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Measurement of nerve growth factor in induced sputum and exhaled breath condensate

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Measurement of Nerve Growth Factor in Induced Sputum and
Exhaled Breath Condensate

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science in Public Health
Department of Environmental and Occupational Health
College of Public Health
University of South Florida

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Measurement of Nerve Growth Factor in Induced Sputum And
Exhaled Breath Condensate

Victor M. Nwiloh, M.D.

ABSTRACT

Several tests are available for evaluation of respiratory disorders but most of them are invasive and associated with some risk or patient discomfort. Examples include bronchoscopy (bronchoalveolar lavage, BAL) [1], venopuncture [2] and sputum induction [3]. Noninvasive sampling of nongaseous substances contained in expired air, collected as exhaled breath condensate (EBC) has been used to detect inflammatory markers and by-products including nitric oxide and arachidonic acid metabolites and proteins [4]. Nerve growth factor (NGF) is a protein that has been implicated in neurogenic airway inflammation and this pilot study aimed to develop a non-invasive approach for evaluation of allergic airway inflammatory disease by measuring and comparing levels of NGF in the induced sputum and EBC of ten (10) asthmatics and ten (10) non-asthmatics.

Though twenty (20) subjects were sampled, an unexpected event due to a defective NGF kit inadvertently resulted in an unsuccessful analysis of fifteen

(15) sets of specimen (6 non-asthmatics and 9 asthmatics), limiting the study.

This study is significant because occupational lung diseases are the number one work-related illness in the United States and occupational asthma is the most common form [9]. Toluene diisocyanate (TDI) is the commonest cause of occupational asthma and workers exposed to TDI vapor may develop inflammatory conditions including asthma, rhinitis and nasal irritation [7].

Results: NGF was detected and measured only in sputum, with a mean NGF level of 210 (210-210, range 0) in asthmatics and 164 (7-280, range 273) in non-asthmatics. Nonetheless, we failed to reject the null hypothesis (number 3).

Conclusion: This limited study did not have adequate power (power 11%) due to the small sample size and thus lacks internal validity. Further studies are needed using a larger sample size.

INTRODUCTION

Diseases and the work environment

Bernardino Ramazzini, the "father of occupational medicine," was the first to write about the effects of workplace hazards in causing some diseases over three hundred years ago [8]. In the 21st century, toxic exposures and other unique hazards found in the workplace continue to have a significant impact on public health.

A disease or disability resulting from the conditions of a person's employment, work, trade or occupation is called an occupational disease, illness or work related injury. It usually but not always occurs following long exposure to a noxious substance or from continuous repetition of certain acts. Occupational lung diseases are the commonest cause of work-related illness in the United States [9]. In 2002, there were about 294,500 newly reported cases of occupational illness in the private industry, and 22,000 newly reported respiratory conditions [9]. Overall, 2.5 per 10,000 full time workers developed nonfatal occupational respiratory diseases [9]. The total cost of occupational injuries and illnesses in the United States exceeds \$170 billion per year [9].

Occupational lung diseases

Occupational lung diseases are the number one work-related illness in the United States based on the frequency, severity, and preventability of diseases [9]. Though these illnesses are usually caused by prolonged exposure to irritating or toxic substances

which may result in acute or chronic respiratory ailments, severe single exposures can also lead to chronic lung disease. Smoking may act synergistically to increase the severity of these diseases.

Occupational lung diseases are often not curable, but are always preventable [9]. Improving ventilation, wearing protective equipment, changing work procedures, and educating workers are the key factors for prevention [9]. Examples of the commoner occupational lung diseases include occupational asthma (commonest), occupational lung cancer, asbestosis, mesothelioma, byssinosis, coal workers' pneumoconiosis, silicosis and hypersensitivity pneumonitis [9].

Occupational asthma

Occupational asthma is defined as asthma caused by work exposure [10]. It is characterized by recurrent episodes of respiratory symptoms, variable airflow obstruction, airway hyperreactivity and chronic airway inflammation [10].

Chronic inflammation may lead to airway remodeling. The term "airway remodeling" refers to structural changes that occur in conjunction with, or because of, chronic airway inflammation and its consequences include incompletely reversible airway narrowing, bronchial hyper-responsiveness, airway edema, and mucus hypersecretion, which may predispose subjects with asthma to exacerbations and even death due to airway obstruction.

Surveillance programs established around the world have determined that diisocyanate chemicals are the most common cause of occupational asthma. In the United States approximately 100,000 workers are exposed to these chemical compounds in the

workplace each year and 5-10% of these workers will develop occupational asthma. In industrialized countries, occupational factors have been implicated in 9 to 15 % of all cases of adult asthma [11, 12].

There are two types: (i) immunologic type, characterized by a latency period before onset of symptoms and (ii) nonimmunologic type, which occurs after single or multiple exposures to high concentrations of a workplace irritant material in the absence of a latency period [13]. Work-aggravated asthma is also well defined, and is preexisting or concurrent asthma exacerbated by workplace exposures [13]. Variant syndromes have been described too [13]. Clinical history obtained with use of questionnaires has a high sensitivity (87-92%) and low specificity (14-32%) for diagnosis, but is less reliable for recognition of airway hyperresponsiveness. A methacholine challenge test is preferred for this. In addition to assessment of airway hyperresponsiveness, noninvasive assessment of airway inflammation can be used to diagnose occupational asthma.

The best treatment for occupational asthma is primary prevention by hazard abatement in the form of removal of the offending agent and substitution of a nontoxic agent. Strict exposure control is needed when asthma is induced by a workplace sensitizer and this can be achieved preferably by engineering control to protect all employees in the work area or respirator (personal protective equipment) use to protect specific individuals. Rhinitis is associated with an increased risk of asthma regardless of atopic status and occupational rhinoconjunctivitis can lead to occupational asthma [14].

Screening and monitoring for rhinitis and or rhinoconjunctivitis during pre-employment physical examinations and as part of a health surveillance program may help

in early detection of sensitized workers and promote early intervention necessary to prevent progression to occupational asthma. This would be expected to result in a significant reduction in the incidence of occupational asthma.

Methods used to study respiratory diseases

Inflammation is a component of several diseases of the upper and lower respiratory tract including rhinitis, asthma, chronic obstructive pulmonary disease and bronchiectasis. Typically it is assessed directly by performing invasive procedures such as bronchoalveolar lavage and bronchial biopsy or indirectly by venopuncture, though the use of these procedures in clinical practice is limited. Another common and well validated method is sputum induction which is non-invasive but often causes great discomfort to patients [10]. As a result of this, researchers continued to search for alternative methods of assessing airway inflammation that is neither invasive nor uncomfortable.

Recently, two new noninvasive methods were introduced. First, is the measurement of exhaled nitric oxide which reveals increased levels in the exhaled air from asthmatic patients that can be decreased by corticosteroid therapy, however, a few occupational studies that have been done to assess the its role in occupational asthma revealed inconsistent results [10]. The usefulness of exhaled nitric oxide is the investigation of occupational asthma is limited by confounding factors such as therapy with inhaled steroids and smoking, thus it has a high sensitivity but low specificity [10].

The second noninvasive method is the detection of markers (e.g. isoprostanes and

aldehydes) and mediators (prostaglandins and leukotrienes) in exhaled breath condensate [10]. Noninvasive sampling of nongaseous substances contained in expired air, which can be collected as exhaled breath condensate (EBC) has been used to detect markers of inflammatory processes including arachidonic acid metabolites and proteins. Because serial measurements can be made with no harmful effects, it is possible that this method could be useful in occupational medicine [10].

Both methods described above use exhaled air, which is usually warm and humidified. Exhaled air contains gases, water vapor, and various volatile substances that come from the airways and the fluid that lines the airways. Several of these dissolved substances have been shown to be markers of airway disease and or inflammation. Because exhaled air is easily obtained in a non-invasive manner with minimal discomfort to patients, it has great potentials for routine use in clinical practice for screening and diagnosing airway inflammation, as well as monitoring patient's response to treatment. However, measurements of markers in exhaled breath can vary based on the parameters used to study them. Results may vary based on type of equipment used, the exhalation duration, the method of acquiring samples, and the expiratory flow rate.

Nerve growth factor (NGF)

NGF is a neurotrophin produced locally in the human airways. Increasing evidence suggests that NGF may also be an important inflammatory mediator [1]. The first report that serum level of NGF was increased in human allergic inflammatory diseases including asthma, rhinoconjunctivitis and urticaria-angioedema was in 1996.

Patients with more than one allergic disease had higher NGF serum values than those with a single disease [2]. Healthy subjects also produce NGF in their airways but at lower levels compared to persons with allergic airway disease.

In vitro studies have shown that NGF is synthesized and secreted by structural cells (human lung fibroblasts, airway epithelial cells and smooth muscle cells) and cells of the immune system (lymphocytes, monocytes, macrophages, eosinophils and mast cells). NGF exerts a central role in the development, differentiation and function of neuronal and immune cells [6]. NGF induces sensory and sympathetic hyper-innervation of the airways, which leads to increased airway reactivity upon provocation with capsaicin or using electrical field stimulation, and increased systemic and airway levels of tachykinins can be induced by an allergen challenge in asthmatics.

Animal studies indicate that NGF alters sensory nerve function and promotes allergic inflammation (via neurokinin 1, NK-1), bronchial hyper-reactivity and airway obstruction (via neurokinin 2, NK-2) and regulation of tachykinin synthesis has been implicated as the mediator [6]. The fact that these effects of NGF can be blocked by a tachykinin receptor antagonist, coupled with the elevated levels of substance P (a tachykinin) found in the lungs of asthmatics during an acute attack further support this. Substance P has a high affinity for NK-1 receptors. Transient increases in substance P protein have been demonstrated in sensory cell bodies innervating respiratory epithelium of the nose or lung following antigen challenge, toluene diisocyanate (TDI) exposure, viral infection and asphalt fume exposure [7]. Previous studies have shown that exposure to TDI results in elevated NGF levels and this precedes the inflammatory changes [7].

NGF measurement in blood, bronchoalveolar lavage (BAL) fluid, nasal lavage fluid, and sputum is well documented in the medical literature using invasive techniques such as endoscopy, venopuncture and sputum induction [1,2,3,5]. However, a literature review revealed no evidence of studies on NGF measurement in exhaled breath condensate.

Research questions

- 1.) Can nerve growth factor (NGF) be measured in induced sputum and exhaled breath condensate (EBC)?
- 2.) If so, how do levels differ between asthmatics and non-asthmatic subjects?

HYPOTHESIS

To study this, NGF is measured in exhaled breath condensate and induced sputum while comparing asthmatics with non-asthmatics. The first Null hypothesis states that NGF is not measurable in induced sputum. The first alternate hypothesis states that NGF can be measured in induced sputum. The second Null hypothesis states that NGF is not measurable in EBC. The second alternate hypothesis states that NGF can be measured in EBC. The third Null hypothesis states that there is no difference in the levels of NGF measured in the sputum of asthmatic persons compared to non-asthmatic persons. The third alternate hypothesis states that higher levels of NGF can be measured in the sputum of asthmatic persons compared to non-asthmatic persons. The fourth Null hypothesis states that there is no difference in the levels of NGF measured in the EBC of asthmatic persons compared to non-asthmatic persons. The fourth alternate hypothesis states that higher levels of NGF can be measured in the EBC of asthmatic persons compared to non-asthmatic persons.

GOALS AND OBJECTIVES

This study aims to develop a non-invasive approach to evaluation of airway inflammation and disease, especially in persons with allergic conditions using exhaled breath condensate.

SIGNIFICANCE AND UTILITY OF RESEARCH

This study is unique because non-invasive techniques are currently not available for routine measurement of NGF. If NGF is detected and consistently measured in EBC, this may represent a safe, easy and non-invasive alternative approach for evaluation of allergic airway inflammation and diseases. Furthermore, since rhinitis is associated with an increased risk of asthma regardless of atopic status, screening and monitoring for rhinoconjunctivitis as part of a preemployment physical and health surveillance program may help in the early detection of possibly sensitized workers and promote early intervention necessary to prevent progression to occupational asthma. The end result will be a significant reduction in the incidence of occupational asthma. Furthermore, measurement of NGF levels may be useful in the assessment of exposure in irritant induced asthma (Reactive Airways Dysfunction Syndrome) and lastly, new drugs that target NGF may be developed in the future for treatment of asthma.

MATERIALS AND METHODS

Overall study design

In this pilot study, the variable studied was asthmatic versus non-asthmatic status and the outcome of interest was levels of the marker nerve growth factor (NGF) in induced sputum and exhaled breath condensate. A cross sectional study design was utilized where individual specimens were collected, processed, stored and analyzed to obtain relevant data.

Twenty people were divided in two groups of ten. The first group was made up of asthmatics with mild disease while the second group constituted non-asthmatics. The study population was otherwise made up of healthy, non-pregnant persons aged 18-50 years old. Health was defined as having a negative history of heart disease, fever or respiratory tract infection within 96 hours prior to participating, chronic lung disease and moderate asthma or worse. Persons on daily maintenance therapy for asthma including beta-2 agonist bronchodilators and steroids were excluded.

Facilities and equipment

The participants were seen at the College of Public Health at the University of South Florida in the Breath Laboratory (MHH Room 323). The records were maintained in a secured cabinet in this room. The key to access the laboratory was distributed by the

University of South Florida only to authorized personnel (obtained through the College of Public Health). The necessary equipment discussed above that was needed to perform this project was available in this room.

Participant recruitment

Healthy subjects were asked to volunteer for the study performed during a single visit lasting approximately ninety minutes at the Breath Laboratory located in the College of Public Health (MHH Room 323). Subjects who expressed a willingness to volunteer were invited to the Breath laboratory and given information about the study aim, procedure, risks and benefits, and the alternatives to the procedure. They were questioned to demonstrate an understanding of all that was discussed. Throughout the encounter subjects were encouraged to ask questions at any time. Afterwards, willing subjects were then required to sign an informed consent before proceeding with the study. They were monitored by a physician throughout the encounter by direct observation. Prior to participating in the study and undergoing spirometry, subjects were advised not to eat or drink anything and to refrain from strenuous exercise as recommended in the American Thoracic Society (ATS) guidelines [15].

Study subjects and restrictions

Only normal healthy, non-pregnant people aged 18-50 years old and those with a self reported history of mild asthma were invited to participate in the study with the following exceptions. A history of heart disease, fever or respiratory tract infection within

96 hours prior to participating, chronic lung disease, moderate asthma or worse and daily maintenance therapy for asthma with bronchodilators and steroids are all exclusion criteria. Subjects were also required to refrain from strenuous exercise, food and drinks at least one hour prior to the test as recommended by the American Thoracic Society [19].

Study questionnaire and eligibility

Each subject was required to fill out a pre-study questionnaire on the day of the study after consenting to participate but prior to commencement of the study. Information included on this form includes age, current symptoms, past medical history, allergies, medications, smoking history and environmental exposure to second hand smoke.

During each visit, subjects were also given a brief exit questionnaire to assess for change in symptoms and possible adverse effects from the study. (See Appendices A and B)

The medical history was elicited to determine health and eligibility criteria.

Subjects who fulfilled the eligibility criteria proceeded to undergo a brief physical examination including auscultation of the lungs and heart. Those subjects who remained eligible after questionnaire, interview, and physical examination were allowed to proceed to get a baseline spirometry. To be eligible to participate in the remainder of the study, all participants were required to have a forced expiratory volume in one second (FEV1) equal or greater than 1.5L on spirometry.

Subject safety considerations

Subject safety was given the utmost consideration in this study. Subjects were advised that they could choose to stop or withdraw at any time without any consequence. All subjects were healthy volunteers. The entire study was non-invasive. Physical examination and initial medical screening questionnaires were reviewed directly by a physician prior to commencement of study protocol. Only subjects who had normal physical examination were allowed to continue in the study. Spirometry, which is commonly used in clinical practice to assess lung function, was obtained as an eligibility criterion. To obtain spirometry, subjects blew out through a disposable filter (to prevent infection) using maximum effort after a maximal inhalation.

Measurement of exhaled breath condensate, which is currently still a research tool, involved tidal breathing into the mouth piece of a cooling tube with a nose clip in place. This results in the trapping of moisture in the breath by condensation. This process was associated with less discomfort than spirometry and sputum induction. During each visit, subjects were also given a brief exit questionnaire to assess for any new symptoms or possible adverse effects from the study protocol.

Participation in this study did not affect the standard care subjects would otherwise receive from their personal physicians unless abnormal findings were detected on physical examination and or spirometry. Under those circumstances, the subject was advised to consult with their personal physician. In the unlikely event that a subject develops an adverse effect the study will be stopped immediately despite the fact that most events would only be transient and subside within a few seconds to minutes of

stopping the activity. In the event of persistent symptoms including chest pain or tightness, wheezing and dyspnea emergency equipment was available at all times during the study to assist the subject until the arrival of appropriate emergency personnel. To ensure patient safety, all subjects were directly observed by a physician who was ACLS-certified during the entire study protocol. The sampling procedure was completely non-invasive.

Physical examination

A brief physical examination with emphasis on auscultation of the lungs and heart was performed on all subjects prior to obtaining spirometry and proceeding with the rest of the study protocol. Auscultation involves listening to the breath and heart sounds with a stethoscope. Person with abnormal finding were deemed ineligible and immediately excluded. Such persons were advised to seek necessary medical care from their personal physician. Auscultation of the heart was performed for the sole purpose of subject safety.

Acquisition of spirometry

Spirometry a common clinical method used to assess lung function, was performed as part of the inclusion criteria for the study. The Koko spirometer was used and calibrated using a standard 3-liter syringe to ambient temperature, humidity, and barometric pressure at least once a day on days when participants were examined. The spirometer was additionally re-calibrated when at least six hours had elapsed since the prior calibration or at the discretion of the examiner. The raw FEV1 and FVC

measurements obtained for each participant were automatically compared to their predicted normal values based on age, ethnicity, weight, height and smoking status in determining their percent of predicted values using parameters as set forth by Crapo et al.

The spirometry parameters examined on each study participant included FEV1, FVC, FEV1/FVC ratio, and the flow-volume loop. At least three spirometric measurements were obtained on each subject, with at least one flow-volume loop showing good effort. Proper technique was ensured by evaluating the flow-volume curve and continuance of the expiratory maneuver for at least six seconds according to ATS criteria. A nose-clip was placed on the subject's nose to occlude the nostrils during spirometry so as to prevent nasal breathing and ensure accuracy. Subjects were asked to forcibly exhale for at least six seconds through a disposable single-use filter after maximal inspiration, followed by another maximal inhalation. The maneuver was demonstrated to them to help achieve consistency.

Subject with abnormal spirometry measurements was notified of this information and advised to consult their personal healthcare provider. They were subsequently excluded from the study. All subjects with an FEV1/FVC ratio greater than 70% of predicted, FEV1 greater than 1.5 L and a normal appearing flow-volume loop were eligible for the study by spirometric criteria. In those participants who did not meet all of these strict criteria, the overall clinical picture, including medical history and physical examination were assessed individually to determine study eligibility. When eligibility due to spirometry did not meet these strict criteria, Dr. Stuart Brooks (faculty advisor) was consulted to determine whether the participant would be eligible for the study.

Adverse effects were unlikely to occur during this phase of the study because subjects identified themselves as being in good health and the test was performed with the patients comfortably seated in a room that was temperature controlled. Nonetheless, there was the slight possibility of unusual symptoms such as lightheadedness, dizziness, chest pains, palpitations, or shortness of breath during the spirometry from overexertion in breathing. As a precaution, subjects were instructed to stop, be seated, and notify the physician examiner immediately if they experienced any symptoms at any time.

Specimen collection

The study was conducted in two parts. First, exhaled breath condensate (EBC) was obtained and afterwards, sputum was collected via induction.

Exhaled breath condensate: The exhaled breath condensate was collected by using a condenser, which permits noninvasive collection of the nongaseous components of the expiratory air (EcoScreen; Jaeger, Wurzburg, Germany). The subjects were instructed to breathe through a device with a mouthpiece and a two-way non-rebreathing valve, which also serves as a saliva trap. Subjects wore a nose clip and were breathing at a normal frequency and tidal volume for 10 minutes. If the subjects salivated they were instructed to swallow. The condensate was collected in a collection cup at the base of the device and transferred into a microtube for immediate storage at -70°C using a micropipette. As the sample was taken non-invasively during tidal breathing, this method of sampling does not influence the sample taken as observed with other methods such as bronchoalveolar lavage or induced sputum.

Induced sputum: Prior to sputum induction, subjects were given clear and detailed instructions because full cooperation was needed to obtain adequate samples. Full resuscitation equipment was made available as recommended for specific and non-specific bronchial challenge procedures. An ultrasonic nebulizer (DeVilbiss Pro-UltraNeb Large volume Nebulizer099HD) with an output of 1ml per minute (1 ml/min) was used. Because of the risk of bronchoconstriction with use of hypertonic saline in asthmatic subjects, pretreatment with a bronchodilator was done on all subjects using a single dose of albuterol 2.5mg given over 3 minutes. Spirometry was repeated to ensure no significant change in FEV1 before proceeding with administration of 3% saline over 7 minutes. 3% saline solution was administered via aerosol inhalation using a nebulizer and afterwards yet another spirometry was done to monitor for FEV1 changes. The process was stopped immediately if there was a fall in FEV1 of 20% or greater compared with the post-bronchodilator value or if subjects developed symptoms including lightheadedness, dizziness, chest pain or tightness, palpitations, dyspnea or wheezing.

Finally, subjects were seated at a negative pressure cubicle where sputum expectoration was performed over a 5 – 10 minute period. The sputum was collected in a petri dish and processed as stated in the literature [16] prior to storage at -70°C . Collected sputum was checked for adequacy prior to processing by preparing a slide. Slide preparation entailed making a sputum smear with a loop and heat fixing it. Slide was stained with 1-2 drops of 1% methylene blue and allowed to stand for 1 minute before washing off with water. Afterwards, the slide was air dried and examined under a light microscope at 40x and 100x magnifications. An adequate specimen was defined as

one with visible inflammatory cells (neutrophils, basophils, monocytes, eosinophils etc) and less than 50% squamous cells. Adequate sputum samples were immediately processed. First, the sample was weighed, inspected grossly and findings were recorded.

The sputum portion of the sample (selected portion) was separated from saliva as much as possible using forceps and transferred to a polystyrene tube and the weight was recorded. 10% dithiothreitol (DTT, Sigma chemical, St. Louis, MO, U.S.A.) equivalent to 4x the weight of the selected portion was added and agitated in a vortex mixer for 15 seconds. Afterwards the mixture was placed on a bench rocker for 15 minutes and DPBS equivalent to the volume of DTT used was added. The mixture was placed on a bench rocker or an additional 5 minutes. The mixture is then filtered through a 70 micrometer nylon gauze/mesh and the weight of the filtrate was recorded. Finally, the filtrate was centrifuged at 500 rpm for 5 minutes and the supernatant was aspirated with a micropipette and transferred to eppendorf micro-tubes for storage at -70°C . The stored samples are later analyzed for measurement of NGF using enzyme-linked immunosorbent assay (Promega, Madison, WI, USA, ELISA).

Measurement of NGF

ELISA: The levels of NGF protein was quantified in 1:2 diluted sputum and EBC samples, using a commercially available, highly sensitive NGF-specific two-site enzyme linked immunosorbent assay (ELISA) – kit according to the procedure indicated by the manufacturer (Promega, Madison, WI, USA). 96-well ELISA plates were coated with an anti-NGF polyclonal antibody in a coating buffer (25mm carbonate buffer, pH 9.7).

Following an overnight incubation at 4 degrees centigrade, the plates were washed (20mM Tris-HCl, 150mM NaCl with 0.05% (v/v) Tween® 20), and incubated in a blocking buffer for 1hour. The diluted sputum and EBC samples and the standard recombinant human NGF was then incubated in wells at room temperature for 6 hours, and washed afterwards. The 2 samples and the recombinant NGF was diluted in the blocking buffer, as recommended by the manufacturer. Rat monoclonal anti-NGF antibody (0.25mcg/ml) was added for an overnight incubation at 4 degrees centigrade, and washed afterwards. After washing, anti-rat horse radish peroxidase-conjugated immunoglobulin G (Ig G) was added and incubated for 2.5 hours at room temperature.

Finally, the substrate (0.02% 3,3', 5,5-tetramethylbenzidine and 0.01% hydrogen peroxidase) was added and incubated for 10 minutes before stopping the colorimetric reaction with 1N hydrochloric acid which changed the solution from blue to yellow. The sample absorbance or optical density was measured at 450NM within 30 minutes of stopping the reaction.

Data collection and analysis

Though a large study population would be required to appropriately assess the research questions, the analysis of exhaled breath condensate (EBC) is an evolving research concept and measurement of nerve growth factor (NGF) in EBC has not been reported in the literature. This study was embarked upon as a pilot study with the intent to follow up with a larger sample size in the event that NGF is detectable in significant amounts in EBC and sputum.

Twenty eligible subjects were studied to provide insight to the following research questions:

(1) Can nerve growth factor (NGF) be measured in induced sputum and exhaled breath condensate (EBC)?

(2.) If so, how do levels differ between asthmatics and non-asthmatic subjects?

A power calculation was performed which was derived using alpha of 0.05 and power of 80%. An estimated sample size of 64 is required to show a significant difference in the mean of the two groups given the above parameters. In any study where there are two groups being compared, the participants are ideally randomly assigned to each group. However, random assignment is not possible when two distinct groups are being used such as males versus females. A similar situation obtains in this study, where the two distinct groups were asthmatics versus non-asthmatics.

The mean of each of the two groups in a study sample may be compared using a Student's t-Test, a parametric analysis, when the population studied is normally distributed. When the population is not normally distributed, a non-parametric analysis must be used. Non-parametric tests include the Wilcoxon Ranked-Sum Test or the Mann-Whitney U Test.

Statistical calculations were performed by inputting the data into Statistical Analysis Software (SAS), which calculated the data automatically. For this study, SAS was utilized to calculate descriptive statistics such as the mean, median, variance, standard error of the mean, standard deviation, range, minimum and maximum values, skewness, and kurtosis for the non-asthmatic group.

STUDY RESULTS

Exclusions

Two asthmatic subjects who expressed their willingness to participate in the study were excluded because they were on daily maintenance therapy for asthma. Another non-asthmatic study volunteer was turned down because of ongoing acute bronchitis which was being treated with antibiotics.

No one was excluded on account of abnormal auscultation during physical exam. All the subjects had a normal cardiac exam with normal first and second heart sounds with regular rate and rhythm and no murmurs, gallops or rubs. The lung exams revealed normal vesicular breath sounds bilaterally with no crackles, wheeze or rhonchi. There was no deformity on chest exam. All subjects had FEV1 greater than 2.5 L.

Study demographics

The following tables demonstrate the results of the demographic information that was collected during the study.

Table 1. Study population

Groups	Numbers	Percentage
Asthmatics	1	20
Non-asthmatics	4	80

Table 2. Age distribution

Age	19	25	29	32	39
Asthmatics	0	0	1	0	0
Nonasthmatics	1	1	0	1	1

Table 3. Sex distribution

	Male	Female	
Asthmatics	0	1	1
Non-asthmatics	3	1	4
Total	3	2	5

Medical Information

The following tables list the history of allergies and smoking, co-morbidities, medications, allergens, physical exam findings and study adverse effects.

Table 4. History of Allergy

	Asthmatics	Non-asthmatics
Positive history of allergy	1	0
Negative history of allergy	0	4

Table 5. Smoking History

	Asthmatics	Non-asthmatics
Never/non-smoker	1	3
Current smoker	0	0
Ex-smoker	0	1

Table 6. Co-morbidities

Medical conditions	Asthmatics	Non-asthmatics
Grave's disease	1	0
GERD	1	0
Hypertension	0	1
Deep Venous Thrombosis	0	1

Table 7. Medications

Medications	Asthmatics	Non-asthmatics
Synthroid	1	0
Albuterol	1	0
Benadryl	1	0
Cimetidine	1	0
Lisinopril	0	1
Aspirin	0	1

Table 8. Nerve Growth Factor Measurement.

	Asthmatic Sputum (pg/ml)	Non-asthmatic Sputum (pg/ml)	Asthmatic EBC (pg/ml)	Non-asthmatic EBC (pg/ml)
1	210	7	0	0
2		171		0
3		280		0
4		198		5

Figure 1: Mean sputum NGF levels in asthmatics and non-asthmatics

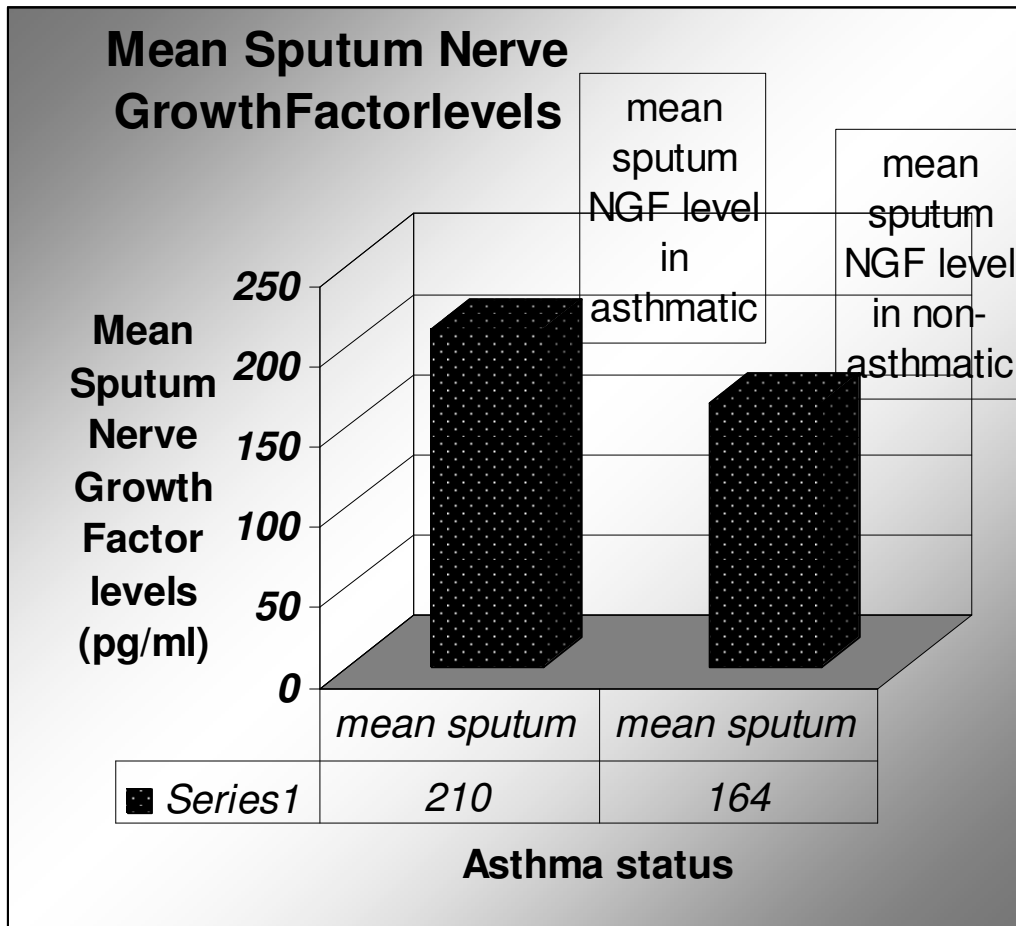


Table 9. Statistical Analysis of Sputum Nerve Growth Factor levels for all subjects

	Asthmatic	Non-asthmatic
N	1	4
Mean	210	164
Median	210	184.5
Standard Deviation		114.5
Variance		13103.3
Standard Error of the Mean		57.2
Skewness		-1.008
Kurtosis		1.837
Range	0	273
Minimum	210	7
Maximum	210	280

t-value 2.87

DISCUSSION

Findings

This study was aimed at determining if NGF is measurable in induced sputum and exhaled breath condensate and if so how do levels differ between asthmatics and non-asthmatics? Demographic data revealed that 5 subjects age 19 – 39 years old participated in the study. Medical information review revealed a history of GERD and Grave's disease in the asthmatic while the non-asthmatics reported hypertension and deep venous thrombosis.

The results revealed that NGF was measurable only in sputum. The mean value in asthmatics was 210 pg/ml while non-asthmatics had a mean value of 164 pg/ml. The independent t-test was used to analyze the data, and a t-value of 2.870 was obtained while the critical value was 2.353 given alpha of 0.05. Though a mean difference of 46 was found between asthmatic and non-asthmatic subjects, this is not statistically significant and we fail to reject the null hypothesis. The power obtained was 0.1135 (11%), however in view of the small sample size used (1 asthmatic and 4 non-asthmatics), the study lacked adequate power. If a sample size of 20 was used as planned initially, the power will only be raised to 23% (0.2288). The prestudy analysis estimated that a sample size of 64 is needed to obtain a power of 80% (0.80) for a mean difference of 0.5, given a standard deviation of 1 and alpha set at 0.05. The type II error, beta (1-power) was 89% (0.8865) but will be reduced to 77% with a sample size of 20.

Implications

The implication gleaned from this study is that there is no significant difference in the sputum NGF levels in asthmatics compared to non-asthmatics and we failed to reject the null hypothesis. However, the study does not have adequate power due to the small sample size. Though a previous study showed no significant difference in sputum NGF levels (pg/ml) in persons with chronic cough including asthma (median 580; inter-quartile range 312 – 880) compared to healthy controls (median 516; inter-quartile range 296-772), some other studies suggest that higher levels of NGF are obtained in asthmatics [19].

Twenty (20) sets of samples were collected and analyzed in 2 batches for this study (Batch A: 5; Batch B: 15). The first batch was analyzed successfully; however the second batch was inadvertently ruined at analysis by a bad NGF kit which was defective from the manufacturer. Unfortunately, a new replacement kit was not immediately available and in view of time constraint under the circumstance, the study had to be reported with the limited data available. This incomplete study lacks internal validity.

Future Directions

In the future, improved micro-concentration collection techniques (lipholization) and analytic chemistry methods could increase the likelihood of detecting NGF in EBC, however NGF may be absent in EBC. Further research in this direction is needed.

Values obtained for NGF in sputum may have been similar to non-asthmatics because only persons with stable mild asthma and normal spirometry were included in

the study except for one subject. In future studies, asthmatic subject selection should be based strictly on spirometric parameters and not just a self reported history.

Finally, future studies should be well designed with emphasis on random selection of subjects, large sample size and objective criteria for defining asthmatic subjects.

Study Limitations

Limitations can have a negative effect on the validity of the study. This incomplete study is limited by its small sample size and thus lacks internal validity. The stronger the association is in nature, the easier it is to find with a small sample size. The closer the true association is to the null value, the larger sample size you need to find it to be statistically significant [17]. When the sample is too small, a study is more likely to miss an association, increasing the likelihood of a Type II error [17]. A Type II error may have been committed in this study where a weaker association could still be present and would not be detected by this study.

Study biases may limit the value of a study. This study may have been limited by selection bias. Though males and females from various racial backgrounds (Caucasian, Blacks, Asians and Hispanics) were used in this study, all subjects were recruited from within the University campus and were current or past undergraduate and graduate students. Observer bias was also present as this was not a blind study. All of these can limit the generalizability of the study [17].

Confounders can also affect a study. Several methods may be used to control for confounders including restriction, randomization, and matching[17]. Multiple restrictions

were used in the study design to attempt to control for confounder but randomization was not done because two distinct groups (asthmatics and non-asthmatics) were being compared. Matching, though beneficial was not used either.

In performing statistical analyses where two distinct groups were compared, three underlying assumptions were made: (1) Assumption of Normality, (2) Assumption of Homogeneity of Variance, and (3) Assumption of Independence [18]. Statistical analyses are most robust to violations of the Normality Assumption. Statistical analyses are moderately robust to the Homogeneity of Variance Assumption. Statistical analyses are the least robust to the Assumption of Independence, the most serious of the violations. In this study, care was taken not to allow people who were genetically related to each other participate in the study.

CONCLUSION

Given the small sample size and the other limitations listed above, this pilot study is limited in its value. NGF was not detected in EBC. Though a higher mean NGF was observed in the sputum of asthmatics, we failed to reject the null hypothesis. NGF may be truly absent in EBC otherwise improved analytic chemistry methods may enhance detection of NGF in the future. Further studies are needed before NGF measurements in sputum can be used routinely in clinical practice. A follow up study using a larger sample size will increase the power and ensure internal validity and generalizability of the results obtained.

Measurement of NGF in sputum may be useful in surveillance programs of occupational asthma and the estimation of dose from exposure with Reactive Airways Dysfunction Syndrome (RADS). Study results observed in sputum may be used in combination with other tools for the diagnosis of occupational asthma.

If elevated levels of NGF is detected in asthmatics, this will conform with other studies done with BAL, nasal lavage and blood. I would expect allergic asthma to be associated with higher levels of NGF compared to irritant induced asthma because it involves sensitization and a latent period. Mild disease may not result in any significant difference in NGF levels compared to non-asthmatics. Furthermore, corticosteroids suppress NGF level elevation / response if given prior to exposure.

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APPENDICES

Appendix A: Pre-study questionnaire

Subject number:_____

PRESTUDY QUESTIONNAIRE

Date _____

- 1) Gender: Male Female (circle one)

- 2) What is your date of birth? _____

- 3) How old are you? _____ years old.

- 4) Do you currently have, or have you ever had any of the following conditions listed below? (Please circle YES or NO)

YES NO CHEST PAIN, PALPITATIONS, IRREGULAR HEART
 BEAT, OR HEART DISEASE?

YES NO HIGH BLOOD PRESSURE?

YES NO ASTHMA

YES NO BRONCHITIS, EMPHYSEMA, OR OTHER
LUNG OR BREATHING DISORDERS?

YES NO DIFFICULT OR HEAVY BREATHING?

YES NO A LARGE AMOUNT OF PHLEGM PRODUCTION

YES NO ANY ALLERGIES?

YES NO ARE YOU PREGNANT?

YES NO HISTORY OF HERNIA?

IF YOU HAVE CIRCLED YES TO ANY OF THE ABOVE QUESTIONS, PLEASE
EXPLAIN: _____

5) Do you have any health problems or past medical history of health problems that
you have seen a physician for? Please list them below.

1. _____

2. _____

3. _____

4. _____

5. _____

6) Are you taking any medications? If so please list them below. (Including over the counter medications)

1. _____

2. _____

3. _____

4. _____

5. _____

6. _____

7. _____

7) At this point in time, to what degree do you note the following symptoms?

(a) Phlegm production:

___NONE ___VERY LITTLE ___MODERATE AMOUNT ___VERY MUCH

(b) Runny or irritated nose or nasal passages:

___NONE ___VERY LITTLE ___MODERATE AMOUNT ___VERY MUCH

(c) Throat irritation or burning sensation:

___NONE ___VERY LITTLE ___MODERATE AMOUNT ___VERY MUCH

(d) Sensation of a “weight” or tightness of the chest:

___NONE ___VERY LITTLE ___MODERATE AMOUNT ___VERY MUCH

(e) Feeling of chest pain, burning, or tightness:

___NONE ___VERY LITTLE ___MODERATE AMOUNT ___VERY MUCH

8) If you have ever smoked, please answer the following:

How many packs per day did you smoke? _____

For how many years did you smoke? _____

When did you stop smoking? _____

9) On what date were you last ill? _____

10) What illness did you have? _____

Please circle YES or NO to the following questions:

a) Are you exposed to second hand smoke at home or at work?

YES or NO

b) Were you or are you exposed to any gases, dusts, or fumes at your job?

YES or NO

If so, please explain: _____

c) Do you ever wheeze or become short of breath?

YES or NO

- This is the end of this questionnaire. Thank you for taking the time to fill it out.
- Special thanks to Dr. Gwyn Crump for allowing the use of his questionnaire in this study.

Appendix B: Post-study questionnaire

Post-Study Questionnaire

SUBJECT NUMBER: _____

Are you currently experiencing any of the following symptoms?

(Please Circle YES or NO)

(1) Difficult or heavy breathing? YES NO

(2) Feeling of chest pain, tightness, pressure, or burning?

YES NO

(3) Wheezing or increased cough? YES NO

(4) Any new symptoms that were not present when you arrived

here today? YES NO

(5) Any other unusual symptoms? YES NO

Special Thanks To Dr. Gwyn Crump for developing the majority of this questionnaire!

Appendix C: Flyer

You're Are Invited To Participate.

What?

A \$20.00 token will be given to each volunteer who completes the study for valuable time spent participating.

This study makes measurements of the air breathed out by subjects. The tests will measure lung function & a constituent of exhaled air and sputum called Nerve Growth Factor (NGF).

This research study involves getting a breathing treatment but is painless and non-invasive.

Who?

Healthy men and women with or without a history of asthma.

Candidates must be 18-50 yrs old and of any race.

Why?

The study is designed to examine whether a certain marker (NGF) found in the air we normally breathe out is different in healthy asthmatics compared to non-asthmatics. Your participation in this study may help develop a less invasive test for routine assessment of allergic airway disorders such as asthma, which may enable healthcare providers to better monitor worsening of disease or exacerbations. Hopefully, the results will benefit science.

When?

The study requires only one visit and will last approximately ninety minutes.

Where?

USF College of Public Health

If you may be interested or have any questions, please contact me (DR. VICTOR
NWILOH) at:

pager number: (813) 332-6925

cell phone number: (678) 472-2387

e-mail: vnwiloh@hsc.usf.edu

Appendix D: Informed consent form

Informed Consent for an Adult

University of South Florida

Information for People Being Asked to Take Part in Research Studies

IRB Study # 103749

Doctors and researchers at University of South Florida (USF) study diseases and other health problems people have. We try to find better ways to help treat these health problems. To do this, we need the help of people who agree to take part in a research study.

Title of Research Study: Measurement of Nerve Growth Factor in induced sputum and exhaled breath condensate.

Persons in Charge of Study: Victor M. Nwilo, MD; Stuart M. Brooks, MD and Thomas Truncala, D.O.

Where the study will be done: University of South Florida, College of Public Health, MHH Room 323

Who is paying for the study: Sunshine Education Research Center

Should you take part in this study?

This form tells you about this research study. You can decide if you want to take part in it. You do not have to take part. Reading this form should help you decide if you want

to take part in the study. If, at any time, you have any questions feel free to ask the person explaining this study to you.

Before you decide:

- Read this form.
- Talk about this study with the study doctor or the person explaining the study. You can have someone with you when you talk about the study.
- Find out what the study is about.

This form explains:

- The purpose of this research study.
- What will happen during this study and what you will need to do.
- The potential benefits of being in this study, if any.
- The risks of having problems because you are in this study.
- The answers to any questions you might have.

You can ask questions:

- You may have questions this form does not answer. If you do, ask the study doctor as you go along.
- You don't have to guess at things you don't understand. Ask the people doing the study to explain things in a way you can understand.

After you read this form, you can:

- Take your time to think about the information that has been provided to you.
- Have a friend or family member read the form.

Talk it over with your regular doctor.

It's up to you:

- If you choose to be in the study, then you can sign the form.
- If you do not want to take part in this study, do not sign the form.

Why is this research being done?

The purpose of this study is to find out if there is a difference between asthmatic and non-asthmatic subjects in relation to a certain substance in the air we breathe out called nerve growth factor which is also found in induced sputum. This research could eventually lead to the development of a non-invasive method for routine assessment of allergic airways diseases such as asthma.

The air we breathe out is currently the subject of intense research. There are certain substances in this air whose levels are affected by specific diseases associated with the lungs. In this study, one of these substances in exhaled breath is called nerve growth factor. Your exhaled breath will be collected into a single-use, clean, disposable tube that has never been used before. Each tube is discarded after you have used it. Sputum is also induced with a nebulizer and collected in a specimen bottle for measurement of nerve

growth factor. Two groups of subjects will be examined in this study because persons with allergic diseases such as asthma have been found to have significantly higher levels of NGF in other tests.

Why are you being asked to take part?

You must be a healthy person within a certain age group to be eligible for the study. You will not be eligible if you have had an acute exacerbation of asthma or febrile illness at the time of evaluation or within the preceding 96 hours or if you are pregnant. You may have had a recent respiratory tract disease if you have had any recent fever, chills, coughing, shortness of breath or difficulty breathing, nasal congestion, runny nose, sinus congestion or headache, colored phlegm or (green, yellow, brown, black, or red), crackling or whistling sounds heard when you breathe, diagnosis by a doctor of nose/sinus/lung infections, or recent treatment with antibiotics for any of these symptoms. You may not be eligible if you have poorly controlled asthma or any other diseases or health problems. We are asking you to take part in this study because you are a healthy young person aged between 18-50 years. We want to find out if there is a difference in the exhaled breath and induced sputum content of NGF between healthy asthmatics and non-asthmatic subjects.

Your regular medical treatment will not actually be part of the research study. In fact you will not be eligible for the study if you suffer from any significant heart or lung disease or other significant medical problems. How long will you be asked to stay in the study? You will be asked to spend no longer than 90 minutes in this study.

During this time you will be required to complete the screening questionnaires, perform a

limited non-invasive physical examination, a breathing test and the study proper.

How often will you need to come for study visits?

A study visit is one you have with the study doctor. This visit is different than the visits you make with your regular doctor. You will need to come for only one visit.

At this study visit, the doctor will obtain a questionnaire both before and after testing, perform a brief physical exam consisting primarily of listening to your heart and lungs using a stethoscope, and perform the breathing tests.

How many other people will take part?

About 30 people will take part in this study at USF.

Will the medical treatment you get from your regular doctor change if you take part in this study?

The kind of treatment you now get from your regular doctor will not change because you take part in this study. You will keep seeing your regular doctor. Your regular doctor will give you the same kind of treatment you would get anyway, whether you take part in the study or not.

If you need it, you can

- Use other medicines prescribed that will help your disease.
- Get any surgery you need.
- You will need to talk to the study doctor about any surgery you have planned.

Talk to your regular doctor about the treatments you may need.

If you have an emergency, you can get emergency care.

Other procedures can be used for measuring airway disease. These include bronchoscopy with bronchoalveolar lavage/ bronchial biopsy (which are invasive), or sputum induction (which can be less comfortable). These procedures, which can be ordered by your regular doctor if needed clinically, will not be performed here.

What other choices do you have if you decide not to take part in this study?

The alternative to being part of this research study is not to participate in this study. There will be no changes in your life. You will continue with your regular life style and are instructed to visit your personal physician for any respiratory or non-respiratory problem you were being followed for before the study. The same is true if you decide to participate in the study.

If you decide you do not want to take part in this study that is okay. There are other choices such as chest X-ray, chest CT, bronchoalveolar lavage, biopsy, or sputum induction that are considered to be the current standard practices for diagnosing airway disease.

How do you get started?

You must be within a certain age group to be eligible for the study. You must also be healthy to participate in this study. Volunteers must be between ages 18 and 50. If you decide to participate in this study, you will be required to review this informed consent, discuss the study and your possible participation with the study doctor and decide

whether you are interested in participating. If you decide to take part in this study, you will need to sign this consent form. This informed consent must be signed before any study-related test or procedure can be done. After signing this informed consent, screening tests will be completed to help determine if you meet the requirements to be in the study. You will be asked to fill out questionnaires, undergo a limited physical examination and perform a limited breathing test called spirometry. Screening tests are tests done to see if you are able to be in the study. In order to qualify, you must record a history of good health. You must record a negative questionnaire response for active respiratory diseases. You must not be suffering from any heart condition.

There are different tests performed. These tests are considered non-invasive and do not involve drawing blood or inserting tubes into your throat or nose.

We will do these screening tests:

1. **Questionnaires:** You will be asked to fill out questionnaires about your health, ethnicity, and socioeconomic status. They will include medical questionnaires. A post-study symptom questionnaire will also be given at the end of each visit.
2. **Physical Examination:** Listening with a stethoscope to your chest and abdomen.
3. **Spirometry:** This is a common, frequently used and accepted medical test for evaluating how your lungs are working. Abnormal spirometry may indicate that you have a lung disease such as asthma, emphysema, chronic bronchitis, and other diseases. The test involves taking in a deep breath and then blowing out into a tube as hard and as fast as you can. The spirometer measures the amount and speed with which you forcibly exhale your deep breath.

You will be notified if your spirometry test results are abnormal and advised to see your personal physician.

The test results will come back right away. At the end of each of these screening tests, you and the research team will decide whether or not you should be in the study.

What will you need to do to get ready for this study?

You will need to refrain from strenuous physical exercise (such as running, jogging, working out with exercise equipment, participating in sports, or doing any heavy physical labor), eating, or drinking for a minimum of one hour prior to the visit.

You must be within a certain age group to be eligible for the study. You must also be healthy to participate in this study. Volunteers must be between ages 18 and 50. If you decide to participate in this study, you will be required to review this informed consent, discuss the study and your possible participation with the study doctor and decide whether you are interested in participating. This informed consent must be signed before any study-related test or procedure can be done. After signing this informed consent, medical tests will be completed to help determine if you meet the requirements to be in the study. You will be asked to fill out a questionnaire, undergo a physical examination and perform a breathing test. In order to qualify, you must record a negative respiratory questionnaire response to treatment for active respiratory diseases. You must not be suffering from any major cardiovascular condition.

There are different tests performed. These tests are considered non-invasive and do not involve drawing blood or inserting tubes into your throat or nose.

What will happen during this study?

Only healthy, non-pregnant subjects without a history of heart or other diseases ages 18-50 years old will be invited to participate. You will be asked to verify that they have not had any signs of recent asthma exacerbation or febrile illness including upper or lower respiratory tract infections. This would include any recent fever, chills, coughing, shortness of breath or difficulty breathing, nasal congestion, runny nose, sinus congestion or headache, colored phlegm or (green, yellow, brown, black, or red), crackling or whistling sounds heard when you breathe, diagnosis by a doctor of nose/sinus/lung infections, or recent treatment with antibiotics for any of these symptoms. At a minimum of 1 hour prior to visit, subjects will be instructed not to eat or drink or participate in strenuous exercise.

If you decide to participate :

During the visit, you will be asked to volunteer for the study. If you volunteer, you will be given information about the study procedure, the risks, the benefits, and the alternatives to the procedure. Then you will be asked if you have any questions and demonstrate an understanding of everything discussed. If you still agree to proceed, you will be asked to sign an informed consent. You will be taken to the Breath Laboratory at the College of Public Health (MHH Room 323) and seated comfortably in an air-conditioned room.

You will be asked to complete a questionnaire including symptoms, medical history to determine “health” and age. Those who fulfill the criteria of age and being “healthy” will be asked to participate in the study. You will then undergo brief physical examination

including auscultation. You will be directly watched by a physician throughout the remainder of the study via direct observation.

Limited spirometry (including FEV1, FVC, and FEV1/FVC ratio), which is part of a common method done in hospitals and clinics to check lung function, **will be done to help verify that your breathing is normal.** A loose-fitting device resembling a clothespin will be placed on the nose during the study to obtain better spirometry results. You will be asked to forcibly exhale into a disposable single-use tube using maximum effort after maximal inhalation. Anyone who has abnormal spirometry measurements will be notified of this information and advised to consult a healthcare provider and disqualified for the remainder of the study.

Adverse effects are extremely uncommon during this phase of the study because subjects are young/ healthy individuals and the room is temperature controlled. You will be seated during the spirometry, and this phase of the study is brief in duration, nonetheless, there is the remote possibility of unusual symptoms such as lightheadedness, dizziness, chest pains, palpitations, or shortness of breath during the spirometry from overexertion in breathing. As a precaution, you will be instructed to notify the physician examiner immediately if you experience any symptoms at any time. Following this, auscultation will be performed again and you will be given another brief symptom questionnaire to make sure you have no adverse effects from spirometry.

Next, the actual study exhaled breath testing, which is strictly part of the research, will be performed to measure the amount of nerve growth factor in the breath. You will be asked to breathe out through a disposable plastic mouthpiece connected to a filter at a constant

rate of flow after a maximal inspiration (which is where you take as deep a breath in as you can to fill your lungs with as much air as possible). Afterwards, sputum induction will be initiated using a nebulizer and samples will be collected in a specimen bottle. Following this, the investigator will again perform a brief physical examination using a stethoscope to listen to your heart and lungs and you will be given another brief symptom questionnaire to make sure you have no adverse effects after the study.

Some study visits may be longer or shorter than ninety minutes depending primarily on both the equipment and subject variability in breathing.

You will be asked to verify the absence of symptoms prior to leaving.

We will study two groups of people:

People in one group will be asthmatics. People in the other group will be of non-asthmatics.

Usually subjects may exhale different levels of markers that we can measure in the breath.

We want to examine whether levels of the marker nerve growth factor in exhaled breath condensate and induced sputum is different between asthmatics and non-asthmatics.

Right now, we do not know for sure if the measurement of nerve growth factor in exhaled breath condensate and induced sputum will accurately measure allergic lung disease. We are doing this study to find out if the measurement of nerve growth factor in your breath is differs amongst asthmatics and non-asthmatics.

If the measurement is not accurate, you may still have airway or lung disease that is

undetected by the study. You should consult your own physician regardless of the study results if you have any concerns or experience any symptoms at any time.

During this study, here is what you will need to do:

Subjects will be instructed to refrain from eating, drinking, or strenuous exercise for at least one hour prior to the visit.

Will you be paid for taking part in this study?

We will pay you for the time you volunteer while being in this study. **You will receive a total of twenty dollars for completion of the study.**

What will it cost you to take part in this study?

The study will pay the costs of limited physical exam (primarily auscultation), limited spirometry, and the experimental nerve growth factor measurement.

You will not have to pay the fees for tests in this study that are not a part of regular medical care for your disease/condition.

Those subjects that do not have a parking permit for on-campus parking will have to pay for any parking costs incurred as required from the University of South Florida by taking part in this study.

You will have to pay for your regular care or any other costs. Your insurance plan should cover your regular costs. Your insurance plan will not have to pay for study costs of limited physical exam, limited spirometry, or nerve growth factor measurement.

What are the potential benefits if you take part in this study?

We don't know if you will get any health benefits by taking part in this study. We do not know if nerve growth factor measurement will help in diagnosing airway or lung diseases. That is why we are doing this study.

This research study should help us learn whether nerve growth factor measurement will help in diagnosing allergic airway or lung diseases.

Since a medical questionnaire, limited physical exam, and limited spirometry will be performed, overt abnormalities detected by your examiner will be relayed to you. You will be instructed to consult your regular doctor regarding these findings.

No matter what, we will learn more about exhaled breath nerve growth factor levels in asthmatics and non-asthmatics. We will learn more about whether differences do or do not exist. What we learn may help others who are actively studying nerve growth factor in exhaled breath for potential use in clinical settings.

What are the risks if you take part in this study?

The treatment might not help.

Right now we do not know for sure if the measurements are consistent. Because of this, you will have to consult your regular physician if you are experiencing any shortness of breath, other symptoms, or have any concerns about disease status. If you do, your condition/disease may get worse.

There may be adverse effects.

You may have problems because of the procedure used in this study. These problems are called adverse effects. Some adverse effects are just a bother. Others could harm you.

There may be some adverse effects that we don't know about yet.

Here are the known adverse effects that could happen with this study:

This study poses minimal risk to you. Spirometry is a standard, routine physical test commonly performed in hospitals and some clinics. It is a noninvasive and relatively safe procedure and involves the requirement for maximal expiratory effort. Thus, there is a minimal risk that you may breathe too hard during the forced expiratory maneuvers.

This could result in: [1]"hyperventilation", where too much carbon dioxide is blown off in a short period of time or [2]excessive physical exertion. Immediate effects you could experience from "hyperventilation" or excessive physical exertion may include lightheadedness, dizziness, chest pain or pressure, rapid heart beat, or shortness of breath.

These usually resolve upon discontinuing the procedure. You are instructed to immediately notify the examiner if any symptoms result. A determination by the examiner of whether or not to continue with the study will be made on an individual basis. At any time, both you and the examiner have the right to stop the study for any reason. If you were to continue despite these symptoms, loss of consciousness or cardiopulmonary arrest could result in rare cases, requiring emergency treatment and/or hospitalization. In such rare cases, permanent effects could result.

Patients with history of any type of hernia are excluded from the study since they could potentially aggravate the hernia using maximal expiratory effort.

The procedure for collecting exhaled breath nerve growth factor is very similar to the procedure for spirometry, except that the subject is asked to breathe out at a slow regular

rate instead of the forced maximal exhalation required in spirometry. Thus, it poses even less risk and discomfort to the subject than spirometry.

Private medical information will be collected during the study to find out your health status. The health information is being collected only as a guide for participation in the study. The questionnaires used in the study must remain confidential. To maintain your confidentiality, the questionnaires and recorded data from the study will be kept in a locked file cabinet. Access to the material will only be made to the investigators of the study. Also, as an additional measure to ensure confidentiality, each person will be given a subject number. This will be the only identifier listed on the questionnaires to link the confidential information to the subject. The master list linking subjects to their subject numbers will also be kept in the locked cabinet, but separately.

During the study, the safety of the normal subjects will be maintained by: [1] the presence of a physician at all times of testing; [2] careful direct physician observation and monitoring of subjects during testing, and [3] instruction of subjects to immediately discontinue the exercise and notify the physician should they experience any shortness of breath, chest pain or pressure, dizziness, lightheadedness, or other unusual symptoms. Emergency equipment will be present, and a physician will be in attendance for every study patient.

If you have any of these or any other problems, notify your study doctor immediately. If these side effects bother or worry you, or if you have other problems, call your study doctor at (813) 974-7545. If you have an emergency, go to the closest emergency room or clinic for treatment.

You may also have problems from the medical treatment you would usually get.

We may need to stop your treatment. If we find that the breathing tests are causing adverse events, we will stop the procedure. Early stopping criteria include:

1. For individual subjects:

The following criteria will be used to discontinue participation in the study:

[1] You wish to withdraw from the study.

[2] You do not meet the criteria for inclusion into the study.

[3] You demonstrate abnormalities on spirometry, physical examination, or monitoring through the study.

[4] You complain of chest pain or pressure, palpitations, or tachypnea.

[5] If you experience other symptoms, they will be assessed by a doctor and assisted in any way needed. A decision will be made whether you may continue to participate in the study provided that your safety has not been compromised. In this case, the decision to continue would require mutual willingness on your part to continue the study.

[6] The safety of the researcher is compromised (for example, a hostile subject).

2. For the study:

The study will be terminated if the health or safety of the volunteers or examiners becomes jeopardized by an unforeseen event.

Is there any risk to your unborn children if you take part in this study?

Pregnant women will not be allowed to participate in the study. There are no adverse effects for men with partners of childbearing age.

If you are a woman

You are excluded from the study if you are or may be pregnant due to the study protocol, since the study results may be affected. If you are pregnant and you inadvertently take part in this study, the risk of adverse effects is still minimal, especially if you are in the first trimester of pregnancy. Nonetheless, it could be that your unborn children may have problems now or in the future.

Tell one of the study doctors right away if:

- You are pregnant
- You are breastfeeding.

If you are a woman

If you take part in this study, you must use a good birth control method, like oral contraceptives or condoms.

What if you get sick or hurt while you are in the study?

If you need emergency care:

- Go to your nearest hospital or emergency room right away. Call 911 for help. It is important that you tell the doctors at the hospital or emergency room that you are participating in a research study. If possible, take a copy of this consent form with you when you go. You should know that the USF does not provide emergency care.
- Call the study doctors as soon as you can. They will need to know that you are hurt or ill. Call Dr. Victor M. Nwiloh, MD or Dr. Stuart Brooks, MD at (813) 974-7545.

If it is NOT an emergency, and you get hurt or sick while you are taking part in this study:

- Go to your regular doctor. It is important that you tell your regular doctor that you are participating in a research study. If possible, take a copy of this consent form with you when you go.
- The USF Medical Clinics may not be able to give the kind of help you need. You may need to get help somewhere else.

If you are harmed while taking part in the study:

The state of Florida enjoys what is called "sovereign immunity." This means that you usually cannot sue the state of Florida. However, the state has waived sovereign immunity (agreed to be sued) in certain situations. One of those situations is if a state employee, such as your study doctor or other USF employee, is negligent in doing his or her job in a way that harms you during the study.

The money that you might recover from the state of Florida is limited in amount.

You can also call the USF Self Insurance Programs (SIP) at 1-813-974-8008 if you think:

- You were harmed because you took part in this study.
- Someone from the study did something wrong that caused you harm, or didn't do something they should have done.
- Ask the SIP to look into what happened.

What will we do to keep your study records private?

Federal law says we must keep your study records private. Private medical information will be collected during the study to ascertain the health status of the subjects. The health information is being collected only as a parameter for participation in the study. It is obtained after discussion with the subjects that the study is only open to healthy subjects. Thus, the questionnaires in the study must remain confidential. We will keep the records of this study private by keeping them in a locked file cabinet. Access to the material will only be made to the investigators of the study. Also, as an additional measure to ensure confidentiality, each subject will be given a subject number. This will be the only identifier listed on the questionnaires to link the confidential information to the subject. The master list linking subjects to their subject numbers will also be kept in the locked cabinet, but separately.

However, certain people may need to see your study records. By law, anyone who looks at your records must keep them completely confidential.

The only people who will be allowed to see these records are:

The medical staff who are taking care of you.

Certain government and university people who need to know more about the study. For example, individuals who provide oversight on this study may need to look at your records. These include the University of South Florida Institutional Review Board (IRB) and the staff that work for the IRB. Other individuals who work for USF that provide other kinds of oversight may also need to look at your records. Other individuals who may look at your records include: the Florida Department of Health, people from the Food and Drug Administration (FDA), the United States Department of Health and Human Services, and the United States Department of Labor. This is done to make sure that we are doing the study in the right way. They also need to make sure that we are protecting your rights and your safety.)

People at the company who paid for this study at the Sunshine Education and Research Center (funded by the National Institute of Occupational Safety and Health, a governmental department under the United States Department of Labor may look at the study records and pertinent portions of your medical records to make sure the study is done in the right way.

We may publish what we find out from this study. If we do, we will not let anyone know your name. We will not publish anything else that would let people know who you are.

What happens if you decide not to take part in this study?

You should only take part in this study if you want to take part.

If you decide not to take part:

- You will not be in trouble or lose any rights you normally have.
- You will still have the same health care benefits.
- You can still get your regular treatments from your regular doctor.

What if you join the study and decide you want to stop later on?

You can decide after signing this informed consent document that you no longer want to take part in this study. If you decide you want to stop taking part in the study, tell the study staff as soon as you can. If you decide to stop, you can continue getting care from your regular doctor.

Are there reasons we might take you out of the study later on?

Even if you want to stay in the study, there may be reasons we will need to take you out of it. You may be taken out of this study if:

- We find out it is not safe for you to stay in the study. For example, your health may worsen. Or we may find out that the device used to measure spirometry or exhaled breath condensate might harm you. Then you may be taken out of the study.
- You are not coming for your study visits when scheduled.

You can get the answers to your questions.

If you have any questions about this study, call Dr. Victor Nwiloh at (813) 974-7545.

If you have questions about your rights as a person who is taking part in a study, call the

Division of Research Compliance of the University of South Florida at (813) 974-9343.

Signatures for Consent to Take Part in this Research Study

It is up to you to decide whether you want to take part in this study. If you want to take part, please read the statements below and sign the form if the statements are true.

I freely give my consent to take part in this study. I understand that this I am agreeing to take part in research. I have received a copy of this consent form to take with me.

Choose to participate in the study : a single visit which will last approximately ninety minutes.

_____ Date
Signature of Person Taking Part in Study

_____ Printed Name of Person Taking Part in Study

_____ Date
Signature of Witness

_____ **Printed Name of Witness**

Statement of Person Obtaining Informed Consent

I have carefully explained to the person taking part in the study what he or she can expect.

I hereby certify that when this person signs this form, to the best of my knowledge, he or she understands:

What the study is about.

What needs to be done.

What the potential benefits might be.

What the known risks might be.

I also certify that he or she does not have any problems that could make it hard to understand what it means to take part in this study. This person speaks the language that was used to explain this study.

This person reads well enough to understand this form or, if not, this person is able to hear and understand when the form is read to him or her.

This person does not have a medical problem that makes it hard to understand what is being explained and can, therefore, give informed consent.

This person is not taking drugs that make it hard to understand what is being explained and can, therefore, give informed consent.

Signature of Person Obtaining Informed Consent	Date
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Printed Name of Person Obtaining Informed Consent

Signature of Witness

Date

Printed Name of Witness]