Nitric Oxide as a Marker of Smoking Abstinence

by

Renata Tramontini Mena Barreto

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for the degree of Masters of Science
Graduate Department of Institute of Medical Science
University of Toronto

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ABSTRACT

Nitric Oxide as a Tool to Evaluate Smoking Abstinence

Renata Tramontini Mena Barreto, Masters of Science, 2010
Institute of Medical Science
University of Toronto

Introduction: To evaluate the effectiveness of smoking cessation intervention, reliable outcome is essential. Exhaled nitric oxide (ENO) is decreased in smokers, tends to normalize after cessation and might be a good tool to evaluate abstinence. Objective: To evaluate changes in ENO after smoking abstinence of 7 or more days. Methods: 58 smokers in a cessation attempt and 12 non-smokers were recruited: 7 visits for smokers and 2 for non-smokers. Carbon monoxide and cotinine were used to detect smoking status. Results: ENO is decreased in smokers compared to non-smokers (10.8 vs. 20.1 ppb, p<0.001). There was no significant difference in ENO pre and post quitting (p=0.080) although there was a trend to increase as early as 3 days after abstinence (10.78 vs. 15.11, p>0.05). There were no differences in nasal NO measurements (p=0.278). Conclusion: ENO doesn’t seem to be a reliable marker of short-term abstinence.

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# Table of Contents

## Chapter 1: Introduction

1.1 Tobacco Smoking and Smoking Cessation (smoking effects in the upper and lower airways) .......................... 1
1.2 Current Methods to Evaluate Smoking Abstinence
   1.2.1 Self-report ............................................. 4
   1.2.2 Carbon Monoxide ..................................... 5
   1.2.3 Urinary Cotinine ..................................... 8
1.3 Nitric Oxide
   1.3.1 Pathways of Production ................................. 11
   1.3.2 Physiologic Roles of NO Production .................. 12
   1.3.3 Tobacco Smoking and Drug Effects on NO Measurement 17
   1.3.4 Smoking Cessation and NO: Current Knowledge ....... 23
   1.3.5 The Two Compartment Model ......................... 25
   1.3.6 Nasal NO ............................................... 28

## Chapter 2: Rationale and Experimental Purpose

33

## Chapter 3: Hypotheses

35

## Chapter 4: Objectives

4.1 Primary .................................................. 36
4.2 Secondary ................................................ 36

## Chapter 5: Methods

5.1 Subjects .................................................. 37
   5.1.1 Inclusion criteria .................................... 38
   5.1.2 Exclusion criteria ................................... 38
5.2 Design .................................................... 38
5.3 Standardized Procedure for the Online Measurement of Exhaled NO in Adults .......................... 40
   5.3.1 General Aspects of ENO Measurements ............... 40
      5.3.1.1 Requirements for clinical use .................... 40
      5.3.1.2 Online Measurements of ENO in Adults ......... 40
      5.3.1.3 Interpretation of Single Breath NO Profiles .... 43
   5.3.2 Equipment Specifications ............................... 45
5.4 Standardized Procedure for the Nasal NO Output Measurement in Adults .......................... 46
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.3.2-1</td>
<td>Major roles of NOS isoforms.</td>
<td>13</td>
</tr>
<tr>
<td>Table 7.1-1</td>
<td>Demographic data (baseline)</td>
<td>50</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 5.3.1.2 – 1</td>
<td>Diagram of a configuration for the breathing circuit used in the ENO measurements.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 5.3.1.3 – 1</td>
<td>Single breath ENO profile.</td>
<td>43</td>
</tr>
<tr>
<td>Figure 5.3.1.3 – 2</td>
<td>Early peak in ENO measurement.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 7.1-1</td>
<td>Exhaled CO Smokers vs. Non-smokers – Baseline</td>
<td>51</td>
</tr>
<tr>
<td>Figure 7.2-1</td>
<td>Comparison of median ENO levels between smokers and non-smokers.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 7.3-1</td>
<td>Comparison of median nasal NO among smokers and non-smokers</td>
<td>53</td>
</tr>
<tr>
<td>Figure 7.4-1</td>
<td>Bronchial NO output Smokers vs. Non-Smokers – Baseline.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 7.4-2</td>
<td>Alveolar NO Smokers vs. Non-Smokers – Baseline.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 7.5-1</td>
<td>ENO before and after quitting for 10 subjects</td>
<td>55</td>
</tr>
<tr>
<td>Figure 7.5-2</td>
<td>ENO before and after quitting for 7 subjects</td>
<td>56</td>
</tr>
<tr>
<td>Figure 7.5-3</td>
<td>ENO&lt;sub&gt;50&lt;/sub&gt; Baseline and after quitting.</td>
<td>56</td>
</tr>
<tr>
<td>Figure 7.5-4</td>
<td>Bronchial NO output baseline, V1 and V2 after quitting.</td>
<td>57</td>
</tr>
<tr>
<td>Figure 7.5-5</td>
<td>Bronchial NO output levels baseline, V1, V2 and V3 after quitting.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 7.5-6</td>
<td>Bronchial NO baseline and after quitting. Each line represents one subject.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 7.5-7</td>
<td>Alveolar NO after smoking cessation – baseline, V1 and V2.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 7.5-8</td>
<td>Alveolar NO in 7 subjects after quitting smoking.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 7.5-9</td>
<td>Alveolar NO baseline and after quitting. Each line represents one subject.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 7.6-1</td>
<td>ENO measurements in non-smokers – baseline and V2.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 7.6-2</td>
<td>ENO levels of all smokers who quit smoking during the study</td>
<td>62</td>
</tr>
</tbody>
</table>
Figure 7.6-3 – ENO of quitters correcting for the number of cigarettes smoked the day before quitting. 63

Figure 7.7-1 – Nasal NO pre and post-quitting – ANOVA – Baseline, V1 and V2 post-quitting 64

Figure 7.7-2 – Nasal NO pre and post-quitting – Baseline, V1, V2 and V3 post-quitting 65

Figure 7.7-3 – Nasal NO measurements per subject – Baseline and post-quitting. 65

Figure 7.8-1 – ENO in smokers who decreased cigarette consumption in 50% or more 66

Figure 7.9-1 – Nasal NO before and after decreasing cigarette consumption in at least 50%. 67
<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Subject Information And Consent Form</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>Questionnaires – First visit questionnaire</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>Questionnaires – Follow-up questionnaire</td>
<td>96</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’-monophosphate</td>
</tr>
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<td>CA,NO</td>
<td>Alveolar NO concentration</td>
</tr>
<tr>
<td>Caw,NO</td>
<td>NO tissue concentration</td>
</tr>
<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Daw,NO</td>
<td>Airway transfer factor (or diffusing capacity) for NO</td>
</tr>
<tr>
<td>ECO</td>
<td>Exhaled carbon monoxide</td>
</tr>
<tr>
<td>ENO</td>
<td>Exhaled nitric oxide</td>
</tr>
<tr>
<td>ENO&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Exhaled nitric oxide 50ml/s</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ETS</td>
<td>Environmental tobacco smoke</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>J’&lt;sub&gt;AW&lt;/sub&gt;</td>
<td>Maximum total airway flow</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NRT</td>
<td>Nicotine replacement therapy</td>
</tr>
<tr>
<td>PALV</td>
<td>Alveolar NO</td>
</tr>
<tr>
<td>PawNO</td>
<td>Conducting airway NO</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>URI</td>
<td>Upper respiratory tract infection</td>
</tr>
<tr>
<td>VawNO</td>
<td>Airway NO production</td>
</tr>
<tr>
<td>VLNO</td>
<td>Alveolar NO production</td>
</tr>
<tr>
<td>V/Q</td>
<td>Ventilation perfusion matching</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Tobacco Smoking and Smoking Cessation (smoking effects in the upper and lower airways)

Tobacco smoking is the most important source of preventable morbidity and premature mortality(1). From the 1.2 billion smokers in the world, half will die from diseases related to cigarette addiction. Smoking is responsible for 5 million deaths per year, and predictions are that 10 million smokers will die per year by 2025. Prevalence of tobacco smoking varies greatly from 5% to more than 55% in different countries. There is also an impressive variation in prevalence between men and women(2).

Cigarette smokers die on average 10 years younger than non-smokers. However, the earlier smoking is stopped the greater the improvement in life expectancy. Even in older age the benefits of smoking abstinence are still present. Doll et al. demonstrated that quitting smoking at the age of 60, 50, 40, or 30 increases life expectancy on average 3, 6, 9, or 10 years, respectively(3).

Many diseases, such as lung cancer, are known to be caused by cigarette smoking, a relationship confirmed about 50 years ago(2). Cigarette smoking has also been linked to the development of laryngeal and nasal neoplasia, chronic obstructive pulmonary disease (COPD), increased airway responsiveness, impaired pulmonary immune function, increased pulmonary infections and, of course, increased cardiovascular mortality(1).

The US Surgeon General’s report published in 1998 – The Health and Consequences of Smoking: Nicotine Addiction – concluded that cigarettes and other forms of tobacco are addictive, and nicotine is the drug that causes the addiction. Pharmacological and behavioural processes that determine tobacco
addiction are similar to those that determine addiction to illicit drugs such as heroin and cocaine(2;4). High doses of nicotine are absorbed rapidly from smoking, so that pharmacologic effects are evident within seconds after the first inhalation. This high dose and the fast absorption are two main factors that promote addiction(2).

Lung function is clearly affected by tobacco smoking. Destruction of alveolar walls and narrowing of small airways occur promoting reduced elastic recoil and increased compliance, increased gas trapping (which contributes to increased physiologic dead space), a greater annual rate of decline in forced expiratory volume in 1 second (FEV\textsubscript{1}) and diminished vital capacity. It also has some effects in the large airways causing impaired ciliary function and hypertrophic mucosal glands in the bronchi. This increased mucosal gland activity with hypersecretion, together with a thickened submucosa and cellular infiltration, are a substantial part of the pathology of chronic obstructive airways disease (COPD) and its sub-category, chronic bronchitis(5).

Cigarette smoke also induces an acute inflammatory reaction in the airways and lung parenchyma. Increase in bronchoalveolar lavage total inflammatory cells and neutrophil counts are seen in smokers. Furthermore, exposure of non-smokers to environmental tobacco smoke (ETS) for 3 hours results in increased circulating neutrophils, neutrophil chemotaxis to the inflamed airways and neutrophil release of oxidants (1;5).

The effects of smoking in the nasal cavity and paranasal sinuses are not well determined. Numerous potential mechanisms through which smoking can affect mucociliary clearance have been reported and include alterations in the quality (viscosity) or quantity of mucus, disruption of ciliary beat coordination, and destruction of ciliated cells. Smoking has been shown to have an adverse effect on
ciliary function in several animal models. However, studies in human smokers have failed to demonstrate consistent changes in nasal histology or in mucociliary transport in vivo. Adult patients who smoke appear to have an increased trend toward requiring initial and revision surgery for chronic sinusitis. Although there is no direct evidence that tobacco smoke precipitates the sinusitis, studies implicate passive smoking as a major influence on patients with sinusitis(6). Smoking is also known to have a role in the development of squamous cell carcinoma of the head and neck, including the nasal cavity.

Smoking cessation therapies are focused on the physical addiction as well as on the psychological and the behavioural component of the addiction(2). Smoking cessation is achieved by only 6% of smokers who try to quit without any medical help(7). Pharmacotherapy can enhance cessation rates two to three fold and should therefore be considered for all smokers who are willing to attempt cessation. Nicotine replacement, Zyban® (bupropion), and Champix® (varenicline) comprise the first line adjunctive therapy in North America, being all FDA and Health Canada approved(2).

It is unclear if a reduction in daily cigarette consumption is of any health benefit. Smokers who decrease their smoking usually engage in a compensatory behaviour taking deeper breaths, holding their breaths longer and/or smoking the cigarette more completely. A 50% reduction in daily cigarette consumption confers only a 30% decrease in biomarkers for toxicant exposure and only modest reductions in cardiovascular risk biomarkers(2).

Pisinger et al. published a review study in 2006 on health benefits of reduced tobacco smoking. They found 31 publications regarding this subject. Despite the limited data, smoking reduction appears to have a small beneficial effect on cardiovascular risk factors, biomarkers of harm and pulmonary symptoms(8).
Controversy exists regarding the effect of smoking reduction on lung cancer but more recent data suggest a positive effect of smoking reduction on the magnitude of approximately 25% disease risk reduction with 50% reduction in number of cigarettes smoked per day showing that the magnitude of smoking reduction is greater than the magnitude of health benefits associated to reduced tobacco smoking consumption(8).

1.2 Current Methods to Evaluate Smoking Abstinence

To evaluate the success of a smoking cessation intervention it is crucial to have reliable methods available to measure either smoking abstinence or reduction in cigarette consumption. These outcomes can be measured by means of self-reporting by the smoker, by measuring biological markers (biomarkers) of smoking, or by a combination of both(9).

1.2.1 Self-report

Self-report measures are accomplished by written or verbal questionnaires about smoking abstinence and cigarette consumption. It is a technically easy and inexpensive measurement strategy. However, it can be inaccurate when not validated with other measures such as biochemical assessment or informant report. This inaccuracy potential is its primary disadvantage(9). Thompson et al, in 1990, found only a modest correlation between self-report and urinary cotinine (r=.33; p<.001)(10). In 1992, Velicer claimed that misreport in self-completed questionnaires was relatively low, between 0 and 5% and Wagenknecht found 4% prevalence of underreported smoking validated by serum cotinine levels(11;12).

It is known that smokers sometimes provide false information about their smoking status especially if they are under pressure, they tend to answer with a socially desirable response when questioned. About 3% to 10% of patients will answer that they are not smoking when they still are(7). Higher prevalence is found in
participants enrolled in smoking cessation programs; they are commonly subject to bias when reporting their smoking status(13).

Murray et al. identified some of the demographic and smoking factors that are predictors of biased self-report: significant others that would likely prefer that the subject quit smoking; people who are married, who live without smokers at home, and who live with a spouse who has quit smoking or has never smoked(13). Other groups that are likely to suffer social bias are pregnant women and adolescents. Boyd et al, in 1998, verified that pregnant smokers under-report smoking status by more than 26%(14).

1.2.2 Carbon Monoxide
Exhaled carbon monoxide (ECO) is the most commonly used biomarker to ascertain smoking status. The detector is portable, easy to measure, requires simple training, is relatively inexpensive and provides immediate, accurate and reliable results(7;15). Immediacy of results is important because it can be used during the smoking cessation intervention. Sharing results with smokers can influence their subsequent smoking behaviour(16).

A high concentration of carbon monoxide (CO) gas is present in the cigarette smoke. CO can be measured in both expired air and blood and its half-life is of 4-5 hours(17). This short half-life is an important disadvantage of this biomarker. If a person abstains from smoking for several hours prior to the test, the CO level could be in the non-smokers range. As reviewed in Stevens et al., another disadvantage of using CO as a biomarker is that sensitivity decreases with infrequent and irregular smoking patterns, making it difficult, if not impossible, to distinguish light or irregular smokers from non-smokers(9).
An additional confounder is that other sources of exposure to CO can occur, such as atmospheric pollution (including exhaust gases), passive smoking, and occupational exposure, and they can all cause elevation in ECO levels unrelated to primary tobacco smoking. Although these other sources can increase ECO levels somewhat, primary tobacco smoking is the exposure most likely to induce high levels of ECO measurement(15). Some smoking behaviours like depth of inhalation, brand of cigarettes, time since last smoked cigarette and body size can also influence measured CO levels(13).

There is no firm consensus in the literature concerning the cut-off point that distinguishes smokers from non-smokers reliably. Levels equal to or greater than 10 ppm are widely considered indicative of current smoking(7;13).

Murray et al. used the cut-off point of 10 ppm to distinguish between smokers and non-smokers, in the Lung Health Study(13). At the chosen cut-off for carbon monoxide of 10 ppm, 1% of participants in the usual care arm reported not smoking but had elevated ECO (>10 ppm) while participants in the intensive smoking cessation intervention arm had a corresponding rate of 4% self-reported non-smoking but with elevated ECO. Moreover, 6% of participants reported smoking but had negative carbon monoxide results in both usual care and special intervention participants. The direction of this difference is consistent with the occurrence of a socially desirable bias in the verbal responses of special intervention participants. The cut-off value of 10 ppm chosen by the Lung Health Study for CO has a sensitivity of 93.7% and specificity of 87.2%(13).

Chatkin et al. suggested estimating the probability of a person smoking based on using the ECO levels as a continuous variable instead of using a single cut-off point once they observed that patients undergoing the smoking cessation process but still suffering occasional relapses had ECO levels lower than 10 ppm. Using
the likelihood method, patients with an ECO level of 7 ppm had a likelihood ratio of 0.39 (0.14-1.14) of being smoking even if they denied it. When a value of 9 ppm was used, there was a likelihood ratio of 1.50 (0.39-5.73) that the patient was still smoking. They suggested that in these borderline groups other biomarkers could be used to determine the smoking status more accurately. With CO levels ≥ 11 ppm the likelihood of an individual still smoking despite stating otherwise is so high that no further tests are necessary to confirm smoking status (LR 63.80, 95%CI 16.1 to 253.1) (7).

Deveci et al. also reported significantly higher levels of CO in healthy smokers compared to healthy non-smokers (17.13±8.50 ppm vs 3.61±2.15 ppm respectively, p<0.001) and compared to passive smokers (17.13±8.50 ppm vs 5.20±3.38 ppm respectively, p<0.001). Passive smokers had higher levels of CO compared to non-smokers but the difference was not statistically significant (p>0.05). They also found a positive correlation between CO levels and the number of cigarettes smoked daily. The cut-off point of 6.5 ppm was chosen to distinguish between smokers and non-smokers because it gave the highest sensitivity (90%) and specificity (83%) (15). Other studies also found a positive correlation between the daily cigarette consumption and CO levels (18;19).

Jarvis et al reported an optimal cut-off point of 8 ppm with sensitivity of 90% and specificity of 89% (20). Crowley also concluded that a CO level > 8 ppm was strongly associated with self-report of current smoking (21).

Researchers have demonstrated high correlations among CO, self-reported smoking, and urinary cotinine. Associations between CO and urinary cotinine have ranged from r=.76 to r=.79; correlation coefficients between CO and self-report have ranged from r=.65 to r=.70 (p < .001 for all) (16). A comparison of cotinine levels with ECO and self-report showed a high correlation among these
markers for a smoking group but low correlation in a non-smoker group exposed to second hand smoke(22). In the general population, false negative rates of CO measurements have been found to range from 2% to 16%(11).

1.2.3 Urinary Cotinine

Cotinine is considered the gold standard biomarker of tobacco smoke exposure(23). It has been regarded as the most useful biomarker to distinguish between smokers and non-smokers, to estimate the nicotine intake in tobacco users and to check for environmental tobacco smoke (ETS) exposure (9;24).

Tobacco smoke inhalation deposits particles of tar and alkaloids in central and peripheral airways(25). Nicotine, the major and most active alkaloid present in cigarette smoke, is then absorbed rapidly. It is estimated that one cigarette delivers approximately 1 mg of nicotine(26). Both smoker’s inhalation behaviour (e.g., deep or long inhalation) and nicotine metabolism influence nicotine absorption(24;26;27).

Once absorbed, nicotine is metabolized in the liver, where it is converted to cotinine, nicotine-10-N-oxide, nicotine glucuronide, and nornicotine(9). Nicotine’s half-life is approximately 2-3 h and it has limited usefulness as a biomarker of tobacco use(11;28).

Cotinine is the main metabolite of nicotine; 70-80% of nicotine is converted to cotinine(9). It can be quantified in blood, urine and saliva and it is superior to nicotine as a biomarker because of its longer half-life and relative lack of contamination sources during the analytical process(29;30). Urinary excretion eliminates from 10 to 15% of the cotinine in the body; the remainder is furtherly broken down into cotinine glucuronide, trans-3-hydroxycotinine, and trans-3-
hydroxycotinine glucuronide (28). Cotinine has a long half-life (15-40 h) and its level is directly correlated to the nicotine intake (9).

Urinary cotinine has some advantages when compared to serum quantification: its collection is not as invasive as blood collection, and has a significant difference between smokers and non-smokers. There are also some disadvantages in using cotinine analysis. Cotinine levels are affected by nicotine replacement therapy (NRT), making this biomarker not as reliable in this population. The time required for specimen collection and storage requirements are also disadvantages. Finally, it is costly if compared to ECO measurement or self-report, and its results are not available immediately delaying the intervention when necessary (9).

The cut off level of 100 µg/g in urinary measurement has been proposed for differentiation between active smokers and non-smokers (31). As reviewed in Haufroid et al., urinary cotinine levels in non-smokers will always be less than 100 ng/ml urine, even in patients exposed to high levels of environmental tobacco smoke (ETS) (31).

Heinrich-Ramm et al. found that cotinine excretion was closely related to the number of cigarettes smoked daily (p<0.0001). One cigarette adds approximately 41µg/g creatinine in the urinary cotinine level. They also reported that few smokers had cotinine values below the cut off point (100 µg/g creatinine) and most were light smokers (2 cigarettes per day on average). Median cotinine value for non-smokers was 200 times below that of active smokers (5.0 µg/g creatinine) (23).

Time since last cigarette, depth of inhalation, brand of cigarettes, and body size can all influence in CO and cotinine measurements (13). Nicotine is present in some foods and these could be a dietary source of cotinine. There are some
common dietary constituents that contain nicotine: eggplants, potatoes, and tomatoes. Urinary cotinine values of 0.6 and 6.2 ng/ml have been calculated to correspond to average and maximum daily consumption of these foods, respectively. Considering the average daily consumption of these foods, this interference remains negligible except in the case of vegetarians(31). However, not all dietary or other nontobacco sources of nicotine have been studied.

Usually, urine cotinine-to-creatinine concentration ratios are used to correct dilution effects of spot urine analysis(32-34). A recent review showed that it may be unnecessary. Jatlow et al. demonstrated that uncorrected urine cotinine had a much stronger correlation with serum concentrations (r = 0.69; p<0.0005) than did the urine cotinine-creatinine ratios (r = 0.41; p<0.0005)(35). Renal handling of cotinine is markedly different than of creatinine. They suggest that correcting spot urine cotinine concentrations for creatinine content, through calculation of a cotinine-to-creatinine ratio, is not useful at least in smokers, and may even be counter-productive(35).

Murray et al. reported that in an intervention group, discrepancy between self-report and cotinine among self-reported non-smokers was more pronounced in older participants, lighter baseline smokers, in those who reported no use of alcohol, and those who were married. In usual-care participants only age was a significant covariate. Using this same model, they found that discrepancy between self-report and CO among an intervention group was a characteristic of men, lower level of education, and those with more smokers at home. They also verified that because of cotinine longer half-life compared to CO, cotinine measures were less subject to behavioural manipulation than CO(36).

As mentioned before, cotinine measurements are costly when compared to ECO, the most commonly used biomarker of smoking abstinence. Besides the equipment
required for testing, a well trained and experienced technician is important in order to have reliable results. Moreover, the cost of storage can be high and often transportation of material (urine) is necessary. These features add costs to the procedure.

A feature that deserves comment is the fact that many smokers who are trying to quit may be using nicotine replacement therapy (NRT) as an adjunctive tool to help with the cessation process. In these subjects, cotinine is not a reliable marker since they can taper the nicotine amount either by the cigarette consumption or by the NRT.

Another important characteristic of cotinine measurements is that results are not available immediately after the test, delaying any possible intervention for future follow-up visits.

1.3 Nitric Oxide

1.3.1 Pathways of Production
Nitric oxide (NO) is a free radical with moderate reactivity which is responsible for a multitude of organ-specific regulatory functions. NO is synthesized by the oxidative process of the guanidine of the amino acid L-arginine by a family of enzymes named NO synthases (NOS’s). This oxidative process produces NO and L-citrulline(37). Three isoforms of this group of enzymes have been identified: NOS1, NOS2 and NOS3. NOS1 and NOS3 are termed constitutive NOS (cNOS) and were previously known as neuronal and endothelial NOS respectively. The other one, NOS2, is termed inducible isoform (iNOS)(38).
The two cNOS are present in neuronal (nNOS) and endothelial (eNOS) tissues. NO production by cNOS is low, short-lasting and calcium dependent. Inducible NOS (iNOS) synthesizes NO in high amounts and enzyme expression is induced by inflammatory mediators such as certain cytokines, microbes and bacterial products(37;39). NO production by iNOS is delayed by several hours after stimulation, but once induced it is active for periods as long as 5 days(37). There seems to be an interaction among the isoforms as NOS2 may down regulate NOS3 activity(39). The three isoforms can either be found in the cytosol or bound to a variety of membranes, depending on conditions and cell type(40). NOS1 and NOS3 control NO concentration under physiological conditions, in all parts of the body, which seems to fluctuate at rather low levels(41). High levels of NO are produced in the body after induction of expression of NOS2 (inducible isoform) under pathological conditions such as inflammation(41).

NO exerts beneficial effects by acting as anti-bacterial, anti-parasital, anti-viral and anti-tumoricidal agent. Nonetheless, it has some detrimental effects if uncontrolled high levels of NO are present. High amounts of NO can react with superoxide anions generating highly toxic compounds(41). In 1993, a new mechanism of NO modulation was described. NO itself was reported to modulate NO production due to its inhibition of iNOS activity. More recently, NO has been shown to suppress the transcription of the gene encoding iNOS in a variety of cells(41). In cells that contain both inducible and constitutive NOS isoforms, NOS1 and NOS3 can regulate (crosstalk with) the machinery that regulates iNOS expression, using NO as a modulator(41).

### 1.3.2 Physiologic Roles of NO Production

NO is a free radical and has long been known as an atmospheric pollutant present in smog and cigarette smoke, a destroyer of ozone, and a precursor of acid rain. It is generated from the exhausts of motor cars and industrial processes(42;43).
About 10 years ago it was discovered to be a potent vasodilator. Since then, the role of NO in many biological processes has been recognized. Its involvement is well recognized in vasodilation, bronchodilation, neurotransmission, tumor surveillance, antimicrobial defense, regulation of inflammatory processes, blood flow regulation and platelet reactivity. NO enzymes are tailored for different locations and stimuli depending on where NO is required. The main areas relate to blood flow, neurotransmission and non-specific immunity. The major roles of each NOS isoforms are presented in the table 1.3.2-1.

**Table 1.3.2-1 – Major roles of NOS isoforms.**

<table>
<thead>
<tr>
<th>NOS1</th>
<th>NOS2</th>
<th>NOS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotransmitter in: GI tract, penile erection, sphincter relaxation, blood flow</td>
<td>Non-specific immune response to microorganisms</td>
<td>Regulates blood flow, blood pressure</td>
</tr>
<tr>
<td>Synaptic plasticity</td>
<td>Part of inflammatory response</td>
<td>Inhibits platelet activation</td>
</tr>
<tr>
<td>Modulates responses to glutamate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Bruckdorfer(42)

In the airways, NOS enzymes are present in different cell types including airway and alveolar epithelial cells, neuronal cells, macrophages, neutrophils, mast cells, and endothelial and smooth muscle cells.

A simple and general rule about NO’s role was established in the early 1990’s. Low levels of NO, as produced by constitutive NOS isoforms (NOS1 and NOS3), are involved in physiological processes whereas high levels, as produced by
inducible NOS isoform (NOS2), are involved in pathological processes(41). NOS1 and NOS3 are constitutively expressed and activated by mediator-induced or stress-induced cell activation. NOS2 is activated by bacterial products and pro-inflammatory cytokines(43).

In a recent review article, Ricciardolo reports that constitutive NOS is expressed in platelets and in neuronal, epithelial, and endothelial cells. These enzymes are calcium and calmodulin dependent and release NO within seconds upon stimulation (e.g., acetylcholine and bradykinin). Inducible NOS is expressed in macrophages, neutrophils, hepatocytes and epithelial, mesangial, endothelial and vascular smooth muscle cells. It is activated by pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interferon (INF)-g and interleukin (IL)-1b(44). NOS2 releases large quantities of NO (20 times more in comparison to constitutive forms) several hours after stimulation reaching a maximum activity after 24 hours. It may continue in a sustained manner for hours or days(42).

NO takes part in the non-specific immune response. Cells’ exposure to invading microorganisms induces NOS2 to produce large quantities of NO and superoxide anions(42).

Since NO bronchodilator effect was discovered, it has been studied in animals and humans. Hogman et al., in 1993, found that inhaled NO at a concentration of 80ppm had no bronchodilator effect in normal individuals and in patients with chronic obstructive pulmonary disease (COPD), but had a small effect in asthmatic patients(45). NO, as neurotransmitter, is involved in the inhibitory non-cholinergic non-adrenergic response in central and peripheral airways(46;47). In allergic airway inflammation, NO induced relaxation is impaired although no changes in nNOS expression were identified, indicating impaired nNOS activity which contributes to exacerbation of asthma(48).
Nijkamp et al. in 1993 demonstrated that NOS inhibitors had the ability to potentiate bronchoconstriction induced by histamine in vivo or histamine concentration dependent contraction of tracheal tubes *in vitro* in guinea pigs. They suggested that NO has a modulator role in airway hyperresponsiveness (49).

Previous *in vitro* and *in vivo* studies have shown that increased hyperresponsiveness induced by allergens is not potentiated by pretreatment with NOS inhibitors and that virus induced hyperresponsiveness is blocked by low doses of inhaled L-arginine suggesting that deficiency of endogenous NO predisposes to airway hyperresponsiveness (44).

Inflammatory respiratory diseases are usually characterized by increased levels of NO due to increased expression of NOS2 in respiratory epithelial cells as well as in inflammatory-immune cells. Excessive production of NO can lead to accelerated metabolism to a family of potentially harmful reactive nitrogen species (RNS) that are thought to be involved in the etiology of inflammatory lung disease (50-52). Despite the evidence of elevated levels of NO in inflammatory lung diseases, its role is still not clear. Exhaled nitric oxide (ENO) does not reflect the actual production of NO in the respiratory tract and is not well correlated with NOS activity in the lungs (53). Increased levels of NO have been described in patients with asthma, atopy, bronchiectasis, rhinitis, and acute lung allograft rejection (44). In patients with asthma, levels of NO may reflect exacerbation and disease severity (54-56).

On the other hand, low levels of exhaled NO are reported in patients with cystic fibrosis, PiZZ phenotype related alpha1 antitrypsin deficiency and pulmonary hypertension (44). Data on ENO in COPD patients are controversial with some studies showing increased levels and others showing decreased levels (44; 57; 58).
Treatment of asthma with steroids results in reduction of ENO due to reduction of underlying inflammation in the airways and to inhibitory effects on iNOS expression itself. Both oral and inhaled steroids have shown to decrease ENO rapidly and dose dependently(44). Even low doses of inhaled steroids can reduce ENO levels to normal in patients with intermittent or mild persistent asthma(59). It is still uncertain if this represents optimal disease control. In patients with more severe persistent asthma, increased levels of ENO are observed even after treatment with high doses of oral or inhaled steroids showing that the airway inflammation exceeds the impact of corticosteroids on ENO in this group(60).

ENO is increased in subjects with chronic cough due to clinical conditions associated with eosinophilic airway inflammation such as cough variant asthma and eosinophilic bronchitis(61-63). Prieto et al. found that 44% of subjects with chronic cough respond well to inhaled corticosteroid (ICS) therapy. They concluded that ENO levels at baseline do not have usefulness in predicting ICS responsiveness in patients with chronic cough. Also, airway hyperresponsiveness (AHR) to methacoline or adenosine 5’-monophosphate (AMP) does not seem to predict response to ICS in this population(64).

Nagaki et al. findings suggest that NO has a stimulatory activity in airway submucosal gland secretion(65). Jain et al. findings suggest that an NO-dependent mechanism upregulates ciliary motility in response to stimulation(66). Ciliary motility is an important component of the unspecific host defense mechanism of the airways and is enhanced by the iNOS inducing alveolar macrophage derived cytokines such as TNF-α and IL-1b(67).

NO also has an important role in the regulation of transepithelial ion movement and changes in its production can play an important part in the pathophysiology of lung disorders characterized by hypersecretion of airways(68).
Recently an antiproliferative effect of NO on airway smooth muscle has been described. In vitro studies have shown that exogenous administration of NO reduces DNA synthesis and proliferation of airway smooth muscle cells. This new NO role may be an important feature to help preventing airway remodeling in patients with asthma and COPD(69;70).

1.3.3 Tobacco Smoking and Drug Effects on NO Measurement

Inhaled cigarette smoke has acute and chronic effects on ENO. Many investigators have observed reduced levels of exhaled NO in smokers(1;5;58;71-75). Its concentration has been shown to increase after smoking cessation(5;73;76). This lower concentration of ENO in smokers has been implicated in the pathogenesis of smoking related diseases(77). Cigarette smoke causes damage to the respiratory epithelium which is responsible for most ENO production. Moreover, smoking related decline in ENO levels may be a marker of respiratory epithelial damage(78).

There are a few possible mechanisms by which ENO levels are reduced in smokers. Cigarette smoke contains high concentrations of NO (74.5-1008 ppm) which may directly lead to a down regulation of NO synthase (NOS), the enzyme responsible for NO formation from L-arginine(79-82). Alternatively, cigarette smoke may inhibit NO production at the NOS gene expression level through as yet unidentified means(72;73;79-81). Other possible mechanisms mentioned in another study are an inadequate supply of cofactors necessary for NO production and an increase in the breakdown of NO(76). Another proposed explanation is that the increased levels of CO in smokers’ airways may decrease expired NO because NOS is a cytochrome P-450-type hemoprotein that could be inhibited by the high concentrations of CO in the lungs of cigarette smokers(83). Persson et al. suggested that smoking may cause toxic damage to NO producing cells(84).
Anatomical studies of the lungs provide some explanation of the lower ENO levels in smokers. Autopsy studies of asymptomatic smokers showed respiratory bronchiolitis, denuded epithelium of the membranous bronchioles, and muscular hypertrophy accompanied by fibrosis of the bronchial walls. A reduction in the number of respiratory tract epithelial cells, NO producers, could explain the reduced NO levels in the airway lumen\(^\text{85}\). The iNOS appears to be the major NOS isoform in the airways and its induction is responsible for a three to fivefold elevation of NO being produced by the conducting airways in inflammatory diseases such as asthma\(^\text{86}\). The down-regulation of iNOS could explain the decreased levels of NO in smokers\(^\text{87}\).

Reduced levels of ENO were first observed in smokers in the early 1990s by Schilling et al. and Persson et al.\(^\text{74;84}\). This effect was described to occur after both acute and chronic exposure to smoking\(^\text{72}\). Lower levels of ENO were also found in healthy subjects and asthmatic children exposed to second hand smoke\(^\text{1;88}\).

Hogman et al., in a study from 2002, found that ENO at an expiratory flow rate of 0.1 litre/s was significantly lower in smokers compared to non-smokers (\(p=0.007\)). Calculations to show the contribution of different areas of the lung showed that NO flux was significantly lower in smokers compared to non-smokers (\(p<0.001\)). On the other hand, alveolar fraction was significantly higher (\(p=0.006\))\(^\text{5}\).

Travers et al., in 2007, found that non-smokers have ENO 1.18 (95% CI, 0.92–1.51; \(p=0.20\)) times higher than current smokers and 1.13 (95% CI, 0.97–1.31; \(p=0.11\)) times higher than ex-smokers. They found a reference range from 7.8 to 41.1 ppb for ENO in normal subjects. Sex, atopy and, to a lesser extent, smoking status influenced normal values, and these factors were included in the reference ranges derived from the multivariate model\(^\text{89}\)
Smoking cessation leads to an increase in ENO levels and, in one report, NO levels normalised after smoking cessation(5;73).

Chambers et al., found an unexpected result while measuring ENO after acute smoke exposure. They found an increase in NO levels one and ten minutes after smoking a cigarette (2.6-4.8, p<0.0001; 2.6-3.2, p=0.003 respectively). They suggest that NO is “trapped” at the epithelial surface of the lower respiratory tract in the form of bioequivalent oxides of nitrogen such as peroxynitrite and S-nitrosothiols, and that this trapping mechanism will be redox sensitive. They further suggest that oxidant stress increases the lower respiratory tract NO concentration and that the chronically reduced state of the epithelial lining fluid in habitual smokers is reflected in a decreased lower respiratory tract NO concentration between cigarettes(77). It contradicts the findings of Kharitonov et al. that showed a reduction in NO levels five minutes after smoking when assessing peak NO concentration in the exhaled breath. This measurement methodology has since been abandoned, as it may include contaminating nasopharyngeal NO(72).

According to Tsang et al., there is no association between age and ENO in non-smoking young adults(90). Sundy et al. suggested that there is an age-related decline in ENO levels that is greatest in older smokers(78). Sundy et al. also found that serum cotinine levels were inversely correlated with ENO in smokers, suggesting that lower levels of ENO were correlated with higher cigarette smoke exposure levels(78).

Kharitonov et al., in 1995, found a strong correlation between the number of cigarettes smoked and ENO(72). Malinovschi et al., on the other hand, didn’t find a dose-response relationship when studying the number of cigarettes smoked and levels of ENO. One possible explanation for those divergent findings is the use of
different ENO parameters and measurement methodologies. While Kharitonov used peak ENO to report their results, Malinovschi used plateau ENO, following the latest recommendations(76).

Malinovschi et al. studied the effects of smoking in flow-independent NO exchange parameters. Using three different flow rates (50, 100 and 500 ml.s\(^{-1}\)) they calculated the three flow-independent NO exchange parameters confined to the two compartments, the conducting airways, which are characterised by the lower mean NO tissue concentration (Caw,NO) and airway transfer factor (or diffusing capacity) for NO (Daw,NO), and the alveoli, characterised by the alveolar NO concentration (CA,NO). They found that current smokers have significantly lower ENO at a flow rate of 50 ml.s\(^{-1}\) (ENO\(_{0,05}\)), lower mean NO tissue concentration and maximum total airway NO flux than never smokers. Current and ex-smokers differed only in terms of lower mean NO tissue concentration (p=0.02). In the multivariable analysis, a significant association was also found between alveolar NO concentration and current smoking. Age was positively associated with alveolar NO concentration (p=0.001). Ex-smokers showed a significantly lower ENO\(_{0,05}\) than never smokers, although the other NO variables didn’t show any significant difference. Non-smokers exposed to second hand smoking had significantly higher alveolar NO concentration than non-smokers not exposed to second hand smoking, whereas, other ENO variables didn’t present any significant differences. The main finding of their study was that current smokers have a lower level of mean NO tissue concentration and alveolar NO concentration(76).

The use of flow-independent NO exchange parameters may aid understanding the location of tobacco-induced changes in airway NO metabolism and exchange(76).
In 2002, Högman and coworkers reported that smokers have decreased levels of ENO, diminished conducting airway NO (PawNO), but normal levels of airway NO diffusing capacity (DawNO) and alveolar NO (PALV). Airway NO production (VawNO) in smokers was 42% lower than in their control subjects(91). On the other hand, another study conducted by the same group found significant elevations of alveolar NO (PALV) and reduction of conducting NO (PawNO) when comparing smokers and non-smokers(5). In a recent study by Brindicci et al. in 2005, they also found a significant elevation of alveolar NO and a reduction of airway NO production in smokers(92).

Pietropaoli et al., in 2007, found that decreased values of ENO in smokers are due to decreased NO production in the conducting airways without significantly decreased production by the alveolar compartment(87). They did not use the recommended technique as stated in the ATS recommendations published in 2005, making comparisons using results from this study more difficult. Pietropaoli et al. found that smokers had a threefold reduction in NO produced by conducting airways. In contrast, NO concentration in the alveoli was only slightly reduced in smokers. It concludes that reduced levels of ENO in smokers results from a threefold reduction in NO production by the conducting airways that enters the airspaces. In contrast, the alveoli of smokers produce the amount of NO observed in age-matched nonsmokers(87).

Four studies that measured flow independent NO parameters in smokers and non-smokers using multiple measurements of expired NO at different constant expiratory flow rates found a 1.5 to 3-fold reduction in NO produced by conducting airways in smokers(5;87;91;92). Only a 4% reduction in diffusing capacity of NO from the walls of the conducting airways into the bronchial lumen was found in smokers but there was a 50% reduction in the concentration of NO in
the walls of the conducting tissues(91;92). Thus smokers have reduced NO production by the airways without impaired diffusion between tissues and airways.

Hogman et al. mentioned that it is known that tobacco smoke affects the surfactant system. It influences the structure as well as the function of the surfactant system which prevents alveolar collapse and protects the lungs from injuries and infections caused by inhaled particles and micro-organisms. The higher levels of alveolar NO seen in Hogman’s et al. could possibly be a sign of such impairment. They speculate that due to partial alveoli collapse, the inhaled NO might not be fully metabolized in the alveoli and therefore will return to exhaled air. Hence an increased alveolar NO might be a prognostic sign of lung damage in asymptomatic smokers(5). One point that has to be considered in smokers is that they have higher risk of developing lung cancer, and high ENO levels have been found in smokers with lung cancer(93). They suggest that NO monitoring can be used to indicate improvements when a smoker decides to stop smoking(5).

The literature on the effects of other drugs on ENO is very limited. Yates et al. found a significant decrement in peak ENO in asthmatic patients after ethanol consumption but this was not observed in a non-asthmatic group(94).

Other studies have measured the effects of alcohol on tissue NO in animal models. Chronic exposure to ethanol led to an increased expression and activity of endothelial NOS as well as an increased release of NO(95). In the brain the effect of ethanol on NOS is diverse and not completely understood(96).

Marijuana and crack-cocaine seems to impair NO production decreasing the bactericidal activity of alveolar macrophages(97). Their effect on ENO hasn’t been studied.
1.3.4 Smoking Cessation and NO: Current Knowledge

It is well established that ENO levels are lower in current smokers. With that, the next question is whether ENO levels would change after smoking cessation. There are only 2 studies that have addressed this issue following healthy smokers during a period of abstinence.

In the first study Robbins et al measured ENO in 14 smokers after 1 week of abstinence and its level had risen in all subjects. They used three different methods to access exhaled NO (peak oral, mean oral and nasal), and found higher NO levels with all methods (63±7 vs. 31±3 ppb, p=0.0029), (9.0±0.8 vs. 5.7±0.4 ppb, p=0.0004), and (41±5 vs. 25±2 ppb, p=0.0025), respectively. The peak oral and mean oral NO levels were still depressed compared to non-smokers’. However, with the nasal method, exhaled NO had risen to a level that was not significantly different than in normal non-smokers. After 8 weeks, 10 smokers were still abstinent and there was a further increase in NO levels when measured by the peak oral method (100±9 vs. 63±7 ppb, p=0.0006) or mean oral method (10.3±0.6 vs. 9.0±0.8 ppb, p=0.043). With the nasal method, NO levels also increased compared to week 1 but this difference was not statistically significant. Despite its limited number of subjects, their study suggests that the effects of cigarette smoking on ENO are reversible(73).

In the second study Hogman et al measured ENO levels in 5 different flow rates to obtain the contribution of both alveolar fraction of NO and NO flux from the airways. They used the model of Tsoukias and George to calculate the alveolar concentration of NO and the flux of NO from the airways(98). ENO 0.1L/s was significantly lower in smokers compared to non-smokers (p=0.007). NO flux was significantly lower (p<0.001) and alveolar fraction was significantly higher (p=0.006) in smokers compared to non-smokers. Nine subjects stopped smoking and after 2 weeks of smoking cessation both alveolar NO and NO flux had risen.
but the difference was not statistically significant. After 4 weeks of abstinence, however, significant increase in both ENO (p=0.03) and NO flux (p=0.02) were seen. They concluded that cigarette smoking results in lower NO flux and that it can be restored to normal values by 4 weeks of non-smoking. There was a slight but not significant increase in ENO just after 1 week(5). They postulate that the increase in NO fraction in the alveolar gas could be a sign of impairment in the surfactant system. Tobacco smoke influences both the structure and the function of the surfactant system. They further suggested that alveolar NO may be a diagnostic tool of lung damage and NO monitoring can be used to indicate improvements when a smoker stops smoking(5).

Another group tried to address this issue in asthmatic and bronchitis/COPD smokers. They found that ENO is highly variable in asthmatic smokers. Possible reasons for this variation were the extent of smoking, the variable severity of asthma and the usage of inhaled corticosteroids (ICS). However, ENO showed a moderate variation in bronchitis/COPD smokers. After a 3 month follow-up after smoking cessation, ENO remained unchanged in both asthmatics and bronchitis/COPD patients. Exact comparisons between the 2 groups are difficult because most asthmatic patients were using ICS whereas most bronchitis/COPD were not. The group concluded that unchanged ENO levels suggest that oxidative/nitrosamine stress does not change markedly during 3 months after smoking cessation. Despite the lack of changes in oxidative stress biomarkers, the group found a decline of symptoms after the 1st month of smoking cessation. It suggests that clinical improvement does not necessarily correlate with objective assessment of asthma/COPD or that these biomarkers may not be the best ones in regard to clinical relevance in COPD and/or that the mechanisms of COPD are still poorly known(99).
1.3.5 The Two Compartment Model

The alveolar and the airways are two different regions in the lungs, each one with its own features. To better understand the contribution of different regions of the lungs in ENO production and metabolism, a model was developed by Tsoukias et al. They suggested that their model may be a simple, effective and reproducible way to determine the contribution of each compartment (airways and alveoli) in ENO(98). It consists of 2 main compartments: a rigid or non-expansible compartment representing the conducting airways, and a flexible or expansible compartment representing the respiratory bronchioles and alveolar region. The most important feature from a gas exchange perspective is the fact that the alveolar volume is expansible and the airway volume is relatively non-expansible. This model describes some basic features of NO exchange observed experimentally: 1) breath holding before exhalation creates an initial spike in NO concentration, 2) exhaled NO concentration is an inverse function of exhalation flow rate and 3) elimination rate of NO is a positive function of expiratory flow(98).

Endogenous NO is produced by cells in the airway mucosa and the alveolar membrane and can follow one of three paths: 1) consumption through reaction with substrates within the tissue compartments, 2) diffusion toward the pulmonary or bronchial circulations, where it reacts instantaneously and irreversibly with the hemoglobin, or 3) diffusion toward the airstream, where it evaporates and enters the alveolar volume or airway volume. Consequently, there will be a net flux of NO between the tissue and the airstream in both compartments(98). Hence, ENO will depend on the exchange of NO in the alveolar compartment and the conditioning of the alveolar gas as it passes through the airway compartment(98).

ENO measurement is performed at a fixed rate of flow and provides a marker of bronchial inflammation. On the other hand, alveolar concentration of NO (CANO), obtained from multiple flow measurements, has been proposed as an
indicator of inflammation in the most distal portions of the respiratory system as well as a reflection of endothelial events(100).

In brief, in the model the volume of ENO is plotted against expiratory flow rate. The slope of the curve represents the alveolar fraction, and the intercept is the NO flux from the airways(98).

The inspired concentration of NO affects the early peak observed in ENO measurements. Inspiration of NO free air obliterates the early peak in the absence of breath hold. Expiratory concentration of NO is independent of inspiratory concentration if the duration of exhalation is greater than 8 seconds(98).

George et al. determined that the flow rates and the methods used to analyze ENO data affect the estimation of NO flow-independent parameters(101). In a recent article that used a linear regression method and ENO measurements at three different flow rates ranging 100 – 200 mL.s$^{-1}$, it was shown that decreasing the highest flow rate increased the estimated CA$_{NO}$(102).

Silkoff et al., in 2000, showed that the diffusing capacity of NO from the walls of the conducting airways into the bronchial lumen (DawNO) and concentration of NO in the walls of the conducting airways (PawNO) could also be calculated from the analysis of multiple expired breaths(103).

Use of flow independent NO exchange parameters may aid understanding the location of cigarette smoking induced changes in airway NO. In theory, Caw$_{NO}$ (airway tissue NO) would be estimated more accurately by using a flow rate as low as possible, since measured ENO would be Caw$_{NO}$ at a flow rate that tends towards 0 ml. s$^{-1}$. Malinovschi et al. found a reduction in airway tissue NO in current smokers which is in accordance with previous study(76;91). They found
that current smoking was associated with reduced alveolar NO concentration. They also found that both current and past smokers have reduced levels of ENO(76).

Hogman et al., using Silkoff’s analysis measuring ENO at 3 different flow rates, reported that smokers have decreased levels of ENO, low concentration of NO in the walls of conducting airways, but normal values for diffusing capacity of NO and alveolar NO concentration(91;103). Another study by the same group showed significant elevations of alveolar NO concentration and reduction of NO concentration in the conducting airway walls(5). Brindicci et al. also found significant elevation of alveolar NO concentration levels in smokers and reduced amount of NO produced by the conducting airways (VawNO)(92).

Pietropaoli et al. concluded that the lower levels of ENO in smokers are caused by decreased NO production in conducting airways without significant decrease in alveolar production. They found that smokers have a three fold reduction in NO produced by conducting airways (VawNO). Meanwhile, concentration of alveolar NO (PALV) was only slightly decreased. Alveolar NO production (VLNO) was equivalent in smokers and non-smokers(87).

A comparison of three studies that used flow independent NO at different flow rates in smokers and non-smokers showed that all investigators found a 1.5- to 3-fold reduction in amount of NO produced by the conducting airways (VawNO) in smokers(5;91;92). There was only a 4% reduction in diffusing capacity of NO from the walls of the conducting airways into the bronchial lumen (DawNO) in smokers but a 50% reduction in the concentration of NO in the bronchial wall (PawNO).
Hogman et al. used the model of Tsoukias and George to calculate the alveolar fraction of NO and the NO flux from the airways(98). Five different flow rates between 0.05 and 0.32 litre/min were used. They found that ENO at 0.1 litre/s was significantly lower in smokers (p=0.007). Calculations for the contribution of different areas showed that NO flux was significantly lower (p<0.001) and alveolar fraction was significantly higher (p=0.006) in smokers compared to non-smokers. In the same study, after four weeks of smoking cessation they found a significant increase in ENO$_{0.1}$ (p=0.03) and NO flux (p=0.02). They suggest that increased alveolar NO in smokers might be a sign of lung damage and NO monitoring could be used to indicate improvements when a smoker stops smoking(5).

Fortuna et al. found that the mean value of CANO is 3.04 (±1.30) ppb (range, 1.45-6.31 ppb), which falls in the range of 1.0 to 5.6 ppb previously described in the literature for healthy persons(100;104;105). These values are also consistent with distal airway NO measurements (CANO) obtained by means of bronchoalveolar lavage during fiberoptic bronchoscopy in healthy individuals(105). CANO quantifies alveolar damage and monitors the course of disease being useful as a marker of alveolar inflammation in diseases involving the lung periphery (severe asthma, interstitial pneumonia, COPD). Lower CANO levels observed in smokers compared to ex-smokers and non-smokers could be explained by the association between endothelial dysfunction and toxic effect of tobacco(106;107).

1.3.6 Nasal NO

The nasal cavities, the nasopharynx, and the paranasal sinuses excrete NO(108;109). In the upper airways, human paranasal sinuses are the major source of NO that passes through the sinus ostia and makes a large contribution to the
levels of NO found in the nasal cavity(109). There is also a smaller contribution of
the nasal mucosa, upregulated via iNOS during inflammation(108).

Silkoff et al. showed that nasal congestion following nasal allergen challenge was
associated with a significant fall in nasal NO which returned to baseline levels
after the congestion resolved. They concluded that it indicates that nasal NO
doesn’t have a role in the control of basal capacitance vessel caliber(110).
Kharitonov et al. reported similar results after allergen challenge(111).

On the contrary, other study demonstrated that ostial occlusion as seen in upper
airway allergy or infection, caused by mucosal swelling, would result in negative
pressure and hypoxia inside the sinus. Hypoxia is a powerful inducer of NOS
resulting in an increased nasal NO production(112).

One possible explanation for this fall in nasal NO after allergen challenge is the
acute congestion and sinus obstruction, as the sinuses are a major contributor of
nasal NO concentration. Alternatively, nasal hyperemia associated with the
congestive response may increase NO absorption, as hemoglobin is a powerful
ligand of NO(110).

In a review study published in 2001, Jorissen et al. comment that nasal NO levels
rise from birth to the age of 10 years, when they reach the normal adult level. This
finding supports the paranasal origin of nasal NO as it rises until the age of 10
years when the sinuses reach their final constitution(113).

On the other hand, recent evidence suggests that the sinuses might not be the main
site of NO production. It is reported in a review study that high levels of NO were
found in neonates right after birth, even before the sinuses have developed(114).
There are different techniques to measure nasal NO and a lack of consensus on measurement techniques. This leads to different findings of nasal NO concentrations in different illnesses such as sinusitis, polyposis, and allergic rhinitis(112). Exceptions are primary ciliary dyskinesia and cystic fibrosis which are known to cause low levels of nasal NO independently of measurement technique. Nasal NO can be used as a screening tool in these diseases(115-117).

Airflow generation through the nasal cavities is required to measure nasal NO. It can be achieved by aspirating or insufflating air via one nostril. Velum closing is necessary to close the pharynx and isolate the nasal cavity from the oral air. It can be achieved by using a positive pressure of 5 to 10 cmH₂O by blowing against a resistor(114). Transnasal airflow produces a washout phase followed by a steady NO plateau(112). In Jorissen’s review, nasal NO levels are reported to vary from 200 to 2000 ppb and are always higher than NO levels from the lower airways. The variety of results in nasal NO measurement is due to different measurement techniques, physiological variations, and pathological changes(113).

Regardless of the technique used to measure nasal NO, it is essential to record NO levels only when a steady state plateau is achieved. Many studies use a low airflow rate which prevents air penetration to the deeper parts of the nose due to laminar flow. Turbulence is essential for achieving maximum nasal NO output. Flows below 1 L/min may not represent the maximum nasal NO output because it fails to mimic the aerodynamics of nasal respiration. Flows between 3 and 6 L/min are similar to the maximum physiologic flow rate (6 L/min at least). Using this flow range, a stable plateau is reached within 20 seconds in most subjects(118;119). NO output is the product of NO concentration and flow. It is essential to measure nasal NO at a known and fixed flow rate. Using flow rates within a flow range that
gives maximal and stable NO outputs makes these measurements at different flow rates become comparable(114).

NO produced in the upper airways has been shown to be a modulator of pulmonary function, improving ventilation perfusion (V/Q) matching. The reduced inhalation of nasally produced NO as in mouth breathing in sleep disorders and after tracheotomy may contribute to the negative effects of these diseases(114). Autoinhalation of NO may represent an important physiologic advantage of nasal breathing compared with oral breathing. Lundberg et al. showed that nasal breathing improves oxygen saturation when compared with mouth breathing(120). Clearly, there is increasing evidence that nasal NO may have beneficial effects on pulmonary function(114).

Studies in sinus mucosa reported NO to be a regulator of mucociliary activity in the upper respiratory tract. Nasal NO strongly correlates with saccharine test changes providing support to previous studies that nasal NO reflects the sinonasal mucociliary function. Low levels of nasal NO correlate with impaired mucociliary activity. Patients with Kartagener’s syndrome have very low nasal NO despite the presence of patent sinuses(121).

Ragab et al. showed that there was no relationship between nasal NO changes and age, sex, smoking, skin prick test positivity, grass pollen sensitivity and family history of allergy(121).

It is also reported that studies in both adults and children showed that topical nasal steroids significantly lowered elevated levels of nasal NO in patients with seasonal and perennial allergic rhinitis(121).
Kharitonov et al. found that untreated rhinitis patients with and without asthma had nasal NO 1.5-fold higher than healthy controls(111). Using the same measurement method, Djupesland et al. found substantially higher NO outputs in symptomatic seasonal allergic patients compared with healthy controls at different aspiration flows(118). Arnal et al. also found a significantly higher nasal NO in patients with perennial and/or seasonal rhinitis with and without active symptoms compared with healthy controls(122). On the contrary, Henriksen et al., using the aspiration method, didn’t find significant difference in nasal NO levels between allergic rhinitis patients and controls during the pollen season(123).

A review study reports that smokers have lower levels of ENO and nasal NO when compared with age- and sex- matched non-smokers. The reason could be a toxic effect of inhaled smoke causing a down regulation in NOS and/or the disruption of NO-producing cells(113). The same study reports that no significant difference in nasal NO levels was found during and after an episode of upper respiratory tract infection(113).
Chapter 2: Rationale and Experimental Purpose

Since it is crucial to confirm abstinence from smoking when testing new smoking cessation drugs or strategies, it is important to develop and study biomarkers that could play a role in this function. From previous research, it is known that smokers tend to under-estimate their tobacco consumption, especially if they are under social pressure to do so.

Biomarkers available in the clinical setting have different sorts of potential problems. As discussed before, CO has a short half-life and smokers can have the measurement in the non-smokers range if their last cigarette was smoked hours before the test. Urinary cotinine has a longer half-life but is still not long enough to measure long term smoking cessation outcomes reliably. Besides that, cotinine is not a reliable tool in patients receiving nicotine replacement therapy (NRT), especially if using gum or inhaler because they can “taper” (control) the nicotine dosage. It is impossible to determine if the urinary cotinine levels found in these patients are related to the NRT or to cigarettes smoked while using NRT. Moreover, urinary cotinine is a relatively invasive test, requires storage facilities and results are not available immediately delaying the intervention when it is necessary.

In order to determine if ENO can be used as a biomarker for long-term smoking abstinence, it is essential to have a better understanding of ENO profile after smoking cessation. Few papers in the literature report that ENO increases after smoking cessation. The methodology used in different publications is different, making comparisons between studies difficult if not impossible.

Furthermore, little is known about the impact of smoking and smoking cessation on different NO compartments, and nothing is known about smoking and nasal
NO. While NO flux seems to be reduced in smokers along with total ENO, alveolar NO has shown to be either normal or increased.

The purpose of the present research protocol is to study the ENO profile after smoking cessation, including measurements of different NO compartments and nasal NO. Measurements were performed in a frequency of twice a week during a three week period whether subjects abstained successfully or not.
Chapter 3: Hypotheses

We hypothesize that NO can be used as a biomarker of smoking cessation and relapse. Like ECO measurements, ENO measurements could be made rapidly in a clinic setting where results could be incorporated into counseling feedback and prescribing decisions. Unlike ECO measurements, ENO measurements appear to change over days to weeks rather than minutes to hours and thus may be more accurate in the face of patient attempts to confound the measurements by short term abstinence prior to clinical visits. Unlike cotinine assay, measurement of ENO would be available rapidly and remain useful in the presence of nicotine replacement therapy.
Chapter 4: Objectives

4.1 Primary
To determine the exhaled NO profile in smokers who are quitting smoking.

4.2 Secondary
To define whether ENO (total) can be a marker of smoking abstinence.
To determine if ENO increases in people who decrease cigarette consumption.
To evaluate alveolar NO in smokers and non-smokers and determine if it changes with cessation or decreased consumption of cigarettes.
To evaluate the effects of smoking cessation or decreased consumption in nasal NO.
Chapter 5: Methods

5.1 Subjects
Study subjects were recruited from the Residential Treatment Program at the Centre for Addictions and Mental Health (CAMH) – Toronto, Canada. This is a 21 day intensive psychosocial treatment program that is initiated after a detoxification period for various drug dependencies. Participants are voluntary, and the program is designed to help them learn how to stop or reduce their drug use and its impact on their life. The use of any kind of drugs but cigarettes was absolutely forbidden during the admission (including any sporadic use of marijuana). All patients in the Residential Program were closely monitored in regards to their previous addictions. They were all periodically assessed with drug screening tests to assure abstinence. Patients who had a positive drug screening were excluded from the Residential Program as well as from the research protocol.

During the 21 day admission period, a smoking cessation program is one of several auxiliary programs offered on a voluntary enrolment basis. On average, 8 new clients are admitted each week, and it is expected that the majority are smokers who will join the smoking cessation program.

Smokers who intended a cessation attempt during this 3 week program and agreed to participate in the study were recruited. The control group consisted of 10 non-smokers who were also taking part in the residential program.

We chose this population because of both the convenience of access and the prolonged admittance period. These characteristics were ideal in order to perform several consecutive measurements in each subject. According to information gathered from program staff, most of these patients were smokers and the majority would voluntarily join the smoking cessation program. All measurements were
conducted at the institution. The length and structure of the program provide the opportunity to follow ENO profile in a relatively stable environment. Moreover, patients with addictions have a high prevalence of smoking and from our knowledge there are no studies in the literature analyzing the ENO in this population after tobacco smoking abstinence.

Concomitant psychiatric disorders were not objectively assessed in the protocol but their presence was not an exclusion criteria.

5.1.1 Inclusion criteria
- Smokers and non-smokers enrolled in the Residential Program at CAMH.
- Smokers must be interested in trying a quitting attempt.

5.1.2 Exclusion criteria
- Asthma diagnosed by a physician.
- COPD diagnosed by a physician.
- Abnormal pulmonary function test results at the baseline visit (FEV1/FVC (forced expiratory volume in the first second/ forced vital capacity) <0.7, FEV1 <70% of predicted and/or FVC <70% of predicted will be excluded).

5.2 Design
This is a cohort study where subjects were followed for up to three weeks.

We intended to compare exhaled NO levels and other biomarkers in smokers who started a quitting attempt while participating in the Residential Program at CAMH. Exhaled NO levels were also compared between smokers and non-smokers. Subjects were recruited at admission to the program. All subjects who provided
informed consent and met the entry criteria were enrolled in the study. There were 7 study visits for smokers and 2 visits for the non-smoking controls.

- **Visit 1 (all participants):**
  Baseline measurements:
  (a) medical history related to lung disease with particular reference to disorders that may alter ENO concentrations
  (b) anterior rhinoscopy (non-invasive exam of the nasal cavities) to evaluate for nasal diseases that can interfere in nasal NO measurements (e.g. nasal polyps) but those subjects were not excluded from the protocol.
  (c) pulmonary function (spirometry)
  (d) exhaled nitric oxide measurement (ENO)
  (e) exhaled carbon monoxide (ECO)
  (f) urinary cotinine
  (g) nasal NO.
  (h) self-report questionnaire administered by an interviewer to assess tobacco consumption and to address addictions history.

- **Visits 2 to 6 (smokers only):**
  Measurements of ENO, nasal NO, ECO, and urinary cotinine, and administration of a self-report questionnaire were repeated two times per week over the 3 weeks admission period.

- **Visit 7 (all participants):**
  Measurements of ENO, nasal NO, ECO, and urinary cotinine, and administration of the self-report questionnaire

Exhaled NO (ENO) profile is the main objective of the present study. ENO measurements were compared to exhaled CO (ECO) and urinary cotinine; the
latter was used as the gold standard for smoking status among subjects who were not using nicotine replacement therapy.

5.3 Standardized Procedure for the Online Measurement of Exhaled NO in Adults

5.3.1 General Aspects of ENO Measurements

5.3.1.1 Requirements for clinical use
Exhaled NO measurements have been primarily used in the research setting but in 2003 the FDA approved the first NO analyzer (Aerocrine AB, Stockholm, Sweden) for clinical monitoring of anti-inflammatory therapy in asthma.

Online measurements of ENO involve real time monitoring of NO breath levels and mouth pressure profiles. Offline measurements of ENO involve collection and storage of exhaled gas in an appropriate vessel for posterior analysis(124).

Many authors have published ENO values in healthy subjects, but variable measurement techniques and methods reduce the usefulness of the data. Ideally, there should be an interinstitutional agreement of mean ENO within 10% for each age group. ENO terminology, units, general principles of measurement and influencing factors are reviewed in: ATS/ERS Recommendations for Standardized Procedure for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005 (124).

5.3.1.2 Online Measurements of ENO in Adults
Online measurements of ENO provide a real time display of ENO concentration versus time or exhaled volume along with other testing variables such as airflow and pressure, whereas offline testing refers to collection of exhalate into suitable
receptacles for delayed analysis. Online sampling permits monitoring exhalation flow and pressure parameters to ensure accurate NO plateau. Inadequate exhalations can be immediately identified and discarded.

Chemiluminescence is one of the methods used to measure NO. It is based on the intensity of fluorescent radiation emitted after chemical reaction oxidation of NO by ozone using a sensitive photomultiplier tube (PMT). This reaction produces NO2* that emits a photon. The total number of photons produced is proportional to the NO concentration(125).

Recommended Standard technique for online measurements of ENO in adults (Figure 5.3.1.2 – 1)

- **Inspired gas source** – Although there is evidence that ambient NO levels do not affect the single-breath plateau levels of ENO ambient NO levels were recorded. An NO filter was used in the inhalation circuit to prevent subjects from inhaling NO in the ambient air. Inhalation of high levels of NO causes an early peak in ENO profile that takes time to wash out increasing the time elapsed until a plateau is reached therefore resulting in prolonged exhalation(126).

- **Inhalation procedure** – Nose clips were not used at any time. Nose clips cause accumulation of nasal NO and promote contamination of exhaled air via the nasopharynx. Subjects were seated comfortably, with the mouth piece at the proper height and position. They were asked to inhale over 2 to 3 seconds through the mouth to total lung capacity (TLC) or near to TLC and then exhaled immediately preventing any breath holding.

- **Exhalation procedure** – To ensure reproducible and standardized measurements it is important to exclude nasal NO and standardize the exhalation flow rate. Nasal NO was excluded asking subjects to exhale
against an expiratory resistance with a positive mouthpiece pressure\(^{(126;127)}\). This pressure was displayed to the subjects so that they could 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 and nasal argon insufflation\(^{(126;127)}\). Mouthpiece pressure was maintained at 20 mm Hg and no greater to ensure velum closure and avoid subject’s discomfort.

**Figure 5.3.1.2 – 1**: Diagram of a configuration for the breathing circuit used in the ENO measurements.

C - computer; ER - expiratory resistance; FM - flow meter; IG - inspired gas; MP - mouthpiece; NO-SL - nitric oxide sampling line; PG - pressure gauge; P-SL - pressure sampling line; V - three-way valve.

- **Standardization of exhalation flow rate**: This study employed five different flow rates. All subjects started their measurements with the lowest flow rate and increased progressively: 50 ml/s, 100 ml/s, 150 ml/s, 200 ml/s and 250 ml/s.
5.3.1.3 Interpretation of Single Breath NO Profiles

Constant flow rate exhalation results in a single-breath NO profile. The profile indicates a plot of ENO levels versus time. It consists of a washout phase followed by an NO plateau which is usually reproducible and relatively flat (Figure 5.3.1.3 – 1).

![Figure 5.3.1.3 – 1](image)

Figure 5.3.1.3 – 1 – Single breath ENO profile.

Figure shows three measurements of ENO each consisting of a washout phase followed by a reproducible plateau.

An early peak may be seen right after the washout phase and followed by the plateau if the subject inhales through the nose or the velum is not completely closed as exhalation begins (Figure 5.3.1.3 – 2). Similarly, this early peak can occur if there is NO in inhaled air or if the subjects held their breaths before exhaling. If a peak was noticed in the profile, the subjects were instructed about the correct maneuver and the test was repeated. If the profile remained unchanged, early peaks were ignored and only the plateaus were interpreted.
Figure 5.3.1.3 – 2 - Early peak in ENO measurement.
Figure shows two measurement curves of ENO. The first curve has a washout phase followed by a plateau. The second curve has a washout phase followed by a peak in ENO levels and then the plateau.

Exhalation was ensured to be sufficiently long to obtain an accurate plateau in the NO versus time profile. The plateau was drawn manually, based on visual inspection, using the computer program tool designed for that purpose. The tool is a straight line placed over the plateau on the curve. Each measurement (curve) is analyzed separately. The profile was at least 6 seconds long to obtain a reasonable plateau. The plateau concentration was then defined over a 3 second window on the plateau profile. Measurements were repeated until reproducible levels were reached. Three NO measurements that agreed within 10% of the mean value were recorded. Final ENO levels were reported as a mean of the plateaus. A minimum of 30 seconds of relaxed tidal breathing off the analyzer was allowed between measurements to allow subjects to rest. Subjects were not overexerted if satisfactory measurements were not achieved.
5.3.2 Equipment Specifications

For this research project we used a chemiluminescence analyzer. The equipment used in the study is not portable, but can be set up in a fairly small space with easy access, facilitating or not causing any extra difficulties for patients.

Ancillary Equipment – The following apparatus are the integral part of the NO analyzer (Figure 5.3.1.2 – 1).

- Output device – The analyzer used in this study possessed both an analog and digital output and RAM storage card. Measurement of ENO was performed on the Sievers 280 (Sievers Co., Boulder, Colorado), a rapid linear-response chemiluminescent analyzer.
- Data collection – Real time monitor display of collection conditions, including NO, pressure, and flow, was used to sense the quality of exhalations and accurate plateau.
- Biofeedback parameters – A display was used for subjects to maintain constant flow rates.
- Sample flow rates – A rotameter was used to display NO analyzer flow rate and was an integral component of the Sievers system.

Calibration Requirements and Procedures

- Zero NO gas – A reliable zero NO gas is essential for ENO measurements. A pure nitrogen gas was used as the zero-NO calibration gas (Praxair, Canada).
- NO standard calibration gas – Specially prepared NO calibration gases are used for this purpose, mostly diluted in nitrogen. In our study we used a gas with 9.75 ppm concentration of NO (Praxair, Canada). A gas of 2% accuracy level is recommended to insure reproducibility of unknown samples. These gases remained stable for over six months.
• Calibration – Calibrations were performed daily using the zero NO gas and the standard calibration gas of known NO concentration. The procedure was performed daily before starting the measurements. It was rechecked during the day if the equipment had been used for longer than four hours.

5.4 Standardized Procedure for the Nasal NO Output Measurement in Adults

5.4.1 General Aspects of Nasal NO Measurement
Measurement of nasal NO requires generation of airflow through the nasal cavities. Airflow generation was created by aspirating air from one nostril while the velum was closed. A constant transnasal airflow was created which produced a washout phase followed by the establishment of a plateau in the nasal NO profile. Nasal NO concentration is inversely related to the created airflow. The transnasal airflow was rigorously controlled since aerodynamics of the flow alters nasal NO output.

Nasal NO output is the product of nasal NO concentration and flow rate. According to Djupesland et al. flows between 3 and 6 L/min are similar to the maximum physiologic nasal airflow (6 L/min at least)(118). Velum closure is required to prevent loss of nasal NO to the posterior velopharyngeal aperture or entry of lower respiratory air into the nasal cavity. It was achieved by asking the subjects to exhale orally against resistance.

5.4.2 Measurement Technique of Nasal NO in Adults
Subjects were seated comfortably to perform nasal NO measurements. A soft silicone tube shaped to fit and occlude most nostrils without traumatizing was adapted to one of the subject’s nostril. A vacuum pump was fitted to the tube to create a constant airflow of 6 l/min.
Subjects were instructed to inhale through the mouth and then exhale, through the mouth as well, into the mouthpiece targeting a pressure of 10 cmH$_2$O (enough for velum closure). While this exhalation was proceeding, air was being aspirated through one nostril as above. A side port just distal to the nasal silicone tube sampled gas for the NO analyzer. A steady plateau was achieved in most subjects within 20 seconds.

Three determinations of steady plateau NO that vary < 10% were performed for each subject during each visit and the mean was reported as the nasal NO output. Nasal NO was always measured after lower airways ENO.

5.4.3 Equipment Specifications

The same analyzer used for lower airway NO measurement in this research project was used for nasal NO measurement. For more information refer to item 3.4.3 – Equipment Specifications for Standardized Procedure for the Online ENO measurement in adults.

For nasal NO measurement we added to this equipment a suction pump and a flowmeter to control the airflow rate.
Chapter 6: Statistics

Normally distributed variables were described as mean and standard deviation. Non-normally distributed variables were described as median (interquartile). Comparison of quantitative data was carried out using Student’s t-test for different groups and Paired t-test for measurements in the same group. When normality test failed, Mann-Whitney Rank Sum test was used.

One Way Multiple Measures ANOVA was used to compare normally distributed data when multiple comparisons were made. Friedman Repeated Measures ANOVA on Ranks was used for data that failed normality test.

For ENO profile after smoking cessation, an exploratory analysis of quitters’ data was performed. We then calculated the 95% confidence interval of non smokers and smokers using all their measurements (multiple observations per subject), and the 95% bound was used to differentiate them from quitters. ENO data for quitters were standardized for the number of cigarettes the subjects smoked. We used the complete data of all smokers to generate a regression equation of NO on number of cigarettes smoked in the previous 24 hours. We then used the residual values from this data set to calculate the upper and lower 95% confidence interval of smokers. Finally, we used the equation to estimate the predicted level of NO for different numbers of cigarettes smoked.

Based on a previous study we estimated to see a 50% increase in ENO levels after 2 weeks of smoking abstinence(5). We therefore calculated that a sample size of 10 subjects would be necessary to detect such change (80% power; p≤0.05).
Chapter 7: Results

7.1 Sample Characteristics
A total of 83 subjects volunteered for the study. Thirteen didn’t meet inclusion criteria and 70 were included. Fifty eight subjects were smokers and 12 were non-smokers. Seven subjects from the smokers group and 2 from the non-smokers group didn’t complete the study (either because of positive screening test for drugs or consent withdrawal), ending the study with 51 smokers and 10 non-smokers who completed the protocol. Non-smokers were defined as either never-smokers or ex-smokers who had not been smoking for at least one year.

A total of 16 subjects were able to abstain from smoking for at least 24 hours during the study. Eleven smokers were able to abstain from smoking for at least 7 days during the study period, 21 decreased their cigarette consumption in at least 50% during three or more consecutive visits, and 19 subjects continued smoking steadily during the whole period.

None of the subjects had past history of lung disease, including asthma or COPD. Two subjects from the smokers group were not able to perform spirometry although they had no history of pulmonary disease and were asymptomatic. They were not excluded from the analysis. There was no statistically significant difference in spirometry between smokers and non-smokers. Ten smokers and 4 non-smokers had a previous clinical diagnosis of allergic rhinitis.

As mentioned before, these subjects were ex drug addicts who were participating in the rehabilitation program. Fifty three percent of them were abstinent for more than 1 month (12% more than 3 months), and the remaining 47% were abstinent for at least 1 week.
Alcohol was abused by 50.8% of smokers (in 14% it was the only abused drug) and 58.3% of non-smokers (in 50% it was the only abused drug). Narcotics were used by 19.3% of smokers and 33.3% of non-smokers. Crack and/or cocaine had been used by 64% of smokers and 30% of non-smokers. Intermittent use of marijuana was reported by 43% of smokers and 40% of non-smokers.

Demographic data of the subjects are summarized in table 7.1-1.

Table 7.1-1 – Demographic data (baseline)

<table>
<thead>
<tr>
<th></th>
<th>Smokers</th>
<th>Non-smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>58</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.2±9.6</td>
<td>47.4±9.6</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>44/14 (75.8% males)</td>
<td>10/2 (83.3% males)</td>
</tr>
<tr>
<td>FEV1 %PRED</td>
<td>92.0±11.0</td>
<td>93.6±14.0</td>
</tr>
<tr>
<td>FVC %PRED</td>
<td>93.0±11.5</td>
<td>92.8±13.1</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>82.0±5.5</td>
<td>82.2±2.7</td>
</tr>
<tr>
<td>Pack-Years</td>
<td>17.3±13.5</td>
<td></td>
</tr>
</tbody>
</table>

CO levels were significantly higher in smokers. Median level for smokers was 26 ppm (15 – 36) whereas for non-smokers was 4 ppm (3 – 5), as shown in figure 7.1-1.
All subjects, including smokers and non-smokers, were able to perform satisfactory and reproducible ENO curve measurements.

ENO$_{50}$ level in non-smokers was two-fold greater than in smokers (figure 7.2-1). Median ENO value for smokers was 10.8 ppb (7.8-15.3) whereas for non-smokers was 20.1 ppb (17.7-27.8) (p<0.001).
Figure 7.2-1 – Comparison of median ENO levels between smokers and non-smokers.

7.3 Nasal NO output Smokers vs. Non-smokers

Nasal NO output was similar between smokers and non-smokers [458.0 (363.0-521.5) and 478.0 (433.5-514.0) nl/minute respectively, P=0.404]. It was not possible to measure nasal NO in 3 smokers and 1 non-smoker. Two subjects had discomfort during the test due to nasal obstruction and the test was interrupted. An adequate plateau level was not achieved in the other two subjects despite various measurement attempts. They had no complaints of nasal obstruction.

As demonstrated in figure 7.3-1, smokers had a wider range of variation in nasal NO output compared to non-smokers. Lower levels (< 300 nl/minute), were only seen in smokers, but there was no statistically significant difference between the two groups. None of the non-smokers had levels bellow 400 nl/minute.
7.4 Bronchial and Alveolar NO Smokers vs. Non-smokers

Smokers had bronchial NO levels two times lower when compared to non-smokers [0.46 (0.34 – 0.64) and 0.97 (0.84 – 1.19) nl/seconds, respectively, $P\leq 0.001$].

Figure 7.4-1 – Bronchial NO output Smokers vs. Non-Smokers – Baseline.
Alveolar NO tended to be lower in smokers than in non-smokers, although this trend was not statistically significant (1.69±0.79 and 1.95±0.69 ppb respectively, P = 0.286).

<table>
<thead>
<tr>
<th>Alveolar NO (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>2.0</td>
</tr>
<tr>
<td>2.5</td>
</tr>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>3.5</td>
</tr>
<tr>
<td>4.0</td>
</tr>
</tbody>
</table>

Figure 7.4-2 – Alveolar NO Smokers vs. Non-Smokers – Baseline.

7.5 ENO Before and After Quitting Smoking

A total of 16 subjects abstained from smoking for at least 24 hours during the study period. From this group, 11 subjects stopped smoking for at least 7 days, the maximum length of cigarette abstinence being 19 days. All subjects were checked for abstinence through a self-report questionnaire, ECO measurement and urinary cotinine measurement in each visit. Subjects who reported not to be smoking, who had ECO levels ≤ 10 ppm, and cotinine levels < 100µg/g urine were considered quitters. When NRT was used as a cessation tool, self-report and ECO were used to check for abstinence.

All subjects in the study had ECO and urinary cotinine measurements performed to confirm their smoking status. From the group of 10 smokers who quit, 7 used NRT as a tool to help them throughout the quitting process. As NRT interferes in urinary cotinine, ECO as well as the self-report questionnaire were used to confirm
their smoking status. All 7 subjects who reported not to be smoking had ECO levels below 10 ppb during their abstinent period. From the 3 subjects who did not use NRT and reported being abstinent from tobacco smoking, 2 had both ECO and urinary cotinine levels that remained in the non-smokers range. The third subject had a relapse detected in visit 4 by a high urinary cotinine level. The ENO levels for this subject during his abstinence period were therefore analyzed up to visit 3.

Ten subjects completed baseline visit, v1 post-quitting (3±2 days) and v2 post-quitting (7±2 days). One subject who stopped smoking for 7 days didn’t attend v1 post-quitting, and is not included in the ANOVA analysis.

In these 10 subjects, the mean ENO increased from 10.78±5.84 ppb to 15.11±8.89 ppb at visit 1, and to 13.90±7.86 ppb at visit 2, but these shifts from baseline were not statistically significant (p=0.080).

![Figure 7.5-1](image)

**Figure 7.5-1** – ENO before and after quitting for 10 subjects

Seven of these patients completed 3 visits post quitting {baseline, v1 post-quitting (3±2 days), v2 post-quitting (7±2 days) and v3 post-quitting (10±1 days)}. Again, there was a trend towards an increase in the mean ENO levels that was not statistically significant (p= 0.277). ENO increased from 9.67 ppb (7.04 - 17.68), to

**Figure 7.5-2** – ENO before and after quitting for 7 subjects

Figure 7.5-3 shows the measurements performed for each subject who stopped smoking for 2 or 3 visits post-quitting.

**Figure 7.5-3** – ENO\textsubscript{50} Baseline and after quitting.

Four subjects from our smoking cessation group got a cold during the study. There was a noticeable increase in ENO levels in the first measurement performed during the symptomatic period compared to the baseline level in three of them.
The other subject didn’t present any change in ENO level. Eight smokers who didn’t quit also developed a cold during the study period. From this group, four had noticeable higher levels of ENO during the symptomatic period. Only one subject from the non-smoking group developed respiratory infection and no change in ENO level was observed.

Two subjects from the smokers group developed allergy symptoms during the study and ENO levels were somehow higher during the symptomatic period. However, this change didn’t seem to be as prominent as the changes seen in subjects who developed respiratory infection.

ANOVA analysis was used for bronchial and alveolar NO separately. Bronchial NO output was analyzed at baseline, V1 and V2 in 10 subjects. Although there was a trend towards an increase in bronchial NO after cessation, it was not statistically significant (P = 0.088).

![Figure 7.5-4](image.png) – Bronchial NO output baseline, V1 and V2 after quitting.

The same type of analysis was used to evaluate bronchial NO output in seven subjects who achieved 10 or more days of abstinence (baseline, V1, V2 and V3...
after quitting). There was no statistically significant difference among bronchial NO output levels during this follow up period, as bronchial NO values ranged from 0.51±0.28 nl/seconds, to 0.64±0.45 nl/seconds, 0.64±0.41 nl/seconds and 0.64±0.29 nl/seconds respectively (P = 0.441). There was an increase in bronchial NO levels in V1 compared to baseline but this level was kept through the following measurements.

![Graph showing bronchial NO output levels](image)

**Figure 7.5-5** – Bronchial NO output levels baseline, V1, V2 and V3 after quitting.

A graph showing the bronchial NO behaviour after quitting in this group of subjects who completed 10 days without smoking is shown in figure 7.5-6.
Figure 7.5-6 – Bronchial NO baseline and after quitting. Each line represents one subject.

There were no changes observed in alveolar NO levels after smoking cessation in 10 subjects comparing baseline, V1 and V2 (P = 0.637). We couldn't identify any trends in alveolar NO levels as for ENO and bronchial NO.

Figure 7.5-7 – Alveolar NO after smoking cessation – baseline, V1 and V2.

A similar finding was seen in the 7 subjects who completed 3 visits post quitting, as demonstrated in figure 7.5-8 (P = 0.478). Median levels of alveolar NO seem to lower in this group of patients after cessation.
Figure 7.5-8 – Alveolar NO in 7 subjects after quitting smoking.

A graph showing the measurements from each subject separately is shown in figure 7.5-9.

Figure 7.5-9 - Alveolar NO baseline and after quitting. Each line represents one subject.
7.6 ENO Profile and Quitters – Correlation Between Days Without Smoking and Increase in ENO

We wanted to see whether there was a value that could be used to distinguish a quitter from someone who continued smoking. We found that there was considerable variation both within and between individuals in the outcome variable NO and that an exploratory type analysis, rather than a confirmatory-type analysis, would be better to study the ENO equilibrium after quitting.

Non-smokers had their ENO levels measured twice during the study period (figure 7.6-1). First measurement was performed during the first 2 days in the program and last measurement during the last 2 days in the program. The interval between the 2 measures was around 18 days. There was no statistically significant difference between the 2 measures in this group.

![Figure 7.6-1 – ENO measurements in non-smokers – baseline and V2.](image)

Data describing ENO changes in quitters is described in figure 7.6-2. This graph shows individual patterns following smoking cessation rather than group means. Also shown in this figure is a line indicating the lower 95% confidence bound of
NO of non-smokers which was 17.94 ppb. The confidence interval was calculated based on all values of all non-smokers. We decided that using multiple measures per subject would be appropriate because of the small sample of non-smokers and our objective of best capturing the range of variation in this measure. With this type of graph we can compare the behaviour of a quitter to a non-smoker. When a quitter has an ENO level above the lower 95% confidence bound, the quitter is statistically indistinguishable from the non-smokers. We noticed that smokers who quit will rarely cross the lower 95% confidence bound of ENO of non-smokers.

Figure 7.6-2 – ENO levels of all smokers who quit smoking during the study

We then compared ENO of quitters to ENO of smokers. We used all data from all smokers to generate a regression equation of ENO on number of cigarettes smoked in the past 24 hours. The residual values from that data set were used to calculate
the upper and lower 95% confidence interval of smokers, which was 10.98 – 12.62. We did it because we wondered if some of the variation in the ENO among smokers might be due to the number of cigarettes they had smoked in the previous 24 hours.

Among quitters, the ENO values shown in figure 7.6-3 are really the deviations of ENO on all days from ENO values from the day they quit (corrected for the number of cigarettes smoked in the previous 24 hours). This is why many of the values in figure 7.6-3 are lower than zero. Adjusting the data in this way did result in some reduction of the variation. As seen in the figure, measurements in most subjects remain indistinguishable from smokers throughout the period they were observed.

**Figure 7.6-3** – ENO of quitters correcting for the number of cigarettes smoked the day before quitting.
7.7 Nasal NO Before and After Quitting

Nasal NO output values were analyzed pre and post-quitting using ANOVA. Nine subjects had their nasal NO measured in the baseline visit, v1 post-quitting (3±2 days) and v2 post-quitting (7±2 days). Repeated Measures ANOVA on Ranks was used for analysis. As seen for ENO respiratory levels, there was a non statistically significant difference in nasal NO output levels before and after quitting smoking during this maximum 7±2 days follow-up (P = 0.278).

![Figure 7.7-1 - Nasal NO pre and post-quitting - ANOVA - Baseline, V1 and V2 post-quitting]

Seven subjects had their nasal NO output measured in the baseline, v1 post-quitting (3±2 days), v2 post-quitting (7±2 days) and v3 post-quitting (10±1 days). One Way Repeated Measures was applied and there was no statistically significant difference between nasal NO levels before and after quitting during this period of follow-up (P = 0.329).
Figure 7.7-2 – Nasal NO pre and post-quitting – Baseline, V1, V2 and V3 post-quitting

A graph showing nasal NO measurements for each subject is shown in figure 7.7-3.

Figure 7.7-3 – Nasal NO measurements per subject – Baseline and post-quitting.
7.8 ENO Before and After Decreasing Cigarette Consumption in at Least 50%

Twenty one smokers decreased their cigarette consumption in at least 50% for at least 3 consecutive visits during the study. We didn’t find a significant difference between ENO levels in the baseline visit and the 3 visits after decreasing cigarette consumption (13.97±7.52 and 13.90±8.38 ppb respectively, P = 0.962).

![Figure 7.8-1](image-url)

Figure 7.8-1 – ENO in smokers who decreased cigarette consumption in 50% or more

7.9 Nasal NO Before and After Decreasing Cigarette Consumption in at least 50%

Nasal NO output was also analyzed in smokers who decreased cigarette consumption in 50% or more. From the 21 smokers in this group, 20 had their nasal NO measured and analyzed. One subject had severe nasal obstruction and couldn’t have the measurement performed. There was no statistically significant difference between nasal NO levels after decreasing consumption for 3 consecutive visits {462 (382 – 498.51) and 444 (365.01 – 508.5) nl/minute, respectively, P = 0.304}.
Figure 7.9-1 – Nasal NO before and after decreasing cigarette consumption in at least 50%.
Chapter 8: Discussion

8.1 Resolution of the Hypotheses

8.1.1 Primary
a. We didn’t find a significant change in ENO levels after smoking cessation in our study group, after a period of 3, 7 or 10 days.

8.1.2 Secondary
a. ENO did not prove to be a reliable marker of smoking abstinence.
b. There were no significant changes in ENO levels in subjects who decreased their cigarette consumption in at least 50%.
c. Smokers and non-smokers have similar levels of alveolar fraction NO. There were no significant changes in alveolar NO levels after smoking cessation or reduced cigarette consumption.
d. There were no significant changes in nasal NO levels after smoking cessation or reduction.

These results were observed in subjects without history of asthma or its symptoms although there were some subjects with history of allergic rhinitis. Subjects who had an upper respiratory infection (common cold) during the period of three weeks prior to the study were excluded. Subjects who developed upper respiratory infection during the study period and were already enrolled were not excluded.

8.2 ENO profile and smoking cessation
First of all, we were able to effectively confirm previous literature data, showing that smokers have lower levels of ENO when compared to non-smokers.
We couldn’t show a significant change in ENO levels of smokers during the first 10 days of abstinence. There was a trend towards increasing values as early as three days after quitting but we could not show statistical significance. One of the possible reasons is that there was a significant range of variation in ENO values between subjects and within subjects.

It is probable that a type II error occurred in our study and a larger sample size would be necessary to show any statistical significance. More subjects might have shown a trend towards higher levels of ENO after one or two weeks of abstinence. This trend could become statistically significant but the data collected in our study were already sufficient to show that individual changes in ENO during the first week or two of abstinence are too small to confirm abstinence or resumption of cigarette smoking. We based the sample size calculation on a paper by Hogman et al. in which they demonstrated a 50% increase in ENO after 2 weeks of abstinence with a relatively small standard deviation (5). If we had based the sample size calculation on the paper by Robbins et al. even a smaller sample size would have been predicted (73). The wide standard deviation in our sample was unexpected and having a larger sample could have brought statistical significance. On the other hand this wide variation is exactly what makes this test unreliable for detecting smoking abstinence in individual patients.

Another possible explanation is the relatively short time of abstinence in this group. ENO failed to show a statistically significant difference after short time of abstinence but this finding does not rule out a monitoring application for ENO in the medium to longer term. These long term abstinence data were not available in the present study and might be a good research question for future studies.

Literature shows that smokers have lower levels of ENO compared to non-smokers (1; 5; 58; 71-75; 84). Furthermore, ex-smokers have ENO levels similar to
never smokers, thereby higher than current smokers (89). Our baseline findings agree with the literature: smokers had ENO levels lower than non-smokers.

Most studies published in the literature regarding this issue use current and previous history of smoking. We found only 2 studies that followed smokers who were trying to quit smoking and measured their ENO levels (5; 73). In these studies they measured ENO levels in intervals of 1 week, 2 weeks, 4 weeks and 8 weeks. Our study differs from these 2 previous publications because we measured ENO in shorter intervals (intervals of 3 or 4 days).

Hogman et al. measured ENO in weekly intervals (week 0, week 1, week 2 and week 4) after smoking cessation. Exhaled CO measured at the same time as ENO was used as an indication of smoking abstinence. Ten smokers signed a contract stating that they would stop smoking for a period of 4 weeks. Hogman didn’t find a significant increase in NO levels after 2 weeks of smoking cessation. However, after 4 weeks there was a significant increase compared to baseline (5).

It is well known that subjects under pressure tend to hide their relapses and state that they are not smoking even if they are still smoking intermittently (7; 13). CO, as discussed earlier, has a short half life and subjects who are smoking sporadically or who haven’t smoked hours before the test will have measurements indistinguishable from non-smokers (9). Some of the subjects may have had a relapse and the authors wouldn’t be able to identify it. These are some potential downsides of this study that could have interfered and biased the results.

Robbins et al. measured ENO 1 week and 8 weeks after smoking cessation (73). Again, CO was used as a marker of abstinence. Moreover, they used a non-standardized technique to measure ENO, making it difficult to compare their
results with other papers that use the standardized technique recommended by the ATS 2005(124).

Robbins found that NO levels, whether measured by peak oral, mean oral or nasal method, rose after 1 week of smoking cessation, and the difference was statistically significant for all three methods. After 8 weeks of smoking cessation, all three NO measures had a further rise but only the peak oral and the mean oral methods were statistically significant compared to the previous visit. Both peak oral and nasal NO levels at this point did not differ statistically when compared to the normal non-smoking controls(73). Robbins et al. used an NO measurement technique that is no longer used. First they used nose clips which are known to increase nasal NO concentration and, consequently increase the risk of contamination of ENO. Second, they didn’t perform online measurements. Exhaled breath was stored in polyvinylfluoride bags and analyzed 5 minutes later. Third, nasal NO levels were measured through air exhaled into a nasal mask, which probably had lower airways air contamination. This reinforces the importance of measuring nasal NO levels in smokers who quit smoking.

While in one study changes could be seen as early as one week, in the other it took, 4 weeks for NO to rise to a significant level, and 8 weeks to rise back to normal or nearly normal levels. One of our aims was to see whether NO would change in a short interval after smoking cessation, and could therefore be used as a tool for abstinence monitoring.

Due to some methodological problems with the former studies, our protocol intended to answer more clearly whether ENO could be used as an early and perhaps more stable marker of smoking abstinence. Some of these methodological issues included the use of CO as the only marker of smoking abstinence and it is well known that smokers tend to hide their relapses. It is impossible to confirm
that these subjects, in both studies, were abstinent for the whole study period just by measuring their CO levels in each visit.

This is one of the strengths of our study. We used the urinary cotinine and CO levels as gold standards so that the chances of misclassifying any subjects were lower. It is true that some of the subjects who were trying to stop smoking were using NRT as an adjuvant tool making it difficult to use cotinine to check for abstinence. In these particular cases, CO was used as gold standard. Another important feature in this regard was the short interval between visits making it more difficult for the study subjects to hide their relapses.

Moreover we measured ENO in accordance to the standardized method published in 2005 and in a shorter interval making it easier to recognize changes. The study by Robbins et al. used a technique to measure ENO that is no longer in use. The technique that has been widely used is the one followed in both Hogman’s study and our protocol.

Another important factor we noticed is that ENO presents a wide variation in consecutive measures in the same subject. Bohadana et al. found a reproducible ENO within-subject between-session measures which conflicts with our findings. They state that the variability increases as ENO level increases. Log-transformation is suggested to minimize this feature(128). We found an expressive variation within-subject between-session in ENO levels. Some of the factors associated with this variability were the presence of allergies, or the development of common respiratory tract infections. In some subjects, however, there was no obvious explanation for this variation. Another interesting feature we could notice is that most smokers have low levels of ENO eventhough they still presented a wide range of variation in between session measures.
Another factor that needs to be considered is the impact of intercurrent illness on ENO levels. Respiratory viruses can increase iNOS expression in airway epithelial cells with increased ENO levels resulting during experimental infections with either human rhinovirus or influenza. In contrast, another paper didn’t find any increase in nasal or lower airway NO during respiratory syncytial virus infection(129). Our experience during this study showed that respiratory infections can raise ENO levels significantly, confounding the effects of smoking cessation in this population.

Other publications regarding smoking cessation and NO measurements do not mention the respiratory tract infection as something that was monitored. We noticed that ENO levels rise right after the symptoms start and maintain high levels throughout the course of the infection. It seems that ENO levels tend to decrease when the symptoms resolve and are close to baseline levels after 3 or more days. There is no precise information in the literature about the ENO changes during the course of a viral respiratory infection and how long these changes can last.

Due to all these features we discussed, and especially to its wide variability between sessions, it might be possible that a type II error occurred and a much larger sample size would be necessary to demonstrate a significant change in ENO levels after smoking cessation. Probably, the time line between event (smoking cessation) and result (increased levels of ENO) is larger than what could be measured in our study. Still, we could show that smokers have lower levels of ENO compared to non-smokers as previously reported in the literature.
8.3 ENO as a marker of smoking abstinence

Our study did show a trend towards higher levels of ENO as soon as 3 days after abstinence. This trend persisted throughout the days, but did not reach statistical significance by day 7 or 10.

ENO didn’t prove to be a reliable marker for smoking abstinence in the short time setting. Our findings show that there is a wide variability within subject which makes it difficult to follow in consecutive measures. There are many extrinsic factors that can interfere in ENO levels. The most significant ones we identified in our study were upper respiratory infections and allergic rhinitis. Our findings do not rule out the application of ENO as a monitoring tool for smoking abstinence for medium to longer term abstinence. We didn’t have long term cessation data available to evaluate this hypothesis.

Studies have shown that respiratory viruses can increase epithelial levels of iNOS (inducible NO synthase) expression. Data supporting increased levels of ENO have been more inconsistent, some studies show increased levels while others show no increase.(129).

Scadding, in a review study published in 2007, found that patients with allergic rhinitis present increased levels of NO if compared to healthy subjects. This may suggest that nonasthmatic patients with allergic rhinitis may have subclinical inflammation in the lower airways(130).

We observed that when subjects developed a respiratory infection (common cold) during the study period their ENO levels increased. A common cold during the previous 3 weeks was one of the exclusion criterium but developing a cold during the study was not. Four subjects of our smoking cessation group developed typical viral URI symptoms during the study. There was a noticeable increase in ENO
levels in the first measurement performed during the symptomatic period compared to the baseline level in three of them. In the remaining subject there was no perceptible change in ENO level. Eight smokers who didn’t quit also developed a cold during the study period. From this group, four had noticeably higher levels of ENO during the symptomatic period. Only one subject of the non-smoking group developed respiratory infection and no change in ENO level was observed.

Our data suggest that ENO cannot be used as an adjuvant tool in the armamentarium of smoking abstinence monitoring, at least in a short term setting. Further studies with larger sample size and longer follow up would be necessary to confirm our findings.

**8.4 ENO levels and decreased cigarette consumption**

One point of interest at present is to understand the effects of smoking reduction. Some studies show that smoking reduction doesn’t strongly interfere in the risks that follow smoking itself. It is very difficult to prove if decreased consumption of cigarettes can bring any benefits. Nowadays, it is faced as a step that can be reached before complete cessation. It could be used as a preparation period for the definite quitting date.

A 50% reduction in cigarette consumption is necessary to decrease biomarkers for toxicant exposure in only 30%. Other than that, only a modest reduction in cardiovascular risk biomarkers is seen (2). This is evidence that there has not been much reduction in toxic inhalation. It is already known that smokers develop compensatory smoking behaviour when they decrease daily cigarette consumption. They acquire habits that allow them to absorb more nicotine from each cigarette smoked.
Our findings agree with the present literature showing that there was no difference in ENO levels after subjects decreased their cigarette consumption in at least 50%. In contrast with the quitters in whom we saw a trend towards higher NO levels, the ones who decreased consumption, had values almost identical to when they started the protocol. It would be interesting to study more subjects who decrease their smoking for a longer period of time and analyze if there is any significant change in ENO levels.

8.5 Alveolar fraction of NO and Smoking

Our findings show that smokers and non-smokers have similar levels of alveolar NO. Although there is a lot of controversy in the literature regarding this issue, our finding goes in accordance with Pietropaoli et al.

Pietropaoli et al. found a significant threefold reduction in NO levels produced by the conducting airways while the NO concentration in the alveoli was only slightly reduced. They found that the alveolar NO production was equivalent in smokers and non-smokers(87).

On the other hand, Mallinovschi et al. found that smokers have reduced levels of NO in the conducting airways as well as in the alveoli(76). Delclaux et al. also found reduced alveolar NO levels in smokers(131).

Another group found that smokers have reduced NO flux levels but increased alveolar NO levels. They postulated that the increased alveolar NO might be a prognostic sign of lung damage in smokers without respiratory symptoms. They further suggest that NO monitoring could be used to indicate improvements when a smoker stops smoking(5).
Although there are controversy regarding alveolar NO levels in smokers, it seems that NO in the conducting airways presents a much more constant pattern. Most studies found reduced levels of NO in the conducting airways.

According to our findings, smokers have a two fold reduction in bronchial NO levels comparing to non-smokers. On the other hand, alveolar NO levels were similar between smokers and non-smokers. It seems that the overall reduction in ENO levels seen in smokers is due to the bronchial fraction and not to the alveolar fraction of NO.

No significant changes in alveolar and bronchial NO levels were observed either after smoking cessation or smoking reduction in our subjects, although bronchial NO showed a trend towards higher levels after quitting smoking, similar to what we found in ENO levels. This trend was not observed in alveolar measures after quitting.

Summarizing, we found a statistically significant difference between ENO levels of smokers and non-smokers. As mentioned before, smokers were proved to have lower levels of ENO when compared to non-smokers. When measuring alveolar and airway NO, it seems clear that this difference in ENO is caused by the decreased levels smokers present in bronchial NO. Alveolar NO was similar between the 2 groups.

### 8.6 Nasal NO and Smoking

We found that smokers and non-smokers have similar nasal NO output measures. No significant changes were seen after smoking cessation or reduced consumption. Very little is known about nasal NO and its relation to smoking status. Few studies demonstrated that smokers have lower levels of nasal NO compared to non-
smokers(113). We couldn’t find studies that analyzed the effects of smoking cessation or reduction in the nasal NO levels.

It is known that cigarette smoking has influence in many nasal and sinuses diseases. Smokers who have chronic sinusitis have higher chances of needing revision surgery for disease control. Also, there is some well established relationship between smoking and sinuses cancer. The exact mechanism that incites these pathologies still needs to be established. Nasal NO may play a role in the physiopathology of some of those diseases.

Nasal NO, as demonstrated in other studies, has a wider range of variation compared to ENO. Its absolute values are much higher as well. In our sample, the range of variation in nasal NO output measures varied from 167.4 to 810 nl/minute. A bigger sample size would be necessary to study the differences in nasal NO in smokers and its possible relation to the development of nasal and sinuses pathologies.

It is quite interesting though to notice that only smokers had levels below 300 nl/min, and that non smokers had levels that were always above 400 nl.minute. The significance of this finding is not known.

In our sample, there was no significant difference between nasal NO measurements before and after quitting smoking. It would be very interesting to study this parameter in a larger sample of subjects abstinent for a longer period of time. Nothing is known about nasal NO and smoking cessation.

It is important to reinforce that very little is known about nasal NO and smoking. We could not show any significant difference in nasal NO measurements in smokers and non-smokers. An interesting finding was that very low levels were
seen only in smokers. We could speculate that these very low levels can be related to the smoking pattern. We know that some smokers exhale their cigarette smoke through the nose and possibly these are the smokers who present very low levels of nasal NO. This is only a speculation, more studies need to be performed in this area to understand the interaction between smoking and nasal NO.

8.7 Improvements and A Look Ahead

Upon completion of this project, we determined that if repeated, some changes would be necessary in order to improve the quality of the results.

One of the potential downsides of the present study is that the population was composed of ex-drug addicts who were going through an abstinence period. They were at CAMH participating in an institutional program and had all been abstinent from their previous drugs of abuse for at least 7 days. Most subjects that came through the program were either alcohol or opioids users, that are not thought to strongly influence ENO. One of the problems is that a high percentage of them also used to consume marijuana and a few other inhaled drugs. Little is known about these drugs’ effects on ENO. Our thoughts are that such factor would be small, and this is confirmed by looking at our non-smokers. Our non-smokers had similar history of drug abuse as the cases, and did not show any significant changes in their NO levels during the study period.

Furthermore, when we compare baseline data of smokers and non-smokers we can see results that are similar to what has been reported in the literature. These 2 groups had been previously exposed to similar types of drugs and if there was any influence on ENO it was small and not relevant.

To be eligible for CAMH rehabilitation program one would have to be free of previous drug addictions for at least one week; most patients enrolled were in fact
abstinent for more than 30 days. This program they joined was not a detoxification program. Relapses from their previous addictions were rare and the few who did relapse were excluded from the study and from the program. Once they were very closely monitored it was very unlikely that a non-identified relapse would have happened.

It is important to emphasize that because of this particular characteristic of our sample, one have to be careful when extrapolating our results to the general population. Moreover, this particular population has the highest prevalence of smoking and we found it would be important to study their ENO behaviour.

Another problem we had during the study period was the low number of patients able to quit smoking during a period longer than 7 days. We know that this population is highly addicted to nicotine and despite all efforts of engaging them in a smoking cessation attempt; many patients had a relapse before completing 7 days of abstinence not being included in the NO profile changes analysis after cessation. Also, we expected that more subjects enrolling in the smoking cessation program would have succeeded. It meant that we ended up needing a much larger population than originally thought, increasing the costs and timeframe of the protocol.

For future studies, it would be interesting to evaluate the ENO changes as well as compartmental and nasal NO changes after a longer period of abstinence, preferably longer than 2 weeks.
References

Reference List


Appendices

Appendix 1

Subject Information And Consent Form

Study Title: Exhaled Nitric Oxide and Smoking Cessation

Study Doctor: Dr. Peter Selby 416-535-8501 ext. 6859
Dr. Kenneth Chapman 416-603-5499
Dr. Renata Barreto 647-202-8190

Purpose: You are being asked to participate in a research study. This form tells about the study. If there is anything you do not understand, please ask the study doctor.

We are doing this study to determine how nitric oxide levels change after smoking cessation.

Nitric oxide is a molecule produced in your lungs and is somehow related to different kinds of lung diseases that can increase or decrease its production. Nitric oxide produced in the lungs can be measured in the air you breathe out. It is known that nitric oxide production is decreased in smokers and it tends to return to normal values after smoking cessation but the reasons for these changes is not known at the moment.

This study will involve 40 people who are currently admitted to the Residential Program at CAMH. There will be 20 smokers enrolled in the cessation of smoking program during their stay and 20 non-smokers. We will perform the measurements in people who succeed but also in those who do not succeed in their smoking cessation attempt.

Procedures: The study will last for up to 21 days (the same duration of your stay at CAMH). If you are a smoker, the tests will be performed during your first day at CAMH and twice a week after that (7 visits in total). If you are a non-smoker, the tests will be performed on the first and last days at CAMH (2 visits in total). The first visit will last for approximately 90 minutes, and the following visits from 30 to 60 minutes each time.

You will be asked to not smoke at least one hour prior to each visit.

Visit 1 (baseline visit):
• You will be asked questions about your smoking habits/abstinence.
• You will be given a general check-up and a medical history will be recorded.
• Your nasal passages (nose) will be examined by having a small instrument placed at the nostril to open it up.
• A breathing test (spirometry) will be performed. The breathing test measures how much air is in your lungs and how well you are able to breathe it out. The test requires you to take a deep breath in and blow out as fast and as hard as you can into a mouthpiece.
• You will be asked to blow into a mouthpiece to measure the nitric oxide and carbon monoxide levels in your lungs.
• Your nasal nitric oxide will be measured by putting a small plastic tube into your nostrils and having some air flow from one side of the nose to the other, while you blow through a mouthpiece.
• A small sample of urine will be collected to measure the cotinine level (a chemical produced by your body when you’ve been exposed to cigarette smoke).

Subsequent Visits:
• You will be asked questions about your smoking habits/abstinence.
• Your nitric oxide and carbon monoxide levels, from the lungs, will be measured.
• Your nasal nitric oxide will be measured.
• Your cotinine levels will be measured by collecting a small sample of your urine.

Eligibility: To participate in this study you must be 18 years of age or older, and enrolled in the Residential Program at CAMH. Smokers must also be enrolled in the smoking cessation program during their admittance period at CAMH. You may not participate in this study if you have previous lung disease (like asthma or COPD) or if your spirometry test performed at the first visit shows some abnormality.

Confidentiality: Your identity will be kept confidential to the full extent provided by law. In addition, neither your name nor other personal identifiers will be used in any reports or publications arising from this study. As part of the continuing review of the research, your study records may be reviewed by the Research Ethics Board and, if applicable, by government regulatory agencies such as Health Canada. A person from the research ethics board may contact you (if your contact information is available) to ask you questions about the research study and your consent to participate. The person reviewing your file or contacting you must maintain your confidentiality to the extent permitted by law.

Compensation: You will receive $5.00 for each visit. You will receive the compensation at the end of all the study measurements. If you decide to withdraw from the study or the study doctor decides to end your participation in the study, you will receive compensation for the number of visits completed.

Risks: There are no risks added in performing these measurements. You may have the inconvenience to collect urine samples during the study visits. You will have to spend some time to have the study measurements done but all testing will be done during your stay at CAMH.
Benefits: You will not have any direct benefits from the present study but your participation may help in future smoking cessation programs by helping other people in their attempt to stop smoking.

Voluntary Participation: Your participation in this study is voluntary. You may choose to withdraw from the study at any time. In addition, the investigators, or their staff, responsible for this study may, at their discretion, end your participation at any time. Your choice to not participate, your choice to withdraw, or removal from the study by the investigator will not affect any treatment needs that you might have at Centre for Addiction and Mental Health now or in the future. Failure to successfully quit smoking will not be a reason for removal from the study.

Additional Information: If you have questions about the study that are not answered in these information sheets, please feel free to ask them. In addition, if you have questions in the future you may contact the investigators at the telephone number given on the first page or contact Dr. Barreto via email: renata.barreto@uhn.on.ca. If you have any questions about your rights as a research subject, please contact Dr. Padraig Darby, Chair, Research Ethics Board, Centre for Addiction and Mental Health, at 416-535-8501 ext. 6876.

Agreement to Participate
I, ________________________________, have read (or had read to me) the Information Sheet for the study named ‘Exhaled Nitric Oxide and Smoking Cessation’. The purpose of this study is not to help me to quit smoking. My role in the study is as a research volunteer to help the investigators collect information about smoking cessation. This information may or may not be useful to develop better smoking cessation programs in the future. My questions, if any, have been answered to my satisfaction. By signing this consent form I do not waive any of my rights.

I understand I will receive a signed and dated copy of this form to keep.

My signature below indicates that I voluntarily agree to participate in this study.

Research Volunteer:

Signature: ______________________________ Date: ______________________________

Name: ______________________________

Please Print

Person Obtaining Consent:

Signature: ______________________________ Date: ______________________________

Name: ______________________________

Please Print
Appendix 2

Questionnaires – First visit questionnaire

Questionnaire - First Visit

Initials:  Gender:  M  F  Subject number:
Age:  Date of birth:

1. Are you a daily smoker?
   Yes  No
2. How many cigarettes a day do you smoke?
3. How long have you been a daily smoker?
4. How many cigarettes did you smoke in the past 24 hours?
5. How many cigarettes did you smoke in the past hour?
6. Do you have asthma diagnosed by a physician?
   Yes  No
7. Do you have COPD/enphisema/ diagnosed by a physician?
   Yes  No
8. Do you have allergic rhinitis diagnosed by a physician?
   Yes  No
9. Do you have nasal polyps diagnosed by a physician?
   Yes  No
10. Did you have a cold or respiratory infection in the past month?
    Yes  No
11. Are you using inhaled steroids?
    Yes  No
12. Are you using nasal steroids?
    Yes  No
13. Are you using nicotine replacement therapy?
    Yes  No
14. If yes, what type(s)?
    patch
    gum
    inhaler
15. What dosage?
Appendix 3

Questionnaires – Follow-up questionnaire

Questionnaire – Follow up

Date:

1. How many cigarettes did you smoke since your last visit? And Past 24h?

2. When did you smoke your last cigarette?
   Today
   Yesterday
   The day before
   > 3 days ago
   >1 week ago
   >2 weeks ago
   >3 weeks ago

3. How many cigarettes did you smoke in the past hour?

4. Are you using nicotine replacement therapy?
   Yes
   No

5. If yes, what type(s)?
   Patch
   Gum
   Inhaler

6. What dosage?