Use of exhaled nitric oxide measurement to identify a reactive, at-risk phenotype among patients with asthma

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Materials and Methods

Subject enrollment and characterization

All subjects were recruited by centers participating in the Severe Asthma Research Program (SARP). All study participants gave written informed consent by signing a consent document approved by the Institutional Review Board at the enrolling center and the SARP Data Safety and Monitoring Board (DSMB). All subjects were screened by history, physical examination, spirometry (before and after 2 puffs of inhaled albuterol), methacholine provocation, and allergy prick skin testing to a standard panel of aeroallergens. All subjects were non-smokers, and classified as healthy controls if they were free of respiratory symptoms, had normal baseline spirometry, a negative methacholine challenge test, and nitric oxide level less than 50 ppb. Asthma was defined by the National Asthma Education and Prevention Program guidelines, which include episodic respiratory symptoms, reversible airflow obstruction (documentation of variability of FEV_1 and/or FVC by 12% and 200 cc either spontaneously or after 2 puffs of inhaled albuterol), and/or a positive methacholine challenge test (1). Up to 8 puffs of albuterol were used to obtain maximal bronchodilator response. Severe asthma was based on the definition used by the proceedings of the American Thoracic Society Workshop on Refractory Asthma (2) with major and minor characteristics. Defining major characteristics include (1) treatment with continuous or near continuous oral corticosteroids, and/or (2) high dose inhaled corticosteroids. The minor criteria are as follow: (1) Daily treatment with controller medication in additional to inhaled corticosteroids; (2) use of short-acting β-agonist on a daily or near daily basis; (3)

Persistent airway obstruction [FEV_1<80% predicted or diurnal peak expiratory flow (PEF) variability >20%]; (4) one or more urgent care visits for asthma per year; (5) Three or more oral corticosteroid bursts per year; (6) prompt deterioration with reduction in oral or inhaled corticosteroid dose; (7) Near-fatal asthma event in the past. Subjects enrolled in SARP were classified as healthy controls, non-severe or severe asthma. Subjects met criteria for severe asthma with at least 1 major and at least 2 minor criteria. Inclusion criteria for control subjects were (1) lack of cardiopulmonary symptoms, (2) normal baseline spirometry, and (3) a negative methacholine challenge test (defined as less than 20% decline in FEV_1 with the maximum dose of methacholine). Exclusion from SARP enrollment for asthmatic and control subjects included current smoking history or smoking history within one year, former smokers with greater then 5 pack-year total history, pregnancy and human immunodeficiency virus infection. Patients prospectively filled in a detailed questionnaire on use of medications including rescue inhalers, control of asthma, medical history and quality of life prior to measure of FE_{NO}. Data was submitted to a data-coordinating center where it was de-identified and entered into a database and later retrieved for statistical analysis.

Lung function

Spirometry was performed on an automated spirometer consistent with American Thoracic Society standards (3). Prior to measurement of baseline pulmonary function and methacholine bronchoprovocation, subjects were instructed to withhold bronchodilators if their asthma symptoms permitted (4 hours for short-acting beta-agonists, 12 hours for long-acting beta-agonists (LABA). The FVC, FEV_1, and FEV_1 to

FVC ratio were collected for each of 3 efforts before and after the administration of albuterol 2 puffs via Aerochamber. Reference equations for spirometry are those of National Health and Nutrition Examination Survey (NHANES III) (4).

Atopy

Allergy skin testing was done once on each subject during the study. Skin prick testing to fourteen common allergens was performed at all SARP sites with the Multi-Test II (Lincoln Diagnostics, Inc). Blood was collected for measurement of total serum IgE and a complete blood count.

Airway reactivity

Methacholine challenge testing was performed in all volunteers. Patients with a baseline %FEV_1 lower than 50% were excluded from methacholine testing because of safety concerns. The dosimeter method in which an electronically controlled valve allows a controlled amount of material from a nebulizer to be inhaled during the course of an inspiratory capacity breath was used in all SARP centers. Increasing concentrations of methacholine were delivered until FEV_1 fell at least 20% when compared to a control (postdiluent) level. The measure used to compare the responsiveness of one individual to another was PC_{20}, the interpolated concentration that was associated with a 20% fall in FEV_1.

Lung volumes

Plethysmographic lung volumes, including total lung capacity (TLC) and residual volume (RV) were measured in 62 Severe and 53 Non-severe Asthma subjects using methods conforming to ATS guidelines (5), and recorded as the percent of predicted values (%Prd) obtained with the equations of Stocks and Quanjer (6), with adjustments for African Americans per ATS recommendations (7).

Exhaled NO (FE_{NO})

All SARP centers performed on-line and/or off-line NO measurements according to the standards published by the American Thoracic Society (ATS)(8). Online FE_{NO} values were used in all data analyses in this report. NO levels were measured online by chemiluminescence at a constant expiratory flow (50ml/sec) in all participating centers. The analyzers were calibrated in accordance with the manufacturer's instructions. Because spirometry can affect the FE_{NO} levels, exhaled gases were collected prior to spirometry, if completed on the same day.

Total NO reaction products (NOx)

NO reaction products in serum samples were measured by an amperometric NO sensor in combination with acidified iodide for the detection of NO derived from total nitrite and nitrate after cadmium/copper-mediated reduction of nitrate to nitrite (ISO-NOP, Nitralyzer II; World Precision Instruments, Sarasota, FL) (9).

Statistical analyses

Categorical data were summarized by frequencies, and statistical comparisons for categorical variables performed using Fisher's Exact Test. Subgroup comparisons within NO level or asthma severity were performed using appropriate contrasts from a logistic regression model including NO level, asthma severity, and their interaction as independent variables. Continuous variables were summarized using the sample size, mean and standard deviation (SD), and alternatively using the median and interquartile range (IQR) for variables with skewed distributions. Group comparisons with respect to continuous variables with roughly symmetric distributions were performed using analysis of variance (ANOVA, i.e. T-tests in the case of two groups). Subgroup comparisons within NO level or asthma severity were performed using appropriate contrasts from an ANOVA model including NO level, asthma severity, and their interaction as independent variables. For continuous data with skewed distributions, comparisons of groups or subgroups of interest were performed using Kruskal-Wallis test (i.e., Wilcoxon rank sum tests in the case of two groups). T-tests were performed at individual significance levels of α=0.05 (i.e., $p<0.05$ was considered significant). Associations between NO levels and other variables were assessed using linear regression for FE_{NO} as a continuous variable and multiple logistic regression for FE_{NO} (high or low) as categorical variables. Multiple logistic regression modeling will be described in more detail in the Results section. All tests and model fitting were performed with the JMP statistical program Version 5.0 (SAS Institute Inc, Gary, NC, USA) and R version 2.4.1 (www.R-project.org) (10).

Multivariate analyses and modeling for determinants of FE_{NO}

Models for FE_{NO} as a continuous outcome in a linear regression model and as a dichotomous outcome classified as high or low in a logistic regression model were created. For multivariate analyses and modeling, parsimonious selection of independent variables was performed in order to avoid confounding that would render the estimated associations with the outcome as non-interpretable or misleading. Similarly, a logistic regression model for which the FE_{NO} outcome would be classified as high or low had to be parsimonious in order to be mathematically stable. Since study variables could be grouped into identifiable categories (domains), a variable selection method was used to identify variables that were most representative of each domain for inclusion into the final multivariate models. Separate multivariate linear regression models of the FE_{NO} measurements were subsequently constructed. Variables with the most significant associations with FE_{NO} in the individual domain models were selected to represent their domains in the final model. The accompanying table lists the domains (in bold) and the variables that were selected from each domain (highlighted) based on a linear regression model of all the variables within each domain. In addition to the selected variables for each domain, age, gender and BMI were chosen as demographic variables for the final models based on their common inclusion in such models. Certain variables that were included in the univariate analyses (like lung volumes and sputum and BAL findings) could not be included in the multivariate analyses due to the limited number of observations compared to other variables in the model.

Supplement references:

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Table: Variables that were selected for multivariate analyses.

General	**Markers of Atopy**
Gender	Serum IgE
Age	Presence of any allergic reaction
BMI	Number of positive skin tests
Asthma Quality of Life Questionnaire	**Inflammatory cells in blood**
Symptom Score	Total WBC
Activity score	Monocytes (%)
Emotional Score	Neutrophils (%)
Environmental score	Lymphocytes (%)
Total Score	Eosinophils (%)
General Respiratory Symptoms	Basophils (%)
Cough	**Medication Use**
Sputum	Inhaled corticosteroids
Chest tightness	Oral corticosteroids
Wheezing	Injectable corticosteroids
Shortness of breath	Inhaled corticosteroids and beta agonist
Nighttime symptoms	Total beta agonists
Pulmonary Function	Total long acting beta agonist
Baseline FVC % predicted	Total inhaled corticosteroids
Maximum FVC % of predicted	Total other corticosteroids
Baseline FEV1 % predicted	Theophylline
Maximum FEV1 % of predicted	**Asthma Medical History**
FEV1/FVC ratio	Age of asthma diagnosis
Maximum FEV1 reversal %	Saw a doctor in the last 12 months due to asthma
PC20	Ever visited ER due to asthma
TLC baseline % predicted	ER in past 12 months
TLC post BD % predicted	Ever Admitted to hospital due to asthma
TGV % baseline predicted	Ever had an ICU admission due to asthma
TGV post BD % predicted	Ever intubated
RV % baseline predicted	History of sinusitis
RV % post BD predicted	Sinus surgery