Elevated Exhaled Nitric Oxide (ENO) in Non-Asthmatic Atopic Adults

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science in Medical Sciences
Graduate Department: Institute of Medical Science
University of Toronto

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Nitric oxide in exhaled air has been proposed as a potential marker of inflammation in the lower airways. Several recent studies have demonstrated that the concentration of exhaled levels of lower airway nitric oxide (ENO) are elevated in asthma, a lung disorder characterized by chronic airway inflammation\textsuperscript{13,42}. There also appears to be a weak relationship between atopy or the predisposition to develop IgE antibodies when exposed to foreign substances, and elevated lower airway ENO.

We measured ENO levels in asymptomatic non-asthmatic atopic individuals and found that levels were significantly elevated when compared to non-atopic healthy individuals. For those atopic individuals sensitized to seasonally variable pollens, we measured ENO levels in and out of the respective pollen seasons. We found there was no significant difference in the ENO levels between out-of-season and during season in the absence of allergic exacerbation and symptoms. Lower airway inflammation may be present in atopic individuals without clinical symptoms of asthma. Therefore, ENO measurement may be able to identify sub-clinical inflammation and predict the risk for developing asthma in the future.
I would like to thank:
First and foremost, Dr. Ivone M. Ferreira for her unending support and guidance. Mrs. K. Khan and Mrs. L. Cleland for their instruction throughout this project. Mr. H. Furlot and the members of our pulmonary function laboratory for their technical support. Dr. Noe Zamel and Dr. Susan M. Tarlo for their clear directions and explanations of the concepts of Respiratory Medicine. My supervisor Dr. Kenneth R. Chapman for his optimism, positivity and guidance: but most of all for this valuable learning experience.
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List of Abbreviations

- BALF  _ Broncho-Alveolar Lavage Fluid
- BHR   _ Bronchial Hyperresponsiveness
- cNOS  _ Constitutive Nitric Oxide Synthase
- ECP   _ Eosinophil Cationic Protein
- EC-SOD _ Extra-Cellular Super Oxide Dismutase
- ENO   _ Exhaled Nitric Oxide
- eNOS  _ Endothelial Nitric Oxide Synthase
- FENO  _ Fractional concentration of exhaled nitric oxide
- FEV1  _ Forced Exhaled Volume in one second
- FRC   _ Functional Reserve Capacity
- FVC   _ Forced Vital Capacity
- GSH   _ Glutathione
- GSNO  _ Nitrosoglutathione
- IFN-γ _ Interferon-γ
- IL-3, IL-5 _ Interleukin-3, Interleukin-5, Interleukin-17
- iNOS _ inducible Nitric Oxide Synthase
- LT    _ Leukotriene
- LTRA  _ Leukotriene Receptor Antagonist
- MDI   _ Metered Dose Inhaler
- NANC  _ Nonadrenergic Noncholinergic
- nNOS  _ neuronal Nitric Oxide Synthase
- NOS   _ Nitric Oxide Synthase
- PC20  _ Provocative Concentration causing a 20% decrease in FEV1
- TLC   _ Total Lung Capacity
- TNF-α _ Tumor Necrosis Factor-α
Chapter 1 - INTRODUCTION

Atopy. is the predisposition to develop IgE antibodies when exposed to foreign substances, that appears clinically most commonly as immediate hypersensitivity responses or "allergies". Asthma may be considered as a specific hypersensitivity reaction localized in the lungs. The American Thoracic Society/American College of Chest Physicians defines asthma as: "A disease characterized by an increased responsiveness of the airways to various stimuli and manifested by slowing of forced expiration which changes in severity either spontaneously or as a result of therapy"\(^1\). Asthma remains difficult to define and in more precise terms may be thought of as a syndrome resulting from multiple patho-physiological processes, which include airway inflammation. It is characterized by intermittent wheezing due to obstruction of the airways that is reversible by pharmacological treatment with beta-agonists or at least partially preventable by anti-inflammatory therapy.

The hypotheses presented in this thesis, to be discussed later, entail analysis of exhaled nitric oxide (ENO) for use in monitoring airway inflammation. This study targets non-asthmatic atopic individuals in an effort to verify the presence of lower airway inflammation as indicated by ENO levels when compared to non-atopic individuals.

1.1. The Human Body's Response to Injury-General Inflammation

Inflammation is the response of tissues of the body to injury or to the presence of otherwise innocuous foreign substances. As a primary line of defense inflammation attracts mediators and cells to the site of damage or intrusion. The inflammatory reaction increases blood supply to the region, increases capillary permeability and encourages the migration of cells from the vasculature into the tissues\(^2\). Mast cells, basophils, eosinophils
and platelets release most active mediators. Vasodilatation, or the dilation of blood vessels due to mediator activity on smooth muscle vessel walls, is the first consequential physiologic response to inflammation. With increased blood flow leukocytes are transported via post-capillary venules into the injured tissue after adhering to endothelial cells.

1.1.1. Chemical Mediators of Inflammation

The backbone of any inflammatory reaction is the release of various mediators that trigger, sustain and regulate the intensity of response. In IgE-mediated allergic responses histamine is one of the primary mediators of inflammation produced and released by mast cells and basophils. It induces increased vascular permeability, increased mucus production, contraction of smooth muscle and alteration of cell migration. Chemotaxis, a directional movement of cells in response to inflammatory mediators, is also initiated. Leukocytes are highly sensitive to and migrate up concentration gradients of chemotactic molecules towards the site of injury. Several mediators produce similar effects and are crucial in establishing the inflammatory cascade.

Arachidonic acid is a fatty-acid released from the membrane of injured cells that acts as a substrate for the production of molecules that further enhance the inflammatory process. Leukotrienes (LT) are produced from the action of lipooxygenase on arachidonic acid. Leukotriene B4 (LTB4) and leukotriene D4 (LTD4) act as neutrophil attractant. Increase mucus production and can induce contraction of smooth muscle. Prostaglandins (PG) are generated by the action of cyclooxygenase on arachidonic acid. They often have a synergistic effect with other circulating mediators producing vasodilation and increased vascular permeability. The action of these molecules reveals the positive and negative
feedback response of enzyme activity. Several other cytokine molecules serve as chemotactic factors that are important in IgE-mediated inflammation\(^6\)\(^-\)\(^7\).

1.1.2. Allergic Responses and Hypersensitivity

Hypersensitivity is the term used to describe an exaggerated or inappropriate immune reaction, in which increased quantities of biochemical mediators and defensive cells produce an injurious response to an otherwise innocuous foreign substance. The response is a complex cascade of antigen recognition, cellular activation and interaction, enzyme catalysis, and negative self-regulating feedback.

Type I, or immediate hypersensitivity is seen in hayfever, some cases of urticaria, anaphylaxis and allergic asthma. Allergy is just one of the many possible explanations for the disease process in asthma. Immediate hypersensitivity (fig. 1) develops within minutes of exposure to foreign antigen and in some cases is followed by a late inflammatory response.

Figure 1.1 Sensitization and triggering of mast cells followed by degranulation and release of chemical mediators.
Mast cells and related basophils bind IgE antibodies to Fce receptors on their surfaces⁸.⁹. Fce receptors are high-affinity sites that stimulate degranulation and release of chemical mediators upon contact with a cross-linked IgE antibody-antigen complex. Concurrently, recognition of antigen by IgE activates phospholipase A₂, a membrane-associated enzyme.¹⁰ Phospholipase A₂ releases arachidonic acid, the initial substrate of the lipo-oxygenase and cyclooxygenase pathways.²

Immunologic mechanisms such as immediate hypersensitivity may lead to development of late-phase allergic reaction (LPRs) and tissue inflammation. Following antigen interaction with IgE antibody, mast cell activation and mediator release, recruitment and activation of other leukocytes and elaboration of cytokines evolves resulting in a reaction several hours later. Late-phase reactions can occur in several organs including the skin, lung and nose, and they are linked to the clinical symptomology of allergic diseases like asthma and allergic rhinitis. Human cutaneous LPRs characterized by burning, pruritis, erythema and induration, may ensue following allergen challenge and development of an immediate wheal and flare reaction. These reactions generally peak at 6-8 hours and may resolve by 24 hours. Similarly, pulmonary LPRs may develop 3 to 4 hours following allergen challenge. Inflammatory cells such as eosinophils and neutrophils are attracted to airway tissue by chemotactic molecules such as eosinophil chemotactic factor (ECF) and neutrophil chemotactic factor (NCF) several hours after allergen challenge. Eosinophils release eosinophil-derived neurotoxin (EDN), eosinophil peroxidase and eosinophil cationic protein (ECP) that contribute to the pathologic changes characteristic of late-phase asthmatic reaction.² It is the presence of these cells and
mediators that induce late obstructive reactions and subsequent airway hyper-responsiveness.

1.2. Allergens

The analysis of foreign triggers and a complete understanding of their chemical and environmental characteristics are important in the assessment and diagnosis of allergic disease. An allergen is any foreign antigen that produces a clinical allergic reaction. Inhalants and foods are most commonly associated with atopic disorders. Common and clinically relevant reactions involve fungal spores, pollens, house dust, dust mites, animal epithelial materials and other substances that reach the respiratory mucosa directly. Drugs, insect venoms, and certain chemicals may also induce similar immediate-type reactions. Most allergens that produce immediate-type reactions are water-soluble. They include proteins, polypeptides and less commonly lipids, and carbohydrates. Protein-rich allergens are usually strongly reactive.

Aeroallergens represent airborne particles that enter the body via inhalation or surface deposition causing respiratory, cutaneous, or conjunctival allergies. Clinically significant aeroallergens must demonstrate buoyancy and allergenicity. Ragweed pollen and fungal spores are typical examples. They are ubiquitous, highly allergenic, and are present in high numbers during the peak of pollen season. Tree pollen, grass pollen, ragweed pollen and fungal particles are seasonally variable and are found in significant numbers in the Toronto area. House dust allergens are omnipresent with exposure that is less seasonally variable. Similarly, animal dander present in some houses can trigger responses in all seasons. Aeroallergen potency is also dependent upon particle size, which ranges from 1 to 60 μm in diameter. Of the common airborne allergens, dust mite particles
range in size from 1 to 10 μm (usually nearer to 10μm), mold spores from 3 to 30 μm, and pollen particles from 20 to 60 μm\(^2\). Penetration of these particles into the various levels of the respiratory tract is dependent on their respective sizes. The nasal mucosa and upper bronchial passages possess protective mechanism that prevent large particles from penetrating any deeper: only those particles 3 μm or smaller reach the alveoli of the lungs\(^3\).

The general mechanism of an inflammatory response in the human body has just been detailed. The presence of specialized cells and biochemical mediators permits a reaction adjusted to each system and its specific requirements. Since the body of this work centers on the respiratory system and disease processes of the lungs, mechanisms of lung injury and pulmonary inflammation are examined next. These topics include and focus on the role of nitric oxide (NO) as a chemical mediator and exhaled nitric oxide (ENO), which is now being researched as a biochemical marker of inflammation in respiratory illnesses.

1.3 Lung Injury and Inflammation

Lung injury leading to inflammation is associated with a complex set of cellular and humoral interactions that may cause tissue destruction. Delayed clearing of foreign substances from the alveolar surface can lead to epithelial cell death and accumulation of lung macrophages and debris. The presence of an inflammatory exudate and the prevention of its removal further precipitate epithelial death. Exposure of the alveolar wall, destruction of the basement membrane, and an amplification of host immune responses\(^2\).

1.3.1 Inflammatory Cells and Biochemical Mediators

Specialized macrophages are known to play a major role in the pathophysiology of lung inflammation\(^1\). A large number of macrophages are present in the alveolar space and
interstitium of individuals with acute lung injury. The presence of activated macrophages initiates a cascade of reactions beginning with the release of cytokines. Systemically, these compounds cause fever and leukocytosis. Locally, they stimulate tissue regeneration, repair and alter the adhesion functions of the endothelium and surrounding fibroblasts. The endothelium releases adhesion molecules that increase the adhesion of neutrophils and lymphocytes to the injured tissue. The reaction begins with binding of mediators to specific cell receptors, signal activation of genes stimulating cytokine-production and up-regulation, and finally protein (enzyme) synthesis. Once activated by antigen-presentation to a specific set of lymphocytes, cytokines like interleukins serve as chemical attractant for neutrophils, eosinophils, basophils, and monocytes to the site of lung injury.

Histamine is a preformed molecule in mast cells, basophils and platelets originating from the action of histidine decarboxylase on histidine. Once released, it binds to H1-receptors on lung endothelia stimulating increased vascular permeability and edema at the site of the injury as well as bronchial constriction. Increased amounts of histamine in lung tissue indicate IgE-mediated antigen-induced injury, as seen in antigen-induced asthma.

Biochemical mediators collaborate with cellular components during an inflammatory reaction. Neutrophils are the first leukocytes to appear at the site of injury and are believed to cause significant tissue damage once sufficiently activated and primed. Injury to the pulmonary vasculature and sequestration of specific cytokines induce alterations in the retentive properties of neutrophils. This process promotes neutrophil adhesion and transmigration across the alveolar/capillary wall into the alveolar
lumen. Primed neutrophils respond several times quicker than unprimed cells, speeding up the inflammatory reaction. Neutrophil-mediated lung injury occurs through the release of oxygen metabolites and proteases. Enzymes produced by the neutrophils reduce molecular oxygen into reactive free radicals that cause injury to lung tissue through peroxidation of lipid membranes.

Eosinophil numbers have been documented as being increased in IgE-mediated allergen-induced injury. These cells are found in the blood, sputum, and airway mucosa of asthmatic individuals. The number of eosinophils and the level of eosinophil cationic protein correlate with the intensity of allergic reactions, the degree of bronchial reactivity, and severity of asthma as indicated by blood analysis and lung lavage. Cells in the lungs release interleukin-3 (IL-3), interleukin-5 (IL-5), and RANTES to stimulate eosinophil migration, differentiation and activation in response to antigen exposure. Similarly, eosinophils release granular compounds such as eosinophil cationic protein, eosinophil-derived neurotoxin, and peroxidase that may cause lung tissue damage. The eosinophil granular proteins cause increased airway contraction and damage respiratory epithelial cells. The overall effect of these reactions is an increase in airway responsiveness.

1.3.2 Inflammation and Persistent Lung Damage

Resolution of inflammation allows the injured tissue to regenerate and returns lung function to normal. An uncontrolled, indefinite inflammatory process leads to scarring, and in severe instances, fibrosis within the lung parenchyma. During the IgE-mediated late asthmatic response, the inflammatory process has been linked to the development of airways hyperresponsiveness, a hallmark of asthma (although present in
several other respiratory diseases). Therefore, if measures of airways inflammation could be accurately defined, assessment of airways hyperresponsiveness and perhaps further damage to the lungs could be analyzed, treated, and possibly prevented.

1.3.3 Established Measures of Airways Inflammation

Current measures of inflammation in the lungs include methods that are invasive and impractical for routine clinical care. The two most commonly accepted methods employ fiberoptic bronchoscopy. Broncho-alveolar lavage requires that patients be placed under intravenous sedation. The flexible bronchoscope is inserted through either the nose or the mouth, and passed into the lower airways\textsuperscript{14}. These airways are inspected and a segment of the lung, usually in the region of the right middle lobe, is selected as the site for sampling. Saline is infused into the sampling region and then removed via suction. This procedure provides analysis of cells and extracellular protein from the airways as well as on the epithelial surface of the alveoli. Similarly, bronchial biopsies sample sections of airway tissue with forceps for analysis of cell content\textsuperscript{15}. Both procedures require laboratory preparation of samples before any analysis can be undertaken. These procedures have revealed that asthma is associated with increased numbers of eosinophils, neutrophils, and lung macrophages\textsuperscript{13}. Biochemical mediators such as histamine have also found to be increased. Although these trends are evident, normal ranges are not well defined and ATS testing criteria for these two methods cautions that fiberoptic bronchoscopy may present some risk to individuals with unstable asthma.

A third method of assessing airway inflammation is the collection of sputum expectorated following the inhalation of hypertonic saline. Careful analysis of sputum reveals increased numbers of inflammatory cells in conditions of atopy\textsuperscript{16}, but the
technique is laboratory intensive and normal standards remain to be defined. Although less invasive than bronchoscopy, the inhalation of hypertonic saline carries with it the risk of triggering bronchospasm. New, simple, and less invasive methods of quantification of airway inflammation could simplify diagnostic and monitoring processes and serve as feasible clinical tools.

1.4 Nitric Oxide and the Human Body

Nitrogen oxides are ubiquitous in the human body. Evidence suggests that there are several biologically relevant forms in human airways. Recent studies demonstrate that human BALF and condensed exhalate contain S-nitrosothiols, nitrate (NO$_3^-$), nitrite (NO$_2^-$), and peroxynitrite (ONOO$^-^-$). Similarly, a study by Gustafsson et al demonstrated the presence of volatile NO gas in the exhaled air of human beings$^{10}$, leading to greater focus on physiological mechanisms that lead to NO production in the lungs. Such analyses are being performed to better understand how NO could be used in disease monitoring and management.

1.4.1 Pathways of Production

Nitric oxide (NO) is one of several chemical mediators involved in the inflammatory process, and has been identified with increased levels in asthmatic airways inflammation. NO and its metabolite, nitrosoglutathione (GSNO) are produced endogenously from the conversion of L-arginine to L-citrulline in the airways by a group of enzymes called nitric oxide synthases (NOS). NOS activity requires the presence of other enzyme substrates such as oxygen and reduced nicotinamide adenine dinucleotide phosphate, and cofactors like tetrahydrobiopterin and flavoproteins$^{31}$. There are two basic classes of NOS, both of which are active in the lungs. The human body produces a
constitutive form (cNOS) that modulates normal physiologic function, and an inducible form (iNOS or type II NOS) that becomes activated during pathological conditions such as inflammation. Physiologically, cNOS has two forms, a membrane-bound endothelial (type III NOS) form, and a neural (type I NOS) form that is involved in the nervous system. Type III NOS produces NO in response to pressure changes in pulmonary and systemic circulation, and type I NOS is linked to central and peripheral neurotransmission. Types I and III NOS are activated by mediator-signaled calcium influxes which lead to calmodulin binding and are present primarily in subepithelial neurons (type I) and vascular endothelium (type III). Type I NOS produces low levels of NO in nonadrenergic, noncholinergic (NANC) neural cells, specifically the iNANC system in human beings. NO is principally involved in iNANC neuro-transmission in human airways.

In comparison, type II NOS is firmly bound to calmodulin following translation and its activity is regulated at the transcription level. It is found primarily in respiratory macrophages, airway epithelial cells, endothelial cells, and fibroblasts. Production of NO by type II NOS activity is dependent upon the rate of gene transcription following tissue injury. The release of cytokines and the presence of endotoxins regulate expression of type II NOS in the respiratory tract. Studies analyzing bronchoalveolar lavage fluid (BALF) indicate that tumor necrosis factor-α (TNF-α) and IL-1β released by alveolar macrophages in the presence of interferon-γ (IFN-γ) may influence iNOS expression. Other studies have shown that the release of IL-4 by macrophages in the presence of IFN-γ may have some role in the regulation of iNOS expression. TNF-α and other cytokines are all found in high concentrations in the BALF of individuals with chronic lung
inflammatory processes like asthma\textsuperscript{25,26}. Inhalation of high concentrations of antigen that often induce asthmatic exacerbations have been linked to increased production and exhalation of NO\textsuperscript{27}. Endobronchial challenge in asthmatic individuals has recently localized this increased source of ENO\textsuperscript{28}.

1.4.2 Physiologic Roles of NO Production

Since the two forms of NOS are produced under varied physiologic conditions they stimulate distinct responses within the body. NO produced from cNOS sources induce neural signal transduction stimulating several respiratory and vascular functions. These changes induce bronchial smooth muscle relaxation resulting in bronchodilatation. Neural control of airway caliber involves cholinergic stimulation, which produces bronchoconstriction by the release of acetylcholine, and NANC stimulation, which stimulating both excitatory (e-NANC) and inhibitory (i-NANC) pathways. NO is one of the primary mediators of the i-NANC pathway leading to smooth muscle relaxation\textsuperscript{23}.

altered pulmonary blood flow, and inhibition of certain components of the inflammatory cascade including leukocyte mobility and adhesion\textsuperscript{29}. Recent studies have demonstrated that NO released from epithelial cells may protect against polymorphonuclear leukocyte-mediated cell injury\textsuperscript{30}.

1.4.3 Adverse Pathological Effects of NO

The pathological changes induced by type II NOS are associated with the ensuing inflammatory response and the increased presence of cytokines in the airways. For instance, during conditions of asthma exacerbation it signifies a large burst in NO and is an important component of the host immune response\textsuperscript{31}. NO has potentially harmful effects on the lungs in such high concentrations. Some deleterious effects may be due to
increased pulmonary blood flow and the exudation of plasma into the airways\textsuperscript{32}. Similarly, the presence of increased levels of NO in the airways has been implicated to have direct cytotoxic effects on epithelial cells\textsuperscript{33}.

As a pro-inflammatory mediator, NO enhances the chemotaxis of neutrophils, monocytes and eosinophils by diffusing to smooth muscle\textsuperscript{34,35}. Direct assays of chemotaxis showed a significant decrease in eosinophil chemotaxis after administration of NOS inhibitors\textsuperscript{36}. Similarly, inhibition of NOS hinders the late cutaneous reaction, which is mediated by the influx of inflammatory cells\textsuperscript{37}. The role of NO in the inflammatory process also includes manipulation of T\textsubscript{H1} and T\textsubscript{H2} lymphocyte balance as well\textsuperscript{38,39}. Subtypes of T-helper cells are characterized on the basis of the cytokines they produce. T\textsubscript{H1} lymphocytes produce cytokines that mediate the classic delayed-type hypersensitivity reaction, whereas T\textsubscript{H2} lymphocytes are thought to contribute to the inflammation in asthma through the release of IL-4 and IL-5. T\textsubscript{H2} lymphocytes mediate immediate-type hypersensitivity reactions, which are associated with allergic diseases. Studies indicate that increased NO suppresses the T\textsubscript{H1} subset of CD4\textsuperscript{+} T cells and conversely favors the T\textsubscript{H2} profile, which is important in prolonging the asthmatic disease process\textsuperscript{38}.

1.5 Mechanisms of Increased ENO: Asthma

It is now established that ENO is elevated in individuals with asthma\textsuperscript{31,39,40}. Since the airways of patients with asthma are chronically inflamed they present a useful model for the analysis of ENO, pathologic reasons for its elevation, and airway responses to treatment. In the last ten years, numerous studies have been conducted to determine the efficacy of measuring ENO as a noninvasive method to assess airways inflammation. If
measurement of ENO is to play a role in the monitoring of asthma and its treatment. its presence and quantity should be related predictably to the degree of airways inflammation. The primary source of the elevated ENO would be the inflamed airway epithelium and/or the pro-inflammatory cells infiltrating the alveoli.

Evidence for the role of NOS, specifically type II NOS, in the elevation of ENO in asthma is numerous. The difference in levels of ENO between healthy individuals and individuals with asthma is due primarily to variations in type II NOS activity. Increased expression of type II NOS has been shown by immunohistochemical staining in the bronchial epithelium of asthmatic individuals. These findings corroborate an in-vitro study indicating increased expression of type II NOS in human epithelial cells in the presence of pro-inflammatory cytokines. A recent study by Yates and colleagues showed that administration of a nonselective NOS inhibitor (aminoguanidine) caused a decrease in ENO in both asthmatic and non-asthmatic individuals. But, when a selective inhibitor of type II NOS like aerosolized aminoguanidine was administered, a decrease in ENO was only observed in asthmatic individuals and not in non-asthmatic individuals. Therefore, NOS plays an important role in the production of NO and each isoform of the enzyme has a different contribution to ENO in healthy individuals and individuals with inflammatory processes in their lungs.

1.5.1 ENO: The Effect of Medication

Multiple studies concur that both inhaled and oral corticosteroids induce reductions in ENO to levels that are very similar to healthy, non-asthmatic individuals. Conversely, reduction of the dose of corticosteroids allows levels of ENO to increase. Specifically, ENO levels return to pathologic levels within two weeks
of discontinuing corticosteroid administration\textsuperscript{46}. Serial measurements of ENO by Massaro et al supported this observation by demonstrating that levels fell when the dose of steroids was increased and rose when the dose was reduced\textsuperscript{47}. Reduction of ENO levels by corticosteroid action is due to inhibition of transcription of type II NOS through blockade of nuclear factor-κB and a subsequent decrease in the synthesis of pro-inflammatory cytokines. Consequently, reduced immunostaining of NO and related nitrogen products has been shown after administration of corticosteroid\textsuperscript{42}. This observation is reasonable because increased levels of ENO in allergic reactions are consistent with increased cytokine production and ensuing expression of type II NOS\textsuperscript{37}. The proposed dual role of NO in the airways as both homeostatic and pro-inflammatory may indicate why the action of corticosteroids is effective in reducing ENO levels in patients with asthma and not in healthy individuals.

The effects of β\textsubscript{2}-adrenergic agonist bronchodilators on ENO levels are variable. Silkoff et al took serial measurements of ENO levels after administration of two puffs of salbutamol (100 µg/puff) by metered-dose inhaler or two puffs of a placebo. The effect of treatment with salbutamol was a significant increase in ENO levels from placebo treatment. The mean increase of ENO levels from baseline was 10%, an effect that persisted for one hour\textsuperscript{48}. Results obtained by Yates and colleagues showed a significant increase in ENO levels in asthmatic individuals on inhaled corticosteroids after treatment with salbutamol. However, the same study found that salmeterol (a long-acting β\textsubscript{2}-agonist) had no effect on ENO levels in asthmatics with or without corticosteroid use\textsuperscript{49}. The findings associated with β\textsubscript{2}-agonist activity may be explained by a mechanical effect on NO exhalation. Changes in airway caliber, specifically the recruitment of airways
following administration of bronchodilators could improve transfer of NO and increase levels during exhalation\textsuperscript{48}.

Similarly, studies have analyzed the effects of leukotriene receptor antagonists (LTRA) on ENO. Bisgaard et al demonstrated that montelukast caused a significant decrease in ENO as compared to a placebo in asthmatic children. The effects of montelukast were rapid in comparison to corticosteroids demonstrating a decrease in ENO within two days rather than within one to two weeks. As indicated by these studies, corticosteroids appear to exert a direct effect on type II NOS as opposed to an indirect effect by LTRA\textsuperscript{50}.

1.5.2 ENO, Lung Function and Markers of Inflammation

Standards for the measurement of airway inflammation and indices of asthma are established. ENO measurements are more useful when used in conjunction with other lung function standards and biochemical markers. As in measures of whole blood or serum, the presence of inflammation can be assessed with testing parameters that may include ENO measurements. The ability to rely on these measures collectively as indicators of general lung health and inflammation requires analysis of underlying relationships. It must be ascertained whether these methods are revealing the same conditions, and whether changes in the results of one testing method can be paralleled by changes in another. Similarly, mechanical maneuvers, such as spirometric procedures may alter ENO levels, and must be fully understood before inclusion of ENO measurements into the current battery of tests.

1. ENO and measures of lung function Recent studies illustrate that there are contrasting results defining the relationship between ENO and measures of lung function.
Lim et al demonstrated that in individuals with mild to moderate asthma there was no significant correlation between ENO levels and conventional indices of asthma control (lung function, symptom scores, medication use, and variability of peak expiratory flow in one month). This study did show that between indices of asthma control there was significant correlation between symptoms and rescue β₂-agonist usage, lung function and β₂-agonist usage, and peak flow variability and β₂-agonist usage. Similar findings by Baraldi et al demonstrate that in grass-pollen allergic asthmatic children there was no discernible relationship between ENO and FEV₁ in each of three seasonal measurements. Though the levels of ENO changed significantly between pre-pollen season and in-season, there was no significant change in FEV₁ or FEF₂₅₋₇₅. Ling-Pei showed similar results in adult asthmatics finding that there was no significant correlation between ENO levels and FEV₁ or the provocative dose causing a 20% decline in FEV₁ for both steroid-treated and steroid-naïve patients. These results seem to indicate that ENO levels are not an indication of asthma control in currently used conventional terms. The internal correlation between the conventional indices of asthma control demonstrated by Lim et al reflect the reliability of these parameters to assess asthma severity. The failure in developing a relationship between trends in lung function and ENO levels may be because they represent different conditions of the pathophysiological processes seen in asthma.

Dupont et al reported that ENO levels reflected bronchial hyperresponsiveness in patients with mild asthma who had not already been treated with inhaled corticosteroids. The study showed a significant correlation between ENO levels and PC₂₀ histamine. The levels of ENO in asthmatic patients on inhaled corticosteroids were found to be
comparable to control subjects. although bronchial hyper-responsiveness, a parameter indirectly linked to inflammation\textsuperscript{13}, could still be demonstrated\textsuperscript{54}. This suggests that ENO reflects the activity of steroids more directly than does the measurement of airway responsiveness or \( \text{FEV}_1 \textsuperscript{51} \) in mild asthmatics, and that objective measures of lung function need to be monitored carefully. These studies also indicate that a concrete and easily definable relationship may not exist between ENO levels and conventional measures of asthma control.

Conventional measures of lung function such as spirometry before and after bronchodilator administration have been found to influence ENO levels. A recent study by Silkoff et al measured ENO levels at baseline and at 1, 5, 15, 30, 45, and 60 minutes following three maximal forced expiratory maneuvers, for both healthy and asthmatic subjects. The results for healthy subjects indicated that ENO decreases to maximal level one minute following spirometry and returns gradually to baseline values by 60 min with a mean fall of 13.0 +/- 9.6%. All timed measurements except 60 min were significantly different from baseline. A similar decline in ENO levels was noticed in asthmatic subjects after spirometry. A maximal 10% decrease was observed at one min and remained significantly lower than baseline until five min at which point it began to rise steadily. One hour post-spirometry, levels were actually found to be significantly higher than baseline in the asthmatic subject group\textsuperscript{48}. A similar study by Deykin et al examined the effects of spirometry on ENO levels in healthy individuals at baseline and every 15 min for one hour. ENO levels were found to decrease significantly from baseline within the first 15 min following forced vital capacity (FVC) maneuvers. In contrast to the previously mentioned study, maximal decrease of 23.4 +/- 5.0% were seen at 60 min. In
addition to deep breath maneuvers, the Deykin group analyzed the effect of plethysmography for the measurement of specific airway conductance or panting on ENO levels. Subjects in a body plethysmograph were asked to pant at a frequency of once per sec and at functional reserve capacity (FRC) against an open and subsequently closed shutter. Collection of ENO was made every 15 min for one hr. In contrast to spirometry maneuvers, serial measurements of ENO levels did not change significantly following the panting procedure. Levels of ENO were significantly lower following spirometry when compared to plethysmography. The findings of these two studies indicate that deep breath procedures like the FVC manoeuver cause a decrease in ENO levels. The continued decrease in ENO level up to 60 min observed by Deykin et al in comparison to the fluctuation seen in the Silkoff study may be explained by differences in the method used to measure ENO. It is important that factors such as expiratory flow rate, lung volume, expiratory pressure, and time of day be controlled when assessing the impact of spirometric procedures on ENO levels. Stromberg et al demonstrated a reduction in ENO following application of positive extrathoracic pressure in a rabbit model. It is interesting to note that positive extrathoracic pressure applied to the lungs would induce volume and pressure changes similar to FVC maneuvers. Similar reduction in ENO levels in both normal and asthmatic individuals indicates that the reduction is not likely due to the pathobiology of asthma, but rather due to a general change in lung pressure and volume. This might implicate a neural pathway in the modulation of such ENO changes.

As an aside, Silkoff et al considered the effect of ventilatory maneuvers used in the measurement of ENO on subsequently measured ENO levels. Measurements were taken five times during one hr in subjects with asthma. The variations in ENO levels were
found to be less than 5% in all subjects and showed no tendency to increase or decrease. Thus, the ventilatory maneuvers used in ENO measurements have no significant effect on ENO measurements made frequently over time\textsuperscript{18}.

\textit{ii. ENO and cellular markers of inflammation} _ Cellular components permeating the lung during an inflammatory process often indicate the degree of injury to the tissue. Among the important cells migrating to the lungs during allergic/asthmatic inflammation are the eosinophils. Eosinophils are found in the blood, sputum, and the airway submucosa of individuals with asthma. They play a predominant role in the inflammation of asthma. The correlation between the number of eosinophils in the blood, sputum, or lung lavage and disease severity indicate the importance of eosinophils during inflammatory processes. Eosinophils also produce granular protein products (ECP) that appear to be abundant in airway-related allergic responses and coincide with eosinophil activity during inflammation\textsuperscript{13}.

Studies are focusing on the relation between the number of eosinophils and levels of ENO as combined evidence of cellular activity during production of elevated exhaled gaseous markers. Eosinophils are primarily counted in the blood and airway biopsy specimens following specific staining, and eosinophil granular proteins by enzyme immunoassay from serum samples. Studies performed by Lim et al found that there is no significant correlation between mucosal eosinophil numbers and ENO levels in asthmatic individuals. Remarkably in the same study, mucosal eosinophils correlated weakly with lung function\textsuperscript{51}. Similar analyses were performed on sputum, bronchoalveolar lavage (BAL), and bronchial biopsy in an effort to determine whether eosinophil counts correlated with ENO. The results of this study showed that both ENO and sputum
eosinophils decreased following administration of corticosteroids, but no significant statistical correlation was found. A significant correlation was found between ENO and BAL eosinophils following administration of corticosteroids. Silvestri et al. reported that blood eosinophils and ENO were both increased in asthmatic subjects, and that there was a significant correlation between the two testing parameters. No such correlation was seen in normal subjects. Lanz et al. also showed that post-corticosteroid treated asthmatics demonstrate a significantly lower amount of ECP. No correlation analysis was performed between ENO and ECP, but both decreased rapidly following administration of corticosteroids.

The variable correlations and weak associations with ENO suggest that changes in the number of eosinophils represent diverse mechanisms of the same inflammatory process. The number of eosinophils in the BAL and sputum may indicate different stages of inflammatory infiltration. The asthmatic patients in the study by Lim et al showed the possibility of such conditions because they revealed mucosal inflammation despite low levels of ENO. An increase in blood eosinophils and the correlation with ENO illustrates a systemic increased proliferation of these inflammatory cells following allergen challenge. Localized tissue permeation and activity vary, perhaps providing a basis for the differences in numbers with each method of collection. Despite the utility of bronchial histology to the analysis of asthma and airway diseases, less invasive methods are needed.

1.6 NO Production and Diffusion: A Model for $F_{ENO}$

Recent studies have explored the mechanics of NO diffusion into the airways and the impact of multiple flow rates on the concentration of ENO. Silkoff et al determined
that there is a negative correlation between expiratory flow rate and ENO concentration. As the concentration of NO in exhaled air decreases due to increased flow rates, the gradient for diffusion of NO from airway walls to the lumen increases\(^{60}\). An emphasis is placed on the rate of NO transfer in the airways in addition to increased production in both normal and asthmatic individuals. In airways inflammation, increased levels of ENO may be due to increased production, but may also be due to more effective transfer from the walls of the airways to the lumen\(^{60}\).

ENO is generated from the convection of NO in the alveoli, and diffusion of NO in the conducting airways. NO produced in the naso-oropharynx also contributes to the level of ENO, but is usually excluded during the measurement procedure\(^{61}\). Levels of NO in the alveoli are constant at relatively low concentrations due to rapid binding to hemoglobin in the pulmonary capillaries. The concentration of NO in the alveoli is derived from the amount present in inhaled gas, NO diffusing from airway walls into inhaled gas, and NO produced by alveolar cells which may include certain leukocytes\(^{43}\). As the gas ascends the airway towards expiration, NO concentrations in the airway lumen begin to increase because of increased diffusion from airway walls.

Diffusion of NO from the airway walls to the lumen is dependent upon the relative concentration gradient and the surface area across which it is taking place. NO concentration in the lumen rises towards equilibrium with airway wall concentrations of NO as the air gets closer to exhalation. A final exhaled concentration of NO is reached at the airway exit.

Silkoff et al. studied the contribution of NO in airway walls and the diffusion capacity of NO from the airways to expired gas to ENO levels. This was accomplished by
analyzing the amount of NO exhaled per unit of time. the concentration of exhaled NO, and the relative relationship between the two factors in normal individuals. Following development of a mathematical model, similar measurements were performed on asthmatic subjects before and after administration of steroids. The results of this study indicated that the increase in NO concentration in the exhaled air of asthmatic individuals was due to increased diffusion. Repeated measurements following administration of inhaled corticosteroids indicated that decreases in exhaled concentrations of NO was due primarily to decreased airway wall concentrations of NO. The product of the diffusing capacity of the airways and the relative concentration of NO in the airway walls measured in asthmatic subjects following administration of steroids was positively correlated with the provocative concentration of methacholine causing a 20% decline in FEV₁ (PC20) and FEV₁/FVC measured before and after steroids. This result possibly reflects maximum diffusion of NO produced in airway walls by cNOS. Therefore the rate of NO diffusion may indicate cNOS activity in the lungs and may reflect stimulated activity of NANC (NO-producing) nerves in the airways compensating for decreased sensitivity of airway smooth muscle to the relaxant effects of NO.

1.7 ENO and the Effects of Atopy

As stated in the first section, mast cell degranulation and release of chemical mediators initiate the IgE-mediated inflammatory process. Sensitization involves synthesis of IgE antibodies, signaled by the release of cytokines (IL-4) and proportionate T-B cell interaction following initial allergen uptake. Subsequent exposure to allergen and cross-linking of cell surface IgE induces triggering of mast cells, resulting in degranulation. release of chemical mediators and frequently, ensuing inflammation. The
importance of IgE antibodies in the manifestation of disease is seen primarily in asthma and other allergic conditions.

Recent studies have demonstrated a relationship between inflammation, atopy, and NO. This relationship is based on the involvement of NO as a pro-inflammatory mediator. Inhibition of NOS hinders the late cutaneous reaction, which is mediated by the influx of inflammatory cells. Similarly, studies indicate that increased NO suppresses the T_{H1} subset of CD4+ T cells and conversely favors the T_{H2} profile, which mediates the immediate-type hypersensitivity reactions promoting the allergic disease process with eosinophilia. Despite the preceding findings, a concrete study detailing the connection of atopy with elevated lower airway-generated ENO remains to be performed. As with asthma, further studies are required to better understand the role of exacerbation of allergies in the elevation of ENO. There are no universally accepted theories as to the underlying mechanism associating non-asthma allergic disease, the presence of bronchial hyperresponsiveness and ENO, and whether the presence of one necessitates the others.

Even in the absence of clinical asthma, atopy may lead to airway hyperresponsiveness. It would be advantageous to make early assessments of airway inflammation, as it is often present in atopic individuals before the onset of clinical symptoms of asthma. It was established early on that ENO levels are elevated in asthma. Even within asthmatic individuals, extended focus is being directed towards the allergic nature of the disease and its role in the elevation of ENO. Ling-Pei et al showed that ENO levels correlated positively with the number of positive skin-prick tests in asthmatic adults. This study also found that ENO values correlated more closely with atopy than with bronchial hyperresponsiveness and lung function. A similar study by Gratziou et al
found that increased levels of ENO were detectable in atopic patients with asthma and/or rhinitis and not in non-atopic patients. These findings suggest that it may be the allergic nature of airways inflammation that is mainly responsible for elevated ENO levels.

Recently, data has also been compiled that shows ENO levels are increased in non-asthmatic, atopic individuals as well. Horvath et al showed that ENO levels were significantly increased in atopic individuals when compared to non-atopic, healthy individuals. Henriksen et al confirmed this finding in individuals with allergic rhinitis and proposed the importance of allergen exposure and its relationship to lower airway inflammation. In healthy children, ENO is associated with skin prick test positivity. The significance of elevated ENO levels in non-asthmatic atopic individuals must still be clarified. Based on these results and the findings of other similar studies, it may be postulated that it may be the presence of atopy that causes increases in ENO levels. Lower airway inflammation may be present sub-clinically in non-asthmatic atopic individuals. Berlyne et al showed that eosinophil % in sputum (another parameter of inflammation) was elevated in non-asthmatic atopic individuals when compared to healthy non-atopic individuals, although in that study, ENO levels were not found to be significantly different. This study also demonstrated that ENO levels were elevated in some non-asthmatic, non-atopic healthy subjects and asthmatic subjects who had normal % eosinophil values. Other reports indicate that ENO and bronchial hyper-responsiveness represent different aspects of the same inflammatory process. Berlyne et al explains that the results from his study show that ENO may not represent eosinophilic activity in inflammation. Elevated levels of ENO observed in respiratory viral infection and bronchiectasis illustrate a relationship of ENO also to non-eosinophilic
inflammation\textsuperscript{67,68}. Therefore, ENO can also represent a different facet of inflammation from eosinophil presence and activity that may be present in non-asthmatic, atopic conditions. ENO measurements may reveal inflammation that is not apparent in other methods\textsuperscript{66}.

1.7.1 Hypotheses for Elevation of ENO in Atopy

Several hypotheses have been suggested to explain why upper airway inflammation results in lower airway NO activity in the absence of clinical asthma. First, the significance of elevated ENO levels in non-asthmatic, atopic individuals may represent a sub-clinical airway inflammation. Djukanovic et al. showed that histological analysis of atopic individuals with no evidence of clinical asthma revealed the presence of airway inflammation\textsuperscript{99}. In addition, the dispersal of such inflammation may be throughout the respiratory tract due to eosinophilic infiltration. The circulation of IgE antibodies in atopic individuals may be sufficient to induce increased production of NO in the airways. Higher-than-normal levels of IgE with relevant allergen exposure stimulate mast cell activation and degranulation. Mast cells would subsequently release TNF-\(\alpha\), IL-1 and IFN-\(\gamma\), which have already been discussed as possible initiators of type II NOS activity in the lungs.

There are several mechanical facets to explaining this phenomenon as well. Nasal challenge with methacholine has shown an increase in lower airway resistance, as well as nasal resistance in atopic individuals\textsuperscript{70}. In relation, increased bronchial responsiveness to methacholine has been documented following nasal challenge with allergen\textsuperscript{71}. These results point to a nasal-bronchial reflex that may explain why rhinitic reactions lead to higher ENO levels originating from the lower airways. Other simple explanations focus
on the consequences of inflammation within the nose. Obstruction in the nose may lead to mouth breathing, impaired filtration, and increased inhalation of aeroallergens. Similarly, aspiration of inflammatory mediators from the nose into the lower airways could cause perfusion of inflammation.

It may also be reasonable to assume atopy acts independently and causes direct stimulation of type II NOS, regardless of the status of the lower airways. This relates to the presence of asymptomatic bronchial hyperresponsiveness (BHR) in atopic individuals, who demonstrate the presence of histologic inflammation in their airways and some airway remodeling. Underlying (sub-clinical) respiratory inflammation and constant exposure to allergens may proclaim the pathogenesis of asthma as well as the predisposition to developing clinical asthma. Therefore, ENO may be an early indicator of asthma in those individuals with atopy, as well as a marker of inflammation.

1.8 Rationale and Experimental Purpose

Levels of ENO have been found to be elevated in individuals with atopy, regardless of the presence of asthma. Many studies are investigating mechanisms of production of ENO in both asthma and atopy, focusing on specific segments of the inflammatory reaction. The results, though varied, seem to indicate that the machinery that leads to an exaggeration of ENO may not be as intrinsically intertwined with the inflammatory process as once thought. The bulk of data up to date on the role of ENO has focused on individuals with known asthma and presenting with typical disease symptoms. Atopic individuals may lack clinical symptoms of asthma, but still reveal underlying inflammation as evidenced by elevated ENO levels. Thus the measurement of nitric oxide
in exhaled gas may prove useful in predicting the state of lung health for individuals susceptible to future disease.

The focus of the current study is measurement of ENO in healthy and atopic individuals. This study will endeavor not only to show that ENO levels are high in atopic individuals when compared to healthy individuals, but will also examine the effect of allergy symptoms, history and seasonal variations in allergies on ENO levels. Nonspecific bronchial hyperresponsiveness to methacholine will also be assessed in relation to ENO levels in atopic individuals, with the intention of demonstrating that atopy and bronchial hyperresponsiveness are separate phenomenon with different relationships to ENO. In comparison to prior studies, atopic subjects who lack allergy symptoms and are not on any anti-inflammatory medications during the time of ENO measurement will be recruited. The importance of this factor will be aimed primarily at the examination of variations in ENO levels during pollen season and out of pollen season. Studies like the one proposed in this thesis will contribute to identifying a normal range for ENO by accounting for asthma and atopy, which may not have been done previously.

1.9 Hypotheses

Following examination of past studies and established criteria, the following assumptions were adopted, and the following hypotheses were tested (please consult Chapter 2: Methods for testing criteria):

1. Atopic individuals, whether showing reactivity to methacholine or not, will exhale higher quantities of NO than non-atopic, non-hyperresponsive individuals.
2. ENO levels will not be significantly different between atopic individuals with bronchial hyperresponsiveness to methacholine and those without.

3. ENO levels will not be significantly different when comparing non-atopic hyperresponsive individuals with non-atopic, non-hyperresponsive individuals.

4. Individuals sensitive to seasonal allergens will have corresponding fluctuations in exhaled levels of NO with changes in pollen concentration.

5. The normal range of ENO levels for non-atopic, non-asthmatic individuals will be lower than those previously described in the literature.
Chapter 2 - METHODS

2.1 Subjects

We included subjects from each of the following groups:

- Non-atopic, non-bronchial hyperresponsive individuals (control group)
- Atopic, non-bronchial hyperresponsive individuals
- Atopic, bronchial hyperresponsive individuals (but without a history of asthma)

Meeting the following criteria:

1. Age between 18 and 65 years, men or women:
2. Atopy, defined as at least one skin prick test with diameter of wheal reaction equal to or exceeding 3mm in the presence of a positive histamine control reaction and a negative control reaction:
3. Able to give informed consent.

We excluded subjects:

1. Doctor-diagnosed asthma:
2. Taking oral or inhaled corticosteroids and/or anti-inflammatory (anti-histamines) medication or having taken any of these medications during the last four weeks before the study:
3. Taking nasal steroids, anti-leukotrienes:
4. Suffering significant illness (including other respiratory diseases):
5. Taking medication for non-asthma/non-allergy illnesses:
6. Pregnant or lactating women:
7. Current smokers or former smoker with an exposure greater than 10 pack years (average pack per day consumption times the number of years smoked).
2.2 Design

The study design was approved by the University Health Network Ethics Committee. The following testing procedures were used for each subject. The study spanned 26 months (August 1999-October 2000). The order in which each test was employed was designed to prevent any interference between tests and to ensure subject safety. Time allotment for each test and spacing between tests was arranged to minimize cross-reactivity and false-positive or false-negative results. Such considerations also allowed subjects sufficient time to rest between breathing maneuvers.

1. Questionnaire- The questionnaire was of original design. Since most subjects were from outside our clinic setting, a complete history was required for each subject. The questionnaire focused on establishing whether subjects had any atopy or asthma. These issues were examined by inquiring about specific known allergies, related symptoms and rates of occurrence. Each subject was asked if he or she had ever experienced episodes of allergic rhinitis, eczema, allergic conjunctivitis, etc (See appendix 2.1). These conditions were explained in lay terms with complete description of symptoms. Subjects were also questioned for the presence of allergies to certain substances like insect bites, food or drinks, medicines, or animals, and were asked to provide specific cases. Subjects were also questioned for the presence of asthma symptoms (chest tightness, wheezing and/or shortness of breath) and whether they had ever experienced an asthmatic attack. Since we wanted to exclude individuals with recent (4wks) or current respiratory tract
infections, inquiries were made into relevant symptoms and the use of antibiotics. Finally, all subjects were questioned thoroughly about smoking history.

2. Exhaled nitric oxide measurements- ENO measurement was performed before the methacholine challenge test and the allergy skin prick test to avoid any false results. The measurements were performed at two time points (in and out of pollen season) atopic subjects sensitized to seasonal allergens. Please consult section 2.3.

3. Methacholine Challenge test- Please consult section 2.4.

4. Allergy skin test- Please consult section 2.5.

2.3 Standardized Procedures for the Online Measurement of Exhaled Lower Respiratory Nitric Oxide in Adults- Official Statement of the American Thoracic Society

2.3.1 General Aspects of Exhaled Nitric Oxide (ENO) Measurements

1. Requirements for Clinical Use:

A Standardized protocol for the online measurement of ENO has been developed to ensure agreement between research institutions of mean ENO values within 5% for each age group being studied. If ENO measurements are to have clinical applicability beyond the research setting adoption of these standardized procedures and collection of normative data is required. Offline measurement of ENO involves collection and storage of exhaled gas in an appropriate vessel for delayed analysis. Online measurements of ENO involve real-time monitoring of gaseous levels and mouth pressure profiles.

For details on ENO terminology, units and general principles of measurement, and influencing factors please refer to:

2.3.2 Online Measurements of ENO in Adults

Online measurements of ENO provided a real-time display of ENO concentration versus time or exhaled volume along with other testing variables such as airflow and pressure. Exhalation with this method allowed the expirate to be continuously sampled by the NO analyzer. Online sampling permitted the monitoring of exhalation flow and pressure parameters to ensure an accurate NO plateau. Since exhalation was performed under computerized supervision, any deviation or sub-optimal performance could be rectified.

*Recommended standard technique for online measurements of ENO in adults*

1. *Inspired gas source._ Ambient NO levels were recorded. During inhalation of gas containing high NO concentrations for online measurements of ENO levels an immediate peak was observed in the ENO vs. time profile. This peak indicated ambient levels of NO present in the NO analyzer and in subject dead space. NO-free air (< 5 ppb NO) was used for inhalation.

2. *Inhalation procedure._ during measurement of any ENO levels a nose-clip was NOT used. Use of a nose-clip would cause accumulation of nasal NO and promote contamination of exhaled air via the nasopharynx. The subjects were seated at the appropriate height and position. The mouthpiece was inserted completely, maintaining a tight seal with the subject’s lips. The subjects were asked to inhale to total lung capacity (TLC) over 2 to 3 seconds and then exhale immediately preventing any breathholding. Recommendations for exhalations from TLC have provided the most constant point in the respiratory cycle.
3. **Exhalation procedure**

Exclusion of nasal NO: Contamination of lower airway NO with nasal NO were controlled, as the levels of nasal NO are significantly higher than lower airway levels\(^{40,74}\). Subjects were made to exhale against an expiratory resistance with a positive mouthpiece pressure\(^{61,75}\). These pressures were displayed to the subject so they may control their breathing and maintain a steady expiratory flow. It is currently accepted that this process causes closure of the velum as indicated by nasal CO\(_2\) measurement\(^{61}\) and nasal argon insufflation\(^{75}\). Mouthpiece pressure was maintained at 20 mm Hg and no greater to ensure velum closure and avoid subject discomfort. A simple apparatus for such maneuvers is shown in figure 2.1.

![Figure 2.1 Equipment configuration for the measurement of ENO: C-computer; NOA-nitric oxide analyzer; NO SL-nitric oxide sampling line; MP-mouthpiece; P-SL-pressure sampling line; PG-pressure gauge; IG-inspired gas; ER-expiratory; V-2-way valve.](image)

Standardization of exhalation flow rate: This study employed a low flow rate of 0.046 L/sec.
2.3.3 Interpretation of Single-Breath NO Profiles

The advantage of constant expiratory flow measurements of ENO is a single NO profile. The profile indicates a plot of ENO levels versus time that consists of a surge during the washout phase followed by the NO plateau, which should be reproducible and relatively flat (Figure 2.2).

![Graph](image)

**Figure 2.2 NO concentration (ppb) and airway opening pressure versus time for three exhalations by the same subject, showing reproducibility of ENO profiles and plateaus.**

An early NO peak may be seen in the profile immediately following the washout period and just before plateau if the subject inhales through the nose or if the velum is not sufficiently closed as exhalation begins (Figure 2.3). Similarly, if there is NO in the inspired air source or if the subject pauses following inhalation to TLC, NO accumulation can cause a peak that precedes plateau. If peaks were noticed in the NO profile, the subjects were instructed of the correct breathing maneuver and the test was repeated. If the profile remained unchanged, early peaks were ignored and only the plateaus were interpreted.
Figure 2.3 NO concentration and airway opening pressure versus time. The left-hand profile was performed with an oral inspiration of gas containing < 5 ppb NO. The right-hand profile shows an early peak that is generated due to nasal inhalation, which causes contamination of exhaled air. The ENO plateau returns to its unaltered form once the peak has washed out.

Exhalation was ensured to be sufficiently long to obtain an accurate and steady plateau in the NO versus time profile. The profile was at least 10 seconds long to obtain a reasonable plateau of 6-8 seconds. Measurements were repeated until reproducible levels were attained. Three NO plateaus were recorded that agreed within 10% of the mean value. The final exhaled value of NO was then reported as the mean of the three plateaus. A minimum of 30 seconds of relaxed tidal breathing was allowed to the subject off of the analyzer apparatus in between trials. This allowed subjects to rest. Subjects were not overexerted if satisfactory measurements were not being achieved.

2.3.4 Equipment Specifications for the Measurement of Exhaled Nitric Oxide

Our NO analyzer performed gas measurements using the principle of chemiluminescence.

*Calibration Requirements and Procedures*

1. Zero NO gas - A reliable zero NO gas is essential for ENO level measurements. A pure N₂-gas was used as the zero-NO calibration gas (Praxair, Canada).
2. NO standard calibration gas- Specially prepared NO calibration gases are used mostly in nitrogen. This study employed a gas with 10.1 ppb concentration of NO. A gas of 2% accuracy level is recommended to insure reproducibility of unknown samples. These gases remained stable for over six months.

3. Calibration- Calibrations were performed daily using the zero NO gas and a standard calibration gas in the expected range of sample values.

_Ancillary Equipment_- The following apparatus are an integral part of the NO analyzer.

1. Output device- The analyzer used in this study possessed both an analog and digital output and a RAM storage card. Measurement of ENO was performed on the Sievers 280 (Sievers Co., Boulder, Colorado), a rapid linear-response chemiluminescent analyzer.

2. Data collection- Real-time monitor display of collection conditions, including NO, pressure, and flow was used with an automatic system to sense the quality of exhalations and an accurate plateau.

3. Biofeedback parameters- A display was used for subjects to maintain constant flow rates.

4. Sample flow rate- A rotameter was used to display NO analyzer flow rate and was an integral component of the Sievers system.

2.4 Guidelines and Procedure for Methacholine Challenge Testing

Methacholine inhalation tests are respiratory testing procedures used in clinical and research laboratories to assess airways hyperresponsiveness. This test measures the dose-response ratio to a known bronchoconstrictive agent. Spirometric alterations indicate the response to the provoking doses of methacholine, and are applicable to adults.
and children. The use of the methacholine challenge test in this study was to determine whether subjects had any nonspecific bronchial hyperresponsiveness and/or to verify the absence of asthma.

2.4.1 Spirometry

*Equipment and testing technique*- This study used a volume-displacement, 8-liter spirometer (Morgan Spiroflow) since they are the "gold-standard" in assessing lung function. All flow-volume curves were plotted on an x-y plotter. The flow-volume curve is a graphic representation of the relationship between airflow and lung volume. The volume displaced during the maneuver was plotted in liters on the x-axis and the flow of exhaled gas generated by the subject was plotted in L/sec on the y-axis. Throughout the testing procedure, subject effort was the single most important indication of a good result. Such results were produced by enthusiastic coaching and often times a raised voice. These test conditions were sought to ensure maximum expiration from beginning to end so that a high quality baseline could be obtained. The quality of each flow-volume curve was examined following each effort. The best three maneuvers with less than 5% variation in FEV₁ were averaged to produce the baseline.

*Definition of terminology*- The following terms are measurement parameters used to describe specific portions of the flow-volume curve and certain segments of the spirometric maneuver.

- Total Lung Capacity (TLC)- total amount of air in the lungs following maximal inspiration.
- Vital Capacity (VC)- maximal amount of air that can be inhaled and exhaled.
- Residual Volume (RV)- amount of air remaining in the lungs following a maximal expiration.

- Tidal Volume (TV)- air volume normally inhaled and exhaled during each breath.

- Forced Vital Capacity (FVC)- measured in liters. The maximal volume of air exhaled using maximal effort following maximal inspiration.

- Forced Expiratory Volume in one second (FEV₁)- measured in liters. The volume of air exhaled forcefully during the first second. This is the most widely used measurement for detecting and quantifying the severity of obstructive lung diseases. The normal range is ≥ 80% of the predicted normal value.

- FEV₁/FVC Ratio- used to detect airway obstruction. A ratio of less than 70% usually indicates an obstructive disorder.

2.4.2 Challenge Test

Choice and preparation of methacholine- Methacholine (acetyl-ß-methylcholine chloride, ACIC Inc.) solutions were prepared from a dry, crystalline powder by the Toronto General Hospital pharmacy staff. The vials were labeled as methacholine with the appropriate concentration in mg/ml and an expiration date. All solution sets were stored in a refrigerator at 4°C. All solutions were warmed to room temperature before administration. The solutions were replaced every three months. The use of methacholine was preferred over histamine because the latter was associated with more systemic side effects.

Nebulizer for the tidal-breathing method- The Bennett Twin (Puritan Bennett Corp of California) nebulizer was used in this study. It delivered a standard aerosol with a particle mass median diameter (MMD) between 1.0 and 3.6 μm. The Bennett Twin nebulizer was
analyzed by Cockroft et al and was found to have good reproducibility. The Bennett Twin nebulizer required a flow rate of 5.5 liters/min to obtain a methacholine output of 0.13 ml/min. When using this nebulizer, the subject performed tidal breathing through a mouthpiece. Aerosolization of methacholine into the laboratory air was prevented with the use of viral filters (Trudell Medical, Mississauga, Canada). Other necessary equipment included a source of compressed air (from a hospital source), a pressure-compensated flow meter (Puritan-Bennett) for turning the aerosol on and off between inhalations and adjusting the flow rate and tubing. Ancillary equipment included a stopwatch, a 3 ml syringe with a needle, and a calculator.

*Spirometric maneuver (measurement of the response)* - The subjects were seated for the test. All subjects wore nose-clips to prevent any leakage of air. The testing procedure and duration were fully explained to all subjects. Each subject performed the spirometric maneuvers while breathing from the mouth. This was performed with a mouthpiece connected to 0.5 meters of corrugated tubing that was attached to the flow port of the dry spirometer. As mentioned earlier, since subject effort was the single most important factor determining the accuracy of the testing, each was instructed to follow the tester's voice.

The subjects were asked to begin by breathing normally. After one normal exhalation the subjects were prompted to inhale rapidly to TLC and then immediately exhale ("blast") the air out forcefully, sustaining the forced expiration for six seconds. Forceful expiration for 6 seconds was performed so that an accurate FVC could be determined and was repeated for baseline determinations only. Following baseline
spirometry. A target 20% fall in FEV\textsubscript{1} (baseline FEV\textsubscript{1} x 0.80) was determined for each subject for verification.

Two-Minute tidal breathing method- 3 ml of methacholine solution, beginning with the lowest concentration was inserted into the nebulizer using a sterile syringe. The lowest dose of methacholine administered was 0.03 mg/ml with doubling doses up to a maximum of 16 mg/ml. The subject was asked to relax and breathe quietly (tidal breathing) into the mouthpiece while the flow was adjusted to 5.5 L/min. The subjects were asked to breathe like this while wearing nose-clips for two minutes. The stopwatch was used to monitor time. The subjects were asked to keep the nebulizer upright, with the mouthpiece constantly in their mouths. All subjects were monitored for comfortable and quiet breathing, and that they were not tipping the nebulizer. After 2 min the flow meter was turned off and the nebulizer was taken away from the subject. Once methacholine doses were administered, forceful expiratory maneuvers were shortened to 2 seconds since only an FEV\textsubscript{1} was needed. Spirometry and determination of FEV\textsubscript{1} was performed at 30, 90, 180, and if necessary 300 seconds after administration of methacholine was stopped. Again, each maneuver was examined for optimum performance and each FEV\textsubscript{1} was checked with the mean baseline value to determine if a percentage drop had occurred and if so, how much. Maneuvers after 90 seconds post-methacholine administration were stopped if the FEV\textsubscript{1} was greater than at 30 seconds post-methacholine. If this was not the case, the maneuver was repeated at 180 seconds to see if the FEV\textsubscript{1} was equal to or greater than at 90 seconds, and so on. We administered increasing double concentrations of methacholine by first discarding the used solution and then adding 3 ml of the next concentration. This procedure continued until a 20% decrease in the FEV\textsubscript{1} was noticed.
from baseline, or once a dose of 16 mg/ml was completed. Subjects were monitored and asked after each dose of methacholine for symptoms of bronchial hyperresponsiveness, such as wheezing, chest tightness, or coughing. As standard protocol in our hospital, each subject was given two puffs (200 µg) of salbutamol (Airomir, 3M Canada) by metered dose inhaler at the end of the test and regardless of bronchial responsiveness. Those subjects who experienced 20% or greater reduction in FEV$_1$ were given two puffs of salbutamol and were asked to perform spirometry after 15 minutes to ensure the FEV$_1$ returned to baseline values.

2.4.3 Acceptability and Reproducibility Criteria

Standard criteria developed by the American Thoracic Society (ATS) were adopted for this study. Please consult:


2.4.4 Measurement and Interpretation of Flow-Volume Curves

Since all measurements were taken on the same x-y plotter, all calculations used the same formula. The y-axis, or the axis displaying the flow of air was recorded on a 1:1 flow to unit of measurement (cm) ratio. Thus a curve 5 cm in the y-direction would translate as a flow of 5 liters/sec. a curve of 3 cm would translate as a flow of 3 liters/sec. and so on. The x-axis, or the axis displaying the volume of air displaced was recorded on a 1:2 volume to unit of measurement (cm) ratio. Thus a curve 10 cm in the x-direction would translate as a volume of 5 liters expired from the lung. a curve of 7 cm would translate into a volume of 3.5 liters expired. and so on.
Data presentation and $PC_{20}$- The results were reported by FEV$_1$ responses for each concentration of methacholine administered. This report included a percent decrease from baseline and a post-bronchodilator response. The $PC_{20}$ was calculated from the change in FEV$_1$ and was used to summarize all data and to give a definition of subject bronchial hyperresponsiveness. If the FEV$_1$ did not fall below 20%, the $PC_{20}$ was reported as greater than 8 mg/ml. Thus airway responsiveness was reported as that concentration of provoking agent to cause a fall in FEV$_1$ of 20%. The following equation was used to calculate the interpolated $PC_{20}$:

$$PC_{20} = \text{antilog } \left[ \log C_1 + (\log C_2 - \log C_1)(20 - R_1) / R_2 - R_1 \right]$$

Where:

$C_1$ = second-to-last methacholine concentration (concentration preceding $C_2$)

$C_2$ = final concentration of methacholine

$R_1$ = percent fall in FEV$_1$ after $C_1$

$R_2$ = percent fall in FEV$_1$ after $C_2$

The provocative concentration that caused a 20% decrease in FEV$_1$ ($PC_{20}$) was used as the outcome variable because calculations were simple and did not involve the complications and estimation required to calculate PD$_{20}$.

2.4.5 Supplementary Concerns

Medications- Subjects were asked to withhold the following medication for the duration of their action prior to testing to avoid interference with the provocation test.

- Inhaled $\beta$-agonists (short acting) 8 hours
- Oral $\beta$-agonists (short acting) 12 hours
- Nonspecific H1 Antihistamines 4 days
Safety- Since inhaled methacholine may cause bronchoconstriction, safety of both subjects and testers was considered and insured. The Medical Director of the Asthma Centre or another physician was always present in case of acute bronchospasm, if there was an emergency, or there was a need for resuscitation. Subjects were not left unattended at any point once methacholine administration had begun.

Medications to treat severe bronchospasm were present in the laboratory area. These included epinephrine and or atropine for injection, and salbutamol or ipratropium in MDI for inhalation. Oxygen was available. A stethoscope and pulse oximeter were also available.

2.5 Allergen Skin Prick Testing—Adopted from the Position Statement from the American Academy of Allergy and Immunology (AAAAI)—(Board of Directors)

2.5.1 Method of Skin Testing

The testing method employed for this study used the percutaneous route, or the prick testing method. The method was appropriate for our setting because it is rapid, causes less discomfort to subjects than intradermal methods, allowed testing of a greater number of allergens per session, and was less expensive per test than other methods. Most important, this testing method is less apt to give rise to systemic reactions. Finally, this method correlates better with actual clinical sensitivity than intradermal methods.

2.5.2 Appropriate Handling of Allergenic Extracts

Since all allergenic extracts were prepared in glycerin, solution stability was longer than if they were prepared in saline. Fifty percent glycerin was used to prevent loss of potency rather than 0.03% human serum albumin. All allergenic extracts were stored at 4°C when not being used and were not allowed to remain at room temperature for long periods.
2.5.3 Positive and Negative Controls

A negative saline control was used to test for non-specific reactivity of the skin to the diluent or to the needle used to deliver the diluent or allergen solution. A positive histamine control was used to test for hyporesponsiveness of the subjects' skin. All subjects were questioned for use of any antihistamines taken within four days of testing and the use of Hismanal within six weeks.

2.5.4 Placement of the Test

Allergy skin testing was performed on the volar surface of the forearms. Each subjects' arms were inspected for allergic rash or presence of tuberculosis inoculate before testing. We insured the subject was comfortable. The area of testing was cleaned with isopropyl alcohol and allowed to air dry completely. The allergen solutions were placed in small drops on the skin. The prick tests for each solution were placed 4cm from neighboring drops to prevent any coalescence or cross-reactivity. Using a drawing needle, the skin was punctured through the test solution. Care was taken not to draw blood. The needle was cleaned and wiped with alcohol after each puncture. After all solutions were pricked into the skin, the remaining drops were wiped off. The test reactions were read after 20 minutes.

2.5.5 Allergens Tested (Omega labs)

Table 2.1 Allergens common to the Greater Toronto Area

<table>
<thead>
<tr>
<th>ALLERGEN</th>
<th>SIZE (μm)</th>
<th>SEASON</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria</td>
<td>10-30</td>
<td>-</td>
<td>Spores</td>
</tr>
<tr>
<td>Aspergillus f.</td>
<td>5-30</td>
<td>-</td>
<td>Spores</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>10-30</td>
<td>-</td>
<td>Spores</td>
</tr>
<tr>
<td>Cat</td>
<td>25-40</td>
<td>-</td>
<td>Dander</td>
</tr>
<tr>
<td>Dog</td>
<td>25-40</td>
<td>-</td>
<td>Dander</td>
</tr>
<tr>
<td>Horse</td>
<td>25-40</td>
<td>-</td>
<td>Dander</td>
</tr>
<tr>
<td>Feathers</td>
<td>30-50</td>
<td>-</td>
<td>Particles</td>
</tr>
<tr>
<td>Cockroach</td>
<td>15-25</td>
<td>-</td>
<td>Cast-skin</td>
</tr>
<tr>
<td>Dust</td>
<td>1-10</td>
<td>-</td>
<td>Particles</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>Dust-mites</td>
<td>10-40</td>
<td>-</td>
<td>Feces</td>
</tr>
<tr>
<td>Trees</td>
<td>20-60</td>
<td>mid-April-May</td>
<td>Pollen grain</td>
</tr>
<tr>
<td>Grasses</td>
<td>20-60</td>
<td>May-July</td>
<td>Pollen grain</td>
</tr>
<tr>
<td>Ragweed</td>
<td>20-60</td>
<td>mid-Aug.-Oct.</td>
<td>Pollen grain</td>
</tr>
</tbody>
</table>

### 2.5.6 Quantitative Assessment

The size of each reaction was graded as the longest and transverse diameter of the wheal and flare in millimeters. Each reaction was also graded from 0 to +4. Negative (-) reactions signified a no wheal. Wheals 1 to 2mm in size were graded as 1+. 3 to 5mm as 2+. 6 to 9mm as 3+. and greater than 9mm as 4+. Both size in millimeters and the grade of reaction were reported.

### 2.6 Statistical Analysis

Sample sizes necessary to demonstrate a significant difference with a probability of 0.80, accepting a type I error of 0.05 (α = 0.05), were determined to be 15 subjects per group (control and atopic). Preliminary data was adopted from previous studies conducted at the Asthma Centre ENO research laboratories.

Association of bronchial hyperresponsiveness with allergen sensitization (atopy) was assessed by the Chi-squared ($\chi^2$) test. Data on lung function parameters between groups were compared by the unpaired t-test. Since exhaled nitric oxide levels were not normally distributed, they were analyzed with the Mann-Whitney U-test and the Kruskal-Wallis test. ENO values for out-of-season and in-season was compared with the paired t-test. Correlation analysis of the relationship between ENO and PC$_{20}$ methacholine, and ENO and the number of positive wheal reactions was performed with the Spearman correlation coefficient. A p-value ≤ 0.05 was considered to be statistically significant.
Appendix 2.1

CONSENT FORM FOR:

Elevated Exhaled Nitric Oxide (ENO) in Non-Asthmatic Atopic Adults

I have been asked to participate in a study which is designed to investigate the levels of nitric oxide in the air I exhale. Taking allergies into account, the study will determine a normal range.

The study will be conducted at the Asthma Centre of the Toronto Western Hospital.

I understand the study will require two visits to the laboratory. A typical visit will require about 1-2 hours of my time. The initial visit will consist of a methacholine challenge test, allergy skin test, and exhaled nitric oxide measurements. The second visit will only consist of repeat exhaled nitric oxide measurements. I also understand that I must refrain from taking alcohol on the night before the visit, and that the principal investigator may ask me to refrain from certain medications if this is deemed possible from a medical point of view.

I understand that I will undergo a methacholine challenge test during the first of the two visits. This safe, routine test involves breathing increasing concentrations of a solution called methacholine in the form of a mist and then exhaling forcefully into a tube which will measure my lung function. The test proceeds until a certain concentration of the methacholine is reached, or when there has been a certain narrowing of my airways. At a certain concentration of methacholine, narrowing of my airways may cause tightness in my chest and wheezing or even breathlessness. These symptoms are as observed in asthma from time to time. At such a time, a bronchodilator (salbutamol) will be administered, which will stop the symptoms within a few minutes. I may also experience a dry mouth during inhalations. I understand that the test is always performed with a physician in the immediate vicinity.

I understand that I will undergo skin testing to determine whether I have allergies following the methacholine challenge test during the initial visit. This safe, routine procedure involves the production of an allergic reaction by intentional exposure to minute amounts of specific allergen. The test will be carried out on the skin, specifically the inside surface of the forearms. Twelve small drops of allergen will be placed on the skin, after which the skin will be pricked with a tiny needle through the allergen solution. Skin will be observed after 10 minutes. I understand that the test is always performed with a physician in the immediate vicinity. I understand that I may be asked to return for repeat exhaled nitric oxide measurements in and out of my allergy season.

I understand that in order to measure exhaled nitric oxide (ENO) in my breath, I will be asked to inhale humidified air from a mouthpiece and then exhale into the same mouthpiece. I know that no drug or substance is administered to me, and thus there is no
reason for any adverse effects of this procedure. I understand that I may be required to repeat the procedure several times. I know that the test requires me to breathe through a small tube and that I may feel resistance as though I am holding my breath for a short period. This test will be performed during the two visits.

I understand that I may cease my participation in this study at any time without any advance notice, and that of course this will in no way prejudice my future care. I understand that the study may not benefit me specifically but that it should forward understanding of this medical condition and of its treatment.

I have been informed that all information will be kept strictly confidential, and that my name will not be associated with the published data in any way.

I have had an opportunity to have all my questions answered. I have been given a copy of this consent form.

I have been asked to consult Dr. Kenneth Chapman (416-603-5499) at any time in the event of any problems concerning this study.

NAME: (please print)______________________________________________________

SIGNATURE:_________________________________________________ DATE:________

WITNESS:_______________________________________________________________
Appendix 2.2 Sample Questionnaire

Name: ______________________
Date: ______________________

Questionnaire For:

**Elevated Exhaled Nitric Oxide (ENO) in Non-Asthmatic Atopic Adults**

Date of assessment (dd.mm.yy): __-__-__

Are you male or female? Male □ Female □

Date of birth (dd.mm.yy): __-__-__

Do you have, or have you ever had allergic rhinitis (episodes of stuffy, runny, itchy nose and sneezing besides at times of colds), hay fever? No □ Yes □ Not certain □

Do you have, or have you ever had eczema (an itchy rash on your skin)? No □ Yes □ Not certain □

Do you have, or have you ever had allergic conjunctivitis (eyey that itch, water, swell, or redden)? No □ Yes □ Not certain □

Do you have generalized allergic reactions to insect bites? If YES, please specify: __________

Do you have allergic reactions to any foods or drinks? If YES, please specify: __________

Do you have allergic reactions to medicines (such as penicillin, aspirin)? If YES, please specify: __________

Do you have allergic reactions to animals (cats, dogs, horses)? If YES, please specify: __________

Do you ever wheeze?

- Never
- Occasionally
- Frequently
- Sometimes during the night
- During pollen season
- During cold weather
- When in contact with pets
- After exercise
In a smoking environment □

Do you regularly bring up sputum/phlegm?
   Never □
   Only with a flu, cold. □
   Chest infection □
   More than 3 times per year □
   Regularly every morning □

Do you usually have a cough? □
Do you have episodes of shortness of breath? □
Do you have episodes of chest tightness? □
In a usual month (averaged over the last 12 months) do you wake through the night with wheezing or shortness of breath? □
Have you ever had an asthmatic attack? □
Do you have heart burn? □
Nasal polyps? □
Does your nose plug up frequently? □
Do you feel mucus at the back of your throat? □
Have you ever smoked? □
Have you smoked cigarettes in the last month? □

Has your doctor ever told you that you had any of the following respiratory diseases?
   No □
   If YES, check all that apply □
   Chronic obstructive pulmonary disease (COPD), chronic bronchitis or emphysema □
   Tuberculosis □
   Cystic fibrosis □
   Other (specify): □
Has your doctor ever told you that you had any of the following chronic diseases?

No

If YES, check all that apply

Diabetes

Rheumatoid arthritis

High blood pressure

Other (specify):
Chapter 3 - RESULTS

3.1 Sample Characteristics

A total of 37 non-atopic, non-asthmatic (non-bronchial hyperresponsive) subjects participated in the study. All of these subjects were non-atopic as shown by negative skin prick tests. Six other non-atopic subjects had bronchial hyperresponsiveness but with no history of asthma. Twenty-four atopic subjects were tested who produced positive skin prick tests. In this group, 15 subjects mentioned a history of allergic symptoms including rhinitis, conjunctivitis, and/or eczema, but no history of asthma. The other nine subjects were asymptomatic and had no history of allergy symptoms. Bronchial hyperresponsiveness was present in 12 of the 24 atopic subjects. The remaining 12 atopic subjects had PC20 values greater than 16 mg/ml. Of the 24 atopic subjects, five were sensitized to only perennial allergens, nine were sensitized to only seasonal allergens, and ten were sensitized to both seasonal and perennial allergens. All subjects, non-atopic and atopic, were free from any rhinitic, conjunctival or eczema symptoms relating to allergy at the time of testing. All subjects were also free from any asthma-like symptoms (chest tightness, wheezing, shortness of breath) at the time of testing, and had no history of asthma or its symptoms.

Demographic characteristics of the subjects are summarized in Table 3.1. The groups were not significantly different in age or male to female ratio.

3.2 Spirometry

The mean baseline spirometric characteristics of the study participants are shown in Table 3.1. The mean FEV1 of atopic subjects with bronchial hyperresponsiveness was significantly lower than that of either atopic subjects without bronchial hyper-
responsiveness or non-atopic non-hyperresponsive subjects. The mean baseline FEV$_1$/FVC was lower in non-atopic hyperresponsive subjects than in non-atopic non-hyperresponsive subjects.

Table 3.1 - Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Non-atopic</th>
<th>HR/Non-atopic</th>
<th>Atopics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-HR</td>
<td>Non-HR</td>
<td>Hyperresponsive</td>
</tr>
<tr>
<td>N</td>
<td>37</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.4 +/- 9.8 (20-55)</td>
<td>31.8 +/- 5.5 (23-40)</td>
<td>33.3 +/- 11.0 (19-53)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>17/20</td>
<td>3/3</td>
<td>5/7</td>
</tr>
<tr>
<td>FEV$_1$ %pred</td>
<td>100 +/- 15 (78-143)</td>
<td>88 +/- 20 (62-119)</td>
<td>100 +/- 11 (80-113)</td>
</tr>
<tr>
<td>FEV$_1$/FVC</td>
<td>81 +/- 7 (58-89)</td>
<td>73 +/- 8** (60-81)</td>
<td>78 +/- 10 (59-91)</td>
</tr>
<tr>
<td>PC$_20$ Methacholine (mg/ml)</td>
<td>≥ 16</td>
<td>2.4 +/- 2.0</td>
<td>≥ 16</td>
</tr>
</tbody>
</table>

*p = 0.017 versus non-atopic, non-HR subject group; p = 0.035 versus atopic-non-hyperresponsive group
**p = 0.011 versus non-atopic, non-HR subject group
HR- hyperresponsive

3.3 Methacholine Provocation and Spirometry

3.3.1 Bronchial hyperresponsiveness proportions_ Compared with non-atopic subjects, atopic subjects had a higher prevalence of bronchial hyperresponsiveness to methacholine (p = 0.004). There were no other significant findings to report.

Table 3.2 - Proportion of bronchial hyperresponsiveness in non-atopic and atopic subjects (χ²-test)

<table>
<thead>
<tr>
<th></th>
<th>Non-atopic</th>
<th>Atopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>43</td>
<td>24</td>
</tr>
<tr>
<td>Bronchial Hyperresponsiveness</td>
<td>6</td>
<td>12*</td>
</tr>
</tbody>
</table>

*p = 0.004
3.3.2 Comparison of atopic subjects sensitized to both perennial and seasonal allergens to those subjects sensitized to only seasonal or only perennial allergens revealed no significant difference in any of the lung function parameters or bronchial provocation testing. Comparison of these atopic groups to the non-atopic, non-hyperresponsive subject group showed the same result.

3.4 Exhaled Nitric Oxide and Atopy

All non-atopic subjects, those with and without bronchial hyperresponsiveness, and atopic subjects had satisfactory and reproducible ENO curve measurements. Since data from all the subject groups was not normally distributed, the Mann Whitney U test was used to compare ENO levels.

<table>
<thead>
<tr>
<th>Table 3.3 - Exhaled NO Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mean ENO_{avg}(ppb)</td>
</tr>
<tr>
<td>14.1 +/- 6.4</td>
</tr>
<tr>
<td>20.6 +/- 16.3</td>
</tr>
<tr>
<td>29.2 +/- 34.2</td>
</tr>
<tr>
<td>31.7 +/- 19.5</td>
</tr>
<tr>
<td>Std Error</td>
</tr>
<tr>
<td>1.1</td>
</tr>
<tr>
<td>6.7</td>
</tr>
<tr>
<td>9.9</td>
</tr>
<tr>
<td>5.7</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>12.7</td>
</tr>
<tr>
<td>16.0</td>
</tr>
<tr>
<td>19.8</td>
</tr>
<tr>
<td>26.0*</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>4.9-31.0</td>
</tr>
<tr>
<td>7.3-51.7</td>
</tr>
<tr>
<td>5.0-129.4</td>
</tr>
<tr>
<td>16.1-66.4</td>
</tr>
</tbody>
</table>

*p < 0.001 versus non-atopic, non-HR subject group
HR- hyperresponsive

The ENO level in atopic subjects (non-hyperresponsive and hyperresponsive) was twofold greater than in non-atopic, non-hyperresponsive subjects (median 19.8 and 26.0 versus 12.7 ppb, p < 0.001). The mean ENO values for the respective groups were 29.2 +/- 34.2 ppb, 31.7 +/- 19.5 ppb and 14.1 +/- 6.4 ppb.
3.5 Exhaled Nitric Oxide, Atopy, and Bronchial Hyperresponsiveness

The ENO level in atopic subjects with bronchial hyperresponsiveness was two-fold greater than ENO levels found in non-atopic, non-hyperresponsive subjects, a statistically significant increase ($p < 0.001$). The median (range) and mean values for the atopic subjects presenting with bronchial hyperresponsiveness were 26.0 (16.1-66.4) and 31.7 +/- 19.5 ppb. When the ENO level found in this subject group was compared to atopic subjects without bronchial hyperresponsiveness, no statistically significant difference was found ($p = 0.19$).
The levels of ENO in non-atopic subjects with bronchial hyperresponsiveness (median (range) 16.0 (7.3-51.7) and mean 20.6 +/- 16.3 ppb) were not found to be significantly different from non-atopic, non-hyperresponsive subjects (p = 0.46).

ENO did not correlate with the PC\textsubscript{20} methacholine for any of the subject groups. Below is the correlation (Spearman analysis) figure (Fig 3.3) for the non-atopic, non-hyperresponsive subject group (R = -0.182, p = 0.282) and the correlation figure (Fig 3.4) for the atopic subject group (R = -0.274, p = 0.203).

3.6 Exhaled Nitric Oxide and Atopic Variations

The levels of ENO found in atopic subjects sensitized to perennial and seasonal allergens were significantly higher than non-atopic, non-hyperresponsive subjects (p = 0.001). The mean (range) for the atopic group was 47.3 +/- 35.9 ppb (8.7-129.4). The ENO levels for atopic subjects sensitized to only perennial allergens (mean (range) 19.7 +/- 10.5 ppb (5.0-31.9)) and those sensitized to only seasonal allergens (mean (range) 18.3 +/- 5.9 ppb (9.6-29.6)) were not found to be significantly different from the non-
atopic, non-hyperresponsive subject group. ENO levels were found to be significantly higher in atopic subjects sensitized to perennial and seasonal allergens when compared to atopic subjects sensitized to only seasonal allergies ($p = 0.037$). No such difference was found when atopic subjects sensitized to perennial and seasonal allergens were compared to atopic subjects sensitized to only perennial allergens.

Table 3.4 Exhaled NO Levels and Atopic Variations

<table>
<thead>
<tr>
<th></th>
<th>Non-Atopic Non-HR</th>
<th>Atopics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Perennial Only</td>
<td>Seasonal Only</td>
</tr>
<tr>
<td>Mean (ppb) ENO$_{avg}$</td>
<td>14.1 +/- 6.4</td>
<td>19.7 +/- 10.5</td>
<td>18.3 +/- 5.9</td>
</tr>
<tr>
<td>Std Error</td>
<td>1.1</td>
<td>4.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Median</td>
<td>12.7</td>
<td>22.6</td>
<td>17.6</td>
</tr>
<tr>
<td>Range</td>
<td>4.9-31.0</td>
<td>5.0-31.9</td>
<td>9.6-29.6</td>
</tr>
</tbody>
</table>

*p = 0.001 versus non-atopic, non-HR subject group; $p = 0.037$ versus seasonal only atopic subject group

Figure 3.5 Comparison of median ENO levels among atopic subjects with perennial and seasonal allergies (P&S) versus non-atopic, non-HR subjects and atopic subjects with only seasonal allergy (S)

In 13 atopic subjects sensitized to seasonal allergens (regardless of sensitization to perennial allergens). ENO levels were measured twice, once out of pollen season and once during peak pollen season. Exhaled NO levels did not differ significantly between
non-pollen and pollen seasons (mean (range) 37.6 +/- 34.4 ppb (8.7-129.4) versus 33.8 +/- 29.4 ppb (6.9-117.9), p = 0.225). All subjects were free from any rhinitic/conjunctival or asthma symptoms on the day of testing and a minimum of four days prior to testing. None of the subjects tested were on any β2-agonist medication, inhaled or oral corticosteroids, or antihistamines (anti-inflammatory).

Table 3.5 Exhaled NO Levels and Atopic Seasonal Variations

<table>
<thead>
<tr>
<th></th>
<th>Seasonal Atopics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In-Season</td>
<td>Out of Season</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Mean ENOₙₑₓ (ppb)</td>
<td>33.8 +/- 29.4</td>
<td>37.6 +/- 34.4</td>
</tr>
<tr>
<td>Std Error</td>
<td>8.9</td>
<td>10.3</td>
</tr>
<tr>
<td>Range</td>
<td>6.9-117.9</td>
<td>8.7-129.4</td>
</tr>
</tbody>
</table>

3.7 Exhaled NO and Allergy Skin Prick Test Reactivity

The number of positive wheal reactions in atopic subjects correlated ENO levels (p < 0.001: R = 0.64). No other significant correlation was found.

Figure 3.6 Correlation using Spearman correlation coefficient between ENO levels and the number of positive wheal reactions in atopic subjects. p < 0.001: R = 0.64.
Table 3.6 Exhaled NO Levels and the Number of Positive Wheal Reactions

<table>
<thead>
<tr>
<th>Subject</th>
<th>Number of positive wheal reactions</th>
<th>ENO_{ex} (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>41.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>17.6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>17.7</td>
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<tr>
<td>4</td>
<td>2</td>
<td>17.2</td>
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<tr>
<td>5</td>
<td>3</td>
<td>9.4</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>41.8</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>24.4</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>16.1</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>48.5</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>31.9</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>129.4</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>82.5</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>25.4</td>
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<tr>
<td>14</td>
<td>2</td>
<td>21.9</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>22.7</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>9.6</td>
</tr>
<tr>
<td>17</td>
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<td>5.0</td>
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<tr>
<td>18</td>
<td>2</td>
<td>13.8</td>
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<td>21</td>
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<td>22</td>
<td>3</td>
<td>8.7</td>
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<td>23</td>
<td>1</td>
<td>22.6</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>12.5</td>
</tr>
</tbody>
</table>
Chapter 4 - DISCUSSION

4.1 Resolution of the Hypotheses

1. Higher levels of ENO were found in subjects with atopy (regardless of the presence of hyperresponsiveness to methacholine) when compared to non-atopic subjects.

2. Levels of ENO were not found to be statistically different between atopic subjects with and without bronchial hyperresponsiveness to methacholine.

3. Non-atopic subjects with bronchial hyperresponsiveness to methacholine did not produce significantly higher levels of ENO when compared to non-atopic subjects without hyperresponsiveness.

4. ENO levels were not found to be different between out-of-season and during pollen season in atopic subjects sensitized to seasonal allergens. ENO levels in atopic subjects sensitized to perennial and seasonal allergens were significantly elevated compared to non-atopic non-hyperresponsive subjects.

5. In contrast to our hypothesis, the normal range of ENO, 14.1 +/- 1.1 ppb, was found to be higher than ranges reported previously.

These results were observed in the absence of asthma or its symptoms (chest tightness, wheezing and/or cough). The subjects with PC<sub>20</sub> methacholine less than 8 mg/ml showed non-specific hyperresponsiveness to methacholine and had never experienced any symptoms prior to entering the study. Similarly, all ENO measurements were made in the absence of any allergy symptoms (rhinitis, conjunctivitis and/or eczema) and in the absence of any medication use that might have influenced the analysis. Examination of ENO levels may be used for early detection of developing airways inflammation in atopic individuals.
4.2 Exhaled NO and Atopy

The increased concentration of ENO in all atopic subjects when compared to non-atopic, non-hyperresponsive subjects is in agreement with numerous studies that have been published lately. Two studies are of particular interest. Horvath et al. demonstrated that exhaled NO levels were higher in asymptomatic atopic individuals when compared to healthy individuals. In the analysis of ENO levels, this study combined atopic subjects who were hyperresponsive to methacholine with those who were not. In comparison, our study separated the two groups of atopic subjects to determine the influence of bronchial hyperresponsiveness on ENO levels. Henriksen and colleagues showed that ENO levels were significantly higher in atopic individuals who were experiencing allergy symptoms and who were being treated with anti-histamines when compared to healthy individuals. Our analysis included only those atopic subjects who were free from any symptoms at the time of testing. As in the preceding studies, the elevation of ENO levels observed in our study cannot be explained by asthma because all subjects denied asthma-like symptoms prior to and during the study. Similarly, other factors known to influence the concentration of ENO such as current smoking and chronic lower respiratory tract infection were absent in both the control and atopic groups. Contamination of ENO levels by nasal NO is unlikely because the soft palate elevates when expiration is performed against resistance. As validated in a procedure by Silkoff et al., subjects were made to exhale against an expiratory resistance with a positive mouthpiece pressure. Nasal CO₂ measurements have shown that the velum is closed and that there is no contamination of NO of exhaled air.
Continual exposure of atopic individuals to even low doses of allergen has been shown to cause increases in bronchial hyperresponsiveness to histamine\textsuperscript{78}. The basis behind such an assumption is that nonspecific hyperresponsiveness, as in hyperresponsiveness to methacholine or histamine, is nonetheless an abnormal sensitivity of the lungs to external stimuli. Such hypersensitivity is characteristic of asthma and its underlying disease\textsuperscript{13}. Since the presence of hyperresponsiveness is an established measure for asthma and other obstructive diseases of the lungs, it is only reasonable to equate them with developing lower airway inflammation when observed with elevated ENO levels in non-asthmatics.

In this study, ENO levels did not correlate with the $\text{PC}_{20}$ methacholine. Atopic subjects with bronchial hyperresponsiveness to methacholine had significantly lower % predicted FEV\textsubscript{1} when compared to the non-atopic, non-hyperresponsive group and when compared to atopic subjects without bronchial hyperresponsiveness (Table 3.1). These findings are in agreement with Henriksen et al. who found that % predicted FEV\textsubscript{1}, though within the normal range, was lower in atopic subjects when compared to control subjects\textsuperscript{64}. There was a significantly greater proportion of subjects who displayed bronchial hyperresponsiveness in the atopic subject group as a whole as compared to the non-atopic group. Though this degree of lung reactivity was not clinically indicative of any disease process, the manifestation of significantly elevated levels of ENO in conjunction with lower than normal % predicted FEV\textsubscript{1} may suggest the possibility of chronic lower airway inflammation in atopic individuals. Furthermore this may represent a form of airway inflammation that is yet sub-clinical in this group of individuals. Persistence of aero-allergen exposure into the upper or lower airways might therefore set
the stage for development of hyperresponsiveness to methacholine. Evidence for a nasal-bronchial reflex has already been given in the Introduction section and further evidence may be seen in our atopic subjects.

In lieu of the preceding discussion, the relationship of ENO levels with atopy and bronchial hyperresponsiveness to methacholine remains to be elucidated. Airway inflammation, bronchial hyperresponsiveness and elevated ENO levels obviously do not always coincide. Our results suggest a stronger relationship between ENO and atopic status than between ENO and responsiveness. There was no statistically significant difference between NO levels exhaled by atopic subjects with bronchial hyperresponsiveness to methacholine when compared to those without bronchial hyperresponsiveness. Baraldi et al. showed in a study of pollen-allergic asthmatic children that there was an increase in ENO levels during the pollen season in which there was increased exposure to allergens, even in the absence of significant changes in lung function. Two other studies have demonstrated similar findings. Lim et al. showed that in mild to moderate asthmatics there was no correlation between ENO and conventional indices of asthma control. Ling-Pei et al. showed there was no correlation between ENO and FEV₁ or the provocative concentration causing a 20% fall in FEV₁ in atopic adults with asthma. It may be said of our study that the lack of a relationship or correlation in the pattern of response between ENO levels, mechanical responsiveness of the airways and the presence of lower airway inflammation is due to a recruitment bias towards individuals with milder to non-existent lower airway disease. This is entirely possible because Henriksen et al. found significant differences in ENO levels between atopic subjects with bronchial hyperresponsiveness to methacholine when compared to
atopic subjects without hyperresponsiveness. Similarly, one model of asthma infers that it is a combination of airway inflammation and BHR that causes lung disease. Either one alone is insufficient to produce asthma. Because we aimed to recruit subjects free of asthma and atopy symptoms and excluded subjects taking medications, we may have failed to detect an effect of marked bronchial hyperresponsiveness on ENO. Subjects with advanced lower airway disease and greater involvement of the lung mechanically and physiologically in the manifestation of inflammation would likely be taking medications that would exclude them from the study.

However, it may not be necessary that bronchial hyperresponsiveness be present for atopy to induce elevation of ENO. The presence of lower airway inflammation even in the absence of airway hyperresponsiveness has been proved by Berlyne et al. This study showed that individuals with eosinophilic bronchitis without asthma and without bronchial hyperresponsiveness had elevated ENO levels when compared to healthy control subjects. Elevated % eosinophil numbers compared to healthy control subjects demonstrates evidence of underlying inflammation in the lower airways. Therefore, it still remains reasonable to assume that sub-clinical lower airway inflammation is present in non-asthmatic atopic individuals who may not manifest bronchial hyperresponsiveness. Degrees of airways inflammation as indicated by a trend in ENO levels can be seen in the four groups of subjects participating in our study. The median values (Table 3.3) of ENO levels for the non-atopic non-hyperresponsive, non-atopic hyperresponsive, atopic non-hyperresponsive, and atopic hyperresponsive subjects were 12.7 ppb, 16.0 ppb, 19.8 ppb and 26.0 ppb, respectively. The presence of higher ENO levels in atopic non-hyperresponsive subjects when compared to non-atopic
hyperresponsive subjects show that even though bronchial hyperresponsiveness indicates inflammation of the airways, it may not necessarily need to be present to cause elevations in ENO. The results of our study also show that regarding the population of subjects participating in our study. ENO has a stronger relationship with atopy than bronchial hyperresponsiveness. With progression of uncontrolled inflammation and the nature of allergic rhinitis, it may be that atopic non-hyperresponsive individuals will develop bronchial hyperresponsiveness in the future.

The number of studies examining these relationships in non-asthmatic atopic individuals is few and further research is necessary to determine whether this is actually the case. Nonetheless, it may be hypothesized that in cases of milder atopic disease without evidence of physiologic changes characteristic of asthma, elevated ENO levels and bronchial hyperresponsiveness may initially act as separate pathways of the same inflammatory progression and not as corollaries. Moreover, as demonstrated in this study, despite the coexistence of inflammation and bronchial hyperresponsiveness and their association to disease severity in asthma, ENO may still be a better marker of inflammation.

4.3 Exhaled Nitric Oxide and Atopic Sensitization

Pollen allergen concentrations are high at certain times of the year and absent at others. Thus, exacerbations in individuals with seasonal allergies depend on the annual variability of concentration of pollens in the area of residence. Elevation of ENO levels during pollen season, which is a period of higher allergen exposure, demonstrated by one study has already been discussed. Similarly, Simpson et al. showed that ENO levels were significantly increased among asthmatic individuals who were sensitized and exposed to
indoor allergens when compared to asthmatic individuals who were sensitized but not exposed. In the current study, we analyzed the ENO levels of atopic subjects who were sensitized to only perennial allergens, those who were sensitized to only seasonal allergens, and those who were sensitized to both perennial and seasonal allergens. To reiterate, all subjects were asymptomatic at the time of testing and free from any medications that may have altered the ENO measurement. In comparison, the Simpson study included subjects who were on β2-agonists during the time of testing. The glaring difference between our studies and those mentioned above is the lack of presence and history of asthma in our subjects.

The results of this study indicate that individuals sensitized to both perennial and seasonal allergens have higher levels of ENO when compared to non-atopic, non-hyperresponsive subjects. This finding is in agreement with that reported by Henriksen et al. A higher concentration of ENO was also found in these subjects when compared to atopic subjects sensitized to only seasonal allergens and those sensitized to only perennial allergens, though only the former was statistically significant (p = 0.037 and p = 0.058, respectively). The clinical significance of finding higher ENO levels in atopic individuals sensitized to both perennial and seasonal allergens needs to be clarified. Variation among these subjects has to be attributed to the individual atopic tendencies. It is reasonable to assume that individuals with year-round allergic rhinitis/conjunctivitis have constant exposure, whether heavy or light, to the causative allergens. Thus, there is some response and resulting inflammation that may be present consistently. If these individuals were also allergic to seasonal allergens, at the height of pollen season those allergies would
ostensibly augment the already existing inflammation. Regardless of the presence of allergy symptoms, penetration of additional allergens into the nose and possibly the lower airways in a sensitized individual would cause further immune reactivity. Two of the atopic subjects sensitized to both perennial and seasonal allergens participating in this study were asymptomatic and had never had any prior allergy or asthma symptoms. Yet they demonstrated higher than normal ENO values (129.4 ppb and 51.2 ppb), which indicate the presence of sub-clinical inflammation. This line of thinking is merely a rationalization of the elevation of ENO levels in the absence of clinical symptoms. The key point to be made here is the degree of relevant allergen-IgE antibody interaction in an atopic individual, regardless of symptoms, and the resulting physiologic conditions.

Whether individuals sensitized to perennial allergens are more susceptible to developing lower airway disease (i.e. asthma) than those sensitized to only seasonal allergens is unknown. The effects of exposure need to be further studied. Moreover, whether a combined effect of perennial and seasonal sensitization exists is unclear. Despite the lack of a significant difference in any of the lung function parameters among these groups, ENO levels as determined in this study seem to indicate that lower airway inflammation is higher due to multiple allergen sensitivity and exposure.

The relationship between ENO levels in atopic individuals and exposure to multiple allergen sensitivity was further investigated. As mentioned in the Results section, a significant positive correlation was found between ENO levels and the number of positive skin test wheal reactions in our atopic subjects. Very simply, as a general trend, when the number of positive skin prick reactions increased so did the concentration of ENO. This is in agreement with Stick et al. A similar relationship between ENO
levels and total serum IgE was observed by Simpson et al\textsuperscript{80}. Once a subject has become sensitized to a particular allergen, subsequent exposure of the airways to high enough levels of allergen might induce cytokine-mediated inflammation and elevated concentrations of ENO. Accordingly, the greater the number of allergens to which an individual has sensitivity and developed specific IgE antibodies, the greater the likelihood of a response after exposure. Though clarification through future studies is still required, if a significant relationship can be established with the number of allergens an individual is sensitive to, ENO again may prove to be a sensitive and reproducible index to predict the presence and degree of underlying inflammation and disease.

4.4 Exhaled Nitric Oxide and Seasonal Variation

This study found that ENO levels were not significantly elevated in 13 atopic subjects sensitized to seasonal allergens during the respective pollen seasons when compared to out-of-season levels. This finding is in contrast with results obtained by Henriksen et al who demonstrated that ENO levels were significantly higher between nonpollen and pollen seasons\textsuperscript{64}. Similarly, Baraldi et al showed that ENO levels increased significantly from nonpollen season to pollen season\textsuperscript{52}. There are several possible reasons why the findings stated above differed from our results. The atopic subjects who were tested during pollen season for a change in ENO levels in the Henriksen et al study were experiencing allergy symptoms and were all on treatment with oral antihistamines at the time of measurement\textsuperscript{64}. The individuals taking part in the Baraldi et al study were allergic asthmatics of whom 57% demonstrated asthma symptoms during pollen season\textsuperscript{52}. In contrast, the subjects who participated in our study were non-asthmatic, free from any allergy symptoms and denied use of any type of medication at the time of pollen season.
ENO measurements. Asthma and other allergic diseases involve an inflammatory process that alters physiologic concentrations of biochemical mediators and cells. By performing BAL and endobronchial biopsy, exposure to allergens, whether seasonal or not, in atopic individuals has already been shown to induce an inflammatory response that involves T-cells and macrophages. The generation of cytokines from activated macrophages and other inflammatory cells would stimulate the amplification of the inflammatory response. The worsening of airways inflammation, whether upper or lower, could be responsible for exacerbation of the disease and the resultant symptoms. This might simultaneously induce type II NOS activity and explain the elevation in ENO levels during a period of high allergen exposure. Both the Henriksen et al study and the Baraldi et al study showed that out-of-season concentrations of ENO, though lower than in-season concentrations, were still significantly higher than the levels found in control subjects. Therefore, even when not exposed to significant levels of allergens and in the absence of symptoms, these allergic subjects still had some lower airway inflammation as shown by elevated ENO levels. These out-of-season values were very similar to the values found in our seasonal atopic subjects out of and during the pollen season. The lack of change in ENO levels in our asymptomatic subjects as opposed to the further elevation of ENO levels in their symptomatic subjects indicates the sensitivity of the ENO response to inflammation. It may be said that the subjects in those studies had more severe allergic conditions than those in our study. Thus when pollen counts increased, the subjects with greater sensitivity responded more strongly, manifested the characteristic symptoms, and exhaled higher levels of NO. Our subjects, though sensitized to the seasonal pollens, probably did not have as severe a response to the allergens, or the disease had not progressed as far as
those mentioned above. Another influencing factor may be the level of pollen for the year in which the study took place. Pollen counts for the Greater Toronto Area were only mild to moderate during the year of study. Though even a slight increase in the pollen load was sufficient to cause most allergic-asthmatics visiting our clinic to develop symptoms and begin to feel discomfort. Regardless of the levels during pollen season, the proposition to remember is that our subjects were not experiencing exacerbation of disease and lacked symptoms during ENO measurement and that may be the reason for contrasting results to the Henriksen et al and Baraldi et al studies. In light of what has just been discussed, ENO measurement may not only be used to reveal lower airway inflammation, but also to define allergic disease severity when allergen exposure is high.

4.5 Exhaled Nitric Oxide: Normal Range

For the last ten years researchers have been trying to establish the nature of ENO levels produced by human beings. The underlying assumption has always been that if concrete testing parameters can be established, ENO measurements can become a rapid addition to existing methods in the assessment of airways inflammation. It has already been demonstrated that asthmatic individuals exhale elevated concentrations of NO. To expand the applicability of this disease marker, a standardized normal range among healthy individuals needs to be determined. Since elevated levels of ENO have now been observed in other respiratory diseases, these conditions must be accounted when establishing a normal range.

The ENO normal range for control (non-atopic, non-asthmatic, non-hyperresponsive) subjects during the course of this study was found to be 14.1 +/- 6.4 ppb (10.0-18.2) (mean (95% CI)). All subjects had a medical history free from lung
disease and negative skin prick tests. were nonsmokers, and did not have any respiratory infections six weeks prior and during the time of ENO measurement. All control subjects had normal lung function and did not have any bronchial hyperresponsiveness. To provide a perspective comparison of this range to the concentrations found in various diseases, ENO levels are provided from measurements performed in our laboratories. Atopic individuals free from anti-histamine or other medicinal treatment were found to have an ENO level of 30.5 ppb (5.0-129.4). Asthmatic individuals who were not on corticosteroid treatment had a level of 51.0 ppb (32.0-87.0). Individuals with chronic obstructive pulmonary disease (COPD) had a mean value of 36.3 ppb (19.3-54.8). A distinct disparity can be seen in the ENO concentrations seen in healthy individuals when compared to individuals with lung diseases or those manifesting an inflammatory process. Accordingly, ENO concentrations also show that asthma may be the airway disease that produces the most severe inflammation followed by COPD and non-asthma allergies. A recent abstract by Ozkan et al. corroborates this assumption and found that asthmatic individuals exhaled the highest concentrations of NO, followed by COPD and then healthy controls. The levels found in the Ozkan et al study were lower than those measured in our laboratories, possibly because they used different measurement system and/or flow rates.

The ENO profiles measured in our control subjects ranged from values that were very low, 4.9 ppb, to those that resembled levels found in atopic individuals, 31.0 ppb. In the past several years, researchers have made significant strides in determining the physiologic character of NO. Though the general issues of a cellular source, inductive triggers, enzymatic catalysis and inflammatory progression are largely resolved, the
behavioral pattern of NO in each individual still may vary. Inflammation in the lower airway other than that evident from asthma may exist in healthy individuals such that it indirectly influences exhalation of NO and is not measurable by conventional methods. Though most findings are in agreement that ENO is one possible method to measure lower airways inflammation, there are still some discrepancies among studies analyzing samples from the same population. ENO levels found in healthy control subjects in one study are often quite different from those found in another. Our levels were found to be slightly higher than those reported in other studies. Some of the incongruity may be attributed to variable testing methods and conditions and the sample population, which in regard to such a sensitive marker would be crucial. Despite standardization of methodology by both the American Thoracic Society (ATS) and the European Respiratory Society (ERS), a common normal range has not been established. This may be because ENO is very sensitive to each human being's individual physiologic characteristics. A joint multi-laboratory study involving samples from several populations might answer some of these questions.

Nonetheless, measurement of exhaled NO may prove to provide a rapid, easily reproducible and noninvasive method to assess lower airway inflammation to determine the disease status of the lungs. However, substantive data is lacking on the applicability of ENO and to which disease conditions it is sensitive. Further scrutiny of this marker is still required before considering its use in the clinical setting. Recent studies have focused largely on asthma and have showed the feasibility of ENO measurement in monitoring lung stability, exacerbations and treatment. This study focused on non-asthmatic atopic adults to ascertain whether inflammation existed in the lower airways. Non-asthma
allergies are usually localized in the upper airways and will often induce some systemic reaction. If, as indicated by elevated ENO levels, non-asthmatic atopic individuals have lower airway inflammation, then ENO may be an early indicator of that inflammation. Measuring ENO levels in atopic individuals may reveal presence of a sub-clinical lower airway inflammatory process.

4.6 Improvements and A Look Ahead

Upon completion of this project, we determined that if ever repeated, there would need to be some changes to improve the quality of the results. First, recruitment of atopic individuals would have to include separate categorization for those with a history of symptoms and those without. Analysis of both symptomatic and asymptomatic subjects under similar conditions would likely clarify the dynamics of ENO with regards to disease severity. Although our self-administered questionnaire examined the history of respiratory illness and allergies, an accurate system to score symptoms and disease triggers would better illustrate the variations in ENO levels according to individual conditions when symptomatic individuals are included. This is important because we are attempting to better understand all factors that may potentially influence ENO measurement. In relation, cross-sectional analysis of perennial and seasonal tendencies would become better focused if a parameter such as a symptom score were added.

The analysis of ENO concentrations in seasonal atopic subjects was performed for three allergens (trees, grasses, and ragweed) with three different pollen seasons (mid-April-May, May-July, and mid-August-October, respectively). The changes that occurred between non-pollen season and pollen season for these subjects were combined into one class. In retrospect, it is clear now that the variations in the nature of the pollens, their
seasonality, and the reactions they produce in human beings would make analysis as a whole somewhat irregular. A far more accurate design would have been to collect sufficient subjects in each pollen group and analyze the fluctuations in ENO levels within a group and then comparatively among groups.

Supplementary testing may also have improved the scope of our results. A repeat methacholine challenge test during pollen season would have indicated any change in bronchial hyperresponsiveness as a result of increased allergen exposure. With this data, not only would we monitor changes in ENO levels, but also changes in lung function. Thus a dynamic relationship between ENO levels and bronchial hyperresponsiveness to methacholine could be established. Midway through this study, equipment for nasal NO was set up, but no measurements were taken in from our subsequent subjects. Nasal NO level measurement in all subjects in conjunction with ENO would have added another dimension in the investigation of airway inflammation. Since most atopic individuals suffer rhinitic symptoms during exacerbations of their allergies, nasal NO levels may have provided an insight into the origin of inflammation and its relation to lower airway inflammation. Lastly, eosinophil counts may have been performed to further explain allergic tendencies and severity of disease. Since the measurement of expired NO principally provides a profile of inflammation in the respiratory system, investigation into the patterns of eosinophil counts might have offered an interactive representation of systemic inflammation.

Future studies that relate to ENO and atopy may be directed towards determining the effects of anti-inflammatory medications such as anti-histamines and nasal corticosteroids. With accurate monitoring of symptoms and administration of drugs
during periods of high allergen exposure, such a study, like those performed in asthmatics, would indicate the efficacy of these anti-inflammatory drugs in controlling the progression of lower airway inflammation. The elevation of ENO may be one of several early signs of development of lower airway inflammation, thus research of its characteristics should continue.
REFERENCES


