

Oxidative Stress and Regional Airway Remodeling and Fibrosis in Obese Asthma

Research Protocol

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Study Summary:

Title	Oxidative Stress and Regional Airway Remodeling and Fibrosis in Obese Asthma
Short Title	Oxidative Stress in Asthma
IRB Protocol Number	Pro00104900
Study Duration	52 months
Study Center(s)	Duke
Study Aims	<p>The human component of the study has two aims:</p> <p>Aim 1. Identify the pathology, structural cell profile (airway fibroblast and epithelial cell) and redox status corresponding to regional areas of fixed and reversible post-bronchodilator defects (BD) in obese asthmatics.</p> <p>Aim 2. Define the cellular requirement for redox-mediated TGF-β signaling between airway epithelial cells and fibroblasts driving regional remodeling in obese asthma.</p>
Number of Subjects	<p>Targeted enrollment number - 50</p> <p>Enrollment will consist of approximately:</p> <ul style="list-style-type: none">• 15-20 “Early Onset T2 high obese” asthma patients (diagnosed at age < 12 years, and FeNO\geq25 ppb)• 15-20 “Late onset obese, non T2” asthma (diagnosed at age \geq12 years, FeNO < 25ppb, obese (BMI\geq30kg/m²) patients will be enrolled• 10 non-asthma obese controls who demonstrate no evidence of atopy or bronchodilator responsiveness will be also be enrolled <p>Recruitment will include adults (18-65 years old) with physician-diagnosed asthma</p>

<p>Asthma Cohort Inclusion/Exclusion Criteria</p>	<p>INCLUSION</p> <ol style="list-style-type: none"> 1. Adequate completion of informed consent process with written documentation 2. Male and female patients, 18 - 65 years old, inclusive 3. Physician diagnosis of asthma for > 1 year 4. Able to perform reproducible spirometry according to ATS criteria 5. All racial/ethnic backgrounds may participate 6. BMI \geq 30 kg/m² 7. Regular treatment with ICS or ICS/LABA and/or LAMA combination medication for at least 3 months; on a stable dose for the 4 weeks prior to Visit 0 8. Smoking history <10 pack years and no smoking in the last 3 months 9. Late onset asthma: Age of asthma onset (diagnosis) \geq12 years; 10. FeNO < 25 ppb at Visit 0 11. Early onset asthma: Age of asthma onset (diagnosis) <12 years <ul style="list-style-type: none"> • FeNO \geq 25 ppb at Visit 0 12. Positive Methacholine challenge- Confirmation of asthma: Either (1) 12% or greater bronchodilator response (BDR) in FEV1 to albuterol at Visit 0 OR (2) PC20 methacholine \leq 16 mg/ml at Visit 0 (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) AND/OR Positive PD40 to X5 and R5 on IOS. <p>EXCLUSION:</p> <ol style="list-style-type: none"> 13. Respiratory tract infection within the 4 weeks prior to Visit 0 14. Oral or systemic corticosteroid burst (for any indication) within the 4 weeks prior to Visit 0 15. One-time doses, such as intra-articular injections into a shoulder or knee joint, require a 4-week washout prior to Visit 0 16. Asthma-related ER visit within the previous 4 weeks of Visit 0 17. History of ICU admission/intubation due to asthma in the past 1 year 18. Three or more asthma exacerbations requiring treatment with systemic corticosteroids in the past year consistent with severe asthma 19. Asthma exacerbation requiring systemic corticosteroids within the 4 weeks prior to Visit 0 20. Significant concomitant medical illness, including (but not limited to) heart disease, cancer, uncontrolled diabetes, other chronic lung diseases 21. Chronic renal failure (creatinine > 2.0) at Visit 0 22. Positive urine pregnancy test at Visit 0 or at any time during the study 23. Untreated sleep apnea 24. Participation in an intervention study (including, bronchoscopy) or use of investigative drugs within the past 30 days or plans to enroll in such a trial during the study 25. Unable or unlikely to complete study assessments in the opinion of the Investigator 26. Study intervention poses undue risk to patient in the opinion of the Investigator 27. Negative Methacholine challenge (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) and negative PD40 to X5 and R5 on IOS
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<p>Inclusion/Exclusion Criteria for Non-asthmatic controls</p>	<p>INCLUSION</p> <ol style="list-style-type: none"> 1. No history of asthma or other chronic lung diseases 2. Male and female patients, 18 - 65 years old, inclusive 3. Not currently smoking or using other forms of tobacco-related products (including vaping) 4. Smoking history <10 pack years and no smoking in the past 3 months 5. FEV1 > 80% of predicted and FEV1/FVC > lower limit of normal. 6. Ability to sign consent 7. BMI \geq 30 kg/m² 8. Negative Methacholine challenge (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) and negative PD40 to X5 and R5 on IOS <p>EXCLUSION</p> <ol style="list-style-type: none"> 9. Respiratory tract infection within the 4 weeks prior to Visit 0 10. Oral or systemic corticosteroid burst (for any indication) within the 4 weeks prior to Visit 0 11. One-time doses, such as intra-articular injections into a shoulder or knee joint, require a 4-week washout prior to Visit 0 12. Significant concomitant medical illness, including (but not limited to) heart disease, cancer, uncontrolled diabetes, other chronic lung diseases 13. Chronic renal failure (creatinine > 2.0) at Visit 0 14. Positive urine pregnancy test at Visit 0 or at any time during the study 15. Untreated sleep apnea 16. Participation in an intervention study (including bronchoscopy) or use of investigative drugs within the past 30 days or plans to enroll in such a trial during the study 17. Unable or unlikely to complete study assessments in the opinion of the Investigator 18. Study intervention poses undue risk to patient in the opinion of the Investigator 19. Positive Methacholine challenge- Confirmation of asthma: Either (1) 12% or greater bronchodilator response (BDR) in FEV1 to albuterol at Visit 0 OR (2) PC20 methacholine \leq 16 mg/ml at Visit 0 (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) <p>AND/OR Positive PD40 to X5 and R5 on IOS.</p>
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<p>Additional Inclusion/Exclusion Criteria for MRI All participants</p>	<p>INCLUSION</p> <ol style="list-style-type: none"> 1. Outpatients of either gender, age > 18 2. Willing and able to give informed consent and adhere to visit/protocol schedules. (Consent must be given before any study procedures are performed.) 3. Women of childbearing potential must have a negative urine pregnancy test prior to MRI. <p>EXCLUSION</p> <ol style="list-style-type: none"> 4. Medical or psychological conditions which, in the opinion of the investigator, might create undue risk to the subject or interfere with the subject's ability to comply with the protocol requirements 5. Conditions that will prohibit MRI scanning (metal in eye, claustrophobia, inability to lie supine, shoulder circumference >140 cm*.) *This measurement is not an absolute as it can vary based on weight distribution.
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Study Schedule	<p>Following prescreening for eligibility, participants will provide consent, and undergo a screening visit (V 0) which includes:</p> <p><u>Screening visit (V 0)</u></p> <ul style="list-style-type: none"> • Urine pregnancy for women of child-bearing potential • Anthropometrics and vital signs • Medical history including current and past medication review • Asthma control questionnaire (ACQ) • Skin allergy testing, • Blood draw for labs: <ul style="list-style-type: none"> ○ CBC with diff (LAB1748) 4.0 mL ○ fasting blood glucose (LAB81) 4.0 mL ○ total cholesterol (LAB60) 4.5 mL ○ triglycerides (LAB134) 4.5 mL ○ chemistries (LAB15) 4.5 mL, and ○ serum IGE (LAB74) 3.5 mL ○ CPT 8.0 mL ○ Total blood volume = 33 mL • Impulse oscillometry (IOS) measurements • Pulmonary function testing will be performed including: <ul style="list-style-type: none"> ○ exhaled nitric oxide (FeNO), <ul style="list-style-type: none"> ▪ May be repeated once if results are high ○ spirometry ○ Lung volumes via body plethysmograph ○ Methacholine challenge <p><u>Visit 1</u> (1-2 weeks after visit 0)</p> <ul style="list-style-type: none"> • Urine pregnancy for women of child-bearing potential • Interim medical history and medications will be reviewed, and any AE's evaluated. • Vital signs • ACQ • The participants will have a standard dose chest CT • Hyperpolarized Xe-MRI performed pre and post bronchodilator. • Approximately 24-hours after MRI, a follow up phone call. <p><u>Visit 2</u> (1-2 weeks after visit 1)</p> <ul style="list-style-type: none"> • Urine pregnancy for women of child-bearing potential • Standard of Care (SOC) Bronchoscopy consent • Vital signs and brief physical exam • Interim medical history and medications will be reviewed, and any AE's evaluated. • The participants will have a bronchoscopy with broncho-alveolar lavage (BAL), brushes and biopsy. <p><u>Visit 3</u></p> <ul style="list-style-type: none"> • A follow up phone call.
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Statistical Methodology	<p>For the <i>in vivo</i> assessments of airway remodeling and fibrosis (bronchodilator reversibility on $^{129}\text{XeMRI}$), we will compare biomarkers of fibrosis (e.g., trichrome or percent elastic fiber staining) between BD types (fixed and reversible airway segments) both within and between obese asthma groups (early onset, late onset and non-asthma control) using a repeated measures two-way ANOVA with appropriate distributional assumption. Main effects of BD type, obese asthma group and their interaction will be evaluated with repeating BD type within subject (random effect) with compound symmetry variance structure. If significant main effect differences are found, pairwise comparisons between BD types and obese asthma groups will be done using Tukey's post-hoc test. Assuming a 50% decrease in fibrosis between BD fixed and reversible types, a sample size of 10 per group provides our study with $\geq 80\%$ power to detect an effect size of 0.53 within and 0.60 between groups, where effect size (f) is the size of the mean differences relative to the standard deviation and assumed correlation (ρ) within group of 0.30. A p-value < 0.05 will be considered statistically significant and all analyses will be performed using SAS version 9.4 or higher (SAS Institute, Inc., Cary, NC). Blinding to conditions and tissue sources will occur across the experimental design. In particular, tissues samples (post-BD fixed and BD reversible tissue based on region) will be blinded to the individual performing the experiments and the individual assays. The key for the tissue samples will be maintained by the program statistician and un-blinding will occur during data analysis by the statistician. The statistical models and analyses that are planned for the primary and secondary outcomes assume that the data are missing-at-random (MAR).</p> <p>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 (Albert '19, Gottfredson '14), will be applied.</p>
Data Collection	<p>Data will be captured in REDCap, except for patient consent forms and AE/SAE forms, which will be on case report forms (CRFs) collected by the coordinators. REDCap is a software tool that does not require client local software and can be accessed from anywhere on the Internet and is secured on a Duke Health Technology Services (DHTS) server. Established systems for data entry, verification, and error-checking will be followed.</p>

Schedule of Events:

Week	-1	0	1	1+
Visit number	V0	V1	V2	V3
Study ICF	x			
Bronchoscopy SOC consent			x	
Vital signs	x	x	x	
Baseline medical history, including current and past medication review	x			
Interim medical and medications review and any AE's evaluated		x	x	
Brief physical exam			x	

Week	-1	0	1	1+
Visit number	V0	V1	V2	V3
ACQ	x	x		
Spirometry	x			
Impulse oscillometry (IOS)	x			
FeNo	x			
Lung volumes via body plethysmograph	x			
Methacholine challenge	x			
Blood draw for clinical labs*	x			
Skin Allergen panel	x			
Anthropometrics	x			
Pregnancy test**	x	x	x	
CT Scan		x		
¹²⁹ XeMRI pre and post bronchodilator		x		
Bronchoscopy			x	
Follow-up phone call		X (24 hours after MRI)		X (24 hours after bronchoscopy)

*Clinical labs: CBC with diff (LAB1748) 4.0 mL, fasting blood glucose (LAB81) 4.0 mL, total cholesterol (LAB60) 4.5 mL, triglycerides (LAB134) 4.5 mL, chemistries (LAB15) 4.5 mL, and serum IGE (LAB74) 3.5 mL, CPT 8.0 mL

**for women of child-bearing potential

The **central hypothesis** is that sites of abnormal ventilation on ¹²⁹XeMRI represent areas of airway remodeling and fibrosis and are enriched with fibroblasts that are invasive, proliferative and fibrogenic. We further hypothesize that regional alterations in oxidant stress driving the production of transforming growth factor-beta (TGF-β) direct pro-remodeling fibroblast functions. Lastly, we hypothesize that ¹²⁹XeMRI will be a sensitive and specific biomarker of airway remodeling and fibrosis in obese asthmatics and rat models of obese asthma. By leveraging our excellence in clinical asthma, bronchoscopy, and translational expertise in cell function/signaling and 3D MR imaging in both patients and animal models, we will conduct both *ex vivo* cell-specific mechanistic studies and *in vivo* animal model studies to uncover the mechanisms of molecular and cellular function through the following **Specific Aims**:

Aim 1. Identify the pathology, structural cell profile (airway fibroblast and epithelial cell) and redox status corresponding to regional areas of fixed and reversible post-bronchodilator defects (BD) in obese asthmatics.

We hypothesize that post-BD fixed defects identified on ¹²⁹XeMRI correspond to sites of increased oxidant stress and airway remodeling and fibrosis. We will use ¹²⁹XeMR imaging to guide bronchoscopy sampling of airways in both BD fixed and BD reversible ventilation defects. We will relate the contribution of the oxidant milieu, structural cell phenotypes and histopathologic composition of airways to changes in ventilation defects observed on ¹²⁹XeMRI.

Aim 2. Define the cellular requirement for redox-mediated TGF-β signaling between airway epithelial cells and fibroblasts driving regional remodeling in obese asthma. We hypothesize that regions of fixed defects on ¹²⁹XeMRI are composed of airway fibroblasts that are more proliferative, invasive and fibrogenic than fibroblasts in BD reversible defects and respond to the increased oxidative milieu and TGF-β signaling. We will assess epithelial cell signaling, airway fibroblast migration/invasiveness, proliferation and extracellular matrix (ECM) protein secretion with *ex vivo* cell culture models. These assays will establish significant associations between airway epithelial signaling and fibroblast function, changes in airway ventilation, and asthma endotypes.

Aim 3 Define regional differences of spatial interactions of mesenchymal cells with epithelial cells in the airway submucosa.

3A - Determine how transcriptional profiles change between lean vs obese asthma and controls;

3B - Determine how transcriptional profiles change in BD fixed vs responsive regions in obese asthma.

We hypothesize that local gene expression changes and cell signaling among both structural cells and immune cells in airway tissue contributes to airway remodeling in asthma patients with obesity. We plan to utilize spatial transcriptomics techniques to analyze endobronchial tissue samples isolated from asthma and non-asthma participants with and without obesity to compare specific airway tissue gene expression patterns in asthma vs non-asthma participants, participants with obesity vs non-obesity, and combined conditions. These gene expression patterns will be localized to pathologic features of asthma and obesity, including accumulation of submucosal eosinophils, fibrosis, smooth muscle and basement membrane thickening, etc in the tissue sections.

We are collaborating with Dr. Alexander Misharin at Northwestern and Dr's. Victor Roggli and Jeffrey Everitt (Duke pathologists) for this aim. Cryopreserved airway tissues from deidentified participants will be used for **Aim 3A** and include tables containing the demographics, physiology, FeNO, ACQ and atopy status. Endobronchial biopsies obtained from participants in this study will be deidentified and included in **Aim 3B**.

Purpose of the Study:

The proposed study will determine if sites of abnormal ventilation on $^{129}\text{XeMRI}$ represent areas of airway remodeling and fibrosis enriched with fibroblasts that are invasive, proliferative and fibrogenic. We hypothesize that regional alterations in oxidant stress drive the production of transforming growth factor-beta (TGF- β) which directs pro-remodeling fibroblast functions. We predict that $^{129}\text{XeMRI}$ will be a sensitive and specific biomarker of airway remodeling and fibrosis in obese subjects with asthma.

Primary outcomes:

- We will identify the pathology, structural cell profile (airway fibroblast and epithelial cell) and redox status corresponding to regional areas of fixed and reversible post-bronchodilator defects (BD) in obese asthmatics;
- We will define the cellular requirement for redox-mediated TGF- β signaling between airway epithelial cells and fibroblasts driving regional remodeling in obese asthma;
- We will develop non-invasive 3D imaging techniques to assess airway regional remodeling in obese asthma.

Secondary outcomes:

- We predict that $^{129}\text{XeMRI}$ will be a sensitive biomarker of asthma control and severity of disease.

BACKGROUND & SIGNIFICANCE:

Obese asthma. Obese asthma is a major public health concern. The prevalence of obesity continues to rise at an alarming rate and has reached epidemic proportions over the past several decades. Obesity is a risk factor for the development of asthma and is associated with approximately 250,000 cases per year in the U.S. ¹. In patients with established asthma, obesity is associated with increased risk for exacerbations, worse respiratory symptoms and poor control ^{2,3}. Obesity and weight gain are associated with increased asthma severity and a nearly 5-fold risk of hospitalization ⁴. *This is a major public health concern, given that the Centers for Disease Control has estimated that 38.8% of asthmatics are obese.*

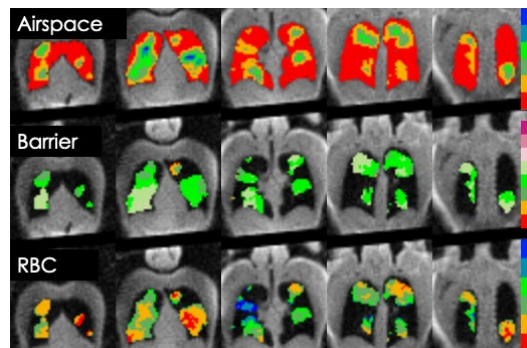
Obese asthma is broadly categorized into two distinct endotypes, early vs. late onset disease. Early onset obese asthma is defined as being diagnosed at <12 years of age, associated with type 2 (T2) high disease, an allergic phenotype, and the development of obesity as a consequence of asthma. Late-onset obese asthma occurs after 12 years of age or in adulthood ⁵ and is associated with the development of asthma subsequent to obesity ⁶. In early onset asthma, obesity contributes to disease severity and is associated with an increased incidence of fixed airway obstruction ⁷; this fixed obstruction is also referred to as airway remodeling and/or fibrosis (structural changes of the airway wall), and manifests as bronchodilator non-responsiveness. In contrast, the late onset obese asthmatic is a common and unique clinical phenotype that is not defined by T2-related airway inflammation. Despite having relatively preserved lung function and lower fractional exhaled nitric oxide (FeNO) levels, cluster analyses show that those asthmatics characterized by body mass index (BMI) >30 kg/m² with late onset disease have a high symptom burden and high utilization of healthcare resources ⁸⁻¹⁰. New biologics targeting T2 high asthma are less effective in obese asthmatics compared to lean patients and are not efficacious in non-T2 disease ^{11,12}. Therefore, we need to better understand how asthma differs in the obese population. Only with such understanding will it become possible to develop effective therapies that benefit these patients.

Airway Remodeling and Fibrosis. Airway remodeling and fibrosis in asthma represents a major cause of morbidity and mortality. It is notoriously difficult to diagnose with precision, to treat effectively, and to monitor effectively as the disease progresses. Clinical assessment of airway remodeling in the obese asthmatic patient is especially challenging as asthma in the obese is often attributed to mechanical effects caused by chronic lung compression and/or airway reactivity induced by breathing at low lung volumes. Airway remodeling can result from either accelerated, uncontrolled inflammation, as a response to injury or as a result of abnormal growth and development¹³. Despite intensive research efforts, treatments for remodeling remain limited, reflecting a poor understanding of the common mechanisms underlying these processes. A central issue is that airway remodeling is heterogeneous. Not only can remodeled tissue exhibit unique mechanisms based on the maturity of the remodeling, but also the extent of remodeling can differ across the landscape of the lung. This regional heterogeneity limits detection of airway remodeling and fibrosis and hampers the ability to test the efficacy of early interventions as global measures of lung function do not detect regional changes in air flow or functional compensation from surrounding tissues. Both these aspects of heterogeneity are addressed in this application.

Obese asthma is associated with more severe airway remodeling and fibrosis than lean asthma^{14,15}. The epithelium is hypothesized to orchestrate the inflammatory and remodeling responses of the airway, as it serves as a rich source of TGF- β ^{16,17}, a profibrotic growth factor. The epithelium also potentially serves as a source of myofibroblasts via epithelial-to-mesenchymal transition (EMT)¹⁸. Fibroblasts and myofibroblasts are the main structural cells of the mesenchyme that mediate the majority of the events contributing to the subepithelial fibrosis in remodeled airways¹⁹⁻²¹. Following injury to the airway, epithelial cells secrete growth factors (e.g., TGF- β and epidermal growth factor) and become migratory by undergoing EMT. In parallel, resident fibroblasts in the airway migrate to sites of airway injury, proliferate, differentiate to myofibroblasts and deposit matrix²²⁻²⁴. Indeed, fibroblasts isolated from the airways of asthmatic subjects make greater amounts of ECM proteins, including collagen²⁵ and hyaluronan²⁶, with lesser amounts of elastin²⁷, compared with fibroblasts isolated from non-asthmatics²⁸. Work by Hackett *et al.* suggests that asthmatic epithelium responds inappropriately to challenge resulting in dysregulated repair and airway remodeling and fibrosis²⁹. In T2 high disease, interleukin-13 (IL-13) has been shown to potentiate airway fibroblast invasion in the asthmatic airway through a mechanism involving TGF- β 1 and metalloproteinases³⁰. Mechanisms regulating remodeling and fibrosis in nonT2 disease are not known, but this knowledge would greatly aid in the development of novel strategies to treat this predominant asthma endotype.

Oxidative stress in Obese Asthma. Oxidative stress is increased in the obese patient because adipose tissue serves as a source of pro-inflammatory cytokines such as leptin, tumor necrosis factor-alpha (TNF- β , interleukin-1beta (IL-1 β and interleukin-6 (IL-6)³¹ which promote increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress is believed to play a critical role in the pathogenesis of asthma and can result from an imbalance between the production of ROS and RNS and/or a loss of antioxidant defense mechanisms. However, to date it remains unclear whether oxidative stress in obese asthma is secondary to increased inflammation or if it is causative to the development of asthma.

Clinical Assessment of Airway Remodeling and Fibrosis in Obese Asthma. Conventional lung function testing lacks sensitivity to evaluate airway remodeling and fibrosis. Spirometry, though considered to be the gold standard, is limited in its use as it does not accurately measure airway resistance in peripheral small airways, the major site of airflow obstruction in obese asthma patients³². Clinically the presence of fixed airflow obstruction as measured by lack of bronchodilator responsiveness (<200 cc or <12% improvement in forced expiratory volume (FEV1) or forced vital capacity (FVC) post-bronchodilator) is considered to be a late and irreversible manifestation of airway remodeling. To overcome this, forced oscillation technique (FOT) has been used to improve the sensitivity to detect small airways dysfunction, but FOT cannot quantify regional changes in small airway disease, which limits our ability to effectively monitor progression of disease and response. These limitations in assessing regional structure-function abnormalities have created a **critical barrier** to the advancement of our understanding of mechanisms driving airway remodeling in the obese asthmatic patient.



Hyperpolarized ^{129}Xe MRI to Assess Lung Structure Function. Hyperpolarized helium (^3He) and xenon (^{129}Xe) MR imaging of the lungs have emerged as novel mechanisms to probe changes in pulmonary ventilation, microstructure and gas exchange. ^{129}Xe has a high solubility in blood and lipid rich tissue. Inhaled ^{129}Xe is absorbed quickly into the blood stream and distributed rapidly throughout the body by circulation ³³. A unique quality of ^{129}Xe is that, once absorbed, it demonstrates distinct chemical shifts in the gas-phase, pulmonary barrier tissue, and red blood cells (RBCs) ³⁴, so combining visualization of gas phase and dissolved phase allows us to simultaneously assess ventilation, interstitial abnormalities, and lung microstructure (**Figure 1**). ^{129}Xe MRI has been used to identify ventilation defects induced by exercise, methacholine challenge and those reversed by bronchodilators ³⁵; these defects increase with asthma severity ³⁶. Moreover, studies using methacholine to induce bronchoconstriction or albuterol as a bronchodilator demonstrate that some regional changes of airflow obstruction are relatively fixed in the lung while others are much more dynamic (months-years) ^{37,38}. ^{129}Xe MRI has also been used to assess small airway dysfunction in asthma ^{33,39} and can identify and localize regional ventilation defects, even in asthmatics with normal spirometry, suggesting a potential role for ^{129}Xe MRI in preclinical detection of disease and as a biomarker of disease activity. We and others have found that ^{129}Xe MRI has a strong safety and tolerability profile in subjects with obstructive airway diseases; indeed, only transient hypoxia, dizziness, paresthesia/hypoesthesia and euphoria have been reported ⁴⁰. Moreover, the test-retest variability of ^{129}Xe MRI is exceptionally good ($\pm 1.5\%$) relative to that of the spirometric measure, FEV1 ($\pm 7.2\%$) ⁴¹. *These studies support safety, tolerability and reproducibility of ^{129}Xe MRI.*

Figure 1. ^{129}Xe Gas Exchange MRI in a severe asthmatic. While the most prominent features are substantial ventilation defects (red), it is also noteworthy that numerous areas of the lung exhibit poor RBC transfer, while others exhibit exceptionally high transfer

Biomarkers in obese asthma. New precision medicine approaches to diagnose and monitor disease activity in the obese asthmatic patient are needed. Despite significant advances in our understanding of the pathobiology of asthma, most of what we have learned about asthma has been obtained studying lean asthmatics. In patients with early onset disease, T2 inflammatory biomarkers are being used to identify eosinophilic asthma phenotypes and to predict response to therapy ⁴²⁻⁴⁵. However, these biomarkers are poorly predictive of disease activity in obese asthma. This problem is amplified in the late onset obese asthma patient for whom there are no other biomarkers and very limited treatment options available. Indeed, biomarkers used to identify T2 driven disease in lean asthmatics have been found to be poorly predictive of eosinophilic airway inflammation in the T2 high obese asthmatic ⁴⁶. As a result, a major limitation in studying obese asthma is lack of the ability to monitor and identify sites of disease activity. This project will advance our understanding of the different asthma endotypes and provide a noninvasive modality to regionally assess lung structure/function and response to therapy.

Bronchoscopy and Clinical Phenotyping.

By using ^{129}Xe MRI to identify BD-fixed and reversible ventilation defects, this study will be the first to objectively evaluate the contribution of structural cell phenotypes, epithelial cell RNA transcriptomes, immune cells profiles and oxidant burden to obese asthma endotypes. Our use of co-registered chest CT/ ^{129}Xe MRI to identify BD fixed and reversible regional changes in ventilation and to guide airway tissue sampling for the study of underlying histopathology, cell composition and oxidative milieu is an innovative approach to studying the biological underpinnings regulating airway remodeling and fibrosis in obese asthma.

All subjects enrolled will undergo bronchoscopy with endobronchial biopsy, brushings and bronchoalveolar lavage (BAL) and blood serum collection. Six endobronchial biopsies and brushings per airway segment will be performed under direct visualization. Biopsy locations will be ^{129}Xe MRI-guided to airway regions of BD fixed and BD reversible defects. Two endobronchial biopsy specimens per airway segment will be fixed in 4% paraformaldehyde and embedded in paraffin. Two or three specimens per airway segment will be used for airway fibroblast culture. Epithelial cells harvested from airway brushes will be grown on air liquid interface for cell culture assays and airway epithelial cell RNA

We will determine airway fibroblast phenotype (i.e. myofibroblast differentiation) in persistent MRI defects post-BD using several methods:

- 1) Airway fibroblasts will be cultured from fresh airway biopsy pieces following Ingram *et al* (34). Immuno-cytochemistry will be performed on cells at passage 0 cultured on collagen-coated chamber slides to determine reactivity with antibodies specific for myofibroblast markers (alpha smooth muscle

actin (α SMA) and amine oxidase, copper containing 3 (AOC3) (77) as well as the fibroblast markers, Fibroblast specific protein-1 (FSP-1) and vimentin, and an epithelial cell marker, E-cadherin.

- 2) Total RNA and protein lysates will be isolated from cells grown at air liquid at Passage 0. Western blotting and quantitative RT-PCR will be used to confirm protein and mRNA expression of α SMA (29) and AOC3 in myofibroblasts, and to rule out expression of epithelial cell protein, E-cadherin.
- 3) To control for cell culture artifacts, fresh airway biopsy tissue will be digested with *ethylenediaminetetraacetic acid disodium* (EDTA) and we will perform fluorescence-activated cell sorting (FACS) for AOC3+ myofibroblasts and AOC3- fibroblasts. Cells will be fixed and stained with anti-vimentin and α SMA to confirm fibroblast and myofibroblast phenotype.

1.3 We will perform single cell ribonucleic acid (RNA) sequencing (scRNAseq) analysis on brushings from airway segments corresponding to post-BD fixed and reversible defects on $^{129}\text{XeMRI}$. This will allow us to assess cellular heterogeneity and to determine its contribution to ventilation heterogeneity. We have performed scRNAseq of bronchial brushings previously using the Drop Seq method (78, 79). Drop-seq permits highly parallel genome-wide transcriptome profiling using unique barcodes that tag cellular mRNAs from individual cells. These unique barcodes will then be used to *in silico* reconstruct the single cell RNAs from tens of thousands of cells. For scRNA seq analysis, the FASTQ files will be processed using dropSeqPipe v0.3 and mapped on to the human genome. Unique molecular identifier (UMI) counts will then be further analyzed using an R package Seurat v3.0.6. UMI counts will be normalized using SCTransform v0.2. Principle components that are significant based on Jackstraw plots will be used for generating t-SNE and UMAP plots. Cell barcodes of clusters of interests will be extracted and utilized for *velocity run* command in velocity.py v0.17.15 as well as generating RNA velocity plots using velocity.R v0.6 in combination with an R package SeuratWrappers v0.1.0.

1.4 Determine the association between ventilation heterogeneity and airway physiology (pulmonary function testing (PFT) and impulse oscillometry) (80, 81).

DESIGN & PROCEDURES:

Subject Recruitment and Screening: We anticipate 40 early and late onset obese asthma patients and 10 obese non-asthma volunteers will undergo Hyperpolarized $^{129}\text{XeMRI}$ and bronchoscopy after screening over the course of this study. Patients will undergo, twice in succession, an approximately 30-min MRI protocol, consisting of several breath-hold scans after hyperpolarized ^{129}Xe or room air administration. The imaging will be repeated pre- and post-bronchodilator (BD), thus requiring 60-min to complete. The results of a co-registered CT of the chest and the pre-post bronchodilator Hyperpolarized $^{129}\text{XeMRI}$ will be reviewed by the Duke Imaging Team to identify segmental persistent post-BD ventilatory defects in obese asthmatics. The CT will provide precise structural information for segmental delineation, while MRI informs regional ventilatory function, interstitial uptake and lung microstructure and function. For these purposes, we need a single high-res (standard dose) CT scan at full inspiration. The Duke Imaging Team will dictate to the bronchoscopist in a blinded fashion which segments contain BD fixed and BD reversible ventilatory defects. Bronchoalveolar lavage (BAL), endobronchial biopsies and brushings will be obtained from these segments.

Study participants will be enrolled at the Duke University Health System from clinics, hospitals and communities via various IRB-approved recruitment methods, including Protocol Pro00102890; Pulmonary Medicine Healthy Volunteer Data Repository (HVDR) and Pro00103507; Pulmonary Medicine Lung Disease Volunteer Data Repository (LDVDR).

Preliminary eligibility asthma diagnosis, medical history will be determined by pre-screening procedures that include a review of medical records (site specific electronic medical record (EMR) reports and search tools such as DEDUCE, slicer dicer and Mychart invitations, and manual review) and clinic schedules to identify potentially eligible participants. Each participant who is pre-screened (e.g. medical chart review is performed to assess potential eligibility prior to approaching the participant), approached for recruitment and screened for study participation will have the following information entered in a screening log: name, MRN, date of birth, race, ethnicity, contact information, date of pre-screen review, and enrollment status, along with the reason(s) for not enrolling in the study, if applicable. Participants will be enrolled into one of the three groups (late onset asthma, early onset asthma, and non-asthma controls) based on their profiles.

Informed Consent Process: Participants will receive an overview of the study during an in person or phone pre-screening process. A copy of the ICF can be sent to the potential participant via e-mail or mail prior to the screening visit. Prior to the initiation of any clinical research procedures at study visits, designated research staff will describe the study's purpose, all procedures, and risks/benefits and review the informed consent document with the potential participant. Subjects will be informed that their decision to participate in the study will have no impact on their medical care. Participants will be provided ample time to read the consent, ask questions, and deliberate their decision.

Patients who are deemed eligible after pre-screening will be invited and scheduled for enrollment at Duke. A verbal consent will be performed to allow for placing orders in the EMR prior to written consent. Willing participants will undergo informed written consent before any study-related procedures take place. Following consent, they will be assigned a study ID in consecutive order and record the assignment for ID on the log.

The coordinator or PI will conduct the consent process with prospective participants. Dr. Driehuys will not personally obtain consent due to his conflict of interest.

Enrollment, Study Visits & Procedures:

Screening Visit 0 – 3-4 hours (week -1)

Following Informed Consent, participants will be enrolled and will be assigned a study ID in consecutive order from the assignment log provided record the assignment on the study log. All enrolled participants will then perform:

- Urine pregnancy for women of child-bearing potential
- Anthropometrics and vital signs
- Medical history review (including the following: age of asthma onset, prior hospitalizations, ER visits, steroid use, frequency of asthma exacerbations, antibiotic use, nocturnal awakenings, frequency of SABA use, immune desensitization, current and past medication use, self-described smoking history)
 - Demographics (race, ethnicity, gender, age)
- ACQ
- Skin allergy testing,
- Blood draw for labs:
 - CBC with diff (LAB1748) 4.0 mL
 - fasting blood glucose (LAB81) 4.0 mL
 - total cholesterol (LAB60) 4.5 mL
 - triglycerides (LAB134) 4.5 mL
 - chemistries (LAB15) 4.5 mL
 - serum IGE (LAB74) 3.5 mL
 - CPT 8.0 mL
 - Total blood volume = 33 mL
- IOS
- Pulmonary function testing will be performed including:
 - exhaled nitric oxide (FeNO),
 - May be repeated once if results are high
 - spirometry
 - Lung volumes via body plethysmography
 - Methacholine challenge

Visit 1 – 4 hours (week 0)

- Urine pregnancy for women of child-bearing potential
- Interim medical history and medications will be reviewed, and any AE's evaluated
- Vital signs

- ACQ
- Standard dose chest CT (SDCT)
- Hyperpolarized ^{129}Xe MRI pre and post bronchodilator.
- Participants will receive a follow up phone call approximately 24 hours after the MRI with the coordinator, including a review of interim medical history and medications and any AE's evaluated

Visit 2 – 2 hours (week 1)

- Standard of Care (SOC) Bronchoscopy consent
- Urine pregnancy for women of child-bearing potential
- Vital signs and brief physical exam
- Interim medical history and medications will be reviewed, and any AE's evaluated.
- Bronchoscopy with BAL 90 ml per airway in two airways for a total of 180 ml, and up to 6 brushes per airway (12 total) and endobronchial biopsies per airway segment (up to 12 total).
- Post-procedure observation in the recovery area of 25 minutes to 1 hour.

The participant will not be allowed to proceed with the bronchoscopy visit which includes moderate conscious sedation without an adult that comes with them during the check in process and is available to escort the participant home.

Visit 3 – follow up (1 week after visit 2)

- Participants will receive a follow up phone call with the coordinator, including a review of interim medical history and medications and any AE's evaluated

Asthma Cohort Inclusion / Exclusion Criteria are as follows:

INCLUSION

1. Adequate completion of informed consent process with written documentation
2. Male and female patients, 18 - 65 years old, inclusive
3. Physician diagnosis of asthma for > 1 year
4. Able to perform reproducible spirometry according to ATS criteria
5. All racial/ethnic backgrounds may participate
6. BMI ≥ 30 kg/m²
7. Regular treatment with ICS or ICS/LABA and/or LAMA combination medication for at least 3 months; on a stable dose for the 4 weeks prior to Visit 0
8. Smoking history <10 pack years and no smoking in the last 3 months
9. Late onset asthma: Age of asthma onset (diagnosis) ≥ 12 years;
 - FeNO < 25 ppb at Visit 0
 - Negative allergen skin test
10. Early onset asthma: Age of asthma onset (diagnosis) <12 years
 - FeNO ≥ 25 ppb at Visit 0
 - Positive allergen skin test
11. Positive Methacholine challenge- Confirmation of asthma: Either (1) 12% or greater bronchodilator response (BDR) in FEV1 to albuterol at Visit 0 OR (2) PC20 methacholine ≤ 16 mg/ml at Visit 0 (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) AND/OR Positive PD40 to X5 and R5 on IOS.

EXCLUSION:

1. Respiratory tract infection within the 4 weeks prior to Visit 0
2. Oral or systemic corticosteroid burst (for any indication) within the 4 weeks prior to Visit 0
 - One-time doses, such as intra-articular injections into a shoulder or knee joint, require a 4-week washout prior to Visit 0
3. Asthma-related ER visit within the previous 4 weeks of Visit 0

4. History of ICU admission/intubation due to asthma in the past 1 year
5. Three or more asthma exacerbations requiring treatment with systemic corticosteroids in the past year consistent with severe asthma
6. Asthma exacerbation requiring systemic corticosteroids within the 4 weeks prior to Visit 0
7. Significant concomitant medical illness, including (but not limited to) heart disease, cancer, uncontrolled diabetes, other chronic lung diseases
8. Chronic renal failure (creatinine > 2.0) at Visit 0
9. Positive urine pregnancy test at Visit 0 or at any time during the study
10. Untreated sleep apnea
11. Participation in an intervention study (including, bronchoscopy) or use of investigative drugs within the past 30 days or plans to enroll in such a trial during the study
12. Unable or unlikely to complete study assessments in the opinion of the Investigator
13. Study intervention poses undue risk to patient in the opinion of the Investigator
14. Negative Methacholine challenge (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) and negative PD40 to X5 and R5 on IOS.

Inclusion Exclusion Criteria for Non-asthmatic controls

INCLUSION

1. No history of asthma or other chronic lung diseases
2. Male and female patients, 18 - 65 years old, inclusive
3. Not currently smoking or using other forms of tobacco-related products (including vaping)
4. Smoking history <10 pack years and no smoking in the past 3 months
5. FEV1 > 80% of predicted and FEV1/FVC > lower limit of normal.
6. Ability to sign consent
7. BMI \geq 30 kg/m²
8. Negative allergen skin test
9. Negative Methacholine challenge (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives) and negative PD40 to X5 and R5 on IOS.

EXCLUSION

1. Respiratory tract infection within the 4 weeks prior to Visit 0
2. Oral or systemic corticosteroid burst (for any indication) within the 4 weeks prior to Visit 0
 - One-time doses, such as intra-articular injections into a shoulder or knee joint, require a 4-week washout prior to Visit 0
3. Significant concomitant medical illness, including (but not limited to) heart disease, cancer, uncontrolled diabetes, other chronic lung diseases
4. Chronic renal failure (creatinine > 2.0) at Visit 0
5. Positive urine pregnancy test at Visit 0 or at any time during the study
6. Untreated sleep apnea
7. Participation in an intervention study (including bronchoscopy) or use of investigative drugs within the past 30 days or plans to enroll in such a trial during the study
8. Unable or unlikely to complete study assessments in the opinion of the Investigator
9. Study intervention poses undue risk to patient in the opinion of the Investigator
10. Positive Methacholine challenge (historical results from previous studies or documented in clinical records within preceding year are acceptable alternatives)
AND/OR positive PD40 to X5 and R5 on IOS.

Additional Inclusion/Exclusion Criteria for MRI

INCLUSION

1. Outpatients of either gender, age > 18

2. Willing and able to give informed consent and adhere to visit/protocol schedules. (Consent must be given before any study procedures are performed.)
3. Women of childbearing potential must have a negative urine pregnancy test prior to MRI.

EXCLUSION

1. Medical or psychological conditions which, in the opinion of the investigator, might create undue risk to the subject or interfere with the subject's ability to comply with the protocol requirements
2. Conditions that will prohibit MRI scanning (metal in eye, claustrophobia, inability to lie supine, shoulder circumference >140 cm*). *This measurement is not an absolute as it can vary based on weight distribution.

Descriptions of Evaluations and Measures:

- **Anthropometrics, height, weight and vital signs** – Blood pressure is a determinant in classifying and understanding relative risk of cardiovascular disease. Blood pressure will be recorded using an automated blood pressure cuff. If, due to medical reasons, the participant cannot be measured for blood pressure on either arm, a brachial blood pressure reading with an appropriately sized cuff may be taken by experienced staff. To assess abdominal obesity, waist measurement should be made at the midpoint between the lower margin of the last palpable rib, and top of the iliac crest (the hip bone). Hip measurement will be measured at the widest point of the buttocks, with the tape measure parallel to the floor. Height will be recorded to the nearest 1 mm. Weight will be recorded to the nearest 100 g or 0.1 kg and repeated 2 times. If the two readings are within 100g of each other, then these readings will be averaged and recorded.
- **Venipuncture** - Blood samples will be obtained by venipuncture of an antecubital vein to determine the levels of study-related biomarkers and clinical labs.
- **Spirometry**- a measure of pulmonary lung function.
- **Exhaled Nitric Oxide**-This procedure involves exhaling gently into a small, handheld device that measures FeNO. Participants will also undergo measurement of exhaled nitric oxide according to standardized techniques. The risks of this test are minimal. Collection of FeNO involves inhalation to total lung capacity while in a seated position followed by slow exhalation against resistance for 10 seconds. Both children and adults can perform this without difficulty. A trained technician will be present to supervise and support the participant in order to minimize discomfort.
- **Impulse oscillometry:** Using this procedure, the patient breathes through a pneumotachograph which generates a sound wave by a loudspeaker that is superimposed over the subject's normal, quiet, tidal breathing. The patient's airflow and sound wave response is transmitted to the apparatus and used to calculate the various components of resistance to breathing. The risks of this procedure are minimal. Both children and adults can perform the procedure without difficulty.
- **Lung volumes via body plethysmograph pre-bronchodilator** - Body plethysmography Lung volumes will be used to assess the following lung volume parameters: Functional Residual Capacity (FRC), Vital Capacity (VC), and then Inspiratory Capacity (IC)
- **Methacholine challenge**-Participants will be asked to come in for a methacholine challenge after consenting. This test is often used to support the diagnosis of asthma. Methacholine is a chemical that may cause the airways of asthmatics to narrow, but does not affect the airways of people without asthma. The test consists of inhaling increasing doses of methacholine through a nebulizer to assess how quickly your airways narrow. Your airways may tighten slightly as the test proceeds. We are performing the test to see how quickly your airways narrow. We will start with very small doses, gradually increasing the dose of methacholine. Spirometry will be performed after each dose to look for changes in lung function. The procedure will be terminated if you demonstrate a significant change in your pulmonary function test (20% decrease in FEV1) or you reach the highest concentration of methacholine. You will receive albuterol (an inhaler medicine that relaxes the airways) after the test is done to reverse any asthma symptoms. The methacholine challenge test will be done by trained personnel and can take up to two hours to complete.

Before the test, you will be asked to withhold all short-acting bronchodilators for 4 hours, long-acting bronchodilators for 12 hours, and Tiotropium for 24 hours. You will also be asked to avoid caffeine and alcohol for 8 hours.

For challenge testing, methacholine will be administered via AeroEclipse BAN II nebulizer following guidelines recommended by the American Thoracic Society (ATS), with 1 additional dose, will be used: 0.03125 mg/mL, 0.0625 mg/ml, 0.1250 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL.

Solutions are prepared using **Provocholine 100 mg in 20 ml**, 1 vial will be required.

Vial	Concentration	Prev	Dil	CalcConc	VolStart	VolEnd
A	16	0	6.25	16	6.25	3.25
B	8	3	3	8	6	3
C	4	3	3	4	6	3
D	2	3	3	2	6	3
E	1	3	3	1	6	3
F	0.5	3	3	0.5	6	3
G	0.25	3	3	0.25	6	3
H	0.125	3	3	0.125	6	3
I	0.0625	3	3	0.0625	6	3
J	0.03125	3	3	0.03125	6	6

The vials will be used in reverse order, starting from the lowest concentration.

- Discard vials A and B.
- Vials are prepared in the order A, B, C, D, E, F, G, H, I, and J but administered to the participant in the order J, I, H, G, F, E, D, and C.
- **Allergy testing** - A skin prick test, also called an allergy skin test, checks for immediate allergic reactions to different substances at once. This test is usually done to identify allergies to trees, grasses and weeds.
- **Chest SDCT**
SDCT (**Standard dose computed tomography**) is a standard medical procedure in lung imaging, widely used clinically and in research. Risks to participants are minimal. The study will involve a small amount of radiation exposure to the subjects. There will be one SDCT scan performed during the study. The scan protocol will be adjusted based on the participant weight. The average weight adult radiation dose is 4 mSv per scan, (8mSv) for the study. This compares to the US limit on radiation workers which is 50 mSv.
- **¹²⁹XeMRI**
Hyperpolarized ¹²⁹Xe is treated as a drug by the FDA and is covered by our existing Investigational New Drug Filing (IND# 109,490), which has been active since 2011 and has reported no SAEs that required medical intervention. The proposed studies will use hyperpolarized ¹²⁹Xe prepared in accordance with the Drug Master File that is part of the IND held by Polarean, Inc. We have been granted the rights by Polarean to cross-reference this IND for our own institutional IND filings. We will continue to follow a ¹²⁹Xe administration protocol that is well established in our hands.

- **Bronchoscopy with Bronchial alveolar lavage and Biopsy**

Bronchoscopy is an established clinical procedure that has been used as standard for diagnosis of lung lesions. Overall, research bronchoscopy in adult asthmatics is considered to be a safe and well-tolerated procedure⁴⁷. Furthermore, bronchoscopy with bronchoalveolar lavage, endobronchial biopsies and airway brushings have been extensively used as clinical research tools in the last decade with complication rates not more than 2-3%.

The Imaging Team will dictate in a blinded fashion to the bronchoscopist where the samples will be obtained based on the results of the co-registered CT and pre-post bronchodilator ¹²⁹XeMRI ventilation images. Samples will be obtained from lung segments containing bronchodilator fixed and bronchodilator reversible defects in the following order to ensure standardization: 1) bronchial brushings, 2) bronchoalveolar lavage (BAL), 3) endobronchial biopsies.

- **Bronchoalveolar Lavage:** The purpose of the BAL is to evaluate the inflammatory cell profile. ¹²⁹XeMRI images will be used to guide where the bronchoscope will be placed and wedged for BAL collection. Three 40 ml aliquots of 37°C sterile 0.9% saline solution are instilled after the bronchoscope is wedged into each subsegment. The bath water temperature is continuously measured. The BAL fluid is harvested by immediate gently hand suction applied to each instilling syringe. Syringes are placed immediately in ice. The volume of the effluent is measured after the procedure and samples are combined with the exception of the first aliquot return which represents the “bronchial” portion of the lavage⁴⁸. Reduction in oxygen saturation following bronchoalveolar lavage is common for several hours. Supplemental O₂ will be administered as required. Our standard method will be used to count and differentiate cell types in the airway and alveolar fraction of the BAL^{49 50 51}.
 - 120 mL instilled in BD reversible and 120 mL instilled in BD fixed airways
- **Bronchial brushings:** The purpose of this procedure is to obtain airway epithelia for *ex vivo* culture. A sheathed biopsy brush (ConMed Disposable Cytology Brushes, Cat #149) will be introduced through the bronchoscope into the appropriate subsegmental bronchus and the bronchial wall will be brushed. Site of brushings will be dictated by the Imaging Core in consultation with Dr. Mammarappalil, our radiologist. and the bronchial wall will be brushed. The brush will be re-sheathed, withdrawn, and cut off into a sterile tube containing 10 ml RPMI medium for further processing. Our previous experience indicates that we are able to retrieve approximately 1.5 million cells/brush (approximately 95% epithelial) using this technique. Cytospin preps will be prepared to evaluate the cell content. A small aliquot of cells will be stained for cytokeratin to determine the percentage of epithelial cells with this technique.
 - up to 6/BD reversible airway and up to 6/BD fixed airway
- **Biopsy:** Endobronchial Biopsy: The purpose of this procedure is to obtain tissue for immunohistochemical staining and for isolation of fibroblasts for *ex vivo* cell culture experiments. Endobronchial biopsies are planned for Projects 1 and 4 and will be performed under direct visualization from the second to fourth generation airways. Site of biopsy will be dictated by Dr. Driehuys’ team. Up to twelve biopsies will be taken from the subcarinae with an “alligator” type forceps.
 - 6 endobronchial biopsies in BD reversible and 6 endobronchial biopsies in BD fixed airway segments

Descriptions of Questionnaires

- **Asthma Control Questionnaire (ACQ):** This questionnaire asks about asthma symptoms during the past 2 weeks.

Subject’s Capacity to Give Legally Effective Consent: Participants incapable of providing informed consent will not be enrolled.

Costs to the Subject: There will be no costs to subjects.

Subject Compensation:

- Screening Visit (V0): \$75
- Visit 1: \$100
- Visit 2: \$450
- Unscheduled visit: \$25
- Total Potential Compensation: \$650

Compensation will be in the form of a ClinCard where compensation will be submitted at the completion of each visit. In addition, parking passes will be provided for visit performed at the main Duke campus for the radiologic and bronchoscopy procedures.

Study Interventions: Study procedures include pulmonary function testing to include methacholine challenge, allergy testing, urine pregnancy tests, clinical interviews, questionnaires, a standard dose chest CT scan, Hyperpolarized ¹²⁹XeMRI, and a venipuncture for clinical labs.

RISK / BENEFIT ASSESSMENT:

The risk/benefit ratio for this study is relatively low. All data collection procedures are minimally

Description of and Steps to Minimize Study Risks:

The potential risks associated with this study are as follows:

1. Confidentiality

Risks: Confidentiality is of critical importance, and we will take many precautions to protect against the possibility of a breach of confidentiality.

Managing risk: In order to protect participants, we have obtained a Certificate of Confidentiality from DHHS. Thus, while we acknowledge that a breach of confidentiality is possible, the likelihood is very low with the above policies and procedures in place.

2. Pulmonary function testing

- ***Spirometry-***

Risks: The risks of spirometry are minimal. The possible risks include fatigue and precipitation of bronchospasm and light-headedness from repeated blowing attempts.

Managing risk: 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 Visit 0 (if applicable) and to qualify participants for bronchoscopy at the relevant 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. The patient may stop the spirometry procedure at any time if he/she should feel shortness of breath or fatigue.

- ***FeNO***

Risks: The risks of this procedure are minimal. Both children and adults can perform the procedure without difficulty. Possible risks include fatigue and precipitation of bronchospasm and light-headedness from repeated blowing attempts.

Managing risk: 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 for subjects who experience bronchospasm. 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. The patient may stop the procedure at any time if he/she should feel shortness of breath or fatigue.

- ***Forced Oscillation***

Risks: There are no risks associated with this procedure. Both children and adults can perform the procedure without difficulty.

Managing risk: None.

- ***Methacholine Challenge***

Risks: The major risk of methacholine challenge is the induction of severe bronchoconstriction. As a precaution, participants will not undergo a methacholine challenge if their FEV1 is less than 60% of predicted, or 1.5 liter.

Managing Risk: 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 for subjects who experience bronchospasm. 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. The patient may stop the procedure at any time if he/she should feel shortness of breath or fatigue

3. *Venous phlebotomy*

Risks: Risks associated with drawing blood include momentary discomfort, bruising, and/or dizziness. While infection, excess bleeding, clotting, and fainting are possible, these are unlikely to occur. To minimize these risks, staff will follow all laboratory safety procedures.

Managing risk: 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 enrolled subjects are in a seated position and medical and nursing personnel will be available at the study sites to treat and manage vasovagal episodes.

4. *Allergy Testing*

Risks: Risks from skin testing include allergy symptoms might occur during the test. The most common symptoms are itching and swelling of the skin where the tests are. In rare cases, shortness of breath can occur.

Managing risk: To minimize the risk of bronchoconstriction, subjects are pretreated with albuterol and the procedure halted.

5. *Hyperpolarized ¹²⁹Xe MRI safety.*

a) Claustrophobia and anxiety

Risk: Due to the size of the bore of the MRI scanner, there is an infrequent occurrence of claustrophobia (fear of being enclosed in tight spaces). These occurrences may be more likely in larger subjects. Claustrophobia is the most likely discomfort.

Managing risk: Individuals with a self-reported history of claustrophobia will be assessed and a mock scan will be performed in cases where symptoms or history are of a concern. Also, technologists will do their best to help comfort any subject who is claustrophobic but chooses to continue, by using a cloth over the subject's eyes or a fan to provide cool air to the subject. Technologists will provide subjects with a squeeze ball alarm and instruct them to use it in case of any discomfort. During the actual MRI scan, staff will speak with subjects between scan sequences. Participants will know that study staff are continually present, and help can be immediately summoned. The technologist will also inform the subject that he or she is free to stop at any time, for any reason. Given the loud noises associated with scanning, hearing protection is provided. Participants will be told to alert the MRI technician immediately if the scanner is ever uncomfortably or painfully loud.

b) Risks related to ferromagnetic objects

Risk: Subjects will be comprehensively screened for objects within their bodies or on their persons that are ferromagnetic. Everyone must remove all metal objects before entering the scanner.

Managing risk: To reduce risk, participants must remove any potentially dangerous objects prior to scanning.

c) Risks related to inhaling hyperpolarized ¹²⁹Xe

Risk: Hyperpolarized ¹²⁹Xe is treated as a drug by the FDA and is covered by our existing Investigational New Drug Filing (IND# 109,490), which has been active since 2011 and has reported no SAEs that required medical intervention. ¹²⁹XeMRI is a non-invasive imaging modality that involves no ionizing radiation. Our center has to date conducted over 500 ¹²⁹Xe MRI exams in patients and volunteers, all of whom received at least 3-4 doses of ¹²⁹Xe gas during the exam. The proposed studies will use hyperpolarized ¹²⁹Xe prepared in accordance with the Drug Master File that is part of the IND held by Polarean, Inc.

Managing risk: The risks of $^{129}\text{XeMRI}$ are minimal. Xenon is a general anesthetic when breathed continuously at concentrations greater than 70% for extended periods of time. In the proposed study, xenon will be delivered in a single breath, with alveolar concentrations below 25%. At these concentrations, subjects may experience transient effects including dizziness, slight tingling or numbness of the extremities, nausea, smelling of flowers, or a feeling of well-being and euphoria. These effects will wane within 1-2 minutes of exhaling the xenon and are documented in the consent forms.

d) Incidental findings on $^{129}\text{XeMRI}$

Risk: Since the MRI methods being tested are experimental, the MRI images will not be formally reviewed for incidental findings, abnormalities that are not expected to be seen that are identified by a reviewer.

Managing risk: However, if there is a finding of concern noted by the study team, then the PI will approach the IRB for guidance on how to proceed on a case-by-case basis.

*The consent will clearly state that the MRI images will **not** be evaluated for incidental imaging findings.*

6. *Standard dose CT (SDCT) Safety*

SDCT is a standard medical procedure in lung imaging, widely used clinically and in research. Risks to participants are minimal. The study will involve a small amount of radiation exposure to the subjects. There will be one SDCT scan performed during the study. The scan protocol will be adjusted based on the participant weight. The average weight adult radiation dose is 4 mSv per scan, (8mSv) for the study. This compares to the US limit on radiation workers which is 50 mSv.

Risk: The SDCT (lung scan) will be reviewed by a qualified person. There is a possibility that while reviewing the SDCT an incidental finding will be identified.

Managing risk: The patient will be informed about the incidental finding. With permission from the patient, the information about the incidental finding will be forwarded to the primary doctor or the patient can be referred to an appropriate doctor for further evaluation.

The patient will be provided the following information:

- An incidental finding may cause you to feel anxious.
- Since an incidental finding will be part of your medical record, it may affect your current or future life or health insurance coverage. This risk will vary depending on the type of insurance plan involved.
- The costs for any care that will be needed to diagnose or treat an incidental finding would not be paid for by this research study. Any additional tests or treatments will be your choice; you or your insurer will be responsible for additional costs.

7. *Bronchoscopy:*

Risks: There is the small risk of bleeding, bronchospasm, irregular heartbeat, shortness of breath, infection or pneumonia in approximately 1-5% of individuals with asthma undergoing bronchoscopy^{47,52}. There is a possibility that bronchospasm can worsen by this procedure. If this occurs, appropriate treatment including albuterol nebulization will be given. If the subject's asthma symptoms continue, the procedure will be terminated, and additional medications will be given if clinically indicated. During the procedure, there may be a temporary drop in oxygen levels. Subjects will be given supplemental oxygen to keep levels normal. If a subject experiences difficulty necessitating continued observation, he/she will be hospitalized. Unstable patients will be transferred to the Medical Intensive Care Unit. Other risks of bronchoscopy include hemoptysis (occurs 10-25% of the time and does not exceed more than 2 tablespoons of coughed-up blood in twenty-four hours), epistaxis and fever (occurring in less than 10-25% of subjects). One death has been reported in the literature, which was attributed to excessive lidocaine administration.

Managing risk: Prior to the bronchoscopy, participant may be given an aerosolized lidocaine treatment by nebulizer or the nose, throat, and vocal cords will be sprayed with lidocaine to minimize coughing during the procedure. During the bronchoscopy, patients are placed on supplemental oxygen and monitored continuously with an oxygen saturation monitor as well as electrocardiogram. The topical anesthesia and sedation dosages are monitored, and limits set and adhered to closely. Research bronchoscopies will be done with subjects with a pre-bronchodilator FEV1 > 50% of predicted. Patients will be instructed to remain on their long-term asthma medication controllers prior to undergoing bronchoscopy. Pre- and post-bronchoscopy care will be provided in

each participating institution and will include adequate nursing staff and standard post-sedation recovery care with direct PI oversight. Duke and University of Colorado have extensive training and experience in performance of such procedures both in the clinical and research setting. The expected duration of a bronchoscopy with brushing and biopsies is not more than 30 minutes. Based on our experience and published recommendations, we will limit the dose of administered lidocaine to a maximum of 9 mg/kg⁵³.

8. Risks of Principal Investigator Conflict of Interest:

Dr. Driehuys is a founder of Polarean, Inc, a company that is commercializing hyperpolarized gas MRI technology. He and the company therefore potentially stand to benefit if the present work shows hyperpolarized ¹²⁹Xe MRI to be useful in detection or monitoring of gas exchange impairment. This potential conflict of interest is being actively managed by the Duke Research Integrity Office. Any risk specific to the proposed study is being managed in the following manner. Image reads will be done independently by the radiologists participating in the study, not Dr. Driehuys. Moreover, those readers will be blinded to clinical outcomes when they do their image assessments. Therefore, Dr. Que will serve as principal investigators on all institutional review board applications at Duke. Hence, Dr. Driehuys will serve only in technical and study oversight roles, but not in image analysis or statistical analysis. Data generated under this protocol, will be published regardless of outcomes, and those efforts will be led by investigators other than Dr. Driehuys.

DATA ANALYSIS & STATISTICAL CONSIDERATIONS: For the *ex vivo* studies, conditions will be performed in triplicate and experiments will be independently replicated to ensure reproducibility. For the *in vivo* assessments of airway remodeling and fibrosis (bronchodilator reversibility on ¹²⁹XeMRI), we will compare biomarkers of fibrosis (e.g., trichrome or percent elastic fiber staining) between BD types (fixed and reversible airway segments) both within and between obese asthma groups (early onset, late onset and non-asthma control) using a repeated measures two-way ANOVA with appropriate distributional assumption. Main effects of BD type, obese asthma group and their interaction will be evaluated with repeating BD type within subject (random effect) with compound symmetry variance structure. If significant main effect differences are found, pairwise comparisons between BD types and obese asthma groups will be done using Tukey's post-hoc test. Assuming a 50% decrease in fibrosis between BD fixed and reversible types, a sample size of 10 per group provides our study with ≥80% power to detect an effect size of 0.53 within and 0.60 between groups, where effect size (*f*) is the size of the mean differences relative to the standard deviation and assumed correlation (*p*) within group of 0.30. A p-value <0.05 will be considered statistically significant and all analyses will be performed using SAS version 9.4 or higher (SAS Institute, Inc., Cary, NC). Blinding to conditions and tissue sources will occur across the experimental design. In particular, tissues samples (post-BD fixed and BD reversible tissue based on region) will be blinded to the individual performing the experiments and the individual assays. The key for the tissue samples will be maintained by the program statistician and un-blinding will occur during data analysis by the statistician.

Because of Dr. Bastiaan Driehuys's conflict of interest related to ¹²⁹XeMRI, and as required by his Polarean management plan, data analysis and image analysis will be conducted by un-conflicted individuals.

We hypothesize that the scRNA sequencing performed on epithelial brushings in Aim 1 will identify differentially expressed proteins in the airway epithelial cell secretomes (85) of early and late onset obese asthma and obese non-asthma subjects, which drive differential fibroblast function. To perform this analysis, we will derive candidate proteins from pathway analysis in the epithelial brushings, correlate these to the secreted epithelial proteins and then confirm that pathways regulated by these proteins are active in either stimulating or suppressing fibroblast migration and invasion using transwell assays. Candidate genes in fibroblasts harvested from early and late onset obese asthmatics and non-asthma obese controls will be validated using RT-PCR. These studies have the potential to yield novel mechanistic insights into the pathogenesis of airway remodeling and fibrosis in obese asthma.

We hypothesize that post-BD fixed defects identified on ¹²⁹XeMRI in obese asthmatics correspond to regions of airway remodeling and fibrosis. ¹²⁹XeMRI imaging-guided bronchoscopic brushings and biopsies will be used to obtain primary airway epithelial cells and fibroblasts to model early onset obese asthma (T2 high disease) *ex vivo* in cell culture. Epithelial cells will be grown in triplicate at air liquid interface (ALI) in 6-well transwells (86) at 37°C in 5% CO₂ until fully differentiated with a mucociliary phenotype then challenged *in vitro* with 50 ng/mL IL-13 ± 20 ng/mL leptin for 24-48 hours in serum free media (34) to mimic physiologic conditions of the T2 high obese asthmatic *in vivo*. To model nonT2 disease in the late onset non-allergic obese asthmatic primary airway

epithelial cells harvested from BD fixed and BD reversible ventilatory defects on $^{129}\text{XeMRI}$ will be grown in triplicate in transwells at ALI and challenged with 200 ppm ozone (O_3) for 1 hour \pm 20 ng/mL leptin (O_3 dose and timing are based on prior work). In T2 high disease, $\text{TGF}\beta 1$ is upregulated and associated with eosinophil burden and asthma severity(89-91). $\text{TGF}\beta 1$ mRNA expression is also upregulated by O_3 in human fibroblasts (92). These experiments will allow us to determine the contribution of airway epithelial cells from post-BD fixed and post-BD reversible regions on $\text{TGF}\beta 1/2$ expression and apical reactive oxygen species (ROS) production (DCFH-DA, Cell BioLabs, Inc.). Basolateral cell supernatants will be assessed for secreted proteins (85, 93) to gain insight into which proteins are potentially involved in ECM degradation, matrix organization, and cell-cell interactions and how these are altered in the different asthma endotypes.

Primary airway fibroblasts obtained by endobronchial biopsy from post-BD fixed and post-BD reversible regions will be challenged with 50 ng/mL IL-13 \pm 20 ng/mL leptin (T2 high disease) vs 200 ppm ozone \pm 20 ng/mL leptin (non-T2 disease), respectively. Cell culture media will be assessed for ROS production (DCFH-DA, Cell BioLabs, Inc.) and specific secretion of $\text{TGF}\beta 1/2$, IL-13, elastin, and Type I collagen ($\text{Col1}\alpha$) (ELISA, R&D Systems) and fibroblast secretome to discern relative expression levels of ECM proteins as well as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which are important for pro-fibrotic airway fibroblast functions (29, 31, 34). Fibroblast invasion, proliferation and migration will be assessed using previously described methods (34, 74). These studies will allow us to determine the fibroblast specific responses contributing to VDP heterogeneity.

Primary airway epithelial cells and fibroblasts will be grown in co-culture and challenged with either 50 ng/mL IL-13 \pm 20 ng/mL leptin for 24-48 hours or 200 ppm O_3 \pm 20 ng/mL leptin for 1 hour to mimic early (T2 high) vs late (non-T2) onset obese asthma. ROS production and gene expression in airway epithelial cells will be measured and fibroblast invasion, proliferation and migration assessed as described above. To then determine if airway fibroblast functions (invasion vs. proliferation vs. migration) are dependent on oxidative stress, we will incubate cells for 4 hours with different concentrations (1, 3, 10 mM) of N-acetylcysteine (NAC) to alter the oxidative milieu. Cells will then be washed and incubated with 10 μM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate dye; Molecular Probes) in culture media for 30 min. Cells are then analyzed for intracellular ROS production by flow cytometry. Studies will be repeated using different $\text{TGF}\beta$ (SB431542; Stem Cell Technologies and LY-364947; Sigma Aldrich) and Nox4 (GKT137831; Medkoo Biosciences, Inc) inhibitors to determine if fibroblast function and oxidative milieu are dependent on $\text{TGF}\beta$ signaling and/or Nox4 expression/activity.

Both epithelial cells and fibroblasts produce $\text{TGF}\beta 1$ and ROS under profibrotic conditions (76). We hypothesize that airway post-BD fixed regions on $^{129}\text{XeMRI}$ are comprised of a subset of fibroblasts that are aggressively more invasive, highly proliferative and more pathogenic than fibroblasts from post-BD reversible regions. To determine if the changes in regional remodeling and fibrosis are cell specific, BD reversible epithelial cells will be grown in co-culture with BD fixed fibroblasts and vice versa, epithelial cells from BD fixed regions will be grown in co-culture with fibroblasts from BD reversible regions. Cells will be challenged with 50 ng/mL IL-13 + 20 ng/mL leptin or 200 ppm O_3 \pm 20 ng/mL leptin as described in Aim 2.1. Endpoints to be measured include fibroblast functional assays (invasion, proliferation and migration) and gene and protein expression (Nox4, $\text{TGF}\beta 1/2$, IL-13, elastin and type 1 collagen) and ROS production.

Data analysis will be performed using a suite of python-based tools suitable for handling large-scale datasets (>5M cells), including scVI-tools, SquidPy and CellCharter. Briefly, after data QC and filtering out cells with low number of transcripts, we will integrate data from all samples together, and perform clustering, similar to single-cell RNA-seq analysis. The resulting cell clusters will be annotated using manual review of marker genes or label transfer from single-cell RNA-seq atlases using transfer learning tool scArches (part of scVI-tools and CellCharter), and these clusters will be projected back into spatial context. We will compare the relative abundance and absolute density of specific cell types between groups. We will also extract additional features, such as cell size or morphological characteristics. We will use CellCharter to perform unbiased identification of spatial neighborhoods and test their association between groups with adjustment for demographic covariates.

DATA & SAFETY MONITORING:

Patient safety and the integrity of the study will be monitored by an appointed university DSMB via review of adverse events and other safety parameters at their regularly scheduled meetings.

Data Storage and Handling and Record Keeping:

Any CRFs will be entered directly into REDCap by the study coordinator, with the support of Duke's office of clinical research.

Data will be captured in REDCap, except for patient consent forms, and AE/SAE forms, which will be on case report forms (CRFs) collected by the coordinators. REDCap is a software tool that does not require client local software and can be accessed from anywhere on the Internet and is secured on a Duke Health Technology Services (DHTS) server. All collected data are backed up daily, both on the local server and by the DHTS enterprise backup system.

Source Documents and Records Retention. Original source documents (consents) for study participants will be maintained at the study site at the Duke Asthma Allergy Airway Center research offices in a badge accessed office in locked cabinets and will be accessible only to the study staff.

Privacy, Data Storage, & Confidentiality: The following precautions will protect the privacy of participants and maintain confidentiality of research data: (1) All staff will be well trained in confidentiality and data security procedures. (2) Privacy will be maintained by conducting all study procedures in closed rooms. (3) Each participant will be assigned a unique study ID number, and all data will be de-identified and coded with ID numbers only. The key linking participant names and ID numbers will be stored in a separate password protected document on Duke OIT approved servers and electronic storage locations, and only essential study staff will have access to it. (4) Documents such as informed consents, copies of laboratory results, and outside medical records obtained as part of the study will be securely stored in locked file cabinets in locked offices and in password protected documents on password protected computers and secure servers. Access to data storage areas and computers will be restricted to designated key personnel. (5) Analysis will occur on de-identified data only. (6) Data will only be stored for as long as necessary to complete the study, and for adherence to university, hospital, and federal regulations.

The risk of loss of confidentiality is inherent in all research studies, and a statement to this effect will be included in the informed consent. Confidentiality will be assured by a number of procedures. First, all staff receive careful training in the importance of participant confidentiality, the separation of data from participant's tracking information, and the use of locked offices, file cabinets, and computer accounts to prevent unauthorized access to the data or identifying information. Second, all study data will be treated confidentially. All questionnaires, and forms will be identified by participant ID (PID/SID) only. No identifying information will be included in the analytical database, nor in any presentations nor publication. All study materials and surveys will be maintained in locked filing cabinets in a secured office at all times. All records with personally-identifying information will be kept in a locked, limited access area (such as a locked file cabinet) available only to site principal investigators and designated study personnel.

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