly, the low dose theophylline study of the American Lung Association (ALA) Research Network reported that obese asthmatics had a paradoxical response to the controller agent theophylline compared to normal weight asthmatics. When normal weight asthmatics were given theophylline in addition to their standard asthma regimen, the rate of asthma exacerbations decreased compared to a rise in obese asthmatics exacerbations (27). In a prospective study, Chanez et al reported that asthmatics with a BMI ≥ 25 had a substantially lower probability of achieving asthma control despite being treated according to asthma guidelines (28). Furthermore, in a study where mild to moderate asthmatics were randomized to inhaled budesonide or budesonide + salmeterol, obese and overweight participants had lower odds ratios for achieving asthma control at 12 weeks irrespective of treatment compared to leaner asthmatics (29). Also, compared to their leaner counterparts, obese asthmatics have a lesser improvement in FEV1 following treatment with inhaled steroids (30). In these studies obese asthmatics were included regardless of age of asthma onset and other phenotypical features.

Mechanistically, there is evidence for steroid resistance in obese asthmatics. Increasing BMI has been shown to blunt dexamethasone induced mitogen-activated protein kinase phosphatase-1 (MAPK) and tumor necrosis factor-α (TNF-α) production by peripheral blood mononuclear cells (31). In bronchoalveolar lavage cells, dexamethasone-induced MAPK expression was also reduced in overweight/obese versus lean patients with asthma although the mechanisms for these differences are not known. Taken together, these studies show that obesity in asthma is associated with reduced clinical response to ICS. Therefore, alternative approaches, both pharmacologic and non-pharmacologic, for the treatment of patients with asthma who are either overweight or obese are needed.

In obese asthmatics, additional co-morbidities can play an important role in explaining increase morbidity. In addition to changes in NO metabolism and steroid resistance, a potential largely unexplained link between asthma severity and obesity, is the MS. This co-morbidity is present in over 2/3 of all obese subjects, and is characterized by abdominal obesity, dyslipidemia (low HDL, high triglycerides), hypertension and insulin resistance. MS, independent of other comorbidities, is a risk factor for having a steeper loss of FEV_1/time among adult non-smokers. Cross sectionally, all the MS components are associated with increased

odds for having an FEV_1 < lower limit of normal; however, in multivariable analysis the strongest associations are observed with abdominal obesity. Among 17,400 children participating in the Coronary Artery Risk in the Appalachian Community survey, increased triglycerides and reduced HDL were associated with greater asthma prevalence. These lipids derangements have also been linked to increased wheezing risk among adults, even when adjusting for adiposity. Multiple lines of evidence suggest that insulin resistance is another potential mechanism for MS-associated changes in lung function in asthmatics. In experimental models, insulin has been shown to induce airway smooth contractility and proliferation. In humans, poor glycemic control has been associated with increased asthma prevalence; further, inhaled insulin can lead to reduced lung function, and therefore is contraindicated among patients with chronic airway diseases.

Additional treatments focusing on inflammatory and oxidative pathways are needed to improve the health of obese asthmatics with metabolic syndrome. Several of the pharmacological properties of nitrite, nitrate and CLA make them ideal candidates to overcome many of the metabolic and inflammatory factors associated with obesity, especially when coexisting with MS. For example, nitrate has been shown in to improve endothelial function, reduce blood pressure and insulin resistance in diabetic patients (32). Another relevant aspect of NOx therapy is that it may increase airway NO bioavailability by providing an alternative source of NO other than NOS, which could be particularly useful in situations, such as obesity, when the L-arginine/ADMA balance favors iNOS uncoupling. CLA is a polyunsaturated fatty acid found in dairy products and ruminant meat (i.e. beef) and an average American diet supplies 15-174 mg of CLA daily. Moreover, CLA is formed in the body following conversion of linoleic acid to CLA by bacteria of the gastrointestinal tract. CLA has pleiotropic anti-inflammatory actions, including partial PPARγ agonist activity, NF-κB inhibition and Nrf2 activation. Clinically, CLA has been associated with a wide range of salutary effects including: decreased body fat mass in obese and healthy overweight patients (33, 34), decreased the leptin/adiponectin ratios, and reduced risk of colorectal cancer. Of particular relevance to obesity and asthma, CLA has been shown to suppress Th-1 and Th17-mediated inflammatory responses in dendritic cells (14). This effect could be relevant to obese late –onset asthmatics who have a greater Th1 polarization and in whom Th17 responses appear to play a major a role. Also, its ability to decrease the leptin/adiponectin ratio could ameliorate airway inflammation. Clinically, CLA has been shown to be potentially effective in improving bronchial hyperresponsiveness in obese and overweight mild asthmatics randomized to receive CLA 4,560 mg/d for 12 weeks. Compared to placebo,

those receiving CLA, had a significant reduction in the average methacholine dose to achieve a 20% reduction in FEV_1 (change in methacholine dose from pre to post treatment was 5mg/ml [1.9] in the CLA arm vs 1.2 [1.2] in the placebo, p=0.02). Interestingly, this occurred in the absence of any significant changes in systemic biomarkers of airway inflammation; however, the study did not include any airway measurements (10).

3 STUDY HYPOTHESES

3.1 Primary Hypothesis

We hypothesize that in obese asthmatics; treatment with NO_x + CLA is well tolerated, safe and will increase eNO while reducing airway oxidative stress.

3.2 Exploratory Hypothesis

We hypothesize that in obese asthmatics, treatment with NOx + CLA will reduce airway oxidative stress in airway epithelial cells.

4 STUDY OUTCOMES

4.1 Primary Outcome

To determine how CLA + NOx affect airway NO bioavailability and oxidative stress

4.2 Secondary Outcomes

To determine whether, compared to baseline, treatment with NOx + CLA can:

a) Increases eNO levels
b) Quantify the concentrations of nitrate/nitrite in plasma and urine, free cLA in plasma, and NO_2-cLA in BALF, plasma, and urine
c) Reduce airway oxidation and inflammation
    a. Western and RTPCR NF-κB and Nrf2 pathways in airway macrophages and PBMCs
    b. Airway leptin and adiponectin
    c. Proportion of airway TH-17 cells/responses

d) Airway XO activity determined in endobronchial biopsies
e) Measurement of $^{15}NO_2$-cLA and endogenous $^{14}NO_2$-cLA in urine, plasma and BAL
f) Decrease production of anion superoxide in fresh airway epithelial cells;
g) Mitochondrial ROS production and bioenergetics in fresh and cultured airway epithelial cells. As a potential source for airway / epithelial oxidative stress

4.3 Exploratory Outcomes

To determine whether treatment with NOx + CLA can:

a) Explore pre and post bronchoscopy changes in airway microbiome.
b) Compare outcomes obtained at baseline between healthy controls participating in EFADA and obese asthmatics with metabolic syndrome

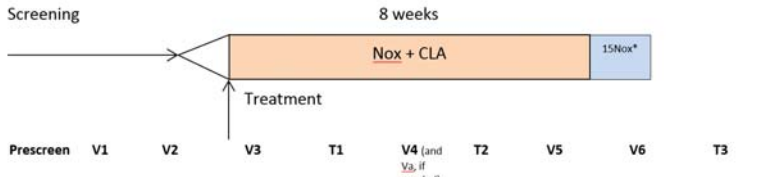
5 RESEARCH DESIGN AND METHODS

5.1 Study Design

This is an open-label, single center, proof of concept pilot study in obese asthmatics.

5.2 Description of the Study Design

Potential subjects will be recruited from the Asthma Institute registry and the CTSI registry, and throughout the community at large. After an initial screen visit, those who sign the consent and meet eligibility will proceed to have methacholine testing; subjects with a > 12% bronchodilation in FEV_1, or achieving a 20 % reduction in FEV_1 from baseline (PC20) after methacholine, will receive ***NOx + CLA*** for a period of 8 weeks.



Prescreen	V1	V2	V3	T1	V4 (and Va, if needed)	T2	V5	V6	T3
Pre-screening; inclusion & exclusion criteria	Consent Spirometry (pre & post) H&P ACQ, ACT eNO	Methacholine eNO	Bronchoscopy Microbiome samples (stool, tongue, BAL) Blood Urine Spirometry Physical Exam ACQ eNO	Diary review Compliance ACQ Review any concerns & health care utilization	Compliance check	Diary review Compliance ACQ Review any concerns & health care utilization	Methacholine eNO ACQ	Bronchoscopy Microbiome samples (stool, tongue, BAL) Blood Urine Spirometry Physical Exam ACQ, ACT eNO	Diary review Compliance ACQ

* 15Nox dose will be taken 24 hours prior to V6 bronchoscopy

**An additional visit might be needed in some participants to perform pre- and post-spirometry after an adequate medication withhold

Prior to treatment, participants will undergo a baseline bronchoscopy at UPMC Montefiore, after which they will be instructed to begin taking the assigned intervention for 8 weeks. At the completion of the treatment period, participants will undergo a second methacholine challenge and a final bronchoscopy.

5.3 Rationale for Dose Selection

The daily study drugs are:

Sodium nitrite and stable isotope labeled sodium 15N nitrite

Standard sodium nitrite will be supplied as capsules for daily oral administration at the dose strength of 20 mg (study dose: 20 mg). The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) proposed an Acceptable Daily Intake (ADI) for sodium nitrite, which corresponds to 0.1 mg/kg of body weight. Using the ADI for sodium nitrite (0.1 mg/kg of body weight) and a body weight of 95 kg, the calculated ADI of sodium nitrite would be 9.5 mg. Much higher doses, of oral sodium nitrite solutions ranging between 140-380 mg are well tolerated acutely with minimal side effects characterized by short-term nausea and headaches, and mild transient reductions in blood pressure with concomitant increase in heart rate. Based on the Joint FAO/WHO ADI for nitrite, the fact that this dose has been approved by the FDA for studies in humans, it is believed that a safe and tolerated dose for sodium nitrite is 20 mg (~0.2 mg/kg body weight per day for a 95 kg subject). This dose selection is roughly equivalent to 2.1 times the ADI.

Sodium nitrate and stable isotope labeled sodium 15N nitrate

Standard sodium nitrate will be supplied as capsules for daily oral administration at the dose strength of 500 mg (study dose: 1,000 mg). In a recent study, single dose potassium nitrate capsule ingestion in healthy adult volunteers in doses of 2,424 mg KNO_3 (or 1,488 mg nitrate ion) and 1,212 mg KNO_3 (or 744 mg nitrate ion) resulted in dose dependent reductions in BP over a 24 hour period without adverse effects (35). Current studies from this group are on-going with long term therapy for hypertension. Short term dietary supplementation with sodium nitrate at 0.1 mmol/kg of body weight per day divided up over 3 times daily for 3 days has been studied by Lundberg et al (36-38). The daily nitrate dose administered corresponds to the amount found in 100-300 grams of nitrate-rich vegetables such as spinach, beetroot or lettuce. Many of these studies demonstrated that dietary nitrate reduced systolic and diastolic blood pressures, mean arterial pressure (MAP), and oxygen cost during physical exercise and improved mitochondrial

efficiency without adverse effects. Several human studies have examined the effect of single ingestion (35, 39), 6 day(40, 41) and 15 day (42) supplemental courses of nitrate in the form of beetroot juice on blood pressure in healthy adults. Systolic (SBP), diastolic (DBP) and mean MAP were significantly reduced with beetroot juice in these trials with no adverse events noted (35, 39-42).

Potential Toxicity: For nitrate, some toxicity studies suggest a positive association with risk of non-Hodgkin lymphoma, gastric and colon cancers with years of exposure. However, these were mostly studies with weak study design and limited strength of evidence. Other case-control studies and cohort studies, which provide stronger evidence, have found no increased risk of human cancer with increasing nitrate intake from diet or drinking water after multivariate adjustment. Taking all studies together, the CONTAM panel (Contaminants in the Food Chain) of the European Food Safety Authority (EFSA) reports evidence does not suggest that nitrate intake from diet or drinking water is associated with increased cancer risk (43). In addition, there is no clear evidence of an effect of nitrate or nitrite on non-cancer human health effects. In the current study, we will use much lower doses of nitrite and nitrate than used safely in any of these studies. Our sodium nitrate of 1gm represents a nitrate ion dose (i.e. 744 mg) that is less than the nitrate ion dose (i.e. 933 mg) associated with 1.488 gm of potassium nitrate used by Webb and colleagues. This medication will be provided by Dr. Gladwin, under IND (115,926).

Conjugated Linoleic Acid

Will be supplied as capsules for daily oral administration at the dose of 3,000 mg/day from GNC. Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid found in dairy products and ruminant meat (i.e. beef) and an average American diet supplies 15-174 mg of CLA daily. Moreover, CLA is formed in the body following conversion of linoleic acid to CLA by bacteria of the gastrointestinal tract. The predominant CLA species found clinically are octadeca-(9Z,11E)-dienoic acid and octadeca-(10E,12Z)-dienoic acid (44). Because linoleic acid cannot be synthesized in the body, it must come from the diet, as it is an essential fatty acid. Multiple clinical trials support that CLA promotes a broad range of physiological benefits (45). Additionally, previous reports demonstrated the ability of CLA to induce adipocyte lipolysis, fatty acid oxidation of adipocytes and significantly decrease body fat mass in obese and healthy overweight patients (33, 34). The benefits of CLA on inflammation have also been described, specifically through reducing macrophage-mediated infiltration into the adipose tissue and cytokine/leptin secretion, ultimately maintaining insulin sensitivity in a high-fat diet murine model of obesity (46). Furthermore, CLA

administration has been positively associated with reduced risk of colorectal cancer in human studies, and through animal studies, this effect was a result of reduced inflammation via PPARγ ligation (47, 48).

6 STUDY ASSESSMENT AND ORDER OF PROCEDURES

Pre-screening

- Based on an IRB-approved script, coordinators will pre-screen by phone or in person to determine if a potential participant should proceed to visit 1 based on major inclusion criteria such as asthma diagnosis, active smoking, presence of other lung diseases or exclusion criteria, etc.
- Participants that meet pre-screening criteria will be verbally consented to perform medication withhold to determine bronchodilator response.

Run-In Period

The run-in period is from the time of consent (V1) to the first bronchoscopy (V3).

Visit 1

- Sign consent form
- Review Inclusion and Exclusion criteria.
- Pre and post (4 puffs of albuterol) spirometry if patient has had adequate withhold (4 hours for SABA, 12 hours for LABA, 24 h for Tiotropium).
- Complete history and physical examination to include body weight, height, vital signs, waist and hip circumference
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure).
- Exhaled NO. Niox mino. (No food, drink (other than water), or strenuous exercise 1 hour prior to visit)
- Asthma Control Test (ACT) and Asthma Control Questionnaire (ACQ)

Visit 2

- Urine pregnancy test in females of child-bearing potential

- Exhaled nitric oxide test
- Brief physical exam
- Methacholine testing- This is a common test often used to confirm a diagnosis of asthma. Methacholine is a chemical that causes the airways to react (narrow) and is easily reversed with the administration of albuterol. Prior to the test, a urine pregnancy test will be performed on women of childbearing potential and a blood pressure reading will be taken. An exhaled nitric oxide test and a brief physical exam will also then be performed. The challenge test consists of inhaling very low, but increasing doses of methacholine through a nebulizer to assess how quickly the airways narrow. Spirometry (focusing on the FEV-1) will be performed approximately 30 and 90 seconds after each dose to look for changes in lung function. If/when a 20% decrease in spirometry (FEV-1) is observed the challenge test will be stopped. Multiple measures are in place to perform this test safely and comfortably. Subjects will receive albuterol after the test is done.
- If a subject's methacholine challenge test result is within 3% of what is positive (17% to 19% change), the participant can be re-tested within 14 days and enrolled accordingly (if 20% change or PC20 occurs). Additionally, if the subject's FEV1 is not 50% of predicted – participant can return within 7 days, and challenge can be done if pre-testing parameters are met. They can then continue with the study accordingly.

<u>Visit 3</u> (Week 0) (Please see Bronchoscopy Procedure description below)

- The research coordinator will contact the subject 24-48 hours prior to Visit 3 in order to verify the medication withhold and readiness for the bronchoscopy procedure.
- Pt arrives to CTRC – MUH after an 8 hour fast
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure).
- A physical exam will be completed
- The subject must have used less than 16 puffs of albuterol in the last 48 hours in order to proceed with bronchoscopy procedures
- Pre – bronchoscopy spirometry (pre- and post-bronchodilator) (safety assessment)
- Asthma Control Questionnaire (ACQ)& pre-bronchoscopy safety checklist
- Tongue scrape x microbiome
- Bronchoscopy for a) brushings , b) BAL, c) endobronchial biopsies; d) microbiome
- Blood work for metabolic syndrome (fasting blood glucose, insulin levels, serum creatinine) lipid profile (HDL, triglycerides, total cholesterol, LDL and VLDL), CBC with differential, and Hgb A1C.

- Plasma collection
- Urine collection X Prostaglandin D2 (PGD2) metabolites and isoprostanes
- Exhaled NO (No food, drink (other than water), or strenuous exercise 1 hour prior to visit)
- Stool x microbiome
- Post bronchoscopy spirometry
- Dispense study drug supply- 4 week supply
- Distribute study Diary Card and review instructions

Phone call visit T1 (2 weeks after Visit 3)

- Review diaries & compliance
- ACQ
- Review any concerns and health care utilization

Visit 4 (4 weeks after Visit 3)

- Short Physical Exam
- Review of diaries and compliance
- Niox Mino (No food, drink (other than water), or strenuous exercise 1 hour prior to visit)
- Pre and Post spirometry
- ACQ & ACT
- Dispense 4 week supply of study drug

Visit 4A (if necessary)

If it is determined by the study coordinator at Visit 4 that the subject has not taken at least 75% of the study drug, the coordinator will provide a 1 week supply of the study drug and ask the subject to return to the Asthma Institute for a compliance check (visit 4A). If the subject is not compliant at Visit 4A, the subject will be withdrawn from the study. If the subject has improved compliance after 1 week, the remaining 3 week supply of the study drug will be dispensed.

Phone call visit T2 (6 weeks after Visit 3)

- Review diaries & compliance
- ACQ
- Review any concerns and health care utilization

Visit 5 (after 8 weeks of treatment)

- ACQ
- Urine pregnancy test in females of child-bearing potential
- Exhaled nitric oxide test
- Brief physical exam
- Methacholine testing- This is a common test often used to confirm a diagnosis of asthma. Methacholine is a chemical that causes the airways to react (narrow) and is easily reversed with the administration of albuterol. Prior to the test, a urine pregnancy test will be performed on women of childbearing potential and a blood pressure reading will be taken. An exhaled nitric oxide test and a brief physical exam will also be performed. The challenge test consists of inhaling very low, but increasing doses of methacholine through a nebulizer to assess how quickly the airways narrow. Spirometry (focusing on the FEV-1) will be performed approximately 30 and 90 seconds after each dose to look for changes in lung function. If/when a 20% decrease in spirometry (FEV-1) is observed the challenge test will be stopped. Multiple measures are in place to perform this test safely and comfortably. Subjects will receive albuterol after the test is done.
- Patient is instructed to take Nitrogen 15 sodium nitrate and 15 sodium nitrite in substitution of regular NOx supplements (same dose) with food approximately 12 h prior to bronchoscopy. This dose is in a form that is easily observed in study samples.
- Dispense study drug supply

Visit 6 (End of study- 24 hours to 1 week after visit 5)

- Pt arrives to CTRC – MUH after an 8 hour fast
- Pre – bronchoscopy spirometry (pre- and post-bronchodilator) (safety assessment)
- Pre-bronchoscopy questionnaires: ACQ, ACT, and pre-bronchoscopy safety checklist
- A brief physical exam will be completed
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure).
- Tongue scrape x microbiome
- Bronchoscopy for a) brushings , b) BAL, c) endobronchial biopsies; d) microbiome
- Blood x plasma collection Urine x PGD2 metabolites and isoprostanes
- Exhaled NO (No food, drink (other than water), or strenuous exercise 1 hour prior to visit)
- Stool x microbiome
- Post bronchoscopy spirometry

Phone call visit T3 (1 week after the 2nd bronchoscopy)

- ACQ
- Review any concerns, healthcare utilization.

Following the initiation of the study treatment, a ± 5 days schedule variance will be allowed for subsequent study evaluations.

End of Study Visit

If a subject is withdrawn from study participation, an End of Study Visit will be scheduled. This visit will take place at the Asthma Institute and will consist of the following procedures:

- Physical Exam
- Spirometry
- ACQ
- Review of diaries

Bronchoscopy Procedure (Visits 3 & 6)

Before the bronchoscopy, a tongue scrape will be performed. The subject will be asked to rinse their mouth with saline and spit out the remaining fluid. Using a plastic cell scraper, the subject's tongue will be gently scraped to collect cell samples. Following collection, the samples will be brought to the PI's lab and used to develop cell cultures for research purposes.

The bronchoscopy procedure will take approximately 45 minutes. While in the CTRC, an IV needle will be inserted by CTRC staff and approximately 6 tablespoons of blood will be drawn. The IV will be used to give medications during the bronchoscopy. Depending on the physician's preference, subjects may be given a mild sedative at this time. Subjects will also perform pre and post spirometry in the CTRC and they will be asked to inhale a medicine (albuterol - given via neb) to open up airways.

Once subjects are in the bronchoscopy suite, the nose and throat will be sprayed with lidocaine (a numbing medication or anesthetic) to reduce feeling and coughing. A maximum of 600mg of lidocaine will be used above and below the vocal cords. If the subject has a history of nausea and vomiting from anesthesia, Zofran may be given before the bronchoscopy. Subjects will be given a sedative (Versed-not to exceed 20 milligrams and/or Fentanyl-not to exceed 500 micrograms) in the IV. The subject's respiratory rate will be monitored by pulse oximentry, and

supplemental oxygen will be provided. A bronchoscopy nurse that is specially trained in sedation will do the monitoring and is qualified to administer the sedatives.

Finally, the bronchoscope will be passed into the windpipe. The tube is small enough that the subject can breathe around it, and is designed so that the study doctor can look through it into the lungs. Secretions can be removed by suction through it.

While the tube is in the participant's lung, the following procedures will be performed.

• Pulmonary Lavage. During pulmonary lavage, five 50 cc (a little over 16 Tablespoons) washes of sterile salt-water solution will be injected through the bronchoscope and then immediately sucked out. This allows the doctor to remove some lung cells and secretions for research study. If less than15cc return from the first 100cc of lavage, the lavage will be stopped.

• Bronchial Brushing. A small brush will be introduced through the bronchoscope and gently rubbed over a very small part of the surface of the lungs. The brush will collect cells, known as epithelial cells, which then can be taken to the laboratory for further study. This is done with 10 brushes.

• Endobronchial Biopsy. While the bronchoscope is inserted, another small instrument will be passed through the bronchoscope to take small samples (biopsies) of tissue from the walls of the airways. Up to 8 samples may be taken during this procedure.

The bronchoscopy procedure will take about 30-45 minutes, but the preparation and recovery time will usually make the visit about 4-8 hours total. If the subject is feeling nauseous after the bronchoscopy, Zofran may be given at the discretion of the physician investigator. An albuterol treatment may be given at the discretion of the physician investigator. Occasionally, the subject's asthma may worsen during or after the procedure. In those cases, we may ask them to spend the night in the Clinical Translational Research Center (CTRC). If subjects are asked to spend the night in the CTRC, subjects will be monitored as described below in the POST Bronchoscopy monitoring procedures.

POST Bronchoscopy Monitoring Procedures:

For this research study, the monitoring/follow-up procedures include:

Subjects will be monitored at UPMC until the sedation has worn off. After the bronchoscopy is completed, subjects will wait in the bronchoscopy recovery area or the clinical research unit for at least 1 hour (but commonly 4-6 hours) following the bronchoscopy to make sure breathing has not been affected by the procedure and to be sure that the anesthesia has worn off. Subjects may be given Tylenol prn if they have a temperature above 38° C. Breathing tests (spirometry) will be done when subjects are awake enough to perform the test and then every 1-2 hours after to make certain breathing capacity is returning to normal level. If subject is not able to breathe as well as before the bronchoscopy we will provide an albuterol dose (can be either neb or MDI) and check spirometry again. If breathing does not return to 85% of baseline after about 6 hours, subject will be evaluated by investigator to determine if further observation (up to 24 hours) is needed. Additional spirometry may be performed for patient safety. It is also possible (if clinically indicated), that subjects receive a prednisone burst to help asthma symptoms improve. However, this is rare since subjects are mild/moderate asthmatics. Subjects will not be allowed to leave the clinic until breathing is back to normal level.

Subjects will not be allowed to drive home after the bronchoscopy. This is because the sedatives used during the procedure may decrease driving ability. We will ask for the contact name and number of the person that will pick up the subject after the bronchoscopy.

The research coordinator will call the subject 24 hours following the bronchoscopy procedure to monitor symptoms. The research coordinator will continue to make follow-up calls as needed for patient safety. At the discretion of the PI, it may be recommended that subjects return to the Asthma Institute for clinical evaluation of breathing issues (respiratory status) in the post bronchoscopy period. This visit would include baseline spirometry and a brief physical exam.

7 HUMAN SUBJECTS

7.1 Recruitment and Selection of Patients

Study participants will be enrolled at Asthma Institute, from clinics, UPMC-affiliated hospitals and communities via various IRB-approved recruitment methods.

7.2 Inclusion Criteria

1) Adequate completion of informed consent process with written documentation
2) Male and female patients, ≥ 18 - 65 yrs old
3) Diagnosis of asthma: based on previous physician diagnosis and either baseline pre-bronchodilator FEV_1 50% or greater predicted with a 12% or greater

bronchodilator response to 4 puffs of albuterol or PC20 methacholine (16 mg) if no BD response. If the subject is not currently on an ICS or ICS-LABA combination, PC20 should be < 8 mg, if no BD response. Spirometry results within the prior 24 months located in the subject's medical records can be used to determine eligibility, if available.

4) From all racial/ethnic backgrounds with a diagnosis of asthma for ≥6 months;
5) Smoking history ≤10 pack years and no smoking in the last year;
6) BMI ≥ 30
7) If subject is on ICS or ICS/LABA therapy- 30 days on a stable dose (up to 1,000 mcg daily fluticasone equivalent)

7.3 Exclusion Criteria

1) Respiratory tract infection within the last 4 weeks;
2) Oral or systemic CS burst within the last 4 weeks;
3) Asthma-related hospitalization within the last 2 months;
4) Asthma-related ER visit within the previous 4 weeks;
5) Significant or uncontrolled concomitant medical illness including (but not limited to) heart disease, cancer, diabetes;
6) History of ICU admission/intubation due to asthma in the past year;
7) More than three systemic corticosteroid requiring asthma exacerbations in the past year;
8) Current statins use (statins lower ADMA levels), patients may stop and re-enroll after 2 weeks of stopping statins;
9) Positive pregnancy test;
10) Intolerance or allergy to the intervention drugs;
11) Current or recent participation (within the past 30 days) in an investigational treatment study;
12) Unable or unlikely to complete study assessments or the study intervention (i.e. bronchoscopy) poses undue risk to patient in the opinion of the Investigator;
13) Any kind of oral nitrates such as nitroglycerin or already taking supplements in this study

14) Systemic steroid dependent asthma (no daily oral steroids- short term therapy for asthma exacerbation is permitted)
15) Chronic renal failure (creatinine ≥ 2.0) at screening (associated with higher ADMA levels)- self-reported and/or verified by medical records.
16) Use of mouthwash containing chlorhexidine (lowers NO) within 1 week prior to screening and throughout the study.
17) Untreated sleep apnea
18) Hgb A1C ≥7- self-reported and/or verified by medical records.
19) Daily use of PPI's (Proton Pump Inhibitor) or H2 Blockers for GERD (it is permitted to take on an occasional basis- no more than 1x per week. If participants wash out of these meds for 1 week, they can enroll)
20) Use of biologics for asthma/allergies unless there is a 4 month washout prior to enrollment
21) Drug and/or alcohol abuse for ≥ 1 year
22) Breastfeeding
23) Any other condition and/or situation that causes the investigator to deem a subject unsuitable for the study (e.g. due to expected study medication non-compliance, inability to medically tolerate the study procedures, or a subject's unwillingness to comply with study-related procedures.

7.4 Criteria for Initiating Treatment

Inclusion criteria

No exacerbations during the run in. Patients, who exacerbate during the run-in, may re-enroll 4 weeks after the exacerbation has resolved (completed course of systemic corticosteroids).

Completed 1st bronchoscopy and absence of complications or serious adverse events following the procedure (i.e. hospitalizations)

8 STATISTICAL ANALYSIS AND POWER CALCULATIONS

8.1 Sample Size Determination

To ensure 90% power to detect a difference in the change in the methacholine dose required to produce a PC20 before and after treatment, a sample size of n=12 participants will be required. This estimate assumes an alpha level of 0.05, and a 2-sided test. The estimate of variability and effect size used was by McRedmond et al, in which the PC20 effect size associated with CLA administration was 3.8 (SD 1.9). There are no preliminary data on how CLA or NOx + CLA affect exhaled NO and therefore are unable to reasonably anticipate a sample size for adequate power.

8.2 Study Conduct Analyses

Deviations will not be distinguished from protocol violations; any failure to follow the protocol will be defined and recorded as a protocol violation. Such violations should be reported to the local Investigational Review Board (IRB) as per required practice. No exceptions to the eligibility criteria for the study are permitted.

8.3 Efficacy Analyses

Primary analysis: The primary analysis will test whether there is a significant difference in exhaled NO and measures of airway oxidative stress (superoxide production, XO activity in biopsies) before and after taking either of the intervention arms. This analysis will utilize an approach of comparing average values pre and post intervention, using matched paired comparisons.

Matched analysis are very efficient in controlling for confounders, as the analysis uses the same individual as case and control under different treatment conditions. Secondary outcomes will be tested following the same scheme of pre and post matched comparisons. To determine the relevance of measured variables, these will be compared to results from healthy controls undergoing bronchoscopy as part of the Electrophilic Fatty Acid Derivatives in Asthma (EFADA) study (IRB#: PRO11010186). This analysis will allow cross sectional comparisons and determine, for example, how obesity influences some of the baseline study variables.

8.4 Safety Analyses

Due to the relatively short duration of this POC, we do not expect to see a large number of exacerbations during the treatment period. However, the rate of exacerbations will be monitored and reported to the DSMB at their regularly scheduled meetings to ensure the safety

of study participants. In addition, as CLA and NOx have not been used in combination for the treatment of asthma, therefore as a POC we will monitor lung function and bronchial hyperresponsiveness.

8.5 Handling Missing Data

Because of the possibility of drop-outs and other missed visits, there will be some missing data. The statistical models and analyses that are planned for the primary and secondary outcomes assume that the data are missing-at-random (MAR). Because likelihood-based methods will be applied, MAR data still yield valid estimates. Although not expected, if it appears that the MAR assumption is not reasonable, then non-ignorable statistical analyses, such as shared parameter modeling will be applied.

8.6 Data Management

Data will be collected on CRFs and transferred to an electronic database following a double entry protocol. Entry fields will include pre specified data ranges and distributions will be regularly checked for the presence of outlier or extreme values.

9 POTENTIAL RISKS AND BENEFITS

9.1 Potential Risks

9.1.1 Risks related to the procedures

Venipuncture

Blood samples will be obtained by venipuncture of an antecubital vein to determine the levels of CLA NO_2-cLA, nitrite, and other inflammatory biomarkers.

Risks: The risks of venipuncture are minimal. The possible risks include bruising and/or infection at the site of the venipuncture and vasovagal episodes experienced by the blood donors. Pressure will be applied to the venipuncture site to prevent bruising. Aseptic technique will be used to prevent infection. Blood will be obtained while the donors are in a seated position and medical and nursing personnel will be available at the study sites to treat and manage vasovagal episodes.

Benefits: There are no direct benefits to the participant

Pulmonary Function Testing (spirometry)

Spirometry will be performed to determine the participants' pulmonary function.

Risks: The risks of spirometry are minimal. The possible risks include precipitation of bronchospasm and light-headedness from repeated blowing attempts. Medical and nursing personnel and medications will be available at the study sites to treat and manage bronchospasm. Inhalation of a short-acting beta-2 adrenergic agonist (such as albuterol) will be used to assess reversibility at some visits. The possible risks of inhaled beta-2 adrenergic agonists include tachycardia and hand tremors. These side effects are non-life threatening and are short-lived.

Benefits: Spirometry with assessment of reversibility to a short-acting beta-2 adrenergic agonist will be used to determine if the participants meet the inclusion criteria for this study; it will also serve as a secondary outcome for the study.

The potential benefits justify the potential risks.

Methacholine Inhalation Challenge

Methacholine challenge will be used to assess airway hyper-responsiveness at baseline and after treatment. This measurement is being made primarily as function of safety to ensure that CLA + NOx do not potentially enhance airway hyperresponsiveness in these patients.

Risks: The major risk of methacholine challenge is the induction of severe bronchoconstriction. As a precaution, participants will not undergo methacholine challenge if their FEV_1 is less than 50% of predicted or 1.0 liter. Medical and nursing personnel, medications and equipment will be available at the study sites to treat and manage any bronchoconstriction episodes.

Benefits: There are no direct benefits to the participant. This procedure is considered necessary for the protocol as a safety measure for this pilot, POC study.

Bronchoscopy

Risk from bronchoscopy are hemoptysis, epistaxis, and local discomfort, fever and a sore throat. Sedation will be given as part of the bronchoscopy and the medications used for this can cause hypotension, lightheadedness or dizziness; these side effects are somewhat common but very short in duration. Appropriate monitoring equipment and medications will be used to promptly identify and manage complications if they occur. Bronchoscopy may cause coughing and occasionally gagging. Fever or chills may occur, especially following a lavage. Chest pain from a collapse of a small segment of the lung (atelectasis) may occur. Some people experience temporary vomiting, soreness of the nose and throat Mild to moderate cough can be

observed for 24 hours after the procedure. The greatest risk of bronchoscopy is transient worsening of asthma. This rarely occurs in mild-moderate asthmatic subjects (<5%), but can occur in up to 25% of severe asthma subjects. Every precaution is taken to prevent this, including giving 40-60 mg of prednisone to severe asthmatics prior to discharge. The subject's asthma may worsen during or after the procedure. In those rare cases, we may ask them to spend the night in the Clinical Translational Research Center (CTRC). There has been only one death (worldwide) associated with a research bronchoscopy so this is very, very rare event. Overall, research bronchoscopy in adult asthmatics is a safe and well-tolerated procedure (49).
Protection against Risk: First during the bronchoscopy, patients are placed on supplemental oxygen and monitored continuously with oxygen saturation monitor as well as electrocardiogram. Due to the potential of increased risk of complications with asthmatic subjects undergoing a bronchoscopy, subjects are pretreated with beta-agonists. The topical anesthesia and sedation dosages are monitored and limits set and adhered to closely. Research bronchoscopies will only be done on subjects with a post bronchodilator FEV_1 predicted of ≥50%.

Urine Collection

Urine will be collected to assess overnight urine cortisol, to perform pregnancy tests at specified visits, and for future exploratory assessments of urine biomarkers.
Risks: There are no risks associated with urine collection.
Benefits: This test will be used to determine PGD2 metabolite levels and isoprostanes.

Tongue Scrape

The subject's tongue will be gently scraped to collect cell samples. Following collection, the samples will be brought to the PI's lab and used to develop cell cultures for research purposes.
Risks: The main risk associated with this procedure is minor discomfort and/or irritation of the tongue.
Benefits: There are no direct benefits to the participant.

9.1.2 Study Specific Risks

Treatment with CLA

CLA isomers have been designated as "generally regarded as safe by the Food and Drug Administration", based on human and animal data that show that there is no indication of

significant CLA-related adverse effects. Human studies have shown that continuous administration of CLA in the dose range proposed in this study, is well tolerated and does not result in serious adverse effects. Doses of up to 10 g / day for short periods have resulted in no adverse events in healthy subjects (www.fda.gov/downloads/food/ingredientspackaginglabeling/gras/noticeinventory/ucm408896.pdf).

Nitrite

Dr. Gladwin, a collaborator in this study, has previously held an IND for sodium nitrite (IND # 70,411) for cardiovascular applications and currently has an approved IND for the use of sodium nitrite for lung transplant recipients (IND # 111,643). The cardiovascular IND involved the administration of sodium nitrite to 69 normal volunteers in 4 phase I-II clinical trials without observed adverse effects. He has also treated 11 subjects with sickle cell disease on this IND without observed adverse effects. The low doses of nitrite used in these investigational treatment regimens – less than 75 mg or 25% of the dose (300mg) used in the emergency treatment of cyanide poisoning – do not produce methemoglobin levels greater than 3% and have not been associated with clinically significant hypotension. There have been no adverse events noted in the 80 treated normal human volunteers and patients with sickle cell disease disease (50-52). In another study by Gladwin et al., 18 healthy adults received an infusion of sodium nitrite totaling 75 mg (15 minutes each x 2 infusions). This was associated with a 7 mm Hg decrease in mean arterial pressure, a peak methemoglobin of less 3% and no other significant effects(51). Note this dose is 3.75 times the dose we plan to use in this trial.

In an open-label three-way crossover study, 9 healthy adult subjects received two single high dose oral sodium nitrite aqueous solutions (0.12 and 0.06 mmol $NaNo_2$/mmol Hb, equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, depending on the total body hemoglobin level of the person) and one intravenous sodium nitrite dose (0.12 mmol $NaNo_2$/mmol Hb)(53). Mild headache occurred in 44-55% of subjects and was the most frequent complaint during each treatment session, which the authors ascribed to the sodium nitrite, not methemoglobinemia, as the percentage of methemoglobinemia stayed below clinically toxic levels (<15%). By report, up to 22% experienced nausea, which subsided within half an hour(53). The pharmacokinetic analysis of this study indicated similar bioavailability of oral and IV delivery of nitrite, as well as similar side effect and safety profiles.

Nitrate

For nitrate, some toxicity studies suggest a positive association with risk of non-Hodgkin lymphoma, gastric and colon cancers with years of exposure. However, these were mostly studies with weak study design and limited strength of evidence. Other case-control studies and cohort studies, which provide stronger evidence, have found no increased risk of human cancer with increasing nitrate intake from diet or drinking water after multivariate adjustment. Taking all studies together, the CONTAM panel (Contaminants in the Food Chain) of the European Food Safety Authority (EFSA) reports evidence does not suggest that nitrate intake from diet or drinking water is associated with increased cancer risk. In addition, there is no clear evidence of an effect of nitrate or nitrite on non-cancer human health effects(43).

In the current study, we will use much lower doses of nitrite and nitrate than used safely in any of these studies. Our sodium nitrite dose of 20 mg per day is <30% of the dose used on the cardiovascular IND and <7% of the dose used for cyanide poisoning. Our sodium nitrate of 1,000 mg represents a nitrate ion dose (i.e. 744 mg) that is less than the nitrate ion dose (i.e. 933 mg) associated with 1,488 mg of potassium nitrate used by Webb and colleagues(39)

To summarize, We anticipate the following symptoms by organ system and likely frequency of risk during the 6 week treatment in this study:

Gastrointestinal

- Common: none
- Frequent: none
- Rare: nausea, abdominal pain and vomiting

Hematologic

- Common: none
- Frequent: none
- Rare: methemoglobinemia

Cardiovascular

- Common: none
- Frequent: none
- Rare: flushing, tachycardia, hypotension

Neurologic

- Common: none
- Frequent: none
- Rare: headache, dizziness, seizure, coma

Respiratory

- Common: none
- Frequent: none
- Rare: tachypnea, dyspnea, cyanosis

Prednisone Burst

Oral prednisone may cause nausea, vomiting, loss of appetite, heartburn, trouble sleeping, and rise in blood sugar or increased sweating.

9.2 Plan for Patient Safety

Study population: To enhance safety we will exclude patients with: a) history of ICU admission/intubation due to asthma; b) More than three systemic corticosteroid requiring asthma exacerbations in the past year; c) an asthma exacerbation requiring systemic corticosteroids within 30 days preceding study enrollment; d) systemic steroid dependent asthma and e) FEV_1 < 50 % predicted.

9.3 Routine Safety Monitoring

Potential risk mitigation will be achieved through monitoring including study visits and telephone contacts every 2 weeks will help identify participants who experience worsening of control or have an asthma exacerbation during the study. Between in-person study visits, participants will be contacted by telephone by the clinic coordinator to ensure appropriate compliance with the study protocol, to answer any questions, and to ensure their asthma is not worsening, as assessed by the participant. We will follow criteria established by Asthmanet, which proposes and algorithm based on measures of asthma control and healthcare utilization, to determine whether a participant's asthma is worsening or he/she is experiencing an asthma exacerbation (REF). The PI or investigator will determine whether the participant is showing signs of treatment failure using criteria identified below. If it is determined that the participant fulfills criteria for treatment failure or an asthma exacerbation, they will be advised to visit the study center within 24 hours for evaluation and initiation of treatment as specified herein. In the absence of these criteria, arrangements will be made for the participant to have telephone contact or return to the clinic at the next regularly scheduled interval.

If, between phone contacts or in-person visits, an asthma exacerbation occurs, the participant should be evaluated at the nearest medical emergency facility as quickly as possible. Participants will be instructed to call the coordinator or the investigator within 24-48h of

receiving care for an exacerbation. The PI may determine whether a participant needs to be seen at the study center following an exacerbation.

Treatment failure (defined for safety purposes) or significant worsening of asthma status will be defined as the identification of the occurrence of one or more of the following while enrolled in the study (these parameters do not apply to the 72 hour post-bronchoscopy period when these may be an expected adverse event following the procedure):

1. An increase in PRN albuterol/levalbuterol use of ≥ 6 puffs (1 neb equals 4 puffs) per 24 hours over baseline use for 48 hours;
2. Scheduled or unscheduled visit to the study center for worsening of asthma symptoms with a change in pre-bronchodilator FEV_1 ≤ 80% of the baseline on 2 consecutive measurements;
3. Additional inhaled (doubling or more) of baseline dosing or oral corticosteroids for more than 3 consecutive days, or more than one parenteral corticosteroid dose due to asthma;
4. Need for emergency treatment at a medical facility that is related to asthma, or results in hospitalization for an acute asthma exacerbation;
5. Participant refusal to continue study drugs because of lack of satisfaction with treatment; or
6. An increase of the ACQ score of 1.5 from pre-treatment, or an absolute score of 2.5.

Participants will be provided with action plans to communicate with the study investigators if any of these events occur. The study investigator will determine whether the participant should follow up with a visit after patient has received care for an exacerbation in a healthcare facility.

10 DATA AND SAFETY MONITORING PLAN

10.1 Data Safety and Monitoring Board

A Data Safety and Monitoring Board (DSMB) will be created to review this study. The DSMB will contain at least 3 members or experts in pulmonary diseases. None of the DSMB members will be affiliated with this study or have a conflict of interest.

10.2 Data Safety and Monitoring Plan

The DSMB will meet at periodic intervals during the course of the study. The DSMB will be expected to convene as needed, but not less than every 6 months to review the progression of the study including subject enrollment, protocol compliance, and adverse event reports. The DSMB will conduct interim monitoring of accumulating data from research activities to assure

the continue safety of human subjects, relevance and appropriateness of the study, and the integrity of research data.

The study Principal Investigator has primary responsibility for the oversight of the data and safety monitoring. The Investigator will review subject safety data as it is generated. The Investigator, sub-investigators, and the research staff will meet on a four week interval to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The Investigator will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re-evaluated and changed.

The study investigators will evaluate all adverse events (AEs). All subjects who have AEs, whether considered associated with the use of the study medication or not, must be monitored to determine the outcome. The clinical course of the AE will be followed up according to accepted standards of medical practice, even after the end of the period of observation, until a satisfactory explanation is found or the Principal Investigator considers it medically justifiable to terminate follow-up.

10.3 Reporting of Serious Adverse Events and Unanticipated Problems

Investigator's Reporting Requirements to the IND Sponsor

Investigator will report all serious, unexpected, and study-related adverse events to the IND Sponsor within 24 hours. The investigator will then submit a detailed written report to the IND Sponsor. The DSMB, all investigators, and IRB will also be promptly notified in accordance with the respective policies and procedures.

IND Sponsor Reporting Responsibilities

The IND sponsor is required to report certain study events in an expedited fashion to the FDA. It is the responsibility of the IND Sponsor to notify all investigators, in a written IND Safety Report, of any adverse event associated with the use of the drug that is both serious and unexpected. Additionally, Sponsors are also required to identify in IND safety reports all previous reports

concerning similar adverse events and to analyze the significance of the current event in light of the previous reports.

The following outlines the safety reporting requirements by timeline for reporting associated type of events:

Within 7 Calendar Days

Any suspected adverse reaction that is:
– associated with the use of the investigational drug and
– unexpected, and
– fatal or life-threatening

Within 15 Calendar Days

Any suspected adverse reaction that is:
– associated with the use of the investigational drug and
– unexpected, and
– serious, but not fatal or life-threatening

Any significant risk findings from other clinical studies or animal or in vitro studies that
– suggests a significant risk would ordinarily result in a safety-related change in the protocol, informed consent, or other aspects of the overall conduct of the clinical investigation.

Additional IND Reporting Requirements

The IND Sponsor will report any clinically important increase in the rate of a serious suspected adverse reaction over that listed in the protocol. A baseline incidence rate may not always be available, but when one is available, a clinically important increase from that rate will be reported. The decision about when to report is a matter of judgment based on a variety of factors including the study population, the nature and seriousness of the reaction, and the magnitude of the observed increase in the rate.

The IND Sponsor will submit annually an Annual Report to the FDA. The Annual Report will include a written summary of the status of all clinical studies being conducted under the IND

application. The IND Sponsor will work with the reporting investigators to prepare a detailed written summary of serious, unexpected, and treatment related adverse events, and will compare, and contrast the event with prior events. The detailed written summary will also be provided to the DSMB and the IRB.

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