H#: 10-01191
NCT02740543

Title: Biomarkers of Irritant-Induced and Allergic Asthma – Phase I and Phase II

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Specific Aims

Asthma is a heterogeneous disease, and although much is understood about mechanisms of inflammation in allergic asthma, less is known about mechanisms of irritant-induced asthma (IA) (5, 7). Understanding the underlying similarities and differences in mechanisms of these two types of asthma will help focus current treatments and lead to development of new therapies. We have a longstanding NYU/Bellevue Asthma registry (NYUBAR), with a large population (N = 900) of asthma cases and controls, a program that has been housed at the CTSI (formerly GCRC). The destruction of the World Trade Center (WTC) resulted in massive dust, gas and fume exposures to local residents, workers and cleanup workers and individuals involved in rescue and recovery and adverse respiratory health effects of this disaster are reported more than 7 years after 9/11 (1-4). Many responders, as well as those exposed as residents or local workers, have developed IA, asthma that arises after a lag from an environmental exposure (1, 5, 6). The WTC Environmental Health Center (WTC EHC) is one of the three New York City (NYC) WTC Centers of Excellence and the only one that focuses on treatment and monitoring of local workers and residents. As such, it has a large population of individuals with irritant-induced asthma. We now propose to use participants from the NYUBAR and the WTC EHC to expand our knowledge of irritant and allergic asthma. Non-invasive studies allow for the assessment of airway inflammation, a non-specific response to environmental exposure and injury. Recent technologies also allow for assessment of microRNA (miRNA), small RNAs that regulate gene expression at the post-transcriptional level and thus serve as a pathway to regulation of inflammation. **We will test the hypothesis that airway inflammation in irritant and allergic asthma may be similar, but result from divergent miRNA regulatory pathways expressed in sputum cells.** These studies will provide preliminary data for future studies that will help identify biological pathways to categorize these asthma phenotypes and target future treatment interventions. We propose the following aims:

Specific Aim 1: To test the hypothesis that irritant–induced asthma is characterized by elevated markers of inflammation in a manner similar to that described for allergic asthma.
SA1.a We will identify and phenotype individuals with WTC dust cloud-exposed irritant-induced asthma (IA), individuals with allergic asthma (AA) and healthy unexposed individuals (Controls).
SA1.b We will measure biomarkers of inflammation using noninvasive techniques for exhaled nitric oxide (ENO) and 8-isoprostane from exhaled breath condensate (EBC) in WTC irritant-induced asthma (IA), individuals with allergic asthma (AA) and in healthy unexposed individuals (Controls).
SA1.c We will measure inflammatory cells in induced sputum from individuals with IA, AA and Controls.

Specific Aim 2: To test the hypothesis that the miRNA expression in airway epithelial cells will differ between IA, AA and Control individuals.
SA2.a. We will isolate RNA from epithelial cells and inflammatory cells in induced sputum and assess the miRNA expression profile using microarray technology among individuals with irritant-induced asthma (IA), individuals with allergic asthma (AA) and healthy unexposed individuals (Controls).
SA2.b. We will identify the mRNA targets of the differentially expressed miRNAs by using publicly available databases and correlate miRNA and mRNA expression.

Specific Aim 3: To test the hypothesis that levels of RNA of targets in human sputum cells will differ before and after the administration of inhaled corticosteroids in asthmatics (both IA, AA). The importance of this hypothesis in the characterization of irritant induced and allergic asthma is to further characterize the regulatory pathways expressed in sputum cells by identifying which inflammatory markers are steroid sensitive and steroid resistant.

SA3.a. We will isolate RNA targets in discrete sputum cells from induced sputum in IA and AA individuals and compare levels before and after administration of inhaled corticosteroids.

I. Background and Significance

The destruction of the World Trade Center (WTC) resulted in massive dust, gas and fume exposures to local residents, workers and cleanup workers as well as to individuals involved in rescue and recovery. Local residents and workers were caught in the massive dust cloud as the buildings collapsed and a major proportion of individuals returned to work or their homes one week after the event when many office buildings and residences were only incompletely cleaned. Responders, local workers, and residents had potential for ongoing dust and fume exposure as indoor and outdoor WTC dust was recirculated and the fires burned for four months. Adverse respiratory health effects from this unprecedented disaster continue to be reported more than 7 years after 9/11 by the Centers of Excellence in New York (1, 3, 8). Data from firefighters who worked at the site demonstrate persistent respiratory symptoms and airway hyperresponsiveness one-year post 9/11 suggesting that they initially developed reactive airways disease (3). Data from subsequent studies of occupationally-exposed individuals, as well as those exposed as residents or local workers, suggest that many have also developed irritant-induced asthma, a form of asthma that can arise after a lag from an environmental exposure (1, 5, 6).

Why study irritant-induced asthma (IA)

Asthma, a complex syndrome with many clinical phenotypes, is characterized by a variable degree of airflow obstruction, airway hyperresponsiveness, and airway inflammation which results in symptoms of cough, shortness of breath, wheezing and chest tightness (9). Asthma is a heterogeneous disease with some patients having a clear allergic component, whereas others non-allergic asthma, some of which arises after irritant exposure (dust, gas, vapors or fumes). In the WTC population there is a high prevalence of asthma-like symptoms that developed after 9/11 which are treated with typical asthma medications despite the uncertainty of the underlying mechanisms and pathophysiology. We know from individuals under treatment in the WTC EHC that respiratory symptoms continue to persist more than 5 years after the event and although most individuals with respiratory symptoms have normal screening spirometry, there is a high rate of airway hyperresponsiveness consistent with irritant-induced asthma (1, 10). The underlying pathology and inflammatory response in irritant-induced is unclear and may differ from classic allergic asthma. Little is known about mechanisms of irritant-induced asthma.

Why study non-invasive markers of inflammation

There are ongoing efforts to define biomarkers to characterize airway inflammation in asthma. Presently the "gold standard" to measure airway inflammation and biomarkers of inflammation in humans involves direct airway sampling by bronchoscopy (11). The invasive nature of bronchoscopy makes it impractical and thus there is much interest in implementation of noninvasive methods. Approaches using exhaled nitric oxide (ENO), exhaled breath condensate (EBC) and induced sputum (IS) as surrogates of the lung have many advantages including sampling simplicity, subject comfort, analytical ease and safety for longitudinal studies. Few studies have been performed on non-invasive markers of irritant-induced asthma. A recent report demonstrated a similar inflammatory cell profile of increased eosinophils and neutrophils in induced sputum in individuals with acute irritant-induced asthma from accidental inhalational injury compared to individuals with allergic occupational asthma (12).

Exhaled NO increases in individuals with asthma, decreases with inhaled corticosteroid treatment and asthma control and correlates with eosinophil count in induced sputum and bronchoscopic sampling of airway inflammatory cells (13-19). ENO has been suggested as a more sensitive and reliable screening tool for the diagnosis of asthma than spirometry(11, 20). Whether ENO levels in irritant-induced asthma are also elevated has not been studied therefore making this noninvasive biomarker an ideal start in characterizing the inflammatory response in this type of asthma.

Exhaled breath condensate (EBC) is collected by the condensation of warm breath in a cold condensing tube. Levels of select markers in the condensed phase breakdown products reflect changes in the composition of the airway lining fluid. Biomarkers from EBC have been used to study asthma, chronic obstructive lung disease (COPD) and lung cancer (11, 21-26). The application of EBC in human exposure studies is sparse. Markers of inflammation and oxidative stress that have

been identified in EBC include isoprostanes and pH (11)(27, 28). The measurement of EBC components (pH, and 8-isoprostane) may help characterize injury in response to inhalational exposure from WTC fumes and dust.
Induced sputum (IS) can be collected after inhalation of hypertonic saline. Measurements of IS, including cell counts, cytokines and chemokines have been used to assess lung inflammation extensively in individuals with asthma and COPD (11, 29, 30). Measurement of eosinophils in IS has been proposed as a diagnostic tool in occupational asthma (31, 32)(33-36). IS contains exfoliated airway epithelial cells and is easily accessible and can serve as a direct measure of the lower respiratory tract. A simple inexpensive and effective method to limit the cellular heterogeneity of IS by enriching airway epithelial cells in a sample has been described by our group and others (37, 38). Only one study has examined the ability to both isolate and analyze microRNA from sputum in patients with lung cancer suggesting a need for further investigation of other lung diseases, particularly asthma (38).

Why study microRNA and mRNA

Studies suggest that the end result of airway injury, whether due to an allergic or irritant process is similar, and is characterized by airway eosinophilia and markers of "remodeling." We and others have described that epithelial cells participate in the development and persistence of airway inflammation due to allergic and environmental exposures (39-41).The biologic pathways that produce airway inflammation, although mediated by airway epithelial cells, may differ between the irritant and allergic asthma phenotypes. MicroRNAs (miRNAs) are a class of small non-protein-coding RNAs that posttranscriptionally regulate the expression of hundreds of target genes, thereby controlling a wide range of biological functions including cellular proliferation, differentiation and apoptosis (42, 43). MiRNAsdownregulate gene expression by suppression of translation or mRNA degradation. Identification of aberrant expression of miRNA may serve as valuable diagnostic or prognostic disease markers (42). Most studies to date have linked miRNAs to cancer because of their potential to function as tumor suppressors or oncogenes (44). Airway biopsies in individuals who smoke demonstrate miRNAs that are differentially expressed and inversely correlated with their predicted mRNA targets when compared to never smokers suggesting that miRNAs may be contributing to smoking-induced mRNA expression changes in airway epithelial cells (45). A recent study failed demonstrate a difference in miRNA expression from bronchial biopsies in mild asthmatics compared to those of healthy individuals but the possibility exists that differences may have been masked by the degree of cellular heterogeneity in bronchial biopsies (46). We propose to examine miRNA expression in epithelial cells derived from induced sputum to directly evaluate regulation of epithelial cell-directed lung inflammation.

Our studies of irritant-induced asthma and allergic asthma using non-invasive methods (ENO, EBC and IS) and miRNA expression will characterize similarities and differences in these asthma phenotypes that can lead to improved biologic understanding of disease and potential targets for treatment interventions.

Preliminary data

WTC and asthma cohorts

The WTC EHC is a program developed for the evaluation, treatment and monitoring of individuals with presumed WTC illness. Initiated as an unfunded community collaboration, it eventually received funding from the American Red Cross, and subsequently from the City of New York and most recently, federal funding from the National Institute of Occupational Safety and Health (NIOSH). The program targets local workers, residents and clean-up workers and as such, is the only one in NYC with this population. We have recently described the respiratory symptoms and findings in this population of individuals with diverse exposures Fig. 1 (1). Although most of the WTC EHC population had normal lung function, clear evidence of airway obstruction was noted in others (6 %), and some had reduced lung volume (20%). These findings suggest that exposures to WTC dust, fumes and gasses resulted in heterogeneous patterns of injury. To date, there are over 3,000 patients enrolled in the Bellevue component of the program, who have signed consent to be recontacted and have information available to us on our WTC EHC database. The NYUBAR has over 900 asthma cases and controls enrolled and phenotyped with consent to be recontacted. Thus the populations described are available to us.

Irritant asthma in the WTC-exposed population

Initial and persistent airway hyperresponsiveness has been well-described in the firefighter population involved in WTC rescue and response (3, 4, 47) To date, our WTC EHC data suggest that respiratory symptoms have persisted for more than 5 years after the event (1, 2). Our preliminary data show that among individuals in our WTC clinic with persistent lower respiratory symptoms that began after 9/11 and had normal spirometry (n=103), 50% demonstrate a positive methacholine challenge test. Individuals who were caught in the WTC dust clouds created as the towers collapsed on 9/11 were at highest risk for a positive methacholine test (odds ratio = 4.1; $p<0.006$). These data suggest persistent irritant-induced asthma in a large population of symptomatic WTC EHC patients.

Non-invasive biomarkers in the WTC-exposed population

An early case-report of a firefighter with severe lung disease showed a remarkable eosinophilic pneumonia (48). An analysis of IS performed on firefighters 10 months after their WTC exposure showed an increase in inflammatory cells consisting of neutrophils and eosinophils(33). Few other studies of biomarkers have been performed in the WTC population. Our initial studies suggest that there is an increase in peripheral blood eosinophils in our patients who have persistent symptoms of wheeze (submitted as an abstract to American Thoracic Society). These data suggest that our patients with irritant-induced asthma may have an inflammatory response characterized by an increase in eosinophils.

Isolated epithelial cells

We will use isolated epithelial cells from IS. We have ample experience isolating bronchial epithelial cells from *in vitro* cultures of epithelial cells and dendritic cells. Fluorescence-activated cell sorting (FACS) analysis of these cells confirms our ability to separate human bronchial epithelial cells and to confirm their purity. We have experience with induced sputum and subsequent cell isolation, as described in our recent publication (Bleck et al., 2015)

Microarray

We propose microarray analysis of miRNA. We have previously published microarray analysis of alveolar macrophages obtained from smokers and nonsmokers (49) as well as those obtained from small airways (50). We have previously published microarray analysis demonstrating upregulation of MER tyrosine kinase (MERTK) apoptotic cell removal receptor in smokers and confirmed upregulation with RT-PCR (49).

Research Design and Methods

Specific Aim 1: To test the hypothesis that irritant–induced asthma is characterized by elevated markers of inflammation in a manner similar to that described for allergic asthma.

SA1.a We will identify and phenotype individuals with WTC dust cloud-exposed irritant-induced asthma (IA), individuals with allergic asthma (AA) and healthy unexposed individuals (Controls) (N=90).

We will study two asthma phenotypes and a control population. Our asthma phenotypes and control population will be defined in the following manner:

IA: Patients will be recruited from the WTC EHC (N = 30) and will have WTC dust cloud exposure, New symptoms after 9/11, and symptoms of wheeze and shortness of breath > 2x / week) in the 4 weeks before inclusion (persistent symptoms).

AA: These patients will be recruited from the NYUBAR (N = 30) and advertising and will have asthma as defined by NIH guidelines (51), persistent symptoms, and absence of WTC dust exposure.

Control: These patients will be recruited from the NYUBAR (N = 30) and advertising and will have no respiratory sx, no asthma diagnosis, no WTC dust exposure. Additional appropriate inclusion and exclusion criteria will be developed for each group including tobacco use, other lung disease, timing of inhaled corticosteroids etc.

The study will entail two to three visits. On visit 1 (V1) all individuals will sign informed consent to participate in the study under an NYU IRB approved protocol. A questionnaire will be completed with standardized questions that include information on WTC exposures, demographics, presence and severity of respiratory symptoms, tobacco history and past medical history. Individuals will undergo spirometry with inhaled bronchodilator. Individuals will undergo methacholine challenge test (visit 1a) if they have normal spirometry or no bronchial hyperresponsiveness. On visit 2, individuals will return to undergo ENO, EBC and spirometry with pre and post bronchodilator maneuvers and induced sputum. Blood will be obtained for CBC with differential cell count, and assessment of total IgE and allergen-specific IgE. Blood will also be stored for future analysis of inflammatory markers. Based on our experience, we have had individuals unable to produce enough sputum and thus yield too small a number of cells. These individuals are excluded from data analysis. In addition, we also have subjects who are able to produce sputum and return for repeat sputum testing.

SA1.b We will measure biomarkers of inflammation using noninvasive techniques for exhaled nitric oxide (ENO) and 8-isoprostane from exhaled breath condensate (EBC) in WTC IA, AA and controls.

We will use the handheld NIOX MINO® to measure ENO (Aerocrine, Sweden) (17, 18, 20, 52). The NIOX MINO® fulfills the ATS recommendations and provides reliable readings and is portable and we have experience with it (53). Most healthy individuals have ENO levels in the range of 5-35 ppb (some variation with age, gender, race/ethnicity) (54). Individuals with asthma have ENO levels that can range from 25-80 ppb and even higher (18, 61, 62).

EBC will be collected using a commercially manufactured condenser R tube® (Respiratory Research, Inc., Charlottesville, VA) for this study because of its transportability and price. Again, we have experience with this technique. Measurements will be made according to published methods (28). Samples will be transported immediately

and frozen (-70˚ C) to improve stability. Our initial studies will focus on measurements of 8-isoprostane, a reliable biomarker of oxidative stress (24, 64). 8-isoprostane will be measured by commercially available ELISA. We will also measure pH of EBC (28, 67). EBC pH is reduced in individuals with asthma and COPD. Changes in pH may be used as diagnostic and monitoring modalities in inflammatory airway diseases (68). We will measure pH in degassed samples as described (69). Samples will also be stored for future studies of inflammatory mediators including cytokines/chemokine studies.

SA1.c We will measure inflammatory cells in induced sputum from individuals with IA, AA and Controls.

Increased numbers of eosinophils and neutrophils have been identified in IS in allergic asthma and occupational asthma and have been used to monitor asthma disease activity (31, 33, 34, 36, 70). We propose to perform sputum induction, collection and processing according to established protocols on each individual (71, 72). IS will be collected after inhalation of hypertonic saline (3%, every 2minute intervals for up to 20minutes) with repeated monitoring of FEV_1 as a safety measure. Sputum will be processed immediately to obtain total and cell differential cell counts (73). Our initial evaluationincluded analysis of cell counts, with measurements of eosinophils and neutrophils (Bleck et al., 2015). We consider significant sputum eosinophilia when sputum eosinophils are $\geq$ 2% and significant sputum neutrophilia when sputum neutrophils are $\geq$ 60%, cut off values, that have been described in the literature (12). We will also examine cell counts as continuous variables.

Specific Aim 2: To test the hypothesis that the miRNA expression in airway epithelial cells will differ between IA, AA and Control individuals.

SA2.a. We will isolate RNA from epithelial cells and inflammatory cells in induced sputum and nasal brushing and assess the miRNA and mRNA target genes and expression profile using microarray technology among individuals with IA, AA and Controls.

The use of sputum for molecular genetic analyses is limited by its cellular heterogeneity which includes 1% of bronchial epithelial cells and greater than 95% macrophages and neutrophils (74). Enrichment of bronchial epithelial cells has been achieved by magnetic cell sorting (MACS) or fluorescent cell sorting techniques. We will perform positive selection of epithelial cells and inflammatory cells using magnetic bead preparations or fluorescent cell sorting techniques. Human epithelial cells will be purified from sputum according to the expression of CD326 (human epithelial antigen, HEA, or epithelial cell adhesion molecule (EpCAM) using the magnetic bead CD326 (EpCAM) Tumor Cell Enrichment and Detection Kit (Miltenyi Biotech). To confirm purity of our isolation for selected samples, epithelial cell identification will be demonstrated by intracellular pan-cytokeratin staining. Cells will immediately be placed in RNAprotect Cell Reagent (QIAGEN) and RNA isolated and fractionated into low molecular weight (LMW) and high molecular weight (HMW) using the miRNeasy mini kit (QIAGEN). RNA will be quantified and quality assessed. We will collaborate with the NYU Cancer Institute (CI) Genomics Services. We will have access to high-throughput genomic and computational methodologies specifically for microRNA. We will use the GeneChip® miRNA Array. Briefly, LMW RNA will be labeled with a FlashTag labeling kit (Genisphere, Inc.) according to the manufacturer's protocol. Samples will be incorporated into a hybridization mix and applied to an affymetrix chip and scanned. Computational analysis with techniques such as principal component analysis will be performed with analytic services provided through the NYU CI Genomics core facility. Data will be log transformed and only miRNA replicates that are detected at levels of 2 standard deviations above the average background will be considered for further analysis. These studies will provide us with distinct miRNAs that may differ between IA, AA and controls, or IA and AA. If specific miRNAs are identified that are different between IA, AA and controls, or IA and AA, we will further validate expression of the miRNA using quantitative real time PCR.

SA2.b. We will identify the mRNA targets of the differentially expressed miRNAs by using publicly available databases and correlate miRNA and mRNA expression.

Most miRNA bind to target-3'UTR and function as translational repressors most often by promoting the degradation of mRNA. Each miRNA may regulate up to 200 target genes (42). We therefore propose to correlate patterns of miRNA changes in our defined populations, with mRNA expression arrays. HMW RNA from isolated epithelial cells will be used for hybridization to Affymetrix HG-U133 Plus 2.0 microarrays. Images will be analyzed in the core facility and data will be normalized as follows: (1) per array, by dividing the raw data by the 50th percentile of all measurements; and (2) per gene, by dividing the raw data by the median expression level for all the genes across all arrays in a data set. We will determine whether up or down-regulated mRNAs are potential targets for previously identified miRNAs.

Specific Aim 3: To test the hypothesis that levels of RNA of targets in human sputum cells will differ before and after the administration of inhaled corticosteroids in asthmatics (both IA, AA). The rationale for this aim of the study is to

investigate which markers are steroid sensitive and which are steroid resistant which would provide information for therapeutic targeting in asthma.

SA3.a. We will isolate RNA targets in discrete sputum cells from induced sputum in IA and AA individuals and compare levels before and after administration of inhaled corticosteroids.

In this second phase of the study, (Phase II) we will enroll AA or IA individuals who have fit enrollment criteria for the phase I part of the study and participated in phase I of the study and were able to provide us with an acceptable sputum sample. These participants will be recontacted for participation in Phase II if they signed permission to be re-contacted. We will obtain a repeat sputum sample as baseline for the Phase II study. An inhaled corticosteroid (Fluticasone HFA 220 mcg, 1 puff twice daily) and large volume spacer will be provided. Inhalation technique and a daily diary will be reviewed. Subjects will be instructed to use Fluticasone propionate 220 mcg q puff twice daily and to record their use in a written diary A repeat sputum sample will be obtained after 2 weeks of Fluticasone propionate use.

Sputum samples will be processed for discrete cells and RNA levels (mRNA, miRNA) will be analyzed to understand the effect of ICS use on RNA expression in discrete sputum cell populations.

Statistical Analysis:

We were able to develop basic techniques and measurement from 10 patients in each group as initially proposed in the previous version of this protocol. We now propose to increase our enrollment from 30 to 50 subjects in each group based on the recruitment and enrollment data of about 30% of screen failures (33 subjects screen failed out of 108 total). In addition, there have been subjects who have not been able to produce sputum or provide adequate number of sputum cells for analysis (38 subjects out of 65 accrued gave adequate sputum samples). This represents about 60% success rate for sample quality. Therefore, we will need to increase our enrollment numbers to achieve an n=30 per group.

Power Analysis: Based on a similar rate of screen failures and inadequate samples, the analysis is now based on 50patients in each group. Our group sample sizes of 50 and 50 achieve 90% power to detect a difference of 0.73 given the null hypothesis that the two-group difference is 0 and the estimated group standard deviations are 1.0 and 1.0, with a significance level (alpha) of 0.05 using a two-sided two sample t-test. We will work with Dr. Mengling Liu, our biostatistician for our statistical analysis.

For ENO and EBC analyses, we will express data as means $\pm$ SD for normally distributed variables. For variables that are not normally distributed we will use medians and the interquartile range. We will use statistical analysis including unpaired *t* tests, Kruskal-Wallis test, and regression coefficients as appropriate. P values of < 0.05 will be considered significant. Differential expression of miRNA and hierarchical clustering will be performed with the genomics core. Statistics using *t* tests or algorithms designed specifically for data mining and biochip interpretation will be used. Pathway analyses will also be performed by the genomics computational core.

Anticipated results and alternative approaches

We will continue to recruit patients with WTC exposure or with asthma based on our experience in our asthma registry or our WTC clinic, in which most people are willing and anxious to participate in any study that might help characterize their symptoms.

We plan to also submit an advertisement to the IRB for approval and to use research match (researchmatch.org) a national registry which is a secure, web-based approach as a recruitment method, and will post the advertisement.

We have chosen to use individuals with WTC dust cloud exposure as our model of initial high dose exposure to enhance our ability to identify patients with irritant asthma. We suspect that other more prolonged exposures also induce irritant asthma, but our previous studies and those of other investigators suggest that this exposure provides the highest risk.

We have proposed a positive selection strategy for isolation of bronchial epithelial cells from IS. The possibility exists that this technique may provide too low a yield of cells. Should that be the case, we can use a negative selection strategy using anti-CD14 and anti-CD16 antibody beads to specifically deplete macrophages and neutrophils respectively (37). We may also need to obtain duplicate sputum samples from patients, i.e. have patients return for a second sputum induction.

Our initial analyses will be between our healthy controls and asthma in general (AI and AA) and subsequently between each type of asthma (AI and AA) to optimize our ability to detect differences in sputum markers and miRNA. The

possibility exists that we will find no difference between measurements of markers of inflammation in exhaled breath between AI and AA. Should that be the case, we will have to examine whether our pilot study was powered to identify a small difference.
We are basing our miRNA and mRNA studies on those that have been performed in epithelial cells derived from cigarette smokers (45). These studies have shown a correlation between mRNA and miRNa expression and thus there is precedent for our studies. Because of the high price of each of these chips, and the cost of core analysis time, we initially propose to use 5 subjects for each condition to obtain a global pattern scale. These studies will subsequently be extended to get a reasonable statistical power.

From our pilot studies and recent publication we will continue to investigate mechanisms of asthma and irritant-induced asthma. The studies are high risk but have potential for high yield, and will provide data for subsequent NIH applications for replication and further evaluation for a junior faculty member. In addition, they have potential to provide information that could help target specific interventions for asthma of different phenotypes as we proposed in phase II of the study. As such, they fit the mandate of the CTSI grants.

II. Characteristics of the Research Population

1. Number of Subjects: 90 (this includes the total number of subjects enrolled in phase I and II of the study that are able to produce sputum)
   [Number of subjects in Phase II: 40]
   The estimated number of subjects to be enrolled (signed consent, including screen failures) is 300.

2. Gender of Subjects: Men and Women (50 % each)

3. Age of Subjects: 18-80 years of age

4. Racial and Ethnic Origin: The sample population of approximately 80 participants is expected to include a distribution of subjects by race/ethnicity in relation to our local population which includes a high percentage of minority subjects (36% White, 35% Black, 50% Hispanic)

5. Inclusion Criteria:
   Inclusion for the WTC population with Irritant-Induced Asthma (IA):
   Patients will be recruited from the WTC EHC and will have WTC dust cloud exposure, new symptoms after 9/11, and symptoms of wheeze and shortness of breath (> 2x / week) in the 4 weeks before inclusion (persistent symptoms).We will be recruiting new subjects for phase II of the study but participants who will have completed Phase I of the study and were able to produce adequate sputum samples may also be contacted for Phase II.

   Inclusion for Allergic Asthma population(AA)**:** Patients will be recruited from the NYUBAR or advertisement and will have asthma as defined by NIH guidelines, persistent symptoms, absence of WTC dust exposure. Participants who will have completed the Phase I of the study and were able to produce adequate sputum samples may also be contacted for Phase II.

   Inclusion of Control Population: Patients will be recruited from the NYUBAR and will have no respiratory symptoms, no asthma diagnosis, no WTC dust exposure, no current tobacco use, $\leq$ 5 p-y history of tobacco use, and normal spirometry with no bronchodilator response and negative methacholine challenge in past 6 months.

   Additional inclusion criteria for individuals with IA and AA:$\geq$ 18 years of age, current nonsmoker, $\leq$ 5 pack year (p-y) history of tobacco use, spirometry in the past 6 months or on day of evaluation with a bronchodilator response of $\geq$ 12% and 200 ml improvement in FEV_1 or a positive methacholine challenge test (decrease in FEV_1 $\geq$ 20% (PC20) after inhalation of $\leq$ 16 mg/ml of methacholine), inhaled corticosteroid use in previous 1 month or more will be allowed.

   Inclusion criteria for Phase II
   Successfully completed Phase I
   Has asthma according to Phase I diagnostic criteria

Signed consent to be re-contacted

6. Exclusion Criteria:
   Exclusion criteria: current smoker, pulmonary diseases such as Chronic Obstructive Pulmonary Disease (COPD) or Interstitial Lung Disease , cardiac disease, inability to perform lung function or other maneuvers, upper respiratory tract infection within the last 4 weeks, FEV1 <60% predicted normal pre-bronchodilator, oral corticosteroid treatment within the last 4 weeks

7. Vulnerable Subjects: No vulnerable subjects will be part of this study.

III. Methods and Procedures

1. Methods and Procedures:
We will use the handheld NIOX MINO® to measure **ENO** (Aerocrine, Sweden) (17, 18, 20, 52). The NIOX MINO® fulfills the ATS recommendations and provides reliable readings and is portable and we have experience with it (53). Most healthy individuals have ENO levels in the range of 5-35 ppb (some variation with age, gender, race/ethnicity) (54). Individuals with asthma have ENO levels that can range from 25-80 ppb and even higher (18, 61, 62).

We propose to perform **sputum** induction, collection and processing according to established protocols on each individual (71, 72). IS will be collected after inhalation of hypertonic saline (3%, every 4 minute intervals for up to 12 minutes) with repeated monitoring of FEV_1 as a safety measure. Sputum will be processed immediately to obtain total and cell differential cell counts (73). Our initial evaluations will include analysis of cell counts, with measurements of eosinophils and neutrophils. We consider significant sputum eosinophilia when sputum eosinophils are $\geq$ 2% and significant sputum neutrophilia when sputum neutrophils are $\geq$ 60%, cut off values, that have been described in the literature (12). We will also examine cell counts as continuous variables.

The use of sputum for molecular genetic analyses is limited by its cellular heterogeneity which includes 1% of bronchial epithelial cells and greater than 95% macrophages and neutrophils (74). Enrichment of bronchial epithelial cells has been achieved by magnetic cell sorting (MACS). We will perform positive selection of discrete sputum cell populations including epithelial cells and dendritic cells using magnetic bead preparations or via flow cytometry. Human epithelial cells will be purified from sputum according to the expression of CD326 (human epithelial antigen, HEA, or epithelial cell adhesion molecule (EpCAM) using the magnetic bead CD326 (EpCAM) Tumor Cell Enrichment and Detection Kit (Miltenyi Biotech). To confirm purity of our isolation for selected samples, epithelial cell identification will be demonstrated by intracellular pan-cytokeratin staining. Cells will immediately be placed in RNAprotect Cell Reagent (QIAGEN) and RNA isolated and fractionated into low molecular weight (LMW) and high molecular weight (HMW) using the miRNeasy mini kit (QIAGEN). RNA will be quantified and quality assessed. We will collaborate with the NYU Cancer Institute (CI) Genomics Services. We will have access to high-throughput genomic and computational methodologies specifically for microRNA. We will use the GeneChip® miRNA Array. Briefly, LMW RNA will be labeled with a FlashTag labeling kit (Genisphere, Inc.) according to the manufacturer's protocol. Samples will be incorporated into a hybridization mix and applied to an affymetrix chip and scanned. Computational analysis with techniques such as principal component analysis will be performed with analytic services provided through the NYU CI Genomics core facility. Data will be log transformed and only miRNA replicates that are detected at levels of 2 standard deviations above the average background will be considered for further analysis. These studies will provide us with distinct miRNAs that may differ between IA, AA and controls, or IA and AA. If specific miRNAs are identified that are different between IA, AA and controls, or IA and AA, we will further validate expression of the miRNA using quantitative real time PCR.

We will obtain epithelial cells from the lining of the nose using a technique called nasal brushing. This is a simple, nonsurgical procedure and is well tolerated by most people. We will gently brush the inside of the lateral wall of each nostril with a small, delicate, sterile brush. This procedure takes about 5 seconds for each nostril.

We also propose to correlate patterns of miRNA changes in our defined populations, with mRNA expression arrays. HMW RNA from isolated epithelial cells will be used for hybridization to Affymetrix HG-U133 Plus 2.0 microarrays. Images will be analyzed in the core facility and data will be normalized as follows: (1) per array, by dividing the raw data by the 50th percentile of all measurements; and (2) per gene, by dividing the raw data by the median expression level for all the genes across all arrays in a data set. We will determine whether up or down-regulated mRNAs are potential targets for previously identified miRNAs.

Phase II

Only subjects with Irritant asthma or Allergic asthma who have successfully completed the first phase of the study and have signed consent to be re-contacted will be recruited for the second phase of the study (Phase II). Phase II will involve two visits over a 2 week time period. For Phase II, we will first obtain sputum samples for a baseline analysis of cells. Study subjects will be provided with an inhaled corticosteroid, Fluticasone propionate HFA 220 mcg, 2 puffs twice a day (Treatment), or no treatment (No treatment). Fluticasone HFA will be provided by the study physician through the CTSI and the Bellevue Pharmacy. Treatment cases will be instructed to self administer the medication with a large volume spacer that will be provided to them. Inhaler teaching will be performed by study physician or coordinator at the time that the subject is provided the inhaler. Treatment Cases and No treatment controls will be provided a diary to record fluticasone inhaler. Treatment cases and No treatment controls will be allowed to use their usual rescue bronchodilator (albuterol) on an as needed basis. Treatment Cases and No treatment controls will return after 2 weeks for a repeat sputum induction.

2. Data Analysis and Data Monitoring:

We will start with 10 patients in each group to develop the basic techniques and measurements. Our group sample sizes of 10 and 10 achieve 80% power to detect a difference of 1.3 given the null hypothesis that the two-group difference is 0 and the estimated group standard deviations are 1.0 and 1.0, with a significance level (alpha) of 0.05 using a two- sided two-sample t-test. Based on results from these studies we will work with Dr. Mengling Liu, our biostatistician and the NYU CI Genomics core for our statistical analysis. We put in a small number of patients as this is a pilot study to develop information that will allow for a subsequent appropriate power analysis.

For ENO and EBC analyses, we will express data as means $\pm$ SD for normally distributed variables. For variables that are not normally distributed we will use medians and the interquartile range. We will use statistical analysis including unpaired *t* tests, Kruskal-Wallis test, and regression coefficients as appropriate. P values of < 0.05 will be considered significant. Differential expression of miRNA and hierarchical clustering will be performed with the genomics core. Statistics using *t* tests or algorithms designed specifically for data mining and biochip interpretation will be used. Pathway analyses will also be performed by the genomics computational core.

For Phase II, the primary outcome is the change in TSLP gene expression in epithelial cells after 2 weeks of treatment of inhaled corticosteroid compared to no treatment. We hypothesize that a significant reduction in the gene expression will be observed in the treatment group of inhaled corticosteroid compared with the untreated group. The difference between two randomized groups will be compared using the two-group t-test if data are normally distributed, and the nonparametric Wilcoxon test will be used for skewed data. The effect of inhaled corticosteroids on newly identified epithelial targets (gene expression array) will also be examined. This is an exploratory goal and will be performed after cell stabilization and short-term culture cells to insure appropriate RNA quality.

Sample size and power: Group sample sizes of 15 and 15 achieve 80% power to detect a difference of 1.3SD against the null hypothesis that both group means are equal with a significance level (alpha) of 0.01 using a two-sided two-sample t-test assuming equal variance.

3: Data Storage and Confidentiality:

Care will be taken to ensure confidentiality. Research records will be held in locked cabinets or secure storage rooms. All transmission of data to the coordinating center for analysis will be by study ID code only.

Sample specimens will be stored for up to 10 years for future use or until the end of the study. Participants will be able to decide if they want any of their specimens stored for research done at a later time.

IV. Risk/Benefit Assessment

1. Risk: Minimal Risk

Spirometry, ENO, IOS and EBC.

Participation in these studies will pose minimal risk of physical or psychological harm. Spirometry, impedance oscillometry, ENO and exhaled breath condensate collection are minimally invasive.

Methacholine testing: MCT has been performed with no serious side effects. Participants may develop transient side effects such as cough, mild dyspnea, and chest tightness although many experience no symptoms. Inhaled methacholine can cause bronchoconstriction and safety of each subject is considered prior to testing. A physician and the medical director of the pulmonary lab are trained to treat acute bronchospasm, including appropriate use of resuscitation equipment, and a physician is present during the procedure. Our pulmonary function lab in Bellevue Hospital is well trained in performing MCT in the clinical and research setting. If bronchospasm occurs appropriate medications will be administered as per the lab protocol (oxygen, nebulizers, subcutaneous epinephrine). If a participant in the study develops an acute asthma episode during any part of this procedure, the procedure will be stopped and albuterol will be administered to improve breathing. The bronchodilator will be provided by a physician involved in the study and the participant will be monitored until their breathing has returned to its baseline.

For all women of childbearing ability, a pregnancy test will be performed before the methacholine challenge study. Pregnant subjects will be excluded from the study.

Induced sputum: Sputum induction involves inhalation of hypertonic saline aerosol, a stimulus known to cause bronchoconstriction in asthmatic subjects. To ensure participant safety during sputum induction we will follow the following protocol: procedure will be performed in a hospital setting by highly trained and experienced staff, administration of a bronchodilator can be given to inhibit hypertonic saline-induced bronchoconstriction, we will limit the sputum induction to 20 minutes and monitor the participant's symptoms closely with repeat spirometry every 2 minutes after induction. If bronchoconstriction occurs we will follow the protocol discussed under MCT.

Nasal brushing: Nasal brushing. We do not anticipate any untoward risk of performing this procedure in normal individuals of in those with asthma. This procedure is not usually painful but some people experience a moment of discomfort for about 5 seconds during the brushing. There may be minor irritation such as reflex tearing from your eyes, or sneezing. There may be a rare chance that there is a small amount of bleeding as if you had a scrape. These possible discomforts resolve on their own.

Administration of inhaled corticosteroid –We are providing high dose inhaled corticosteroid (*Fluticasone propionate HFA 220 mcg*) for a short (2 week) period of time. This medication poses no more than minimal risk to research subjects and the risk is minimized by the short treatment period and the use of a large volume spacer. This is an approved treatment for asthma. The most common symptoms experienced by patients who have taken fluticasone are: upper respiratory infections (25-29%), hoarseness or dysphonia (1-4%), inflammation of throat, nose or sinuses (1-12%), oral candidiasis (2%) , nausea and vomiting (1%), other gastrointestinal symptoms (1-2%), and muscle or bone pain (3%). Subjects will be instructed to on avoidance of oral candidiasis by rinsing their mouth after administration of the medication.

2. Protection against Risks:

All participants will be provided with a contact physician capable of providing sources of appropriate medical or professional intervention in the event of adverse effects to the subject. This person will be available on a 24 hour contact basis through the New York University/Bellevue Hospital Asthma Clinic hot line.

3: Potential Benefit to the Subjects:

The subject will be informed that the proposed research will be of no immediate benefit to him/her but may help advance our knowledge of irritant and allergic asthma and help others in the future.

V. Investigator Qualifications and Experience

Joan Reibman, MD. Dr. Reibman is a Professor of Medicine and Environmental Medicine and will oversee in procedure, analysis and will be responsible for overall analysis and presentation of data.

Angeliki Kazeros, MD, Dr. Kazeros is a junior faculty member in the Division of Pulmonary, Critical Care and Sleep Medicine. She will supervise patient recruitment, obtain consent, and oversee the noninvasive procedures.

Bertram Bleck, PhD. Dr. Bleck will oversee cell studies including cell analysis, epithelial cell isolation, FACS analysis and will perform ELISAs.

Karen Carapetyan, M.A. Ms. Carapetyan is a clinical coordinator for the asthma and WTC studies. She will help with recruitment of patients, maintenance of IRB forms and in the performance of non-invasive procedures.

Maria L Cotrina, Ph.D. is a clinical coordinator for the asthma and WTC studies. She will help with recruitment of patient, maintenance of IRB forms and in the performance of non-invasive procedures.

Mengling Liu, PhD. Dr. Liu is a biostatistician in the Department of Environmental Medicine. She currently works with the WTC EHC and will now help in analysis of biologic data.

VI. Subject Identification, Recruitment and Consent/Assent

1. Method of Subject Identification and Recruitment

The WTC-EHC clinic and the asthma clinic at Bellevue will be the recruitment source. The physicians will screen the patients and refer them to the study.

Investigators will also reach out to potential participants via ResearchMatch.Org, which is a national registry developed by institutions affiliated with the Clinical and Translational Science Awards (CTSA). This provides a secure, web-based approach to address a key barrier to advancing research: participant recruitment. We also have an IRB-approved advertisement and will post this at various sites.

2. Process of Consent

The consent form will be written and oral. When it is needed the services of a translator will be provided. Attached is a copy of the informed consent that will be presented to subjects.A second consent form will be provided to asthmatic patients who have completed Phase I of the study and wish to continue on to Phase II of the study with administration of inhaled corticosteroid. Attached is the copy of the consent form for phase II of the study that will be presented to subjects. The consent process will take place in the CTSI rooms. Confidentiality and privacy of the patients will be maintained. The patient will have the opportunity to ask questions to the physician. The Clinical Coordinator and the physician will obtain consent from the subjects. Time will be allotted for a period of Question and Answer in which the patient will have the opportunity to have all their concerns addressed by the physician regarding the study. Primary recruitment will be with patients who understand English. The questionnaires are only available in English, and if required translations services will be requested.

3. Subject Capacity: 90 subjects (accrued and able to be included in the data analysis)

4. Subject /Representative Comprehension: Time will be allotted for a period of Question and Answer in which the patient will have the opportunity to have all their concerns addressed by the physician regarding the study.

5. Debriefing Procedures: no information will be withheld from the subject since no psychological studies will be performed.

6. Consent Forms:

All participants will have consent obtained according to the NYU-IRB consent form. Consent will be sought by a clinical coordinator and signed consent forms will be maintained for all subjects. Complete information on the procedure and potential risks of the procedure will be provided to the patient. The participant will be informed that the procedure will be of no immediate benefit to him/her but may help advance our knowledge of mechanisms of airway inflammation

7. Documentation of Consent: All consents will be signed.

8. Costs to the Subject: There will be no cost to the subject.

9. Payment for Participation: The subject will receive $50.00 per visit.

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