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Non-invasive method of measuring airway inflammation: exhaled nitric oxide

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Abstract

Background

Nitric oxide (NO) was well known to be a component of air pollution, often in the form of nitrogen dioxide (NO$_2$). However its importance in biological systems altered dramatically with the discovery in 1987 that it was the 'endothelial-derived relaxing factor'. Since then there has been an explosion of research on NO demonstrating that this gaseous molecule was a widespread physiological mediator and was simultaneously recognised as a vital component of immune function contributing to macrophage-mediated cytotoxicity. NO was therefore a key molecule in modulating inflammation, including airway inflammation.

The aim of this thesis was:

1. To adapt a NO chemiluminescence analyser from measuring airway pollution to measuring exhaled air in human subjects.
2. To measure NO levels in exhaled air in adult subjects.
3. To evaluate whether altering measurement parameters altered the levels of NO obtained.
4. To adapt this technique from adults to measure exhaled NO in children.
5. To compare levels of NO from healthy children to groups of asthmatic children on either bronchodilator therapy only, or on regular inhaled corticosteroids.
6. To compare the levels of NO in a pilot group of asthmatic children before and after commencement of inhaled corticosteroids.

Methods

A Dasibi Environmental Corporation Model 2107 chemiluminescence analyser was adapted specifically requiring a reduction in response time, which was achieved by modification of the circuitry and re-routing of the analogue signal directly to a chart recorder, achieving a reduction of the response time by 80%. Addition of a number of analysers allowed the measurement of exhaled NO, carbon dioxide (CO$_2$), mouth pressure and flow for each exhalation from total lung capacity. Twenty adult subjects (in total) were then studied looking at direct (NO, CO$_2$, mouth pressure) versus t-piece (with the addition of flow) measurements making five exhalations from total lung capacity, at 3-minute intervals (direct/t-piece/direct or t-piece/direct/t-piece in series). The area of NO under the curve versus the peak of the NO trace was compared and the exhalation pattern of NO versus CO$_2$ was compared. Measurement conditions were altered to evaluate the effect of individual parameters on the exhaled NO result. This included separately assessing different expiratory flows, different expiratory mouth pressures, the effect of a high versus a low background NO level and the
effect of drinking water (of varying temperatures) prior to exhalation. Healthy control children were then enrolled to the study from a local school (Park Walk Primary School) and compared with asthmatic children enrolled from outpatient clinics at the Royal Brompton Hospital. The asthmatic children were further divided into those on bronchodilator treatment only and those on regular inhaled corticosteroid therapy. NO was also measured before and two weeks after commencing inhaled corticosteroid therapy in previously steroid-naive asthmatics.

**Results**

It was possible to modify a chemiluminescence analyser to enable measurement of exhaled NO. In 12 healthy subjects (mean age 32 years, 6 males) peak direct NO levels were 84.8 parts per billion (ppb) (standard error of the mean (SEM) 14.0ppb), significantly higher than 41.2ppb (SEM 10.8ppb) measured via the t-piece system. The exhaled NO rose to an early peak and plateau while the CO₂ levels continued to rise to peak late in the exhalation. The mean times to peak NO levels were 32.2 seconds (s) (direct) and 23.1s (t-piece), which was significantly different from the mean times to peak CO₂ levels at 50.5s (direct) and 51.4s (t-piece, p<0.001). At peak NO level, the simultaneous CO₂ level of 4.9% (SEM 0.14%, direct) and 5.2% (SEM 0.18, t-piece) were significantly lower than the peak CO₂ achieved of 5.8% (SEM 0.21%, direct, p< 0.001) and 6.2% (SEM 0.28, t-piece, p<0.001). There was no difference between repeat direct or t-piece measurements.

With regard to varying measurement conditions, the mean peak concentrations of NO decreased by 35ppb (95% confidence intervals 25.7-43.4) from a mean of 79ppb (SEM 15.4ppb) at an expiratory flow rate of 250mls/min to 54.1ppb (SEM 10.7ppb) at 1100mls/min. The mean peak concentration of NO did not change significantly when mouth pressure was increased in eight of ten subjects, although in two it did decrease in the highest pressure. The mean NO concentration with machine and subjects sampling from a low NO reservoir was 123ppb (SEM 19.4), which was an increase from results obtained before at 81.9ppb, SEM 10.2ppb, p=0.001 95%, CI -19.9 to -62.7) and after at 94.2ppb (SEM 18.3ppb, p=0.017, 95% CI 6.0-5.18) sampling with high ambient NO levels. The mean peak NO concentration decreased from 94.4ppb (SEM 20.8) to 70.8ppb (SEM 16.5, p=0.002 95% CI 12.9-33.1) with water consumption.

In 39 healthy pre-pubertal children with a mean age of 9.9 years (range 9-11 years, 23 girls) the mean direct exhaled NO level was 49.6ppb (SD 37.8ppb, range 11.5-197.2ppb) compared with mean exhaled NO via t-piece sampling of 29.2ppb (SD 27.1ppb, range 5.1-141.2ppb).
There was no significant difference between boys and girls for either the direct or the t-piece recordings. In comparison with normal children, 15 asthmatic children on bronchodilator therapy only had much higher levels of exhaled NO at 126.1ppb via the direct system (SD 77.1ppb, p<0.001) and 109.5ppb via the t-piece system (SD 106.8ppb, p<0.001). In 16 asthmatics on regular inhaled corticosteroids the mean peak exhaled levels were significantly lower at 48.7ppb via the direct method (SD 43.3ppb, p<0.001) and via the t-piece system at 45.2ppb (SD 45.9ppb, p<0.01). There was no difference between the normal children and the asthmatic children who were on regular inhaled corticosteroids (p=0.9 direct, p=0.2 t-piece). There was no significant difference in CO₂, mouth pressure, duration of expiration and expiratory flows between the three groups or between the two methods (direct and t-piece). In six asthmatic children the mean peak exhaled NO levels fell from a medium peak level of 124.5ppb to 48.6ppb when measured before and two weeks after commencement of inhaled corticosteroids on treatment.

Discussion

This research showed it was possible to modify an NO chemiluminescence analyser to enable measurement of exhaled NO in adult and paediatric subjects. Furthermore, it was possible to measure both healthy and asthmatic children. There were significant differences between the exhalation pattern of NO and CO₂ suggesting that NO was produced in the airways, not at alveolar level, unlike CO₂. The measurement of exhaled NO required a standardised approach as exhaled NO levels decreased with increasing expiratory flow, when measuring at a time of high ambient NO concentration, and with consumption of either hot or cold water immediately preceding exhalation (such as might be given if a subject was coughing). The findings with expiratory mouth pressure were less certain, with a difference seen in only two of ten subjects.

The levels of exhaled NO measured in children aged 9-11 years were lower than that measured in the adult subjects. There was no difference between boys and girls, or with other parameters such as having a personal history of atopy, a family history of atopy, or the presence of a smoker or furry pets within the house-hold. These findings may have altered with increased numbers in this group and could possibly be a type two statistical error. The results of exhaled NO in asthmatic children on bronchodilator therapy only were significantly elevated compared to both normal children and asthmatic children treated with regular inhaled corticosteroids. The exhaled NO level also fell significantly by two weeks following the commencement of inhaled corticosteroid treatment in steroid-naive asthmatic children. These
results suggested that the methods of measuring exhaled NO required standardization and that it could potentially be a non-invasive measure of airway inflammation to follow - particularly in children with asthma who were commencing inhaled steroid treatment.
Dedication

This is Dedicated:

To the strong women in my family from my great, great grandmother

[Image]

to my sister Angela who always believes in me.
Acknowledgements

United Kingdom

Professor Andrew Bush invited me to a research position at the Royal Brompton Hospital and has been supportive from day one for both my research career and clinical training. His own approach I seek to emulate with his excellent clinical acumen, approach to children and their families. In addition, he maintains a huge research output and when working with him in this capacity his attention to detail and editing ability is superb. I, like most of the paediatric respiratory fraternity, count Andy, and his wife Sue Bush, as friends.

Professor Peter Barnes from the then National Heart & Lung Institute contributed valuable advice and supervised other research projects that I conducted. He rather bravely allowed me to join his laboratory as one of only three ‘medics’ among the 25 scientists from whom I learnt about statistics, information technology and attention to detail.

Dr Seinka Dinarevic assisted with the studies on the children and provided a link to the local school from where the children were recruited.

Park Walk Primary School, London who were approachable regarding the research and interested in assisting the study. The children enrolled from the school and from the Brompton Hospital clinics were terrific – humorous, enthusiastic, willing to help and could always be depended upon to question some factor about the testing or the research that I had not covered with them. They loved all the switches even more than I did.

Caroline Busst was the biomedical engineer who provided the main assistance with regard to modifying the analyser as we went through and assisted with troubleshooting whenever that was necessary. She brought completely different knowledge to mine to this project from an engineering and particularly electrical engineering capacity, which complimented the clinical and practical knowledge that I was able to offer.

I would like acknowledge the great ‘Fellows’ that I worked with at the Royal Brompton Hospital. We were embarking on clinical and research careers and it was great to be part of the group and they remain friends to this day; Paul Munyard, Lara Shekerdemian, Jane Davies, Clare Hogg, Kate Brown and Adam Jaffe.
New Zealand

The Paediatric Department at the Faculty of Health and Medical Sciences, University of Auckland all contributed particularly in the final days of submission assisting with proof-reading and formatting.

A very special thank you to Jan Tate the CF nurse specialist and friend who has always been very supportive and made my daily working life better, particularly at times of clinical overload. She also helped take the photos used in this thesis – late into the night.

Professor Innes Asher has always been supportive of myself in the clinical, research and teaching arenas and has helped greatly with her capacity, despite the many hats she currently wears, in offering advice and editing comments.

Dr Elizabeth Edwards who was my first research fellow, the first Ph.D student that I supervised which she completed before me and now both colleague and friend as well as fellow netball enthusiast.

Merrin Harger, project manager of subsequent research with whom I shared an office. She was amusing every day and left me one of her inspirational artworks when she left to embark on a new career as an artist (that we all were so talented).

Mirjana Jaksic, always generous with her own clinical time, I thank for supporting me in particular by picking up the occasional extra clinic, so that I could catch up.
Personal

My family has always been completely supportive in everything that I do and my parents, Daphne Mary Pemberton Byrnes and Brian Liston Dominic Byrnes, were thrilled to see me start at medical school all those many years ago, although, sadly, were not alive to see me graduate. And my special Aunt, Veronica Commins, who has supported my sister and myself through our careers and also, sadly, died before seeing this dedication to her years of generosity. My brothers, sisters and their families all offer their special supports – I feel lucky to be part of a large whanau, and I hope they will be pleased to see me emerge from my study.

It is a rare person who believes in you so wholeheartedly, even at times when you yourself have misgivings and I would like to thank my sister, Angela Platt-Byrnes, for ringing me every Saturday and every Sunday to ensure that I was … and to encourage me to be … sitting at my desk working on this thesis.

And to my own support network: Dr Sue Armstrong-Wahlers, Dr Michael Wahlers and Trudi Fava.
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<td>allergic bronchopulmonary dysplasia</td>
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<td>ATS</td>
<td>American Thoracic Society</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
</tr>
<tr>
<td>C₃, C₅</td>
<td>complement factor 3, complement factor 5</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 3', 5' monophosphate</td>
</tr>
<tr>
<td>CAT₁, CAT₂, CAT₂B, CAT ₃</td>
<td>cationic amino acid proteins</td>
</tr>
<tr>
<td>CB</td>
<td>chronic bronchitis</td>
</tr>
<tr>
<td>CD₃⁺</td>
<td>cell marker for T lymphocyte</td>
</tr>
<tr>
<td>CD₄⁺</td>
<td>cell marker for T helper lymphocyte</td>
</tr>
<tr>
<td>CD₈⁺</td>
<td>cell marker for T cytotoxic lymphocyte</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cNOS</td>
<td>constituent nitric oxide synthase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Co</td>
<td>cobalt</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSF</td>
<td>central spinal fluid</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECMO</td>
<td>extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophil cationic protein</td>
</tr>
<tr>
<td>ECRHS</td>
<td>European Community Respiratory Health Survey</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelial derived relaxing factor</td>
</tr>
</tbody>
</table>
ELISA Enzyme-linked immunosorbent assay
eNOS endothelial nitric oxide synthase
EPN/EPX eosinophilic neuraminidase
EPO eosinophilic peroxidise
ERS European Respiratory Society
ERSTF European Respiratory Society Task Force
FAD flavin adenosine dinucleotide
Fe iron
FEF_{25-75\%} forced expiratory flow at 25 to 75 percent of forced vital capacity
FEV\textsubscript{1} forced expiratory volume in 1 second
FEV\textsubscript{0.5} forced expiratory volume in half a second
FMN flavin mononucleotide
FVC forced vital capacity
GINA Global Initiative for Asthma
GM-CSF granulocyte macrophage - colony stimulating factor
GTP guanosine 5-triphosphate
H\textsubscript{2}O water
H\textsubscript{2}S hydrogen sulphide
HNO\textsubscript{2} nitrous acid
HRCT high resolution computerised tomography
ICAM1 intracellular adhesion molecule 1
IFN\gamma interferon gamma
IgA immunoglobulin A
IgG immunoglobulin G
IgE immunoglobulin E
IHCS inhaled corticosteroids
IL1, IL2, IL3, IL4, IL5, IL6, IL8, IL9, IL10, IL11, IL12, IL13, IL17 interleukin 1, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, interleukin 8 interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, interleukin 17
iNOS inducible nitric oxide synthase
ISAAC International Study of Asthma and Allergies in Childhood
Kb kilobases
L/min litres per minute
LABA long acting \beta_2 agonist
LADA: N^N dimethyl L-arginine
LFA1: lymphocyte function-associated antigen 1
L-NAME: N^w arginine methyl ester
L-NMMA: N^w monomethyl L-arginine
L-NOARG: N^w-nitroarginine
LNIO: N- imino-ethyl-ornithine
LPC: lysophosphotidylcholine
LPS: lipopolysaccharide
LTC4, LTD4, LTE4: leukotriene C4, leukotriene D4, leukotriene E4
MBP: major basic protein
mg/ml: milligrams per millilitre
mls/min: millilitres per minute
Mn: manganese
mRNA: messenger ribonucleic acid
N2: nitrogen
N2O: nitrous oxide ("laughing gas")
N2O3: dihydrogen trioxide
N2O4: dihydrogen tetraoxide
NADPH: nicotinamide adenosine di-nucleotide phosphate
NANC: non adrenergic, non cholinergic (nerves)
NF-kB: nuclear factor-kappa B
NHANES 1: National Health and Nutrition Examination Survey 1
nL/min: nanolitres per minute
nNOS: neuronal nitric oxide synthase
NO: nitric oxide
NO+: nitrosium cation
NO-: nitroxyl anion
NO2: nitrogen dioxide
NO2-: nitrite
NO3-: nitrate
NO2Tyr: 3-nitrotyrosine
NOS: nitric oxide synthase/s
NOx: nitrogen oxides (usually NO, NO2 and NO3)
NZPAG: New Zealand Paediatric Asthma Guidelines
O2: oxygen
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>O$_3$</td>
<td>ozone</td>
</tr>
<tr>
<td>OH$_-$</td>
<td>hydroxyl anion</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>ONOOH</td>
<td>peroxynitrous acid</td>
</tr>
<tr>
<td>PaO$_2$</td>
<td>pulmonary artery oxygen</td>
</tr>
<tr>
<td>PCD</td>
<td>primary ciliary dyskinesia</td>
</tr>
<tr>
<td>PC$_{20}$</td>
<td>provocation concentration producing 20% fall in the forced expiratory volume in one second</td>
</tr>
<tr>
<td>PD$_{20}$</td>
<td>provocation dose producing 20% fall in the forced expiratory volume in one second</td>
</tr>
<tr>
<td>PEF</td>
<td>peak expiratory flow</td>
</tr>
<tr>
<td>Pmo</td>
<td>mouth pressure</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PM10</td>
<td>particulate matter with a diameter of less than 10µm</td>
</tr>
<tr>
<td>redox</td>
<td>reduction oxidative reactions</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver-operator curve</td>
</tr>
<tr>
<td>RS</td>
<td>sulphur thios</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SIGN</td>
<td>Scottish Intercollegiate Guidelines Network</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosis</td>
</tr>
<tr>
<td>SO$_2$</td>
<td>sulphur dioxide</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>T$_{H1}$</td>
<td>T helper lymphocyte cell type 1</td>
</tr>
<tr>
<td>T$_{H2}$</td>
<td>T helper lymphocyte cell type 2</td>
</tr>
<tr>
<td>T$_{H3}$</td>
<td>T helper lymphocyte cell type 3</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFβ</td>
<td>tumour necrosis factor beta</td>
</tr>
<tr>
<td>type I (nNOS)</td>
<td>Type I neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>type II (iNOS)</td>
<td>Type II induced nitric oxide synthase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>type III (eNOS)</td>
<td>Type III endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>µg/ml</td>
<td>micrograms per millilitre</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations International Children's Fund</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV light</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>V/Q</td>
<td>ventilation and perfusion ratio</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
</tr>
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</table>
Chapter 1: The burden of respiratory disease in New Zealand

1.1 Introduction

This opening chapter will set the scene as to why I felt that this area of research was of importance and of interest. The burden of respiratory disease in New Zealand is high, and clearly apparent to anyone who trains or works here in the field of medicine. It was an obvious problem as I came through the Auckland School of Medicine, it was obvious when I chose a rural setting to do my first house year, and it remained obvious when I commenced training in paediatrics. The burden appeared to be borne disproportionately by children, and by children of two specific community groups — Maori and Pacific Island. In the 1980s and 1990s, New Zealand was leading the world in both the incidence of asthma and, more appallingly, asthma mortality. Although the diagnosis of asthma in children was not always clear, we embarked on anti-inflammatory drug treatment, possibly for years. While asthma was thought to be a reversible disease, it has since transpired that an element of irreversibility could develop which has been termed ‘airway remodelling’.

This thesis commenced ten years ago and is based on work I conducted while employed as a fellow and senior registrar and lecturer at the Royal Brompton Hospital in London. My interest, then as now, was in paediatric respiratory disease. I was concerned that we seemed to be making treatment decisions regarding the use of potential toxic anti-inflammatory agents, specifically the use of steroids, without measuring any inflammatory parameter. Rather, we were using history, examination and other surrogate measures such as x-rays and lung function (where possible and where children were old enough) to determine correct diagnosis and response to treatment. So for the individual child; was the diagnosis correct? Was the child on too much or unnecessary medication? — a treatment with potentially significant side effects, such as adrenal suppression and growth failure. Or was the child on too little medication? — suffering symptoms daily with the possible development of irreversible disease, or even death. I saw many children in the clinic that were in both categories; on “too much” steroid therapy for too long incorrectly, on “too little” with unnecessary morbidity, and so very few seemed “just right”. I planned and conducted the research outlined in this thesis, which I saw as a unique opportunity to investigate a possible solution to this quandary. The research presented is thus now historical. It has already contributed to medical literature, including the formulation by the European Respiratory Society and American Thoracic Society of standardized procedures for the measurement of exhaled nitric oxide (NO), the marker I chose to explore to indicate airway inflammation. I have continued to research this
area, some of it has been expanded from the work presented here, and in these experiments I dotted the ‘i’s, crossed the ‘t’s and was hands on adapting the individual machinery. It remains eligible to present and I have lived with it so long that I am pleased to do so. This thesis presents the research leading to the experiments that I undertook and describes the development of NO as a measure of inflammation during this time and subsequently to the end 2006.

In this chapter I review the burden of respiratory disease in paediatrics in New Zealand. This review was part of a monograph edited by myself and Professor Innes Asher, and in which I also wrote two chapters. This will be followed by a brief overview of asthma, the difficulty of accurate diagnosis and the background to it being designated an inflammatory disease. The following chapter will present how NO was considered a pollutant, then became recognised as a vital biological agent, and then known as an inflammatory marker. In Chapter 3, the characteristics of NO will be examined as these make it a difficult gas to measure. Chapter 4 will discuss the possible options for measuring NO and in Chapter 5 I will describe how a machine that measured airway pollution became adapted to measure exhaled air. In Chapters 6 and 7 the feasibility of measuring NO in exhaled air in adults was assessed; particularly exploring what factors affected the NO readings. These then describe the standardized method I developed which was used as presented in Chapter 8 to enable exhaled NO in healthy children to be measured and in asthmatic children on bronchodilator or steroid treatment, as well as a smaller group that was commencing steroid therapy. The penultimate chapter, Chapter 9 will be devoted to reviewing the literature regarding NO to the present day, and where this investigation is now best utilized. The final chapter, Chapter 10, will be a brief commentary what I have learnt through the process of this research and writing.

I hope you enjoy this story.

1.2 The burden of paediatric respiratory disease in New Zealand

The ‘Trying to Catch Our Breath’ monograph (Anonymous 2006) published last year was an initiative by twelve leaders from the New Zealand paediatric health community, supported by the Asthma and Respiratory Foundation of New Zealand (Te Taumatua Huango, Mate Ha o Aotearoa), to document and present the alarming data that existed on the incidence, prevalence and the severity of respiratory disease in children. It was presented to the Right Honourable Mr Peter Hodgson, Minister for Health in April 2006 by the Asthma and Respiratory Foundation of New Zealand (Te Taumatua Huango, Mate Ha o Aotearoa). The title of the document was deliberately ambiguous as the group also feel breathless in trying to
deal with the sheer numbers of children requiring assessment and treatment with these disorders. The rates of most respiratory conditions are higher than comparable westernized countries and the severity of these conditions more significant. Much of this is preventable. In 1999, 'The Top Ten Report' (Graham, Leversha et al. 2001) detailed the top ten issues affecting the health and well-being of children and young people in Auckland and Waikato, including a review of the top ten causes of potentially avoidable hospital admissions in New Zealanders aged 0-24 years (see Figure 1.1).

Figure 1.1: Top 10 causes of avoidable admissions, 0-24 years, 1999

Over half of these potentially avoidable admissions were respiratory or ear, nose and throat conditions. Disease is exacerbated by the proportion of children living in poverty (which is defined as living in a household with an income below 60% of the median family income net of housing costs). This has increased from 16% in 1987/1988 to 29% in 2000/2001 (Ministry of Social Development 2002). Indeed New Zealand has one of the worst rates of child poverty in rich countries (UNICEF 2005). The burden of disease particularly impacts on certain ethnic groups within New Zealand. Maori children currently make up 25% of all children living in New Zealand and, as a collective group, Pacific peoples make up 6.5% of the New Zealand population. However both of these groups have significantly higher proportions of children aged less than fourteen years of age – 35% of Maori children (Te Puni Kokiri 2002) and 39% of Pacific children (Statistics New Zealand 2002) compared to 20% of total New Zealanders.
In addition, both of these groups are disadvantaged with regard to disposable income, adequate housing, educational opportunities, unemployment rates and access to healthcare (Ministry of Health 2002; Statistics New Zealand 2002; Asher 2006).

A review of the statistics for the different paediatric respiratory conditions follows. Nationally the admission rate for children < one year of age for lower respiratory tract infection, predominantly bronchiolitis and pneumonia, is 102.6/1000 but up to 176.5/1000 in certain regions (Graham, Leversha et al. 2001). The rates of admission for bronchiolitis for children < one year of age have increased from 26.6/1000 children in 1988 to 58.1/1000 in 1998, an increase of 118% (Vogel, Lennon et al. 2003). In addition, this increase in admission rates is accompanied by more severe disease in those admitted in comparison to the previous decade and in comparison to paediatric admissions of bronchiolitis in other developed countries. Of the children admitted; 59% required oxygen, 21% required nasogastric fluids, 22% intravenous fluids, 8% were admitted with apnoea and 3.1% required ventilation (Vogel, Lennon et al. 2003). Pneumonia continues to be a worldwide problem with acute lower respiratory tract infections being an important cause of mortality in children < five years of age and remains one of the leading causes of disability-adjusted life years lost worldwide. This largely reflects children in developing countries where it is estimated that 4.3 million children aged < five years die annually from lower respiratory tract infections (Garenne, Ronsmans et al. 1992). The incidence tends to be higher in children < five years of age at 34-40 cases per 1000 compared to any other age group except possibly for adults > 75 years (McIntosh 2002). A recent study conducted in New Zealand ascertained that the national pneumonia admission rate for children 0-14 years of age was 4.0/1000 based on the discharge diagnoses for pneumonia in 1998 to 1999 (Milne, Lennon et al. 2003). These admissions were skewed toward the younger age group. This equated to 1,534 admissions per 100,000 for children aged < two years, 562 admissions per 100,000 for children aged 2-4 years and 170 admissions for children aged 5-9 years. Extrapolation of this data amounts to approximately 3000 paediatric hospital admissions for pneumonia in New Zealand per year. Furthermore, from 1988 to 1995 there was an annual increase of 5% in the hospital admission rate. This increase was not due to admitting children with less severe disease; indeed the disease severity also seemed to be on the rise (Ministry of Health 1998; Grant 2006). Even pertussis, which is largely preventable by immunisation, continues to be a significant problem. Reported coverage of pertussis immunisation shows that only 60% of children are fully immunised, with only 42% of Maori and 45% of Pacific children completing the recommended
immunisation schedule (Ministry of Health 1992; Grant 2000; Grant, Pati et al. 2001; Turner 2006).

Bronchiectasis has been decreasing in most developed countries but this does not appear to be the case in New Zealand. In studies that I led, subsequent to the research presented in this thesis, a prospective investigation was conducted utilising the ‘New Zealand Paediatric Surveillance Unit’, covering 94% of paediatricians nationally. This requested notification and subsequent data on any new diagnosis of bronchiectasis made in children < 15 years through 2001 and 2002 (Twiss, Metcalfe et al. 2005). The incidence from this study put bronchiectasis at 3.7/100,000/year, which is seven times greater than the only other comparable national study done in Finnish children (Sayajakangas, Keistinen et al. 1998). This equates to 1/1700 live born children being diagnosed with bronchiectasis before the age of 15 years. If the incidence rate was to remain static and all these children survived to 15 years of age, this would equate to a prevalence of 1/3000 children overall. Again a disparity is seen with the incidence found to be three times higher in Maori children (1/1700) and twelve times higher in Pacific Island children (1/650) than those with European ethnicity. As well as being common, the disease was severe as demonstrated in both the Auckland study (Edwards, Asher et al. 2003) and the National study (Twiss, Metcalfe et al. 2005) with 83-93% having bilateral disease, and 61-64% having three or more of the six lobes of the lung involved. In addition the incidence of tuberculosis (TB) has decreased for adults in New Zealand over the recent years, but disturbingly there has been no such reduction in paediatric rates as demonstrated by our review of paediatric TB cases through the 1990s (Howie, Voss et al. 2005). Again significant ethnic disparities occur in these rates; in children < 15 years, Pacific children account for 17% of all cases of TB and Maori children for 15% compared to European at 3%. Unsurprisingly, this condition is also associated with socioeconomic deprivation (Ministry of Health 2002).

These reports all confirm the extent of paediatric respiratory disease in New Zealand and the fact that they do not appear to be on the wane despite the improving statistics seen in other developed and comparable countries.

1.3 The burden of asthma disease in New Zealand

Asthma is one of the most common chronic diseases in the world with an estimated 300 million people affected and the number of disability-adjusted life years lost estimated at approximately 15 million per year (GINA 2004; GINA 2005). Asthma has also had a demonstrated increase in prevalence in the 1980s and 1990s as evidenced by a number of
studies conducted throughout the United Kingdom, Australia and New Zealand (Robertson, Heycock et al. 1991; Peat, van den Berg et al. 1994; Rona, Chinn et al. 1995; Mitchell and Asher 1997; Asher and Grant 2006). In the 'Global Burden of Asthma' report (GINA 2004) the proportion of the population said to have 'clinical' asthma in New Zealand is given as 15.1% and in England (the country I did the research reported in this thesis) is given as 15.3%. The case fatality rate for asthma is 4.6/100,000 asthmatics in New Zealand and 3.2/100,000 asthmatics in England. In the 2006 ranking for asthma mortality from one (highest) to 68 (lowest) New Zealand appears at 17 and England at 26. The International Study of Asthma and Allergies in Childhood (ISAAC), (Weiland, Bjorksten et al. 2004) and the European Community Respiratory Health Survey (ECRHS), (Anonymous 1996) asked about self reported wheezing in the previous twelve months as one of their core questions, having demonstrated this had good specificity and sensitivity for diagnosis of asthma. In 13-14 year old children, England and New Zealand were ranked six and seven out of 84 countries where the listing was from one being the highest percentage of positive responses to 84 being lowest percentage of positive responses (Anonymous 1998). Furthermore, New Zealand ranked second when the symptom prevalence of wheeze in 13-14 year olds was determined by responses to a video questionnaire in 44 countries (Anonymous 1998).

In addition to community prevalence, asthma is a major cause of hospital admissions for children, particularly noticeable in younger age groups. Admission rates had been increasing through the 1970s, 1980s and early 1990s (Anderson, Bailey et al. 1980; Jackson and Mitchell 1983; Mitchell 1985; Hyndman, Williams et al. 1994) with possible stabilisation or reduction in the most recent years (Kemp and Pearce 1997; British Thoracic Society 2001; Akinbami and Schoendorf 2002). There were two major epidemics of asthma mortality and New Zealand featured highly in both. The first mortality increase was observed in several countries through the 1960s and included Australia, England, Norway, Scotland, Wales and New Zealand. The second epidemic was seen in New Zealand alone when mortality rates reached 4.1/100,000, which appeared linked to the use of the relatively non-selective β-2 agonist 'Fenoterol'. A 'Fenoterol' case control study was undertaken which looked at 117 patients between 5-45 years dying of asthma between 1981 and 1983. Each index case was matched with four controls and the use of 'Fenoterol' gave a relative risk of death of 1.59, slightly higher in females at 1.65 compared to males at 1.44. However it was greater in the patients who were less than 20 years of age with an odds ratio of 2.08 (Crane, Pearce et al. 1989). Fenoterol was subsequently withdrawn from the drug tariff and, coinciding with the greatly reduced use after 1990, there was a significant reduction in the number of deaths (Crane,
Pearce et al. 1995). Despite the stabilization in the last few years and the reduction in previous high mortality, asthma remains a prevalent paediatric disease (Kercsmar 2006; Weinberger and Abu-Hasan 2006).

1.4 The diagnosis of asthma – the quandaries in children

1.4.1 The asthma diagnosis in national and international guidelines

Prior to the difficulties regarding treatment, particularly in children, the first hurdle is making a correct diagnosis of asthma which commences by having an appropriate definition. The 2005 ‘British Guideline on the Management of Asthma’ from the British Thoracic Society & Scottish Intercollegiate Guidelines Network (SIGN) guideline states; “The diagnosis of asthma is a clinical one; there is no confirmatory diagnostic blood test, radiographic or histopathological investigation. In some people a diagnosis can be corroborated by suggestive changes in lung function tests” (SIGN 2005). The problem of accurate diagnosis appears to have existed for many decades. “The clinical diagnosis of asthma is not always simple and the absence of an agreed definition of the disease is a problem with many descriptions existing” appeared in 1959 from a conference on ‘terminology’ (Anonymous 1959). The difficulties of diagnosis and differential diagnoses are apparent in the paediatric section of the SIGN guideline and in two paediatric asthma guidelines published in 2005; ‘Pocket Guide for Asthma Management and Prevention in Children’ from the ‘Global Initiative for Asthma’ (GINA) (GINA 2005), and the ‘Management of Asthma in Children aged 1 – 15 years’ from the Paediatric Society of New Zealand (NZPAG) (Paediatric Society of New Zealand 2005). Ultimately these publications agree that it is a clinical diagnosis based on a history of appropriate symptoms of wheeze, which varies over time, improving either spontaneously or as a result of treatment and with wheeze heard during times of exacerbation. However all indicate this should occur in the absence of other features that could suggest congenital, suppurative or atypical respiratory disease, or is not responsive to asthma treatment.

1.4.2 What is meant by ‘wheeze’?

Even here there are at least two difficulties. Firstly, what is meant by ‘wheeze’ and secondly, that not all wheeze is asthma. Parental reporting of wheezing does not always coincide with what doctors mean by wheezing (Cane, Ranganathan et al. 2000; Cane and McKenzie 2001). Of parents presenting with infants with a problem of ‘noisy breathing’, 59% used the term ‘wheeze’ to describe their concern. However, after being shown video clips illustrating wheezing and other airway noises, only 36% continued to describe their infant as wheezing.
Video clips of infants with a variety of breathing noises led to 30% of respondents using other words to describe wheezing and 30% using the term ‘wheeze’ incorrectly (Elphick, Sherlock et al. 2001).

In addition, all that wheezes is not asthma (see Table 1.1). Up to 40% of infants who have been hospitalised with bronchiolitis may have subsequent wheezing episodes, usually in association with viral infections, up to five years of age (Martinez, Wright et al. 1995) and approximately 10% continue to wheeze after age five years (Noble, Murray et al. 1997; Sigurs, Bjarnason et al. 2000; Hall 2001). In the Tucson study of over 1200 children, wheeze was reported in almost 50% at some time between birth and six years (Martinez, Wright et al. 1995). Similar studies from Europe suggest 15-32% of children had wheezing in the first five years of life (Strachan 1985; Park, Golding et al. 1986). Sixty to 80% of infants who start wheezing in their first two years of life do not go on to have asthma. This group is found to have diminished airway function present prior to the onset of wheeze, and wheeze often ceases around the age of 3-5 years (Morgan and Martinez 1992; Holberg, Wright et al. 1993; Wilson 1994; Martinez, Wright et al. 1995; Clough, Keeping et al. 1999) which confirms the findings of a much earlier study (Boesен 1953). However, a minority of wheezy infants will develop later asthma (Wilson 1994; Martinez, Wright et al. 1995; Cochran 1998). These children continued to have wheezy episodes, developed an increased total serum immunoglobulin E (IgE) by age 9 months, developed sensitivity to a panel of aeroallergens by age 6 years, and had significantly lower lung function by age 6 years (Martinez 2002). In prospective follow up studies from childhood to adolescence, up to 80% of asthmatic children were reported to lose their symptoms during puberty (Balfour-Lynn 1985; Peat, Salome et al. 1989; Nicolai, Illi et al. 1998). Thus asthma may then remit in later childhood or may continue throughout childhood and into adult life (von Mutius 2001; Weinberger 2003).

Table 1.1: Alternative diagnoses in children with wheeze

<table>
<thead>
<tr>
<th>Acute wheeze</th>
<th>Recurrent or chronic wheeze</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaphylactic reaction</td>
<td>including foreign bodies</td>
</tr>
<tr>
<td>Angio-oedema</td>
<td></td>
</tr>
<tr>
<td>Aspiration syndromes</td>
<td></td>
</tr>
<tr>
<td>Bacterial tracheitis</td>
<td>respiratory syncytial virus, metapneumovirus, parainfluenza, influenza, adenovirus</td>
</tr>
<tr>
<td>Bronchiolitis</td>
<td></td>
</tr>
<tr>
<td>Acute wheeze</td>
<td>Recurrent or chronic wheeze</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><strong>Infection</strong></td>
<td><strong>syndromes</strong></td>
</tr>
<tr>
<td>Pertussis, mycoplasma, tuberculosis, any other bacterial infection</td>
<td>Bronchiolitis — recurrent</td>
</tr>
<tr>
<td>Bronchiolitis obliterans</td>
<td>Respiratory syncytial virus, metapneumovirus, parainfluenza, influenza, adenovirus</td>
</tr>
<tr>
<td>Cardiac conditions</td>
<td>Congestive heart failure, vascular compression (ring or sling)</td>
</tr>
<tr>
<td>Chronic lung disease of prematurity</td>
<td>Bronchiectasis, chronic bronchitis</td>
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<tr>
<td>Chronic suppurative disease</td>
<td>Anatomic lesion: cystic malformations, foregut malformations, granulation tissue, tracheoesophageal fistula</td>
</tr>
<tr>
<td>Congenital abnormality</td>
<td>+/− aspiration</td>
</tr>
<tr>
<td>Eosinophilic bronchitis</td>
<td>Cystic fibrosis, primary ciliary dyskinesia</td>
</tr>
<tr>
<td>Gastro-oesophageal reflux</td>
<td>Smoke, glue, drugs</td>
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<tr>
<td>Genetic disorders</td>
<td>Tracheomalacia, bronchomalacia</td>
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<td>Immune deficiencies</td>
<td>Adenovirus</td>
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<td>Inhalational irritants</td>
<td>Pertussis</td>
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<td>Malacic syndromes</td>
<td>Mycoplasma</td>
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<tr>
<td>Post-infectious</td>
<td>Wegener's granulomatosis, others</td>
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<tr>
<td>Sarcoidosis</td>
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<td>Tuberculosis</td>
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<td>Vasculitic disorders</td>
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<td>Vocal cord dysfunction</td>
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Despite these two confounding issues, wheeze remains a key element in the diagnostic criteria cited for asthma in all the guidelines (Paediatric Society of New Zealand 2005), (GINA 2005), (SIGN 2005) and confirmed by surveyed clinician opinion (Werk, Steinbach et al. 2000). There is no standard way of estimating the frequency or severity of episodes of wheeze to identify that which is purely consistent with asthma and classical diagnosis usually relies on associated symptoms and findings (GINA 2005). In addition, the performance of a β-2 agonist treatment trial for acute wheeze are described in these guidelines and also in two recent bronchiolitis guidelines, ‘Wheeze and Chest Infection in Infants under One Year’ from
the Paediatric Society of New Zealand which I co-chaired (Byrnes, Vogel et al. 2005) and a report from the Agency for Healthcare Research and Quality, from the United States Department of Health and Human Services (Viswanathan, King et al. 2003).

1.4.3 Where does cough fit in?

The use of cough as a diagnostic symptom is another area that is not straightforward. When asked what clinical factors were associated with establishing an initial diagnosis of asthma, 96% of the responding clinicians thought the requirement was recurrent wheeze, 90% symptomatic improvement with a bronchodilator, and 89% recurrent cough, but there was disagreement over what combinations of these factors were important (Werk, Steinbach et al. 2000). “Troublesome cough is a characteristic of asthma” (Li, Lex et al. 2003) and “cough is as frequent a symptom as is wheezing” (Weinberger and Abu-Hasan 2006) has been reported. Controversy has focused on whether ‘cough variant asthma’ (sometimes called ‘cough equivalent asthma’) truly exists, with researchers both for and against.

It is thought that asthma could occur without wheezing if the obstruction involves the small airways predominantly, and then coughing or shortness of breath may be the only complaint (Kercsmar 2006). In a follow-up study of 125 preschool children with recurrent cough but no other symptoms at the time of enrolment, 56% of them were symptom free at follow-up while 36.8% continued to have recurrent cough in the absence of a ‘cold’, and 7.2% had developed recurrent episodes of wheeze (Brooke, Lambert et al. 1998). One hundred children with a mean age of 5.7 years with chronic cough were followed for three years. Of the 75 deemed as having ‘cough variant asthma’, 28 (37%) went on to develop more ‘classical’ asthma symptoms (Todokoro, Mochizuki et al. 2003). Bronchial hyper-reactivity, nocturnal cough and developing cough at an earlier age may each be predictors of those who go on to get more typical asthmatic symptoms (Brooke, Lambert et al. 1998), (Todokoro, Mochizuki et al. 2003). It has been suggested in adults that those with cough who go on to develop classical asthma have a threshold for airway hyper-responsiveness somewhere between asthmatic and normal subjects (Koh, Jeong et al. 1999; Mochizuki, Arakawa et al. 2005) and 30% of those with cough and bronchial hyper-responsiveness will go on to develop asthmatic symptoms (Fujimura, Nishizawa et al. 2005). Eosinophilic inflammation (eosinophils, eosinophil cationic protein (ECP), interleukin 5 (IL5)) has been demonstrated in some patients with persistent cough; a pattern of inflammation similar to asthmatics seen in adults (Lee, Cho et al. 2001; De Diego, Martinez et al. 2005) and in children (Yoo, Koh et al. 2004). Basement membrane thickening, a pathological feature of asthma, was demonstrated in ‘classical’
asthmatics at 8.6 microns, in ‘cough variant asthmatic’ subjects at 7.1 microns and both were significantly different than healthy controls at 5.0 microns (Niimi, Matsumoto et al. 2000).

Others are less enthusiastic for this diagnostic category – given that cough is a very common symptom. A diagnosis of cough variant asthma could result in a significant increased number of children being treated. “Isolated chronic cough in children is rarely asthma, and the term ‘cough-variant-asthma’ should not be used” (Chang, Landau et al. 2006). Wheezing reported by children had a very good discriminating ability for asthma whereas cough less so in over 1600 school children aged 8-12 years (Yu, Wong et al. 2004). In asthmatic children who had cough as a predominant symptom, the cough occurred early and heralded the onset of an exacerbation. Asthma scores did relate to cough scores, although neither cough receptor sensitivity nor cough scores related to any specific inflammatory marker (Chang, Harrhy et al. 2002). In 1245 children 6-12 years of age comparing symptoms of persistent cough with symptoms of wheeze, the former had less demonstrable atopy and less morbidity. The authors concluded that while ‘cough variant asthma’ was not shown, there remained a significant number of children with persistent cough diagnosed and treated as having asthma (Faniran, Peat et al. 1999). So in the presence of persistent cough, if history, examination, chest x-ray and spirometry are normal, the cough could be regarded as non-specific and the child regarded as in the category of ‘normal’, as opposed to heralding a morbid or pre-morbid condition (Chang and Powell 1998; Bush 2002).

Finally, treatment trials for cough using inhaled corticosteroids (IHCS) (Dicpinigaitis 2006; Matsumoto, Niimi et al. 2006) and a leukotriene receptor antagonist (Spector and Tan 2004) showed an improvement in symptoms. However the natural history of post-viral cough is that most will have resolved in 3-4 weeks and only 10% will continue for more than 25 days, with gradual resolution. Improvement could therefore have erroneously been attributed to treatment. The Cochrane Systematic Review of the efficacy of IHCS for non-specific cough in children > two years of age evaluated two trials enrolling a total of 123 children. The first trial using beclomethasone dipropionate 400μg/day (metered dose inhaler via a spacer) found no difference from placebo in reducing cough frequency measured objectively or scored subjectively. The second trial using fluticasone propionate 2mg/day for three days followed by 1 mg/day for 11 days (metered dose inhaler via a spacer) showed a significant improvement in nocturnal cough frequency after two weeks, but also with a significant (though smaller) improvement with placebo (Tomerak, McGlashan et al. 2005). There was no difference between the use of inhaled salbutamol and placebo (Tomerak, Vyas et al. 2005) or
inhaled anti-cholinergic agents and placebo (Chang, McKean et al. 2004) in children with persistent non-specific cough.

1.4.4 Investigations

There are few investigations that are specific to asthma in adults and children. There are no diagnostic chest x-ray findings; the film may be normal, or there may be hyperinflation, interstitial infiltrates or atelectasis with mucus plugging (Arthur 2000; Kercsmar 2006). Hyperinflation, the most common pattern associated with asthma, can also be seen in bronchiolitis and cystic fibrosis (CF) (Arthur 2000). Similarly, studies have shown that chest x-rays are not useful in determining the difference between bronchiolitis and pneumonia (Khamapirad and Glezen 1987; Davies, Wang et al. 1996; Byrnes, Vogel et al. 2005). In a survey of school age children referred to a respiratory clinic, 30% had prior diagnoses of pneumonia on chest xray appearances but presented with symptoms identical to those subsequently associated with their asthma diagnoses (Castro-Rodriguez, Holberg et al. 1999; Mahabee-Gittens, Bachman et al. 1999).

Both total IgE and skin prick testing to common allergens have been used as a method of detecting atopy, which overlaps the asthma syndrome. IgE levels appear to have been increasing over the last decades coinciding with the increase in asthma (Burrows, Martinez et al. 1989; Burney, Malmberg et al. 1997). Higher levels of IgE have been associated with increased atopy and an increased incidence of wheeze (Call, Smith et al. 1992; Heymann, Carper et al. 2004). An association has been demonstrated between the level of IgE and airway hyper-responsiveness as measured by bronchial challenges (Muranaka, Suzuki et al. 1974; Burney, Britton et al. 1987). In a long term infant follow up study, the mean total serum IgE at nine months of age was significantly higher among those who became persistent wheezers compared to those who did not wheeze. Within the group of early wheezers, those who were wheeze free by ages 11 and 16 years had total IgE levels similar to those who never wheezed (Martinez 2002). However, the findings are not always consistent. Total IgE levels were found to be higher in some cohorts of children than others despite similar asthma prevalence, and the total IgE was higher in countries where house dust mite was the dominant source of allergen (Platts-Mills and Heyman 2006). The ECHRS study involving 37 centres in 16 countries demonstrated variation in the prevalence of response to at least one specific IgE which ranged from 60% in Albacete (Spain) to 45% in Christchurch (New Zealand) and was not always consistent with asthma prevalence. The study also showed significant variations between countries for total IgE and for prevalence of any specific IgE. At all ages women had
a 26% lower total serum IgE than men, which is surprising when asthma tends to be more prevalent in women in adulthood. There was a lack of association between the prevalence of atopy (defined as a positive response to any of the four specific allergens used in skin prick testing in all centres) and the geometric mean of total serum IgE. It is probable that the total and specific IgE are influenced differently and while raised IgE is associated both with atopy and with asthma, the relationship is not clear (Burney, Malmberg et al. 1997). Furthermore, IgE antibody production is T cell dependent, and the immune response to allergens can also include other immunoglobulins such as immunoglobulin G (IgG) and immunoglobulin A (IgA) which may give further variations (Platts-Mills and Heyman 2006). It is true to say that if a child is thought to have bad asthma, but is non-atopic, then the search for an alternative diagnosis should be undertaken immediately.

Pulmonary function tests have been one of the mainstays of assessing respiratory disease in older children and adults. A measurement in forced expiratory volume in 1 second (FEV₁) alone can miss small airway obstruction in patients and the forced expiratory flow rate over 25-75% of forced vital capacity (FEF25-75%) may well be a more sensitive indicator, particularly in children and/or in mild disease; however, the measurement may be very variable. Subjects can have a reduction in this parameter of more than two standard deviations below predicted norms even with no clinical symptoms (Kercsmar 2006). There is a significant overlap with measurements between healthy children and those with episodes of wheeze (if not current), and the diagnostic value of baseline lung function tests is generally thought to be poor (Dundas and McKenzie 2006). The guidelines suggest a test of bronchodilator responsiveness for asthma either clinically and/or utilising improvement in lung function (usually FEV₁) by an arbitrary amount, sometimes 15%. Despite this, the SIGN guideline states “a definitive diagnosis of asthma can be difficult to obtain in young children. It is often not possible to measure airway function in order to confirm the presence of variable airway obstruction” (SIGN 2005).

The other option for possible diagnosis and monitoring of asthma is to use peak expiratory flow (PEF). This is defined as the largest expiratory flow achieved with a maximal forced effort from a position of maximum inspiration sustained for longer than 10 milliseconds (Wright and McKerrow 1959). PEF reflects a range of physiological characteristics including lung elastic recoil, large airway calibre and lung volume (Ruffin 2004). The original ‘Peak Flow Meter’ was pioneered by Martin Wright in 1959 (Wright and McKerrow 1959) and is now supplanted by newer models but these have retained the advantages of the original in being low cost and easily portable. In the late 1970s the use of PEF recordings was first

However, there are issues with using PEF as this predominately reflects alteration in large airway calibre which is different to FEV₁ that reflects changes in calibre of both large and medium sized airways (Osmanliev, Bowley et al. 1982; Robinson, Chaudhary et al. 1984; Dolyniuk and Fahey 1986; Gregg 2000). PEF is effort dependant so is susceptible to both respiratory muscle strength and patient motivation (Tzelepis, Pavleas et al. 2005). While the reproducibility of PEF has been reported to be good with a coefficient of variation in well trained subjects of between 5 to 14%, still more than 50% of asthmatic patients show a 10% difference and 33% of patients show more than a 20% difference between the percent predicted value of FEV₁ and PEF (Kelly and Gibson 1988; Paggiaro, Moscato et al. 1997). In addition, the standard deviation for PEF readings is consistently greater than that of FEV₁. In another study, the coefficient of PEF repeatability was 10% for healthy adults but 17% for healthy children, 17% in asthmatic adults and 28% in asthmatic children (Enright, Sherrill et al. 1995). This poorer repeatability in asthmatic subjects has been confirmed by other studies (Meijer, Postma et al. 1996; Timonen, Nielsen et al. 1997; Holcroft, Eisen et al. 2003). In addition, significant differences in variability were also described between encouraged PEF readings done in a laboratory and those done at home (Reddel, Ware et al. 1998; Gannon, Belcher et al. 1999).

The two methods of using PEF to detect or monitor asthma was either by determining daily PEF variability or by using the comparison of the current PEF to a subject’s best PEF (Jain, Kavuru et al. 1998). PEF variability is associated with acute asthma exacerbations (Bellia, Cibella et al. 1985; Beasley, Cushley et al. 1989; Jindal, Aggarwal et al. 2002). Cross sectional studies have demonstrated significant (albeit weak) correlations between PEF variability and overall severity of inflammation, symptoms scores, FEV₁ and bronchial hyper-responsiveness (Higgins, Britton et al. 1992; Brand, Duiverman et al. 1997; Douma, Kerstjens
et al. 2000; Jindal, Aggarwal et al. 2002) with similar (but weaker) correlations found in longitudinal studies (Brand, Duiverman et al. 1999; Kamps and Brand 2001). However, the degree of variability considered ‘abnormal’ is difficult to determine (Jindal, Aggarwal et al. 2002) with a difference of 20% between repeated PEF recording suggested as necessary to identify asthma (Hetzel 1981; Jamison and McKinley 1993; National Asthma Education and Prevention Program 1997). Studies have shown no clear demarcation between normal individuals and asthmatics (Higgins, Britton et al. 1989; Parameswaran, Belda et al. 1999), with the greatest overlap between groups occurring in children (Albertini, Politano et al. 1989; Goldberg, Springer et al. 2001). PEF variability of 20% gave a sensitivity of 51.7%, specificity of 82.4%, positive predictive value of 13.9% and a negative predictive value of 95.5% for detecting asthma in one study (Goldberg, Springer et al. 2001). In a second study 20% variability gave a sensitivity of 36%, specificity 90%, positive predictive value of 16.4% (Kunzli, Stutz et al. 1999) while this cut off missed three quarters of the asthmatic patients in a third study (Goldstein, Veza et al. 2001). The relationship between the magnitude of PEF variability and response to bronchodilators is poor on or off IHCS therapy (Connolly 1979; Kerstjens, Brand et al. 1994; Douma, Kerstjens et al. 2000; Iwasaki, Kubota et al. 2000) and the relationship between PEF measurement when on long acting β2 agonist treatment is complex depending on the timing of the last dose (Reddel, Jenkins et al. 1999; Reddel, Ware et al. 1999). Also both daily peak flow levels and mean peak flow may fall so that the variability may not change (Reddel, Jenkins et al. 1999). In addition, there appears to be a circadian rhythm for PEF in all individuals (Hetzel and Clark 1980; Hetzel 1981) but exaggerated in patients with asthma (Turner-Warwick 1977; Connolly 1979; Bagg and Hughes 1980; Hetzel and Clark 1980).

The other option recommended is to use a percentage of maximum PEF value for an individual to discern asthma. A reduction from best PEF of 16.5% gave a sensitivity of 51% and a specificity of 98.7%, while a reduction of 20% gave a sensitivity of only 40% and specificity of 99.3% (Aggarwal, Gupta et al. 2002). In children, percent predicted PEF only weakly correlated symptoms or airway responsiveness (Kolbe, Richards et al. 1996; Brand, Duiverman et al. 1997; den Otter, Reijnen et al. 1997). This also assumes the patients will know their best PEF measurement but only 29% of 104 subjects were able to report this (Diner, Brenner et al. 2001). This group also showed that over subsequent days, 45% of patients had a measured PEF greater than their reported personal best. Thus if calculated on the reported figure both over-treatment in as many as 30% of clinically stable patients (Douma, Kerstjens et al. 1998) and inappropriate emergency department discharges based on
improvement to 70% PEF would have occurred (Diner, Brenner et al. 2001). Predicted PEF values has also been shown to be alter with age, sex, race, height and smoking making advice based on “predicted values” difficult (Gregg and Nunn 1973; Nunn and Gregg 1989; Higgins, Britton et al. 1992; Higgins, Britton et al. 1993; Boezen, Schouten et al. 1994; Boezen, Schouten et al. 1995; Bellia, Cuttitta et al. 1997; Jain, Kavuru et al. 1998; Bellia, Catalano et al. 2004). In addition, significant variation in predicted PEF values has been demonstrated even within the same populations (Jindal, Aggarwal et al. 2002).

The number of PEF measurements also contributes to accuracy and studies have used between two and twelve readings (Gannon, Newton et al. 1998; Jindal, Aggarwal et al. 2002). Taking twelve measurements daily as the gold standard, using four of these detected only 60-80% of the variability and using two measurements detected only 20-45% of the variability (D’Alonzo, Steinijans et al. 1995). Another study showed that four, three and two daily measurements explained 90-95%, 70-82% and 55% of the diurnal variability respectively (Gupta, Aggarwal et al. 2000). Patients also need to repeat the measurements several times on each occasion as, on review of 5,809 test sessions, it was the third manoeuvre that most frequently (40% of the time) gave the highest reading (Gannon, Belcher et al. 1999). Practically, it would be difficult to request patients to do anything beyond two readings per day over several days, let alone weeks or months. With a circadian rhythm playing a part, ideally but also difficult would be to request the PEFs be done regularly at specific times of the day. Finally, the age at which the child becomes able to use a peak flow effectively is also variable (Clough 1996).

Overall the recommendation is not to use PEF for diagnosis, but international guidelines continue to recommend PEF monitoring in the assessment of asthma, although more recently for selected groups; those with severe asthma, poor perceivers, as a short term monitoring procedure for exacerbation or to assess the adjustment of the medication dose (Reddel 2006). PEF monitoring did reduce symptoms and improve other parameters such as days lost from work, physician consultations and hospital visits in some studies (Ignacio-Garcia and Gonzalez-Santos 1995; Cowie, Revitt et al. 1997). However, others showed no major changes in outcomes in adults (Jones, Mullee et al. 1995; Turner, Taylor et al. 1998; Adams, Boath et al. 2001; Tierney, Roesner et al. 2004) or children with asthma (Mortimer, Fallot et al. 2003). In children, it was difficult to perform (Gorelick, Stevens et al. 2004) and was not useful in dictating management during acute asthma (Wensley and Silverman 2004). A Cochrane review in 2004 concluded there was not enough data even combining seven trials (921 adults and 46 children) to show that personalised self management plans with or without PEF
monitoring improved asthma outcomes (Toelle and Ram 2004). A further Cochrane review (Bhogal, Zemek et al. 2006) in 2006 assessed four trials with 355 children comparing symptom based written action asthma plans to PEF monitoring and found no differences in the rate of exacerbation, oral steroid courses, admissions, symptoms, lung function, school absenteeism or quality of life (Charlton, Charlton et al. 1990; Yoos, Kitzman et al. 2002; Leta, Schlie et al. 2004; Wensley and Silverman 2004). Symptom monitoring was actually preferred over peak flow monitoring by the children. The final conclusion from this analysis was “symptom based written action plans are superior to peak flow written action plans for preventing acute care visits.” (Bhogal, Zemek et al. 2006). The daily use of PEF in a large prospective study was not perceived to be useful by most families and therefore unlikely to be adhered to by many (McMullen, Yoos et al. 2002).

Electronic recording spirometers were compared to hand written diaries in 61 subjects aware of the recording. Adherence to PEF monitoring over 72 weeks gradually declined from 96% to 89% with 13% of participants ultimately withdrawn because of poor adherence (Reddel, Toelle et al. 2002). When participants were unaware of electronic recording and completed a pen and paper diary over 3 month periods, 64% and 44% adherence to monitoring was noted (Chowienczyk, Parkin et al. 1994; Verschelden, Cartier et al. 1996). Two groups of children reported 96.6% and 94.8% PEF monitoring compliance while the simultaneously monitoring electronic device recorded compliance at 73.4% and 80.9%. Compliance decreased significantly from week one to week four such that overall the compliance was less than 50% in 12.5% of the children and 50-75% in 20% of the children. Invention of some PEF measurements occurred and increased threefold during the study time (Kamps, Roorda et al. 2001). This adds to the difficulty of giving action points, especially if figures are made up and/or it is not perceived as useful.

In addition to these difficulties, differences have also been demonstrated when using different PEF techniques. These include fast or slow exhalation (Richards 1993; Tzelepis, Zakythinos et al. 1997), a breath-hold at total lung capacity (Matsumoto, Walker et al. 1996), if the position of the instrument is changed in relation to the mouth (Nolan, Tolley et al. 1999) or with posture differences (Bongers and O’Driscoll 2006; Lin, Parthasarathy et al. 2006). Increased air trapping also reduced the ability of a normal PEF to predict a normal FEV₁ or FEF₂₅-₇₅% from 83 to 53% potentially falsely reassuring an individual of having better pulmonary function than is correct (Eid, Yandell et al. 2000).
Furthermore, investigators have also found a poor agreement between the brands of peak flow metres when tested at sea level (Jackson 1995), at altitude (Gardner, Crapo et al. 1992) and in the clinic (Gregg 1991; Quanjer 1992; Burge 1993; Dahlqvist, Eisen et al. 1993; Gregg 1998; Nolan, Tolley et al. 1999) even after the technical requirements for meters were established by the National Heart, Lung and Blood Institute in 1991 (National Heart Lung and Blood Institute 1991). Differences have also been seen between the values obtained from new or old meters of the same type (Douma, van der Mark et al. 1997; Nazir, Razaq et al. 2005).

Finally there is also a report of adverse reactions to peak flow monitoring with two patients having a herniation of abdominal content and three having depression or neurotic preoccupation with their PEF values which the authors calculated as a side effect incidence of 1.1 cases per thousand patients in the study population (Schoch, Nierhoff et al. 1998). There has been a report about the potential of fungal contamination of peak flow devices (Ayres, Whitehead et al. 1989). So PEF monitoring is also not the answer for accurate diagnosis and may not offer significant advantage for long term monitoring in most asthmatic patients over symptom assessment.

Airway challenges (both direct and indirect) have also been used to make an asthma diagnosis, although this happens less often in paediatric field outside of the research arena. Airway direct challenges have been used widely and are well standardised most commonly using methacholine or histamine. These act as direct stimuli on the effector cells, predominantly airway smooth muscle, but also on mucus glands and airway microvasculature, causing airflow limitation (Adelroth, Hargreave et al. 1986; Woolcock, Anderson et al. 1991). The responses follow a continuous distribution within the population, which leads to difficulty when determining a normal, versus an asthmatic response (Dehaut, Rachiele et al. 1983). This has been arbitrarily defined (currently set at <8mg/ml⁻¹) so that the majority of asthmatics are identified at the selected cut off when the provocation concentration (PC₂₀ or provocation dose, PD₂₀) produces a 20% fall in the FEV₁ (Tashkin, Altose et al. 1992; Sterk, Fabbri et al. 1993; Lotvall, Inman et al. 1998; American Thoracic Society 2000). This has allowed a sensitivity of almost 100% for clinically current asthma (symptoms within a few days of the testing) but is less sensitive to epidemiological asthma (symptoms within the past year) (Townley, Bewtra et al. 1979; Hopp, Bewtra et al. 1984; Backer, Groth et al. 1991). At a PC₂₀ of <8mg/ml⁻¹, the positive predictive value is actually less than 50% (Cockcroft, Murdock et al. 1992). If this arbitrary point is reduced, the specificity and positive predictive value can approach 100%, such as at a PC₂₀ of less than 1mg/ml, but the sensitivity and negative predictive value are then poor. The current values have thus been selected to be
sensitive and to have good negative predictive value in getting a response in asthmatics who
have recent or current symptoms. This comes at the cost of being less specific and having less
positive predictive value or being useful for population screening for asthma (Cockcroft,
Murdock et al. 1992). As part of the ECRHS, bronchial hyper-reactivity using standardised
methacholine challenges was tested in over 13,000 adult subjects in 35 centres from 16
countries showing considerable variation in incidence (Chinn, Burney et al. 1997). In some
this mirrored their known rates of asthma disease, such as being high in New Zealand, Great
Britain, Australia and the USA, and low in Iceland and Switzerland. There was less of an
obvious connection in others, for example showing high rates of hyper-reactivity in Denmark
which has far lower documented asthma prevalence than, for example, Italy, Spain or Sweden
which all had low reactivity rates. In an early study of 307 adults, bronchial reactivity was
noted in 3% of normal subjects, 100% of symptomatic asthmatics, 69% of currently
asymptomatic asthmatics (Cockcroft, Killian et al. 1977). In other studies, the proportion of
individuals with bronchial hyper-responsiveness was only about 40-60% of those reporting
current wheeze (Backer, Dirksen et al. 1991; Backer, Groth et al. 1991; Backer and Ulrik
1992; GINA 2005).

As well as some 'normals' responding in what is deemed the asthmatic range (Cockcroft,
Killian et al. 1977; Cockcroft, Murdock et al. 1992), the direct airway challenges are also non-
specific, as subjects with other respiratory diseases also react, most notably cough (Brooke,
Lambert et al. 1998), chronic obstructive pulmonary disease (COPD) and/or chronic
bronchitis (CB) (Ramsdell, Nachtwey et al. 1982; Ramsdale, Roberts et al. 1985; Calverley,
Burge et al. 2003).

Direct airway challenges in children are limited to older age groups. In over 2000 children
aged 7-10 years tested with a cumulative dose of 3.9\mu mol of histamine, only 58% of the
children with asthma and current symptoms responded. While 52% of those diagnosed with
asthma responded overall, 53% of subjects demonstrating bronchial hyperactivity had no
asthma diagnosis (Pattemore, Asher et al. 1990). In three regions with similar asthma
admissions (Auckland, inland New South Wales and coastal New South Wales) between 700
to over 1,000 children were tested at each centre and while two groups had similar rates of
bronchial hyper-reactivity to methacholine challenges, the third had a much lower rate (Asher,
Pattemore et al. 1988). The hyper-reactivity to direct airway challenge has a similar sensitivity
and specificity profile for asthma diagnosis as questionnaire data (Asher, Pattemore et al.
1988; Backer, Dirksen et al. 1991; Bakke, Baste et al. 1991; Backer and Ulrik 1992; Joos,
O'Connor et al. 2003). In one of these studies, the level of bronchial hyper-reactivity was a
poorer predictor of a diagnosis of asthma than responses to the question ‘has your child had wheezing in the past 12 months’ on written questionnaires (Pattemore, Asher et al. 1990). In 495 children aged 7-16 years, the subjects with asthma represented a subgroup within the responsive end when inhaling increasing concentrations of histamine rather than the asthma and the ‘normals’ having separate distribution peaks. Selecting a low dose of 2.4mg/ml\(^1\) as the cut off point gave a specificity of 98% for asthma, but a positive predictive value of only 60% and a sensitivity of only 57% (Backer, Groth et al. 1991).

Using indirect challenges is a more recent phenomenon from the late 1980s and early 1990s (Manning, Watson et al. 1993; Joos, O'Connor et al. 2003). These are termed indirect as the triggers act on cells, which then release mediators or cytokines to cause a secondary bronchoconstriction and airflow limitation. They include physical stimuli such as exercise, osmotic stimuli such as hypertonic saline or mannitol, or pharmacological stimuli such as adenosine or bradykinin. These challenges are thought to be a better reflection of the active airway inflammation as observed in asthma (Joos, O'Connor et al. 2003). Also IHCS reduce responsiveness to these indirect challenges, as in asthma, while in contrast they only have a small effect on histamine and methacholine challenges. The indirect challenges have demonstrated less sensitivity but more specificity in differentiating asthmatics from ‘normals’ (Vasar, Braback et al. 1996; Godfrey, Springer et al. 1999; Joos, O'Connor et al. 2003). In addition in children, an exercise challenge has also been shown to be better at distinguishing asthma from other chronic airway disorders such as CF, bronchiolitis obliterans, ciliary dyskinesia and bronchiectasis (Avital, Springer et al. 1995; Godfrey, Springer et al. 1999). There is a statistical, albeit weak, correlation between the methods; however there are individuals who have positive exercise challenges and negative histamine or methacholine challenges. A hypertonic saline challenge in adults has been shown to identify exercise induced asthma (Belcher, Lee et al. 1989; Boulet, Turcotte et al. 1989; Smith and Anderson 1990; Brannan, Koskela et al. 1998). The possibility of having exercise induced asthma and not responding to hypertonic saline or mannitol challenges occurs, but only in those with very mild asthma (Brannan, Koskela et al. 1998). In children, those with a history of current wheeze were seven times more likely to have a positive response to hypertonic saline than asymptomatic children (Riedler, Reade et al. 1994). However the use of osmotic challenges to date have been seen more frequently as a treatment to improve mucociliary clearance and lung function, predominantly in children with CF (Eng, Morton et al. 1996; Rodwell and Anderson 1996; Robinson, Daviskas et al. 1999).
Thus determining the presence or absence of bronchial hyper-responsiveness to a trigger at a certain concentration is not necessarily diagnostic of asthma. The direct challenges lack specificity, while the indirect challenges lack sensitivity. Both challenge types are required to be done in the laboratory and while they are achievable with children, there is both a safety component and a significant time commitment to be considered. As repeated measures to monitor response to treatment or progression of asthma, they are less than satisfactory for these reasons.

In summary while the diagnosis of asthma may seem straightforward in some who fit the 'classical' asthma profile, there are many difficulties to be faced when testing for asthma. In addition, the investigations are more suited for asthma determination in adults than in children. A diagnosis of asthma in children remains difficult, when in this group getting the correct diagnosis is important if the usual asthma treatment is to be recommended.

1.5 Treatment and concerns

1.5.1 Possible adverse effects of asthma treatment in children

Much is known about the underlying pathology of asthma, and this will be discussed in depth in the next section of this chapter. This has resulted in all the guidelines (GINA 2005; Paediatric Society of New Zealand 2005; SIGN 2005) recommending anti-inflammatory treatment; with IHCS at step two as the first preventer and the introduction of oral corticosteroids at steps four or five with unresponsive and difficult asthma, and for acute treatment. The balance is between the correct diagnosis, appropriate management and to prevent irreversible airway thickening on the one hand, against the development of side effects with treatment on the other.

The adverse effects as listed in the guidelines for IHCS include; adrenal cortical insufficiency, growth failure, dysphonia and oral candidiasis. The adverse effects listed for oral corticosteroids is somewhat lengthier; adrenal insufficiency which may cause devastating hypoglycaemia, adrenal suppression, growth failure, hypertension, diabetes mellitus, reduction in bone mineral density, skin atrophy, striae, poor healing, immunosuppression and cataracts. Doses of IHCS of greater than 400µg/day of beclomethasone dipropionate or equivalent in children have been associated with side effects (Calpin, Macartthur et al. 1997; Sharek and Bergman 2000). There have been reports of IHCS affecting short term growth (Agertoft and Pedersen 1997), intermediate term growth over months to a year (Tinkelman, Reed et al. 1993; Doull, Freezer et al. 1995; Simons 1997; Verberne, Frost et al. 1997; Allen,
Bronsky et al. 1998), and a long-term effect over a period of years (Anonymous 2000). However, observational studies have suggested that despite long term IHCS in moderate doses, final adult height is not affected (Agertoft and Pedersen 2000; Peters 2006). While all guidelines recommend regular monitoring of the child’s height, isolated growth failure is not a reliable indicator of adrenal suppression (Dunlop, Carson et al. 2004).

On higher doses (800μg/day of beclomethasone dipropionate or equivalent) of IHCS or oral steroids, blood pressure should be monitored, bone density assessed and cataracts should be screened for, if being used for greater than a few months. Children may be more at risk of developing cataracts and glaucoma (Renfro and Snow 1992; Nootheti and Bielory 2006), though one study disagreed (Simons, Persaud et al. 1993). IHCS at the recommended levels, and in some studies even high doses of fluticasone, had minimal effects on bone mineral density (Hopp, Degan et al. 1995; Kelly, Clee et al. 1995; Agertoft and Pedersen 1998; Griffiths, Sim et al. 2004). However others found that after years of treatment, asthmatics had lower total body bone mineral density than controls (Boot, de Jongste et al. 1997; Allen, Thong et al. 2000) with a negative relationship between this density and the total cumulative doses of IHCS used (Wong, Walsh et al. 2000). Oral candidiasis has been found in up to 5% of adult patients with asthma with positive mouth cultures up to 25% and dysphonia is also said to occur in up to 58% of patients at some time (Barnes, Pedersen et al. 1998).

The issues regarding side effects of these medications should also be considered in two other populations. Firstly, healthy subjects (for example if the diagnosis is not secure) may have greater systemic side effects to IHCS and oral steroids than asthmatics (Brindley, Falcoz et al. 2000; Brutsche, Brutsche et al. 2000; Falcoz, Oliver et al. 2000; Mackie, McDowall et al. 2000). Secondly, in the last years, treatment with IHCS has been assessed in younger children; those with preschool and infant wheeze and/or bronchiolitis. Most of the studies in the preschool group have tried to target those who are more likely to develop asthma over time – with either recurrent wheezy episodes, personal history of atopy, a family history of asthma or a positive asthma predictive index (Castro-Rodriguez, Holberg et al. 2000; Guilbert, Morgan et al. 2006). These have enrolled between 31 and 625 children aged six to 47 months have received doses from 40μg/kg to 1000μg via nebuliser or 300-800μg/day via metered dose inhaler with a spacer and face mask for between 6 and 26 weeks. Improvements were seen in symptom free days (Bisgaard, Gillies et al. 1999; Roorda, Mezei et al. 2001; Teper, Colom et al. 2004; Guilbert, Morgan et al. 2006), in lung function parameters (Pao and McKenzie 2002; Teper, Colom et al. 2004), in bronchial hyper-reactivity (Stick, Burton et al. 1995) and in use of additional ‘rescue’ medication (Bisgaard, Gillies et al. 1999; Teper,
Colom et al. 2004; Guilbert, Morgan et al. 2006). Side effects noted were growth velocity (Guilbert, Morgan et al. 2006), adrenal suppression, increased cough and hoarseness, and cataract formation (Bisgaard, Allen et al. 2004). An earlier Cochrane review in 2000 had suggested that episodic high dose IHCS was partially effective. However, they concluded that “there is no current evidence to favour maintenance low dose IHCS in the prevention and management of episodic mild viral wheeze of childhood” (McKean and Ducharme). In addition, more recent Cochrane reviews do not support the use of IHCS in acute bronchiolitis or used to prevent wheezing post acute illness in 2006 (Blom, Ermers et al.) or the use of oral steroids for bronchiolitis in 2004 (Patel, Platt et al.). So while at the current time steroids are not recommended in this age group, the increased use exposes many more children to corticosteroid therapy, often before a definitive diagnosis of classic asthma is able to be made (de Blic and Scheinmann 2000).

In fact it is interesting to note within the guidelines, the significant difference in the level of evidence available for the different age groups. For example in the pharmacological management chapter of the SIGN guidelines (SIGN 2005), the graded evidence for the recommendations is presented in age brackets (>12 years and adults, 5-12 years and < 5 years). Across these age groups the evidence frequently drops from ‘1++’ (strong evidence with high quality meta-analyses and systematic reviews of randomised controlled trials) down to ‘4’ (expert opinion) in the youngest age group.

1.5.2 A marker for inflammation would be useful

There is a need to minimize side effects, while preventing morbidity (and mortality). This is important for better individual health, and is also important financially – reducing work and school days lost. Other financial burdens can be experienced by a family with a child with uncontrolled asthma as revealed in this quote taken from the ‘Trying to Catch Our Breath’ monograph (Anonymous 2006):

“I visited a family who had almost no furniture in the house. They had taken their child to the doctor one evening in the previous week for an asthma attack. They spent $80 for the visit and the medications. This was their entire food budget for the following week. They were eating white bread and butter.” (Claire Richards, Asthma Nurse Educator, Porirua Asthma Service, New Zealand)

In 2005, the GINA guideline (GINA 2005) suggested to “titrate the dose of inhaled corticosteroid to the lowest dose at which effective control of asthma is maintained”. The
SIGN guideline (SIGN 2005) stated "the smallest dose of inhaled steroids compatible with maintaining disease control should be used. At higher doses add on agents for example long acting β-2 agonists should be actively considered". In the NZPAG (Paediatric Society of New Zealand 2005) again the recommendation is to "titrate the dose of inhaled corticosteroids to the lowest dose at which effective control of asthma is maintained". The difficulty here is how to measure what the appropriate low dose is and whether control has been achieved. Ultimately, treating the pathological changes rather than being guided by symptoms alone might well be useful. In the next section, I will present that data suggesting that asthma is an inflammatory disease, and why an easy to measure, non invasive, marker of airway inflammation might be of great value.

1.6 **Asthma as an inflammatory disease.**

During the last decades there has been a gradual move from the concept of asthma as a disease of bronchoconstriction to that of an inflammatory disorder, and from the concept of complete reversibility to accepting that some irreversible airway damage can occur. The current definition of asthma proposed by the GINA committee is "Asthma is a chronic inflammatory disorder of the airway in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes" (GINA 2005). In the following sections, I will briefly summarise findings from autopsy studies, then biopsy and lavage, induced sputum, blood and urine samples in asthmatic patients compared to normal subjects. While a full description of the research that has resulted in these two major premise alterations is beyond the scope of this thesis, the purpose of the next sections are two-fold; to background the concept of asthma as an inflammatory disease and to show that many of these samples are difficult to obtain. This makes them more appropriate for single or pre and post intervention determinations rather than repeated longitudinal or population assessments. I have also concentrated on what was known at the time of my research commencing but have included how this is viewed to date. NO will be discussed in the next chapter.

1.6.1 *What has been learnt from autopsy studies?*

The hint that asthma was an inflammatory disease occurred early. In the late 19th century Charcot-Leyden crystals (crystalline structures shaped as double narrow pyramids), Curschmanns spirals (coiled fibrils of mucus) and eosinophilia in sputum were recognised during severe exacerbations of asthma (Osslter 1892). This was followed early and mid last century by autopsy studies of adults dying from asthma (Huber and Koessler 1922; Bullen 1952; Dunnill 1960) and much later by autopsy studies in children (Cutz, Levison et al. 1978).
These described gross pathological changes with diffuse mucus plugging of the airways, sloughing of the epithelial lining, an increase in airway smooth muscle and oedema of the peribronchial tissues. Light microscopy showed mucus plugs, goblet cell hyperplasia and apparent thickening of bronchial basement membranes with increased smooth muscle. There was an infiltration of inflammatory cells with a marked increase in eosinophils. The later paediatric study (Cutz, Levison et al. 1978) also examined the specimens under electron microscopy. This again demonstrated mucus plugs, which consisted of epithelial, macrophage and eosinophil cells and cell fragments. A thickened basement membrane appeared to be composed of collagen deposits and the submucosa was infiltrated by a mixture of inflammatory cells, predominantly eosinophils. Recent autopsy studies have compared pathology specimens between patients with fatal asthma, non-fatal asthma and controls (Koshino, Teshima et al. 1993; Kepley, McFeeley et al. 2001; Chen, Samson et al. 2004). Significant smooth muscle shortening, increased submucosal gland area, increased mucus plugging and an increase in basophils was seen in the cases of fatal asthma compared to the other groups. These contributed to airway wall thickening with smooth muscle hypertrophy and increased collagen along the basement membrane. Compared to ten controls, 18 adults who had died of asthma had proteoglycans prominent in the extra cellular matrix creating most of the basement membrane thickening seen, with differing types of proteoglycans expressed in the two groups (de Medeiros Matsushita, da Silva et al. 2005).

1.6.2 Studies of inflammation using bronchoscopy and biopsy

The use of bronchoscopy was developed initially as a therapeutic measure by Dr Chevalier Jackson in 1904 (Reynolds 1987), and later became a way of obtaining airway samples. The flexible fibroptic bronchoscope was introduced into medical centres in the early 1970s (Ikeda 1970) with appropriate guidelines (Smiddy, Ruth et al. 1971; Sackner, Wanner et al. 1972), which were later modified to include standards for bronchoalveolar lavage (BAL) (Bernstein, Boushey et al. 1985; Reynolds 1987; Turner-Warwick and Haslam 1987) and detailed indications and protocols for the procedure were subsequently published (Klech and Pohl 1989; Kelch and Hutter 1990; Bleecker, McFadden et al. 1992; Rennard, Aalbers et al. 1998; Haslam and Baughman 1999; Reynolds 2000). The development of paediatric bronchoscopy commenced a decade later (Wood and Sherman 1980; Wood 1985) when construction of smaller bronchosopes became possible and these entered clinical practice in the mid 1990s (Perez and Wood 1994; Wood 1996; Rennard, Aalbers et al. 1998; ERS 2000; Wood 2001; Midulla, de Blic et al. 2003). Thus these technical advances allowed biopsy and BAL studies in ‘living’ patients to be undertaken.
In brief, the human airway can be divided into three layers; the inner wall (epithelium, basement membrane and the submucosa), the outer wall (loose connective tissue), and the smooth muscle layer (Bergeron and Boulet 2006). All layers have shown some alterations in obstructive respiratory disease compared to normal lung. With regard to the presence of inflammation, biopsy studies have shown increases in eosinophils, eosinophilic proteins, T lymphocytes (Foresi, Bertorelli et al. 1990; Poston, Chanez et al. 1992; Laitinen, Laitinen et al. 1996; Carroll, Cooke et al. 1997) and certain cytokines (or their messenger ribonucleic acid (mRNA)) such as interleukin 2 (IL2), interleukin 3 (IL3), IL5, granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNFα) and rantes when compared to both normal subjects and patients with other respiratory diseases (Azzawi, Bradley et al. 1990; Ackerman, Marini et al. 1994; Humbert, Durham et al. 1996; Powell, Humbert et al. 1996; Vrugt, Wilson et al. 1999). The presence of inflammation was confirmed even in mild asthma (Beasley, Burgess et al. 1993; Laitinen, Laitinen et al. 1996; Grootendorst, Sont et al. 1997; Laitinen, Karjalainen et al. 2000; Ward, Reid et al. 2005) and in patients during disease remission (Foresi, Bertorelli et al. 1990). A positive correlation with each of these parameters to the severity of asthma has been demonstrated by some studies (Beasley, Burgess et al. 1993; Carroll, Cooke et al. 1997; Vrugt, Wilson et al. 1999; Amin, Ludviksdottir et al. 2000; Barnes, Burke et al. 2000; Benayoun, Druilhe et al. 2003). In addition, some markers more commonly associated with infective diseases, such as interleukin 8 (IL8) and eotaxin, have also been demonstrated to be positively correlated with asthma severity (Lamkhioued, Renzi et al. 1997; Pepe, Foley et al. 2005). However, the correlations of these markers with severity have not been unanimous (Benayoun, Druilhe et al. 2003; Shahana, Bjornsson et al. 2005) and overall no unique inflammatory profile has been demonstrated for all asthmatic patients (Kavuru, Dweik et al. 1999).

In addition to the increase in inflammation demonstrated in asthma, there have also been increases noted in the airway wall components. These include airway smooth muscle, mucus glands, and submucosal and extra-cellular matrix tissue which have also been related to symptom severity and bronchial hyper-responsiveness (Saetta, Di Stefano et al. 1991; Chetta, Foresi et al. 1997; Benayoun, Druilhe et al. 2003; Chen, Samson et al. 2004; Pepe, Foley et al. 2005). The presence of high numbers of fibroblasts has been the most consistent feature demonstrated to relate to severity of disease and poor response to treatment (Wenzel, Schwartz et al. 1999; Boulet, Turcotte et al. 2000). In the last decade, these findings have taken on new importance as to their relevance to possible remodelling of the airway resulting in irreversible changes occurring (Jeffery, Laitinen et al. 2000; Wilson and Bamford 2001;
Black and Johnson 2002; Jeffery 2004; Kariyawasam and Robinson 2005; Ward and Walters 2005). This airway remodelling in asthma has been found in both large and small airways (Saetta, Di Stefano et al. 1991; James, Maxwell et al. 2002) and the changes are the same regardless of the asthma being atopic, occupational or intrinsic (Homer and Elias 2000). The inner wall components include loss of epithelial integrity (Naylor 1962; Laitinen, Heino et al. 1985; Jeffery, Wardlaw et al. 1989) and hyperplasia of the goblet cells in the sub-epithelium (Aikawa, Shimura et al. 1992; Ordonez, Khashayar et al. 2001). While thickening of the basement membrane was also described in the original autopsy and other studies above, it now appears that the true basement membrane (the ‘lamina rara’ and ‘lamina densa’) is not grossly altered (Roche, Beasley et al. 1989; Homer and Elias 2000) with the actual fibril content of the membrane found to be similar in asthmatic and control groups (Saglani, Molyneux et al. 2006). It is the area that sits immediately below the true basement membrane, ‘the lamina reticularis’ that is increased and this has been termed ‘subepithelial fibrosis’ or ‘reticular or sub-basement membrane thickening’ (Boulet, Laviolette et al. 1997; Elias, Zhu et al. 1999; Beckett and Howarth 2003). Here, enhanced collagen deposition has been demonstrated with subtypes I, III and IV, fibronectin, tenascin, lumican and biglycan (Roche, Beasley et al. 1989; Laitinen, Laitinen et al. 1996; Laitinen, Altraja et al. 1997; Wilson and Li 1997; Huang, Olivenstein et al. 1999; Benayoun, Druilhe et al. 2003; Karjalainen, Lindqvist et al. 2003). Myofibroblasts are specialized cells that have features of both myocytes and fibroblasts and are sources of interstitial collagens so are likely contributing to this deposition (Homer and Elias 2000). Myofibroblast number has been demonstrated to correlate directly to the thickness of this layer (Brewster, Howarth et al. 1990; Hoshino, Nakamura et al. 1998). However, it is the increased thickness of the smooth muscle layer that contributes most to the overall thickness of the airway. This has variably been described as due to hypertrophy and/or hyperplasia (Hossain 1973; Ebina, Takahashi et al. 1993; Cho, Seo et al. 1996; James 1997). The smooth muscle contraction has been shown to particularly narrow the airway where the entire lumen is surrounded as in the smaller bronchi prior to respiratory bronchioles. This is consistent with computer modeling of airway function, showing that the smaller airways are the site of the greatest increase in airway resistance in asthma (Hogg 1997). Finally, changes have also been described in the airway vasculature with an increase in both size and number of vessels and this angiogenesis also contributes to airway oedema seen (Li and Wilson 1997; Tanaka, Yamada et al. 2003). Given the variable nature of the findings it is difficult to show the exact nature of the progression, and this is the subject of ongoing research (Boulet 2000).
The ability to biopsy has led to studies of the early (Gonzalez, Diaz et al. 1987; Georas, Liu et al. 1992) and late asthma reactions (Robertson, Kerigan et al. 1974; Crimi, Chiaramondia et al. 1991) and a comparison of both (Metzger, Richerson et al. 1986; Metzger, Richerson et al. 1986; Aalbers, Kauffman et al. 1993) in response to deliberate allergy provocation. These demonstrated an influx of neutrophils at 6-8 hours followed by an influx of eosinophils at 24-48 hours which continued more than a week but seemed to resolve by day 16. An increase in the cellular expression of the mRNA of IL5 and GM-CSF occurred at 18 to 48 hours (Ohnishi, Sur et al. 1993; Tang, Rolland et al. 1997) with an increase in total proteins and airway permeability also in the late phase (Taylor, Hill et al. 1996; Teeter and Bleecker 1996).

In assessing response to treatment in asthmatic patients treated with moderate to high doses of IHCs (400mcg to 1600mcg/day beclomethasone equivalent) for between two weeks and up 5-10 years, most studies have reported a decrease in T-lymphocytes, eosinophils, and mast cell numbers in the airway wall (Lundgren, Soderberg et al. 1988; Burke, Power et al. 1992; Booth, Richmond et al. 1995; Djukanovic, Homeyard et al. 1997; Olivieri, Chetta et al. 1997; Barnes, Burke et al. 2000). As well, decreased dendritic cell number (Burke, Power et al. 1992; Trigg, Manolitsas et al. 1994; Bentley, Hamid et al. 1996) and reduced inflammatory cell activation measured as less granule secretory production and less cell membrane activation markers have also been noted (Bentley, Hamid et al. 1996; Olivieri, Chetta et al. 1997; Barnes, Burke et al. 2000; Ward, Reid et al. 2005). The expression of mRNA for IL4, IL5 and GM-CSF within cells also decreased with steroid treatment (Sousa, Poston et al. 1993; Bentley, Hamid et al. 1996). Interestingly, the effects have been more marked in the biopsy samples than the corresponding lavage studies (Booth, Richmond et al. 1995; Thompson, Teschler et al. 1996; Djukanovic, Homeyard et al. 1997; Olivieri, Chetta et al. 1997). The effect of steroids on the thickness of the epithelium and basement membrane have been less conclusive; some studies showing improvement (Olivieri, Chetta et al. 1997; Ward and Walters 2005) that has not been demonstrated in others even after years of treatment (Lundgren, Soderberg et al. 1988; Wenzel, Szefler et al. 1997; Wenzel, Schwartz et al. 1999; Barnes, Burke et al. 2000).

Paediatric studies have been more recent, in keeping with the later technical bronchoscopy development and the additional concerns regarding safety in this group. These have shown similar findings to the adult asthma studies with eosinophilic and lymphocytic inflammation, and an increase in the same cytokines (interleukin 4 (IL4), IL5, rantes) (de Blic, Tillie-Leblond et al. 2004; Payne, Qiu et al. 2004; Pohunek, Warner et al. 2005). The findings associated with airway remodeling (smooth muscle hypertrophy and reticular basement
membrane thickening) were present early in a study in children with an age range down to 1.2 years (Pohunek, Warner et al. 2005) and did not show differences between those with few symptoms compared to those with persistent symptoms or 'difficult to control' asthma (Cokugras, Akcakaya et al. 2001; Jenkins, Cool et al. 2003; Payne, Rogers et al. 2003; de Blic, Tillie-Leblond et al. 2004; Fedorov, Wilson et al. 2005). In addition, the findings were not associated with age, length of symptoms, lung function or bronchial reactivity (Payne, Rogers et al. 2003). Bronchial wall thickening on high resolution computerized tomography (HRCT) scan was shown to correlate with eosinophils numbers, ECP and sub-basement membrane thickening (de Blic, Tillie-Leblond et al. 2005).

1.6.3 Studies of inflammation using bronchoalveolar lavage

In adults the standard bronchoscope wedges between the 4th and 6th order bronchus (Hunninghake, Gadek et al. 1979) typically localising a lung zone with $10^6$ alveoli or a volume of 165ml at total lung capacity and 45ml at residual volume. In the 1960s, two studies initially started using catheters to do small volume bronchopulmonary lavage for assessment of disease (Finley, Swenson et al. 1967; Pratt, Finley et al. 1969). Standard guidelines for clinical, diagnostic and research practices have been developed for use in adults (Klech and Pohl 1989; American Thoracic Society 1990; Rennard, Aalbers et al. 1998; Reynolds 2000), and more recently for use in children (ERS 2000; Midulla, de Blic et al. 2003). Lavage studies have confirmed many of the biopsy study findings in asthmatics when compared to normal subjects. Increases are seen in eosinophils, T-lymphocytes, eosinophilic proteins and cytokines such as IL4, IL5, rantes and chemo-attractant factors with excellent recent reviews detailing these findings (Barrios, Kheradmand et al. 2006; Graham 2006; Tulic and Hamid 2006). An antigenic or exercise challenge resulted in an observed increase in eosinophils (Kirby, Hargreve et al. 1987; Wardlaw, Dunnette et al. 1988; Bousquet, Van Vyve et al. 1993; Krug, Tschernig et al. 2001), T cell lymphocytes (Virchow, Walker et al. 1995; Schuster, Tschernig et al. 2000), mast cells (Kirby, Hargreve et al. 1987; Bousquet, Van Vyve et al. 1993; Olsson, Rak et al. 2000), basophils and neutrophils (Kirby, Hargreve et al. 1987; Mattoli, Mattoso et al. 1991; Bousquet, Van Vyve et al. 1993), complement factors C3A and C5 (Krug, Erpenbeck et al. 2001) and eosinophilic proteins (Wardlaw, Dunnette et al. 1988).

Many more proteins can be measured in lavage fluid and this can demonstrate the effect of cells, even if the latter are not as visible. For example, mast cells account for up to 20% of the inflammatory cells and basophils are also prominent in biopsy studies, but both are only a
small proportion of cells recovered by BAL (Dunnill 1960; Foresi, Bertorelli et al. 1990; Koshino, Teshima et al. 1993; Macfarlane, Kon et al. 2000). Provocation studies have shown the early asthma response is associated with increased histamine, tryptase, prostaglandin D2, prostaglandin F2, thromboxane and leukotrienes which are released by these two cell types (Murray, Tonnel et al. 1986; Wenzel, Fowler et al. 1988; Liu, Hubbard et al. 1991; Chilton, Averill et al. 1996; Kavuru, Dweik et al. 1999). Adhesion molecules such as intracellular adhesion molecule 1 (ICAM 1) and lymphocyte function-associated antigen 1 (LFA 1) were also increased (Williams, Johnson et al. 1992). The cytokines associated with an allergic profile – IL3, IL4, IL5 and GM-CSF – and their receptors in cell surfaces were measured in high amounts (Virchow, Walker et al. 1995; Chung and Barnes 1999; Hamid, Tulic et al. 2003). Increases have also been seen in IL9 and IL13 which have been associated with mucus hypersecretion and IgE regulation (Louahed, Toda et al. 2000; Shimbara, Christodoulopoulos et al. 2000; Louahed, Zhou et al. 2001) as well as increased interleukin 11 (IL11) and interleukin 17 (IL17) that have pro-fibrotic activity (Einarsson, Geba et al. 1995; Minshall, Chakir et al. 2000; Molet, Hamid et al. 2001). On the other hand, decreased interleukin 10 (IL10), an anti-inflammatory cytokine, was seen during acute asthma and this returned to levels similar to controls with recovery (Borish, Aarons et al. 1996). Chemokines have been demonstrated as present and rising with airway challenges including eotaxin, monocyte chemoattractant protein and rantes as well as TGFβ which has been noted to have a role in the subepithelial fibrosis (Hamid, Tulic et al. 2003). With specific challenges, such as using ragweed antigen, specific IgE and IgA have also been shown to be increased in bronchoalveolar lavage fluid (Peebles, Hamilton et al. 2001). Similar to the findings from the biopsy studies, with treatment of either IHCS or oral corticosteroids, while there was a reduction of eosinophil, mast and epithelial cell numbers in lavage fluid, the eosinophilic proteins did not reduce and there was not a direct correlation between the inflammatory cell decrease and improvement in symptoms or bronchial hyper-responsiveness (Duddridge, Ward et al. 1993; Booth, Richmond et al. 1995; Djukanovic, Homeyard et al. 1997; Olivieri, Chetta et al. 1997).

The paediatric literature in this area has been more recent but has largely been in agreement with the findings in adults. The increase in eosinophilic inflammation and eosinophil proteins were seen (Barbato, Panizzolo et al. 2001; Just, Fournier et al. 2002; Najafi, Demanet et al. 2003), as well as increased neutrophils which were associated with persistent symptoms (Just, Fournier et al. 2002), ‘difficult to treat' asthma (de Blic, Tillie-Leblond et al. 2004; Payne, Qiu et al. 2004) and status asthmaticus (Lamblin, Gosset et al. 1998; Tonnel, Gosset et al.
In paediatrics, BAL has also been used to compare inflammatory profiles with other diseases that present with wheeze. An increase in eosinophil cell and proteins was seen in a group of asthmatics compared to control and bronchiolitis groups (Kim, Chung et al. 2000; Marguet, Dean et al. 2001; Kim, Kim et al. 2003; Kim, Kim et al. 2005). However, IL8 levels also correlated with the eosinophil level in the asthmatic group (Kim, Kim et al. 2003) and the number of neutrophils appeared to reflect severity of asthma (Marguet, Dean et al. 2001). A recent summary of these studies into airway inflammatory profiles in paediatric asthma has been published (Warner 2003).

1.6.4 Inflammatory markers in induced sputum

1.6.4 (i) Studies in adult subjects

Non-isotonic ultrasonically produced aerosols were first used more than fifty years ago to examine bronchial reactivity (Cheney and Butler 1970). Currently, nebulised hypertonic saline is used for three main reasons. Firstly, it can act as an airway challenge to evaluate hyper-responsiveness. Secondly, it can be utilised as a treatment to aid expectoration of the thickened sputum produced in some respiratory diseases, particularly CF (Eng, Morton et al. 1996; Wark, McDonald et al. 2005; Donaldson, Bennett et al. 2006; Elkins, Robinson et al. 2006). Thirdly, it has been used to induce sputum for microbiological assessment (for example in CF and TB) and/or for the harvest of cells and cytokines for research. The technique consists of inhaling saline at 3-7% concentration generated by an ultrasonic nebuliser, which is necessary to give a sufficient output for saline aerosol generation, for 10 to 30 minutes (Belda, Hussack et al. 2001). Patients or research subjects are requested to cough and try to expectorate sputum into a container every 3-5 minutes (Paggiaro, Chanez et al. 2002). Dithiothreitol 0.1% is used to break down mucus sulphhydryl bonds and to disperse cells. The samples are then centrifuged with slides made of the cellular component and stained to identify cell viability, total cell counts, the percentage of squamous cells and differential percentage or numbers of the other cell types. The sputum supernatant following a cytocpin preparation is then used for measurement of soluble mediators. The mechanism by which hypertonic saline causes sputum production is uncertain, but thought to be a combination of the osmotic movement of water into airways, the induction of cough and the increased rate of mucociliary clearance (Umeno, McDonald et al. 1990; Holz, Kips et al. 2000; Paggiaro, Chanez et al. 2002). This technique was first described in the early 1990s to obtain sputum samples from asthmatic and normal adult subjects (Fahy, Liu et al. 1993). A task force of European Respiratory Society members developed the "Standardised
methodology of sputum induction and processing” (Djukanovic, Sterk et al. 2002) which has a detailed report on sputum induction (Paggiaro, Chanez et al. 2002), methods of processing (Efthimiadis, Spanevello et al. 2002) and measurement of fluid-phase mediators (Kelly, Keatings et al. 2002) concentrating on techniques and findings in asthma and COPD subjects. Pre-treatment with β₂ agonists as appropriate and lung function and/or oxygen saturation monitoring is recommended throughout the procedure (Paggiaro, Chanez et al. 2002).

The early studies confirmed and enlarged on the inflammatory profiles seen in asthmatics compared to normal subjects as had been described in the biopsy and lavage studies above. An eosinophilic dominant inflammation was seen with increases in total cell numbers, as well as individual eosinophil and neutrophil counts, eosinophil derived proteins [ECP, eosinophil derived neurotoxin (EDN)¹ and major basic protein (MBP)], (Pin, Gibson et al. 1992; Fahy, Liu et al. 1993; Foresi, Leone et al. 1997; Louis, Shute et al. 1997; Bacci, Cianchetti et al. 1998; Park, Whang et al. 1998; Rosi, Ronchi et al. 2000) the allergic profile interleukins (IL3, IL4, IL5, IL10) and certain proteins such as tryptase, eosinophilic chemo-attractant factors, and adhesion molecules (Shoji, Kanazawa et al. 1998; Hamzaoui, Brahim et al. 2000).

Correlations between these sputum inflammatory cells and markers and clinical parameters have been investigated. The strongest associations appear to be during an acute asthma exacerbation with increases in eosinophils, activated eosinophils and ECP (Jang and Choi 2000; Jang, Choi et al. 2000), basophils and mast cells (Pin, Freitag et al. 1992; Gauvreau, Lee et al. 2000). During times of increased symptoms, eosinophil number, ECP, IL5 and albumin were all found to independently contribute to abnormalities of FEV₁ and FVC in asthmatics (Fujimoto, Kubo et al. 1997; Bacci, Cianchetti et al. 1998; Shoji, Kanazawa et al. 1998; Grebski, Wu et al. 1999; Woodruff, Khashayar et al. 2001; Bartoli, Bacci et al. 2004). Severe asthma was predicted by high eosinophil numbers, ECP and albumin concentrations, though there was no such gradation in mild to moderate asthmatics (Fujimoto, Kubo et al. 1997; Bartoli, Bacci et al. 2004). The eosinophil counts gave a sensitivity of 68.3%, and a specificity of 55.3% for moderate to severe asthma compared to mild, and was of a higher diagnostic value for asthma than sputum ECP or methacholine challenge (Park, Whang et al. 1998; Rosi, Ronchi et al. 2000).

¹ 'Eosinophil protein X' and 'eosinophil derived neurotoxin' is the same protein but referred to by these two different names throughout studies, and denoted as either 'EPX' or 'EDN'. I have elected to refer to it as 'eosinophil derived neurotoxin' and 'EDN'. Eosinophil cationic protein, eosinophil derived neurotoxin, major basic protein and eosinophil peroxidase are all separate proteins.
The correlations are weaker when looking at asthmatics during times of stability (Grootendorst, Sont et al. 1997; Ronchi, Piragino et al. 1997). No relationship was demonstrated with steroid dose or lung function test results in over 100 stable asthmatics and 40 normal subjects in one community sample of adults (Louis, Sele et al. 2002). Eosinophils were frequently within the normal range and not a useful diagnostic tool in a population divided into ‘normal subjects’, ‘asthmatics’, ‘wheeze but no asthma’ and ‘industrial exposure to irritants’ (Lemiere, Walker et al. 2001). Eosinophils, ECP, MBP, EDN and IL5 correlated with methacholine challenge and FEV₁ in adult asthmatics (Pizzichini, Pizzichini et al. 1996). However, the eosinophil numbers only accounted for 16% of the variance of the methacholine challenge across one group, while the TNFα levels correlated more closely (Obase, Shimoda et al. 2001). Other studies showed no correlation between eosinophilic inflammatory parameters and lung function (Hashimoto, Minoguchi et al. 1999; Rosi and Scano 2000), methacholine (Rosi and Scano 2000) or histamine challenges (Iredale, Wanklyn et al. 1994; Hashimoto, Minoguchi et al. 1999). Eosinophils, leukotriene E₄ (LTE₄), ECP and rantes were all increased in poorly controlled asthmatics rather than being consistently high in severe asthmatics, for example if patients were well controlled (Romagnoli, Vachier et al. 2002). In addition, they were high only with symptoms even in long-term corticosteroid dependent patients (Tarodo de la Fuente, Romagnoli et al. 1999). Considerable heterogeneity in sputum counts were seen in over 250 adult asthmatics and normal subjects with eosinophils of all amounts (Green, Brightling et al. 2002).

Many studies have used sputum to determine the best predictor of response to steroids. When commencing longer term IHCSt treatment in placebo controlled trials, sputum eosinophils correlated to the response in a number of studies (van Rensen, Straathof et al. 1999; Aldridge, Hancox et al. 2002; Deykin, Lazarus et al. 2005; Brightling 2006) and was a better marker than ECP levels in either sputum or blood (van Rensen, Straathof et al. 1999; Rosi, Ronchi et al. 2000; Aldridge, Hancox et al. 2002; Deykin, Lazarus et al. 2005), baseline lung function (Deykin, Lazarus et al. 2005) or methacholine challenge results (van Rensen, Straathof et al. 1999; Deykin, Lazarus et al. 2005). Acutely, sputum eosinophils and ECP fell with oral or intravenous steroid treatment (Claman, Boushey et al. 1994; Rosi, Lanini et al. 2002) with the eosinophil count giving a positive predictive value of 68%, sensitivity of 54% and specificity of 76% for an increase in FEV₁ >15% with steroid therapy (Little, Chalmers et al. 2000). Eosinophils, neutrophils, ECP, IL5 and fibrinogen all increased when treatment was reduced but the eosinophil count was consistently the most accurate, across a number of studies, to predict when treatment reduction would lead to symptom development (Pizzichini, Pizzichini
et al. 1999; Giannini, Di Franco et al. 2000; Jatakanon, Lim et al. 2000; Green, Brightling et al. 2002). The results seen across all the studies suggest that the inflammation measured in this way reflects current asthma control rather than grading chronicity or severity per se.

As indicated in the biopsy and lavage studies above, increased neutrophil counts may also be a marker for poorly responsive disease. In severe asthmatics, there were more neutrophils and less eosinophils compared to mild asthmatics (Loh, Kanabar et al. 2005) and neutrophilic inflammation was dominant in the minority of subjects from over 250 adult asthmatics who demonstrated less corticosteroid response (Green, Brightling et al. 2002). Both eosinophilic inflammation and neutrophilic inflammation were found to independently contribute to abnormalities of FEV₁ in 205 adult asthmatics (Woodruff, Khashayar et al. 2001). Treating the neutrophil count resulted in better control than monitoring lung function or symptoms (Chlumsky, Striz et al. 2006). Neutrophils were also increased acutely, making up more than 75% of the sputum cells in 10 of 18 adult subjects during acute asthma while eosinophils made up more than 75% of the cells in only three subjects (Fahy, Kim et al. 1995).

1.6.4 (ii) Induced sputum in children

Sputum induction in children developed more recently and therefore in some of the studies from the 2000s it has been combined with NO measurement. As this will be covered in depth in later parts of the thesis, I will only discuss the cell and cytokine inflammatory markers from induced sputum in this section. The technique has been used for getting bacterial, viral, fungal and tuberculosis cultures to determine infective aetiology (Merrick, Sepkowitz et al. 1997; Utsunomiya, Ahmed et al. 1998; Ordonez, Henig et al. 2003; kiso, Mudido et al. 2005; Ratjen 2006). Using induced sputum to assess inflammatory disease was first described in 1980 when presence of an eosinophilia was used to diagnose co-existent asthma in children with CF was investigated (Sly and Hutchison 1980). In 1995 sputum and nasal smears taken from 111 young children presenting acutely with wheeze showed increased eosinophils, neutrophils and basophils in those deemed ‘asthmatic’ compared to those who were wheezy but unresponsive to asthma treatment (Twaddell, Gibson et al. 1996). Increased eosinophils at 3.8% and epithelial cells at 11.5% were seen in 16 children with uncontrolled asthma compared to 15 with controlled asthma at 2.5% and 10.5% and 72 healthy non-asthmatic children at 0.3% and 1.5% respectively (Cai, Carty et al. 1998). Mast cells, prominent in adult asthma studies, were found in only 4 of the 42 asthmatic children (Cai, Carty et al. 1998). An increase in eosinophil percentage and ECP was seen in 50 asthmatic children compared to fifteen children with chronic bronchitis (CB) and 25 healthy children (Yazicioglu, Ones et al.
In 146 children with asthma and 37 controls, the percentage of eosinophils increased from a mean of 1.5% in children who had infrequent asthma to 2.3% with frequent asthma and to 3.8% if they had persisting symptoms, compared to controls at 1%. Similarly, ECP levels increased from 113ng/ml in infrequent asthma to 220ng/ml in frequent asthma and 375ng/ml in the persistent asthmatics compared to 139ng/ml in the control group. While there were significant differences between the asthmatic groups, no difference was demonstrated between the ‘infrequent exacerbation’ and ‘control’ children (Gibson, Simpson et al. 2003).

However, in 27 healthy and 60 asthmatic children, no correlation was seen with any marker of airway inflammation and asthma severity as measured by lung function (Wilson, Bridge et al. 2000). In addition, in 58 asthmatic children, it was the methacholine challenge rather than the sputum eosinophil counts or ECP that related to the presence of recent symptoms (Wilson, James et al. 2001). No correlation was demonstrated between sputum eosinophils and FEV₁ or sputum ECP in 25 asthmatic children (Piacentini, Bodini et al. 1999). Further, no correlations were demonstrated in 32 children with stable asthma between ambulatory cough frequency, sputum inflammatory parameters or FEV₁ (Li, Lex et al. 2003). In 40 children with difficult asthma, only nine children had abnormal sputum cytology; six had a predominant sputum eosinophilia while three had a predominant neutrophilia (Lex, Payne et al. 2005).

Within one hour of arriving in the emergency department with acute asthma, eight children in one study and 38 in a second took less time to produce an induced sputum sample than when done during a time of stability. The sputum showed higher numbers of total cells, eosinophils, neutrophils, basophils and mast cells compared to macrophages which was the dominant cell during the recuperative phase when the sampling was repeated two weeks later with symptom resolution (Twaddell, Gibson et al. 1996; Norzila, Fakes et al. 2000). The second study also demonstrated reductions in ECP, myeloperoxidase, IL5 and IL8 by the recovery sample. The primary cell of inflammation was either eosinophil or neutrophil or showed a co-dominant pattern (Norzila, Fakes et al. 2000). Following a treatment with single nebulised glucocorticoid dose, there was a reduction in eosinophil percentage in 30 asthmatic children but ECP, IL5, GM-CSF and albumin levels, as well as FEV₁, remained unchanged (Oh, Lee et al. 1999).

A number of studies have looked at the inflammation in induced sputum before and after commencing IHCS treatment for more chronic asthma symptoms. In 14 children reduced sputum and serum eosinophils and ECP levels were seen after two weeks of IHCS treatment (Sorva, Metso et al. 1997). And in 60 children, there was reduction in sputum eosinophils
over a six month treatment programme, with no corresponding reduction in those on disodium cromoglycate (Rytila, Pelkonen et al. 2004). In the ‘Childhood Asthma Management Program’ (CAMP) study, IHCS treated patients had a lower sputum percentage of eosinophils at 0.2% versus 0.8% than those treated with nedocromil sodium or placebo. In repeated sputum inductions during reduction of medication, sputum eosinophil counts predicted an exacerbation (Covar, Spahn et al. 2004).

1.6.4 (iii) Technical aspects of bronchoscopy, bronchial biopsy, bronchoalveolar lavage and/or induced sputum

Despite the increasing ability to sample from the lung with the use of bronchoscopy, airway biopsy, BAL and induced sputum, there remain questions over the success, reproducibility and the safety of these techniques. To start with, there is significant variability both within and between biopsy samples even when taken from one individual (Richmond, Booth et al. 1996; Sont, Willems et al. 1997; Jeffery, Laitinen et al. 2000). The parameters from biopsy measurements are less reproducible than physiological tests, with even diurnal variation in number and function of inflammatory cells (Poulter, Burke et al. 2000). Biopsy specimens are also likely to be taken from quite different sites, from both proximal and distal airways, which may not be comparable (Balzar, Wenzel et al. 2002; Boulet 2002; James and Carroll 2002). BAL variability was investigated by doing 180 procedures in 20 clinically stable but symptomatic asthmatic adults. The standard deviation for total cell counts was $78 \times 10^3$ cells/ml and for macrophages was 16%, lymphocytes was 13% and eosinophils 1% (Ward, Gardiner et al. 1995; Ruffin 1996). The lavage profile seen in asthma, has also been seen in allergic rhinitis (Gutierrez, Prieto et al. 1998; Alvarez, Olaguibel et al. 2000; Polosa, Ciamarra et al. 2000; Boulay and Boulet 2002; Beeh, Beier et al. 2003; Wilson, Duong et al. 2005; Bonay, Neukirch et al. 2006; Hara, Fujimura et al. 2006) and in subjects with atopic dermatitis (Kyllonen, Malmberg et al. 2006).

The initial success rate of hypertonic saline in obtaining samples in adults was 75% (Pin, Gibson et al. 1992; Foresi, Leone et al. 1997; Hashimoto, Minoguchi et al. 1999) but improved to 83% to 93% with refinement of the procedure (Vlachos-Mayer, Leigh et al. 2000; Bartoli, Bacci et al. 2004). The success of sputum induction in children has been given as 60 to 74% in controls (Wilson NM 2000) and 61% to 94% in asthmatics (Norzila, Fakes et al. 2000; Wilson, Bridge et al. 2000; Li, Lex et al. 2003; Covar, Spahn et al. 2004; Rytila, Pelkonen et al. 2004; Lex, Payne et al. 2005). It is more successful in acute than chronic asthma (Norzila, Fakes et al. 2000; Rytila, Pelkonen et al. 2004) and more successful in the
older children, particularly those > 12 years of age (Norzila, Fakes et al. 2000; Covar, Spahn et al. 2004; Rytila, Pelkonen et al. 2004; Lex, Payne et al. 2005). In the CAMP study, 90 out of 117 children (75%) with mild to moderate asthma across eight centres provided an adequate sputum sample for analysis (Covar, Spahn et al. 2004). The total cell counts were the only similar result when induced sputum samples were examined on two separate days, two weeks apart in the same 37 subjects (Thomas, Yates et al. 1999). Considerable heterogeneity was also documented, particularly in eosinophils, in samples from over 250 adult asthmatics and normal subjects (Green, Brightling et al. 2002). Some of the variability seen between groups may be due to differing techniques. A range of 0.9 to 12% concentration of saline has been used, with the measurement being of either the whole expectorated sample or a sample separated from saliva or by picking out mucus plugs. The plugs were thought to provide more cells with better cell viability (Gershman, Wong et al. 1996; Pizzichini, Pizzichini et al. 1996; Spanevello, Beghe et al. 1998) while selected sputum had higher concentrations of soluble markers such as ECP (Gershman, Wong et al. 1996; Spanevello, Beghe et al. 1998). Using 0-5 minutes of hypertonic saline gave more neutrophils, while using a longer period of 10-15 minutes gave higher concentrations of eosinophils, IL4 and IL5 with lymphocytes, macrophages and epithelial cells remaining unchanged (Taha, Hamid et al. 2004). A comparison of induced and spontaneous sputum samples showed an increased number and more viable cells in the former process (Pizzichini, Pizzichini et al. 1996).

In addition, the induction procedure itself may alter findings. Consecutive sputum samples during one sputum induction displayed an increasing neutrophil gradient (Holz, Jorres et al. 1998; Nightingale, Rogers et al. 1998; Richter, Holz et al. 1999; Belda, Hussack et al. 2001) which occurred within six hours and lasted for 24 (Holz, Jorres et al. 1998; Nightingale, Rogers et al. 1998) with decreasing mucin and increasing concentrations of surfactant (Gershman, Liu et al. 1999). This effect was observed with both high and low output nebulisers (Berlyne, Lemiere et al. 1998). Repeated sputum induction over days also resulted in increased neutrophils with decreasing macrophage and eosinophil numbers (Belcher, Murdoch et al. 1988; Holz, Richter et al. 1998) and the effect of hypertonic saline on nasal mucosa resulted in increased histamine and leukotriene release (Silber, Proud et al. 1988). The exact cause of this inflammatory response is unknown but the additional fluid, hypertonicity, frequent coughing, or possibly bacterial contamination could all be contributory (Holz, Richter et al. 1998; Nightingale, Rogers et al. 1998; Sacco, Fregonese et al. 2000).
There are other studies suggesting sample repeatability was acceptable, with correlation coefficients between 0.7-0.87 for macrophage, lymphocyte, neutrophil and eosinophil counts in asthmatic, rhinitis, CF and normal subjects (Pin, Gibson et al. 1992; in 't Veen, de Gouw et al. 1996; Spanevello, Migliori et al. 1997; Bacci, Cianchetti et al. 2002; Beier, Beeh et al. 2004; Smountas, Lands et al. 2004). The repeatability of the soluble markers albumin, fibrinogen, IL8 and ECP were also thought to be acceptable in adult subjects with mild to moderate asthma (in 't Veen, de Gouw et al. 1996).

Performing all of these procedures requires highly experienced staff, particularly with children, also recognising the need for general anaesthesia for bronchoscopy, as well as highly qualified laboratory technicians (see Section 1.6.4 (iii) below regarding safety). The procedures are time-consuming which limits their use in clinical practice. For sputum induction, it is estimated that should a patient produce a suitable sample within 10 minutes of starting the procedure (and it can take up to 30 minutes) in a well practiced laboratory, the total time required for processing through to a result is 100 minutes and the sample requires processing within 2 hours of collection (Holz, Kips et al. 2000). The time spent to make the procedure ‘child friendly’ is likely to be longer. The CAMP researchers across eight centres commented “this procedure still remains a research tool in asthma because of its requirements for technical expertise” (Covar, Spahn et al. 2004).

1.6.4 (iv) Safety aspects of bronchoscopy, bronchial biopsy, bronchoalveolar lavage and/or induced sputum

In 159 asthmatic adults undergoing 273 bronchoscopies in six studies, there were 34 adverse event episodes which included bronchospasm, pleuritic chest pain, shortness of breath, fever, flu-like symptoms and haemoptysis (Elston, Whittaker et al. 2004). Another safety review reported that after six to eight biopsies taken per procedure in 57 patients; 40% had cough, 12% cough and bronchospasm and 3.5% required additional rescue medication (Tapanainen, Lindqvist et al. 2002). Arterial saturation decreased in 50 asthmatic patients from a mean of 97% to a mean of 92% and from 98% to 93% in 25 normal subjects during bronchoscopy and biopsy, without correlation to the pre-operative lung functions tests (Van Vyve, Chanez et al. 1992). Bacteraemia is also described with fibrooptic bronchoscopy, with 26 of 200 consecutive patients having positive blood cultures after the procedure despite the majority of patients having no prior respiratory illness (Yigla, Oren et al. 1999). A prospective analysis of the financial cost of complications from flexible bronchoscopy over a 30 month period revealed of the 1,009 bronchoscopies performed in 660 adults as an outpatient procedure,
complications occurred in 5% necessitating hospital admission in 0.5%, resulting in an additional cost of US $6,996 to treat complications but with an additional US $34,500 in those requiring hospitalisation (Colt and Matsuo 2001).

The largest study looking at side effects with flexible bronchoscopies in children, was a retrospective analysis of 2,836 procedures over 21 years, which concluded that it was a safe procedure. However 21 had life threatening hypoxaemia, 17 had laryngospasm or bronchospasm and 4% had nasopharyngeal bleeding (Nussbaum 2002). A large prospective study of 1,328 procedures (excluding intensive care examinations) recorded at least one complication in 6.9% of cases, of which 5.2% were mild and 1.7% were major with one pneumothorax (de Blic, Marchac et al. 2002). In 170 children with respiratory symptoms having bronchoscopy and lavage plus at least three endobronchial biopsies, fluctuations of oxygen saturation and end tidal carbon dioxide (CO₂) were seen in all patients but only one had a prolonged desaturation episode. In addition, minor bleeding was demonstrated at the site of the biopsies but no other side effects occurred (Salva, Theroux et al. 2003). Of 38 asthmatic children undergoing bronchoscopy, one had desaturation, two had fever and in four their asthma worsened over the following week. Of 35 non-asthmatic children undergoing bronchoscopy for another reason (not ‘normals’), there were there were 17 adverse events which were predominantly laryngospasm and apnoea (Payne, McKenzie et al. 2001). In 66 children < 5 years of age a comparison was made between having bronchoalveolar lavage alone and the addition of endobronchial biopsies with approximately half the group in each. Complications occurred in 24% and 18% respectively including cough, desaturation, apnoea and laryngospasm (Saglani, Payne et al. 2003). In 42 CF children and 39 with other indications for bronchoscopy, the complication rate was 13.3% and 17.9% respectively (Molina-Teran, Hilliard et al. 2006).

So despite the progress in knowledge from the biopsy and lavage studies – they are still invasive, require bronchoscopy, and sedation in adults and often general anaesthesia in children. It is therefore particularly difficult to justify use of these techniques in early age groups and/or repeatedly which is where the most knowledge regarding progression of disease is likely to come. Discussions about safety in the literature has presented both pro and con arguments (Connett 2000; Larsen and Holt 2000; Shields and Riedler 2000) ending with one commentator suggesting “because of the invasive nature of the investigation there are few conditions for which repeat sampling can be justified” (Connett 2000). The debate has continued more recently with a series of letters discussing the appropriateness of biopsy in particular as a research tool in children (Mallory 2006; Bush and Davies 2007).
So is induced sputum in adults and children safe? Particularly those with asthma? In 34 asthmatic and normal subjects, a mean fall in FEV$_1$ of 5.3% was seen, but with a maximum of 20% (Pin, Gibson et al. 1992). Pre-treatment with a $\beta_2$ agonist did not prevent bronchoconstriction in all the asthmatic subjects, especially in those with a baseline low FEV$_1$ (Wong and Fahy 1997). Such pre-treatment in mild asthmatics resulted in an unchanged FEV$_1$, but with a reduction observed in the normal subjects (Thomas, Yates et al. 1999). One group also documented that an overuse of $\beta_2$ agonists the day before sputum induction reduced the protective effect of the pre-medication (Pizzichini, Pizzichini et al. 1997). While samples were successfully obtained even in subjects with 'difficult to control' asthma, severe bronchoconstriction occurred in 22% with development of symptoms and a reduction in FEV$_1$ of $>15\%$ (ten Brinke, de Lange et al. 2001). Another study noted that the procedure had to be stopped because of side effects in 12% overall and in 17-18% of those with severe asthma (de la Fuente, Romagnoli et al. 1998). When performed as part of their routine clinical visit, the procedure was well tolerated in over 300 asthmatics, even among those with more severe respiratory obstruction with an FEV$_1$ down to 60% predicted or one litre. However in patients with a baseline FEV$_1$ between 40% and 59%, there was a mean 8% reduction in FEV$_1$ and in patients with a baseline FEV$_1$ of $<40\%$ predicted, 6% fell by 20% (Vlachos-Mayer, Leigh et al. 2000). A decrease in arterial oxygen saturations of 6% in asthmatic patients, 5.3% in smokers and 6% in healthy subjects has been reported suggesting that patients needed monitoring during this procedure (Castagnaro, Chetta et al. 1999).

Reviewing the safety in children; in 53 sputum induction alone was tolerated in 98%, with 94% completing the procedure, 92% providing an adequate sample but 4% experienced a $>15\%$ fall in FEV$_1$ (Jones, Hankin et al. 2001). In 182 children who had combined sputum induction and bronchial provocation testing using hypertonic saline, the procedure was completed by 90% with a distressing cough in 13% and mucosal irritation with wheeze in 1% and fewer, 70%, successful samples obtained. So while the two investigations can be combined, this was less well tolerated and resulted in less sampling success (Jones, Hankin et al. 2001). In 40 children with a baseline FEV$_1$ $>65\%$ predicted, seven (18%) had symptoms of shortness of breath and wheezing and of these three (8%) had a significant fall in FEV$_1$ of greater than 20% despite pre-treatment with a bronchodilator (Lex, Payne et al. 2005). In the CAMP study, nine (8%) of 117 children with mild to moderate asthma developed significant bronchospasm requiring treatment (Covar, Spahn et al. 2004).
1.6.5 Studies of inflammatory markers in blood and urine

Inflammatory markers have also been studied in blood and urine and I am going to briefly review what has been found in this area. Blood and urine samples are relatively easy to obtain when compared to the general anaesthesia or sedation required for bronchoscopy, lavage and biopsy, are more consistently obtainable than induced sputum and less likely to cause side effects than these other procedures. However blood testing is still seen as invasive by children and most adults. Also, these compartments are removed from the direct area of interest and reflect systemic findings, which may or may not be related to the lung.

As with results from sampling the lung, monitoring of inflammatory markers in blood have shown an increase in eosinophils, ECP and EDN levels in children and adults with asthma compared to control groups. In some studies these correlated with asthma severity or current symptomatology (Kristjansson, Shimizu et al. 1994; Parra, Prieto et al. 1996; Rao, Frederick et al. 1996; Remes, Korppi et al. 1998; Yazicioglu, Ones et al. 1999; Stelmach, Gorski et al. 2002), and although other studies also found higher levels, no such correlations were found (Pizzichini, Pizzichini et al. 1997; Bacci, Cianchetti et al. 1998; Niimi and Matsumoto 1999; Stelmach, Jerzynska et al. 2001; Stelmach, Jerzynska et al. 2001; Aldridge, Hancox et al. 2002; Reichenbach, Jarisch et al. 2002) or even mixed results were obtained (Currie, Syme-Grant et al. 2003). In adults, a large cross sectional study of almost 7000 subjects found physician diagnosed asthma was significantly associated with serum eosinophil count (Schwartz and Weiss 1993). Serum eosinophil and ECP levels during acute asthma were correlated with more severe exacerbations and less bronchodilator responsiveness in 48 adults (Lee, Lee et al. 1997). Plasma total IgE, plasma specific IgE, blood eosinophil percentage and exhaled NO were thought to account for 55.5% of the variance seen in bronchial hyper-reactivity (Leung, Wong et al. 2005). Urinary levels of EDN did increase at time of asthma exacerbation (Cottin, Deviller et al. 1998).

In children, ECP and eosinophilic peroxidase (EPO) in both serum and urine showed reduced gradation in levels from asthmatics with current symptoms to symptom free asthmatic children and to healthy children (Lonnkvist, Hellman et al. 2001). Both urinary EDN and serum ECP were significantly higher in children with atopic asthma than control subjects (Kristjansson, Strannegard et al. 1996). In both studies levels decreased with IHCS treatment (Kristjansson, Strannegard et al. 1996; Lonnkvist, Hellman et al. 2001) and in one increased if treatment was reduced (Lonnkvist, Hellman et al. 2001). Urinary EDN levels were increased in 80 asthmatic children compared to 24 controls, and were higher in symptomatic than
asymptomatic patients independent of the presence of atopy or the treatment modality. Urinary EDN levels in the asthmatics correlated to lung function, and in the subgroup of 15 children re-examined two months after commencement of treatment, the reduction of EDN also correlated with the changes in lung function (Lugosi, Halmerbauer et al. 1997). While no correlation was found between urinary EDN values and lung function in two other studies of 155 and 39 children, there was a similar reduction of EDN three months after commencing IHCS treatment (Kristjansson, Strannegard et al. 1996; Labbe, Aublet-Cuvelier et al. 2001). Circadian variations of the eosinophil proteins in urine samples have been demonstrated with high early morning and nocturnal peaks compared to lower levels in the afternoon in both asthmatic and non-atopic, non-asthmatic groups of children (Storm van's Gravesande, Mattes et al. 1999; Wolthers and Heuck 2003).

A more explored area using these samples has been in measuring the levels of the leukotriene group of proteins, particularly in urine samples. These proteins were shown to increase early in adult asthmatic groups following an airway challenge (Westcott, Smith et al. 1991; Kumlin, Dahlen et al. 1992; O'Sullivan, Roquet et al. 1998; Bancalari, Conti et al. 1999) with peak excretion occurring at two hours (Kumlin and Dahlen 2000; Mai, Bottcher et al. 2005). The results were more variable when assessing whether levels remained elevated during the late asthmatic response – not seen in one study (Manning, Rokach et al. 1990) but documented in two others (O'Sullivan, Roquet et al. 1998; Bancalari, Conti et al. 1999). LTE4 was high in 184 adults presenting with moderate to severe acute asthma and reduced two weeks later in the 146 followed up during recovery (Green, Malice et al. 2004). Compared to control subjects, there was an increase in the LTE3 level in asthmatics with nocturnal asthma exacerbations but not in asthmatics without, although the numbers in each group were less than ten (Bellia, Bonanno et al. 1996). Patients with asthma did excrete more LTE4 over a 24 hour period than normal subjects, again with less than ten subjects in each group (Asano, Lilly et al. 1995). In 40 severe asthmatics, 25 mild to moderate asthmatics and 20 non-asthmatic controls, there was a gradation LTE4 levels despite treatment with high dose IHCS in the severe group (Vachier, Kumlin et al. 2003). LTE4 in urine was higher in 35 atopic asthmatic than 32 non-atopic children with RSV bronchiolitis and 23 controls (Oh, Shin et al. 2005).

Other studies have been less positive about the correlations between inflammatory markers measured in blood and urine samples and asthmatic disease. In adults, no correlation between clinical status, functional status and serum ECP was seen during five months in asthmatics either admitted for acute asthma or who were seasonally sensitised to birch and tree pollens.
and had intermittent symptoms (de Blay, Purohit et al. 1998). The combination of serum ECP with peak expiratory flow did not translate into a useful combination to determine IHCS adjustment and was less useful than symptomatology alone or combined with other lung function parameters (Lowhagen, Wever et al. 2002). Also, the blood inflammatory markers did not reflect health related quality of life scores in subjects with mild asthma (Ehrs, Sundblad et al. 2006).

In children, serum ECP levels followed during acute exacerbation and recovery of asthma in 11 asthmatics did not show a uniform pattern of increasing then decreasing values over this period (Niggemann, Ertel et al. 1996). In 34 children with moderate asthma, none of the markers measured reflected asthma activity, including serum eosinophils, serum ECP, serum EDN, urinary EDN or urinary histamine levels (Hoekstra, Grol et al. 1998). Serial measurements of ECP and EDN in urine samples taken monthly did not provide additional information in monitoring childhood asthma or contribute to practical management in a prospective study that followed children over a six month period measuring diary symptomatology and peak flow (Wojnarowski, Roithner et al. 1999).

In addition, the markers when raised were not found to be specific for asthma. Considerable overlap was seen in blood eosinophil and ECP levels in asthmatics and controls, with no difference in the mean levels measured in adults with asthma or with allergic rhinitis or in those who had both (Sin, Terzioglu et al. 1998). While physician diagnosed bronchitis was more significantly associated with neutrophil count, it was also associated with eosinophil count and, as well, chronic sputum production was also associated with both cells (Schwartz and Weiss 1993). In children, serum ECP and EDN was higher in the 36 asthmatic than 166 healthy children but higher rates were also seen in those with allergic rhinoconjunctivitis, atopic dermatitis and/or allergic skin sensitization. An elevated ECP gave an odds ratio of 2.3 for asthma, but 2.9 for atopic dermatitis. Similarly, an elevated EDN gave an odds ration of 2.61 for asthma, but 5.23 for allergic rhinoconjunctivitis (Remes, Korppi et al. 1998). Considerable overlap existed in another study comparing serum ECP levels in 21 children with asthma and/or atopic dermatitis (Kristjansson, Shimizu et al. 1994). In 207 children aged 24-41 months and 76 aged 0-23 months in whom repeated samples were obtained in a large longitudinal childhood asthma study, the serum ECP was shown to be influenced by a number of factors including age, the presence of active eczema and the presence of maternal smoking in a dose dependent fashion (Lodrup Carlsen, Halvorsen et al. 1998). In 72 infants with recurrent wheezing, there was no correlation between serum ECP and the development of asthma and similarly no correlation between serum ECP and
bronchial hyper-responsiveness (Reichenbach, Jarisch et al. 2002). Neither eosinophil numbers nor ECP levels could predict the intensity of response to bronchial inflammatory reaction in 46 house dust mite allergic children (Vila-Indurain, Munoz-Lopez et al. 1999).

Negative findings also occurred in studies into the leukotrienes. While there were significant associations between lung function parameters and urinary LTE₄, it did not distinguish between groups of 49 mild and 31 moderate to severe asthmatics (Severien, Artlich et al. 2000). A single urine sample did not predict bronchial hyper-responsiveness or degree of airflow obstruction in 41 asthmatics (Smith, Hawksworth et al. 1992). There was no increase in LTC₄, LTD₄ or LTE₄ in urine after an exercise challenge in adults (Taylor, Wellings et al. 1992). The measurement of urinary EDN in children with either acute or chronic asthma, lower or upper respiratory tract infections or controls did not show significant differences between these groups (Oymar and Bjerkenes 2001).

So in all age groups blood and urine inflammatory parameters have failed to show consistent discrimination between asthma and other allergic diseases, and in children between asthma and other respiratory diseases, and also failed to determine asthma severity. The cells and proteins in these systemic samples may not necessarily indicate what is happening in the lungs as they are being measured from more distant sites. In addition, the measurement made from blood samples by the time these reach the laboratory is made up of both the circulating ECP and what is released from the eosinophils after the blood has been drawn into the tube (Venge 1995). While these are easier samples to obtain with the benefit of fewer side effects than sampling nonproductive respiratory secretions directly, blood testing is still perceived as an invasive procedure and urine samples can be tiresome to obtain, particularly in children.

1.6.6 Comparison of inflammation results between the different samples

Comparisons have been made between the levels of inflammatory parameters obtained through the different tissue or fluid samples. The cellular composition of induced sputum correlates well with bronchoalveolar lavage and wash, but to a lesser extent with bronchial biopsies (Fahy, Wong et al. 1995; Maestrelli, Saetta et al. 1995; Grootendorst, Sont et al. 1997; Keatings, Evans et al. 1997; Pizzichini, Pizzichini et al. 1998). For example, mast cells usually make up only a small proportion of cells recovered by lavage but are as much as 20% of the inflammatory cells in biopsy studies (Dunnill 1960; Foresi, Bertorelli et al. 1990). Likewise there are more basophils in the biopsy specimens than in the lavage specimens (Koshino, Teshima et al. 1993; Macfarlane, Kon et al. 2000). On the other hand as induced sputum is rich in neutrophils and eosinophils but poor in lymphocytes, the origin of the
sample is thought to be from the larger airways (Keatings, Evans et al. 1997; Nocker, OUt et al. 2000). Induced sputum eosinophilic measures did predict exacerbations better than bronchoalveolar lavage samples (Nocker, OUt et al. 2000). While correlations were identified between blood eosinophils and serum ECP, and sputum eosinophils and sputum ECP, in a number of studies it was the sputum ECP that best correlated to bronchial obstruction, and the sputum ECP and eosinophils that best correlated to methacholine challenge in asthmatics (Sorva, Metso et al. 1997; Grebski, Wu et al. 1999; Piacentini, Bodini et al. 1999). Sputum eosinophils and ECP were related to the symptom scores and FEV₁, while no relationship was demonstrated between blood eosinophils and symptom scores (Bacci, Cianchetti et al. 1998). Finally, the measurement of ECP was easier to do from induced sputum than from blood or lavage fluid, required less technical input and time and had better correlation to disease activity (Barck, Lundahl et al. 2005). In a comparison between blood and urine samples, one study showed a better correlation between lung function and the inflammatory parameters measured in the urine samples (Labbe, Aublet-Cuvelier et al. 2001).

1.7 Chapter summary

So in this introductory outline; the burden of paediatric respiratory disease in New Zealand is great and asthma in both adults and children is a major cause of morbidity, stimulating my interest in conducting research in this area. The diagnosis of asthma, particularly in children, can be difficult with no single diagnostic or prognostic marker. Asthma continues to be a clinical diagnosis based on history of symptoms, few examination findings and response to treatment, with possible confirmation from lung function testing in older children. However the mainstay of treatment is anti-inflammatory medication, which is associated with significant side effects such as adrenal cortical insufficiency, growth failure, dysphonia and oral candidiasis for IHCS use, and adrenal insufficiency which may cause devastating hypoglycaemia, adrenal suppression, growth failure, hypertension, diabetes mellitus, reduction in bone mineral density, skin atrophy, straie, poor healing, immunosuppression and cataracts for oral corticosteroid use (GINA 2002; GINA 2005). We do not routinely measure a marker for inflammation, but rather base decisions and treatment regimes on surrogate markers such as symptoms, lung function, chest xray appearances, bronchial reactivity – to determine successful outcomes.

The theory that asthma is an inflammatory disease was first indicated by autopsy studies in early and mid last century. With the development of bronchoscopy, biopsy studies became possible and these consolidated the pathogenesis of asthma as an inflammatory disease.
led to research which looked for less invasive methods of assessing the inflammatory component of asthma and bronchoalveolar lavage and this led to the development of the ability to induce sputum using nebulised hypertonic saline. These samples allowed increasing measurement not only of the cellular component, but also of the proteins that formed the asthmatic inflammation. These measurements could be done repeatedly so parameters were followed longitudinally to determine what happened during acute exacerbations of asthma, in response to treatment and over longer periods of time during chronic asthma. Studies were also undertaken using blood and urine samples. In the main there has been a recognition that the majority of asthma and other allergic diseases have an increased allergic inflammatory profile with an eosinophilic dominant pattern, raised levels of eosinophilic proteins (ECP, EPO, EDN and MBP), and cytokines (IL3, IL4, IL5, rantes). While over the last decades this was thought to be an imbalance between T lymphocyte classes, between the T helper type 2 (Th2) and T helper type 1 (Th1), (Colavita, Reinach et al. 2000; Holgate, Davies et al. 2000; Busse and Lemanske 2001; Peters 2003), very recently it has been thought there may be upregulation of both classes with a third type, the T lymphocyte regulatory cells playing a pivotal role (Lazarus, Raby et al. 2004; Goldman 2007). Certainly some asthma is known to have a neutrophil dominant or neutrophil present pattern, with the markers more commonly associated with infective conditions. This is seen more often in severe persistent asthma (Wenzel, Szeffler et al. 1997; Jatakanon, Uasuf et al. 1999), associated with a poor response to treatment (McDougall and Helms 2006; Wenzel 2006), has been demonstrated in some acute exacerbations (Martin, Cicutto et al. 1991; Fahy, Kim et al. 1995; Ordonez, Shaughnessy et al. 2000), and in sudden fatal asthma (Sur, Crotty et al. 1993). While the eosinophils and neutrophils themselves and the proteins they release have shown the best association with asthma exacerbations and response to treatment – neither have been completely consistent. In addition, obtaining the appropriate samples can be difficult.

So, while contributing enormously to our understanding of asthma, the use of bronchoscopy, biopsy and BAL, induced sputum, blood and urine testing all have their disadvantages, particularly when assessing disease in children. The first three options remain invasive, require anaesthesia in children and are associated with risks and side effects, especially in children with respiratory disease, and therefore cannot be done frequently. They also require specialised personnel in terms of theatre and bronchoscopy staff. Induced sputum results in less satisfactory sample success in children than in adults, although successful samples can be obtained in up to 75% of control children. It also can be associated with side effects, as nebulised hypertonic saline is also used as an airway challenge. This test also requires
experienced staff to undertake the procedure, and laboratory staff to process samples within a short time frame. It also represents a significant time commitment - even in the most experienced hands and a sample taken in the shortest time, the minimum time to from start to getting a result for one sample is estimated to be 100 minutes (Holz, Kips et al. 2000). Both blood and urine sampling have been found to be less useful, probably because the site of measurement is distant from the site of interest, the lung. In addition, apart from obtaining urine samples, the other sampling procedures all require a hospital or clinical setting.

These studies started to come about at the same time as my own research commenced. In view of all these difficulties a simpler, direct, non-invasive technique for measuring airway inflammation was sought, that could be achieved easily by children, and could be repeated regularly with time. Because measurement of lung function is a common procedure in children and does not involve collecting specimens and there is no anaesthesia or needles, the result is instant - a test analogous to this would seem ideal.

In 1987 came the announcement that a gaseous molecule, NO, was responsible for acting as both a widespread physiological mediator and was also involved in host defense (Hibbs, Taintor et al. 1987; Ignarro, Buga et al. 1987; Khan and Furchgott 1987). This resulted in an explosion of research in the early 1990s identifying both NO and the enzymes that produced it - the nitric oxide synthases (NOS) in all biological systems. NO was shown to play a major role in host defense and inflammation within the lung as in other systems. Toward the end of 1995, I also went to a one day seminar on NO at the Royal Society of Medicine in London to hear Professor Salvador Moncada, one of the major early researchers into NO in the cardiovascular system, talk. I was struck by the importance of this one tiny molecule, and the unfolding story with more to come. This seemed like a great opportunity to further explore the possibility of NO as a potential marker for airway inflammation; however there were a number of hurdles to overcome. Measuring NO was going to be difficult given its gaseous, short lived and highly reactive nature. As well there was the difficulty of measuring levels in exhaled air from human subjects, especially in children.

This research was conducted at the Royal Brompton Hospital in London from the mid 1990s. On returning to New Zealand it was acceptable to submit this research over time as a thesis. Some of the findings within this thesis are now well-known, but were unknown, new and exciting when first demonstrated. The findings contributed to the knowledge as to what technical aspects were important in the measurement of NO, to the standardising of protocols for measurement, and to what were important capabilities to be developed for the newer NO
analyser machines. This thesis presents one researcher negotiating research in one area of medicine – which will be presented how it unfolded at the time and will be reviewed with the up to date NO literature in the penultimate chapter.

In the chapter to follow, I will review what was known about NO and nitrogen dioxide (NO₂) in pollution where it was recognised to be a major component, and its effects at the population level on respiratory disease. The study of pollution was where the machines were first developed in order to analyse levels. The chapter will then describe the recognition of NO as a physiological mediator and its key role in inflammation. Chapter 3 will review the actions and interactions of NO as well as the NOS enzymes that produce this molecule in vivo. Chapter 4 will elaborate on the methods of measuring this short lived, reactive molecule. Chapter 5 will discuss how the chemiluminescence analyser, the method of choice for the subsequent experiments, could be modified to measure NO in exhaled air in humans using an analyser developed for NO measurement in the environment. Chapter 6 is the commencement of the research in normal, control adult subjects looking at the measurement of exhaled NO through two different methods, either directly into the NO analyser which also allowed the measurement of mouth pressure and CO₂, and through a t-piece system which enabled the measurement of flow in addition to the other parameters. Chapter 7 describes the research undertaken to see what technical aspects of measurement altered the NO levels obtained. Chapter 8 describes the research as I measured exhaled NO in normal and then asthmatic children. It briefly reviews the effect of age, atopy, the presence of pets or the presence of smoking in the home on the NO obtained in a group of healthy pre-pubertal children. There is a comparison of results from this control group to a group of asthmatics on bronchodilator therapy only and a group of asthmatics who were on chronic inhaled corticosteroid therapy. A small number of steroid naive asthmatics were measured before and after commencing inhaled corticosteroids. Chapter 9 summarises what was learned about measurement of exhaled NO from these studies and how that has contributed to the literature. This is followed by a further review of the literature to the current time and the place of NO measurement as it currently stands. Finally chapter 10 will present the final thoughts with regard to this research project and 'where to from here'.

NB: Some of the work presented in this opening chapter formed the monograph for the Asthma and Respiratory Foundation of New Zealand; (Anonymous 2006) in which I contributed two chapters and edited in conjunction with Professor Innes Asher. This was presented to The Honorable Mr Peter Hodgeson, Minister of Health April 2006, and is available on two websites: www.asthmanz.co.nz or www.paediatrics.org.nz.
Chapter 2: Nitric oxide: pollutant to mediator

2.1 Introduction

There are two lines of research, from quite disparate areas, that came together to make the studies described in Chapters 6, 7 and 8 possible. The first is the measurement of air pollution, which has been increasingly important due to recognition that different pollutants cause or contribute to human disease, including nitrogen oxides; and the second is the increasing recognition of the importance of nitric oxide (NO) in biological systems in the last 25 years. I will examine the developments in both of these areas that led to the commencement of the research described in this thesis.

2.2 Pollution, nitrogen oxides and disease

2.2.1 Concerns regarding pollution

There is now no doubt that episodes of air pollution have resulted in excess deaths. Concerns with regard to pollution and its effects on health date back to early last century, although the detrimental effects of poor air quality and poor housing have been recognised for far longer. Indoor air pollution can be traced to some 200,000 years ago, when humans first moved to colder climates necessitating the construction of shelters and use of indoor fires for cooking, warmth and light. These mostly ‘inside’ fires likely resulted in an excess exposure to levels of pollution as evidenced by the carbon soot found in prehistoric caves (Brims and Chauhan 2005). Severe outdoor air pollution episodes have been documented since the early 17\textsuperscript{th} century but these became more frequent and more severe throughout the 19\textsuperscript{th} century. The most definitive and well documented episodes were the great smogs that occurred in London, United Kingdom, in 1948, 1952 and 1962, with the most severe occurring in December 1952 (Brims and Chauhan 2005). The smoke concentration at this time was 50 times above the average level, and this rise in pollution was followed by an equally sharp rise in mortality (Ministry of Health, UK 1954). An estimated human death toll of 4,000 occurred during this period which was three times the expected mortality for that time of year. Most deaths were among infants, the elderly and those with chronic respiratory disease (Anderson, Ponce de Leon et al. 1996). In addition, emergency hospital admissions tripled for respiratory disease, and doubled for cardiovascular disease over the same period (Schwartz 1991). These led to the Clean Air Act in 1956 and 1968 in the United Kingdom (UK), with further modifications to the Act made in 1993.
The pollution of the 'great smog of London' and contemporaneously elsewhere was to do with the burning of coal and generation of 'black smoke' (Ministry of Health 1954). Smog has become the common name given to the "soup" produced from photochemical reactions in the atmosphere. Because of the role that heat and sunlight play in its production, the highest levels of smog are recorded on hot, sunny days. Summer smog is composed mainly of ground level ozone and particulate matter. Because ozone is not produced at high levels in cold weather, winter smog is composed mainly of particulate matter and sulphur dioxide (World Health Organisation 2003).

However with the increase in mechanisation and industrialization, particularly with increasing motor vehicle use, the relative contribution of pollutants from the different sources has changed over the last few decades. There has been a reduction from industrial sources and domestic heating, and an increase from car exhaust emissions. The combustion of these fossil fuels produces carbon monoxide, nitric oxides, benzene, sulphur dioxide, and particulate matter. In addition, diesel engines, which are also on the rise, emit much higher emissions of the gases and 100 times more particles compared with gasoline engines equipped with modern exhaust treatment systems (Riedl and Diaz-Sanchez 2005).

Reflecting the increasing pollution problem, 172 individual compounds and 17 compound classes as hazardous air pollutants of concern in the USA in 1990 (Clean Air Act 1990). Of these, the compounds that have been associated with respiratory and/or cardiovascular disease are particulate matter with a diameter of less than 10μm (PM10), ozone, nitric oxides and nitrogen dioxide (NO₂) in particular, sulphur dioxide (SO₂), carbon monoxide (CO), lead and volatile organic compounds (Anonymous 1995; Anonymous 1996).

This association between large specific pollutant episodes and increased disease and/or deaths is well documented for these early episodes. It has subsequently been more difficult to tease out whether less, but more continuous, pollutant exposure had similar health effects and has resulted in support both for (Bates 2000) and against (Gamble 1998) and necessitated large committee reviews of the topic (Anonymous 1995; Anonymous 1996). The suspicion that there was an adverse effect arose because of the noted increasing rate of allergic disease occurring in parallel and geographically coincident with the increased industrialisation. Even in 1873 when the first report of environmental exposure causing disease was documented (Charles Harrison Blackley), that ‘hay fever’ or ‘hay asthma’ was caused by grass pollen, it was even then observed to be more common in urban than in rural settings (Brims and Chauhan 2005). The difficulties in confirming the effects of a lower pollution dose over a
longer period of time were manifold. Firstly, there were many differing pollutants available to measure. Secondly, there was likely to be an interaction with pollutants and other environmental and/or infective triggers for disease. Thirdly, there was uncertainty that an increase in disease rates could occur at levels that were below World Health Organisation (WHO) recommendations for safety of the time (World Health Organisation 2003; World Health Organisation working group 2003).

Analysis of the London data from 1958 to 1972 reveals a pattern that the daily mortality was indeed associated with pollution levels on the previous day. This was especially so for particulate matter, SO\(_2\) and acidic aerosol pollution (Mazumdar, Schimmel et al. 1982; Schwartz and Marcus 1990), but it was difficult to determine whether a single pollutant was responsible and authors concluded it may be the interactions between the three that were important (Ito, Thurston et al. 1993). A further large study analysed air pollution and daily mortality in London between 1982 and 1992 as part of an across Europe project. The strongest association was found with ozone recorded the same day, and particulates recorded the previous day with small but positive effects also seen with NO\(_2\) and SO\(_2\) on all causes of death (excluding accidents), cardiovascular mortality and respiratory mortality (Anderson, Ponce de Leon et al. 1996). Even relatively low levels of air pollution have been shown to be associated with increased health problems and with day to day variations in mortality (Schwartz 1993). From the 1960s, through three decades of London winters, there was a highly significant association between mortality and levels of particulate matter and/or SO\(_2\) comparing levels and mortality (Schwartz and Marcus 1990). In the USA, an association between levels of air pollution and overall mortality was also demonstrated (Ostro 1993; Schwartz 1994), as well as between particulate matter (PM10) and an observed increase of respiratory disease within the levels usually measured in urban areas (Dockery and Pope 1994).

### 2.2.2 Disease and pollution

So what has been discovered in terms of disease? Some subgroups of population appear more sensitive to the effects of air pollution; these include young children, the elderly and people with pre-existing chronic cardiac and respiratory disease such as chronic obstructive pulmonary disease (COPD) and asthma (Anonymous 1995; Anonymous 1996). For example, the analyses of fine particulate air pollution and mortality in nine counties across California showed that the particulate level was related in an increase in overall mortality, as well as for deaths out of hospital, respiratory disease, cardiovascular disease, diabetes and deaths in
adults greater than 65 years (Ostro, Broadwin et al. 2006). Increased rates of myocardial infarction, dysrhythmias, increased blood pressure, heart failure and hospitalisation for cardiac events have been associated with higher air pollution levels (Pope, Verrier et al. 1999; Peters, Liu et al. 2000; Linn and Gong 2001; Peters, Dockery et al. 2001; Pope, Burnett et al. 2004).

The 'Swiss Study on Air Pollution and Lung Diseases in Adults' involving over nine thousand subjects showed positive correlations of levels of particulate matter, ozone and NO$_2$ with chronic cough, chronic sputum production, and shortness of breath during the day, during the night and on exertion, but not with other respiratory symptoms (Zemp, Elsasser et al. 1999). This study also showed reduced lung function parameters for FVC, FEV$_1$ and FEF$_{25-75}$ which negatively correlated with the same three pollutants (Ackermann-Liebrich, Leuenberger et al. 1997, Schindler, 2001 #652). A major study in six cities in the USA demonstrated a three fold increase in chronic cough with increased exposure to particulate pollution (Dockery and Pope 1994). Throughout California from 1973 to 1987 site and season specific particulates were monitored and showed correlations between the development of symptoms of airway obstructive disease, increased severity of obstructive disease, chronic productive cough and increased asthma (Abbey, Hwang et al. 1995). Stronger associations were seen with those who were also occupationally exposed to dusts and fumes. Increased symptoms of cough, bronchitis, asthma, and COPD were associated with increased air pollution (Schwartz 1993; Souza, Saldiva et al. 1998). Particulate matter was found to be associated with respiratory diagnoses made by a physician and chronic bronchitis in a study involving 53 urban areas throughout the United States with graduated levels of pollution (Schwartz 1993). In Australia, ozone levels, particulate matter and the concentration of SO$_2$ had strong associations with emergency department review of respiratory illnesses overall, and for asthma exacerbations in twelve hospitals, though it proved difficult to detect out differences between the pollutants (Atkinson, Anderson et al. 1999). Particulate matter levels were also positively associated with hospital admissions for asthma and for COPD (Erbas and Hyndman 2005).

Histopathologic changes have been shown in lung tissue samples of individuals who died due to violent causes depending on their pollutant exposure history. With the increased pollution, there was increased presence of inflammatory reaction, wall thickening, and secretory hyperplasia. There were also increased carbon deposits along the regional lymphatic drainage (Souza, Saldiva et al. 1998).
Studies on traffic exposure, (including detailed studies on occupational exposure to increased vehicular emissions (Raaschou-Nielsen, Nielsen et al. 1995; Zagury, Le Moullec et al. 2000; Roegner, Sieber et al. 2002; Seshagiri 2003; Lai, Liou et al. 2005) and occupational NO exposure (Azari, Williams et al. 1996; Markhorst, Leenhoven et al. 1996; Olin, Ljungkvist et al. 1999; Phillips, Hall et al. 1999; Qureshi, Shah et al. 2003; Maniscalco, Grieco et al. 2004)), have also demonstrated that there is a consistent effect of long term exposure to car traffic on non-specific respiratory symptoms (Abbey, Hwang et al. 1995) and lung function (Ackermann-Liebrich, Leuenberger et al. 1997). Proximity to traffic exposure was a risk factor for wheezing, asthma severity and prevalence (Nitta, Sato et al. 1993; Oosterlee, Drijver et al. 1996, Brunekreef, 1997). In three Japanese cross sectional questionnaire studies in five thousand women participants, the estimated odds ratio for chronic cough, chronic sputum production, chronic wheeze and chest infections with sputum were increased the closer the subjects lived to roadways with heavy traffic (Nitta, Sato et al. 1993). Air pollution appears more likely to exacerbate existing asthma rather than generate new cases, although it is associated with reduced lung function in healthy children (Barnes 1994; Segala 1999). There are fewer studies that have looked at the effects of air pollution on upper airway disease rates, but general practitioner consultations due to upper airway symptoms increase on days of higher concentrations of particulate matter and SO$_2$ (Gordian, Ozkaynak et al. 1996; Hernandez-Garduno, Perez-Neria et al. 1997; Hajat, Anderson et al. 2002).

Only a few authors have looked specifically at the effects of air pollution on children, where both outdoor air pollution and indoor air quality have been implicated as causal factors for respiratory diseases and respiratory symptoms (Nicolai 1999). An increase in mortality in children during severe episodes of air pollution has been shown and this includes an increase in the general rate of mortality, an increase in respiratory mortality and an increase in perinatal mortality (Bates 1995; Anderson, Ponce de Leon et al. 1996). As well as mortality, an increase in respiratory morbidity has been shown. The respiratory illnesses sensitive to increased pollutants in children include acute bronchitis, cough, asthma and pneumonia. Similarly to adults, this is most often attributable to an increase in particulate matter (Aunan 1996). In the ‘Six Cities Study of Air Pollution and Health’ reported rates of chronic cough, bronchitis and chest illness were positively associated with all measures of particulate pollution and positively, though less strongly, associated with SO$_2$ and NO$_2$ (Dockery, Speizer et al. 1989). Particulate matter in air pollution has been shown to triple the prevalence of chronic cough, nocturnal cough and bronchitis in a cross sectional study of over 4,000 Swiss children aged six to 15 years from ten different communities with the highest PM$_{10}$
levels compared to those children living in less exposed areas (Braun-Fahrlander, Vuille et al. 1997). In a prospective, observational study over one year and comparing 1025 children presenting with wheezy episodes with other admissions as a control group, the day to day variations in the concentration of ozone were associated with an increased incidence in acute wheezy episodes (Buchdahl, Parker et al. 1996). Children living along a busy street were found to have a higher prevalence for most respiratory symptoms than children living along a quiet street. This was most significant for medication use and for episodes of wheeze (Oosterlee, Drijver et al. 1996). Children admitted with asthma were more likely to live in an area of high traffic flow compared to admissions for non-respiratory reasons or a community sample of children with a significantly linear trend observed for traffic flow for children living less than 500 metres from a main road (Edwards, Walters et al. 1994). This study also showed that children were more likely to be admitted for non-respiratory reasons compared to the community sample of children if they lived within 200 metres of a main road. Similarly results for atopy and road traffic have also been demonstrated with, for example, higher rates of allergic sensitization found in children playing more than one hour per day near major traffic thoroughfares (Brunekreef, Janssen et al. 1997).

Some studies have incorporated lung function testing to look at the effects of air pollution. The lungs develop throughout childhood, with peak lung function occurring between 20 and 25 years, followed by a plateau of ten years before beginning to decline. A deficit in growth caused by air pollution effects, for example, would most likely translate into a reduced baseline function that would be carried throughout life (Gilliland, Gauderman et al. 2002; Gauderman 2006). Air pollution data from monitoring stations in twelve California communities demonstrated that a greater proportion of young adults with and FEV\textsubscript{1} less than 80% predicted lived in the areas of higher carbon and PM10 (Gauderman, Vora et al. 2007). An inverse dose dependent relationship was found between the carbon content of airway macrophages in induced sputum and FEV\textsubscript{1}, FVC and FEF\textsubscript{25-75} in healthy children living in an area with a variation of PM10, primarily due to emissions from road traffic (Kulkarni, Pierse et al. 2006). Reviewing the age group six years to 24 years of age, FVC, FEV\textsubscript{1} and PEF all showed statistically significant negative correlations with the annual concentrations of total suspended particles, NO\textsubscript{2} and ozone (Schwartz 1989). The relationships still persisted when children with pre-existing respiratory illnesses and smokers were included. Furthermore in a longitudinal three year study, children exposed to high PM10 levels during the summer had a reduced growth of FEV\textsubscript{1} and FEF\textsubscript{25-75} (Horak, Studnicka et al. 2002). A reduction in lung function with higher levels of ozone was also demonstrated in children from three schools in
Mexico (Castillejos, Gold et al. 1992), with atopic status being an additional risk factor (Jorres, Nowak et al. 1996). In children with moderate to severe asthma attending summer camps over three years, air pollution levels (predominantly ozone) were associated with acute asthma exacerbations, chest symptoms and a reduction in lung function (Thurston, Lippmann et al. 1997).

Increased absences in school and kindergarten or playschool attendances have also been associated with an increase in particulate matter – which was more pronounced in six to nine year olds than older age groups (Pope, Schwartz et al. 1992; Ransom and Pope 1992). There has also been an association with rises in levels of pollutants on prolonging episodes of infection (Bates 1995). Increased levels of pollutants have also been associated with an increased risk of developing upper respiratory tract infections and symptoms in children (Jaakkola, Paunio et al. 1991, Ostro, 1999 #657), particularly with high concentrations of SO2, and moderate levels of NO2 and particulate matter (von Mutius, Sherrill et al. 1995).

Clearly, it would be helpful to confirm these findings using more specific experiments to determine a dose response and the mechanism of damage which causes disease. This has been done in a few animal models which have demonstrated the development of infectious and allergic lung disease with increased severity when exposure to air pollutants (ozone, NO2, SO2). These pollutants increased damage in infection and inflammation in mouse and guinea pig populations predominantly but also in a dog and a monkey model (Gilmour 1995; Miyabara, Takano et al. 1998; Ng, Kokot et al. 1998; Selgrade 2000; Whitekus, Li et al. 2002). While it is more problematic to confirm a dose response in humans, it has been possible to do nasal challenges with allergen, with or without the addition of diesel exhaust particles, showing exaggerated responses in the presence of the particles (Diaz-Sanchez, Tsien et al. 1996; Diaz-Sanchez, Jyrala et al. 2000; Bastain, Gilliland et al. 2003; Gilliland, Li et al. 2004). It is now thought that oxidant stress is the mechanism that underlies the toxic effect of most forms of air pollution (Nel, Diaz-Sanchez et al. 2001; Kelly 2003; Kelly and Sandstrom 2004; Gauderman 2006). The lung is actually well equipped to deal with oxidative stress as the lung lining fluid is rich in enzymatic and low molecular weight non-enzymatic antioxidants. In addition, there are intracellular defences with glutathione based enzymes that use a variety of the products of oxidative stress as substrates and therefore prevent their build-up. However individuals have differing genotypes of this enzyme family, which appear to make them more or less susceptible to damage from air pollutants, including active or passive smoke inhalation (Miyabara, Yanagisawa et al. 1998; Mudway and Kelly 2000; Gilliland, Rappaport et al. 2002; Bastain, Gilliland et al. 2003; Gilliland, Li et al. 2004).
More recently 'Air Quality Indexes' were developed to better inform the public about air
quality or the degree of pollution on any given day. These are composite measures of
common air pollutants for which there are demonstrable adverse effects on health and the
environment. At the current time these include CO, NO\textsubscript{2}, SO\textsubscript{2}, ground level ozone, suspended
particulate matter (sometimes expressed as a coefficient of haze) and total reduced sulphur
compounds. The Index is reported on a scale of 0 to 100 (or more) with scales of 'very good'
air quality being 0-15 and 'very poor' near the 100 mark (Hiltermann, Stolk et al. 1998;
Abelsohn, Stieb et al. 2002).

2.2.3 Nitrogen oxides in pollution

NO is a potentially toxic molecule; and both NO and its interrelated forms were discovered as
components in pollution. Space shuttle and jet plane exhaust, lightening and smog are all
known to have a high proportion of NO (Nathan 1992). Nitrogen oxides were detected in the
evaluation of air pollution from the Titan II firings (Diamond and Johnson 1965). NO forms
part of the origin of the shuttle glow which was observed in 1983, and was carefully examined
because of concern that it would interfere with space-based spectroscopy. It was found to be
the reaction between NO and O\textsubscript{2} forming an excited form of NO\textsubscript{2} which released light as it
desorbed (Viereck, Murad et al. 1991). Increases in air traffic have resulted in increases in
nitrogen oxides in the troposphere, again causing concern about the effects in atmospheric
chemistry (Johnson, Henshaw et al. 1992). There exists an array of compounds that includes
NO, nitrosium cation (NO\textsuperscript{+}), nitroxy anion (NO\textsuperscript{-}), peroxynitrite (ONOO\textsuperscript{-}) and hydroxyl
anion (OH\textsuperscript{-}). Most of these are inherently unstable compounds and interact with other
elements particularly ozone and oxygen, so the main compound measurable in pollution from
this class of gases is the stable gas NO\textsubscript{2}. The ambient NO\textsubscript{2} levels in London vary between 25-
58ppb (24 hour average), European air levels are 78ppb (24 hour average) and the level in the
USA is 53ppb (one year average) (Nicolai 1999).

Many epidemiological studies of outdoor pollution have found associations between NO\textsubscript{2}
exposure and disease, even at levels below the current WHO guidelines. The 'Air Pollution on
Health: European Approach' studies incorporated data from 15 cities with a total population
of greater than 25 million. A rise in NO\textsubscript{2} over the one hour safety maximum of 50 \(\mu g/m^3\) was
associated with a 2.6% increase in asthma admissions and a 1.3% increase in daily all cause
mortality (Touloumi, Katsouyanni et al. 1997). In Toronto, from the total number of
premature deaths related to air pollution, NO\textsubscript{2} was assessed to be responsible for almost 40%,
and, of the hospital admissions, 60% was also thought to be attributable to NO\textsubscript{2} levels, with
the other main pollutants studied being CO and SO2 (Abelsohn, Stieb et al. 2002). Three studies have looked at the effect of ambient NO2 concentrations on respiratory illnesses in children. Firstly, a significant association between the frequency of croup and the daily measured level of NO2 for the peak periods (September and March) of disease rate was found in five German communities (Schwartz, Spix et al. 1991). Secondly, the risk of admission to hospital with wheezing bronchitis was significantly related to outdoor NO2 exposure in girls (although not boys) in Stockholm, however the presence of a gas stove in the home was also a significant risk factor (Pershagen, Rylander et al. 1995). A third study showed worsened virus-induced asthma in children following exposure to higher NO2 levels (Chauhan, Krishna et al. 1998).

However examination of the potential harmful effects of the pollutant NO has been investigated down to the individual cigarette. NO exists in an English cigarette “at a level of 1,000 parts per billion with the level of a French cigarette being one million parts per billion” (Borland and Higenbottam 1987). NO corrodes metals and interacts with plastics. There are particular problems with the development of even more dangerous combinations as listed above in the presence of higher concentrations of oxygen (see Chapter 3 and Chapter 4 regarding toxicity).

So by the time this research began, the nitrogen oxides were known to contribute to pollution, to contribute to the excess of cardiovascular and respiratory disease and mortality seen with increased air pollution and studies were well underway using analysers to measure daily levels for air quality recordings and to ascertain their continued association with disease.

2.3 The discovery of nitric oxide in biological systems

2.3.1 Vascular control

It is increasingly rare to discover something completely novel. The discovery of NO and its importance in biological systems, in particular the opening up of a new concept of the use of gases as biological mediators, was truly new. Initially the exact nature of NO as a mediator was not recognised and it was labelled as ‘Endothelial Derived Relaxing Factor’ (EDRF).

In 1916 Mitchell observed that oxides of nitrogen were produced in mammals showing more nitrogen in urine than consumed (Mitchell, A. et al. 1916). In 1928 Tannenbaum confirmed that mammals produced nitrogen oxide by showing production occurring in the intestine (Mitchell 1928; Tannenbaum, Fett et al. 1928). In the 1970s research was undertaken on categorising biological ‘amines’ such as adrenaline, noradrenaline, histamine and
acetylcholine. These were known to contribute to both cell to cell and nerve cell to terminal tissue interactions. One of the main investigators in this area, Professor R. F. Furchgott, was studying the mechanism of contraction and relaxation of isolated of blood vessels. Using identical chemicals, the studies initially yielded conflicting results. Laboratory investigations subsequently revealed that the results were dependent on which technician conducted the experiment. One technician found that acetylcholine always relaxed the vessels and another found that it always contracted the vessels. Investigating further, Furchgott discovered that the reason for this anomaly was the presence or absence of the endothelial monolayer, as one technician meticulously kept the endothelium intact and the other always removed the endothelium in preparation. The presence or absence of this endothelium resulted in quite different reactions of the blood vessel to the applied mediator (Furchgott, Jothianandan et al. 1984). This also explained a somewhat surprising finding which had been made many years earlier in his research career. He found that acetylcholine (a well known vasodilator in animals and in perfused organ studies) elicited contraction rather than relaxation on an aortic strip. However, the preparation technique unknowingly stripped the specimen of endothelial cells (Furchgott and Bhadrakom 1953). It was this that led to the coining of the term for the substance responsible as the 'EDRF' (Furchgott and Zawadzki 1980).

Advances in this area occurred with the development of perfusion-bioassay procedures which allowed more direct studies on the properties of EDRF than the previous experiments which had been carried out in organ chambers. Intact endothelial cells were held upstream, a section of blood vessel wall stripped of endothelium was held downstream so substances could be perfused in turn to see whether each test substance inhibited EDRF by interfering with its synthesis, its release or inactivated EDRF after release. This approach was also used to assess the rate of decay of EDRF by altering the transit time of the perfusate (Furchgott 1998). Initially this experiment resulted in confusion, as estimations of the EDRF half life were estimated from four to 50 seconds. However later this was able to be explained by the differences in the superoxide anion (O$_2^-$) concentration in the perfusion fluid as this rapidly inactivates EDRF (see Chapter 3.2 ‘Reactions of nitric oxide’), (Gryglewski, Palmer et al. 1986; Rubanyi and Vanhoutte 1986). These perfusion bioassay studies also allowed demonstration of the release of EDRF from vessels with increased shear stress, usually secondary to increased flow thereby suggesting that EDRF had a continuous physiological role in the control of regional blood flow and regulation of blood pressure.
These and other researchers continued pursuing the identity of this substance with the evolving characteristics becoming increasingly similar to a compound of nitrogen, resulting in experiments comparing ‘EDRF’ with nitrogen oxides. Furchgott initially announced that EDRF could actually be the inorganic gas ‘NO’ at a conference in July 1986 (Khan and Furchgott 1987). This same conclusion was proposed at the same conference by Ignarro as a result of his experiments on bovine pulmonary arteries (Ignarro, Buga et al. 1987; Ignarro, Byrns et al. 1988). Similarly in 1987, Palmer, Ferrige and Moncada showed that vascular endothelial cells generated NO and that the amounts generated accounted for the biological activity of the named EDRF mediator (Moncada, Palmer et al. 1988). This was a novel suggestion as – until this time – gases had not been thought capable of acting as biological mediators.

It had been recognised that endothelium dependant relaxation of blood vessels was associated with increased cyclic guanosine 3', 5' –monophosphate (cGMP) levels in vascular muscle. This was demonstrated earlier in a number of studies using rat aorta (Rapoport and Murad 1983), rabbit aorta (Diamond and Chu 1983, Furchgott, 1983 #415), bovine coronary arteries (Holzmann 1982) or bovine pulmonary arteries (Ignarro, Burke et al. 1984). Rises in cGMP are produced by activation of soluble guanylate cyclase (sGC), a magnesium sensitive hexodimer protein which contains two heme molecules. Under basal conditions, the guanylate
cyclase does not produce significant levels of NO, but the relaxant factor, EDRF, was thought to alter the binding in so much that it increased the activation of the enzyme by 400 fold. However it is now known that it is nitrosylation of the guanylate cyclase that results in high levels of cGMP which both prevents entry and promotes movement out of the cell of calcium resulting in vasodilatation. The Moncada research group then identified that the substrate for NO synthesis was L-arginine and also went on to synthesise an inhibitor of NO formation by producing a false competitive inhibitor of L-arginine; N\(^{\text{\#}}\) monomethyl L-arginine (L-NMMA), (Gardiner, Compton et al. 1990). It was by using this inhibitor that further clarification of the role of NO was possible.

### 2.3.2 Immune function

At the same time that EDRF was being identified as NO, other research groups were looking at NO in a different biological system – that of host immune defence.

Macrophages were known to be able to inhibit tumour growth and induce tumour cell death (Nathan 1992). In 1987 (Hibbs, Vavrin et al. 1987) it was suggested that the macrophage cytotoxicity might in part be due to the ability of the macrophages to synthesise NO, nitrite and nitrates from L-arginine. At the tissue level, NO was known specifically to be toxic to cells. A number of bacteria were also known to be able to generate reactive species of nitro-oxygen suspected as occasionally causing lung disease. However the main interest in these bacteria was not in the medical arena but centred on the importance of bacteria using these pathways causing meat to degenerate. Organisms that had demonstrated the production of nitro-oxygen compounds included *Achromobacter cycloclastes*, *Alcaligenes faecalis*, and *Bacillus halodenitrificans* (Godden, Turley et al. 1991).

The suggestion that the use of NO in this way could occur within living animals came from the demonstration that germ free rats excreted more nitrate than they ingested (Green, Tannenbaum et al. 1981). This was also found in humans in a stable state but further investigations showed that the amounts increased dramatically during intercurrent infection (Green, Ruiz de Luzuriaga et al. 1981). Other researchers confirmed this by injecting rats and mice with pro-inflammatory or infectious agents and showing that there was an increase in the amount of nitrates that were excreted (Wagner, Young et al. 1983, Stuehr, 1985 #580). In part this was described by using macrophages from these rats *in vitro* and showing that these cells could produce nitrite and nitrate by oxidizing L-arginine (Iyengar, Stuehr et al. 1987) to yield citrulline and a compound with the ability to react with amines and generate nitrosamines (Miwa, Stuehr et al. 1987). It was thought that a compound with such ability had to be an
oxide of nitrogen which was more reactive than nitrite or nitrate. It was also shown that macrophages used an oxidative form of injury associated with iron loss and involving inhibition of iron-sulphur enzymes to inflict damage on tumour cells and fungi. L-arginine was then identified as the necessary metabolite in the extracellular medium to sustain this form of macrophage-mediated cytotoxicity (Hibbs 1991). Substituting L-arginine analogs blocked the cytotoxicity in a stereospecific manner and blocked the production of nitrite by activated macrophages. Nitrite or nitrate substances alone could not replicate the cytotoxicity of the nitrite-producing macrophage except when in an acidic pH – a condition coincidentally in which nitrite can generate NO (Hibbs, Vavrin et al. 1987; Stuehr, Gross et al. 1989). NO (rather than other nitro-oxygen species) specifically mimicked the pattern of macrophage-mediated cytotoxicity and scavengers of NO blocked the cytostatic effect of macrophages. Further investigation showed that this cytostatic effect used by the macrophages was also operational against yeasts, helminths, protozoa and mycobacteria (Nathan and Hibbs 1991).

2.3.3 Nitric oxide – a common pathway

These two separate areas of investigation into immune function and vasomotor control came together in 1989, when Stuler showed that the compound secreted by activated alveolar macrophages with L-Arginine availability, demonstrated the same bioactivity as that described for EDRF and coincided with the chemical reactivity profile of NO (Stuehr, Gross et al. 1989).

When these two major discoveries came together, it suggested NO synthesis from L-arginine might in fact be a widespread pathway for the regulation of cell function and communication. NO has the lowest molecular weight of any known bioactive mediator that cells produce. The molecule’s chemical reactivity means that its half life is short and interaction specificity is minimal. Therefore, it was surprising that such a simple, fleeting, indiscriminating reactant could convey enough information in a regulatory manner to help control vital mechanisms such as vascular tone and neurotransmission (see below Section 2.3.4). It was also surprising that a mediator involved in homeostasis, was also involved in host defence to destroy micro-organisms and tumour cells (Nathan 1992). Following the demonstration that both endothelium cells and macrophages were able to synthesis this new mediator, there was an explosion of research demonstrating that many other cells were able to produce NO. This molecule was shown to have physiological roles in many neuronal tissues; including the cerebellum, the cerebral cortex and the hypothalamus, as well as in ganglion cells of the autonomic nervous system (Garthwaite, Charles et al. 1988; Bredt and Snyder 1990;
Garthwaite 1991), and the non adrenergic, non cholinergic (NANC) peripheral nerves (Bredt, Hwang et al. 1990; Rand 1992).

NO was subsequently found, with likely mediator roles, in the renal cortex epithelial cells, adrenal glands, gut, genital system and has also been found in amniotic fluid (Nathan 1992). Thus NO was realised to be an important gas used in biological systems in many species including humans, and within humans NO was seen in a wide variety of tissues as a gaseous mediator. The type of roles it fulfils in three of these key systems (cardiovascular, neurological and immunity) will be discussed briefly below, while the role of NO in the lung is discussed in more depth in Chapter 4, preparatory to measuring levels from the airway.

2.3.4 Nitric oxide in physiological roles

2.3.4 (i) Cardiovascular

NO is produced by nitric oxide synthases (NOS) which generate NO from the semi-essential amino acid L-arginine and release picomolar amounts of NO in response to receptor stimulation (see Chapter 3 and 4 for detail on chemical reactions and pathways). In this way NO is responsible for the basal vasomotor tone and essential for the regulation of blood flow and blood pressure. NO dependent vasodilator tone is in part maintained through the release of NO in response to physical activation of endothelium cells by stimuli such pulsatile flow and shear stress. This role was in part determined by the studies described above before NO was so-named, and in part been determined subsequently by the use of the non-functioning competitive inhibitor of this enzyme, L-NMMA. A potent vasoconstrictor, L-NMMA constricts vascular beds and produces a hypertensive response in animals with high potency (Elsner, Muntze et al. 1992) and has been shown to cause vasoconstriction in the forearm circulation in humans (Gardiner, Compton et al. 1990). As documented below, NO is also released by non-adrenergic non-cholinergic (NANC) nerve terminals and this may also contribute to the regulation of blood flow and pressure. The success of compounds used in angina and hypertension such as nitro glycerine and sodium nitroprusside are now known to be because these compounds imitate endogenous nitro-vasodilator compounds by relaxing blood vessels (Doel, Godber et al. 2000).

NO also inhibits platelet aggregation and adhesion. This occurs with the increase in cGMP activity within platelets and subsequent phosphorylation of proteins that regulate platelet activation (Radomski, Palmer et al. 1987; Radomski, Palmer et al. 1987). It can also be generated either by the endothelium or the platelets themselves generating NO to act as a
negative feedback mechanism to inhibit platelet activation (Mehta, Chen et al. 1995). NO is also involved in the reaction of leukocytes with vessels walls, again inhibiting activation. NO inhibits vascular smooth muscle cell hyperplasia which may reduce the development of pulmonary hypertension (Papapetropoulos, Garcia-Cardena et al. 1997; Ziche, Parenti et al. 1997). Endothelial dependant relaxation demonstrated in human vascular tissue is greater in the arteries than the veins, suggesting that arteries may generate more NO. This relaxation is inhibited in a number of patient groups with pulmonary vascular disease. The response is decreased in atherosclerotic coronary arteries compared with normal coronary arteries (Egashira, Suzuki et al. 1995), in children and adult patients with hypercholesterolemia (Quyyumi, Mulcahy et al. 1997), and in patients with essential hypertension (Panza, Garcia et al. 1995). It is also lower in the vessels of diabetic animals (Sobrevia, Nadal et al. 1996) and in pulmonary arteries obtained from patients undergoing heart lung transplantation (Dusting and Macdonald 1995). NO dysfunction has also been noted as contributing to heart failure (Hanssen, Brunini et al. 1998; Sharma, Coats et al. 2000), and to the pathogenesis of sickle cell disease (Enwonwu, Xu et al. 1990; Waugh, Daeschner et al. 2001). Finally, it has been shown when solutions of L-arginine (substrate for NO generation) are exposed to cigarette smoke, there is a depletion of L-arginine and the formation of cyanomethyl-L-arginine and this is likely to act as a competitive inhibitor and has been shown to have an inhibitory effect on NOS. This may contribute to the vascular compromise seen in long term smokers (Wong 2000).

NO is also associated with significant pathology within the vascular system in regards to sepsis (Vallance and Moncada 1993). In endotoxin shock in animals, the generation of NO is directly related to the degree of systemic hypotension. This is also seen as a characteristic of septic shock in humans and it is also suggested as being responsible for the hypotension induced by chemo/radiation therapy seen in patients with cancer who undergo such treatment. Endotoxin also induces NOS production and activity in venous smooth muscle cells and increased NO here may play a role in the hypotension seen with endotoxaemia. Similarly endotoxin induces NOS production in the myocardium, which may contribute to the dilated cardiomyopathy development. There have been trials using inhibitors of NO to reverse the hypotension which has been induced in animal models by lipopolysaccharide or endotoxin via TNFα. However the level of inhibition of NOS is crucial for outcome, since high doses cause severe vasoconstriction and end organ damage in multiple organs and thus causes rapid death. With titration being the key factor and difficult to control in experiments to date without
significant complication, this treatment has not yet been able to become part of standard clinical management strategies (Petros, Lamb et al. 1994; Grover, Zaccardelli et al. 1999).

2.3.4 (ii) Nervous system – central and peripheral

NO has become accepted as a non-endogenous messenger, signal mediator and non classical neurotransmitter with NO synthases (see Chapter 3.4 ‘Nitric oxide synthase isoenzymes’) detected in varying amounts in all areas of both animal and human brain (Bredt and Snyder 1990). NO synthases are highly similar to NADPH diaphosphorase which is found in about 2% of the neurons in the cerebral cortex (Bastian and Hibbs 1994), and the two enzymes are closely co-localised in both the brain and the peripheral nervous system. Glutamate is the main excitatory neurotransmitter and an interaction between this and NO was demonstrated early (Garthwaite, Charles et al. 1988). After specific receptor stimulation, NO is released from a postsynaptic source to act at a presynaptic region on one or more neurons in any direction. This results in an increase in glutamate and a stable increase in synaptic transmission, a phenomenon known as long-term potentiation (Bliss and Collingridge 1993), which is linked to memory formation (O’Dell, Hawkins et al. 1991). Experiments in animals have shown that inhibiting NO synthesis by analogue competitive inhibitors impairs learning behaviour (Son, Hawkins et al. 1996). NO has also been shown to have a role in feeding behaviour, nocioception and olfaction (Bagetta, Iannone et al. 1993). An increasing body of work has continued to demonstrate the importance of NO in both short and long term memory (Susswein, Katzoff et al. 2004). In addition, the NO produced by the perivascular nerves of the cerebral arteries directly modulates vascular control (Gonzalez, Barroso et al. 1997). NO may also have a role in modulating pain (Moore, Babbedge et al. 1993).

At low concentrations, NO plays a role in vasodilatation and neurotransmission, however at higher concentrations it can be neurotoxic. It also may be that NO can possess either neurodestructive or neuroprotective properties depending on its oxidation reduction status (see Chapter 3 ‘Reactions with nitric oxide’). Microglial cells, which are of monocyte-macrophage derivation, can express the form of NOS which gives very high levels of NO (see Chapter 3: inducible NOS) within the central nervous system. These cells are implicated in the pathogenesis of neurodegenerative disease with excess generation of NO and high glutamate levels acting via receptors shown to mediate cell death in focal ischemia, Krabbe’s disease, Huntington’s disease and Alzheimer’s disease (Choi 1988; Meldrum and Garthwaite 1990; Snyder 1993; Vodovotz, Lucia et al. 1996; Dawson and Dawson 1998; Akiyama, Barger et al. 2000). For example, NOS inhibitors blocked ischemic damage following middle
cerebral artery ligation in several animal models (Dawson, Dawson et al. 1992; Yun, Dawson et al. 1997) and the NOS knock out mice had smaller infarcts after cerebral ischaemia (Iadecola 1997). The neurons which actually have high levels of both the NOS and the NADPH enzymes appear resistant to a variety of toxic insults including Huntington's disease, Alzheimer's disease and vascular stroke (Ferrante, Kowall et al. 1985; Koh, Peters et al. 1986). These neurons are also rich in manganese super oxide dismutase, and if this contributes to the neuroprotection, it may be similar to the reason for the variation of the half life initially given in the cardiovascular experiments reported above – i.e. that it depends on the extent of super oxide anion availability. Excess NO levels have also been implicated in demyelinating conditions such as multiple sclerosis (Parkinson, Mitrovic et al. 1997) and periventricular leukomalacia (Koprowski, Zheng et al. 1993). There is evidence showing that the production of NO is significantly raised within the multiple sclerosis lesions, but also in the cerebral spinal fluid, blood, and urine of these patients (Lin, Lin et al. 1993). In addition, the lack of ability to generate NO is important, such as seen in patients with Duchenne Muscular Dystrophy in whom skeletal muscle lack expression of the neuronal NOS (Brenman, Chao et al. 1995).

As mentioned, NO is also found in peripheral nerves where it contributes to sensory transmission and is a transmitter and/or modulator in non-adrenergic non-cholinergic (NANC) nerves, although there is considerable variation depending on both experimental conditions and the different species in which the experiments are carried out. The NANC nerves cause vasodilatation and relaxation of animal and human tracheal muscle via NO release, and this is not inhibited by NOS inhibitors (Stuart-Smith, Bynoe et al. 1994; Baba, Yoshida et al. 1998; Sipahi, Ercan et al. 1998). In human airway specimens, the NANC NO mediator relaxation is more important in the distal airways (Belvisi, Stretton et al. 1992; Ellis and Undem 1992). This response is reduced in recipient transplanted human bronchial tissue and in specimens from patients with CF (Stretton, Mak et al. 1990).

In the gastrointestinal tract, NO seems to mediate relaxation including dilatation of the stomach, the sigmoid colon and the internal anal sphincter in humans. Selectively blocking the NANC mediated relaxation of the gastrointestinal tract in mice resulted in marked stomach enlargement and inner circular gut muscle layer hypertrophy, thought to be compensatory for the inability of the pyloric sphincter to relax (Huang, Dawson et al. 1993; Bredt 1999). Histochemical studies of biopsy specimens from infants with hypertrophic pyloric stenosis and biopsy studies from adults with cardiac aplasia seem to confirm this with reduced NOS enzyme demonstrated (Vanderwinden, Mailleux et al. 1992). Also the gene
locus for the enzyme is a susceptibility locus for infantile pyloric stenosis (Chung, Curtis et al. 1996).

NO also has many roles in the reproductive system (Rosselli, Keller et al. 1998), an example being a reduction of NO being associated with pre-eclampsia and premature delivery. NO functions as the NANC transmitter which leads to relaxation of the corpus cavernosum and thus the development of penile erections with its effect able to be blocked in animal studies (Burnett, Lowenstein et al. 1992). A local reduction in NO is associated with decreased bladder capacity and hyperactivity (Burnett, Calvin et al. 1997) with knock-out mice displaying hypertrophied urinary bladders and loss of neurally mediated relaxation of urethral and bladder muscle, which provides a model for voiding disorders in humans. Studies in animals show that there is increased expression of NOS during pregnancy with increased urinary excretion of nitrite and nitrate (Conrad, Joffe et al. 1993; Conrad, Vill et al. 1993). The vasodilatation and decrease in blood pressure during pregnancy is likely to be also due to increased NO secretion with uterine NO synthesis preventing myometrial contraction.

2.3.4 (iii) Host defence

That NO was used in host defence was discovered in one of the original areas of research that delineated the importance of NO. The use of NO may have originated in evolution as a first line defence against invading micro-organisms (Hibbs 1991; Bogdan 2001). Resistance to tumour cells and cancer development was shown to be enhanced in a non-specific way by bacterial products (Nathan 1992). Activated macrophages were demonstrated to synthesise nitrite and nitrate, with generation dependant on L-arginine and inhibited by analogues of L-arginine (Hibbs, Vavrin et al. 1987). The generation of NO is now a known feature of many immune cells (dendritic cells, NK cells, mast cells, monocytes, macrophages, microglial cells, Kupffer cells, eosinophils and neutrophils) as well as other cells involved in the immune reaction (such as endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial cells and Schwann cells), (Bogdan 2001). The NO produced in these cells comes from the inducible form of NOS having the ability to generate much larger amounts of NO than the other enzyme types; the local concentration of NO synthesis is an important determinant of cytotoxicity. NO is a ubiquitous pathogen-killing agent and it is truly a generalist with a non-specific response to infection. It can be toxic to many kinds of pathogen including viruses, bacteria and parasites (both intracellular and extracellular) as well as some metazoan parasites (James 1995; Bogdan 2001; Colasanti, Gradoni et al. 2002). NO mediated killing of bacteria is thought to have been
developed as a two step process. Firstly, interferon gamma (IFγ) and TNFα activate macrophages and promote NOS synthesis of NO. The definitive second step is the respiratory burst during phagocytosis. It could be an adaptive host defence mechanism in humans. For example, in African children a mutation in the promoter gene seems to be associated with increased NO production which appears to provide significant protection against malaria (Hobbs, Udhayakumar et al. 2002).

NO also regulates lymphocyte function and may have a role in inhibiting subsets of T helper cells. However most of the normal host cells are susceptible to necrosis or apoptosis from the inhibition of mitochondrial enzymes and DNA damage caused by this molecule, particularly if the cells producing NO are over stimulated. NO is not restricted to a single defined receptor but can act widely. It is present in both acute and chronic inflammation. In these areas NO is likely to have a number of roles ranging from enhanced vasodilatation, the formation of the oedema, modulation of sensory nerve endings and increased leukocyte activity that possibly induces higher NO levels to be produced as a reaction to organisms and therefore causes increased tissue cytotoxicity. NO in respiratory inflammation will be elaborated on in Chapters 3, 4 and 5.

There are many other examples. Increased NO production was noted in inflammatory disease (Boughton-Smith, Evans et al. 1993; Tran, Visser et al. 1993; Alican and Kubes 1996; Levine, Pettei et al. 1998), particularly in ulcerative colitis (Lundberg, Hellstrom et al. 1994) though less convincingly for Crohns disease (Rachmilewitz, Stamler et al. 1995). This has been confirmed in animal models with Macaque monkeys (Ribbons, Zhang et al. 1995), guinea pigs (Miller, Thompson et al. 1995) and rats (Kankuri, Vaali et al. 2001). Increased urinary nitrite is higher at times of disease exacerbation in humans (Goggins, Shah et al. 2001). Inhibitors have been shown to ameliorate induced chronic ileitis in animal models. There are also increased nitrite concentrations in plasma and synovial fluid in patients with rheumatoid arthritis, osteoarthritis, (Stefanovic-Racic, Stadler et al. 1993; Amin, Di Cesare et al. 1995; St Clair, Wilkinson et al. 1996; Stichtenoth and Frolich 1998), type 1 diabetes (Eizirik, Flodstrom et al. 1996), giant cell arteritis, systemic lupus erythematosis (SLE), spondolarthropathy and Sjogrens syndrome (Belmont, Lewartovsky et al. 1997; Strand 1997; Strand, Leone et al. 1998). In SLE, for example, serum nitrite levels correlated with level of antibodies to double stranded deoxyribonucleic acid (DNA) and to symptom scores although the highest correlation was between serum nitrite and renal disease. Biopsies in non-lesional skin showed there were increases NOS expression during periods of active SLE (Belmont, Lewartovsky et al. 1997). In animal models of arthritis, a competitive NOS inhibitor blocked
NO synthesis and in so doing blocked paw swelling and histopathological changes in the joint (Stefanovic-Racic, Meyers et al. 1994).

Sepsis is a special picture. Sepsis begins with the exposure to an infectious agent which induces a cascade of events that initially tries to compensate for the problem with tachycardia, peripheral vasoconstriction, fever (usually) and increased polymorphonuclear cells. This is followed by progressive vasodilatation with a high cardiac output and decreased vascular resistance resulting in hypovolaemic shock and decreased venous return to the heart. This can progress to a state which is resistant to vasopressor agents and is often combined with a pro-coagulant state. Increased levels of serum nitrates were found to be correlated with the degree of systemic vasodilatation (Ochoa, Udekwu et al. 1991; Yoshizumi, Perrella et al. 1993). In animal studies, NOS inhibitors were used to treat induced sepsis but this was far from universally successful. The studies did not show improved haemodynamic parameters such as mean arterial pressure, but there was a further derangement of local blood flow (Booke, Meyer et al. 1995) resulting in worsened renal (Schwartz, Mendonca et al. 1997) and liver impairment (Gundersen, Corso et al. 1997) and worsened inflammation generally (Aaron, Valenza et al. 1998). The timing and dosage were found to be critical, as their use preventatively or early in shock resulted in worse outcomes (Cohen, Huberfeld et al. 1996; Strand, Leone et al. 1998). In one study of 12 patients with severe sepsis and hypotension, low doses of L-NMMA (NOS inhibitor) did result in an increase in pulmonary vascular resistance but also led to a decrease in cardiac output causing concern that this would result in poorer tissue perfusion (Petros, Lamb et al. 1994). In a further pilot study involving 32 patients with septic shock, the infusion of L-NMMA also resulted in an increase in vascular tone and a decrease in cardiac index within the first hour of therapy. The infusion continued for up to eight hours and mean arterial pressure was sustained with a 60-80% reduction of noradrenaline use (Grover, Zaccardelli et al. 1999). However a much larger multi-centre trial was then undertaken enrolling 797 patients with septic shock allocated to receive the NOS inhibitor or placebo for up to 7 days or 14 days in addition to standard therapy. It was terminated early because of a trend to higher mortality in the treated group by day 28 from multiple organ failure (Serrano, Casas et al. 2004).

2.4 Chapter summary

The idea for doing research into NO in the lung in human adult subjects; in healthy subjects and in those with respiratory disease, in particular asthma, came at a time when the areas of environmental pollution and mediator research were rapidly developing. The first area
included the expanding knowledge and measurement of airway pollution and the connection between higher or longer duration of daily exposure and increased cardiovascular and respiratory mortality and morbidity. Through the 1990s increasing effort was made to clarify the effects of the different pollutant components. Particulate matter, especially respirable particles at less than 10 microns in diameter, and ozone have been consistently associated with excess disease and mortality. However the nitrogen oxides have also had associations with increased respiratory disease (bronchitis, COPD, pneumonia and asthma) and for more episodes of wheeze with increased symptoms and lower lung function in adults and children with asthma.

The second area led from the startling discovery of the gas NO as a ubiquitous, biological messenger acting as a physiological mediator, a neurotransmitter and a mechanism of host defence. Since this was realised, research into NO in every biological system flourished. However there were a number of hurdles to overcome in measuring this molecule. Measuring NO was going to be difficult given its gaseous, short lived and highly reactive nature, and there were also the difficulties of measuring levels in exhaled air from human subjects.

In 1992 NO was named Science Magazine’s “Molecule of the Year”. In 1998 Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were winners of the 1998 Nobel prize in Medicine for their research in identifying firstly EDRF, and then renaming and identifying NO as the mediator in vascular epithelium.

The following chapter will discuss the synthesis and control of NO production in detail, the interactions of NO with other chemical and biological compounds, and the synthesis and control of the nitric oxide synthase enzymes that produce it. This knowledge is preparatory to discussing how to measure NO concentrations in the respiratory system.
Chapter 3: The synthesis, reactivity and control of nitric oxide

3.1 Introduction

The previous chapter detailed the two areas where nitrogen compounds were being increasingly recognised for their importance; air pollution and biological systems. In air pollution the nitrogen oxides were associated with excess mortality, in particular respiratory and cardiovascular morbidity; and in the biological systems, NO was found to be a widespread fundamental messenger. In this chapter, I will detail how NO is produced and cover its highly reactive and interactive properties. I will discuss the nitric oxide synthase (NOS) enzymes and how they are controlled in the physiological roles and the host defence roles of NO. The properties of NO needed to be understood when preparing to design experiments to measure it in vivo.

3.2 Properties of nitric oxide

Figure 3.1: The molecular structure of NO

\[
\text{\textbullet N = O\textbullet}
\]

NO is a clear, colourless gas. It is a free radical with an unpaired electron (\(\text{\textbullet N=O}\), abbreviated NO, see Figure 3.1) and therefore it is highly reactive with a half life of seconds and readily combines with other free radicals (Beckman and Crow 1993). However it does not self react, possibly because its bond length is intermediate between double and triple bond lengths (Braker and Mossman 1975). Its small size and the fact that it is a relatively non-polar molecule means that it moves readily through hydrophobic lipid membranes (Shaw and Vosper 1977; Malinski, Taha et al. 1993; Liu, Miller et al. 1998). Its reactivity means that it binds quickly with transition metals such as iron, copper, cobalt, or manganese that are central ions to many cytochromes and oxidases, and in the case of iron, haemoglobin. NO can be an oxidant or a reducing agent depending on the ‘redox’ environment. NO is soluble in water up to 2 millimoles per litre at 20°C in one atmosphere, but has a high partition coefficient so it usually exists as a gas. NO reacts rapidly with oxygen in air producing nitrogen dioxide (NO\(_2\)) (Vallance and Collier 1994). NO is very soluble in lipid and water and is therefore fully diffusible in the environment of the cell (Archer 1993; Henry, Lepoivre et al. 1993).
Table 3.1: Properties of nitric oxide

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>30.006 (1 mol = 0.030006 kg)</td>
</tr>
<tr>
<td>N...O bond distance</td>
<td>1.1508 angstroms</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td>approximately 20</td>
</tr>
<tr>
<td>Saturated NO solution</td>
<td>approximately 3mM</td>
</tr>
<tr>
<td>Oxidation products</td>
<td>Nitrite, nitrate</td>
</tr>
<tr>
<td>Dissociation t½ Hb-NO</td>
<td>approximately 3 hours</td>
</tr>
<tr>
<td>Absolute density</td>
<td>101.325 kPa at 25°C</td>
</tr>
<tr>
<td>Solubility in H₂O at 0°C</td>
<td>7.34 ml/100ml</td>
</tr>
<tr>
<td>Solubility in H₂O at 20°C</td>
<td>4.6 ml/100ml</td>
</tr>
<tr>
<td>Solubility in H₂O at 60°C</td>
<td>2.37 ml/100ml</td>
</tr>
</tbody>
</table>


NO is generated by the stereospecific enzyme NOS which acts on L-arginine cleaving the terminal guanidino nitrogen to produce NO and L-citrulline. This is accomplished via five separate steps and requires 3 co-factors [tetrahydrobiopterin (BH₄) and flavoproteins; flavin adenosine dinucleotide (FAD), flavin mononucleotide (FMN)], and 2 co-substrates [oxygen and nicotinamide adenosine dinucleotide phosphate (NADPH)] in the presence of calmodulin and calcium (Kwon, Nathan et al. 1990; Stuehr, Kwon et al. 1991; Barnes and Belvisi 1993; Knowles and Moncada 1994). L-arginine is a semi-essential non-aromatic amino acid and is present in nuts (especially brazils and almonds), shellfish, and meat (bacon, beef and game) (Vallance and Collier 1994). The necessary elements are demonstrated in figure 3.2.

Figure 3.2: The reaction to generate nitric oxide

The formation of NO by NO synthase involves the conversion of L-arginine to L-citrulline with several co-factors including the flavones and the tetrahydrobiopterin. There are a number of known competitive inhibitors for the NO synthase enzymes. NO itself can oxidised to nitrite, nitrate or peroxynitrite.
BH₄ = tetrahyrobiopterin, FAD = flavin adenosine dinucleotide, FMN = flavin mononucleotide, H₂O = water, L-NAME² = N⁵-nitroarginine methyl ester, L-NMMA = N⁶-mono-methyl-L-arginine, L-NOARG = N⁶-nitroarginine NADPH = nicotinamide adenosine nucleotide phosphate, NO = nitric oxide, NO₂⁻ = nitrite, NO₃⁻ = nitrate NO synthase = nitric oxide synthase, O₂ = oxygen, ONOO⁻ = peroxynitrate.


<table>
<thead>
<tr>
<th>Table 3.2: Reactions with nitric oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once NO is formed, there are a number of possible pathways of reactions (Nathan 1992; Stamler, Jaraki et al. 1992; Stamler, Simon et al. 1992; Henry, Lepoivre et al. 1993; Anggard 1994; Gaston, Drazen et al. 1994; Vallance and Collier 1994; Eiserich, Patel et al. 1998);</td>
</tr>
<tr>
<td>1. Redox reactions in the presence of O₂:</td>
</tr>
<tr>
<td>(a) To create reactive nitrogen species</td>
</tr>
<tr>
<td>→ nitrate (NO₂⁻)</td>
</tr>
<tr>
<td>→ nitrate (NO₃⁻)</td>
</tr>
<tr>
<td>→ nitrous acid (HNO₂)</td>
</tr>
<tr>
<td>→ nitrogen dioxide (NO₂)</td>
</tr>
<tr>
<td>(b) To create superoxides:</td>
</tr>
<tr>
<td>→ peroxynitrite ONOO⁻</td>
</tr>
<tr>
<td>→ peroxynitrous acid (ONOOH)</td>
</tr>
<tr>
<td>(both strong oxidising agents)</td>
</tr>
<tr>
<td>2. (a) Reactions with transition metals: cobalt (Co)</td>
</tr>
<tr>
<td>copper (Cu)</td>
</tr>
<tr>
<td>iron (Fe)</td>
</tr>
<tr>
<td>manganese (Mn)</td>
</tr>
<tr>
<td>zinc (Zn)</td>
</tr>
<tr>
<td>(b) Reactions with haem and nonhaem metalloproteins:</td>
</tr>
<tr>
<td>ascorbate oxidase</td>
</tr>
<tr>
<td>catalase</td>
</tr>
<tr>
<td>ceruloplasmin</td>
</tr>
<tr>
<td>cytochrome c</td>
</tr>
<tr>
<td>haemoglobin</td>
</tr>
<tr>
<td>lipo-oxygenase</td>
</tr>
<tr>
<td>myoglobin</td>
</tr>
<tr>
<td>succinate dehydrogenase</td>
</tr>
<tr>
<td>tyronase</td>
</tr>
<tr>
<td>3. (a) Interactions with nitrogen thiols and amines:</td>
</tr>
<tr>
<td>→ nitrosothiols</td>
</tr>
<tr>
<td>→ nitrosamines</td>
</tr>
<tr>
<td>(b) Interactions with sulphur:</td>
</tr>
<tr>
<td>→ sulphur thiols</td>
</tr>
<tr>
<td>5. Reactions with amino acid</td>
</tr>
<tr>
<td>5. Reactions with lipids</td>
</tr>
</tbody>
</table>

² These compounds are more often designated as N⁵ than N⁶ and the former is used in the text but the latter was used in this diagram.
3.3 Reactions of nitric oxide

The reaction of NO at any given time is dependent on a number of factors; the rate of reaction and therefore the concentrations of the species with which NO reacts the fastest, the concentration of NO, the reduction-oxidation (redox) environment in which it is found and therefore the presence and concentration of O₂, the availability of other interacting compounds and the pH (see Table 3.2). As well as local reactions, it is now believed that the interaction between NO and thiols and amines may be a way of stabilising NO in a bioactive form, potentially facilitating NO transport in tissue (Gaston, Drazen et al. 1994). I will review the different pathways briefly below.

3.3.1 Reactive nitrogen species and superoxide reactions

High levels of NO exposure can cause significant damage. It can damage all classes of macromolecules including DNA. It can destroy mitochondrial enzymes, prevent DNA synthesis, and inhibit protein synthesis (Hibbs, Taintor et al. 1988; Curran, Ferrari et al. 1991; Kwon, Stuehr et al. 1991; Stadler, Billiar et al. 1991; Lancaster 1992; Lepoivre, Flaman et al. 1992).

However it has become increasingly obvious that reactions with NO in the presence of O₂ forming NO derived reactive nitrogen species are equally important in mediating toxic injury. These compounds include nitrite (NO₂⁻), nitrate (NO₃⁻), NO₂, nitrous acid (HNO₂) or dinitrogen trioxide (N₂O₃). It also interacts with O₂ to produce superoxides such as peroxynitrite (ONOO⁻) and peroxynitrous acid (ONOOH), both strong oxidising agents. These can continue to react with all classes of biomolecules including lipids, DNA, thiols, amino acids and metals leading to the two mechanisms of damage which are oxidation and nitration (Eiserich, Patel et al. 1998).

NO₂ is produced by the reaction of NO with O₂ and oxidation of NO₂ to NO₂⁺ by myeloperoxidase (Ignarro, Fukuto et al. 1993). This reaction will therefore occur in areas of high macrophage numbers and high O₂ concentration and therefore is commonly seen in the lung. The exposure of human plasma to NO₂ causes rapid loss of ascorbate, uric acid, α-tocopherol, bilirubin, and protein thiols as well as increasing lipid peroxidation (Halliwell, Hu et al. 1992). Exposure of lung lining to NO₂ leads to loss of ascorbic acid, uric acid, and glutathione (Postlethwait, Langford et al. 1995; Kelly and Tetley 1997). Under acidic conditions NO₂ becomes protonated to form HNO₂. At least two biological compartments experience pH low enough to allow HNO₂ formation in vivo; the stomach with a pH 2.5-4.5
(Knowles, McWeeny et al. 1974) and within neutrophil phagocytes with a pH 3.0-6.5 (Cech and Lehrer 1984). This makes the production of this acid possible in high inflammatory conditions and after dietary intake of certain nutriments such as smoked and cured foods. High levels of both NO and NO₂ during inflammation in the presence of the inducible nitrogen synthase enzyme (see below) forms dinitrogen trioxide (N₂O₃). Both NO₂- and NO₃- are moderately stable and can be released on tissue contact (Moro, Darley-Usmar et al. 1994; White, Moellering et al. 1997). NO can undergo electron oxidation and reduction reactions generating nitrosonium cation (NO⁺) and nitroxyl anion (NO-) and these can participate in numerous other redox reactions. NO can also regulate apoptotic signals through formation of reactive NO species (Tamir, Lewis et al. 1993; Kim, Talanian et al. 1997; Nakano, Terato et al. 2003).

Peroxynitrite anion (ONOO⁻) and peroxynitrous acid (ONOONO) are oxidants which can also react with numerous targets. The formation requires NO and O₂- anion produced by many cell types – neutrophils, macrophages, smooth muscle cells endothelial cells and fibroblasts (Ischiropoulos, Zhu et al. 1992; Kooy and Royall 1994; Boota, Zar et al. 1996; Thom, Xu et al. 1997). They interact with glucose, fructose, and mannitol (White, Moellering et al. 1997; Skinner, White et al. 1998), and DNA bases. They hydroxylate aromatic amino acids (such as tyrosine, tryptophan, phenylalanine), and oxidise thiols and lipids (Beckman, Ischiropoulos et al. 1992; Alvarez, Rubbo et al. 1996; Beckman 1996). Peroxynitrite can directly inhibit oxidation reactions by chain terminating lipid peroxy and alkoxyl radicals or through regulation of cell signalling pathways that lead to induction of antioxidant enzymes ((Rubbo, Radi et al. 1994; O'Donnell, Chumley et al. 1997; Moellering, McAndrew et al. 1998). It can nitrate both free and protein bound tyrosine residues to give a 3-nitrotyrosine compound found in high levels in many inflammatory conditions (see below) (van der Vliet, O'Neill et al. 1994; Alvarez, Rubbo et al. 1996). Addition of ONOO to red blood cells results in methaemoglobin formation. ONOO also reacts with iron-sulphur enzymes and can lead to inactivation (Castro, Rodriguez et al. 1994; Bouton, Hirling et al. 1997).

While responsible for many toxic reactions – it is possible that this also provides a mechanism for removal as the ONOO compound isomerises into NO₃- and NO₂- at neutral pH (Lewis, Tamir et al. 1995; Munzel, Sayegh et al. 1995; Pfeiffer, Gorren et al. 1997). It is also possible that the ONOO- reaction with CO₂ which then degrades which may also be a protective mechanism (Lyman and Hurst 1996; Uppu and Pryor 1996).
3.3.2 Reactions with transition metals and metalloproteins

The biological chemistry of NO is strongly mediated through reaction with transition metals. The reaction is with catalytic metal centres such as iron (Fe) in both haem and nonhaem proteins (Tsai 1994). The high affinity of NO for the ferrous iron (Fe⁡²⁺ in the reduced state) in haemoglobin leads to a key interaction (Sharma, Traylor et al. 1987; Eich, Li et al. 1996). In vivo, this reaction of NO and oxyhaemoglobin to form methaemoglobin and nitrate (NO₃⁻) represents the major pathway for scavenging the endogenous NO production and is a significant route of NO removal (Lancaster, Langrehr et al. 1992). While this reaction is rapid, in fact it would be too rapid to allow NO to interact with the subendothelial layer in the vascular compartment (Lancaster, Langrehr et al. 1992). However, it is now known that when the haemoglobin is in erythrocytes, the reaction with NO is limited by diffusion into the cell and the half life is increased (Liu, Miller et al. 1998). Studies have shown that 70% of the NO in humans is recovered as NO³⁻ excreted through the urine (Westfelt, Benthin et al. 1995). Oxyhaemoglobin can also directly oxidase NO₂ and NO₃ forming methaemoglobin.

NO reaction with other Fe containing proteins can lead to either activation or inactivation depending on the protein. For example, the reaction with soluble guanylyl cyclase (sGC) leads to a conformational change which results in up to a 300 times increase in activation of the enzyme. Activation of sCG results in the formation of cyclic guanosine monophosphate (cGMP) from guanosine 5-triphosphate (GTP) (Ignarro, Degnan et al. 1982) and accounts for major signal transduction activity of NO. It is likely to be this mechanism that is the primary one mediating vessel relaxation and the inhibition of platelet aggregation (Moncada and Higgs 1991). In contrast, formation of a nitrosyl complex with cytochrome c oxidase leads to reversible inhibition which is competitive with O₂ binding (Eiserich, Patel et al. 1998).

NO also interacts with other metals such as zinc and copper. Formation of nitrosyl compounds with co-ordinating cysteine residues results in release of zinc (Kroncke, Fehsel et al. 1994). Formation of nitrosyl compounds with copper also occurs, and is particularly important in copper containing enzymes such as the cytochrome c oxidase (Radi 1996). It is through this type of interaction with metal ion containing proteins (metalloproteins) that NO effects the enzymes of mitochondrial respiration and this is also a mechanism of the macrophage mediated defence and involves, for example, cytochrome c oxidase and catalases (Cleeter, Cooper et al. 1994; Torres, Darley-Usmar et al. 1995). Part of this is an NO and O₂ competition at the cytochrome c oxidase binding site for O₂ and therefore NO inhibition is
more potent at low oxygen tensions (Brown and Cooper 1994). Again the most dramatic effects will occur in high levels of NO seen in inflammation.

There are also regulatory proteins in which the interaction of NO can modulate translation of mRNA sequences which contain Fe responsive elements such as ferritin and transferrin receptors (Pantopoulos, Weiss et al. 1996). In this way NO can also interact to regulate Fe levels in cells.

3.3.3 Nitrogen thiols and amines

S-nitrosothiols (S-nitrosoglutathione, S-nitrosoalbumin, S-nitrosohaemoglobin as examples) are stable molecules that can potentially transfer NO+ to other thiols or transport and release NO in distant areas and in other tissues (Stamler, Simon et al. 1992; Jia, Bonaventura et al. 1996; Funai, Davidson et al. 1997). This provides another mechanism by which NO regulates protein function. It has long been demonstrated that nitrosothiol compounds are generated during the curing process of meat with the interaction of nitrite (Emi-Miwa, Okitani et al. 1976). Exactly how the NO is released from these compounds and when is unclear; however these compounds have been suggested to account for some of the NO biological actions (Stamler 1994). For example, S-nitrosation has been shown to confer vasodilatory properties on other specific proteins. On the tissue type plasminogen activator enzyme, which dissolves fibrin in blood clots, s-nitrosation confers vasodilatory and anti-platelet functions (Stamler, Simon et al. 1992). The effect of shear stress to increase NO may also operate via s-nitrosation of plasma proteins. The vasodilatation effects of organic nitrates used in treatment such as nitroglycerin may also occur through the intermediate formation of these compounds (Fung, Chong et al. 1988). They are therefore potential therapeutic agents for pharmacological NO delivery (Butler, Flitney et al. 1995; Stamler 1995; Upchurch, Welch et al. 1996). S-nitrosothiols have been detected in the nervous system where they may function as neurotransmitters and use in animal models mimic NANC actions (Barbier and Lefebvre 1994; Liu, Gillespie et al. 1994; Slivka, Chuttani et al. 1994). Several reports have identified proteins whose activity changes upon S-nitrosation in vitro (Stamler 1994; Stamler, Toone et al. 1997).

These compounds can also allow NO to inhibit cytokine dependent signalling in vascular endothelial and smooth muscle cells. Both s-nitrosoglutathione and sodium nitroprusside prevent activation of the transcription factor NF-κB and expression of vascular cell adhesion molecule-1. Nitrosothiols can also regulate calcium transits and again can alter signal
transduction pathways in this manner by altering ion homeostasis (Stoyanovsky, Murphy et al. 1997; Xu, Ea et al. 1998).

### 3.3.4 Reaction with amino acids

The importance of the interaction of NO species with amino acids is exemplified by the reaction with tyrosine to form 3-nitrotyrosine. This reaction can severely compromise function of proteins with tyrosine residues in the active site, disrupting structure and activity (Eiserich, Patel et al. 1998). This reaction was first described in 1990 (Ohshima, Friesen et al. 1990) and the level of 3-nitrotyrosine is now commonly used diagnostic marker of NO derived oxidants in both human disease states and animal models (Schmidt, Hofmann et al. 1996). An “overwhelming body of evidence has amassed in the last several years revealing that NO₂Tyr (3-nitrotyrosine) is a reliable footprint of reactive nitrogen species production that spatially and temporally parallels tissue and cellular injury” (Eiserich, Patel et al. 1998). Although a direct relationship between 3-nitrotyrosine formation and pathological outcomes remains poorly characterised, it is dramatically elevated in diverse diseases such as atherosclerosis, sepsis, acute and chronic lung disease, neurodegenerative diseases, inflammatory bowel disease, chronic organ rejection, myocardial inflammation and tobacco smoking (Ischiropoulos, Zhu et al. 1992; Eiserich, Patel et al. 1998). The compound can be incorporated into tuberculin which disrupts the cell cytoskeleton (Eiserich, Hristova et al. 1998) and can attenuate adrenoreceptor agonists in vivo (Kooy and Royall 1994). The formation of 3-nitrotyrosine is dependent on increased NO production and frequently associated with activated phagocytes (neutrophils, monocytes, eosinophils, macrophages). NO can also interact with amino acid radicals in proteins (Eiserich, Cross et al. 1996).

### 3.3.5 Reaction with lipids.

The reactive nitrogen species (NO, NO₂, ONOO⁻) also interacts with unsaturated lipids which leads to either initiation of oxidation, altering the rates and products of lipid oxidation products or inhibition (see below). For example, the unsaturated free fatty acids arachidonic acid and linoleate are substrates for the enzymatic synthesis of bioactive medicators such as prostaglandins and leukotrienes through the activities of lipo-oxygenases, cyclo-oxygenases and cytochrome P₄₅₀. These play a role in regulation of blood pressure, platelet aggregation, and bronchosconstriction. Free unsaturated lipids and those esterified to phospholipids are a significant component to biomembranes pulmonary surfactant and plasma lipoproteins. Uncontrolled oxidation as can be caused by NO and related species can lead to changes in integrity and fluidity of biomembranes. The interaction can also lead to the formation of other
toxic products such as isoprostanes and reactive aldehydes (Eiserich, Patel et al. 1998). Lipid peroxidation is a characteristic feature of nearly all inflammatory diseases (Kuhn, Belkner et al. 1994; Quinlan, Lamb et al. 1996; Kuhn, Heydeck et al. 1997; Li, Maher et al. 1997).

On the other hand, NO can also serve as a protective factor. There are also lipid derived radicals both free and membrane bound which contribute to inflammatory conditions by inducing membrane damage again resulting in alteration of fluidity and integrity, as well as the production of eicosanoids and eicosonoid-like isomers which possess potent bioactivity. Direct reaction of NO with radical lipids results in inhibition of lipid oxidation and in this way may prevent lipid oxidation induced tissue damage (Rubbo, Radi et al. 1994; Gutierrez, Nieves et al. 1996; Wink, Cook et al. 1996; O'Donnell, Chumley et al. 1997).

3.3.6 Reactions with genes

Transcriptional regulation of several genes by NO have been reported. Interestingly, these are in general anti-inflammatory (Naruse, Shimizu et al. 1994; Pilz, Suhasini et al. 1995); examples include regulation of adhesion molecules, the haem metabolising enzyme, haemoxygenase (Foresti, Clark et al. 1997) and glutathione synthesis (Moellering, McAndrew et al. 1998).

3.3.7 Making sense of the reactions

In essence, the reactions that are important in biological systems depend on the amount of NO and where the NO is formed. The short half life and reactivity means NO is likely to act as a local messenger molecule, transferring messages within and between individual cells. In biological systems, when small amounts are formed as a mediator for physiological processes, the NO preferentially binds to haem, hence in the blood stream most NO is quickly bound to haemoglobin. From here it rapidly decomposes to yield predominantly nitrate (NO$_3^-$) and some nitrite (NO$_2^-$) and the compounds are eliminated in the urine with a half life of five to eight hours. NO will also interact with other heme containing proteins such as enzymes particularly sGC which results in a rapid increase in activity and production of cGMP. Excess NO is mopped up by other nitrosation reactions, for example nitrosothiols and these are now known to be active in their own right, and a way of stabilising NO in a bioactive form and potentially facilitating NO transport in tissue.

When released in much larger amounts, it is likely that NO more often reacts with other elements such as metals, for example copper, iron and zinc proteins releasing free Cu$^{++}$, Fe$^{++}$ and Zn$^{++}$ and generating O$_2$ and highly toxic hydroxyl radicals leading to effect massive
oxidative injury. High levels of NO disrupt DNA and cause both genotoxic and cytotoxic
damage. If there are changes in the pH, in the thiol content or in the redox state, this can result
in increased damaging and carcinogenic potential. Of interest is the reaction of NO with
amino acids such as tyrosine, which in itself is now used as a marker in several inflammatory
diseases.

Finally, there is now discussion that the original EDRF concept is made up of the actions of
NO and the actions of some of the post NO production of other compounds, in particular the
s-nitrosothiols (Myers, Minor et al. 1990; Vanin 1991; Jia, Bonaventura et al. 1996; Liu,
Miller et al. 1998).

3.4 Nitric oxide synthase isoenzymes

The nitric oxide synthases are large complex proteins that unusually contain both oxidative
and reductive domains. They have homology with cytochrome P_{450} which is also unique
(Bredt, Hwang et al. 1991; Vallance and Moncada 1994). They are synthases rather than
synthetases as they do not use adenosine triphosphate (ATP) in their reactions (Knowles and
Moncada 1994). NOS needs the substrate (L-arginine), the co-substrates (O_{2} and NADPH)
and the co-factors (BH_{4}, FAD, FMN) (see Chapter 3.2 above). The reaction is a five electron
mono-oxygenate oxidation pathway which involves two separate mono-oxygenation steps in
sequence (see Figure 3.2, Knowles, 1994 #712). In step one N^\wedge-\text{hydroxyarginine} is an
intermediate species formed (Stuehr, Kwon et al. 1991) in the first reaction by acquiring one
\text{O}_{2} molecule and one NADPH molecule (Kwon, Nathan et al. 1990) and the presence of BH_{4}
(Kwon, Nathan et al. 1989; Tayeh and Marletta 1989). The second step is the oxidation of N^\wedge-
hydroxyarginine to form citrulline and \text{NO}. Flavin coenzymes are involved in the transfer of
electrons to form a reduced oxygen species (Stuehr, Cho et al. 1991; Stuehr, Fasehun et al.
1991). There are consensus binding sites for FAD, FMN and NADPH located in the carboxyl
terminal portion of the NOS protein, and also a consensus binding site for calmodulin. The
other co-factor BH_{4} binds to NO on a 1:1 stoichiometry basis and has a redox role in enzyme
activity (Hevel and Marletta 1992; Knowles and Moncada 1994). All three isoenzymes are
phosphorylated (Nathan and Xie 1994).
There are two forms of NOS; the constitutive form (cNOS) and the inducible form (iNOS). The constitutive form is in turn made up of two types – the endothelial NOS (eNOS) and the neuronal NOS (nNOS), although there is only one form of the inducible NOS. They have also been classified as type I (nNOS), type II (iNOS) and type III (eNOS) which was the order in which they were first purified with the first isolation of their DNA (Bredt, Hwang et al. 1990; Bredt and Snyder 1990; Janssens, Simouchi et al. 1992; Lammers, Barnes et al. 1992; Lowenstein and Snyder 1992; Lyons, Orloff et al. 1992; Xie, Cho et al. 1992; Forstermann, Closs et al. 1994). The homology between the isoforms in humans is between 51-58% with approximately 51% between nNOS and iNOS, and 54% homology between eNOS and iNOS. (Nathan 1992; Chartrain, Geller et al. 1994; Forstermann, Closs et al. 1994; Marsden, Heng et al. 1994; Nathan and Xie 1994). Across species, the amino acid sequences for each isoform is well conserved at greater than 90% for the constitutive forms and greater than 80% for the inducible forms (Forstermann, Closs et al. 1994). They predominantly operate in the areas for
which they are named; eNOS in vascular cells, nNOS in the CNS and peripheral nerves, and iNOS within the immune cells, although these divisions are not so strict as first thought and some cells therefore can produce two different types of the enzyme (Nathan 1992). The enzymes are coded for on differing chromosomes; chromosome 7 for eNOS (21-22 kb, 26 exons), chromosome 12 for nNOS (150 kb, 29 exons) and chromosome 17 for iNOS (37 kb, 26 exons) (Marsden, Heng et al. 1993). The characteristics of the NOS enzymes are listed in Table 3.3 (Byrnes, Bush et al. 1996).

Table 3.3: The characteristics of the nitric oxide synthase isoenzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Constitutive/inducible</th>
<th>Type</th>
<th>NO or production</th>
<th>Calcium/calmodulin</th>
<th>Chromosome location</th>
<th>Cells in which enzyme is found</th>
<th>Enzyme stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>constitutive</td>
<td>I</td>
<td>picomoles</td>
<td>dependent</td>
<td>7: 26 exons span 21 kb</td>
<td>CNS: especially cerebellum; PNS: gut, bladder, reproductive organs; NANC nerves</td>
<td>acetylcholine, bradykinin, Ca&lt;sup&gt;2+&lt;/sup&gt; ionophore, histamine, leukotriene, PAF</td>
</tr>
<tr>
<td>eNOS</td>
<td>constitutive</td>
<td>III</td>
<td>picomoles</td>
<td>dependent</td>
<td>12: 28 exons span &gt;100 kb</td>
<td>endothelial cells, mast cells, platelets, smooth muscle cells, neutrophils</td>
<td>serotonin, thrombin, shear stress</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible</td>
<td>II</td>
<td>nanomoles</td>
<td>independent</td>
<td>17: 26 exons span 37 kb</td>
<td>macrophages, neutrophils, airway epithelial cells, fibroblasts, mast cells</td>
<td>lipopolysaccharide, γ-interferon, TNFα, TNFβ, IL-1, IL-2, leptochoic acid, picolinic acid</td>
</tr>
</tbody>
</table>

a CNS, central nervous system; PNS, peripheral nervous system; NANC, inhibitory noradrenergic noncholinergic
b TNF, tumor necrosis factor; IL, interleukin.

3.4.1 Constitutive nitric oxide synthases

Activation of the two constitutive isoenzymes (nNOS and eNOS) depends on the levels of calmodulin and calcium. The NOS enzyme lies dormant until an increase in cellular calcium in the presence calmodulin occurs. A calcium concentration of 200 to 400 nanomoles allows half the maximum activity of the enzyme. This results in a sustained release of NO over several minutes, with picomole amounts of NO released, and this acts locally. The enzymes are stimulated by a number of mediators, depending on where they are situated, and these include bradykinin, acetylcholine, calcium ionophore, histamine, leukotriene, platelet activating factor (serotonin/thrombin) and exercise. In this way these enzymes participate in maintaining physiological balance within systems as discussed in the previous Chapter 2.3.4. The nNOS was the first of the isoenzymes to be purified and cloned (Bredt, Hwang et al. 1991; Schmidt, Pollock et al. 1991). There is wide expression and high activity of nNOS isoenzyme in the brain and throughout the peripheral nervous system in the NANC nerves,
providing NO as a neurotransmitter (Forstermann, Closs et al. 1994). However it has also been found in the spinal cord (Dun, Dun et al. 1992), in sympathetic ganglia and adrenal glands (Dun, Dur et al. 1993; Sheng, Gagne et al. 1993), in the epithelial cells of the lung, uterus and stomach (Schmidt, Gagne et al. 1992), in pancreatic islet cells (Schmidt, Warner et al. 1992) and in human skeletal muscle (Forstermann, Closs et al. 1994). While in the main nNOS NO production functions mostly in neurotransmission, it is also utilised to maintain muscle tone, for example, in the gastrointestinal tract and skeletal muscle. These enzymes are involved in homeostasis throughout the body from memory, behaviour, circadian rhythms, to the gastroenterology tract, renal function and reproductive activity. It is likely that this isoenzyme form is the largest proportion of constitutive NOS in humans (Forstermann, Closs et al. 1994; Knowles and Moncada 1994). The eNOS isoenzyme within arteries give a basal level of NO production continuously, which maintains the vascular blood flow and blood pressure. This isoenzyme is largely responsible for the NO which inhibits platelet aggregation and platelet adhesion.

3.4.2 Inducible nitric oxide synthase

The main method of activation, the amount of NO produced and the regulation of this form of the enzyme is very different from the constitutive forms. The iNOS isoform is regulated at transcriptional level with activation requiring both a primary and a secondary signal before the mRNA for the enzyme is produced. The priming agent is interferon gamma (IFNγ) or an interferon inducing agent like bacterial lipopolysaccharide (LPS) (Weinberg, Chapman et al. 1978; Stuehr and Marletta 1985; Drapier, Wietzerbin et al. 1988; Sherman, Loro et al. 1991; Munoz-Fernandez, Fernandez et al. 1992). The category of factors which can act as the secondary agent continues to be enlarged by every new study. These include interleukin 1 (IL1), IL2, TNFα, tumor necrosis factor β (TNFβ), muramyl dipeptide, lipoteichoic acid, picolinic acid (metabolite of L-tryptophan), ozone, cAMP elevating agents, ultraviolet light, ozone and trauma (Ding, Nathan et al. 1988; Drapier and Hibbs 1988; Lorsbach, Murphy et al. 1993; Bastian and Hibbs 1994; Nathan and Xie 1994). In addition, a number of viral and antimicrobial products from mycobacterium tuberculosis, salmonella typhimurium and protozoan parasites have also been found to stimulate this form of the enzyme (MacMicking, North et al. 1997; Shoda, Kegerreis et al. 2001; Thoma-Uzynski, Stenger et al. 2001). Some food components are also capable of inducing NO and may be the mechanism of action of some food carcinogens, eg soybean trypsin inhibitor, beta amylase (Nathan 1997). For example, nitrites and nitrates are important antimicrobial, flavouring and colouring agents in meat and fish products (Chow and Hong 2002).
The induction requires gene transcription and mRNA production, and therefore occurs several hours after the exposure to the activating agents. However this also means that the production of NO can go on for days until the enzyme is broken down. Also important is that when activated, nanomole levels of NO are produced; ie 100 times that of the endothelial or neuronal isoenzyme production. NO exposure can damage all classes of macromolecules including DNA, it can destroy mitochondrial enzymes, inhibit protein synthesis, and cause apoptosis of cells (Hibbs, Taintor et al. 1988; Curran, Ferrari et al. 1991; Kwon, Stuehr et al. 1991; Stadler, Billiar et al. 1991; Lancaster, Langrehr et al. 1992; Lepoivre, Flaman et al. 1992; Tamir, Lewis et al. 1993; Nakano, Terato et al. 2003). So the regulation of this enzyme is extremely important given its unique role in host defence but also ability to inflict significant host damage (Nathan 1997).

Under certain conditions such as in the absence L-arginine or if BH₄ is limited (or in the presence of adriamycin which may in part explain the toxicity of this drug), all the NOS enzymes, but more often seen with this inducible form of the enzyme, can reduce O₂ via the flavin cofactors to O₂⁻ (Xia, Dawson et al. 1996; Cosentino, Patton et al. 1998; Vasquez-Vivar, Kalyanaraman et al. 1998). The simultaneous generation of O₂⁻ and NO then results in producing ONOO⁻ causing further cellular injury.

3.4.3 Control of the nitric oxide synthase isoenzymes

3.4.3 (i) The constitutive forms

All of the enzymes can be regulated at pre-transcriptional, post transcriptional and post translational levels (Papapetropoulos, Rudic et al. 1999). However the main control factors appear to be different for each. The control of the constitutive enzymes is predominantly maintained by the need for the presence of substrate and cofactors, while the control of the inducible form is predominantly by the need for two signals for transcription.

The constitutive enzymes require both the presence of calmodulin and a certain level of calcium from 200 to 400 nmols to commence production, and if the calcium concentration falls below this level then production ceases. The substrate L-arginine is required and L-arginine exists within and outside the cell. A low substrate amount can occur as a result of the absolute availability of L-arginine, or the ability of the cell to take up L-arginine, or the ability of the cell to regenerate L-citrulline back to L-arginine to act as further substrate. Vascular cells, for example, are able to perform this regeneration with the use of an enzyme arginosuccinate synthetase. BH₄ is also required, and this is produced by another pathway.
The enzyme of this second pathway – guanosine triphosphate cyclohydrolase I – is in turn affected by certain cytokines and inflammatory products. So while this operates as a control for the isoforms, in fact the limiting effect may be more important for the iNOS enzyme which is present in higher amounts, particularly in infection and inflammation when much greater levels of these cytokines are around and when the production of NO is higher (Stuehr 1999; Werner-Felmayer, Golderer et al. 2002). This will be discussed in more depth below in Section 3.4.3 (ii).

The rates of formation of NO and L-citrulline are non linear for all isoenzyme forms which demonstrates there is negative feedback inhibition and this is also a mechanism to control overproduction (Rogers and Ignarro 1992; Assreuy, Cunha et al. 1993; Bastian and Hibbs 1994).

In addition, there are some differing control mechanisms operating for each of the constitutive isoenzymes. Firstly, the nNOS form can be regulated by alternative splicing to give multiple transcripts and molecular diversity. This means that the protein can be spliced in different places giving different molecular weights which possibly fulfil a differing role. There are two major transcriptional clusters identified within the human nNOS gene, with one form lacking approximately 315 base pairs, or 105 amino acids and this smaller version is seen predominantly in the peripheral nervous system, renal tract and in the male reproductive tract (Papapetropoulos, Rudic et al. 1999).

Secondly, regarding the eNOS form, it was noted that some physiological situations led to increased eNOS expression such as shear stress and exercise training (Nishida, Harrison et al. 1992; Sessa, Pritchard et al. 1994; Uematsu, Ohara et al. 1995), and situations of local hypoxia (Marsden, Schappert et al. 1992). While both iNOS and nNOS exist in soluble forms, eNOS exists in a particulate form (Nathan 1992). The importance of this is that the proper localisation of eNOS is a pre-requisite to enable it to interact with specific proteins that will allow full activity. Post translation, eNOS undergoes a process of acylation (Shaul, Smart et al. 1996) which appears necessary to anchor the enzyme to the membrane in the Golgi apparatus, and in the plasmalemmal vesicles (the caveolae). There are some regulatory proteins that play a role in promoting this within the cells (G-protein coupled receptors, caveolin or Hsp90), that contribute to the correct localisation and that can also be individually influenced therefore contributing to more or less NO being produced. For example, Hsp90 increases with increased histamine or fluid shear stress (Garcia-Cardena, Fan et al. 1998). Changes in phosphorylation of eNOS have been observed with shear stress (Corson, James et
al. 1996; Eng, Morton et al. 1996; Fleming, Bauersachs et al. 1998), secondary to calcium mobilising agents (Michel, Li et al. 1993), tyrosine phosphatase inhibitors (Garcia-Cardena, Fan et al. 1996), and a number of protein kinases (Hirata, Kuroda et al. 1995; Chen, Mitchelhill et al. 1999). The phosphorylation of the enzyme makes it more sensitive to calcium necessary for the active pathway.

The most potent activator is lysophosphatidylcholine (LPC), a major phospholipid found in oxidised low density lipoproteins and this allows up to an 11 fold increase in mRNA induction and eNOS production, although there is a varying discrepancy between the levels of the mRNA and both the absolute protein levels and the levels of activity pointing to continued control later in the pathway (Papapetropoulos, Rudic et al. 1999). Interestingly, the content of LPC in atherosclerotic vessels is higher than normal vessels. Statin-based cholesterol lowering drugs result in a modest increase in the eNOS mRNA levels by a post transcriptional mechanism involving stabilisation (Laufs and Liao 1998). Oestrogens are capable of modestly increasing eNOS expression (Wiener, Ikokazu et al. 1995; Kleinert, Wallerath et al. 1998). The activity of the enzyme is reduced by the presence of TNFα which destabilises the eNOS mRNA and reduces the half life of the enzyme from 48 to 3 hours (Yoshizumi, Perrella et al. 1993).

3.4.3 (ii) The inducible form

Control of the iNOS isoenzyme can also operate at pre-transcriptional, post transcriptional and post translational levels, and drugs also interact to affect enzyme generation. However, different to the constitutive forms, control, particularly of gross overproduction, is extremely important to host survival.

Firstly, looking at the pre-transcriptional level; the activation of the iNOS promoter region is the main control for this isoenzyme. Most of the work looking at effects at a transcriptional level has been done in explanted mouse macrophages or macrophage cell lines (MacMicking, Xie et al. 1997; Papapetropoulos, Rudic et al. 1999). This has revealed a number of binding sequences including interferon gamma (INFγ), TNFα, TNFβ, interferon alpha (IFNα), NF-κB, gamma activated sites, interleukin 6 (IL6), activating protein sites and a basal transcription site. However the importance of only 2 of these has been clearly documented. The NF-κB site is important for iNOS induction (Nunokawa, Ishida et al. 1994) and LPS induction also operates through this site (Nathan 1992), and a cluster of four sites for IFNγ are important for NOS transcription (Xie, Kashiwabara et al. 1994). The need for second signal as well as IFNγ or an IFNγ-like substance to activate iNOS is probably an important control to prevent
inappropriate production from commencing (Lorsbach, Murphy et al. 1993). Another possible protective mechanism is, while INFγ induces iNOS, it also induces production of IL10 an anti-inflammatory cytokine. Pre-treatment of macrophages with IL10 inhibits their ability to express iNOS or to produce NO, although once iNOS exists then it has no effect (Cunha, Moncada et al. 1992). IL4 added with IL10 acts even more strongly in inhibiting the production of the enzyme (Oswald, Gazzinelli et al. 1992).

Secondly, examining the post transcriptional control, certain cytokines can have either stabilising or destabilising effects on the mRNA for iNOS, thus greatly altering its half life. For example, IFNγ stabilises the mRNA prolonging its action while transforming growth factor beta (TGFβ) can destabilise the mRNA and reduce its transcription as well as accelerating its breakdown (Vodovotz, Bogdan et al. 1993; Imai, Hirata et al. 1994; Sirsjo, Soderkvist et al. 1994; Perrella, Patterson et al. 1996; MacMicking, Xie et al. 1997). While this inhibits the production of iNOS in this way, it does not effect cNOS production (Ding, Nathan et al. 1990).

Thirdly, post translational control, as mentioned above, includes the availability of the substrate and cofactors. However the inducible form is independent from some of the regulations described for the constitutive forms. While all the isoenzymes require calmodulin as a cofactor, it is tightly bound to this form of the enzyme (Xie, Cho et al. 1992). In addition, the constitutive enzymes require calcium at levels of 200 nanomoles, whereas the inducible form can activate with calcium at much lower levels down to 39 nanomoles and notably the cellular basal level of calcium is nearer 70 to 100 nanomoles. This also explains why this form of the enzyme can keep producing NO when the constitutive enzymes have stopped production once the calcium falls below a certain level (Bastian and Hibbs 1994). A low amount of the substrate L-arginine acts as a control mechanism, there are three possibilities as mentioned above. There may be an absolute low availability of L-arginine. The uptake of arginine into the cell occurs through a sodium and pH dependent pathway, mediated by a family of cationic amino acid proteins (CAT1, CAT2, CAT2B, CAT3), (Closs, Scheld et al. 2000; Nicholson, Manner et al. 2001), but this can be up-regulated by the presence of LPS. External to the cell, the arginine levels are controlled by the enzyme arginase which degrades arginine to urea and ornithine (Gotoh and Mori 1999; Munder, Eichmann et al. 1999; Rutschman, Lang et al. 2001). Macrophages contribute to this, possibly also developed as a protective mechanism, as they release an abundance of arginase. However macrophages and vascular smooth muscle cells can also regenerate arginine from citrulline and therefore re-utilize the citrulline amino acid. This is with the use of another enzyme arginosuccinate
synthetase present in these cells, again upregulated by LPS and IFNγ (Hattori, Campbell et al. 1994; Nussler, Billiar et al. 1994; Nagasaki, Gotoh et al. 1996). The availability of cofactor BH₄ may also affect iNOS activity (Stuehr 1999; Werner-Felmayer, Golderer et al. 2002). One of the key enzymes (guanosine triphosphate cyclohydroylase I, as mentioned above) which generates this cofactor is affected by the presence of certain cytokines with IFNγ, TNFα, IL1 and LPS increasing its activity, while the protective cytokines IL4, IL10 and TGFβ suppressing activity. IL8 also causes a concentration dependent inhibition of iNOS (McCall, Palmer et al. 1992). Fibroblast growth factors inhibit NO synthesis and this may be a particular protective factor for the retina against damage (Goureau, Lepoivre et al. 1993).

Finally, in keeping with the other isoenzyme forms, the rates of formation of NO and L-citrulline from iNOS are non linear which suggests there is negative feedback inhibition (Rogers and Ignarro 1992; Assreuy, Cunha et al. 1993; Connelly, Palacios-Callender et al. 2001).

Studies recently have suggested an “adaptive NO resistance” where some cells likely exposed to high levels of NO have an inducible NO resistance mechanism (Demple 2004) and on subsequent exposure to high levels of NO the loss of cells reduces from 80 to 20%. These resistance mechanisms also operate against other free radicals (Kim, Bergonia et al. 1995; Bishop, Marquis et al. 1999).

Some of the effects of iNOS have been clarified by the use of NOS knock out mice – demonstrating both beneficial and detrimental roles. Mice with no iNOS have altered immune responses and decreased survival to bacterial, viral and parasitic infection (MacMicking, Nathan et al. 1995; Wei, Charles et al. 1995). They also have increased leukocyte adhesion to endothelium during toxaemia, poor wound repair and incomplete regeneration e.g. to liver biopsies and resections (Hickey, Sharkey et al. 1997; Rai, Lee et al. 1998; Yamasaki, Edington et al. 1998). However they are also resistant to endotoxin induced mortality, end organ damage after haemorrhagic shock, or hypoxic injury, and develop less eosinophilia in allergic airways disease (Wei, Charles et al. 1995; Nathan 1997; Hierholzer, Harbrecht et al. 1998; Ling, Gengaro et al. 1998).

3.4.4 Nicotinamide adenosine di-nucleotide phosphate oxidase and inducible nitric oxide synthase

These are the two key enzymes involved in host defence. There is 36% homology between them and both are present in macrophages, one of the key defence cells.
Nicotinamide adenosine dinucleotide phosphate oxidase (NADPH) is involved in the respiratory burst generating O$_2^-$ as a result of phagocytic triggering (Segal 1989). The O$_2^-$ produced is a precursor of other reactive oxygen species such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH*). NADPH oxidase is produced primarily in polymorphonucleocytes, monocytes and macrophages – all expendable cells in host defence. It is membrane bound but has membrane and cytoplasmic subunits and is responsible for defence against extracellular pathogens and engulfed organisms accessible to phagocytic cells. Activation and priming occurs with INF$\gamma$, PAF, GCSF, GMCSF, IL1, IL6, IL8, TNF$\alpha$ and substance P. Activation can also occur with complement factor CF5a, chemotactic factors, phorbol esters and fatty acids.

In comparison, iNOS can be induced in virtually all nucleated somatic cells and is responsible for the defence against pathogens that survive and proliferate in the intracellular environment. It lies within the cytoplasm and kills pathogens that enter the cells (Nathan 1992). When it was realised that biosynthesis of NO was possible by the same cells, it meant that these cells were capable of the production of a range of reactive potentially toxic oxynitrogen species such as nitrosium (NO$^+$), nitroxyl ions (NO$^-$), NO$_2$, and S-nitrosothiols (Stamler, Jaraki et al. 1992). However, although macrophages can have both NADPH oxidase and iNOS present, O$_2^-$ and NO are not often simultaneously produced thus avoiding the formation of peroxynitrite (ONOO$^-$). They appear to be independently regulated although INF$\gamma$ induces both enzyme systems. It may in part depend on the presence of L-arginine and the isomeric form in which it is found (Ding, Nathan et al. 1988; Martin and Edwards 1993; Bastian and Hibbs 1994). In isomerism the ‘cis’ form is when the two substituent groups are orientated in the same direction, while the ‘trans’ form is when the substituents are oriented in opposing directions. In the trans form, ONOO$^-$ undergoes homolytic cleavage to form highly reactive molecules HO$^*$ and NO$_2$ which can produce significant and irreversible damage to microbes, but also to host cells. However the cis form, ONOO$^-$ rearranges to form a non-toxic nitrate and it may be that the conditions for formation of these cis and trans isomers are what determine their formation in these cells.

3.4.5 Drugs and other agents that affect the isoenzymes and nitric oxide production

3.4.5 (i) Drugs

Corticosteroids inhibit the expression of inducible but not constitutive forms of NOS. This occurs at transcriptional level, with the ability to inhibit the induction of iNOS, but they are ineffective once the enzyme is synthesised. There are no recognisable steroid responsive
elements in the iNOS promoter region. Similarly the antifungal imidazoles drugs inhibit the induction of NOS. Methotrexate inhibits the synthesis of BH₄, therefore limiting the availability of this as a cofactor (Werner-Felmayer, Werner et al. 1990; Gross, Jaffe et al. 1991).

Diphenylene iodonium is an aromatic compound that binds to the flavoprotein cofactors, but is not a therapeutic option, because it was realised that very high doses would be required (Stuehr, Fasehun et al. 1991). Carbon monoxide binds to the haem in NOS and inhibits enzyme activity (White, Moellering et al. 1997). Compounds that bind calmodulin can affect cNOS but not iNOS (Bredt and Snyder 1990; Stuehr, Cho et al. 1991). Antioxidants protect NO.

Calcium mobilising agents (Michel, Li et al. 1993) and tyrosine phosphatase inhibitors (Garcia-Cardena, Fan et al. 1996) both increase the rate of phosphorylation of eNOS resulting in increased sensitivity to calcium and increased production. Statin-based cholesterol lowering drugs result in a modest increase in the eNOS mRNA levels by a post transcriptional mechanism involving stabilisation (Laufs and Liao 1998). Oestrogens are capable of modestly increasing eNOS expression (Weiner, Knowles et al. 1994; Kleinert, Wallerath et al. 1998). The angiotensin converting enzyme inhibitors inhibit the breakdown of bradykinin, and bradykinin stimulates particularly the endothelial cells to produce NO, so act to increase activity. Finally NO is the active moiety of the glyceryl trinitrate and other agents that have been trialled as anti-hypertensives and for bronchodilator properties.

3.4.5 (ii) Nitric oxide synthase inhibitors

The NOS inhibitors have been essential for evaluating the role of NO in physiological and pathophysiological processes, and have been used by infusion in both laboratory animals and humans to judge effects. L-arginine analogues have been created to act as competitive false substrates to inhibit NOS (see Chapter 2.3). They have now been widely used to study the NO generating NOS pathways which has been helpful in delineating the effects of NO in a variety of systems (Hibbs, Taintor et al. 1987; Hibbs, Vavrin et al. 1987; Palmer, Rees et al. 1988). This was first accomplished by the modification of guanidino group of L-arginine to create a series of inhibitors. These include L-NMMA (N⁷ monomethyl L-arginine) which has substituted a methyl group, L-NAME (N⁷ arginine methyl ester) which has substituted a nitro group and similar substitutions for the related compounds; L-NNA (N⁷ – nitro – L-arginine), LADMA (N⁷N⁷ dimethyl L-arginine), and L-NIO (N⁷ – iminoethyl-L-ornithine) (Knowles and Moncada 1992) (see Figure 3.3). They are stereo specific, acting at the level of the
enzyme and are competitively reversed by the normal substrate L-arginine, but not D-arginine. With prolonged incubation, the inhibitors appear to exhibit non-competitive properties (Marletta, Tayeh et al. 1990). Ebselen is a selenium containing antioxidant (2-phenyl, 2-benzisoselenazol-3-(2h)-one) which is synthetic analog of glutathione peroxidase and is able to inhibit both iNOS and cNOS (Hibbs, Taintor et al. 1990).

Figure 3.4: The chemical structures of the nitric oxide synthase inhibitors

The structures of arginine and of the arginine analogues most frequently used as inhibitors of the NO synthases with the predominant ionic species at neutral pH shown.


Some NOS inhibitors occur naturally such as aminoguanadine a competitive inhibitor, and two of the compounds discussed above (L-NMMA and L-NAME) also occur naturally in very small amounts, and so may also operate as a control at substrate level. Interestingly, the
Inhibitor compounds do not have equal responses with the 3 isoenzymes, for example cNOS is more sensitive to L-NAME and iNOS is more sensitive to L-NMMA. Aminoguanadine has been shown to have 10 to 100 times the affinity for iNOS compared to cNOS. LNIO is a particularly potent inhibitor of the neutrophil form of iNOS suggesting subclasses of the enzymes. Selective inhibitors of cerebral NOS have also been described, possibly utilising the differing length transcriptions described for this iso-enzyme (Moore, Babbedge et al. 1993).

3.5 Chapter summary

NO is a free radical with an unpaired electron which is highly reactive with a half life of seconds. It is produced by the NOS enzymes which exist as constitutive and inducible forms. They require L-arginine as a substrate, plus co-substrates (NADPH and O₂) and co-factors (BH₄ and flavoproteins). The neurological and endothelial constitutive forms generate continuous low levels of NO to maintain physiological processes and are largely controlled by calcium levels and available factors to be active. Activation of the inducible form requires a primary and a secondary signal before transcription occurs. However it then goes on to produce far greater amounts of NO for far longer than the other forms and is free from some of the controls of the other enzymes. It is this isoenzyme that is responsible for the host defence availability of this molecule.

NO reacts to form a number of related compounds known as the reactive nitrogen species (nitrate, nitrite, nitrous oxide and nitrogen dioxide) and in the presence of higher concentrations of oxygen, it forms superoxides (peroxynitrite and peroxynitrous acid). These compounds in turn are highly reactive and operate to kill intracellular and extracellular pathogens, but also to cause toxicity and destruction to the host cells and host DNA. NO also reacts with thiol compounds (S-nitrosoglutathione, S-nitrosocysteamin, S-nitrosohaemoglobin) and these have been increasingly recognised as a mechanism by which NO can be stabilised, transported and transferred to other compounds and other tissues. The compounds have also been demonstrated to have active roles regulating protein function and to have their own vasodilatory properties. A key interaction of NO is the reaction with metals in the centre of metalloproteins, and this forms the main method of excretion of NO. NO reacts with the ferrous iron in haemoglobin to form methaemoglobin and nitrate, the latter of which is then excreted in the urine. NO and related compounds can in addition react with genes, amino acids and lipids. NO itself is a clear and colourless gas. Having examined the properties of NO and the production pathway – the next chapter will discuss the technical options of how to measure NO levels.
Chapter 4: Methods to measure nitric oxide

4.1 Introduction

The previous chapter reviewed the production of NO, its reactions and interactions in vivo. This chapter will examine the available methods of measuring NO. There were three issues that I believed needed to be addressed; firstly, how the production of this evanescent molecule could be measured as accurately as possible, secondly, which technique was the most appropriate for measuring the production of NO in the lung and thirdly, how could it be done non-invasively such that it could be used with children – so preferably no venepuncture, no biopsy or bronchoscopy and lavage required. A technique based on the way it was measured in airway pollution but adapted to exhaled air in a manner analogous to lung function was sought.

From the previous chapter (see Chapter 3.1 ‘Introduction’), it is known that NO is formed stereo-specifically from the guanidino nitrogen of L-arginine and oxygen. This is accomplished via five separate steps (see Figure 3.3) and requires cofactors (tetrahydrobiopterin and flavins FAD, FMN) and cosubstrates (O₂, NADPH) as well as calmodulin and calcium.

Therefore the possible factors that could be measured to assess the activity of this pathway are:

- The decrease in L-arginine
- The formation of L-citrulline
- The activity of cyclic guanosine monophosphate (cGMP)
- The formation of NO
- The formation of methaemoglobinuria
- The formation of nitrite (NO₂⁻)
- The formation of nitrate (NO₃⁻)

These, plus the measurement of co-factors and the other nitrogen compounds have all been explored. A detailed review of all methods to measure NO in all compounds and in all tissues is presented in “Methods in Nitric Oxide Research” edited by Martin Feelisch & Jonathan J Stamler, published by John Wiley & Sons Ltd, Baffins Lane, Chichester, West Sussex, England, 1996. It is immediately obvious that many of these compounds will be inappropriate to use for detection in lung exhalate. I will briefly review the options before reviewing in more depth the technique of NO measurement with the use of a chemiluminescence analyser.
4.2 L-arginine, L-citrulline and cyclic guanosine monophosphate

The use of L-arginine as a substrate for NO production is a relatively specific reaction. This allows the production of NO to be correlated to the loss of L-arginine and the gain of L-citrulline, and it is possible to measure both of these in a laboratory setting (Senshu, Sato et al. 1992). However the baseline concentrations of L-arginine need to be known, which is difficult in vivo. Also, while the formation of L-citrulline can be measured (Hecker, Sessa et al. 1990, Senshu, 1992 #898; Bredt and Schmidt 1996), this is made more difficult in vivo where certain cells such as vascular endothelial cells and macrophages (and likely others) are able to regenerate L-citrulline back to L-arginine at a variable rate to act as further substrate (see Chapter 3.4.3 ‘Control of the nitric oxide synthase isoenzymes’). This recycling clearly precludes the use of measurement of the levels of these compounds to accurately reflect the production of NO.

The NOS enzymes stimulate soluble guanylate cyclase (sGC) with subsequent accumulation of cyclic guanosine monophosphate (cGMP). Again, it is possible to measure the levels of this protein, however, this requires access to, or the releasing of, the protein from its usual intracellular site. This is also non specific for NO as both sGC and sGMP operate in many cellular enzyme pathways (Schultz, Bohme et al. 1969; White and Aurbach 1969; Archer 1993; Craven and Ignarro 1996).

4.3 Methaemoglobin

NO can be measured by the transformation of reduced haemoglobin (Fe $^{2+}$) as it is oxidised to methaemoglobin (Fe $^{3+}$) which can then measured by spectrophotometry (detection threshold = 1 nmol). The reaction is rapid and will be almost stoichometric under most experimental conditions, with the only interfering compound likely to be superoxide anion production (Sutton, Roberts et al. 1976). The advantages are that spectrophotometers are widely available, there is no need to acidify the sample and that methaemoglobin remains relatively stable. Accumulation of oxyhaemoglobin is not a problem due to the much higher affinity of haemoglobin for NO than $O_2$. The disadvantages are that it is more useful for biological samples other than exhalate, considerable expertise is required and the assay will detect other nitrosyl groups in any given sample (Noack, Kubitzek et al. 1992; Murphy and Noack 1994; Feelisch, Kubitzek et al. 1996).
4.4 Nitrite and nitrate

The measurement of NO in biological tissues has been difficult in the past because of the short half-life, the small amounts produced, and its lability in the presence of O₂.

By contrast nitrite and nitrate are stable metabolites whose presence in blood, urine and tissue in humans was demonstrated many years ago (Mitchell, A. et al. 1916; Mitchell 1928; Tannenbaum, Fett et al. 1928). The diazotization assay is the standard technique for measuring inorganic nitrite and was described more than 150 years ago by Greiss (Greiss 1864; Greiss 1879). A two step assay is carried out with the reagents sulphanilic and N-(1-naphthyl) ethylenediamine first mixed and then incubated with a nitrite containing sample which interacts in a 1-1 ratio generating a purple azo dye which can be monitored by spectrophotometry at a wave length of 546nm (Greiss 1864; Green, Wagner et al. 1982). This remains the most common method of measuring nitrite and nitrate (Schmidt and Kelm 1996) in fluid such as blood and urine (Wishnok, Tannenbaum et al. 1993; Baylis and Vallance 1998). Studies have suggested that it is circulating nitrite measurable in serum or plasma rather than nitrate that more reflects the NO synthesis in both humans and animals (Bode-Boger, Boger et al. 1999; Lauer, Preik et al. 2001; Kleinbongard, Dejam et al. 2006). However, the excretion rate in urine of nitrate is a more non-invasive method to measure whole body NO synthesis and is particularly useful for obtaining baseline results and then assessing responses to physical and pharmacological treatment (Kanno, Hirata et al. 1992; Bode-Boger, Boger et al. 1994; Borgonio, Witte et al. 1999; Bode-Boger, Boger et al. 2000).

There are some difficulties in making these measurements in biological assays as NO decomposes to nitrite and nitrate at different rates depending on the ambient conditions and on the redox environment of the fluid being measured. As well, these two compounds are present at different concentrations in urine, serum and/or blood therefore requiring the ability to measure and calculate across a wide concentration range potentially from low nanomolar to high micromolar levels (Schmidt and Kelm 1996). Although adaptation to enable this has occurred as studies on the nitrogen compounds have progressed (Green, Wagner et al. 1982; Cortas and Wakid 1990; Ohta, Araki et al. 1994; Tsikas 2005). However even a very recent comprehensive review of the topic states "Despite the chemical simplicity of nitrite and nitrate, accurate and interference-free quantification of nitrite and nitrate in biological fluids as indicators of NO synthesis may be difficult" (Tsikas 2005). The Greiss reaction gives an assay that is designed to measure single samples, although it can be modified to give online
results (Tracey, Linden et al. 1990), but may not be sensitive or specific enough to measure NO alone (Schmidt and Kelm 1996).

NO will rapidly convert to nitrite and nitrate in biological samples and by adding acids or reducing substances these can be transformed back to release the NO molecule (Verdon, Burton et al. 1995; Kelm, Dahmann et al. 1997). However acidification and/or reduction will release NO from all nitroso compounds, alkyl or inorganic nitrites, the nitrosamines and nitrosothiols. If the sample contains compounds other than nitrite and nitrate, then there may be overestimation of the NO that existed as a biologically active or inflammatory mediator.

4.5 Nitric oxide

It has become possible to measure NO directly down to the cellular level by using microelectrodes. This commenced with the modification of a miniature O₂ electrode, and sealing it so that only low molecular weight gases could enter. By introducing a positive voltage, NO was oxidised on the surface of the electrode and could be measured over 1-3 μmol range (Shibuki 1990). The probe was approximately 2 mm in diameter. Modification of this then allowed a much smaller microsensor to be developed using a semiconductor polymeric porphyrin and a cationic exchanger on a sharpened carbon tip which reduced the size to 5 microns (Malinski and Taha 1992; Taha, Kiechle et al. 1992) and could measure NO as an electrical current. This has allowed NO measurement in cardiac cells (Xian, Zhang et al. 2000; Kanai, Pearce et al. 2001; Katrilik and Zalesakova 2002), brain and nerve cells (Shibuki 1990; Taha, Kiechle et al. 1992; Malinski, Bailey et al. 1993; Kumar, Porterfield et al. 2001), osteoclasts (Silverton, Adebanjo et al. 1999), trachea and main bronchi from an animal model (Ricciardolo, Vergnani et al. 2000) and in suspensions of mitochondria and cells such as platelets, leukocytes and in cell cultures (Wadsworth, Stankevicius et al. 2006). The detection rate has been as low as 10⁻²⁰ Mol, with a linear response between 8.0 to 4.8 x 10⁻⁶ mol/L. This low detection limit is also matched by high sensitivity and selectivity although it does also detect catecholamine activity (Tu, Xue et al. 2000). It has also been achieved as an online measurement so release of NO could be followed in activated macrophages, which correlated well with the nitrite concentration determined by the Greiss assay (Cserney and Gratzi 2001). However a recent review suggested that further methodological development of these microsensors was needed in order to avoid the influence of changes in temperature, pH, and oxygen on the measurements (Wadsworth, Stankevicius et al. 2006).

My interest was to look at measurement in exhaled breath. A number of very early methods of detection depended on colorimetric analysis but for exhalate these were too slow and too
insensitive (Braker and Mossman 1975; Archer 1993). For example, the use of moist iodide paper to detect the oxidation products of NO in air has a sensitivity of 300ppm and has a response time of 5 minutes. When collecting samples to measure the NO concentration, the presence of oxygen will continue to degrade the sample. This means the sample needs to be measured quickly, oxygen contamination must be avoided (which is difficult in biological specimens), or the loss of NO with these reactions needs to be compensated for by converting the other compounds back to releasing NO just prior to measurement.

It is possible to use collected expirate which can be bubbled through degassed water and the NO can be trapped by nitroso compounds or reduced haemoglobin to form stable adducts that can then be detected by electron paramagnetic resonance (detection threshold = 1 nmol). Electron paramagnetic resonance spectroscopy allows a specific assessment of molecules whose energy levels are altered in the presence of a magnetic field and this property is typical of molecules with an uneven number of electrons (Henry, Ducrocq et al. 1991; Henry, Lepoirve et al. 1993; Singel and Lancaster 1996). Usually a continuous wave of electromagnetic radiation in the microwave frequency is applied to the sample. When the frequency of this is equivalent to the energy difference of the electron spin energy levels, which is known as the ‘resonance’, the radiation is absorbed and the absorption leads to the generation of a signal which is measured. This technology has proved useful in detecting the movement of NO, for example determining the specific parentage of L-arginine to NO to methaemoglobin, determining the active site for NO or the binding site with other compounds. Interestingly, despite the unpaired electron, the NO molecule is only detectable in an excited state, while at a ‘ground’ state (unexcited state) the coupling of electron spin and the orbital angular momentum makes it resonant silent on the spectroscope. It is possible to identify signals at different frequencies which allow different molecules within compounds to be identified. This is unless a wide band of frequency is occupied as is seen with the iron in the haem proteins and from some of the other compounds used to trap NO such as metals or thiols. The compounds used for trapping NO in this way are also sensitive to pH. At acidic pH levels, the nitroso and nitrone compounds are unstable and can yield NO-type spectra even in the absence of NO (Arroyo and Kohno 1991). At alkaline pH there can be inhibition of the reaction between NO and haemoglobin (Feelisch, Kubitzek et al. 1996; Hakim, Sugimori et al. 1996). Finally, the equipment required to perform electron paramagnetic resonance was, and remains, expensive and considerable expertise is necessary (Archer 1993). In addition, more direct methods of measuring exhalation were becoming available.
NO can be detected by standard gas chromatograph techniques (Pai, Payne et al. 1987; Tsikas, Boger et al. 1994), although initially this was found to be much less sensitive than most of other methods discussed here, partly because of the necessity for periodic manipulation and thus disturbance of the reaction mixture during the sample processing. It is now increasingly combined with scanning mass spectrophotometers where NO in air can be detected (Kelm, Feelisch et al. 1988). Sensitivity has been limited by the resolving power (13500) required to differentiate between $^{15}\text{N}_2$ (m/z 30.0022) and NO (m/z 29.99799). Quantification was further complicated by the vast difference in the concentration of these two gases, namely parts per thousand for $^{15}\text{N}_2$ and parts per billion for NO. Specific experiments can be designed using the isotope of $^{15}\text{N}$ as a specific label for NO. In the mass spectrometer, gases are admitted under very low pressures into an ion source where they are ionized in a high energy electron flux. The positive ions formed are accelerated in an electric field and then deflected in a magnetic field. The radius of the deflection is inversely proportional to the number of the particles. A multi-collector device for a number of particles can be used. It can also be used to measure the evolution of gas from a liquid sample. The reaction mixture is ‘sparged’ with an inert compound such as argon (sparging is to agitate by introducing a compressed gas), a plunger is lowered to the liquid surface and closed to the atmosphere. The reaction is then commenced with injections of reagents, inhibitors, enzymes or cells – and in the case of NO stripping, obtaining NO back from the compounds to which has bonded by acidification. The gas then diffuses into a vacuum line through a cold trap which removes water vapour, and the remaining gases carrying NO and other compounds of interest are scanned and analysed at brief intervals (Payne, Le Gall et al. 1996). This has been used to study nitrogen metabolism, not only in biological fluids but also, for example, to assess bacterial activity in river water etc. However using labelled arginine, it has been used to measure NOS activity (Tsikas 2004) and very recently to assess whole body NO synthesis in healthy children after subjects had oral doses of the labelled substrate with the measurement of the subsequently collected urine (Forte, Ogborn et al. 2006). For all these measurements, isotope labelling is required, and therefore they are not so useful for repeated exhalation measurement under different conditions.

4.6 Chemiluminescence

The method that held the most promise for measuring NO in direct gas samples such as exhaled air was chemiluminescence.
A group of instruments have been developed to enable the measurement of certain compound concentrations within samples by using light reactions. These include:

- ‘Spectrophotometers’, mentioned above, which measure how light is absorbed by the specimen with the light being generated by the meter itself.
- ‘Fluorometers’ which measure light emitted by the specimen after excitation of the sample generated by the meter.
- ‘Luminometers’ which measure light generated with no light or excitation input – this makes them comparatively simple compared to the other devices as they only require a reaction chamber, a light detector and a recorder (and for the measurement of NO, a photomultiplier).

The chemiluminescence analysers (see Figure 4.2) were originally developed to measure NO as an atmospheric pollutant as discussed in Chapter 2.2. The measurement is based on the observation that the reaction of NO with ozone produces light (see Figure 4.1).

**Figure 4.1: Chemical reaction between nitric oxide and ozone**

\[
\begin{align*}
\text{NO} + \text{O}_3 & \rightarrow \text{NO}_2^* + \text{O}_2 \\
\text{NO}_2^* & \rightarrow \text{NO}_2 + h\nu^a \\
\end{align*}
\]

\(\text{(}\ast = \text{unstable electron})\)

\(\text{(}h\nu^a = \text{light})\)

The interaction of ozone with gases such as NO, NO\(_2\), CO and SO\(_2\) was described in the early 1960s (AIHA 1966), although ozone reacts most readily with NO. In the excited state the electrons are unstable and they dissipate energy as they regain their original state. This light is sufficient to make chemiluminescence one of the most sensitive NO assays available. The chemiluminescence reaction of NO and O\(_3\) is very fast and has a low activation energy of 10.5 joules (Johnston and Crosby 1954). At room temperature the rate constant of the reaction (both steps) is \(10^{-7} \text{ mol}^{-1} \text{ s}^{-1}\) (Clyne, Thrush et al. 1964). This high speed means that the chemiluminescence assay is able to detect rapid changes in NO concentration and therefore can be adapted for on-line measurement. It was, for example, used early to monitor NO concentrations when this was delivered as a treatment trial in intensive care for pulmonary hypertension (Pepke-Zaba, Higenbottam et al. 1991; Kinsella, Neish et al. 1992; Roberts, Polaner et al. 1992; Gerlach, Rossaint et al. 1993). NO\(_2\) reacts with ozone more slowly and the reaction requires higher activation energy so the chemiluminescence levels of NO are not affected by NO\(_2\), even if the latter is present in high concentrations (Johnston and Crosby 1954; Fontijn, Sabadell et al. 1970).
The first chemiluminescence analyser to measure NO was built in 1970 utilizing these principles of chemical reaction. A vacuum of reduced pressure (approximately negative 1-15 mmHg) is used to draw the gaseous sample into a reaction chamber through a valve where ozone is generated by electrical discharge. The vacuum is also necessary to evacuate gases that could potentially absorb energy from NO\(_2^*\), and to stabilise NO by removing O\(_2\), to prevent any O\(_2\) reacting with the NO to produce NO\(_2\) which does not emit light (Hampl, Walters et al. 1996). The NO and O\(_3\) are then mixed in front of a photomultiplier tube sensitive to low levels of light at the red sensitive end of the spectrum (660 – 900 nm). The photons from the reaction strike a photosensitive surface and the impact releases electrons which are accelerated toward an electron sensitive surface (the first dynode) by an electric field. Each electron impact on this first dynode then causes emission of several electrons and these are accelerated to a second dynode. This step is repeated but the electrons are attracted to the terminal electrically charged element – the anode – and the resulting current is measured. This amplification achieved by the photomultiplier is necessary to measure the signal as the NO\(_2^*\) reaction emits a relatively weak red light (Hampl, Walters et al. 1996) and means that each electron emitted from the original photosensitive surface becomes a signal from millions of electrons at the anode (Turner 1985).

Figure 4.2: Diagram of the chemiluminescence analyser

NO chemiluminescence analyser. A vacuum pump draws ozone and sample into the chemiluminescence reaction chamber. In the reaction chamber, NO sample reacts with ozone and the emitted light is detected by a cooled photomultiplier and recorded. Ozone is created by electrical discharge in the ozone generator. The inflow rate of the sample gas is regulated by the needle valve with the aid of an optional flowmeter.

The original group demonstrated that the light emitted was indeed directly proportional to the linear content of the specimen and I have included the graph of their results (see Figure 4.3). They used increasing volumes from one to one hundred micro-litres of a commercially available gas in gas tight, N₂ flushed syringes. The chemiluminescence signal was recorded as the peak height at an integration time of two seconds. This showed a linear relationship of chemiluminescence measured in millivolts to NO concentration measured from zero to fifty picomoles.

Figure 4.3: Relationship between chemiluminescence in millivolts to nitric oxide concentration in picomoles.


The background output of the photomultiplier is relatively stable, unlike the other light source meters which can have surges. The dark current (as it is known) can be affected by changes in temperature, light and alterations in voltage, and by very high levels of NO. Large disturbances in the dark current may take days to stabilise. For this reason most are equipped with a cooler as cooling the photomultiplier tube improved the signal/noise ratio. Preferably the analyser should also be kept in a temperature controlled room and away from any other equipment that potentially generates heat.

A red cut off filter separates the reaction chamber and the photomultiplier to prevent the detection of light with wavelengths below that of the NO/O₃ reaction. These include blue or ultraviolet emission of alkenes and sulphur containing species such as the reaction of hydrogen sulphide (H₂S). These reactions are unlikely to be a major problem in practice as the compounds are present on a greatly reduced scale compared to NO with far less or no biological activity, and they are not volatile. As the NO/O₃ reaction takes place in the gas phase, this is ideal for measurement of gaseous samples such as exhaled air. The resulting
NO₂ produced can be removed by a soda lime column and resulting O₃ can be removed using a charcoal column; or the exhaust can be immediately directed outside. I opted to use columns to ‘scrub’ the compounds from the exhaust air. The luminescence signal as measured by a photon counter and transmitted as an electrical signal and (in the early adapted machine which we used) this was then transmitted to a chart recorder. In the later versions of the machine the signal went to an analogue digital computer.

Sensitivity: The detection threshold of NO is 20-50pmol. In aqueous solutions the chemiluminescence assay has been reported to detect as little as 10⁻¹³ M of NO (Zafiriou and McFarland 1980). The chemiluminescence/NO curve has been found to be linear between NO doses of 0-50pmol (Fontijn, Sabadell et al. 1970) and 300-3000 pmol (Menon, Wolf et al. 1989; Brien, McLaughlin et al. 1991).

Specificity: Chemiluminescence is almost exclusively due to NO. As mentioned above, there are few other substances that react to produce light and these are either non-volatile or are not biologically important such as the production of H₂S and alkenes. One author (Archer 1993) found that at very high levels a solvent used for many drugs (dimethyl sulfoxide – ‘DMSO’) could also cause a chemiluminescent signal. This is unlikely to be a problem in exhalate and pertains more to the use of high dose chemicals on explanted or cultured tissue (Mottu, Laurent et al. 2000). However this chemical is a known antioxidant and more recently has been employed as a treatment in certain inflammatory conditions, most particularly in interstitial cystitis (bladder inflammation), but also for some rheumatologic diseases (Santos, Figueira-Coelho et al. 2003; Chancellor and Yoshimura 2004; Parsons 2004).

The NO chemiluminescence analysers available in 1995 were designed for measuring NO concentrations within 2-4000ppb and 40-400ppm range in a continuous ambient air sample. They had been adapted for online recording and had a stabilised measurement capability as measured by drift without using an auto zero over 24 hours. In most this was cited at between zero and two ppb. They operated in ambient temperatures of 5-40°C and humidity of 0-95%. The estimates of NO concentrations were decreased 10-15% at 100% humidity, however NO pre-drying of the expirate was not thought to be necessary for most environments (Figure 4.2 does have a pre-drying unit added in the diagram). The original response time of these machine developed to measure airway pollution were long, too long for the purpose that we
required it. For example, there was a 90 second delay in the model we used (Model 207, Dasabi Corporation), but modification of the circuitry and the sending of the pre-computer analogue signal directly to the chart recorder (with assistance from the Biomedical Engineering Department at the Royal Brompton Hospital, London, United Kingdom: Carolyn Busst and Ron Sinclair) decreased the response time – a factor checked in the first experiments made to establish the analyser’s capability (see Chapter 5). The option of a reader, chart recorder or an analogue digital computer may seem unusual now in the days of easy, fast computer access, and the new analysers have these inbuilt for signal display. The sampling flow of the NO analyser depends on the vacuum pump rate. The NO analysers listed above at the time that these experiments began sampled at fixed rates between 200 and 800 mls/min. This too was modified for use on the first experiments using this machine (see Chapter 5). The newer purpose built analysers have a wider range of sampling flow options of 25, 50,100, 250 and 500 mls/min.

Table 4.1: The companies providing chemiluminescence analysers adaptable for nitric oxide measurement in 1995

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChemLab Instruments Ltd, Hornchurch, UK</td>
<td></td>
</tr>
<tr>
<td>Columbia Scientific Industries Corporation,</td>
<td>Austin, Texas, USA.</td>
</tr>
<tr>
<td>Dasabi Environmental Corporation, Glendale,</td>
<td>California, USA. (Model 2107)</td>
</tr>
<tr>
<td>Eco Physics, Durnten, Switzerland (CLD 700)</td>
<td></td>
</tr>
<tr>
<td>Lear-Seigler/Monitor Laboratories, Englewood,</td>
<td>Colorado, USA.</td>
</tr>
<tr>
<td>Seivers Instruments, Boulder, Colorado,</td>
<td>United States of America.</td>
</tr>
<tr>
<td>Thermoelectron, Warrington, UK. (Model 42)</td>
<td></td>
</tr>
</tbody>
</table>

In addition to detecting NO in gas samples, I will mention briefly that the technique can be used for liquid samples. At room temperature NO has a partition coefficient of 20; that is in a sample containing both liquid and gas, there is 20 times more NO in the gas phase than dissolved in the liquid phase. So if a fluid with no air contact is then injected into a chamber, the NO quickly escapes into the gas phase and this can be aspirated into the chemiluminescence analyser. The rest of the NO can then be “stripped” from the solution by bubbling the solution with an inert gas under vacuum conditions usually at a rate of between 8-10 mls/min driving it into the gas phase which can then be measured (Chung and Fung 1990; Archer, Shultz et al. 1995; Hampi, Walters et al. 1996). However it is vital to avoid foam or bubbles getting into the reaction chamber as this can then coat the PMT and impair its
function. This is especially important with biological specimens as proteinaceous material (such as albumin and blood) foams during stripping and therefore provides more difficulties.

The chemiluminescence assay can also be used to measure the other intermediates and other end products of NO oxidation such as s-nitrosothiols, nitrite, and nitrate. The compounds can be made to release the NO by reducing them with acid (usually hydrochloric acid, citric acid or glacial acetic acid is used). This gives the nitrosonium ion (NO+) which then can react with anions (such as iodine which is commonly used) which then dissociates to give NO, water and iodine, with the NO able to be measured in the chemiluminescence analyser.

Figure 4.4: Reaction equations used to release nitric oxide from other nitrogen compounds

\[
\begin{align*}
\text{NO}_2^- + 2\text{H}^+ & \rightarrow \text{NO}^+ + \text{H}_2\text{O} \\
\text{NO}^+ + \text{I}^- & \rightarrow \text{ONI} \\
2\text{ONI} & \rightarrow 2\text{NO} + \text{I}_2
\end{align*}
\]

As can be seen, this is more difficult than the direct gas measurement and involves more steps. In addition, all the reagents must be replaced with every sample and extra time required for signal of the acid and iodine compounds alone. It is very temperature and (clearly) pH dependent. However, as mentioned above, when correctly performed it remains very sensitive to NO in small amounts (Zafiriou and McFarland 1980).

4.6.1 Calibration

Machine calibration had to be carried out regularly, and at the time a number of commercial gas companies offered cylinders with graduated NO concentrations:

- BOC Gases, Surrey Research Park, Guildford, Surrey, UK
- Scott Specialty Gases, Troy, Michigan
- Matheson, East Rutherford, New Jersey

(Modified from our original published chapter (Byrnes, Bush et al. 1996)).

These were prepared by oxidation of ammonia at 500°C over platinum gauze or produced by passing an electric arc through the air (Braker and Mossman 1975). Individual calibration samples of NO can be prepared chemically either by adding acids to sodium nitrite (NaNO₂), or mixing NO₂⁻ and a denitrifying enzyme (Pai, Payne et al. 1987). A preparation of a saturated NO solution (NO = 3 mM) is needed. Double distilled cold water is bubbled with
helium for 30 minutes to remove $O_2$. The water is then bubbled with pure NO (>99.0% v/v) for 30 minutes in a glass sampling bulb. Samples can be aspirated through a rubber gas-tight syringe. Before aspiration $N_2$ should be injected into the glass bulb to exclude $O_2$ (Archer 1993). Once a saturated NO solution has been made serial dilutions of the NO gas or solution can be made using deoxygenated $H_2O$. Having reviewed the alternatives, the company that I used was BOC Gases (Surrey Research Park, Guildford, Surrey, United Kingdom) who were local to where the research was taking place and who regularly supplied gases to the Royal Brompton Hospital. Also they could provide NO in cylinders of appropriate concentrations suitable for calibration of the levels we intended to measure (see below).

Whether personal or commercial preparations of NO are being made for calibration purposes it is imperative to avoid contamination with $O_2$ which will reduce the NO in the sample. Zero calibration can be done with NO free certified compressed air. Alternatively, passing room air through the ozone generator of the chemiluminescence analyser, which converts all NO$\textsubscript{x}$ to nitrogen dioxide and then passing the resultant gas through soda lime and activated charcoal to remove the NO$\textsubscript{2}$ and ozone respectively can also produce NO$\textsubscript{x}$ free air. The most useful calibrations gas concentrations are zero and concentrations within the usual levels measured, with the final calibration just above the highest levels likely encountered to avoid the need for linear extrapolation beyond the range. Not all the companies could provide appropriate concentrations or had them in stock. In normal subjects on the current analysers with the current measurement protocols, the range is 0-25 ppb for oral exhaled NO and 250-1000 ppb for nasal NO but the levels on the older machines were often higher (0-80 ppb) for oral exhaled measurements. The reason for these differences will be come clear when reviewing the methodology experiments that I completed (see Chapter 5). I always had a zero and two NO cylinder concentrations for calibration for the experiments described in the work of this thesis. They were either 27 ppb and 103 ppb and 110 ppb or 55 ppb and 118 ppb when the first cylinders were exhausted.

4.6.2 Safety and toxicity

In the final part in this chapter I want to review the toxicity of NO, and the two gases generated using the chemiluminescent technique – $NO_2$ and ozone. The reactions and effect of NO at the cellular level has been covered in Chapter 3 sections 3.2 ‘Properties of nitric oxide’ and 3.3 ‘Reactions of nitric oxide’. NO must be handled with care – the toxicity of NO is due to the gas itself and also the interactions that occur with oxygen when it forms a dimeric form of NO$\textsubscript{2}$ – a reddish brown toxic gas (Budvari, O'Neil et al. 1989) which in high $O_2$
concentration can form the superoxide peroxynitrite (OONO). In part the effects of these gases have been covered when reviewing the noxious effects of air pollution with particular reference to the nitrogen oxides in Chapter 2.2. NO and, even more so, NO₂ are common air pollutants (Morrow 1984; Anonymous 1995; Anonymous 1996) with inspired NO concentrations from airway pollution ranging from 20-500ppb in non-polluted areas to 1.5ppm or greater in polluted areas (Aranda and Pearl 2000). As previously mentioned, cigarette smoke contains NO concentrations in order of 100ppm (Norman and Keith 1965), or higher depending on the cigarette type (Borland and Higenbottam 1987) and cigarette smoke can inhibit NADPH and myeloperoxidase (Nguyen, Finkelstein et al. 2001). While this does not result in an acute respiratory event, it might cause an increase risk of mutations over time and thus it contributes to cigarette smoke carcinogenic potential.

With the use of nitrous oxide (N₂O, “laughing gas”) effects were noted when N₂O cylinders were contaminated with NO showing that high NO concentrations could give rise to acute pulmonary oedema and methemoglobinaemia (Anonymous 1967; Clutton-Brock 1967). High levels of NO were needed to get this response in experiments with animals. For example, exposure of lambs to 80ppm of NO for three hours in 21% oxygen did not result methaemoglobinaemia or any of the acute problems previously documented (Frostell, Fratacci et al. 1991). Exposure of dogs to 20,000ppm did result in this acute respiratory event with high morbidity and mortality (Shiel 1967). Inhalation of 50ppm of NO impairs the performance of learned tasks and prolongs brainstem evoked potential responses in rats, without significantly elevating methaemoglobin levels (Groll-Knapp, Haider et al. 1988). In healthy subjects inhaling 100ppm of NO for three hours, methaemoglobin levels increased by 1.77% of total haemoglobin to a peak at 45 minutes. Serum nitrogen oxides (NO₂, NO₃) increased from 36.7 to 124umol L⁻¹ at 172 minutes and then declined (Young, Sear et al. 1996). Inhaled NO at 5ppm resulted in thickened alveolar membranes in rabbits (Hugod 1979) and NO at 43ppm and NO₂ at 3.6ppm narrowed the surfactant hysteresis, altered epithelium ultra-structure and caused interstitial atrophy (Hugod 1979). Intermittent NO exposure over a 9 week period in rats resulted in degeneration of interstitial cells and the interstitial matrix leading to an emphysematous-like destruction of alveolar septa (Mercer, Costa et al. 1995).

NO₂ is more toxic and has long been known to cause respiratory damage and fatality (Kooiker, Schuman et al. 1963; Wagner, 1965 #1030). It was early recognised as a concern in atmospheric pollution (Kennebeck, Wetherington et al. 1963). NO₂ has been shown to be taken up easily by lung lining fluid at a rate related to its speed of reaction to glutathione
This is increased with alkalosis and hyperthermia (Postlethwait, Langford et al. 1991; Postlethwait and Bidani 1994). A number of studies have been conducted to determine the acute effects of NO\textsubscript{2} exposure at levels seen in air pollution on airway lining (usually the nose), lavage fluid, lung function and airway reactivity in normal and asthmatic adult subjects. The exposures have ranged from two to six hours at levels from 200 to 800ppb, and were to NO\textsubscript{2} alone or combined with either ozone or SO\textsubscript{2} (Devalia, Rusznak et al. 1996; Devalia, Bayram et al. 1997; Jenkins, Devalia et al. 1999). These exposures resulted in disruption of the airway epithelia, resulting in an increase of inflammatory cells with eosinophil 'priming', and a release of inflammatory cytokines such as TNF\alpha and IL8. Increased airway hyper-reactivity in normal (Utell, Frampton et al. 1991) as well as asthmatic subjects was also demonstrated (Devalia, Rusznak et al. 1996; Devalia, Rusznak et al. 1996; Jenkins, Devalia et al. 1999). Cilia were also disrupted and, after exposure to NO\textsubscript{2} at 2ppm for 4 hours, ultra-structurally altered cilia with excess matrix and multiple ciliary axenomes were seen (Carson, Collier et al. 1993). Intermittent NO\textsubscript{2} exposure over a period of weeks in rats also resulted in emphysematous like destruction of alveolar septa with reduction in the ventilatory surface (Evans, Stephens et al. 1972; Freeman, Crane et al. 1972; Mercer, Costa et al. 1995). There was also hyperplasia of the respiratory epithelium with squamous metaplasia (Maejima, Suzuki et al. 1992), disturbed surfactant (Muller, Barth et al. 1992) and aldehyde release (Robison, Forman et al. 1995). Prolonged inhalation of 2ppm NO\textsubscript{2} is associated with terminal bronchial epithelial hypertrophy and alveolar cell hyperplasia (Sherwin, Dibble et al. 1972). Gas mixtures of NO\textsubscript{2} and NO, each in concentrations of less than 1ppm, have been shown to reduce peak expiratory flows and diffusion capacity in beagles (Bloch, Lewis et al. 1972). In studies using lower concentrations (similar to pollution values) the damage in the airway was related to duration of exposure (Maejima, Suzuki et al. 1992), but when exposed to high doses the dose was more damaging than the duration of exposure (Lehnert, Archuleta et al. 1994). NO\textsubscript{2} of 175ppm has been used in rats to create severe lung damage but avoiding death to develop an ARDS model for study (Meulenbelt, Dormans et al. 1992). NO\textsubscript{2} has been recognised as the product of grain fermentation responsible for the syndrome of pulmonary oedema and haemorrhagic bronchiolitis obliterans known as silo fillers disease (Ramirez and Dowell 1971; Horvath, doPico et al. 1978; Leavey, Dubin et al. 2004).

The Occupational Safety and Health Administration guidelines (NIOSH 2005) recommend that the safety limit of mixed nitrogen oxides is below 25ppm. Inhalation at this concentration may cause immediate pulmonary irritation and higher doses may cause haemorrhagic
pulmonary oedema in days. The guideline states that inhaling NO at a dose of 25ppm for 
eight hours is the maximum safe dose and time limit. The level considered an immediate 
damage to life and health is estimated at 100 to 150ppm based on older toxicology studies 
(Carson, Rosenholtz et al. 1962; Sax 1975). For NO₂ the recommendation has been altered 
down from 5ppm to only 1ppm (in 1996) for eight hours as the exposure limit (NIOSH 2005). 
Levels of 10 to 20ppm have demonstrated to be a mild irritant (Patty 1963), levels of 100ppm 
are dangerous causing haemorrhagic pulmonary oedema (NRC 1985), and 150 to 200ppm 
fatal from haemorrhagic pulmonary oedema (Braker and Mossman 1975).

Ozone is a colourless to blue gas with a pungent odour. Again the 'usual' exposure to ozone is 
through airway pollution as mentioned in Chapter 2.2, (Anonymous 1995; Anonymous 1996) 
where it is one of the most toxic elements reaching one hour mean ambient concentrations of 
200-400ug/m³ (AIHA 1966; Lippmann 1989; van Bree and Last 1997). Short term ozone 
exposure causes reduction in lung function, increased airway hyperactivity, increased airway 
inflammation, increased respiratory symptoms and hospital admissions (van Bree, Marra et al. 
1995; Nikasinovic, Momsas et al. 2003). Exposure to long term elevated ozone levels is again 
associated with reduced lung function, increased respiratory symptoms, exacerbations of 
asthma and airway cell and tissue changes (van Bree, Marra et al. 1995; Anderson, Ponce de 
Leon et al. 1998). At the tissue level, ozone with its high reactivity is usually consumed as it 
passes through the first layer of tissue it contacts at the airway/air interface or lung/air 
interface (Pryor 1992; Pryor, Squadrito et al. 1995). Since the lung lining fluid is 0.1 to 20 
microns thick, in the thinnest parts it can react directly with cells and it has been shown, for 
example, to act with the type II macrophages disrupting surfactant activity. The majority of 
the interface area has thicker lining fluid and ozone is transformed into other reactive species 
such as aldehyde and hydrogen peroxide, and these go on to cause the airway damage. This 
causes epithelial disruption and increased permeability with an increase in inflammatory cells. 
The disruption of cell membranes also results in a release of cytokine, cyclo-oxygenase and 
lipoxygenase products. In studies looking at toxicity, differences have been demonstrated 
between species with regards to dose and length of exposure that results in damage (Dormans, 
vан Bree et al. 1999). Inhaled ozone is also used in animals to give a lung fibrosis model for 
study (Cross, Hesterberg et al. 1981; Yu, Song et al. 2001). In direct comparison of toxicity, 
ozone was found to cause four times as much damage to the airways as NO₂ (Chang, Mercer 
et al. 1988) and be ten times more toxic to macrophages (Rietjens, Poelen et al. 1986).

The Occupational Safety and Health administration guidelines recommended exposure limit 
for ozone is 0.1ppm (0.2mg/m³) (NIOSH 2005). The immediate danger to life and health level
is set at 5ppm (AIHA 1966) based on pulmonary oedema that developed in welders who had a severe acute exposure to an estimated 9ppm of ozone (plus other pollutants) (Giel, Kleinfeld et al. 1957). Fifteen to 20ppm is lethal to animals in two hours, exposure to 50ppm for one hour "would likely prove fatal to humans" (King 1963).

4.7 Chapter summary

This chapter has reviewed the possible compounds and methods that could be used to determine the level of NO based around the known pathway of formation from L-arginine to L-citrulline and NO, and the nitrogen compounds. The chemiluminescence technique was the most appropriate for use in measuring exhaled NO in humans. This analysis is based on the reaction of NO with ozone which produces light, and the light intensity has been shown to be directly proportional to the concentration of NO in the sample. The chemiluminescence technique appeared to be both appropriately sensitive and specific for NO for this purpose. The chemiluminescent NO analysers available at that time had been developed to measure NO in air pollution. The job now was to assess their use and examine what technical alterations were required to enable measurement of NO in exhaled air in humans in a reliable and reproducible way. This was needed before discussion as to what these levels meant, and before a trial of measurement in children. Initial testing showed that the response time of the analyser was too slow for our purpose initially, but a rerouting of the signal to a chart recorder decreased the response time and meant that it was practical for use for the studies that follow.

Finally in this chapter, I have reviewed the toxicity of NO, NO₂ and ozone. Although I was only going to be measuring NO in exhalate and therefore it was unlikely to reach high levels, I was using NO calibration gases. NO₂ and ozone are the two gases created by this technique so I wanted to also assess their toxicity, although I was planning to remove them immediately by having them 'scrubbed' with a soda lime column and a charcoal column respectively.

The next chapter examines the chemiluminescent analyser – Model 2107 (Dasibi Environmental Corporation, Glendale, California, USA) – in more detail, and the commencement of the machine and subject methodology studies. What was known about exhaled NO levels at the time of commencement of these studies in 1995 will also be reviewed here.
NB: The review presented in this chapter and the start of the machine methodology presented at the beginning of the next chapter formed the basis of the publication:

Chapter 5: Methodological assessment of the chemiluminescence analyser

5.1 Introduction

The previous chapter reviewed the methods available to measure NO in biological systems and determined that the most appropriate method for measuring NO in exhaled air using a chemiluminescence analyser, originally developed to assess NO pollution levels. The technical aspects of the chemiluminescence analysers were reviewed and the direct correlation between the intensity of light measured and the concentration of NO described. The instrument that I ultimately used was the Dasibi Environmental Corporation 'Model 2107'. In this chapter the characteristics of this analyser will be reviewed, as well as the other equipment. Connections between a number of analysers were developed to enable simultaneous measurement of NO, CO₂, flow and pressure. I will also mention the other people involved in the work and the role each person undertook.

5.2 The equipment and personnel

5.2.1 Chemiluminescence Analyser 'Model 2107'

The chemiluminescence analyser Model 2107 (Dasibi Environmental Corporation, Glendale, California, USA; local supplier Quantitech Ltd, Unit 3 Wolverton Rd, Old Wolverton, Milton Keynes, UK) was designed for measuring NO concentrations within a 2-4000ppb range in a continuous ambient air sample and operated in ambient temperatures of 5-40°C and humidity of 0-95%. It had been adapted for online recording with a sampling rate at 240 mls/min and had a highly stabilised measurement capability (drift without auto zero over 24 hours of 1ppb). The original response time of this machine was long at 90 seconds, too long for the purpose for which it was now required. Modification of the circuitry and the sending of the pre-computer analogue signal directly to the chart recorder decreased the response time to 6.4 seconds (see below). The estimates of NO concentrations were decreased by 10-15% at 100% humidity; however NO pre-drying of the expire was not thought to be necessary (this may have been considered differently if the experiments were conducted in areas of high humidity such as within New Zealand). I did use a water absorber when doing the reservoir set of experiments only (see Section 6.4). This machine operated on the same principles as described in the last chapter. The gaseous specimen was drawn by the vacuum pump into a reaction chamber where the ozone was generated internally by an electrical discharger. The NO and O₃ were mixed in front of a red-sensitive photomultiplier tube, the light emission of this reaction was measured at 660 to 900nm in the red sensitive range, and the signal
amplified and detected as a current. As mentioned in the previous chapter, the dark current of
the photomultiplier can be affected by changes in temperature and this model had an inbuilt
cooler that kept the photomultiplier temperature at -20°C to improve the signal/noise ratio.
The machine itself should ideally also be kept in a temperature controlled room (as was
suggested in the machine manual). In our case it was placed in a small air conditioned room
within the hospital away from any other equipment that potentially generated heat – though
there was one window into the room which resulted in some temperature change. The NO
analyser was re-serviced just prior to our experiments taking place.

The NO analyser was calibrated with a zero and two known NO gas concentrations before and
after every subject during the methodological studies and between every two subjects when
later studies in children were being undertaken. Two of the gases used were commercial
preparations of NO made for calibration purposes by BOC Gases (BOC Gases, 10 Priestley
Rd, Surrey Research Park, Guildford, Surrey, UK) and as the studies progressed included
concentrations of 37ppb, 55ppb 103ppb, 110ppb and 118ppb with the balance being nitrogen
(N₂). Calibration gases at these low levels were extremely difficult to get at the
commencement of these studies as these gas mixtures had no known use other than
calibration, and this was not widely used at this time. The third gas was a zero calibration and
this was prepared by passing room air through the ozone generator of the chemiluminescence
analyser, which converted all NOₓ to NO₂. This was then passed through a soda lime and
activated charcoal column to remove the NO₂ and O₃ respectively and then passed direct into
the analyser. The resulting NO₂ and O₃ from the experiment were removed in the same
manner by passing through the soda lime column and the charcoal column. To minimise NO
loss by absorption or interaction with other surfaces (see Section 5.4), teflon tubing (4mm
external diameter) was used as the most inert substance to connect the machinery and to
sample from the subject.

5.2.2 Capnograph, pressure transducer, flow meter and chart recorder

In addition to measuring NO itself, we wanted to be able to measure other parameters. At the
time, the early publications available on exhaled NO from the lung, which will be reviewed in
the next chapter (see Section 6.3), seemed to show a consistency in the trends found in NO
results in different subject groups but the absolute levels of NO reported between the
investigators were very different. This suggested that some of the measurement techniques
were altering the NO results recorded. There was also discussion in the literature about where
the measured NO from exhalation was anatomically being generated. In view of this, we
wanted to compare the patterns of exhaled NO with CO₂, the latter being largely of alveolar origin. For this purpose, I elected to measure expiratory CO₂, flow and pressure in addition to the expiratory NO levels which meant developing a number of monitors connected in parallel that could operate simultaneously. The equipment used, as described below, was all equipment that was able to be 'seconded' from the adult lung function laboratory and put together by myself and one of the biomedical engineers (Ms Carolyn Busst, see below) and the ultimate format is demonstrated in Figure 5.1.

Figure 5.1: Schematic diagram of how the analysers were placed

This is a schematic diagram of the analysers and connections required to measure nitric oxide, carbon dioxide, mouth pressure and flow. The signals from each of the 4 machines went directly to, and were displayed on, a Linseis 4 channel pen chart recorder (Linseis Limited, Cambridge, United Kingdom) with each signal denoted by a different colour.

The CO₂ levels were measured with a Morgan Capnograph (PK Morgan, Kent, United Kingdom) which sampled at 200 mls/min⁻¹. Calibration of CO₂ was done using a zero and gas cylinder of known CO₂ concentration at 5.9% and later 5.7% also provided by BOC gases (address given above). The mouth pressure was measured using a Medex Straingauge pressure transducer (Medex Medical Incorporated, Rossendale, United Kingdom) which was connected at right angles to the flow via a connector in the mouthpiece. This was then directed through a Galtec pressure amplifier (Galtec Limited, Isle of Skye, United Kingdom) which measured the pressure in mmHg and showed a linear and stable response. The flow was measured via a flow meter to a low flow pneumotachograph (CT Platon Limited, Jays
Close, Viables, Basingstoke, United Kingdom) and a GM electrospirometer (GM Instruments Limited, Kilwinning, United Kingdom) then gave an electrical output that could be sent to the chart recorder. The pneumotachograph was initially calibrated by passing flow from an air cylinder set at known flow rates (100, 250, 500, 750 and 1000mls/min) and was linear over this range but then seemed to read slightly but consistently high after 1100mls/min. In initial self trials (myself and Carolyn Busst) the response from human exhalation seemed unsteady or 'noisy'. This appeared to be the effect of the small but repeated voluntary alterations in expiratory flow by the subject (one of us) trying to maintain a flow without adequate feedback. It disappeared when the steady flow of compressed air was put through the rotameter and the pneumotachograph at designated flow rates. In view of this, we then added the rotameter as part of the routine exhalation. Subsequently, all the subjects were directed to watch the rotameter during exhalation and aim to maintain a set flow rate by keeping a 'bobbin' at a set number on the upright scale. This improved the steady quality of the signal. The rotameter was a Platon glass tube C6 with stainless steel float 6D (Flowmeter Model GTV – CT Platon Ltd, address above).

Figure 5.2: An example of tracing from the testing.

The first thing to note is that the chart record is read from right to left which is how it was generated from the chart recorder. Note the offset of the traces from the different analysers which were displayed.
in different colours that became the standard: NO in green, CO₂ in red, and mouth pressure in blue. As this is an example from the direct method of analysis, flow is not denoted. The measurements of each parameter were calculated from the calibration markings (see Chapter 6).

As can be seen in Figure 5.2, the NO rose to a plateau which often had additional small deviations, the CO₂ continued to rise to a peak throughout the exhalation, the mouth pressure (and flow with the t-piece measurements) were under voluntary control and therefore less exact plateaux markings were seen and the lines of measurement were drawn through the middle to determine that they had been appropriately close to requested levels. Note the offset of the traces from the different analysers which were displayed in different colours that became the standard: NO in green, CO₂ in red, and mouth pressure in blue. As this is an example from the direct method of analysis, flow is not denoted but when measured, as during the t-piece sampling technique, was displayed as a brown trace. The measurements of each parameter were calculated from the calibration markings.

We (Carolyn Busst and myself) checked the reported sampling rates of the NO and CO₂ analysers. As mentioned, the sampling rate was determined by the vacuum pump within the NO analyser and had a fixed rate. Similarly, the sampling rate of the CO₂ analyser was also determined by the vacuum pump which could apparently be adjusted by pressing the calibration button on the front of the analyser panel and the rate changed by pressing the up and down arrow keys. We left the sampling rate at the setting it was on when we received it from the Adult Physiology Laboratory (Royal Brompton Hospital). This was in the middle of the range suggested by the manufacturers and was used for all assessments. The check of the machines' sampling was repeated a number of times prior to development of the protocol for the experiments and again between the methodological experiments conducted with adult subjects and commencement of the studies on paediatric subjects. We listed what was to become our standard procedure as the following steps:

- Connect the outlet line from the mouthpiece t-piece connection to the lower port of the rotameter.
- Turn the rotameter needle valve off to ensure that the room air cannot be sampled via the rotameter.
- Disconnect the pneumotachograph from the rotameter.
- Run the paper to record zero sampling.
- Connect the outlet side of the pneumotachograph to the inlet of the mouth piece.
• Ensure there is no leak in the connections and room air will now be drawn through the pneumotachograph by the NO analyser and capnograph thus measuring their sampling rates.
• Run the chart recorder to mark the level of the combined sampling rates.
• Label the chart record.
• Reconnect the pneumotachograph to the upper port of the rotameter.

5.2.3 Personnel

Adjustments to the original NO analyser circuitry and the sending of the pre-computer analogue signal directly to the chart recorder were made with the assistance of the Biomedical Engineering Department, Royal Brompton Hospital (Mr Ron Logan-Sinclair [later of Logan Research Ltd, Rochester, Kent, UK] and Ms Carolyn Busst). The connections and adaptation of the equipment to do all the measurements simultaneously and the experiments on assessing machine capability were done by myself and Ms Carolyn Busst. I designed and conducted the methodological experiments on the healthy adult subjects. I also designed the studies involving normal and asthmatic children as subjects. Following ethical approval from the Royal Brompton Hospital Ethics Committee, Dr Seinka Dinarevic and I gave presentations to the local primary school and, with individual signed parental or caregiver consent, we enrolled children the school. I also enrolled all the asthmatic subjects from the weekly paediatric asthma and/or the weekly paediatric general respiratory clinic at the Royal Brompton Hospital in which I was working. Dr Seinka Dinarevic helped transporting the children to and from the local school, and we collected the consent and the questionnaire data, did the clinical examination, measured height and weight, performed the lung function and exhaled NO testing on all the children. The manual calculation of the levels for each parameter from the chart recorder using the calibration measurements was completed by us both. I entered the data into a statistical analysis programme (Statistical Products and Services Solutions, SPSS Inc, Chicago, USA – package U 7.0), while Dr Seinka Dinarevic double checked the entries. We shared the drafting of the paediatric paper, while I wrote the other papers in conjunction with contributory editing and suggestions from the other named authors on each. I remained the key driver and contact for all this work. The same equipment was used for all the following experiments and the work was carried out in the Paediatric Department at the Royal Brompton Hospital, South Kensington, London, England. The main supervisor for this work was Professor Andrew Bush. In addition, Professor Peter Barnes was

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2 Now the ‘Royal Brompton & Harefield National Health Trust, South Kensington, London NW3 6NP, U.K.
an advisor for this work and main supervisor for other research done at the time (publications listed as Appendix 1), and Dr Elliot Shinebourne also acted as advisor, particularly in the early stages.

5.3 Direct versus reservoir measurement

The concept of the proposed methodological experiments was to have a subject exhale into teflon tubing connected to NO, CO₂, flow and mouth pressure meters allowing measurement of each. It was established that all the equipment could come directly off the mouth piece, with the exception of flow connector. This was due to the necessity of the addition of a rotameter to provide a visual feedback guide for the subjects regarding expiratory flow and added a level of complexity to the system. In view of this, I elected to do a series of experiments in which one set of exhalations was connected directly to the NO analyser, pressure and CO₂ monitors (known as the ‘direct method’) and a second set of exhalations where a t-piece system enabled the additional measurement of flow (known as the ‘t-piece method’ or ‘t-piece sampling system’). A comparison of the two techniques is shown diagrammatically in the next chapter in Figure 6.5. Below is a photograph of one of the children performing the procedure with the analysers labelled in Figure 5.3. Unfortunately I did not take any photographs of the subjects during the methodological experiments.

Figure 5.3: Photographs of one of the children performing the exhalation
The photographs are taken from behind (a) and from the right side (b) of one of the paediatric subjects ‘Jonathon’ that was testing the procedure (used with permission) as he performs a single exhalation into the mouthpiece. I have labelled the connections and analysers as seen below (not all visible in both photographs):

1. Mouth piece.
2. Connector.
3. NO analyser (Model 2107, Dasibi Corporation).
4. CO₂ analyser (Morgan capnograph).
5. Low flow pneumotachograph (CT Platon Limited).
6. Mouth pressure analyser (Medex Straingauge pressure transducer).
7. Galtec pressure amplifier (Galtec Limited).
8. GM electrospirometer (GM Instruments Limited).
9. Four channel pen chart recorder (Linseis Limited).
10. Timing device.
11. NO zero calibration column.
13. The first of the pages listing instructions on the wall.

5.4 Assessments of the analysers

The response time of each analyser had to be determined to enable direct comparisons. The chart recorder recorded continuously at a paper speed of 50mm/second and was set so that as the recording came off, the signals were read from right to left. An oscilloscope was connected to the system with a three way tap which on turning allowed the test gas to flow down at the apparatus at a rate comparable to the subjects’ expiratory flow rate (500mls/min was chosen) starting at the subject mouthpiece. The output of the analysers was displayed on the same oscilloscope. The measurement of the delay time and the response time was then measured with a stop watch.
Delay time: 

the time between turning on the switch and the onset of the analyser signal

Response time: 

the rise time that from onset of signal to 95% of plateau or a square wave

The experiment was repeated 10 times in all with times recorded for each of the analysers. Results are presented in the Table 5.1.

Table 5.1: The delay and response time of the NO, CO₂, mouth pressure and flow meter analysers used

<table>
<thead>
<tr>
<th>Analyser</th>
<th>Delay time</th>
<th>Response time</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO analyser Model 2107</td>
<td>5.9 seconds (SEM 0.8 s)</td>
<td>Seconds (SEM 0.1 s)</td>
</tr>
<tr>
<td>CO₂ Morgan capnograph</td>
<td>0.11 seconds (SEM 0.003 s)</td>
<td>41 milliseconds (SEM 2.9 ms)</td>
</tr>
<tr>
<td>Medex Staingauge pressure transducer (mouth pressure)</td>
<td>7.3 milliseconds (SEM 0.2 ms)</td>
<td>No appreciable delay</td>
</tr>
<tr>
<td>Platon flow meter</td>
<td>40.2 milliseconds (SEM 0.8 ms)</td>
<td>No appreciable delay</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean, s = seconds, ms = milliseconds

This established that the delay time for the NO analyzer at 5.9 seconds (SEM 0.8s), and 95% response time at 0.5 seconds (SEM 0.1s) showed the NO analyzer responded much more slowly than the CO₂ analyser at a delay time of 0.11 seconds (SEM 0.003s) and 95% response time at 0.41 milliseconds (SEM 2.9ms). It has to be remembered that the delay time was influenced only by the time that it took for the gases, flow or pressure to get to the analysers and the time to commence registering the change. The response time should be a square wave for ‘on/off’ phenomena like the pressure and the flow, but could be influenced by other parameters in the measurement of the gases. The pattern of excretion through the exhaled breath was unknown for NO, and was known to increase with the duration of exhalation for CO₂. So while the response time was measured with these two gases, it was the delay time that was the key factor. In essence it meant that when comparing the NO and CO₂ recordings, an allowance needed to be made for the difference in time delay of the measurements from each machine and for the 2mm offset of the pens on the recorder.

Finally, confirmation of the detection of the appropriate gases was checked for the two analysers. Cylinders of 100% O₂, 5.9% CO₂ and 37ppb of NO were run through the NO analyser and the CO₂ analyser with only the NO gas detected and the others giving a zero reading on the NO analyser machine and only the CO₂ gas detected with the others giving a zero reading on the CO₂ analyser machine.

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When planning the protocol, it was obvious that the response time of the NO analyser limited the possible respiratory manoeuvres. I next considered online measurement of repeated single exhalations analogous to lung function testing versus exhaling into a reservoir that was then fed into the analysers. I briefly explored the use of a reservoir, as this had three theoretical advantages:

- it would allow the measurement of a whole exhaled breath, whereas the online measurement which would control flow may mean that at low flows the subject would not reach complete exhalation before requiring to take another breath,
- it would allow me as operator to put the collected sample through the machine at a constant flow instead of requiring the individual subject’s voluntary but imperfect control,
- performing tidal breathing over a set time or exhaling into a bag may have been easier when it came time to measure the children rather than relying on their being able to do controlled breaths into the analysers.

At the time that I was commencing the experiments, there had been some studies into NO levels in exhaled air where a reservoir system had been used. The problems that had been detected and needed to be overcome involved NO instability – a topic that has been touched on repeatedly. Firstly, although in O₂-free and haemoglobin-free solutions NO may be stable, in the presence of O₂, NO levels rapidly decrease by 50% in 8-20 minutes. Secondly, NO is adsorbed by most plastics giving low and/or variable readings. NO also interacts with transition metals and with the sulfhydryl groups contained in rubber. Reservoir systems that had been used were polyvinyl chloride, rubber and fluoroethylene propylene. Preliminary studies in one group demonstrated that at levels of 5-30ppb there was no NO loss in the polyethylene reservoir at 6 and 12 hours post collection, although they commented that any loss may have been within the noise of the measurement that they were getting at that time of 10% (Schilling, Holzer et al. 1994). Another group had found that using polyethylene tubing compared to Teflon tubing resulted in loss of NO presumably as it adsorbed to the former, particularly at higher (>40ppb) levels of NO (Kharitonov, Logan-Sinclair et al. 1994). One group with a Douglas bag collection (an anaesthetic bag used to collect exhaled air, usually made of rubber) described no perceivable problems, but two other groups found them unreliable at higher NO concentrations (Kharitonov, Logan-Sinclair et al. 1994). Subsequently Mylar foil bags or balloons were used and seem to hold NO levels stable for at least 48 hours (Steerenberg, Nierkens et al. 2000). However, this research group also discarded the first part of exhalation to get lower respiratory tract samples and found that it

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led to an increased water content that decreased measured NO levels, though this could be minimised by exhaling through a water absorber into the reservoir bags (Steerenberg, Nierkens et al. 2000). NO online measurement correlated well with NO measured via a balloon technique (mylar foil) when measured immediately during times of low air pollution and high pollution days (van Amsterdam, Verlaan et al. 1999). Another group disagreed and showed that the online measurement was higher than the mylar bag reservoir results when both were measured immediately (Silkoff, Stevens et al. 1999), though the offline measurements were similar to the end expiratory online measurements (Jobsis, Schellekens et al. 2001). NO concentration in the mylar bag was also shown to actually increase at 24 and 48 hours after collection (Silkoff, Stevens et al. 1999). When the first European Respiratory Society Task Force (ERSTF) recommendations were published regarding the measurement of exhaled air, they recommended the online technique of measurement (Kharitonov, Alving et al. 1997). In some initial testing we did, teflon and “wine cask bags” (which are mylar foil bags) did not seem to absorb NO, but a Douglas bag did seem to absorb NO and the material of the bag became sticky. The use of teflon or teflon coated tubing to connect the reservoir or the subject to the analysers appeared to minimise NO loss.

In view of the purpose of the studies, the online technique seemed more appropriate as it allowed comparison of signals throughout the exhalation and allowed an alteration of the test conditions to investigate how this affected NO results. A slow controlled exhalation from total lung capacity appeared to be the most satisfactory exhalation for observing the results. This manoeuvre yielded obvious NO and CO₂ traces and most people, including most children in the age group studied, could be easily trained to perform this correctly. Thus I could discard the reservoir system for most of the studies which I believe excluded another uncertain element from test measures.

5.5 Other measurements

For all the experiments utilising the healthy adult volunteers and normal and asthmatic children, a respiratory examination was carried out, and weight (minimal clothing, no shoes) and height were recorded on a digital Seca scale (Seca 770 medical scales, Seca Ltd, 4802 Glenwood Rd, Brooklyn NY11234, USA) and a Harpenden stadiometer (Harpenden Portable Stadiometer, Crosswell, Crymych, Pembrokeshire, SA41 3UF, UK) respectively. Lung function was measured according to the American Thoracic Society (ATS) criteria (American Thoracic Society and Association. 1994) on a compact vitalograph (Vitalograph Ltd, Maids
Moreton House, Buckingham, United Kingdom) using the Polgar predictive equation (Polgar and Promadhat 1971) from a standing position for all subjects.

5.6 Chapter summary

This chapter has described the equipment, the sequence of analysers and the capabilities of each that was used for testing in the studies presented in the following chapters (Chapters 6, 7 and 8). Chapter 6 will begin with a brief review of what was known about NO in the lung, the early published articles in the measurement of exhaled NO and describe the first of five methodological experiments carried out in healthy adult volunteers.

NB: The review and data presented in Chapters 4 and 5 formed the basis of the publication:

Chapter 6: Methodological studies of exhaled nitric oxide in healthy adult subjects: direct versus t-piece testing

6.1 Introduction

So far, I have examined why a marker of airway inflammation that was easy to measure, particularly in terms of being non-invasive, would be useful. I have outlined the developing knowledge on NO as a physiological agent and inflammatory marker. The previous chapters then explored how NO could be measured by chemiluminescence and presented data on adapting an appropriate analyser with our own initial methodology testing. In this chapter, I will very briefly review NO in the lung, the literature on exhaled NO available to me at the beginning of my research, and present the methodology experiments themselves. The first experiment, which will be presented in detail in this chapter, was to assess the ability to measure NO from exhalation in adults and to compare NO, CO₂, mouth pressure and flow traces using two methods; direct to analyser and side arm sampling systems. The following chapter will detail further experiments designed to examine a number of technical factors to assess whether they alter the levels of exhaled NO obtained. These investigations were in response to the newly presented and published literature in this area where, although the conclusions were similar from different research groups regarding NO results in different populations, it remained curious that the absolute levels of NO being documented were very different. How the results from these experiments then fit into subsequent research, current literature and new discoveries are detailed in Chapter 9.

6.2 Nitric oxide and the nitric oxide synthases in the lung

From the literature presented in Chapter 2.2 ‘Nitric oxide in biological systems’, and in Chapter 3.4 ‘Nitric oxide synthase isoenzymes’, it was clear that NO and the NOS enzymes were widespread throughout human organ systems. In the lung, NO has four major roles – vasodilator, bronchodilator, neurotransmitter for the NANC nerves and as an immune and inflammatory mediator. By the time of this research all three isoforms of the enzyme had been detected in the human respiratory tract (Barnes and Belvisi 1993; Jorens, Vermeire et al. 1993; Marsden, Heng et al. 1993; Moncada and Higgs 1993; Singh and Evans 1997). Localisation of NOS in lung tissue was demonstrated by immunohistochemical labelling using both polyclonal and monoclonal NOS antibodies (Bredt, Hwang et al. 1991; Schmidt, Gagne et al. 1992; Hamid, Springall et al. 1993; Hattori, Kosuga et al. 1993; Kobzik, Bredt et al. 1993; Forstermann, Closs et al. 1994; Rengasamy, Xue et al. 1994; Kawai, Bloch et al. 1995; Ambalavanan, Mariani et al. 1999). The detection of NOS within the respiratory tree
initially appeared somewhat site-specific. Early studies showed NOS presence in bronchial epithelial cells with little in the respiratory terminal bronchi, smooth muscle cells or alveolar sacs. However, this changed following stimulation of the iNOS enzyme, when there was some mild uniform labelling of epithelium in large cartilaginous airways with iNOS in normal trachea and main bronchi, but with much greater labelling in the more peripheral bronchi. This was first demonstrated when comparing normal and inflamed rat lung samples that had been stimulated with intra-tracheal lipopolysaccharide, and in human tissue from four airway and ten parenchymal specimens obtained from uninvolved areas of surgical tumour resections stimulated with TNFα. While the presence of the constitutive forms along the bronchi before the infective stimulation seemed appropriate given the eNOS and nNOS activity in physiological regulation, it was uncertain why iNOS should be seen there. The researchers suggested that it may be in response to continual low grade induction of iNOS activity in response to airborne toxins (Hamid, Springall et al. 1993; Kobzik, Bredt et al. 1993).

Turning from the presence of the enzymes to NO itself: when administered exogenously, NO gas was shown to relax tracheal and airway smooth muscle in vitro taken from different sources such as bovine trachea, guinea pig trachea and human airway smooth muscle (Jansen, Drazen et al. 1992). The gas also had the capacity to relax tissues that had previously been constricted with methacholine, histamine or leuktriene D₄ (Jansen, Drazen et al. 1992; Gaston, Drazen et al. 1994). NOS inhibition resulted in exaggerated bronchosconstrictor responses in animal models (Ricciardolo, Mistretta et al. 1996; Nogami, Umeno et al. 1998; Boer, Duyvendak et al. 1999) and human specimens (Ricciardolo, Di Maria et al. 1997; Taylor, McGrath et al. 1998). These responses suggested that NO had a role in modulating basal airway tone both directly and via the NANC nerves and, as discussed previously in Chapter 2, is now thought to be one of the main mediators for regional airflow and blood flow matching. The reaction products of NO such as s-nitrosoglutathione and s-nitrosalbumin also had bronchodilator effects (Jansen, Drazen et al. 1992; Asano, Chee et al. 1994; Gaston, Drazen et al. 1994; Gaston, Drazen et al. 1994; Bannenberg, Xue et al. 1995) with lower levels noted in asthmatics (Gaston, Sears et al. 1998). In the lung, low concentrations of NO caused airway smooth relaxation during bronchospasm (Buga, Gold et al. 1989; Jansen, Drazen et al. 1992; Gaston, Drazen et al. 1994). As seen systemically, the NO production from the eNOS isoenzyme was found to maintain vasomotor tone (Stamler, Osborne et al. 1993). Pulmonary arterial endothelial cells released NO in response to acetylcholine and bradykinin agonists which relaxed pulmonary arterial, venous and lymphatic smooth muscle (Crawley, Liu et al. 1990; Dinh-Xuan, Higenbottam et al. 1991). Release of NO from endothelial cells in the
pulmonary circulation appeared to counteract hypoxic vasoconstriction responses to catacholamines and prostaglandin F2α, with NO release decreased in chronic hypoxia (Crawley, Liu et al. 1990). In vivo the NANC nerves were shown to cause vasodilatation and relaxation of animal and human tracheal muscle using NO as the mediator and these were not inhibited by NOS inhibitors (Stuart-Smith, Bynoe et al. 1994; Baba, Yoshida et al. 1998; Sipahi, Ercan et al. 1998). However, human airway specimens suggested this NO mediator relaxation via the NANC nerves was more important in the response of distal rather than proximal airways (Ellis and Undem 1992). In addition, the NO-dependent NANC dilatation of human bronchi was reduced in transplanted tissue and in specimens from patients with CF (Belvisi, Barnes et al. 1995; Belvisi, Ward et al. 1995). Nitrosothiols of low molecular weight, such as S-nitrosoglutathione, and NO₂ were also detected in alveolar fluid from normal individuals at concentrations sufficient to suggest regulation of basal airway tone (Barnes 1993; Gaston, Reilly et al. 1993).

The NOS enzymes were considered to have a role in mucociliary clearance, and even now this remains uncertain as the precise regulation of ciliary beat frequency is still unclear. Similar to NOS activity, ciliary beat frequency is increased by a rise in intracellular calcium with possible intracellular mechanisms involving cAMP, calmodulin, and inositol 1,4,5-triphosphate. Increases of NO have been detected when ciliary beat increases, although no consistent correlation has been shown between NO levels and cilia activity (Wanner, Salathe et al. 1996; Dirksen 1998; Yeates 1998; Uzlaner and Priel 1999). Ciliated cells have been shown to have increased NO production and increased beat frequency after treatment with L-arginine (Li, Shirakami et al. 2000) and L- NMMA caused a 40% decrease in ciliary beat frequency lasting 60 minutes which was reversed with L-arginine administration (Jain, Rubinstein et al. 1993).

Finally NOS activation in macrophages, neutrophils and mast cells exerts bactericidal and fungicidal effects primarily through the oxygen superoxide formation of the NO products such as peroxynitrite as covered in Chapters 2.3.4 and 3.4.2. These are important in host defence but in excess can have similarly detrimental effects on the normal cells. Higher concentrations of NO in combination with oxidant generation leads to inflammation and oedema (Beckman, Beckman et al. 1990; Mulligan, Hevel et al. 1991; Mulligan, Warren et al. 1992) and these potent oxidants deplete glutathione, uric acid and ascorbate in lung lining fluid (Williams, Rhoades et al. 1971; Kelly, Shah et al. 1997). Exposure of alveolar type II cells particularly to peroxynitrite leads to profound inhibition of surfactant synthesis (Gaston,
Drazen et al. 1994), and the reactions can form potentially carcinogenic nitrosamines such as N$_2$O$_3$ and N$_2$O$_4$ (Miwa, Stuehr et al. 1987).

6.3 The need for methodological experiments

In a key development, NO was then detected in exhaled air (Gustafsson, Leone et al. 1991; Archer 1993; Leone, Gustafsson et al. 1994). In 1991 Gustafsson and Leone first showed that NO could be detected in the exhaled air of tracheostomized animals and normal humans (Gustafsson, Leone et al. 1991). Exhaled NO was then measured in single and tidal breathing in eight adult subjects giving levels between 8.3 to 20.3 ppb (Borland, Cox et al. 1993). Exhaled NO was measured at a mean peak of 3.25 ppb in ten subjects with normal breathing, increasing to 100.25 ppb with a sixty second breath hold prior to exhalation, and decreasing to 4.75 ppb with hyperventilation. During exercise, the mean peak levels in five subjects dropped from 9 ppb to 5.6 ppb with 50 and 100 watt exercise on an ergometer cycle, although the total NO excretion (NO concentration times number of breaths) increased (Persson, Wiklund et al. 1993). It was shown to be increased in normal subjects during a time of upper respiratory tract infection to a mean peak of 31.5 ppb during symptoms and reducing to a mean peak of 87 ppb three weeks later during recovery (Kharitonov, Yates et al. 1995). NO was shown in three studies at this time to be lower in chronic smokers, with Persson et al measuring a mean in mixed exhaled air of 3.9 ppb in 6 smokers compared to their 20 normal subjects at 8.4 ppb (Persson, Zetterstrom et al. 1994). Kharitonov et al reporting a lower mean peak of 42 ppb in 41 smokers compared to 88 ppb in 73 nonsmokers in single exhalations (Kharitonov, Robbins et al. 1995). Schilling et al documenting a mean of 16 ppb in 12 smoking females versus 21 ppb in 21 non-smoking females, and a mean of 15 ppb in 24 smoking males versus 19 ppb in 24 non-smoking males by using a reservoir method to collect tidal breathing (Schilling, Holzer et al. 1994). In this paper Schilling et al also demonstrated a reduction of exhaled NO in ten hypertensive patients to a mean of 13.7 ppb (Schilling, Holzer et al. 1994). One study reported varying levels of exhaled NO throughout the menstrual cycle in seven women from a peak exhaled NO at 150 ppb midcycle to a low of 59 ppb during menstruation (Kharitonov, Logan-Sinclair et al. 1994). As by far the greatest amounts of NO in vitro were shown to be produced from iNOS stimulation, (Nathan 1992; Barnes and Belvisi 1993), it had been hypothesised to be a measure of airway inflammation (Barnes 1993; Barnes and Kharitonov 1996). This seemed to be confirmed when higher levels were found in animal asthma models when an exacerbation was induced (Persson and Gustafsson 1993; Endo, Uchido et al. 1995), and then in adult asthmatic subjects compared to normal subjects in a number of early studies. Initially Kharitonov et al found levels of 283 ppb compared to controls at 80.2 ppb, Persson et
al found levels of 10.3ppb compared to controls at 8.4ppb, Massaro et al found levels of 13.2ppb compared to controls at 4.7ppb, and Robbins et al found levels of 174ppb compared to controls of 105.5ppb (Kharitonov, Yates et al. 1994; Persson, Zetterstrom et al. 1994; Massaro, Mehta et al. 1996; Robbins, Floreani et al. 1996). Alving et al demonstrated no overlap between their control group with a range of 5-16ppb and their asthmatic group who had a range measured at 21-31ppb (Alving, Weitzberg et al. 1993). Massaro demonstrated that the NO levels were even higher during a period of acute asthma in seven patients requiring emergency department treatment with a reduction of NO beginning by 48 hours (Massaro, Gaston et al. 1995) and Kharitinov et al showed a reduction over three weeks in eleven asthmatic patients who commenced on IHCs therapy. However, as can be seen above, the absolute mean concentrations of exhaled NO obtained by these separate workers in similar patient groups and normal subjects using similar techniques appeared so disparate as to be confusing.

In addition, the regional source of NO within the respiratory tract was debated. Initially Borland et al suggested that the NO measured in exhaled air from a single full exhalation and during tidal breathing was of alveolar origin like CO_2 (Borland, Cox et al. 1993). Persson et al then suggested that NO was formed preferentially in the small airways such as the terminal and respiratory bronchioles (Persson, Wiklund et al. 1993). By comparing the concentrations of NO exhaled during tidal breathing through either nose or mouth, Alving et al suggested that the major contribution came from the nasal space with a minor addition from the lower airways (Alving, Weitzberg et al. 1993). Lundberg et al demonstrated a decreasing concentration of exhaled NO when sampling progressively down the respiratory tract at the nose, mouth and, in four tracheotomised patients, below the vocal cords (Lundberg, Farkas-Szallasi et al. 1995).

Another group also looked at exhaled concentrations of NO in five adults with tracheostomy during spontaneous breathing where oral NO concentrations were 9 - 25.7ppb while via the tracheostomy this dropped to 1.7 - 6.5ppb. Eleven patients admitted for minor abdominal surgery had similar decreases in NO levels measured under anaesthesia from nasal (mean 54ppb), oral (mean 13ppb) and following intubation (mean 1.3ppb, which was close to the lower detection limit of their analyser). Seven patients intubated and ventilated in intensive care with multiple diagnoses all had NO concentration levels close to the detection limit of the analyser (range of 0.0 - 1.3ppb) (Schedin, Frostell et al. 1995). By isolating the nasal passages from the rest of the respiratory tract with voluntary closure of the soft palate, Kimberly et al showed the release of NO in the nasal passages was approximately seven times greater than
the rest of the respiratory tract (Kimberly, Nejadnik et al. 1996). Higher levels still were demonstrated in the paranasal sinuses with an age-dependent increase in keeping with sinus pneumatisation (Kimberly, Nejadnik et al. 1996). It was postulated that the sinus production of NO diffusing into the nasal spaces gave the high levels of NO then measured in the nasal cavity. Gerlach et al measured NO concentrations in the nasopharynx and trachea showing higher levels during inspiration than expiration (Gerlach, Rossaint et al. 1993). This led to the suggestion that there may be absorption of NO by the lower respiratory tract (giving a concept of auto-inhalation). However they could not exclude an exhaled component coming from pulmonary synthesis of NO.

The variation in NO levels obtained made it difficult to compare results presented by different groups (see Table 6.1).

Table 6.1: The published results of exhaled nitric oxide in adults

<table>
<thead>
<tr>
<th>Authors and Paper</th>
<th>Subject numbers</th>
<th>Method of measurement</th>
<th>Results in controls</th>
<th>Results In disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borland et al, 1993 (Borland, Cox et al. 1993)</td>
<td>8 controls</td>
<td>single exhalation tidal breathing</td>
<td>8.1ppb</td>
<td>14.7ppb</td>
</tr>
<tr>
<td>Schedin et al, 1995 (Schedin, Frostell et al. 1995)</td>
<td>5 controls</td>
<td>mouth breathing tracheostomy breathing</td>
<td>16ppb</td>
<td>4.6ppb</td>
</tr>
<tr>
<td></td>
<td>11 controls</td>
<td>tidal breathing via nose</td>
<td>84ppb</td>
<td>13ppb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tidal breathing via mouth</td>
<td>0.3ppb</td>
<td></td>
</tr>
<tr>
<td>Gerlach et al 1994 (Gerlach, Rossaint et al. 1994)</td>
<td>10 controls</td>
<td>Spontaneous breathing: trachea</td>
<td>89ppb (insp)</td>
<td>42ppb (exp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controlled ventilation</td>
<td>2ppb (insp)</td>
<td>63ppb (exp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trachea</td>
<td>72ppb (insp)</td>
<td>31ppb (exp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controlled ventilation</td>
<td>2ppb (insp)</td>
<td>45ppb (exp)</td>
</tr>
<tr>
<td>Kimberly et al, 1996 (Kimberly, Nejadnik et al. 1996)</td>
<td>5 controls</td>
<td>Bronchoscope sampling: at epiglottis, nose breathing</td>
<td>22ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at epiglottis, mouth breathing</td>
<td>9ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>in trachea, nose breathing</td>
<td>18ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>in trachea, mouth breathing</td>
<td>9ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tidal breathing over 2 minutes:</td>
<td>141nl/min/M²</td>
<td>68nl/min/M²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>during nasal breathing</td>
<td>7ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>during oral breathing</td>
<td>23ppb</td>
<td></td>
</tr>
<tr>
<td>Authors and Paper</td>
<td>Subject numbers</td>
<td>Method of measurement</td>
<td>Results in controls</td>
<td>Results in disease</td>
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<tr>
<td>------------------</td>
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</tr>
<tr>
<td>Jilma et al, 1996 (Jilma, Kastner et al. 1996)</td>
<td>22 male controls, 21 female controls</td>
<td>single exhalations</td>
<td>34ppb, 20ppb</td>
<td></td>
</tr>
<tr>
<td>Persson et al, 1993 (Persson, Wiklund et al. 1993)</td>
<td>10 controls, 5 controls</td>
<td>tidal breathing following breath-hold of 60s during hyperventilation pre-exercise exercise at 50 and 100 watts</td>
<td>3.25ppb, 100.25ppb, 4.75ppb, 9ppb, 5-6ppb</td>
<td></td>
</tr>
<tr>
<td>Sato et al, 1996 (Sato, Sakamaki et al. 1996)</td>
<td>16 controls</td>
<td>reservoir collection no breath-hold breath-hold for 10 seconds breath-hold for 60 seconds</td>
<td>(numbers from graph)</td>
<td>12ppb, 14ppb, 15ppb</td>
</tr>
<tr>
<td>Morris et al, 1996 (Morris, Sooranna et al. 1996)</td>
<td>5 female controls</td>
<td>single exhalations daily for one month</td>
<td>51 ng g-1 No variation across the month</td>
<td></td>
</tr>
<tr>
<td>Kharitonov et al, 1994 (Kharitonov, Logan-Sinclair et al. 1994)</td>
<td>40 male controls, 19 female controls, 7 females through a menstrual cycle</td>
<td>single exhalations ** **</td>
<td>75ppb, 70ppb, midcycle 150ppb menstruation 59ppb</td>
<td></td>
</tr>
<tr>
<td>Trolin et al, 1994 (Trolin, Anden et al. 1994)</td>
<td>27 controls, 8 with exercise</td>
<td>single exhalation total output over one minute, single exhalation total output</td>
<td>8.6ppb, 90.9ppb, 2.8ppb, 171.2ppb</td>
<td></td>
</tr>
<tr>
<td>Robbins et al, 1996 (Robbins, Floreani et al. 1996)</td>
<td>91 controls, 18 asthmatics, 23 smokers</td>
<td>single exhalation reservoir collection</td>
<td>105.5ppb, 14.5ppb, 174.2ppb, 27.2ppb, 39.6ppb, 7.3ppb</td>
<td></td>
</tr>
<tr>
<td>Iwamoto et al, 1994 (Iwamoto, Pendergast et al. 1994)</td>
<td>8 controls with exercise</td>
<td>single exhalation at rest one minute collection: at rest exercise hyperventilation</td>
<td>26.3ppb, 9.5ppb, graphs for individuals 4.8ppb</td>
<td></td>
</tr>
<tr>
<td>Martin et al, 1996 (Martin, Bryden et al. 1996)</td>
<td>18 controls, 32 allergic rhinitis, 18 controls, 32 allergic rhinitis, 18 controls, 32 allergic rhinitis</td>
<td>single exhalation following breath-hold of 10s following breath-hold of 60s</td>
<td>11.1ppb, 15.6ppb, 34.0ppb, 32.1ppb, 62.0ppb</td>
<td></td>
</tr>
<tr>
<td>Alving et al 1993 (Alving, Weitzberg et al. 1993)</td>
<td>12 controls, 8 asthmatics</td>
<td>Tidal breathing: nasal inhalation oral inhalation with URTI oral inhalation</td>
<td>23ppb, 9ppb, 32ppb, 11ppb</td>
<td></td>
</tr>
<tr>
<td>Authors and Paper</td>
<td>Subject numbers</td>
<td>Method of measurement</td>
<td>Results in controls</td>
<td>Results in disease</td>
</tr>
<tr>
<td>----------------------------------------</td>
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<td>--------------------</td>
</tr>
<tr>
<td>Persson et al, 1994</td>
<td>20 controls</td>
<td>mixed exhaled air</td>
<td>8.4ppb</td>
<td>10.3ppb</td>
</tr>
<tr>
<td>(Persson, Zetterstrom et al. 1994)</td>
<td>23 asthmatics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 smokers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kharitonov et al, 1994</td>
<td>67 controls</td>
<td>single exhalation</td>
<td>80.2ppb</td>
<td>283ppb</td>
</tr>
<tr>
<td>(Kharitonov, Yates et al. 1994)</td>
<td>61 asthmatics</td>
<td></td>
<td></td>
<td>101ppb</td>
</tr>
<tr>
<td></td>
<td>52 asthmatics on IHCS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kharitonov et al, 1995</td>
<td>25 asthmatics:</td>
<td>single exhalation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Kharitonov, Yates et al. 1995)</td>
<td></td>
<td>single responders baseline</td>
<td>214ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>single responders 27 hours</td>
<td>233ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>dual responders baseline</td>
<td>258ppb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>dual responders 27 hours</td>
<td>264ppb</td>
<td></td>
</tr>
<tr>
<td>Massaro et al, 1996</td>
<td>5 controls</td>
<td>mixed expired air at carina with bronchoscope</td>
<td>4.7ppb</td>
<td>13.2ppb</td>
</tr>
<tr>
<td>(Massaro, Mehta et al. 1996)</td>
<td>5 asthmatics</td>
<td>mixed expired air at carina with bronchoscope</td>
<td>7.0ppb</td>
<td>40.5ppb</td>
</tr>
<tr>
<td>Massaro et al 1995</td>
<td>90 controls</td>
<td>mixed air</td>
<td>6.2ppb</td>
<td>13.9ppb</td>
</tr>
<tr>
<td>(Massaro, Gaston et al. 1995)</td>
<td>43 asthmatics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kharitonov et al, 1995</td>
<td>73 controls</td>
<td>single exhalation</td>
<td>88ppb</td>
<td>42ppb</td>
</tr>
<tr>
<td>(Kharitonov, Robbins et al. 1995)</td>
<td>41 smokers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schilling et al, 1994</td>
<td>21 female controls</td>
<td>reservoir collection</td>
<td>21ppb</td>
<td>16ppb</td>
</tr>
<tr>
<td>(Schilling, Holzer et al. 1994)</td>
<td>12 female smokers</td>
<td></td>
<td></td>
<td>19ppb</td>
</tr>
<tr>
<td></td>
<td>24 male controls</td>
<td></td>
<td></td>
<td>15ppb</td>
</tr>
<tr>
<td></td>
<td>24 male smokers</td>
<td></td>
<td></td>
<td>13.7ppb</td>
</tr>
<tr>
<td></td>
<td>10 with hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kharitonov et al, 1996</td>
<td>11 asthmatics baseline</td>
<td>single exhalation</td>
<td>203ppb</td>
<td></td>
</tr>
<tr>
<td>(Kharitonov, Yates et al. 1996)</td>
<td>following IHCS</td>
<td></td>
<td></td>
<td>120ppb</td>
</tr>
<tr>
<td>Kharitonov et al, 1995</td>
<td>18 (URTI)</td>
<td>single exhalation</td>
<td>315ppb</td>
<td></td>
</tr>
<tr>
<td>(Kharitonov, Yates et al. 1995)</td>
<td>during recovery</td>
<td></td>
<td></td>
<td>88.3ppb</td>
</tr>
<tr>
<td>Kharitonov et al, 1995</td>
<td>79 (controls)</td>
<td>single exhalation</td>
<td>89ppb</td>
<td></td>
</tr>
<tr>
<td>(Kharitonov, Wells et al. 1995)</td>
<td>20 bronchiectasis</td>
<td></td>
<td></td>
<td>285ppb</td>
</tr>
<tr>
<td></td>
<td>19 bronchiectasis on IHCS</td>
<td></td>
<td></td>
<td>88ppb</td>
</tr>
</tbody>
</table>

This table lists the published results of exhaled NO in adults by the beginning of 1997 showing the wide range of techniques and even with apparently similar techniques the wide range of results reported. The mean results of the exhaled NO for each subject group are presented here. I have listed
only those measured by mouth or lower respiratory tract rather than nasal or sinus measurement which will be discussed in a later chapter.

(exp = expiratory, IHCS = inhaled corticosteroids, insp = inspiratory, ng = nanogram, ppb = parts per billion, s = seconds, single responders = early asthmatic response only, late responders = both early and late asthmatic response, URTI = upper respiratory tract infection).

6.4 The aims of the exhaled nitric oxide methodological experiments

The following methodological experiments were therefore designed and conducted in adult subjects to try and answer two main questions:

1. Were NO and CO₂ produced from the same region within the lung?
2. Which technical factors during measurement led to changes in the NO results and therefore would require standardisation in future research in this area?

The aims of the methodological investigations were:

1. To determine whether exhaled NO could be measured by the chemiluminescence analyser now modified from its original purpose of measuring NO in air pollution.
2. To compare the exhalation pattern of NO versus CO₂ from a series of single exhalations.
3. To compare the results of NO using the peak level versus the area under the curve of the recording trace.
4. To compare the results of NO and CO₂ between two methods of measurement; either direct exhalation to the NO analyser (which also allowed measurement of CO₂, and mouth pressure) or exhalation via a t-piece system (which measured these parameters with the additional measurement of expiratory flow).
5. To assess the reproducibility of the NO and CO₂ measurements.
6. To investigate whether altering the expiratory flow changed the exhaled NO levels measured.
7. To assess whether pressure had any effect on the exhaled NO levels by two methods:
   (a) Delivering the calibration gas at different pressures to the NO analyser.
   (b) Altering the expiratory mouth pressure voluntarily by adult subjects.
8. To investigate whether ambient NO levels affected exhaled NO levels.
9. To investigate whether water consumption immediately before exhalation changed the NO results obtained (this was added in response to a chance finding).

The experiments and results from aims one to five listed above will be detailed in this chapter. The experiments and results conducted to answer aims six to nine where a specific single condition was varied to assess the effect on the exhaled NO levels obtained are detailed in Chapter 7.
6.5 Setting up for the experiments

In the previous chapter, the capabilities and set-up of the analysers was described. In this section, I will outline how the analysers were calibrated prior to each subject in the methodological experiments and the basic procedure to serve as an introduction to all the subsequent experiments on adult volunteer subjects. Changes in this exact procedure and results of each set of studies will be outlined as separate experiments. I will present and then discuss the initial findings after the first investigations which compared the two sampling techniques with NO, CO₂, expiratory mouth pressure, and (in the t-piece system) expiratory flow results.

6.5.1 Set-up and calibrations

These instructions were set out by Carolyn Busst and myself after many individual tests of the machinery and then posted next to the NO analyser to follow each time I did these studies to ensure a standard procedure. I have re-presented them here as they were written in the present tense and with numerical representation of the recorded numbers for times and frequency or repetitions, but have added in italics some additional notes that came to be part of the set-up as I progressed through and became more experienced. There was no change to the nature of the investigations, but made it easier to standardise the machines and have the set-up running efficiently. The purpose of these procedures was two-fold; to calibrate the analysers, in particular the NO and CO₂ analysers, and to mark the levels on the chart recorder to enable calculation of the study parameters.

One hour before the study:
1. Check that the NO analyser and the vacuum pump are switched on and that the ozone generator in the analyser is switched on (toggle switch left hand side, front panel). [NB: I found the analyser and vacuum more stable and took less time to reach a stable level if I let it run continuously Monday to Friday or over weekends as well if experiments were being undertaken. This is in keeping with how it would have been used originally as a continuous monitor of air NO pollution.]
2. Switch on the capnograph (switch located at the back of the machine).
3. Switch on the electrospirometer (switch located at the back of the machine).
4. Check that the calibration gas cylinders and air cylinders are not empty.
5. Check the paper in the chart recorder. If in doubt regarding having sufficient to record the whole next subject experiment, replace with a new roll. Keep the old ones for test runs.
6. Check that the pens work. Fill with ink if necessary, careful not to overfill as this can lead to smudges on the traces.

7. Check that the mouthpiece is clean and that the sample tubing is free from water. If it is not, flush the tubing with compressed air from the cylinder.

8. Do not switch on the pressure equipment as it is battery powered and this will just waste the battery.

Calibration just before the study:

On chart recorder note:

- Date.
- Study name.
- Subject name.
- Parameters and voltage scales [these remained unchanged and would only have mattered if there was a log scale change in the concentrations of the gases, pressure or flow that we were measuring, see NO calibration below].
- Paper speed.
- Barometric pressure.
- Temperature.
- The background ambient NO level.

The procedures for calibrating the analysers:

**Calibration of the NO analyser:**

- Make sure the rotameter valve is closed.
- Zero calibration: connect NO sampling probe to the black, cylindrical NO charcoal scrub unit.
- NO concentration calibration one: connect NO sampling probe to the calibration gas ‘one’ nozzle - turn on the cylinder and record the level noting on the chart record.
- NO concentration calibration two: connect NO sampling probe to the calibration gas ‘two’ nozzle - turn on the cylinder and record the level noting on the chart record.
- Turn off the cylinders.
- Connect the sample port to the mouth-piece.
- Run the chart recorder to record both NO and CO₂ baseline ambient/room air values and note results on the chart record.
Note that ordering of new NO gases cylinders takes 2 months to sort out and arrive so when to a quarter full commence the order for the next cylinder.

NB: The calibration gases used during the time of the studies were 37 ppb, 103 ppb, 110 ppb, 55 ppb, and 118 ppb. For an example of calibration on the chart record see Figure 6.1.

Figure 6.1: Recording of the calibrations for NO, CO2 and pressure analysers

The chart recording is read from right to left and the green tracing shows NO, the red tracing CO2 with the zero, ambient and calibrations for each. The blue tracing is for mouth pressure and this is shown as calibration in sets of 2mmHg.

Calibration of the capnograph:
- Make sure the rotameter valve is closed.
- Press the CAL button on the front of the panel. The pump determines the sampling rate of the CO2 probe. (The sampling rate can be changed by pressing the up and down arrow
keys but it is currently in the middle of the range suggested by the manufacturers, and
used for all the early assessments).

- Zero calibration: connect the CO₂ sampling probe to the NO/N₂ tubing (NO calibration
gas) and press the CAL button to accept the zero calibration – mark on chart record – turn
off the gas.

- CO₂ calibration gas one – connect the CO₂ sampling probe to the CO₂ calibration gas
nozzle used and press CAL button to accept calibration - mark on the chart recorder - turn
off the gas.

- Barometric calibrations: Press the CAL button until the barometric pressure is displayed
- press the up arrow to set the barometric pressure - the CO₂ bobbin will fall for 3 seconds
and then rise back up again and the current barometric pressure will be displayed - make a
note on chart record.

- Connect the sample port to the mouthpiece.

- Press display on the front panel to obtain the end-tidal CO₂ display.

- Run the chart recorder to record both NO and CO₂ baseline values noting the ambient
levels (more important for the NO recordings) (see Figure 6.1).

**Calibration of the flow rotameter:**

- Zero calibration: with the air supply turned off and the rotameter needle valve turned off
- connect the air supply tubing to the bottom inlet of the rotameter - turn the air supply on –
run the chart recorder and label the trace.

- Calibration levels: open the valve until the rotameter bobbin moves and calibrate to
150mls/min, 225mls/min, 325mls/min, 400mls/min, 500mls/min, 600mls/min,
700mls/min, 800mls/min, 900mls/min, 1000mls/min at each level run the chart recorder
each time and note the calibration on the chart record - beyond this range the readings
become less stable when repeated over time and therefore less likely to be accurate.

- Leave the rotameter valve open - turn off the air supply, the bobbin will fall to zero
releasing the pressure in the air line (see Figure 6.2).
The chart recording is read from right to left and the brown tracing shows the levels of flow calibrations in mls/min. The calibrations are worked out on the sheet.

**Mouth pressure.**

- Open the rotameter needle valve.
- Ensure all connections are tight.
- Switch on the pressure generator (small black sliding switch, right hand side).
- Zero the display with the small black screw on the left hand side front panel of the pressure generator.
- Zero calibration: connect the line from the pressure generator to the test point of the Medex pressure transducer and ensure that it is secure – zero the pressure generator using the wheel in the centre of the generator – zero the pressure monitor gauge making sure the needle is on the zero of the scale – record the zero level on the chart record.
Calibration pressures: the test port of the pressure transducer is on the opposite side of the transducer to the mouthpiece — to mimic an increase in mouthpiece pressure it is necessary to create an equivalent negative pressure at the test port — move the wheel in the direction indicated by the vacuum arrow to the right of the wheel — a negative figure is noted on the generator display — increase the vacuum by increments of 2 mmHg from 0-20 mmHg and run the chart paper at each level and mark on the chart record.

- Ensure that when calibrating the trace does not drift down which suggests a leak and in this case recheck the joints.
- At the end, disconnect the generator and switch it off to save the batteries and ensure the needle on the pressure monitor is on zero (see Figure 6.1).

For the testing the chart speed can be set 20 mm/min or 50 mm/min — the latter should be used for the subjects. The scaling factor on the CO₂ channel on the pen recorder should be set to 10 volt full scale deflection (i.e. set the the toggle switch to V and the pointer on the voltage knob pointing to 10 on the V scale), and that of the NO channel to 500 mV. The scale of the NO channel will have to be changed if the calibration gases contain more than 490 ppb NO [not required during this time].

At the end of the study:
- Repeat the calibrations in reverse order immediately the study has finished.
- Check that the gas cylinders are off.
- If no further studies on the day — turn everything off except the NO analyser.
- If there is a gap between studies turn off the pressure monitor only to conserve the battery.
- The ozone generator can be switched off overnight but it should be switched on again an hour before the study to stabilise.
- The NO analyser can be switched off if it is not going to be used for long periods — switch off the ozone generator 1st, then the NO analyser and finally the vacuum pump.

6.5.2 The exhalation protocol

Standard protocol for exhalation is described here and forms the basis for all the subsequent experiments — any deviation in order to assess different factors is documented in each experiment.
Direct to analysers method:
- The subject sits at rest for a minimum of 5 minutes.
- Each subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- 5 exhalations are made consecutively at 3-minute intervals.
- Nose clips are worn 5 seconds before the exhalation, and taken off between measurements.
- Measurements of NO, mouth pressure and CO₂ are made.
- Mouth pressure is standardised to 4 mmHg by a fixed restriction.
- The sampling rate of the combined analysers was 440mls/min.

T-piece sampling system to analysers method:
- The subject sits at rest for a minimum of 5 minutes.
- Each subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- 5 exhalations are made consecutively at 3-minute intervals.
- Nose clips are worn 5 seconds before the exhalation, and taken off between measurements.
- Measurements of NO, mouth pressure and CO₂, and flow are made.
- Mouth pressure is standardised to 4 mmHg by a fixed restriction.
- Flow in the t-piece was standardised by having the subject exhale at a constant rate of 225ml/min. The subjects controlled their expiratory flow rates voluntarily observing the rotameter bobbin level of the pneumotachograph for visual feedback.
- The sampling rate of the combined analysers including the flow was 665mls/min.

Figure 6.3: Schematic diagrams of the two different types of connections: direct and t-piece measurements

Direct:
The first figure shows the connections from the mouthpiece in the direct system to the NO analyser, the CO$_2$ analyser, the pressure analyser and the chart recorder. The second figure shows the t-piece connection allowing additional measurement of flow.

6.5.3 Ethics and Consent

The studies received prospective approval by the Royal Brompton Hospital Ethics Committee and informed consent was obtained from each of the volunteer subjects.

6.5.4 The subjects

In total, twenty healthy volunteers (mean age 35.9 years, range 18-52 years, 9 males) took part in these methodological studies. Following consent, each subject completed a questionnaire (see Appendix 2). The subjects were non-atopic with no history of respiratory or cardiac disease and taking no medication. All had normal spirometry (Compact Vitalograph, Vitalograph Ltd, Buckingham, United Kingdom). Two female subjects involved in the variable flow and variable pressure experiments smoked two cigarettes per day; the rest were nonsmokers.

6.5.5 Statistical analysis

The results were analysed using the Statistical Products and Services Solutions, (Package U 7.0, SPSS Inc, Chicago, USA). This employs the Student’s t-test for matched pairs with mean,
standard error of the mean (SEM) and ranges given. A p-value of less than 0.05 was considered significant.

6.6 Methodological experiment one

- Direct versus t-piece NO measurement
- Peak NO versus area under the NO curve
- Exhalation patterns of NO versus CO₂

6.6.1 Hypotheses

1. The levels of NO measured in exhaled air are dependent on the techniques of measurement.
2. There will be good correlation between the peak NO levels and the area under the recording curve of the NO measurement.
3. Curves for NO and CO₂ in a single exhalation should be identical if they are predominantly being produced in the same department within the lung.

6.6.2 Aims

1. To compare exhaled NO levels measured by two different techniques.
2. To compare results of NO measured as peak level with area under the curve.
3. To compare the peak levels and curves of NO and CO₂ in a single exhalation.

6.6.3 Procedure

Each subject abstained from food and drink for four hours prior to the experiment. The experiments were made if the ambient level of NO was less than 10ppb, and all the testing was done with inhalation from ambient room air. The procedures as described in the previous section for starting and calibrating the analysers were made before and after each subject. The exact procedure followed is as described above in Section 6.5.2. Twelve subjects were enrolled and each subject continued until five sets of measurable exhalations were made for each set of conditions with the first set being determined randomly by tossing a coin (heads - direct, tails - t-piece). This was followed after a five minute break by measuring exhalation under the other set of conditions. Again, after a five minute break, the first conditions were measured again. Expiratory mouth pressure was requested to be voluntarily kept at 4mmHg by the subjects and was measured on the chart recorder. During the exhalations into the t-piece system, the subjects were also requested to maintain a set expiratory flow by watching
the rotameter. Tracings of each parameter (see in previous chapter Figure 5.2; NO in green, CO₂ in red, pressure in blue, flow in brown) were recorded on the chart recorder and each then calculated using the calibration recordings. The NO and the CO₂ peak levels on the recording was measured. The peak NO levels were then compared with the area under the curve. The latter was measured by drawing a baseline along the recording congruent with the zero reading. The baseline and the curved NO record were then traced out and the area within the margins calculated. Each result was the mean of five exhalations. The comparisons presented between the direct and the t-piece measurements are the first measurements of each. The final repeated set was compared against the first set to assess whether there were differences over this time.

6.6.4 Results

Figure 6.4: Example of the chart recording rolls for the direct versus t-piece measurements.

6.6.4 (i) Direct versus t-piece nitric oxide and carbon dioxide measurement

Fifteen measurements were performed successfully on each of the twelve subjects (six male, six female) with their lung function as percent predicted for gender, age and height by the Polgar predictive equation (Polgar and Promadhat 1971) having an overall mean FVC of 107% (range 96-120%) and FEV₁ of 102% (range 93%-115%). The direct mean peak
concentration of exhaled NO was 84.8ppb (SEM 14.0, range 25.1 - 189.0). There was greater variation shown in the female subjects with a mean of 92.4ppb (SEM 36.9, 26.2 - 189.0) than the male subjects with a mean of 71.7ppb (SEM 28.7, range 25.1 - 141.0). The peak exhaled NO levels measured through the t-piece system were lower in all the subjects with a total subject mean of 41.2ppb (SEM 10.8, range 10.00 - 101.6). The mean in males was 33.2ppb (SEM 8.4) versus females of 50.7ppb (SEM 12.3) (see Figure 6.6). The data for one individual as the change occurred from t-piece to direct measurement is shown in Figure 6.7.

Note again that the chart recording is read from right to left so the exhalation commences at the right hand side of each set of tracings. The scales were added for convenience of viewing this single tracing as the scales were worked out on each graph depending on the checks with the calibration gases. The main point depicted is the change with higher NO levels being measured in the direct rather than the t-piece system.

Figure 6.5: Example of recording on one subject — direct and t-piece measurements

Note the chart is read from right to left, NO is recorded in green, CO₂ is recorded in red, mouth pressure is recorded in blue and flow (seen only in the t-piece measurements) is recorded in brown. The exhaled NO levels increase when the trace moves from t-piece to direct recordings. The calculations made for each exhalation can also be seen on the chart.
Comparisons of mean exhaled peak NO by the direct and t-piece system (n=12, each mean of 5 exhalations, SEM = standard error of the mean).

Figure 6.7: Example of a recording for the two systems

This is an example of the raw data from one individual showing the NO and CO₂ and mouth pressure (P_{mo}) with the appropriate scales added along the ‘y’ axes. The main point to note is the difference seen in the exhaled NO concentration between the two methods; higher in the direct versus the t-piece system of measurement.
Table 6.2 shows all the results on one subject to demonstrate that each of the testing parameters are measured five times with the first set of conditions repeated. The parameters of pressure and flow, although standardised voluntarily, were also measured to ensure they were adequately controlled.

Table 6.2: Complete results for an individual subject

<table>
<thead>
<tr>
<th></th>
<th>NO in ppb</th>
<th>CO₂ as % total gases</th>
<th>Mouth pressure in mmHg</th>
<th>Time in seconds</th>
<th>Time NO peak</th>
<th>Time CO₂ peak</th>
<th>CO₂ at NO peak</th>
<th>NO at CO₂ peak</th>
<th>Area under the curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct 1</td>
<td>41</td>
<td>6.2</td>
<td>4.0</td>
<td>58.3</td>
<td>32.4</td>
<td>50.4</td>
<td>5.4</td>
<td>37.7</td>
<td>6.43</td>
</tr>
<tr>
<td>Direct 2</td>
<td>45</td>
<td>5.9</td>
<td>4.0</td>
<td>59.4</td>
<td>32.4</td>
<td>54.0</td>
<td>5.3</td>
<td>42.2</td>
<td>7.71</td>
</tr>
<tr>
<td>Direct 3</td>
<td>50</td>
<td>6.3</td>
<td>4.2</td>
<td>64.8</td>
<td>27.6</td>
<td>60.3</td>
<td>4.9</td>
<td>46.6</td>
<td>8.93</td>
</tr>
<tr>
<td>Direct 4</td>
<td>48</td>
<td>6.4</td>
<td>4.2</td>
<td>64.8</td>
<td>34.8</td>
<td>56.6</td>
<td>5.7</td>
<td>44.4</td>
<td>8.77</td>
</tr>
<tr>
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<td>6.4</td>
<td>4.0</td>
<td>66.0</td>
<td>24.0</td>
<td>60.5</td>
<td>5.7</td>
<td>46.6</td>
<td>9.8</td>
</tr>
<tr>
<td>T-piece 1</td>
<td>10</td>
<td>6.8</td>
<td>4.0</td>
<td>60.6</td>
<td>6.0</td>
<td>48.0</td>
<td>3.7</td>
<td>8.9</td>
<td>1.27</td>
</tr>
<tr>
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<td>6.4</td>
<td>4.2</td>
<td>51.6</td>
<td>8.4</td>
<td>63.6</td>
<td>4.5</td>
<td>11.1</td>
<td>1.6</td>
</tr>
<tr>
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<td>6.6</td>
<td>4.1</td>
<td>58.3</td>
<td>12.0</td>
<td>63.5</td>
<td>4.9</td>
<td>13.1</td>
<td>1.2</td>
</tr>
<tr>
<td>T-piece 4</td>
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<td>4.2</td>
<td>60.0</td>
<td>12.0</td>
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<td>4.9</td>
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<td>1.5</td>
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<td>21.6</td>
<td>49.1</td>
<td>5.9</td>
<td>11.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

I have also included Table 6.3 with the mean results and ranges from all the subjects for exhaled NO and CO₂, each the mean of 5 readings per subject. The coefficient of variation for individuals was 17% for the direct method and 11% for the t-piece method. The background level of NO ranged from 1 to 11ppb; this did not alter the conclusions so was not taken into account in the calculations.

Table 6.3 NO and CO₂ results from single exhalations in twelve adult subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Gender</th>
<th>Peak NO</th>
<th>Range NO</th>
<th>Peak CO₂</th>
<th>Range CO₂</th>
<th>Ratio NO/CO₂</th>
<th>Peak NO</th>
<th>Range NO</th>
<th>Peak CO₂</th>
<th>Range CO₂</th>
<th>Ratio NO/CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>m</td>
<td>29.4</td>
<td>25.3-33</td>
<td>5.6</td>
<td>5.2-6.1</td>
<td>5.3</td>
<td>12.2</td>
<td>12-13</td>
<td>6.7</td>
<td>6.3-6.9</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>m</td>
<td>43.6</td>
<td>41-45</td>
<td>5.8</td>
<td>5.7-5.9</td>
<td>7.5</td>
<td>35.2</td>
<td>34-38</td>
<td>6.2</td>
<td>6.0-6.3</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>m</td>
<td>45.1</td>
<td>42-59</td>
<td>4.4</td>
<td>4.0-4.6</td>
<td>10.3</td>
<td>26.9</td>
<td>18-39</td>
<td>5.3</td>
<td>5.0-5.4</td>
<td>5.1</td>
</tr>
<tr>
<td>4</td>
<td>m</td>
<td>63.6</td>
<td>59-68</td>
<td>6.5</td>
<td>6.3-6.8</td>
<td>9.6</td>
<td>14.2</td>
<td>14-15</td>
<td>7.1</td>
<td>6.9-7.2</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>m</td>
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<td>103-151</td>
<td>6.0</td>
<td>5.8-6.1</td>
<td>19.8</td>
<td>66.2</td>
<td>24-71</td>
<td>6.5</td>
<td>6.4-6.6</td>
<td>10.2</td>
</tr>
<tr>
<td>6</td>
<td>m</td>
<td>129.9</td>
<td>125-141</td>
<td>6.8</td>
<td>6.6-6.9</td>
<td>19.1</td>
<td>30.8</td>
<td>29-33</td>
<td>7.2</td>
<td>7.1-7.3</td>
<td>4.3</td>
</tr>
<tr>
<td>7</td>
<td>f</td>
<td>47.2</td>
<td>41-52</td>
<td>6.3</td>
<td>5.9-6.4</td>
<td>7.5</td>
<td>10.4</td>
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<tr>
<td>8</td>
<td>f</td>
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<td>5.2-5.5</td>
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<td>30.3</td>
<td>29-32</td>
<td>5.4</td>
<td>5.3-5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>9</td>
<td>f</td>
<td>52.6</td>
<td>32-72</td>
<td>5.1</td>
<td>4.1-5.6</td>
<td>10.3</td>
<td>40.8</td>
<td>34-56</td>
<td>4.9</td>
<td>4.4-5.2</td>
<td>8.5</td>
</tr>
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<td>6.8</td>
<td>6.7-6.9</td>
<td>18.5</td>
<td>72.7</td>
<td>69-74</td>
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<td>6.5-6.9</td>
<td>10.7</td>
</tr>
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<td>166.8</td>
<td>143-189</td>
<td>5.5</td>
<td>5.2-5.8</td>
<td>30.3</td>
<td>50.8</td>
<td>46-56</td>
<td>6.2</td>
<td>6.0-6.3</td>
<td>8.2</td>
</tr>
</tbody>
</table>
NO and CO₂ results from single exhalations as the mean of five recordings in twelve adult subjects by direct or t-piece system measurements (m = male, f = female). (a. NO measured in parts per billion. b. CO₂ measured as % of total gas expired).

There was no significant difference between peak CO₂ level by the direct measurements with a mean of 5.81% (range 4.0% to 6.9%) or t-piece measurements with a mean of 6.19% (range 4.4-7.2%, p = 0.66) or between male and female subjects (5.84% versus 5.75% direct, p = 0.64, and 6.41% versus 6.03% t-piece, p = 0.44), (see Figure 6.8). There was no difference in repeated last set of exhalations for either NO or CO₂ whether it was a repetition of the direct or the t-piece measurements, although the coefficient of variation had improved suggesting that there may be some improvement with training.

Figure 6.8: Mean exhaled CO₂ levels measured by direct and t-piece systems

Comparisons of mean exhaled peak CO₂ by the direct and t-piece system (n=12, each mean of 5 exhalations, SEM = standard error of the mean).

6.6.4 (ii) Peak nitric oxide versus area under the nitric oxide curve

The correlation coefficients comparing the results of peak NO level versus using area under the curve for these traces were 0.87 for the direct results and 0.81 for the t-piece results and are shown below in Figure 6.9.
Correlation of the peak NO levels with the measurements of area under the NO curve with a correlation coefficient of 0.87 (direct) and 0.81 (t-piece).\(^4\)

\(^4\) The correlation here is slightly better than that given in the 'Methods to Measure NO' article. This was an invited article and I included the data completed at that time but continued with the study.
6.6.4 (iii) Comparison of exhaled nitric oxide and carbon dioxide

There was a significant difference in the mean time to reach peak NO levels between the direct (mean 32.2 seconds) and t-piece method (mean 23.1 seconds, \( p < 0.001 \)) while there was no difference between the time to reach CO\(_2\) peaks between the direct (mean 50.5 seconds) and t-piece methods (mean 51.4 seconds, \( p = 0.5 \)). In addition, the time taken for the peak NO level to be reached and the time taken for the peak CO\(_2\) level to be reached were compared. In the direct measurements, the mean time to reach peak NO was 32.2 seconds (SEM 4.3), which was significantly less (\( p<0.001 \)) than the mean time to reach peak CO\(_2\) levels at 50.5 seconds (SEM 5.3). This was also less than the time to total exhalation of 56.2 seconds (SEM 5.4, \( p<0.001 \), see Figure 6.10a). In the t-piece measurements the mean time to reach peak NO was 23.1 seconds (SEM 4.4) which was also significantly less (\( p<0.001 \)) than the mean time to reach peak CO\(_2\) levels at 51.4 seconds (SEM 4.6). Again this was less than the total duration of exhalation of 53.5 seconds (SEM 4.7, \( p=0.14 \), see Figure 6.10b).

Figure 6.10a: Time to peak NO and peak CO\(_2\) measured by the direct system

Comparisons of the time in seconds taken to reach peak NO, the time taken to reach peak CO\(_2\), with the total time for exhalation in measurements by the direct system (\( n=12 \), each mean of 5 exhalations).
Comparisons of the time in seconds taken to reach peak NO, taken to reach peak CO₂ with the total time for exhalation in measurements by the t-piece system (n=12, each mean of 5 exhalations).

I also marked the CO₂ level at the time of NO peak to compare the difference between the CO₂ level at this time and at the time of CO₂ peak, which occurred later in the exhalation. For the direct measurements, the CO₂ level measured at peak NO was 4.88% (SEM 0.14) which was significantly lower than the peak CO₂ at 5.81% (SEM 0.21, p< 0.001, see Figure 6.11a). For the t-piece measurements, the CO₂ level measured at peak NO was 5.23% (SEM 0.18) which was significantly lower than the peak CO₂ at 6.19% (SEM 0.28, p< 0.001, see Figure 6.11b).

Comparisons of the CO₂ concentrations in percent of total expired gases at the time of peak NO and at peak CO₂ in measurements by the direct system (n=12, each mean of 5 exhalations).
Comparisons of the CO$_2$ concentrations in percent of total expired gases at the time of peak NO and at peak CO$_2$ in measurements by the direct system ($n=12$, each mean of 5 exhalations).

6.7 Discussion: The origin of the exhaled nitric oxide

The study demonstrated that NO from a single exhalation could be measured by two methods; either by direct connection to the NO analyser or by using a t-piece side arm sampling system. However the results of NO were significantly different with an approximate halving of the peak levels from the direct to t-piece technique, while there was no difference seen in the CO$_2$ results. These methods gave similar individual coefficients of variation as other groups (Kharitonov, Yates et al. 1994; Morris, Carroll et al. 1995; Jilma, Kastner et al. 1996; Massaro, Mehta et al. 1996; Hogman, Stromberg et al. 1997). The NO results from the female subjects showed greater variability, and this has been noted previously as possibly being an effect of the menstrual cycle in one (Kharitonov, Logan-Sinclair et al. 1994) but not a second paper (Jilma, Kastner et al. 1996). Our subjects were chosen to minimise any other confounding factors most notably being non atopic, non-asthmatic and having normal lung function. As noted in the total group of subjects from which all the studies were done, there were two smokers (two cigarettes per day) which was not ideal but I believed controlling for atopy was more important when excluding subjects. As it happened, neither subject took part in this direct versus t-piece experiment but were involved in later experiments.

Previous researchers had been unable to agree whether NO was of alveolar or airway origin (Alving, Weitzberg et al. 1993; Borland, Cox et al. 1993; Persson, Wiklund et al. 1993). It is known that the vast bulk of CO$_2$ is of alveolar origin, having diffused across the alveolar:capillary membrane from the pulmonary circulation. If CO$_2$ and NO were both of alveolar origin, the time to reach their respective peaks and the ratio of peak NO to CO$_2$ would remain the same even if measurement conditions were varied. I thus measured exhaled
CO$_2$ and NO using two different conditions. In the direct experiments the subject exhaled directly into the NO analyzer at 440 mls/min while in the indirect studies exhalation was into a t-piece system at a faster rate of 665 ml/min. The time to reach peak CO$_2$ level, and the level of the peak CO$_2$ were the same in both manoeuvres. However the NO traces were very different. Firstly, the time to reach peak NO was shorter than the time to peak CO$_2$ in both manoeuvres (32.2 versus 50.5 seconds in direct measurements, and 23.1 versus 51.4 seconds in t-piece measurements). Secondly, altering the rate of exhalation altered the time to peak NO independently of the time to peak CO$_2$. Thirdly, at peak NO levels the CO$_2$ levels were below their maximal peak. Fourthly, the ratio of peak NO to peak CO$_2$ differed between the two measurement methods. Thus although it is possible that a small amount of NO is of alveolar origin, the bulk of NO must be being produced proximal to the alveolar membrane.

In any physiological study, it is important to consider whether the choice of apparatus or the manoeuvre selected could have significantly affected the conclusions. Ideally, I would have used fast response time analysers, with identical delay times. Fast-response time analysers for NO had been used by others and are now available in the much later purpose-built machines (see Chapter 9). At this time, Persson et al had used a NO analyser with an integration time of 0.01 second to study the changes in NO at rest and during exercise. They came to similar conclusions, that NO and CO$_2$ were not from identical lung compartments, using a very different analyser and manoeuvre (Persson, Wiklund et al. 1993). Our analyser would have been unable to detect rapid transient changes in NO. However, had these occurred, and they had not been described by other workers, they would have strengthened rather than detracted from these conclusions. Rapid transients have not been described for CO$_2$, and their presence in the NO signal would have implied a different source, which is the main conclusion of this research and our paper.

I considered the possibility that differing pressures exerted on the NO analyser may have accounted for the difference in readings and I describe two experiments in the next chapter (see Section 7.4) specifically to answer this point (Byrnes, Dinarevic et al. 1997). However, I was careful to standardise to a mouth pressure of 4mmHg during the expiratory manoeuvre to eliminate the possibility of this artefact. The pressure drop from mouth to analyser was measured for both direct and t-piece apparatus, and found to be essentially the same. The two manoeuvres used deliberately different expiratory flows to try to separate the CO$_2$ and NO signals. However the sampling flow to the NO analyser remained constant throughout this study.
I also considered whether the different response times of the analysers could have affected our conclusions. Ideally I would have liked equal analyser response times or a single machine (such as a mass spectrophotometer) to differentiate both gases simultaneously. The NO response was slower, but the NO peaked first. Had the NO response time been the same as that of the CO₂ analyser, the NO peak would have been even earlier, thus again the analyser differences would serve to obscure, not enhance our conclusions.

We elected to study a single slow exhalation for the purposes of these studies, accepting it may not be physiological or necessarily the best method for clinical practice. However, it was designed to supply an answer to the question posed. The possibility exists that the results were affected by back diffusion of alveolar NO into the alveolar capillary blood, and by nasal contamination of the exhalate. During a prolonged expiration, if alveolar epithelium were the source of NO, then back diffusion would allow some to be carried away combined with haemoglobin. However, although this could affect the height of the NO peak, it is inconceivable that it could affect the time to reach peak, and thus my conclusions are unaffected by this concern. Furthermore, the total time of expiration was very similar in the two different manoeuvres, suggesting that, were this mechanism to be operative, it would apply equally between the two tests.

Higher levels of NO are produced by the paranasal and nasal spaces when compared to oral exhalation (Alving, Weitzberg et al. 1993; Lundberg, Rinder et al. 1994; Lundberg, Weitzberg et al. 1994; Kimberly, Nejadnik et al. 1996) and these sites contribute a major proportion of the NO concentration measured by oral exhalation. Indeed the paranasal sinuses also contribute a major proportion of the NO concentration measured by nasal exhalation (Lundberg, Rinder et al. 1994; Lundberg, Farkas-Szallasi et al. 1995). However if all of the NO came from the nasopharyngeal region then the pattern of the exhaled NO trace would be expected to both peak and fall off early rather than give the continued plateau seen in this experiment. NO had been measured in exhaled air of tracheostomised rabbits (measured by chemiluminescence), guinea pigs (measured by Quattro mass spectrometry) (Gustafsson, Leone et al. 1991) and in humans (measured by chemiluminescence) (Lundberg, Weitzberg et al. 1994). I attempted to exclude nasal contamination by asking the subject to wear a noseclip, and to exhale against a moderate resistance (4mmHg). One group had used nasal suction combined with an (amplitude unstated) expiratory resistance (Silkoff, Kesten et al. 1995) and another had excluded nasal NO contamination by voluntary closure of the soft palate (Kimberly, Nejadnik et al. 1996) but these methods were more difficult and by no means universally used. Whereas I cannot exclude a degree of nasal contamination, I believe that our
technique will have excluded most contamination, and that in any case the degree of contamination will have been identical because of the very careful standardisation of experimental conditions including expiratory mouth pressure. This study was not designed to differentiate nasal and bronchial sources of NO.

I elected to analyse the peak NO rather than the area under the curve. The two appear to be closely correlated (Kharitonov, Yates et al. 1994; Byrnes, Bush et al. 1996) and had good correlation coefficients greater than 0.8 for both techniques in this study. I aimed to determine the sources of the two gases, not the total amounts produced, which we would expect to be independent of the experimental conditions. Thus the different behaviour of the peak signals as the experimental conditions change gives more useful information than the area under the curve.

It might be argued that if airways of volume 200mls contribute a peak NO at 85 ppb, a total of $10^{-10}$ mols, and the alveolar expirate (four litres) a plateau of 60, a total of $10^{-8}$ mols, then most NO comes from the alveoli. This assumes that subjects exhaled to residual volume, which is not the case. Furthermore, if this model was correct, peak CO₂ (definitely alveolar) and peak NO should move in the same direction as experimental conditions vary. The reverse was seen. In eight subjects peak NO fell using the t-piece while CO₂ rose; in the other four peak NO fell and peak CO₂ also decreased. It is impossible to account for this on a model of major alveolar production of NO. The model that best accounts for our data is continuous airway production of NO, diluted variably in a flow-dependent manner by NO free (or low concentration NO) alveolar gas, analogous with a gaseous phase dye dilution method. This data cannot exclude that alveolar gas contains small amounts of NO.

This study also demonstrated that the conditions of measurement critically affect NO levels obtained. Thus both the time to reach the NO peak and the peak level of NO reached were quite different in the two methods used in this study although the patterns of the traces were similar. I considered whether the difference in results could have been artifactual due to minor differences in the apparatus used in the two techniques. Teflon tubing which does not absorb NO connected the mouth piece to the NO analyzer. In the t-piece system there was a two and a half centimetre extension of plastic tubing leading from the mouth piece to the actual t-piece with the teflon tubing then leading to the NO analyzer off one arm and further tubing off the second arm passing directly to the pneumotachograph for flow analysis. This is unlikely to account for the magnitude of differences obtained. The other alteration was that moving from
the direct to the indirect method led to an increase in flow rate from 440 ml/min to 665 ml/min. This may have been enough to account for the different results.

Clearly, at the time of this research, there were major and unexplained differences between results from different investigators as documented in Table 6.1. These results showed that the levels of NO measured in exhalate were critically dependent on measurement conditions. This may be related to expired flow rates but other factors may be important. It has been suggested that orally-exhaled NO concentrations correlate with the inhaled ambient concentrations of NO (Dotsch, Demirakca et al. 1996), although we found in a subsequent experiment a high ambient NO rendered exhaled NO levels difficult to interpret (Byrnes, Dinarevic et al. 1997). Our current study was only undertaken if the ambient NO levels were low – while we aimed for less than 10 ppb, on one occasion by the end of the session the ambient NO had drifted up to 11 ppb hence the range given from 1-11 ppb in the results. As mentioned the greater NO levels and wider variations seen in females may be due to possible effects of the menstrual cycle (Kharitonov, Logan-Sinclair et al. 1994) and this was not controlled for in the present study. However, as the measurements were done sequentially on each subject on the same day, conclusions as to the origin of NO from this study should not be affected.

As increasing research on NO in exhaled air was being undertaken in subjects in health and different respiratory and vascular diseases at the time, it was increasingly important to understand where and at what level NO was being generated so that abnormal results could be properly interpreted.

NB: The results of this research formed the basis of the following publications:

- **Byrnes CA, Dinarevic S, Busst CA, Bush A, Shinebourne EA. Is nitric oxide produced at airway or alveolar level? European Respiratory Journal 1997 10 (5) 1021-1025**
Chapter 7: Methodological studies of exhaled nitric oxide in healthy adult subjects: investigating test parameters

7.1 Introduction

The previous chapter described the commencement of the methodological studies into exhaled NO in adult volunteers. The first investigation was the measurement and comparison of NO and CO₂ in single exhalations by two methods; direct to analyser and via t-piece sampling systems. In both methods the results and patterns of NO and CO₂ could be determined, as well as expiratory mouth pressure measurement and, in the t-piece system, expiratory flow could also be recorded. This was an attempt to answer the question as to whether NO and CO₂ came from the same areas within the lung. This experiment also demonstrated a significant difference in the peak exhaled NO levels with an almost halving of the NO result when moving from the direct to t-piece measurements with no corresponding reduction seen in the peak CO₂ levels. The main change between these two techniques appeared to be an increase in the expiratory flow which was required for the t-piece sampling. I have already noted in the previous chapter (Section 6.4) the different levels of NO reported by different researchers despite investigating similar population groups (healthy controls, asthmatics, smokers) and summarised these in Table 6.1. I thought it was vital to understand which parameters when altered gave different exhaled NO results. This was needed to be able to recommend standardised procedures so that better interpretation of results would be possible both between different research groups and in comparisons of results in health and disease.

As mentioned in Section 6.4, the aims in designing the methodological experiments were to answer two main questions posed:
1. Were NO and CO₂ produced from the same regions within the lung?
2. Which technical factors during measurements led to changes in the exhaled NO results?

The results from the direct versus t-piece analysis suggested that the exhalation patterns seen were different between the gases. While it was known that CO₂ was of alveolar origin, the pattern of NO exhalation suggested that it came from a more proximal source such as the airways. So I felt that we had answered the first question. The experiments detailed throughout this chapter were to answer the second question. The parameters chosen to examine, by changing each one alone and maintaining an otherwise standardised procedure, were:

- The effect of expiratory flow — an effect of flow altering the level of NO had been suggested by discrepancies in the published literature summarised in Section 6.3 'The
need for methodological experiments' and from the first methodological experiment conducted and described in the last chapter.

- **The effect of pressure** – to be assessed both by applying a calibration gas under pressure to the NO analysers and by looking at the effect of expiratory mouth pressure in NO results.

- **The effect of the ambient NO when high** – this was not always standardised and sometimes not reported in early literature and could have been a source of error in results presented by different groups.

- **The effect of water consumption** – this was due to a chance finding during one experiment, where a person drank some water during a series of test exhalations and the NO results dropped significantly for the next readings. I considered that asking both healthy and, more particularly, subjects with any respiratory disease to inspire and exhale fully may well lead to coughing in some for which a common response is to give a glass of water. If this then resulted in decreasing the levels of subsequent NO measurements, this could easily skew results.

The peak exhaled NO was shown to have a good correlation with the area under the NO curve in the previous experiment, so I elected to proceed with the peak measurements as they were a more straightforward measurement, without the possibility of introducing error when tracing around a curve, drawing a line for zero along the lower edge of the recording and then calculating the area within these boundaries. I will summarise the subjects, ethics and statistics again at the beginning of this chapter pertaining to all the investigations in the adult subjects. For each experiment I will present the hypothesis, aim, procedure and results. I will discuss the results and how they contribute to findings in the literature of all four experiments at the end of the chapter. As the direct and t-piece experiment was deemed ‘methodological experiment one’, these are labelled methodological experiments two to five.

7.2 **Ethics, subjects, calibrations and statistical analysis**

The studies received prospective approval by the Royal Brompton Hospital Ethics Committee and informed consent was obtained from each of the volunteer subjects.

In total, twenty healthy volunteers (mean age 35.9 years, range 18-52 years, 9 males) took part in these methodological studies. Following consent, each subject completed a questionnaire (see Appendix 1). They were non-atopic with no history of respiratory or cardiac disease and taking no medication. All had normal spirometry (Compact Vitalograph, Vitalograph Limited, Buckingham, United Kingdom) and results are presented as percent
predicted or age, sex and height as defined by the Polgar reference equation (see results in Section 6.6.4). Two female subjects involved in experiments two and three smoked two cigarettes per day; the rest were nonsmokers.

The set-up of the analysers and the calibrations before and after each subject doing each set of exhalations for the individual experiments was carried out in the same manner as described in Section 6.5.1 ‘Set up and calibrations’ and Section 6.5.2 ‘The exhalation protocol’ of the previous chapter. Any specific differences from these procedures are documented under the separate experiments when outlined below. The procedures were written out prior to performing them, I have therefore re-presented them here using the present tense and numerical documentation as in the original instructions that I devised.

The results were analysed using the Statistical Products and Services Solutions Package 7.0, SPSS Inc, Chicago, USA). This employs Students t-test for matched pairs. An analysis of variance for repeated measures was used to determine the effects of flow, mouth pressure, NO background and pre/post water consumption using the subjects as a blocking variable. Differences are represented by mean, standard error of the mean (SEM) or 95% confidence intervals (SD) where appropriate. The correlation coefficient for NO levels at the increasing expiratory flows was calculated for each individual subject and is given for the group as a whole.

7.3  Methodological experiment two - effect of expiratory flow

7.3.1  Hypothesis

1. The levels of NO measured in exhaled air will alter with changes in the expiratory flow.

7.3.2  Aim

1. To compare exhaled NO levels measured at different expiratory flows.

7.3.3  Procedure

Ten adult volunteers (six females) were enrolled. All measurements were made by the t-piece system. The subjects performed five exhalations at each of four expiratory flows: 250mls/min, 500mls/min, 750mls/min, and 1100mls/min with a final set of measurements at the first flow to rule out an order effect. Switching the machinery on in advance and calibration between and after each subject occurred as discussed in the previous chapter (Section 6.5.1).
The exhalations were carried out in the standard manner:

- The subject abstains from food and drink for 4 hours prior to the experiment.
- The subject sits at rest for at least 5 minutes.
- The chart recorder is started 30 seconds prior to each exhalation to document baseline pre-exhalation measurements.
- The subject puts on nasal clips 5 seconds prior to each exhalation and exhales by mouth, and removes the clips between exhalations.
- The subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- The exhalations occur 3 minutes apart.
- The subject exhales at a constant mouth pressure of 4mmHg voluntarily controlled by the subject observing the pressure gauge and keeping it at level '10' on the scale.
- The subject exhales at a constant flow for 5 exhalations at each of 4 expiratory flows. The flow is voluntarily controlled by the subject keeping the rotamer at specific levels:

<table>
<thead>
<tr>
<th>Flow</th>
<th>Rotameter level</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mls/min</td>
<td>1.5</td>
</tr>
<tr>
<td>500 mls/min</td>
<td>3.0</td>
</tr>
<tr>
<td>750 mls/min</td>
<td>4.5</td>
</tr>
<tr>
<td>1100 mls/min</td>
<td>6.0</td>
</tr>
</tbody>
</table>

- The flow rate at which participants started, either 250mls/min and increasing consecutively or 1100mls/min and decreasing consecutively, was made by tossing a coin.
- The subject continues until 5 exhalations at each flow level adequate for measurement are made.
- A final set of measurements at the first flow was made.
- The subject was breathing ambient air when the natural background level of NO was less than 10ppb.

7.3.4 Results

The mean peak concentrations of NO at the four expiratory flow settings were 79.0ppb (SEM 15.5) at 250mls/min, 67.8ppb (SEM 13.5) at 500mls/min, 59.1ppb (SEM 11.2) at 750mls/min and 54.1ppb (SEM 10.7) at 1100mls/min (see Table 7.1). There were strong linear relations between the NO concentrations obtained at the four flow levels for each individual subject with a correlation coefficient of 0.85 (see Figure 7.1). The analysis of variance showed a highly significant difference across the flow rates (p<0.001), with wide variation between the
individual subjects (see Figure 7.2 for an example of the recording on one subject). The mean
decrease in exhaled NO was 35ppb (95% CI 25.7, 43.4) from the expiratory flow rate of
250mls/min to 1100mls/min. There were no significant differences in the mean CO₂ levels,
mean mouth pressure (controlled) or mean duration of exhalation in the measurements made
at the differing flow rates. There was no difference between the first baseline and repeated
baseline of NO when the expiratory flow was the same (p=0.9 95% CI -16.9, 17.0), excluding
an order effect (see Table 7.2).

Table 7.1: NO, CO₂ and duration of each exhalation at different expiratory flows

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age</th>
<th>NO</th>
<th>CO₂</th>
<th>Durtn</th>
<th>NO</th>
<th>CO₂</th>
<th>Durtn</th>
<th>NO</th>
<th>CO₂</th>
<th>Durtn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>32</td>
<td>23</td>
<td>4.7</td>
<td>57</td>
<td>17</td>
<td>4.5</td>
<td>59</td>
<td>23</td>
<td>4.5</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>33</td>
<td>102</td>
<td>7.2</td>
<td>52</td>
<td>89</td>
<td>7.0</td>
<td>55.0</td>
<td>73</td>
<td>6.3</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>37</td>
<td>78</td>
<td>6.0</td>
<td>56</td>
<td>68</td>
<td>6.1</td>
<td>56</td>
<td>60</td>
<td>5.9</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>49</td>
<td>85</td>
<td>6.4</td>
<td>66</td>
<td>70</td>
<td>5.7</td>
<td>59</td>
<td>79</td>
<td>5.8</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>43</td>
<td>31</td>
<td>6.2</td>
<td>55</td>
<td>23</td>
<td>6.2</td>
<td>54</td>
<td>19</td>
<td>5.8</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>32</td>
<td>147</td>
<td>6.2</td>
<td>63</td>
<td>115</td>
<td>6.1</td>
<td>64</td>
<td>100</td>
<td>6.2</td>
<td>68</td>
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<td>7</td>
<td>Female</td>
<td>37</td>
<td>83</td>
<td>5.7</td>
<td>50</td>
<td>86</td>
<td>5.8</td>
<td>52</td>
<td>82</td>
<td>5.9</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>35</td>
<td>143</td>
<td>6.4</td>
<td>71</td>
<td>127</td>
<td>6.2</td>
<td>71</td>
<td>95</td>
<td>5.2</td>
<td>67</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>43</td>
<td>67</td>
<td>6.7</td>
<td>84</td>
<td>60</td>
<td>6.9</td>
<td>72.1</td>
<td>43</td>
<td>2.0</td>
<td>71.0</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>36</td>
<td>30</td>
<td>6.1</td>
<td>58</td>
<td>24</td>
<td>6.0</td>
<td>57</td>
<td>18</td>
<td>5.9</td>
<td>55</td>
</tr>
</tbody>
</table>

NO is measured as peak NO in parts per billion, CO₂ is measured as peak CO₂ as the percentage of
total gases, duration is measured in seconds. Each result is the mean of five exhalations for every
subject. a. Durtn = duration of exhalation.

Figure 7.1: Exhaled NO results with increasing expiratory flows

Mean peak exhaled NO levels at different expiratory flows for ten individuals (six females). Each point is the mean of five exhalations.
7.4 Methodological experiment three – effect of pressure

7.4.1 Hypotheses

1. The levels of NO will alter with different pressure.
2. The levels of NO measured in exhaled air will be different with changes in expiratory mouth pressure.

7.4.2 Aim

1. To compare NO levels measured using a calibration gas delivered to the NO analyser at different pressures.
2. To compare NO levels measured at different expiratory mouth pressures.

7.4.3 Procedure

(i) A calibration gas of 110ppb was applied to the NO analyser as measured via the direct system and was delivered to the analyser at pressures from 4 to 40mmHg in increasing increments of 4mmHg determined by the pressure gauge, and the NO level recorded.

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Ten adult volunteers (eight females) were enrolled. All measurements were made by the t-piece sampling system. The subjects performed five exhalations at each of four expiratory mouth pressures of 4mmHg, 8mmHg, 12mmHg and 16mmHg with a final set of measurements at the first mouth pressure to rule out an order effect. Switching the machinery on in advance and calibration between and after each subject occurred as discussed in the previous chapter (Section 6.5.1).

The exhalations were carried out in the standard manner:

- The subject abstains from food and drink for 4 hours prior to the experiment.
- The subject sits at rest for at least 5 minutes.
- The chart recorder is started 30 seconds prior to each exhalation to document baseline pre-exhalation measurements.
- The subject puts on nasal clips 5 seconds prior to each exhalation and exhales by mouth, and removes the clips between exhalations.
- The subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- The exhalations occur 3 minutes apart.
- The subject exhales at a constant flow of 500mls/min voluntarily controlled by the subject observing the rotameter and keeping it at level ‘3’ on the scale.
- The subject exhales at a constant mouth pressure for 5 exhalations at each of 4 different pressures which are voluntarily controlled by the subject keeping the pressure gauge at specific levels:

<table>
<thead>
<tr>
<th>Mouth Pressure</th>
<th>Pressure Gauge Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mmHg</td>
<td>10</td>
</tr>
<tr>
<td>8 mmHg</td>
<td>20</td>
</tr>
<tr>
<td>12 mmHg</td>
<td>30</td>
</tr>
<tr>
<td>16 mmHg</td>
<td>40</td>
</tr>
</tbody>
</table>

- The initial pressure of either 4mmHg and increasing consecutively or 16mmHg and decreasing consecutively was made by tossing a coin.
- The subject continues until 5 exhalations at each pressure level adequate for measurement are made.
- A final set of measurements at the first pressure is made.
- The testing is done at a time when the ambient NO level is less than 10ppb.
7.4.4 Results

There was an increase in the peak NO signal of 5.6ppb seen across the range from 4mmHg to 40mmHg of pressure when the calibration gas was applied to the NO analyser in increments of 4mmHg, with the mean and standard deviation shown for each level in Figure 7.3.

Figure 7.3: The effect of pressure on the calibration gas measurement

![Graph showing the effect of pressure on calibration gas measurement.](attachment:image.png)

The mean and standard deviation of the NO levels of a known calibration gas with a concentration of 110ppb delivered to the NO analyser at incremental pressures with an increase of 5.6ppb demonstrated over this range.

The mean peak concentrations of NO obtained at the four mouth pressure settings were 61.0ppb (SEM 15.1) at 4mmHg, 55.2ppb (SEM 12.3) at 8mmHg, 47.3ppb (SEM 8.4) at 12mmHg and 40.1ppb (SEM 7.2) at 16mmHg (see Table 7.2). There was no significant difference in the exhaled NO levels obtained at each mouth pressure. Figure 7.4 shows that in the majority of patients NO levels did not change. However in two subjects who found the higher pressures difficult to sustain (see reduced duration of exhalation in the Table 7.3 for these two subjects at this higher pressure) there was a drop in NO as mouth pressure increased. There was no difference between the first baseline and repeated final baseline of NO when the mouth pressure was the same (p = 0.44 95% CI -16.7, 15.6) thus excluding an order effect (see Table 7.4). There was no significant difference between the flows measured at the different mouth pressure readings. The peak expired CO₂ at different mouth pressure levels tended to decrease. The mean peak values were 5.90% (SEM 0.83) at 4mmHg, 5.83%
(SEM 0.82) at 8mmHg, 5.69% (SEM 0.86) at 12mmHg and then 5.53% (SEM 0.95) at 16mmHg. There was also a tendency for the duration of exhalation to decrease, from 49.9 seconds (SEM 4.9) at 4mmHg, 49.6 seconds (SEM 5.6) at 8mmHg, 44.2 seconds (SEM 6.3) at 12mmHg to 40.9 seconds (SEM 5.5) at 16mmHg.

Figure 7.4: Exhaled NO results at increasing expiratory mouth pressure

Mean peak exhaled NO levels at different expiratory mouth pressures for ten individuals (eight females). Each point is the mean of five exhalations.

Table 7.2: Comparison of the first and repeated last set of exhalations at the same expiratory flow

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age</th>
<th>Expiratory flow</th>
<th>First Set of Exhalations</th>
<th>Last Set of Exhalations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NO</td>
<td>CO₂</td>
</tr>
<tr>
<td>1</td>
<td>Female</td>
<td>32</td>
<td>250mls/min</td>
<td>23</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>37</td>
<td>250mls/min</td>
<td>78</td>
<td>6.0</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>43</td>
<td>250mls/min</td>
<td>31</td>
<td>6.2</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>32</td>
<td>250mls/min</td>
<td>147</td>
<td>6.2</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>37</td>
<td>250mls/min</td>
<td>83</td>
<td>5.7</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>35</td>
<td>250mls/min</td>
<td>143</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>36</td>
<td>250mls/min</td>
<td>30</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>33</td>
<td>1100mls/min</td>
<td>77</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>49</td>
<td>1100mls/min</td>
<td>70</td>
<td>6.0</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>43</td>
<td>1100mls/min</td>
<td>38</td>
<td>7.0</td>
</tr>
</tbody>
</table>

NO is measured as peak NO in parts per billion, CO₂ is measured as peak CO₂ as the percentage of total gases, duration is measured in seconds. Each result is the mean of five exhalations for every subject. a. Durtn = duration of exhalation.
### Table 7.3: NO, CO₂ and duration of each exhalation at different expiratory mouth pressures

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age</th>
<th>4 mmHg</th>
<th>8 mmHg</th>
<th>12 mmHg</th>
<th>16 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NO</td>
<td>CO₂</td>
<td>Durtn</td>
<td>NO</td>
</tr>
<tr>
<td>1</td>
<td>Female</td>
<td>52</td>
<td>142</td>
<td>6.6</td>
<td>51</td>
<td>112</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>36</td>
<td>46</td>
<td>5.8</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>36</td>
<td>38</td>
<td>4.8</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>30</td>
<td>11</td>
<td>4.6</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>43</td>
<td>59</td>
<td>6.8</td>
<td>81</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>32</td>
<td>107</td>
<td>6.4</td>
<td>62</td>
<td>101</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>43</td>
<td>34</td>
<td>6.1</td>
<td>46</td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>36</td>
<td>51</td>
<td>6.0</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>43</td>
<td>56</td>
<td>5.9</td>
<td>54</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>32</td>
<td>51</td>
<td>5.1</td>
<td>42</td>
<td>58</td>
</tr>
</tbody>
</table>

NO is measured as peak NO in parts per billion, CO₂ is measured as peak CO₂ as the percentage of total gases, duration is measured in seconds. Each result is the mean of five exhalations for every subject. a. Durtn = duration of exhalation.

### Table 7.4: Comparison of the first and repeated last set of exhalations performed at the same expiratory mouth pressure

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age</th>
<th>Expiratory pressure</th>
<th>First Set of Exhalations</th>
<th>Last Set of Exhalations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NO</td>
<td>CO₂</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>36</td>
<td>4 mmHg</td>
<td>46</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>36</td>
<td>4 mmHg</td>
<td>38</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>43</td>
<td>4 mmHg</td>
<td>59</td>
<td>6.8</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>32</td>
<td>4 mmHg</td>
<td>107</td>
<td>6.4</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>36</td>
<td>4 mmHg</td>
<td>51</td>
<td>6.0</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>32</td>
<td>4 mmHg</td>
<td>62</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>Female</td>
<td>52</td>
<td>16 mmHg</td>
<td>142</td>
<td>6.8</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>30</td>
<td>16 mmHg</td>
<td>4</td>
<td>4.3</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>43</td>
<td>16 mmHg</td>
<td>21</td>
<td>5.7</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>43</td>
<td>16 mmHg</td>
<td>51</td>
<td>5.8</td>
</tr>
</tbody>
</table>

NO is measured as peak NO in parts per billion, CO₂ is measured as peak CO₂ as the percentage of total gases, duration is measured in seconds. Each result is the mean of five exhalations for every subject. a. Durtn = duration of exhalation.
7.5 Methodological experiment four - effect of ambient nitric oxide

7.5.1 Hypothesis

1. Exhaled NO measurements will be affected by the ambient NO level.

7.5.2 Aims

1. To compare NO levels measured at a time of high and low ambient NO concentrations with the high level being background and low level provided by breathing from a reservoir system containing a low NO concentration.

7.5.3 Procedure

Three adult volunteers (all females) were enrolled and all measurements were made by the t-piece sampling system.

The first set of exhalations was carried out in the standard manner:

- The subject abstains from food and drink for 4 hours prior to the experiment.
- The subject sits at rest for at least 5 minutes.
- The chart recorder is started 30 seconds prior to each exhalation to document baseline pre exhalation measurements.
- The subject puts on nasal clips 5 seconds prior to each exhalation and exhales by mouth, and removes the clips between exhalations.
- The subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- The exhalations occur 3 minutes apart.
- The subject exhales at a constant mouth pressure of 4mmHg voluntarily controlled by the subject observing the pressure gauge and keeping it at level ‘10’ on the scale.
- The subject exhales at a constant flow at 500mls/min voluntarily controlled by the subject observing the rotameter, keeping it at a level at ‘3’ on the scale.
- At the end of each exhalation the connections are reversed to allow the subject and the analysers to continue to sample from the low NO reservoir.
- The subject was breathing ambient air when the natural background level of NO was high - above the NO concentration normally measured on exhalation.
- The subject continues until 5 exhalations adequate for measurement are made.
The second set of exhalations involved a reservoir system (see Figure 7.5):

- The ambient level is checked to make sure it is high – greater than the levels of known exhaled NO results of the subjects, ambient NO > 75ppb.
- A reservoir bag (made of aluminium) is filled from a tank of NO free air (measured NO level 0-2ppb).
- The reservoir bag is switched on-line to the analysers and the NO level in the bag is checked to be < 5ppb.
- The subject breathes for 5 minutes from the reservoir bag. The subject exhales through a one-way valve to avoid reservoir contamination.
- The analysers sampled from the reservoir system between exhalations.
- For the measured exhalation the connections were turned so that the subject exhaled to the analysers.
- The subject exhales at a constant mouth pressure of 4mmHg voluntarily controlled by the subject observing the pressure gauge and keeping it at level ‘10’ on the scale.
- The subject exhales at a constant flow at 500mls/min voluntarily controlled by the subject observing the rotameter, keeping it at ‘3’ on the scale.
- At the end of each exhalation the connections were reversed to allow the subject and the analysers to continue to sample from the low NO reservoir.
- This means none of the analysers are exposed to the high ambient level of NO during this set of 5 exhalations.

The third set of exhalations was carried out in the standard manner:

- The measurements made were a repeat of the first set detailed above.
- Between exhalations the subject was breathing as normal in the known high ambient NO level.
- Between exhalations the analysers were sampling as usual from the known high ambient NO level.

Note: in this experiment the measurement used was NO plateaux rather than NO peak levels, as the NO dropped from high level during the first and third set therefore a peak was not obvious, while a plateau was more easily established.

7.5.4 Results

The ambient NO concentration was variable, although usually did not change quickly but drifted up and down over hours. To demonstrate the appearance of the changing background
NO level, I have enclosed photographs (see Figure 7.6 of two of the recordings made from the NO analyser over a 24 hour period with a slow paper speed.

Figure 7.5: Measurement of exhaled NO incorporating the reservoir system

Diagrammatic representation of the reservoir system allowing both machines and subject to sample from low NO/NO free air; NO, CO₂, expiratory mouth pressure and expiratory flow are all measured.
The ambient air NO concentration ranged from 91-200ppb during these experiments with a mean background NO concentration of 134.5ppb (SEM 11.9). The background concentration of the reservoir system was 1ppb (range 0-2ppb, SEM 0.2). Three subjects completed the experiment (mean age 37 years, all female). The mean plateau NO concentrations during the first set of exhalations with the high background NO was 94.9ppb (SEM 3.4ppb, range 84-101ppb). The mean plateau exhaled NO concentration in the set of exhalations during which both the machine and subject were sampling from the NO-free reservoir system was 123.1ppb (range 103-164ppb, SEM 19.4). The mean plateau NO concentrations during the last set of exhalations with the high background NO was 99.9ppb (SEM 20.5ppb, range 53-151ppb). Figure 7.7 shows a typical trace for one subject showing the rise to NO plateau during an exhalation made from the reservoir system and the fall to an NO plateau during an exhalation made from the high ambient NO. In the direct method, the NO level measured from the reservoir system was 41ppb (95% CI 19.9, 62.7) greater than the measurements made before and after the reservoir system was used (see Figure 7.8). There were no significant differences
in the NO plateaux seen before and after the set of exhalations done with the reservoir (p=0.2, 7, 95% CI -10.9, 35.8, see Table 7.5). No difference was seen in the CO₂ or duration between the three sets of exhalations (see Table 7.6).

Figure 7.7: Recording from one subject showing NO results with exhalations from low and high background NO

Figure 7.8: The effect of high and low pressure on the calibration gas measurement

Comparison of plateau exhaled NO levels following inhalation of high (ambient) labelled as 'pre' and 'post' or low (reservoir) labelled as 'reservoir' NO concentrations. Each point is a mean of five exhalations.
Table 7.5: Exhaled NO results with high and low background NO concentrations

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Ambient NO</th>
<th>Pre (high NO) mean (range)</th>
<th>Reservoir (low NO) mean (range)</th>
<th>Post (high NO) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One (SD)</td>
<td>200.1ppb</td>
<td>119.6ppb (105.8-135.7ppb)</td>
<td>202.4ppb (154.1-225.4ppb)</td>
<td>162.4ppb (98.9-246.1ppb)</td>
</tr>
<tr>
<td>Two (CAB)</td>
<td>136.1ppb</td>
<td>94.9ppb (84.0-100.8ppb)</td>
<td>134.0ppb (102.9-163.8ppb)</td>
<td>100.0ppb (52.5-151.2ppb)</td>
</tr>
<tr>
<td>Three (CMB)</td>
<td>91.4ppb</td>
<td>30.8ppb (28.9-35.2ppb)</td>
<td>32.9ppb (20.3-38.6ppb)</td>
<td>15.8ppb (16.2-22.3ppb)</td>
</tr>
</tbody>
</table>

NB: The 'high' and 'low' NO note in the column headings refers to the ambient NO concentration during each set of exhalations measured, with each result the mean of five exhalations. NO is measured in parts per billion.

Table 7.6: Exhaled CO₂ results with high and low background NO concentrations

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Pre (high NO) mean (range)</th>
<th>Reservoir (low NO) mean (range)</th>
<th>Post (high NO) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One (SD)</td>
<td>CO₂ 5.2% (5.0-5.5) Duration 52.2 secs (45-60 seconds)</td>
<td>CO₂ 5.1% (4.9-5.2) Duration 54.0 secs (36-63 seconds)</td>
<td>CO₂ 5.1% (5.0-5.2) Duration 51.6 secs (39-63 seconds)</td>
</tr>
<tr>
<td>Two (CAB)</td>
<td>CO₂ 5.7% (5.6-5.8) Duration 66.0 secs (63-72 seconds)</td>
<td>CO₂ 5.7% (5.6-5.8) Duration 65.4 secs (57-69 secs)</td>
<td>CO₂ 5.8% (5.7-5.9) Duration 56.4 secs (42-63 seconds)</td>
</tr>
<tr>
<td>Three (CMB)</td>
<td>CO₂ 5.5% (5.3-5.9) Duration 55.2 secs (48-63 seconds)</td>
<td>CO₂ 5.6% (5.4-5.7) Duration 58.2 secs (54-69 seconds)</td>
<td>CO₂ 5.5% (5.4-5.6) Duration 57.0 secs (54-60 seconds)</td>
</tr>
</tbody>
</table>

NB: The 'high' and 'low' NO note in the column headings refers to the ambient NO concentration during each set of exhalations measured, with each result the mean of five exhalations. CO₂ is measured as percentage of total gases and exhalation duration measured in seconds.

7.6 Methodological experiment five – effect of water consumption

7.6.1 Hypothesis

1. Exhaled NO measurements will be affected by the consumption of water.

7.6.2 Aim

1. To compare NO levels measured by standard procedure before and after a set of testing in which water was consumed immediately before each exhalation.

7.6.3 Procedure

Ten adult volunteers were enrolled (eight females) and all measurements were made by the t-piece sampling system.

The first set of exhalations was carried out in the standard manner:
- The subject abstains from food and drink for 4 hours prior to the experiment.
- The subject sits at rest for at least 5 minutes.
- The chart recorder is started 30 seconds prior to each exhalation to document baseline pre-exhalation measurements.
- The subject puts on nasal clips 5 seconds prior to each exhalation and exhales by mouth, and removes the clips between exhalations.
- The subject inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume over 30-60 seconds.
- The exhalations occur 3 minutes apart.
- The subject exhales at a constant mouth pressure of 4mmHg voluntarily controlled by the subject observing the pressure gauge and keeping it at '10' on the scale.
- The subject exhales at a constant flow at 500mls/min voluntarily controlled by the subject observing the rotameter, keeping it at '3' on the scale.
- The subject was breathing ambient air when the natural background level of NO was less than 10ppb.
- The subject continues until 5 exhalations adequate for measurement are made.

For the second set of exhalations:
- The procedure followed the standard set above.
- The subject consumed 60mls of water 20 seconds to 5 seconds prior to the next exhalation. The water temperature was hot in five subjects and cold for five subjects.

The third set of exhalations was carried out in the standard manner detailed above as for the first set of exhalations.

7.6.4 Results

The mean peak NO concentrations in the 10 subjects was 93.7ppb (SEM 20.8). This decreased by 23ppb (95% CI 12.9, 33.1) to 70.8ppb (SEM 16.5, p=0.002) during the measurements made with the subjects drinking 60mls of water before each exhalation. There was then a significant increase of 17ppb (95% CI -9.7, -24.1) to 87.8ppb (SEM 18.7, p = 0.001, see Figure 7.9) in the subsequent recordings made in the standard way. The consumption of water reduced the levels on NO obtained regardless of the water temperature. In the testing with cold water the mean NO went from 76.9ppb to a mean of 57.3ppb, and then returned to a mean of 72.3ppb in the final set of exhalations. With hot water the mean NO of 110.5ppb dropped to 84ppb and then returned to 102.8ppb. There was no difference in
the NO concentrations taken pre and post the water experiment (p=0.095). There was no difference in the CO₂ or duration of expiration across the three sets of exhalations made.

Figure 7.9: The effect of consuming water on the subsequent exhaled NO levels measured

![Graph showing exhaled NO levels](image)

Comparison of peak exhaled NO levels with the exhalation performed in a standard manner pre and post a set of exhalations done where the consumption of either hot (in red) or cold (in black) took place just prior to each exhalation. Each point is a mean of five exhalations.

7.7 Discussion: which measurement factors alter nitric oxide levels?

The methodological experiments described in Chapter 6 and 7 demonstrated that the measurement of NO in exhaled air was feasible. A number of cross-sectional studies looking at exhaled NO concentrations in different subject groups had been reported. The findings within each research team had been consistent, but the absolute levels of NO reported by the investigators in similar subject groups were very different. All these investigators used NO chemiluminescence analysers which, although developed by different companies, had similar sensitivities and they stated that regular calibration was being performed. The study groups were similar. For example, the results in healthy control subjects through the 1990s were reported with means of 3.25ppb, 4.75ppb and 100.25ppb under different conditions (Persson, Wiklund et al. 1993), 4.7ppb (Massaro, Mehta et al. 1996), 6.2ppb (Massaro, Gaston et al. 1995), 8.1ppb (Borland, Cox et al. 1993), 8.4ppb (Persson, Zetterstrom et al. 1994), 8.6ppb (Trolin, Anden et al. 1994), 9ppb (Alving, Weitzberg et al. 1993), 11.1ppb (Martin, Bryden et al. 1996), 16ppb (Schedin, Frostell et al. 1995), 19ppb and 21ppb (Schilling, Holzer et al. 1994), 22ppb (Kimberly, Nejadnik et al. 1996), 26.3ppb (Iwamoto, Pendergast et al. 1994), 34ppb (Jilma, Kastner et al. 1996), 42ppb (Gerlach, Rossaint et al. 1994), 51 NO g⁻¹ (Morris, Sooranna et al. 1996), 70ppb and 75ppb (Kharitonov, Logan-Sinclair et al. 1994), 80.2ppb (Kharitonov, Yates et al. 1994), 88ppb (Kharitonov, Robbins et al. 1995), 89ppb (Kharitonov, Wells et al. 1995) and 105.5ppb (Robbins, Floreani et al. 1996). Similar ranges in NO results
between these researchers were documented in the literature for other subject groups such as those with asthma, bronchiectasis, hypertension and smokers. The measurement of NO in these studies did vary between mean peak exhaled NO, mean plateau exhaled NO and NO measured during tidal breathing. This led me to the suspicion that the technique of measurement critically affected the NO levels obtained. In the first methodological study in twelve healthy adults (Byrnes, Dinarevic et al. 1997), I demonstrated that the concentration of NO obtained could be halved depending on whether the subject was breathing directly into the NO analyser (mean 84.8 ppb) or breathing through a t-piece system (mean 41.2 ppb). The main difference between these two methods was a change in flow from 440 mls/min to 665 mls/min. The methodology experiments detailed in this chapter were therefore designed to determine what factors altered the concentration of exhaled NO obtained.

In the second methodological study, changing the expiratory flow rate significantly altered the mean concentration of NO obtained. The higher the expiratory flow, the lower the concentrations recorded, with a mean decrease of 35 ppb when moving from 250 mls/min to 1100 mls/min in ten subjects (see Figure 7.1). Most of the literature at the time in the cross sectional studies had usually mentioned the sampling rate of the machine only, or measured tidal volumes over a 1-2 minute time periods with none specifically noting a standard expiratory flow. Alving et al reported that if the airflow was increased in 12 healthy controls from 2 L/min\(^{-1}\) to 5 L/min\(^{-1}\) a slight increase in the levels of NO was noted – the data were not shown and the increase was noted to be insignificant. This proved a different finding from most others (Alving 1993). Imada et al showed a hyperbolic relationship between the NO concentration and the sampling flow rate (see Figure 7.10) with a marked reduction of exhaled NO when increasing flow in one subject (Imada 1996).
Kharitonov et al also showed the concentration of exhaled NO decreased from 78ppb in 46 healthy controls and 301ppb in 30 adult asthmatic subjects at an exhaled flow of 1L/min to 7ppb and 46ppb in the groups respectively when the exhalation rate was increased to 5L/min (Kharitonov, Chung et al. 1996). At the same time that the results from our own studies were being published, there were also two studies published that had similarly examined the effect of flow. Silkoff et al described an increase in the mean plateau NO from 3.2ppb to 110.5ppb when the expiratory flow was increased from 4.2mls/s to 1,550mls/s in ten subjects (Silkoff, McClean et al. 1997). Hogman et al reported a reduction in the NO levels with an increase in the expiratory flow from 50mls/s to 300mls/s in 50ml/s increments (see Figure 7.11).

The changes of NO with flow could be explained if (as is now known) most NO is produced proximal to the alveoli. Thus, analogous to a gaseous-phase dye dilution, at high flow rates the proximal NO signal from the upper and lower airways is greatly diluted by NO free (or at least low NO) alveolar gas. I thought therefore the importance of controlling expiratory flow rates clear-cut.
Figure 7.11: Exhaled NO determined at single breath plateau concentrations at increasing expiratory flows and at increasing expiratory mouth pressures


In the pressure experiment, there were no significant differences between the mean exhaled NO concentrations at 4mmHg, 8mmHg, 12mmHg and 16mmHg. In Figure 7.3 it can be seen that most of the ten subjects appeared to have flat traces across the sets of exhalation but the top two and the bottom one subjects’ traces do appear to have a reduction in NO with the increased pressure settings. There was a trend towards differences between the peak CO₂ levels reached being 5.90% at 4mmHg, 5.83% at 8mmHg, 5.83% at 12mmHg and 5.53% reached at 16mmHg. This is likely to be accounted for by there also being differences in the duration of exhalation with 49.9s at 4mmHg, 49.6s at 8mmHg, 44.2s at 12mmHg and 40.9s at 16mmHg. The differences probably reflect the difficulty for subjects of maintaining exhalation against a strong resistance at constant flow to generate the higher mouth pressures. Subjects found it tiring to maintain this pressure and found it difficult to keep their lips tight around the mouthpiece so as not to allow any air to escape when exhaling. When designing the experiment in the preliminary trials both myself and Carolyn Busst tried a range of expiratory mouth pressures up to 20mmHg, and we quickly dropped this last high pressure as being extremely difficult to exhale against. Two other studies published at the same time showed no difference in the NO with differing pressures. In five subjects there was no difference between an expiratory pressure of 6mmHg and 20mmHg (Silkoff 1997, Marked flow dependence). And in six subjects at expiratory pressures over 0 to 20cmH₂O there was no difference in exhaled NO (see Figure 7.9 above, Hogman). It would appear that mouth
pressure over the likely range encountered (such as the lower three pressure settings that I tested) has no relevant effects on the measurements in most individuals. Correspondingly there was no difference in NO, CO₂, flow or duration of exhalation across these readings. However as two, possibly three, individuals did show an effect, this again suggested the need also to standardise mouth pressure for exhaled NO measurements.

The background NO concentration is variable and occasionally can be extremely high. In many of the published studies, the ambient NO level at the time of testing was not stated and I felt this may be another reason for error in the results obtained between and within groups. This could occur particularly if testing was done on different days pre and post an intervention with a subject group when the background levels may differ over time. In three subjects, I showed that there were large differences in the mean exhaled NO concentration obtained depending on the concentration of the NO being inhaled. In this experiment, the exhaled NO measured was higher when inhaling low reservoir NO than when inhaling high ambient levels. Three studies documented inhalation from NO-free air (Alving, Weitzberg et al. 1993; Schilling, Holzer et al. 1994; Steerenberg, Nierkens et al. 2000) with one having noted that the ambient air contained high and variable amounts of NO concentration (6-192ppb) leading them to prepare and recommend an NO free reservoir for inhalation (Schilling, Holzer et al. 1994). Another study mentioned that the ambient NO air was recorded and the absolute zero was adjusted just before each measurement by flushing the NO analyser with NO free certified air (Kharitonov, Logan-Sinclair et al. 1994). A number of studies recorded levels of ambient air at 0-20ppb (Persson, Wiklund et al. 1993), 5-20ppb (Kharitonov, Yates et al. 1996), 0-38ppb (Kharitonov, Yates et al. 1994; Jilma, Kastner et al. 1996; Kimberly, Nejadnik et al. 1996) and 0-68ppb (Kharitonov, Robbins et al. 1995) but did not seem to affect the readings and therefore were not taken into account. One group had specifically requested two volunteers to inhale an NO concentration of 800ppb, hold their breath for fifteen seconds and then exhale into the NO analyser. As they documented no change in the NO concentration before and after inhalation, they concluded that the inspired NO must disappear from the respiratory tract within that time (Kharitonov, Yates et al. 1994).

While the effect of ambient NO was either not specifically tested or was not published at this time by any of these other groups, some researchers did investigate whether inhalation via the nose or the mouth made a difference to the exhaled NO levels measured. This was an area of variability, now known to be the degree of contamination of exhaled NO by nasal and sinus production. The measured NO levels have been found to be greatest in the paranasal sinuses (Lundberg, Farkas-Szallas et al. 1995) with an age-dependent increase in keeping with sinus
pneumatisation. A progressive decrease in NO concentration had been found when sampling progressively down the respiratory tract from nasal passages (Alving, Weitzberg et al. 1993; Kimberly, Nejadnik et al. 1996), oral cavity (Gerlach, Rossaint et al. 1994), and below the vocal cords (Lundberg, Weitzberg et al. 1994). The slow expiration time, particularly in the direct measurements, did raise the possibility that I was merely recording inspired air, contaminated by NO produced from the nose and sinuses, and exhaled unchanged. Schedin et al showed the difference in exhaled NO in tidal breathing when the inhalation was by nose giving a mean of 64ppb and by mouth giving a mean of 13ppb (Schedin, Frostell et al. 1995).

Similarly when single exhalations were measured, nasal inhalation gave exhaled oral results of 32ppb and 22ppb while inhalation by mouth gave exhaled oral results of 9ppb and 9ppb in two other studies (Alving 1993; Kimberly 1996). However using a faster (t-piece) method in our own study did not reveal an abrupt discontinuity suggestive of emptying unchanged dead space prior to measuring true exhaled NO. The other area of difference between groups that was studied by others at this time, which I did not examine, was the effect breath-holding had on the subsequent exhaled NO. Using collection into a reservoir system, Sato et al showed that the NO levels increased in proportion to the duration of exhalation and to duration of a pre-exhalation breath-hold in 16 controls (Sato, Sakamaki et al. 1996). In 18 controls Martin et al reported the increase of exhaled NO from no breath-hold at 11.1ppb to 15.6ppb and 32.1ppb in exhalations following ten and 60 second breath-holds respectively (Martin, Bryden et al. 1996). The findings were similar in 32 subjects with allergic rhinitis from a baseline exhaled NO at 16.2ppb to 34ppb following a ten second breath-hold and 62ppb following a sixty second breath-hold (Martin, Bryden et al. 1996). Kimberly et al in eight controls showed an increase in exhaled NO from 7ppb to 23ppb with a 30 second breath-hold (Kimberly, Nejadnik et al. 1996). Persson et al recorded the most dramatic increase from 3.25ppb with tidal breathing to 100.25ppb with a breath-hold of 60 seconds (Persson, Wiklund et al. 1993). Kharitonov et al determined that a breath-hold for 20 seconds gave an initial peak of NO but end expiration values were similar to non breath-hold results (Kharitonov, Chung et al. 1996). However, I believed that the finding with the ambient NO in this chapter suggested that measurements of exhaled NO must be done on days with low ambient background NO, or by using an NO-free circuit such as described.

The final experiment may seem an unusual test to have performed, but was based on a chance finding that following drinking a glass of water, the NO levels on the next exhalations dropped dramatically. The reason I thought that this may be significant was that when doing lung function or full inspiratory/expiratory manoeuvres, a common side effect, particularly in
subjects with respiratory disease, is to cough and an equally common response is to give the person water to drink. This may be important if the levels of NO were then reduced. Exhaled NO measured in the standard way did decrease significantly from 93.7ppb to 70.8ppb when 60mls of water was drunk between 20 and five seconds before the exhalation, and was not different whether that water temperature was hot or cold. Many studies did not comment on consumption of food or drink prior to the testing. One group reported asking the subjects not to consume caffeine within two hours before the testing procedure (Kharitonov, O'Connor et al. 1995; Kharitonov, Yates et al. 1996). Another group did mention having a water absorber installed in the proximal expiratory port in collections into a reservoir system (Schilling, Holzer et al. 1994). The mechanism of the fall in the exhaled NO that I documented is unknown. One group reported in two papers that the gastrointestinal tract had higher levels of NO present which could be measured when ‘belching’ occurred (Lundberg, Weitzberg et al. 1994; Herulf, Ljung et al. 1998). The levels cited were far greater than those measured during exhalation – so it is possible that the gastro-intestinal tract may contribute to the exhaled NO levels and was reduced by drinking water. Another possibility is that of quenching of the NO gas. One study showed that prolonged sampling of wet gas resulted in an increased time for the initial calibration to reach stable values from five to 20 minutes. They also demonstrated a reduction in exhaled levels with increased water vapour and concluded that a decrease of NO readings in water-saturated samples may have contributed to variation across studies (van der Mark, Kort et al. 1997).

In conclusion, several factors critically affect the measured mean peak concentration of exhaled NO. An increase in expiratory flow rate resulted in a decreased concentration of NO. Although there was no difference at increasing expiratory mouth pressures for most of the subjects, individuals showed decreased exhaled NO concentrations and across all subjects there was a decreased duration of exhalation and CO₂ measurement. A high inspired background NO made the exhaled NO concentrations difficult to interpret, and drinking water just prior to measurement decreased the exhaled NO obtained. These findings confirmed that the measurement of exhaled NO concentrations in humans should be performed in a standard manner for the levels to have any meaning and to enable different teams of investigators to compare results. These findings may have accounted for some of the discrepant results reported in the literature. Since these results were published there has been a considerable increase in the body of literature on exhaled NO with further studies in all these areas and these will be covered to the present day in Chapter 9.
One aim of investigating the parameters that altered the levels of exhaled NO in these methodological studies was to enable standardisations for future studies. In the next chapter, I will describe the exhaled NO research that I did with healthy and asthmatic children. The results presented here in Chapters 6 and 7 did make up some of the literature reviewed for the first American Thoracic Society and European Respiratory Society recommendations to standardise the methods of measuring exhaled NO.

The work in this chapter formed the basis of the publication:

- Byrnes CA, Dinarevic S, Busst CA, Bush A, Shinebournes EA. Is nitric oxide produced at airway or alveolar level? European Respiratory Journal 1997 10 (5) 1021-1025

In addition Mr Ron Logan-Sinclair went on to develop the LR2000 series of NO analysers using some of the ideas generated from this research (Logan Research Ltd, Unit B2, Spectrum Business Centre, Anthony's Way, Rochester, Kent, United Kingdom), while Carolyn Busst went on to run a ski lodge in Switzerland!
Chapter 8: Exhaled nitric oxide in healthy and asthmatic children

8.1 Introduction

The protocol was now established and I felt we would be able to measure NO in a reproducible manner in children by standardising all of the parameters discovered to alter NO readings. Initially I wanted to measure exhaled NO in healthy children: that is children with no respiratory diagnosis or any acute or chronic respiratory symptoms. This was to confirm that the technique was possible in children and to confirm some of the findings from the adult studies presented in the previous chapters. Included was a comparison of the results from the direct versus t-piece sampling systems that incorporated a change in flow. I then wanted to compare the exhaled NO levels from these healthy 'control' children to those from asthmatic children in two categories; children on bronchodilator therapy only versus those on regular IHCS. Finally I wanted to measure the exhaled NO pre and post IHCS commencement in any child who was on bronchodilator therapy only but who clinically required the introduction of a preventer for asthma control.

At the time of commencement of this research there had been very little published on exhaled or nasal NO in children. There had been a series of studies looking at the levels of NO from nasal and sinus passages. High concentrations of NO were found when aspirating air from the sinuses of five patients undergoing surgery at 9.1 parts per million (note that most of the exhaled NO reported is measured in parts per billion) (Lundberg, Farkas-Szallasi et al. 1995). An age-dependent increase in mean nasal NO plateau concentrations was also demonstrated in 49 subjects with an age range of 0-62 years, thought to be consistent with the development and pneumatisation of the paranasal sinuses (diagram included in the next chapter as Figure 9.2) (Lundberg, Farkas-Szallasi et al. 1995). The NO levels sampled directly from one nostril and in exhaled air were also significantly less in four children with Kartagener's syndrome at 4ppb when compared to 20 healthy children at 221ppb (Lundberg, Weitzberg et al. 1994).

The following experiments ran from 1995-1997. At the time that we published these findings in 1996 and through 1997, a number of research groups also published results of their investigations into exhaled and nasal NO in children. In the main these were comparisons between control subjects and those with respiratory diseases. In the next section, I will present the subjects, protocols and results from the studies we conducted in healthy children and discuss the findings comparing with these other results from the literature. In the second part of the chapter I will present the literature available from studies in groups of adult asthmatics as again these were published ahead of any results in children. The procedures followed were
the same for the children with asthma as those undertaken in the healthy children. I will present the results and discuss the findings comparing our results with those then published looking at similar groups of children.

8.2 Ethics, consent, statistics and subjects

Prospective ethical approval for the study was given by the Royal Brompton Hospital Ethics Committee (Royal Brompton Hospital, Sydney St, London SW3 6NP, United Kingdom; now Royal Brompton and Harefield National Health Trust).

The subjects were recruited from a local school (“Park Walk Primary School”, South Kensington, London) within walking distance of the hospital. Following our initial approach and discussion with the school board, the principal and the teachers of the children in the age groups that we were hoping to enrol, a pamphlet about the study and a questionnaire were sent home with children in 2 classes aged 9-11 years to their parent/s or caregiver/s (Appendix 2). The school was familiar with the hospital and had been involved in previous (completely separate) research. Two of the class teachers had participated in an “Asthma at School” education programme run by myself and the respiratory nurse specialist (Ms Lizzie Webber) at the Royal Brompton Hospital which ran for three years and was open to teachers in primary schools throughout London.

Each child participated if:

- He/she had returned a signed consent and a questionnaire filled out by their parent/s.
- He/she was willing to be part of the study.
- He/she had permission of the class teacher to attend the hospital for an hour.
- He/she had no recorded respiratory, cardiac or other major chronic disease. (The exception was children with asthma who were later recruited into the second part of this study).
- He/she had no upper or lower respiratory tract infection for at least four weeks.

The children were walked by the investigators from the school to the hospital, completed the study and returned to the school always having been in our care. The children attended the hospital in pairs from 1030 hours to 1200 hours and were returned in time for lunch break. The aim was to enrol at least 30 paediatric subjects.

The results were analysed using the Statistical Products and Services Solutions, (Package U 7.0, SPSS Inc, Chicago, USA). This employs Students t-test for matched pairs with mean,
standard error of the mean (SEM), standard deviation (SD) and ranges given where appropriate. In the group of asthmatic children that were measured before and after the commencement of IHCS, medians are give. A p-value of less than 0.05 was considered significant.

8.3 Methodology of exhaled nitric oxide measurement in healthy children

8.3.1 Hypotheses

1. NO can be measured in exhaled air in children.
2. NO levels will be different in the two methods of measurement; via the direct and the t-piece sampling systems.
3. NO levels will be higher in children with current or past personal history of atopy such as allergic rhinitis and/or eczema (excluding asthma in the first part of the study).
4. NO levels will be higher in children with a family history of atopy (allergic rhinitis and/or eczema and/or asthma) in first degree relatives.
5. NO levels will be higher in children who may be expected to have environmental reasons for airway inflammation such as environmental smoke or presence of furry pet/s within the household.

8.3.2 Aims

1. To measure exhaled NO in healthy children by two methods; via the direct and the t-piece sampling systems.
2. To compare the NO levels of children with and without a personal history of atopy (having excluded asthma).
3. To compare the NO levels of children with and without a family history of atopy in first degree relatives.
4. To compare the NO levels of children with and without the presence of a household smoker.
5. To compare the NO levels of children with and without the presence of a household furry pet.

8.3.3 Protocol

The questionnaire is added as Appendix 3. Questions were asked regarding information on the following:
The child had abstained from food and drink for two hours prior to the experiment.

*Height* was measured on a Harpenden stadiometer (Harpenden Portable Stadiometer, Crosswell, Crymych, Pembrokeshire, UK) and weight was measured on a digital Seca scale (Seca 770 medical scales, Seca Ltd, 4802 Glenwood Rd, Brooklyn NY11234). Lung function was measured via spirometry and in accordance to the ATS criteria (American Thoracic Society and Association 1994) and the best of three reproducible flow volume loops was recorded using a Compact Vitalograph (Vitalograph Limited, Buckingham, United Kingdom). The results are presented as percent predicted for age, sex and height as defined by the Polgar reference equation. All measurements were conducted by myself (predominantly taking care of the NO readings) or Dr Senka Dinarevic (predominantly taking care of the lung function testing). All results were then worked out by using the calibrations on the chart recording system by both and entered into the SPSS database by myself and checked by Dr Senka Dinarevic.

The experiments were made if the ambient level of NO was less than 10ppb and all inhalation was done from ambient air. The procedures as described in the previous section (Section 6.5) for setting up and calibrating all equipment were made before and after every two subjects. The procedure listed below is re-presented exactly as it was designed and placed on the wall adjacent to the analysers. It is therefore in the present tense and with numerical rather than written designation of numbers.

<table>
<thead>
<tr>
<th>Direct to analysers method:</th>
</tr>
</thead>
<tbody>
<tr>
<td>The child sits at rest for at least 5 minutes</td>
</tr>
<tr>
<td>Each child inhales to total lung capacity then performs a slow vital capacity manoeuvre to residual volume or for as long as possible</td>
</tr>
<tr>
<td>5 exhalations are made consecutively at 3-minute intervals</td>
</tr>
<tr>
<td>Nose clips are worn 5 seconds before the exhalation, and taken off between measurements</td>
</tr>
<tr>
<td>Measurements of NO, mouth pressure and CO₂ are made</td>
</tr>
<tr>
<td>Mouth pressure is standardised to 4 mmHg by a fixed restriction</td>
</tr>
<tr>
<td>The combined analysers sampled at 440mls/min</td>
</tr>
</tbody>
</table>
8.3.4 Results

We recruited 39 healthy children (23 girls, 16 boys) mean age 9.9 years (range 9-11 years) from the local school. They had no current cardiac or respiratory disease, no upper respiratory tract infection for the preceding four weeks and were on no oral medications. Their lung function showed a mean FVC of 99% predicted (SD 11.3), FEV₁ of 95% predicted, (SD 9.0) and FEF₂₅-₇₅% of 88% predicted for age gender and height (SD 12.8). There was no correlation with exhaled NO results and lung function parameters. The correlation coefficient values for the comparison of peak NO levels to the results obtained by measuring area under the curve of the NO signal was 0.75 for the direct measurements and 0.34 for the t-piece measurements.
The following assessments used peak exhaled NO levels in keeping with our work in adults.

The mean level of NO measured by direct exhalation into the analyser was 49.6 ppb, (SD 37.8, range 11.5-197.2 ppb). The mean level of NO measured via the t-piece system was 29.7 ppb, (SD 27.1, range 5.1-141.2 ppb). The reduction of the exhaled NO peak seen between the two systems with the higher flow required for the t-piece measurement was consistent in all subjects and consistent with the results in the adult experiments (see Figure 8.1).

Figure 8.1: Peak exhaled NO results in healthy children

Comparison of the peak exhaled NO results in the direct to analyser and the t-piece sampling system in 39 healthy children with each data point being a mean of five exhalations.

There was no significant difference between the direct mean NO levels in boys at 43.1ppb (SD 40.5, range 11.5-197.2ppb) and girls with mean NO of 55.2 ppb (SD 35.4, range 17.7-124.8ppb, p=0.43). There was also no significant difference between the t-piece mean NO levels in boys at 25.6ppb (SD 29.2, range 7.3-141.2ppb) and girls with mean NO 33.8ppb (SD 25.1, range 5.1-94.3 ppb, p=0.11), (see Figure 8.2a and 8.2b).
No difference was seen when comparing the peak exhaled NO results between 23 girls and 16 boys aged 9-11 years in the direct to analyser sampling system with each data point being a mean of five exhalations.

No difference was seen when comparing the peak exhaled NO results between 23 girls and 16 boys aged 9-11 years in the t-piece sampling system with each data point being a mean of five exhalations.

There was no significant difference between the mean CO₂ levels by direct and t-piece measurements. The mean CO₂ was 5.4% (SD 0.66, range 3.8-6.2%) direct compared to 5.52% (SD 0.66, range 3.9-6.3%) via t-piece measurements (p=0.44). Duration of the exhalation had
a mean value of 32.9 seconds for the direct method (SD 11.4, range 15.8-65.8s) and a mean value of 28.6 seconds for the t-piece method (SD 7.8, range 14.8-48s). The mouthpiece was standardised at 4.0mmHg in both techniques and the flow to 225mls/min in the t-piece method by voluntary control using visual feedback to the children. There was still some variation around this depending on how successful the children were at maintaining the pressure and flow as requested during a slow exhalation from total lung capacity which is shown in Table 8.1.

Table 8.1: Coefficients of variation of peak NO, peak CO₂ and mouth pressure measurements made by both the direct and t-piece systems, and of flow made by the t-piece system

<table>
<thead>
<tr>
<th>Type of measurement</th>
<th>Coefficient of variation</th>
<th>Coefficient of variation range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct method:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak NO in ppb</td>
<td>25.9</td>
<td>2.6-67.4</td>
</tr>
<tr>
<td>CO₂ % total gases</td>
<td>0.44</td>
<td>0.41-0.81</td>
</tr>
<tr>
<td>Mouth pressure in mmHg</td>
<td>22.4</td>
<td>4.5-57.0</td>
</tr>
<tr>
<td>T-piece method:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak NO in ppb</td>
<td>29.9</td>
<td>0.5-65</td>
</tr>
<tr>
<td>CO₂ % total gases</td>
<td>0.44</td>
<td>0.41-0.81</td>
</tr>
<tr>
<td>Mouth pressure in mmHg</td>
<td>12.5</td>
<td>2.9-36.1</td>
</tr>
<tr>
<td>Flow in mls/min</td>
<td>14</td>
<td>1.8-30.3</td>
</tr>
</tbody>
</table>

Comparisons were made within this group of children of other factors that could result in airway inflammation which may give higher results of exhaled NO. Regarding a personal history of atopy; the question sent home to the parents was:

- Has he/she ever had eczema? Yes/No
- Has he/she ever had hay fever? Yes/No
- Does he/she have eczema/hayfever now? Yes/No

There were eleven children that had a personal past or current history of allergic rhinitis compared to 28 children who did not. At the time of testing none had current nasal symptoms but three did have current eczema and were using appropriate topical creams including hydrocortisone cream. There was no significant difference in the exhaled NO levels in the atopic children at 34.5ppb (SD 11.8) compared to non-atopic children at 45.6ppb (SD 38.5) via the direct method or via the t-piece method measured at 29.6ppb (SD 24) versus 24.8ppb (SD 26.2) respectively. We also looked at whether family history of atopy had any effect on the exhaled NO levels in the children. The question sent home to the families was:
Any first-degree relative (parent or sibling) who was stated to have at least one of the conditions listed was counted as a positive and any more distant relative or no family member having any of the conditions listed were counted as a negative. Of the 39 children, 18 had a positive family history of atopy compared to 21 that did not and there were no differences seen in the direct exhaled NO levels at 40.6ppb (SD 26.7) versus 44.0ppb (SD 38.9), or in the t-piece levels 25.3ppb (SD 21.4) versus 27.0ppb (SD 29.0).

The possibility of smoking causing airway inflammation and affecting the results of exhaled NO was explored. The question sent home to the parents was: “Does anyone in the family smoke? Yes or No” When the questionnaires were checked through with the children it was determined whether the family smoker lived in the household. There were no differences seen in the 19 children who lived with one smoker in the household compared to the 20 children from non-smoking households at 37.1ppb (SD 17.4) versus 49.0ppb (SD 45.8) via direct and 22.2ppb (SD 20.4) versus 31.1ppb (SD 30.3) via the t-piece system. The children were not asked about active smoking.

The possibility of a reaction to pet dander causing airway inflammation and affecting the results of exhaled NO was explored. The question sent home to the parents was: “Do you have any pets? If yes please name type”. When the questionnaires were checked through with the children the type of pet was checked again. Cats, dogs and any furry animal living within the house were counted as positive, no matter the number involved. Fish and birds were counted as a negative, as were not having pets. There were no differences in the peak exhaled NO between those thirteen children who had a household furry pet (or pets) and those 26 that did not at 39.3ppb (SD 29.2) versus 43.9ppb (SD 35.7) via direct and 23.5ppb (SD 23.2) versus 27.6ppb (SD 26.7) via the t-piece system. There was no attempt made to look at possible dose-response for any of these questionnaire replies.

8.4 Discussion: exhaled nitric oxide results in healthy children

This study confirmed that it was possible to measure exhaled NO levels in children aged between nine and eleven years by using these two different techniques. As in the adult studies there was a consistent difference between the NO levels obtained with the direct and the t-
piece methods. The main difference between these techniques is an increased flow of 225mls/min, and the adult technical studies revealed that with increasing flow there was a decreasing level of exhaled NO obtained. In this case, an increase in flow of 52% led to a reduction of exhaled NO of 40%.

The study was not designed to look at age-related differences in exhaled NO, as had been suggested by Lundberg et al as occurring with possible development and pneumatisation of the sinuses (Lundberg, Farkas-Szallasi et al. 1995). The children studied were in a narrow age range to try and standardise the results and to limit other extraneous effects. The age was chosen as those likely to be able to do lung function tests and therefore complete the exhaled NO testing successfully. However the results in these ‘normal’ children were significantly lower than the results obtained from the twelve healthy adults studied initially except for the comparison of t-piece measurement in males. The children had mean exhaled NO level 49.6ppb compared to 84.8ppb in adults via direct measurement and mean NO 29.7ppb compared to 41.2ppb in t-piece measurement. The boys had a mean exhaled NO of 43.1ppb (direct) and 25.6ppb (t-piece) compared to 71.7ppb (direct) and 33.2ppb (t-piece) of male adults. The girls had a mean exhaled NO of 55.2ppb (direct) and 33.8ppb (t-piece) compared to 92.4ppb (direct) and 50.7ppb (t-piece) of female adults. The differences were more significant for the direct than the t-piece technique. There was no difference within this close two year age band, though it was noticeable that the nine year old children found it more difficult to control expiration within the prescribed limits than the older children did. This narrow and young age bracket was also chosen to try and enrol children that were prepubertal following suggestions that exhaled NO may vary through the menstrual cycle (Kharitonov, Logan-Sinclair et al. 1994). This ruled out a confounding factor if it was later discovered that pubertal change had effects on exhaled NO in either male or females, although, in fact, this remains unknown. In both the children and the adults there was no significant difference in exhaled NO between the genders.

There were significant differences between the duration of exhalation with the mean being 32.9 seconds for the children and 56.2 seconds for the adults in the direct measurements, and 28.6 seconds for the children and 53.5 seconds for the adults in the t-piece measurements. Three children had long exhalations of greater than 50 seconds for all of their direct method exhalations and longer than 40 seconds for all the t-piece method exhalations and all had the higher levels of peak CO₂ measured. Interestingly, these three had swimming training and were on representative teams either at the school and/or swim clubs.
There are limitations with regard to the questionnaire data. The questionnaire was developed as one of convenience and piloted on ten families in the paediatric general respiratory and asthma clinics to ensure that it was easy to understand and easy to fill out. Two modifications were made as a result of this — the description of wheeze was added, and the words for describing amount of cough were changed. Park Walk School is an English-speaking school but did have families of many nationalities so English was not necessarily the first language of all the parents who were filling out the questionnaires and signing the consent form. The questionnaire did not go through a validation process, save our own initial pilot. We did not interact with the parents of the children directly. The questionnaires were distributed by the teachers through the two classrooms and returned to the teacher from those happy to participate. We did not have any data on the children who were given the questionnaire and did not return it or did not gain consent for the study. I therefore cannot compare the demographics of those who participated and those who did not to see if there were any major differences between them. Forty-six children attended the hospital for the study from a possible fifty-six in two classes. The classes were not stable through the study period with additional movement of six children moving into and out of the classes because of moving within the school and or two children from one family leaving the school altogether. Two children that attended the hospital were ruled out because of inability to do lung function successfully, although interestingly both were able to complete the exhaled NO tests. Five children had asthma, four on bronchodilator therapy only and one on regular IHCS therapy who briefly attended my asthma clinic — these children were measured but not documented in this segment of the study. The teachers ruled out seven children either because they had special educational needs or because of misbehaviour — I do not know whether their families had or had not consented. This gave us a response of 73% overall or 63% successful 'normal' subject results.

The questionnaire responses were checked with the children on the day that they were brought to the hospital to study. However it was obvious that the children were confident on some of the responses and less confident on others. They tended to be confident on name, age, nationality, current conditions such as asthma, hayfever or eczema, current medications, the presence of family pets or presence of household smokers. They were less confident, and there was more variability among the children about the degree of confidence, on answers regarding recent 'colds', past history of conditions and family history of conditions, and for these we had to rely predominantly on the written responses. We had documented nationality but did not look at differences between them because of low numbers. The six nationalities
were loosely grouped as British, Indian, Arabic, African, European and South American with one to fifteen children in each group.

**We prospectively elected** to enter into the database a positive or negative response to the questions and not categorise answers any further. We therefore did not investigate possible dose-response relationships for atopy (*personal or family*), passive smoking or presence of pets. For example, we did not try to quantify the numbers of cigarettes smoked by the one or more household smokers and look at the exhaled NO levels in this group. Likewise we did not quantify the number of atopic responses within the family and see if there was any correlation between atopy severity and exhaled NO. This was for a number of reasons. Firstly, as mentioned we were relying on questionnaire data which could be variably accurate. Secondly, in the straight ‘positive and negative’ categorisation of responses there were no significant differences that I thought should be further investigated in this setting. Thirdly, the numbers with a positive response to one of these areas ranged from eleven to nineteen so the numbers were becoming small. Finally, it would have been difficult with these small numbers to quantify one response in exclusion of other responses. At the time, our sample of 39 children was the largest study in this area of research but the lack of findings of these factors that might cause some degree of airway inflammation may be secondary to lack of numbers causing a type two statistical error.

Despite the questionnaire and normal lung function, we had one boy who was an outlier with direct peak NO of 197.2ppb and *t-piece peak NO* of 141.2ppb which was 73ppb and 43ppb higher than the next highest result. On history, he occasionally wheezed with viral infections and, following parental permission had 20% fall in FEV1 at 2 mg/ml histamine on challenge. It is possible he had mild asthma or was on the extreme of the normal range of airway reactivity. He was a European boy with no personal or family history, from a non-pet owning, non-smoking household.

By time of publication of this study, other investigators during the same period had also begun to examine the measurement of NO in children, both in normal subjects and those who with a range of respiratory diseases. In the main, the control children studied had no respiratory disease, were on no medications and usually were recorded to have no upper respiratory tract infection for between two and six weeks prior to the studies. Unfortunately, as mentioned in the previous chapters, with all the early research each individual research group developed their own techniques and thus every group utilised a different method of *measurement*. As with the adult data, the absolute results in the healthy children differed
significantly between research groups, partly because of different techniques utilised. Lanz et al reported a mean exhaled NO of 14ppb (+/- 2ppb) in seven children with single exhalations from total lung capacity into a reservoir bag that was then fed through the NO analyser (Lanz, Leung et al. 1997). They also showed that there was no difference in the NO levels when re-measured five days later (Lanz, Leung et al. 1997). Using a similar method of slow exhalation into a reservoir and then put through the analyser, Nelson et al reported a much lower mean exhaled NO of 5.05ppb (+/- 0.4ppb) in 21 children (Nelson, Sears et al. 1997). Baraldi et al showed that NO reached a steady plateau when measured during tidal breathing after one to two minutes and reported mean levels of 5.4ppb in 16 children (Baraldi, Azzolin et al. 1997). Lundberg et al also measured plateau oral exhaled NO levels with tidal breathing and reached a similar figure of 4.8ppb (SD 1.1) in 19 children (Lundberg, Nordvall et al. 1996). Balfour-Lynn et al measured NO from single exhalations taking the point of NO when the end tidal CO₂ reached a plateau in an attempt to compare alveolar levels for both oral and nasal measurement. The 57 control children had a mean oral exhaled NO of 4.8 ppb (range 1.1 to 23ppb), and mean nasal NO of 1024 ppb (range 158 – 2502ppb) (Balfour-Lynn, Laverty et al. 1996). Two studies measured controls across wide age ranges that incorporated an unknown number of children. Dotch et al measured exhaled NO from single exhalations although the response time of the analyser was not fast enough at 25 seconds for a 90% response rate to measure one breath, so the subject was required to exhale four to five times into the analyser via mouth inhalation and with nose clips in place. In 68 controls aged four to 34 years the mean NO result was 3.0 ppb (+/-2.5ppb) by these single exhalations, with a minute ventilation concentration of NO of 25 ppb (+/- 27 ppb) and direct nasal NO level of 96 ppb (+/- 47 ppb) (Dotch, Demirakca et al. 1996).

In another study, Grasemann measured NO by single exhalations to an analyser that had a 10 second response time. The mean exhaled NO level in 30 control subjects aged six to 37 years was 9.1 ppb (+/- 3.6ppb). Lundberg et al measured the plateau NO signal by continuous sampling from oral exhalation or nasal exhalation or by direct nasal measurement in 19 children. The mean oral level was 4.8ppb (SD 1.1), the mean nasal level was 21ppb (SD 9.1) and the mean directly measured nasal level was 239ppb (SD 20) (Lundberg, Nordvall et al. 1996). Most of these researchers therefore reported absolute levels of exhaled NO that were less than the results that I have reported from my studies. However I had elected to use single exhalations measured immediately by the analysers, while these others used tidal breathing or plateau measurements with or without a reservoir technique over several breaths.
In comparison to the early literature in the adults, the research groups studying children appeared to more often consider the effect of ambient NO. Our experiments were only undertaken if the ambient NO was less than 10 ppb, as I had already shown in the previous methodology experiment in adults (Section 7.6) that the ambient NO did affect the exhaled NO levels. Similarly, Grasemann et al commented that “In preliminary experiments we observed that the higher NO concentrations in ambient air were associated with increased exhaled NO concentrations.” (Grasemann, Michler et al. 1997). Ambient NO in this paper was reported at between two and 22 ppb in one paper which authors thought unnecessary to take into account, but the exhaled levels in this paper were 1.1 to 23 ppb in the control children which was exactly this ambient range (Balfour-Lynn, Laverty et al. 1996). One group noted that when measuring the exhaled levels in their control group it did not change when measured in ambient or NO-free air, thus all their subsequent experiments were carried out inhaling room air. However at the time their ambient NO level was reported as 3.95 ppb +/- 0.98 ppb and their NO free air at 3.97 +/- 1.14 ppb so there was no difference in the inhaled levels and this ambient level may have changed over time (Nelson, Sears et al. 1997). Finally one group stipulated that they did all their measurements with the children inhaling NO-free air (Baraldi, Azzolin et al. 1997).

We found no correlation between exhaled NO levels and gender or lung function parameters. Nelson et al also found that there was no correlation between NO and height, weight, gender or FEV1 in either the control or asthmatic children (Nelson, Sears et al. 1997). Balfour-Lynn et al found no correlation between exhaled NO and FEV1 or FVC across control and CF groups of children (Balfour-Lynn, Laverty et al. 1996).

In our study, we have documented that exhaled NO could be measured in children, reporting normal values obtained with these techniques. As the continuation of this research, I then moved on to investigate the exhaled NO levels in asthmatic children.

8.5 Methodology of exhaled nitric oxide measurement in asthmatic children

8.5.1 Background

Presence of higher levels of exhaled NO in asthma were first indicated in two animal models where exhaled NO significantly increased following induction of an asthma exacerbation using ova-albumin sensitised guinea pigs and rabbits (Persson and Gustafsson 1993; Endo, Uchido et al. 1995). There followed a number of publications in adult subjects showing that exhaled NO levels appeared to be raised in patients with asthma when compared to healthy
controls. Kharitonov et al reported levels of 283ppb in 61 asthmatics compared to 67 controls at 80.2ppb when measured with single exhalations (Kharitonov, Yates et al. 1994). Persson et al measuring mixed exhaled air found levels of 10.3ppb in 23 asthmatics compared to 20 controls at 8.4ppb (Persson, Zetterstrom et al. 1994). Massaro et al using a similar technique found levels of 13.2ppb in five asthmatics compared to five controls at 4.7ppb (Massaro, Mehta et al. 1996). Robbins et al measured single exhalations and a reservoir collection in 18 asthmatics and 91 controls, reporting significant differences between the groups with both techniques, of 174ppb compared to 105.5ppb and 27.2ppb compared to 14.5ppb (Robbins, Floreani et al. 1996). Alving et al demonstrated no overlap between their control group with a range of 5-16ppb and their asthmatic group who had a range measured at 21-31ppb (Alving, Weitzberg et al. 1993). Martin et al also found significant differences between 18 control subjects and 32 patients with allergic rhinitis when measured by single exhalation with 11.1ppb compared to 16.3ppb, following a ten second breath-hold at 15.6ppb compared to 34.0ppb and following a 60 second breath-hold at 32.1ppb and 62ppb (Martin, Bryden et al. 1996). Massaro demonstrated that the NO levels were even higher at 13.9ppb during a period of acute asthma in seven patients requiring emergency department treatment. A reduction of NO began after 48 hours which soon became indistinguishable from control NO levels at 6.2ppb (Massaro, Gaston et al. 1995). Kharitonov et al showed a reduction from 203ppb to 120ppb over three weeks in eleven asthmatic patients and an increase on their mean FEV₁ as percent predicted from 92% to 99% after commencing 800μgs budesonide dipropionate twice per day, with no corresponding change when the same patients were randomised to placebo (Kharitonov, Yates et al. 1996). As by far the greatest amounts of NO in vitro were shown to be produced from iNOS stimulation, these high levels in asthma were hypothesised by these groups to be a measure of airway inflammation (Barnes 1993; Barnes and Kharitonov 1996). Following measurement of exhaled NO in healthy children, I was then interested to assess whether these same findings as had been documented in adults could be found in children with asthma.

8.5.2 The asthmatic subjects

The children were recruited from the paediatric respiratory or asthma outpatient clinics at the Royal Brompton Hospital where I was seeing patients. Five children were also enrolled from the Park Walk Primary School (four on bronchodilators only and one on regular IHCS therapy who was subsequently also seen in the asthma clinic). They were presented with the same questionnaire as used for the control children. Following informed consent from their parent/s or caregiver/s, the children were enrolled if:
There was a signed consent form and a questionnaire filled out by their parents.

The child was willing to be part of the study.

The child had a doctor diagnosis of asthma.

The child was on either bronchodilator therapy only or bronchodilator therapy and long term IHCS therapy. No child studied was on any other asthma medications (such as long acting β2 agonist therapy, theophylline or oral steroids). Some of the children were on topical treatments for eczema.

There had been no change in their asthma treatment in the last six weeks.

There was no current or recent upper or lower respiratory tract infection for at least four weeks.

The child had recorded no other respiratory, cardiac or other major chronic disease.

The ethical consent obtained for the study and the statistical analysis have been documented previously in Section 8.2.

8.5.3 Protocol

The protocols for the asthmatic subjects were identical to those used in the control subjects and are listed in Section 8.3.3 above. The two techniques of measurement were used: the direct to analysers method and the t-piece sampling method. The children did one set of five exhalations under each condition and continued until five sets of measurable exhalations were made, or it became obvious that either the procedure was too difficult or it was exacerbating their asthma. As the children were enrolled from either the morning clinics or the school (as previously organised) all the children were measured at the same time during the day – late morning between 1000 hours and 1300 hours.

8.5.4 Results

In total 31 asthmatic children were recruited for the studies of exhaled NO in asthma: fifteen were on bronchodilator treatment only and 16 on regular IHCS therapy. The children with asthma on bronchodilator treatment only had an FVC of 92% (SD 14.5) and FEV₁ of 78% (SD 10.4) percent predicted by the Polgar reference equation (Polgar and Promadhat 1971). The children with asthma on regular IHCS had a mean FVC of 98% (SD 18.5) and FEV₁ of 86% (SD 17.5). The mean peak NO level measured by the direct method in asthmatic children on bronchodilator treatment only was 126.1ppb (SD 77.1, range 14.4-361.1ppb) which was significantly higher than compared to the healthy children (mean 49.6ppb, SD 37.4, range 11.5-197.2ppb see previous results, p<0.001). The mean peak NO level measured by the
The mean peak NO level measured in asthmatic children on bronchodilator treatment only was 109.5ppb (SD 106.8, range 13.4-421.4ppb) which was significantly higher than in the healthy children (mean 29.7ppb, SD 27.1, range 5.1-141.2ppb see previous results, p<0.001). The mean peak NO level measured by the t-piece method in asthmatic children on regular IHCS therapy was 45.2ppb (SD 45.9, range 1.7-198.0ppb) which was significantly lower when compared to the asthmatic children on bronchodilator treatment only (p<0.001). There was no significant difference between the NO levels in normal and asthmatic children on regular IHCS therapy (p=0.21) (see Figure 8.3b).

Figure 8.3a: Mean peak exhaled NO levels in control and asthmatic children measured direct to the analysers
Mean peak exhaled NO levels in healthy children (n=39), asthmatics on bronchodilator therapy only (n=15) and asthmatic children on regular IHCS therapy (n=16) measured by the t-piece sampling system. Note the Y scale is discontinuous to accommodate the outliers.

There were no differences in the CO₂ levels, mouth pressures, and durations of expiration between the different groups or between the two methods within each group as noted in Table 8.2.

Six asthmatic children on bronchodilator treatment only but deemed clinically to require the introduction of IHCS therapy to improve their asthma control were recruited for the longitudinal study. Prior to commencing steroids the median exhaled NO was 124.5ppb (range 67.6-330.6ppb). Following treatment for two weeks on either budesonide dipropionate 400μg twice per day in five subjects or budesonide dipropionate 200μg twice per day in one subject all delivered via a Turbohaler®, NO fell to a median level of 48.6ppb (range 36.8-153.6ppb). This reflected a decrease in all the children with only one subject now having a different result from that observed in normal children (see Figures 8.4a and 8.4b).
8.6 Discussion: exhaled nitric oxide in asthmatic children

Asthma is a chronic disease of airway inflammation that is treated with anti-inflammatory drugs. However in the routine clinical setting we measure lung function rather than any inflammatory parameters. The gold standard of assessment of airway inflammation is bronchoscopy and bronchial biopsy as discussed in Chapter 1. Research then began to suggest that NO could be measured in exhaled air and may be related to airway inflammation in adult asthmatics (Alving, Weitzberg et al. 1993; Kharitonov, Yates et al. 1994; Persson,
Zetterstrom et al. 1994; Kharitonov, Yates et al. 1995; Massaro, Gaston et al. 1995; Kharitonov, Chung et al. 1996; Martin, Bryden et al. 1996; Massaro, Mehta et al. 1996; Robbins, Floreani et al. 1996; Kharitonov, Rajakulasingam et al. 1997). In the experiments above we set out to assess whether the pattern of NO excretion was similar in children as determined in adults. In 39 children with no known respiratory problems it was possible to measure exhaled NO with mean levels of 49ppb (direct) and 29.7ppb (t-piece). I have also shown that there was a significant increase in mean NO concentrations to 126.7ppb (direct) and to 109.5ppb (t-piece) in asthmatic children receiving bronchodilator treatment only. There was a significant decrease in children on regular IHCS therapy with mean NO levels of 48.7ppb (direct) and of 42.5ppb (t-piece). There was no difference between the exhaled NO levels of the healthy children and the stable asthmatics on regular IHCS therapy. There was no significant difference in the mean age of the children in the three groups, although the age range of the children recruited from the outpatient clinics was greater than those in the control group. So while the effect of puberty and age was controlled for the healthy children, it is possible that this may have affected results in the other two groups (Lundberg, Farkas-Szallasi et al. 1995; Franklin, Taplin et al. 1999). The lowest age of a child that we successfully studied was six years. All of the testing was completed in the mornings between 1000 hours and 1300 hours so we could disregard any concerns regarding circadian rhythm effects (Mattes, Storm van's Gravesande et al. 2002). The pattern of NO exhalation appeared to be the same as the patterns seen in the adult subjects, and the pattern was the same in asthmatic children as in healthy children, although the levels seen in children were lower. Similar to the results from the adult subjects in the methodological experiments, we saw a reduction of the NO levels between the direct and t-piece method of sampling in all the groups of children. However the magnitude of the reduction was different. There is an increase from 440mls/min in the direct sampling technique to 665mls/min in the t-piece sampling method, an increased flow of 51%. This led to a 50% reduction of the NO levels in the adult experiment (methodological experiment one described in Chapter 6), a 40% percent reduction in the healthy control children, a 24% reduction in the asthmatic children on bronchodilator treatment only and an 8% reduction in the asthmatic children on IHCS therapy. The variation seen when measuring children was greater than that in adults, and there was a wide range of exhaled NO levels within all three paediatric groups. I have previously discussed one boy who was an outlier in the group of healthy children. One subject recorded as an asthmatic on bronchodilator treatment only had mean exhaled levels of 14.4ppb via the direct method which was 53ppb lower than the next value, and 13.4ppb via the t-piece method which was 17ppb lower than the next value. She had not had any episodes of asthma for two years and
had last used her β2 agonist inhaler ten months previously. While there had been an increase noted of exhaled NO levels with upper respiratory tract infections (Kharitonov, Yates et al. 1995), none of the children tested had had a respiratory tract infection, an exacerbation of asthma within four weeks and any changes in medication for six weeks. Interpretation of single NO results, however, must be made with caution. In a small group of six children measured before and two weeks after starting ICHS, the exhaled NO levels dropped significantly from a median of 124.5ppb to 48.6ppb and in all but one subject had returned to the normal range. This suggested that the exhaled NO could be a useful monitoring tool for airway inflammation in asthma.

A number of groups also published in the area of exhaled NO in paediatric controls, asthma and cystic fibrosis through 1996 and 1997. Lanz et al looked at three groups of children: seven asthmatics, six atopic but non-asthmatic children and seven controls with no asthma all aged between eight to eighteen years with exhaled NO at 52ppb (+/- 5ppb), 16ppb (+/- 2ppb) and 14ppb (+/-2ppb) respectively. The exhaled NO in the asthmatic group decreased to 14ppb (+/-1ppb) when treated with oral steroids for 14 days (Lanz, Leung et al. 1997). The other two groups were re-measured five days later and showed no difference in their exhaled NO readings. By a different method, Nelson et al measured expired NO concentrations via a slow exhalation into a reservoir system, which was then sealed and subsequently run through the NO analyser. Twenty one control children aged five to eighteen years had a mean NO of 5.05 ppb which was significantly lower than thirteen asthmatic children with a mean exhaled NO at 16.3pp. This decreased linearly as airflow obstruction improved with commencement of systemic steroid therapy of prednisone 2-4mg/kg/day. However, even when five of the asthmatic patients had normalisation of their lung function, the exhaled NO was still higher than the control children at 13.5ppb (Nelson, Sears et al. 1997). This group speculated that “NO assays may prove to be a more sensitive measure of childhood asthma than spirometry.”

Baraldi et al measured sixteen children aged six to thirteen years during tidal breathing with children inhaling NO free air with all measurements. NO reached a steady plateau during oral breathing after one to two minutes. The mean level in the normal children was 5.4ppb (+/-0.4ppb) significantly lower than sixteen children measured during an acute asthmatic attack at 31.3ppb (+/- 4.2ppb) and although these levels decreased with a five day course of oral steroids (prednisone 1 mg/kg per day), the mean levels remained significantly higher than controls at 16.5ppb (+/- 2.3ppb) (Baraldi, Azzolin et al. 1997).

Two studies looked at NO levels in children with allergic rhinitis and/or sinusitis. In 36 asthmatic children all taking IHCS, there was no difference with direct nasal sampling of NO
levels between the asthmatics with allergic rhinitis at 252ppb (SD 20) compared to those without rhinitis at 256ppb (SD 26) (Lundberg, Nordvall et al. 1996). In sixteen children with acute maxillary sinusitis the mean nasal concentration was 70 ppb (+/- 8.7ppb) increased to 220ppb (+/- 15ppb) after oral antibiotic therapy. In comparison nine children with upper respiratory tract infections but not thought to have sinusitis had mean nasal NO levels of 249ppb (+/- 32ppb), which did not change after oral antibiotic treatment (Baraldi, Azzolin et al. 1997).

In studies comparing children with asthma and children with CF, Lundberg et al also compared the plateau oral exhaled NO levels demonstrating no difference between 19 control children and eight children with CF at a mean of 4.8ppb (SD 1.2) and 5.8ppb (SD 0.8) respectively. However there was a significant increase in the 36 children with asthma to 13.8ppb (SD 2.5). These higher levels were seen in the asthmatic children despite their taking a range of medication with twelve on low dose IHCS (defined as 0-100µgs budesonide or equivalent per day), 16 on moderate doses of IHCS (defined as 200-400µgs budesonide or equivalent per day) and eight on high doses of IHCS (defined as 600-800µgs budesonide or equivalent per day). I note there appear to be gaps in these ranges i.e. 100-200µgs and 400-600µgs which are not discussed but it is likely that there was no child on treatment in these ranges which would have required odd dosing regimes. With nasal exhalation sampling, there was no difference between the control group or this asthmatic group (recalling that all were on IHCS therapy) at 21ppb (SD 9.1) versus 27ppb (SD 2.6), although a possible trend was noted of a decreasing NO when on the higher steroid doses. There was also no difference in these two groups when having direct nasal sampling (via a nasal olive) with the controls measured at 239ppb (SD 20) and the collective asthmatic group measured at 254ppb (SD 17). However in both the nasal measurements those that were able to perform the technique from the group of children with CF showed significantly lower levels with their nasal breathing giving levels between 9-15ppb and their nasal direct sampling giving levels between 40-105ppb (Lundberg, Nordvall et al. 1996). In 68 controls, 90 asthmatics and 67 subjects with CF with a collective age range from four to 34 years, Dotsch et al used a slowly responding analyser measuring a series of single exhalations and showed a correlation between exhaled NO levels and the ambient NO concentration. They then reported results between the three groups studied only in the subjects that were measured during days of zero ambient NO concentration (Dotsch, Demirakca et al. 1996). The mean level of exhaled NO in 30 asthmatics aged four to fourteen years was 8.0ppb (+/- 6.1ppb), significantly higher than in 37 controls aged four to 34 years at 3.0ppb (+/- 2.5ppb). Twenty three patients with CF aged five to 32 years tended to have a
higher level of exhaled NO at 4.9ppb (+/-2.6ppb) than the controls but this did not reach
significance. They also found no correlation between NO and the lung function in any group
(Dotsch, Demirakca et al. 1996). In another study, Grasemann et al measured NO by single
exhalations to an analyser that had a 10 second response time across a wide age range of
subjects (Grasemann, Michler et al. 1997). The mean exhaled NO level in 30 control subjects
aged six to 37 years was 9.1 ppb (+/- 3.6ppb) which was significantly lower in 27 subjects
with CF aged six to 40 years at a time of disease stability at 5.9ppb (+/- 2.6ppb). This group
commented that: “In preliminary experiments we observed that the higher NO concentrations
in ambient air were associated with increased exhaled NO concentrations.” In view of this,
they went on to calculate the difference between the NO levels taking into account this
ambient NO concentration and still found a significant difference with the control group
having levels of 5.0ppb (+/-3.1ppb) compared to the CF group at 1.5ppb (+/- 1.2ppb)
(Grasemann, Michler et al. 1997). Finally Balfour-Lynn et al measured plateau exhaled NO at
the time of CO2 plateau by placing a plastic nose piece just inside one nostril which was then
connected to the Teflon tubing of the NO analyser. They showed a significant difference
between the nasal measurements from 57 control children at 1024ppb (95% CI 896-1152ppb)
when compared to children with CF; in thirteen on IHCS at 522ppb (95% CI 313-730ppb) or
50 not on IHCS at 460ppb (95% CI 399-520ppb). There was no difference in the exhaled NO
levels in these two groups and the use of IHCS did not affect the exhaled oral levels of NO in
the CF group with the results being means of 4.8ppb (95% CI 3.8-5.8ppb) in control children,
4.7ppb (95% CI 4.0-5.3ppb) in those with CF not on steroids and 3.6ppb (95% CI 2.5-4.8ppb)
in CF children on steroids. There was also no significant difference in the NO levels in the CF
group between those 26 colonised with Pseudomonas aeruginosa compared to the 24 non-
colonised. There did appear to be a difference with a lower level in those 31 colonised with
Staphylococcal aureus at a mean of 4ppb (95% CI 3.4-4.6ppb) and those 19 which did not
have this organism at 5.8ppb (95% CI 4.4-7.2ppb). As there were 63 children in total there
must have been some considerable overlap between having Staphylococcal aureus and/or
Pseudomonas aeruginosa, plus the correct identification of the true presence of lower
respiratory tract pathogens in this age group with CF is known to be difficult so the overlaps
within these groups may have obscured findings (Balfour-Lynn, Laverty et al. 1996).

This completed my work in this area. I had demonstrated some key findings of factors that
affected exhaled NO measurements and would require standardisation for procedures in the
future. These were expiratory flow, expiratory mouth pressure, the need for low ambient NO
levels or to inhale from a reservoir of NO free air, and the need to prevent water consumption
during the testing procedure. I had demonstrated that it was possible to measure exhaled NO in children, both healthy controls and asthmatics. In fact in some of the control children, they found the exhaled NO measurements an easier task than completing lung function testing to ATS criteria standards. I had demonstrated significantly higher levels of exhaled NO was found in children with asthma on bronchodilator therapy only when compared to healthy children and asthmatics on regular IHCS therapy, with no difference between the latter two groups. Finally I had also demonstrated in a small group of asthmatic children the exhaled NO levels reduced significantly following two weeks of IHCS treatment. These findings contributed to the evolving literature to suggest that NO was a marker for airway inflammation in asthma. I was invited with other research groups working in this area to present this data to a European Society Task Force (ESTF) meeting in Stockholm in September 1996 as a satellite working party to the European Respiratory Society Annual Conference where the first discussion regarding standardisation procedures took place. This later became the basis for the first publication setting out the best standard practices based on the data available (Kharitonov, Alving et al. 1997).

In the following chapter I will review how research in exhaled NO and nasal NO measurement has progressed. I will review the findings on factors that altered NO results in both healthy groups and those with respiratory diseases. I will show how the standardisation procedure for testing has continued to evolve to the present day. I will then review the findings in the literature across adults, children and infants with a variety of conditions. I will end with an opinion as to the best use of exhaled and nasal NO testing is at the current time.

The work in this chapter formed the basis of the publications:

Chapter 9: Exhaled and nasal NO to today

9.1 Introduction

Having already presented my own research and preliminary discussion around the findings, this chapter reviews where the research on exhaled NO had reached by 2000 and describes the continued progression in this field to 2006. I will begin with a review of the official statements from two international respiratory societies — the European Respiratory Society (ERS) and American Thoracic Society (ATS) — both of which aimed to standardise the techniques of NO measurement. I will present what is now known regarding NO in health and in respiratory diseases in adults, children and infants. In the final paragraph I will give my opinion as to where I believe this information is most useful.

9.2 Technical factors affecting results across research groups

By the end of the 1990s, exhaled and nasal NO had been measured in many groups; in normal subjects (both adult and paediatric populations), as well as in those with asthma, non-asthmatic atopy, chronic obstructive pulmonary disease (COPD), chronic bronchitis, cystic fibrosis (CF), ciliary disorders, bronchiectasis and interstitial lung disease. As alluded to in previous chapters, some findings remained consistent across the research groups. The best examples of consistency in exhaled oral NO results were the high levels measured in steroid naive asthmatics compared to normal controls which reduced with steroids, and the lower NO levels in asthmatics already on inhaled or oral corticosteroids. For nasal NO measurements, the most consistent findings were the low levels seen in patients with primary ciliary dyskinesia and CF. However, other results were not consistent across research groups and even when conclusions were similar, the absolute levels of exhaled NO were very different. It was this discrepancy that led to the commencement of the methodological studies discussed in the previous chapters. There were many reasons for the differences in the absolute levels that were reported. I will list here what I believe constituted the main sources of variation.

1. Modification of analysers — the early analysers had been modified from machines originally developed to measure NO in pollution using the chemiluminescence reaction, similar to the machine that I adapted. The analysers used at that time included:
   • Aerocrine AB, PO Box 1024, Solna, Sweden.
   • CLA 510s, Horiba, Kyoto, Japan.
   • CLD 700AL, Eco Physics, AG, Basal or Duerten, Switzerland.
• Model 2107, Dasibi Environmental Corporation, Glendale, California, United States of America.
• Model 42, Thermoelectron, Warrington, United Kingdom.
• Sievers Model 280A, GE Analytical Instruments, Boulder, Colorado, United States of America.

These had differing response times ranging from 5-25 seconds while the subsequent purpose built models from 2000 improved this to 0.02-0.3 seconds. The above models all had differing sampling flows which ranged from 50-500mls/s (machine sampling rate), and different sensitivities to NO ranging from 0.3 to 5ppb.

2. Different methods of sampling – the three commonest techniques used for sampling the exhalation were:
   • Direct to the machine.
   • Using a t-piece or side arm sampling.
   • From a reservoir of stored exhalations.

3. Variation of reservoir and tubing materials – the type of material used to collect the sample also varied which may have resulted in some absorption or adsorption of NO as discussed in Chapter 4. This is particularly important in reservoir studies with the use of bags and tubing made of polyethylene, teflon, mylar, teflon coated, metal and ‘Douglas’ bags (a type of anaesthetic bag made of rubber).

4. Method of cleaning and/or filter use – there was also the possibility of interference from hygiene and infection control procedures. This isn’t discussed in the early papers. At the time, as currently, standard infection control procedures were recommended with regard to doing routine spirometry (American Thoracic Society and Association. 1994). It is likely most researchers were following a similar type of infection control when measuring NO and this may have resulted in interference – either with use of inline filters and/or chemicals for cleaning. Data presented in one abstract showed that the NO concentration was greatly augmented by alcohol containing disinfectant (Meijer, Kerstjens et al. 1996) at a time when the use of chlorhexidine (also an alcohol containing disinfectant) was common in usual lung function laboratory hygiene practices. One study showed no differences between a disposable mouth piece (Sievers®) and a mouth piece containing a filter (HEPA) (Leme, Kasahara et al. 2002), but there has been little research in this area.

5. Different exhalation techniques – subjects were asked to perform exhalation differently:
For the oral measurements options included:

- Single exhaled breath.
- Tidal breathing.
- Exhalation subsequent to a breath-hold with the suggested breath-hold ranging from 5 to 30 seconds.
- With or without the use of nose clips.
- Subject sitting or standing.

For the nasal measurements options included:

- Single exhalation via the nose.
- Sampling while tidal breathing.
- Direct sampling by nasal olive.

6. Different ranges of expiratory flows – differing flows were utilized ranging from 10-100mls/min.

7. Arbitrary measures of expiratory mouth pressure – either no specific expiratory pressure was set, or it was not reported. In those that did standardise expiratory mouth pressure, the pressures ranged from 5-20cmH₂O.

8. Differences in recording and reporting of the results – the result reported was a different part of the recorded NO signal, including:

- Peak.
- Plateau.
- Area under the curve.
- The NO measurement at the peak CO₂ level.

Given the multitude of permutations, it was not surprising that different levels and at times different results in similar populations of subjects were being reported in the literature.

9.3 Standardisation

In view of these different levels reported at that time, a European Respiratory Society Task Force (ERSTF) was assembled to review techniques and a meeting organised in Stockholm, September 1996, a meeting I attended. This was the first attempt to standardise measurements for future NO research. This meeting resulted in an “Exhaled and Nasal Nitric Oxide Measurements; Recommendations” (Kharitonov, Alving et al. 1997). Two years later, a task
force from the ATS with many of the researchers from the ERSTF meeting combined to produce "Recommendations of Standardised Procedures for the Online and Off-line Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide in Adults and Children – 1999", which was adopted in July of that year as an official statement of the ATS (American Thoracic Society and Association. 1999). Following this, there have been two further documents written regarding standardisation of NO measurement (Baraldi, de Jongste et al. 2002; American Thoracic Society and European Respiratory Society 2005). The established ERSTF went on to publish "Measurement of Exhaled Nitric Oxide in Children, 2001" (Baraldi, de Jongste et al. 2002). Finally a joint statement was prepared by the ATS and ERS, which was adopted by both societies in 2004 and subsequently published in 2005. This was the "ATS/ERS Recommendations for Standardized Procedures for the Online and Off-line Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide 2005" (American Thoracic Society and European Respiratory Society 2005).

In this section I will review the current ‘standardisation’ documents and discuss some of the studies that led to these recommendations and the developments from 1997 to 2005. I will critique my research to identify if the procedures I developed were correct and if they contributed to the variability seen between research groups in light of subsequent findings. The papers published from the research described in the previous chapters have been cited in these documents.

There are a number of separate acceptable procedures to measure NO in adults and children – each now having a standard method of measurement. These are:

1. Single breath online measurement (see Table 9.1).
2. Online measurement of exhaled NO during spontaneous or tidal breathing (see Table 9.2).
3. Off-line measurement via a reservoir collection system (for delayed analysis).
4. Nasal NO measurements (see Table 9.3).

Following this, I will review the more recent research on measuring exhaled NO in infants using single breath or tidal breathing techniques.

9.3.1 Single breath online measurement

The current technique recommended for single breath online measurement for adults and children (> 6 years of age) who are able to achieve a single exhalation is presented in Table 9.1.
Table 9.1: The recommended standard for single breath online NO measurement

<table>
<thead>
<tr>
<th>Single breath online measurement for adults and children:</th>
</tr>
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<tbody>
<tr>
<td>• the subject should be seated comfortably with the mouth piece at the proper height and position.</td>
</tr>
<tr>
<td>• the subject, particularly a child, should breathe quietly for five minutes to acclimatise.</td>
</tr>
<tr>
<td>• a nose clip should not be used.</td>
</tr>
<tr>
<td>• the use of NO free air (containing less than 5ppb) for inhalation is preferable.</td>
</tr>
<tr>
<td>• the subject inhales to total lung capacity.</td>
</tr>
<tr>
<td>• the exhalation takes place immediately after inhalation with no breath-hold.</td>
</tr>
<tr>
<td>• the subject exhales at a constant flow of 50mls/s and the expiratory flow is maintained at a constant level.¹</td>
</tr>
<tr>
<td>• the expiratory pressure should be maintained between 5 and 20cmH₂O.</td>
</tr>
<tr>
<td>• visual biofeedback should be available to assist subjects in controlling their exhalation rate and pressure.</td>
</tr>
<tr>
<td>• a peak and then a plateau should be seen in the recording.</td>
</tr>
<tr>
<td>• an NO plateau of greater than three seconds identified during an exhalation of six seconds in adults and a plateau of two seconds identified during an exhalation of four seconds in children is required.</td>
</tr>
<tr>
<td>• the plateau level should be used as the recorded measurement.</td>
</tr>
<tr>
<td>• the final result reported should be the mean of three readings within 10% variability or two within 5% variability.</td>
</tr>
<tr>
<td>• separate exhalations should be done at greater than 30 second intervals.</td>
</tr>
</tbody>
</table>

¹. Note: this is the standard recommendation unless using different expiratory flows to enable measurement of NO from different lung compartments, see section below: 9.3.1.(i) Flow.

9.3.1 (i) Flow

Early studies recommended a slow and prolonged exhalation between 10 and 15L/min (16-25mls/s) for a duration of 5-30 seconds (Kharitonov, Alving et al. 1997). However in subsequent statements, a flow rate of 0.05L/s (50mls/s) was been the main recommendation. Based on our own and other studies, it has been repeatedly demonstrated that exhaled NO is markedly flow dependent with a reduction in concentration when the exhalation rate increases (Alving, Weitzberg et al. 1993; Imada, Iwamoto et al. 1996; Kharitonov, Chung et al. 1996; Lundberg, Weitzberg et al. 1996; Byrnes, Dinarevic et al. 1997; Byrnes, Dinarevic et al. 1997; Byrnes, Dinarevic et al. 1997; Hogman, Stromberg et al. 1997; Silkoff, McClean et al. 1997). The 50mls/s flow has been shown in subsequent studies to be acceptable and reproducible in adults (Silkoff, McClean et al. 1997; Deykin, Massaro et al. 2002; Kharitonov, Gonio et al. 2003), in adolescents (Kissoon, Duckworth et al. 2000) and in children (Baraldi, Azzolin et al. 1999; Kissoon, Duckworth et al. 2002; Pedroletti, Zetterquist et al. 2002; Pijnenburg, Lissenberg et al. 2002; Shin, Rose-Gottron et al. 2002; Kharitonov, Gonio et al. 2003). From a practical standpoint, fast exhalations in children result in a rapid decline in lung volume and
difficulty in sustaining exhalations long enough for the NO values to plateau (Kissoon, Duckworth et al. 1999). Correction of expiratory flow for lung size has been debated given the widely different lung volumes throughout early childhood, but this was not shown to reduce exhaled NO variability (Kroesbergen, Jobsis et al. 1999). There is no current recommendation for correcting the expiratory flow for either weight or height in children or adults.

Compartment modeling has further demonstrated the importance of the expiratory flow used – with the flow determining where the exhaled NO measured is being produced within the lung. The exchange dynamics of NO is different from the other previously well studied gases such as O$_2$ and CO$_2$ because of differences in physical and biochemical properties. Firstly, NO is highly reactive. Secondly, it is actively produced basally in response to various stimuli. Thirdly, it binds avidly to haemoglobin with different kinetics. I have alluded to these factors previously in Chapter 6 when comparing the differing NO and CO$_2$ exhalation pattern, where the latter is known to be predominantly produced in the alveolar compartment. We used this finding to suggest that NO was coming from a different part of the lung to CO$_2$.

One research group (Tsoukias and George 1998) used a two compartment model to describe the pulmonary exchange dynamics of NO, and to try and understand the results of the NO levels being presented in the literature (see figure 9.1 below). Tsoukias’ research group depicted the first compartment as a rigid or non-expansile compartment representing the conducting zone; the airways from the trachea through to generation 17 as defined by Weibel (Weibel 1963). The second compartment was described as a flexible or expansile compartment representing the respiratory bronchioles to the alveolar region. Both compartments were surrounded by a layer of tissue representing the bronchial mucosa in the airway compartment and the alveolar membrane in the alveolar compartment. The next layer was blood which represented the bronchial circulation and the pulmonary circulation in the airway and alveolar compartments respectively.

Using this model, the group then derived a series of mathematical equations to describe each of the possible pathways for NO. This included NO production in the tissue, transfer of NO from tissue to blood (and therefore loss of NO) and transfer of NO from tissue to alveolar or airway space. Equations were also derived for the behaviour of NO in intra-thoracic air, assuming a well mixed compartment and accounting for convective flow of NO in and out of the compartments in inspiratory and expiratory manoeuvres, and for diffusion back into tissue. The blood layers were considered an infinite sink for NO. All the parameters were
taken from the available literature including the production rate of NO in the tissue layer. Using these equations they could then explain the pattern of NO seen in single exhalations under different conditions including with and without breath holds and at different expiratory flows. Overall, the model worked well and appeared to mirror actual results from the human studies, although the predictions of the initial peaks seen with single exhalations were of variable accuracy. Generally the model underestimated the peak in single exhalations and overestimated the peak after breath-holds. The researchers stated in their paper that “Initial reports have suggested that ambient levels of NO do not affect the exhalation profile.” I also could not ascertain if they took into account any upper airway contamination or whether, like a later group (Silkoff, Sylvester et al. 2000), they assumed closed vellum. These could be potential sources of error.

Figure 9.1: A two-compartment model of NO exchange

A two-compartment model of NO exchange dynamics using equations to derive the measurement of NO from different lung compartments depending on the expiratory flow. 

These predictions built on work from a previous group who had also derived equations to model NO interactions in the lung (Hyde, Geigel et al. 1997). Their model was able to predict NO results from the lower airway using a one compartment model but was unable to account for the upper airway contamination. A later group also used a two compartment model and reported that a breath hold of 10 to 20 seconds meant that the concentration of NO was equilibrated - "alveolar airway" production of NO now equaled the amount of NO diffusing out of the airways (Pietropaoli, Perillo et al. 1999). The subsequent exhalation could then give the alveolar content of NO. Finally, using similarly derived equations, another group re-analysed their earlier data, from which they had concluded that NO levels correlated with expiratory flow (Silkoff, Sylvester et al. 2000). They determined that a even closer relationship existed between the exhaled NO concentration (defined as the quantity of NO exhaled per unit time) and the expiratory flow. They felt this allowed a better estimation of the quantity of NO diffusing into the exhaled gas and therefore better reflected tissue NO concentration.

In essence, I believe these groups were making the point that studies were presenting NO results in a far too simplistic manner. NO was being presented as a single result from a single compartment when in reality the NO concentration varied in different parts of the airway system. In addition, the levels were always interpreted as if the NO was solely the result of NO production, while in reality the NO reading was a combination of production, diffusion (dictated by the rate of removal by capillary blood throughout the lung) and ventilation. The importance of the modeling described above was to define specifically that different expiratory rates would allow evaluation of different lung compartments. This could be critical in appropriately discerning inflammation in different diseases; for example the conducting airways in the airway disease of asthma or the alveolar compartment in alveolar diseases such as in interstitial lung disease. This theory was tested by measuring 'fractionated' NO in a cross section of subjects using three exhalation flows at 100, 175 and 370ml/s in 40 newly diagnosed, steroid naive asthmatics, 17 patients with allergic alveolitis and 57 healthy controls (Lehtimaki, Kankaanranta et al. 2001). The asthmatic patients did indeed have higher bronchial NO output at 2.5nL/s compared to those with alveolitis or healthy controls which were both measured at 0.7nL/s. On the other hand, the subjects with alveolitis had higher alveolar NO concentrations at 4.1ppb than the asthmatic or healthy subjects which were both measured at 1.1ppb. Thus there was no difference in NO between those with asthma and healthy controls when the alveolar compartment was measured (Lehtimaki, Kankaanranta et al. 2001). Finally they demonstrated that the concentration of NO in the bronchial
compartment correlated with the serum level of the EPX and bronchial hyper-responsiveness in those with asthma, while the alveolar NO concentration in patients with alveolitis correlated inversely with the pulmonary diffusing capacity.

A number of studies have now been conducted assessing NO in this manner. These have demonstrated a higher exhaled NO in the conducting airways in asthmatics compared with controls, with no mean difference of NO measured in alveolar compartment in otherwise stable asthmatics (Hogman, Holmkvist et al. 2002; Lehtimaki, Kankaanranta et al. 2002; Mahut, Delacourt et al. 2004; Shin, Rose-Gottron et al. 2004; Lehtimaki, Kankaanranta et al. 2005). The reduction of NO in response to steroid therapy also occurs in the airway compartment (Silkoff, Sylvester et al. 2000; Lehtimaki, Kankaanranta et al. 2001; Shin, Rose-Gottron et al. 2004). In a cross sectional study of proximal and distal sampling in children, it was the proximal NO output that correlated better with the FEVı (Mahut, Delacourt et al. 2004). However, as no theory is perfect, both high airway and high alveolar NO has been demonstrated on highly symptomatic asthmatics or those with ongoing nocturnal symptoms (Mahut, Delacourt et al. 2004; Shin, Rose-Gottron et al. 2004; Lehtimaki, Kankaanranta et al. 2005). Also, high airway levels of NO were also seen in subjects with allergic rhinitis despite having no airway symptoms (Hogman, Holmkvist et al. 2002). Low airway levels were seen in smokers and patients with COPD, though the alveolar levels in NO were increased in the COPD patients (Hogman, Holmkvist et al. 2002). Airway exhaled NO levels were no different between CF and healthy control adolescents, but lower levels were seen in the alveolar compartment and tissue concentration in the healthy group (Shin, Rose-Gottron et al. 2002). Increased rates of alveolar NO have been described in allergic alveolitis (Lehtimaki, Kankaanranta et al. 2001) and scleroderma (Girgis, Gugnani et al. 2002) with negative correlations between NO levels and the diffusion factor demonstrated in both.

The nomenclature used has also required adaptation as discussed in the 2002 and 2005 standardisation documents (Baraldi, de Jongste et al. 2002; American Thoracic Society and European Respiratory Society 2005). The previous recommendations were to express exhaled NO or nasal NO in terms of ppb (equivalent to nL/L of exhaled air). The standard single exhalation remains at 50mls/s, now suggested to be denoted as FE-no.0.05. NO output representing the rate of exhaled NO is denoted by V-no and calculated from the product of NO concentration in nL/L and expiratory flow rate in L/min in the following equation:

\[ V_{no} \text{ (nL/min)} = \text{NO (nL/L)} \times \text{air flow rate (L/min)} \]
The ATS guideline states "terms such as NO release, NO excretion, NO secretion and NO production are to be discouraged when referring to $V_{\text{no}}$" (American Thoracic Society and European Respiratory Society 2005).

A constant expiratory flow is required with any exhalation measured and has been recommended following the findings from early studies. This is most easily achieved with appropriate biofeedback using a gauge or computer display for subjects to maintain expiratory flow within specified limits. All newer machines as exemplified by the companies that took part in the 2005 recommendations (Aerocrine, Eco Physics, Eco Medics, Ionics Instruments and Ekips Technologies) have these available. However there are other options which include the use of dynamic resisters (Kharitonov, Gonio et al. 2003), operator controlled flow (Baraldi, Scollo et al. 2000), starling resisters (Hogman, Stromberg et al. 1997; Tsoukias, Tannous et al. 1998) and server controlled devices (Silkoff, Bates et al. 2004). These techniques have usually been tried in young children but can also be useful in other subjects that have difficulties controlling their exhalation such as those with neuromuscular disease. In children aged 4-8 years, 50% could not perform the single breath online technique adequately. However, the addition of a dynamic flow resister allowed exhalation with a variable mouth pressure while maintaining constant expiratory flow and resulted in only 7% of the children unable to perform the manoeuvre (Baraldi, Scollo et al. 2000).

9.3.1 (ii) Mouth pressure

An expiratory mouth pressure of between five and 20cmH$_2$O is currently recommended. This degree of resistance is needed for velum closure of the soft palate to prevent nasal contamination of the exhaled NO and has been validated by nasal CO$_2$ measurements (Silkoff, McClean et al. 1997) and nasal argon insufflation (Kharitonov and Barnes 1997). Many studies have confirmed higher sinus and nasal levels when comparing sinus, nasal, exhaled and/or lower airway sampling (Alving, Weitzberg et al. 1993; Lundberg, Rinder et al. 1994; Lundberg, Weitzberg et al. 1994; Schedin, Frostell et al. 1995; Dillon, Hampl et al. 1996; Imada, Iwamoto et al. 1996; Kimberly, Nejadnik et al. 1996). The other options described to prevent nasal contamination has been continuous nasal aspiration (Silkoff, Kesten et al. 1995) or to inflate a balloon in the posterior nasal pharynx to separate the two compartments (Schedin, Frostell et al. 1995; Kimberly, Nejadnik et al. 1996). Clearly, exhaling against an appropriate mouth pressure is the easiest option and has been most widely used.

Unlike the effects of flow on NO, the effects of varying mouth pressure has not demonstrated consistent findings in all studies. Two studies showed no difference in plateau NO
measurement with variable expiratory mouth pressure (Hogman, Stromberg et al. 1997; Silkoff, McClean et al. 1997), but another showed increasing exhaled NO concentrations with increasing expiratory pressure from two to ten cmH₂O (Kondo, Haniuda et al. 2003). Our own findings suggested the opposite with a reduction of exhaled NO with increasing mouth pressure in two of ten subjects (Byrnes, Dinarevic et al. 1997). However, as noted in the 2005 recommendations, a pressure above 20 cmH₂O should be avoided as it may be “uncomfortable for patients or subjects to maintain”. Indeed, I think this may have contributed to the fact that there was a falling off of the exhaled NO in our study in two subjects. Having tried it repeatedly myself, while pressures of 4 mmHg (5.4 cmH₂O) and 8 mmHg (10.9 cmH₂O) were easy to maintain, 12 mmHg (16.3 cmH₂O) became a little more difficult but 16 mmHg (21.8 cmH₂O), the highest expiratory pressure sampled in our study, was very tiring to exhale against, and 20 mmHg (27.2 cmH₂O) almost impossible to maintain and therefore was not used. One needs to recall that in the earlier studies a far longer exhalation time was also required with the older modified analysers.

9.3.1 (iii) Nasal clips and breath-holding

While the early studies varied as to whether or not nose clips were used, the current recommendation is not to wear them when doing exhaled NO in any of the methods of measurement as this may actually increase the risk of having nasal and sinus contamination of the oropharyngeal and exhaled sample (American Thoracic Society and European Respiratory Society 2005). The previous standards suggested that a nose clip may be worn to prevent inhaled air to contaminate the exhaled sample (see below) (Baraldi, de Jongste et al. 2002).

In addition, the inhalation is now recommended as being through the mouth with higher initial exhaled NO demonstrated following an inhaled breath through the nose when compared to inhalation through the mouth (Phillips, Giraud et al. 1996; Robbins, Floreani et al. 1996). Any degree of breath-holding also results in NO accumulation in the nasal and oropharyngeal spaces giving higher exhaled levels (Persson, Wiklund et al. 1993; Lundberg, Weitzberg et al. 1994; Kharitonov, Chung et al. 1996; Kimberly, Nejadnik et al. 1996; Massaro, Mehta et al. 1996; Sato, Sakamaki et al. 1996). Breath-holding was shown to increase NO levels in a time dependent manner in atopic and healthy subjects (Martin, Bryden et al. 1996). Breath-holding appears to affect the peak more than the plateaux levels in healthy and asthmatic subjects (Shinkai, Suzuki et al. 2002). The effect of breath-holding also depended on the ambient NO; when the ambient NO was greater than 10 ppb, exhaled NO was decreased whereas if the
ambient NO was less than 10ppb the exhaled NO was increased after a 10 second breath-hold (Jobsis, Schellekens et al. 2001).

9.3.1 (iv) The recorded measurement

The NO recorded in the literature has been peak, plateau, area under the curve or NO plateau signal at CO₂ peak. The current recommendation is for plateau levels to be reported, unless calculating NO output. This requires a plateau of three seconds following exhalation of at least six seconds for participants older than 12 years of age, or two seconds following exhalation for at least four seconds for children less than 12 years of age. The peak measurement has shown more variability as influenced by the degree of dead space, ambient NO and inspiration taken via nose or mouth. An expiratory flow of 50mls/s ensures an acceptable time to plateau, an acceptable rate of decline in lung volumes and is appropriate for children at less than 12 years of age, as well as those with vital capacities less than one litre (Pfaff and Morgan 1994; Canady, Platts-Mills et al. 1999; Franklin, Taplin et al. 1999; Kissoon, Duckworth et al. 1999).

9.3.1 (v) The effect of ambient nitric oxide

Recommendations since 1997 have been to record the ambient NO as this fluctuates considerably. A note from one of the authors (A Sovijarvi, Helsinki University Central Hospital, Finland) in this document states; the ambient NO was noted to vary between 1 and 600ppb during winter months in their studies (Kharitonov, Alving et al. 1997). Initially it was suggested that measurements should only be performed with an ambient NO less than 40ppb. With improved standardisation and more sensitive and rapidly responding analysers this is now too high. The 2005 recommendation is that the ambient NO be less than 5ppb and/or that subjects inhale NO free air while being tested. The newer NO analysers including the smaller, more transportable units such as the MINO® (Aerocrine AB, Solna, Sweden) or z-700 NO meter (Dasibi Environmental Corporation, Glendale, California, United States of America) have a scrubbing facility within the inhalation limb. This may be more important for reservoir collections during tidal breathing where an increased amount of ambient NO is exhaled and thus collected. In our experiments we elected to measure only when the NO in ambient air was less than 10ppb. However on many days this precluded measurement which delayed the experiments. Unlike other researchers, we found exhalation from a high NO concentration compared to exhalation from a low NO concentration resulted in a drop in the overall NO level as discussed in Chapter 7. On review now, it may have been because we were waiting for a plateau to develop with a drop from the high ambient NO reading and it took some time...
to settle. We may have been measuring the latter part of the exhalation in error and this potentially may give lower levels than the peak or the true plateau. Thus, we may have been comparing the plateau in the exhalation from the low ambient NO with the last part of the exhalation from the high ambient NO. In two studies NO was significantly higher when measured in a total of 215 children including healthy controls, non-asthmatic atopics and asthmatics at times of high ambient NO (>10ppb, mean 19ppb) and at times of low ambient NO (<10 ppb, mean 5.1ppb) (Baraldi, Azzolin et al. 1998; Jobsis, Schellekens et al. 2001). Ambient NO had a different effect on results after a ten second breath hold where measured NO decreased if the ambient NO was higher (again >10ppb was the cut-off used) and measured NO increased if the ambient NO was lower (NO<10ppb) (Jobsis, Schellekens et al. 2001). Nasal NO in healthy children correlated with ambient NO and, in those children greater than 12 years, it was the only factor correlating with the measured expiratory levels (Struben, Wieringa et al. 2005). More recently there has been a study suggesting that even levels of 5-10ppb ambient NO may have an effect on the exhaled results, with no difference seen at less than 5ppb, upholding the most recent recommendations (Franklin, Turner et al. 2004).

Other studies have examined effects of pollution on exhaled NO. Ambient NO levels, as well as ambient CO levels, were positively correlated with the exhaled NO results in 16 non-smoking healthy subjects (Van Amsterdam, Verlaan et al. 1999). Mean air pollution was also correlated with the exhaled NO levels in 16 healthy subjects measured on two separate days (van Amsterdam, Verlaan et al. 1999) and 18 subjects measured on four separate days (Steerenberg, Snelder et al. 1999) at times of differing pollutant levels. At times of high ambient NO, an increased risk of developing respiratory symptoms described as "sore throat, runny nose, having a cold or being sick at home in the following week" was also shown in 68 children (Fischer, Steerenberg et al. 2002).

9.4 **Online spontaneous or tidal breathing measurement**

A single exhaled breath with online measurement remains the measurement of choice. However, in some studies 50% of children aged 4-8 years of age (Baraldi, Scollo et al. 2000), 10% of children aged 9-16 years (Baraldi, Scollo et al. 2000) and 30% of children aged 4-16 years of age (Jobsis, Schellekens et al. 1999) could not perform this technique appropriately. Online measurement during tidal breathing, usually as a mean of three breaths, has been explored as an option for younger children and infants. One study measured exhalation following quiet breathing with the operator controlling the expiratory flow by varying
expiratory resistance. A flexible rubber tube 9cm long with a diameter of 0.9cm and an end resistor of 2mm was placed in the circuit. The operator could bend the flexible device during exhalation to provide resistance and allow manual control of the expiratory flow by direct checking on the monitor without requiring active cooperation from the child. Of the 115 children enrolled, 110 (93%) were able to perform this manoeuvre compared to 73 (63%) who were able to perform the active single exhaled breath (Baraldi, Scollo et al. 2000). In another study, NO was measured online in 67 children during controlled tidal breathing (76% were aged 2-5 years) with flow measured by a pneumotachograph and displayed on the computer screen with a negligible delay of 0.1 second allowing the operator to target the exhaled flow within preset limits of 0.4 to 0.6L/s by continuously adapting the outlet resistance. Of the nine children (13%) who failed the measurement; seven had asthma, two had episodic wheeze, four were aged two, three were aged three and two were aged five. The visualisation of the online measurements allowed breath to breath profiles to be scrutinised to ensure "a stable, calm and reproducible breathing pattern" (Buchvald and Bisgaard 2001).

Tidal breathing results in lower NO levels than single exhalations and this is exaggerated at higher NO readings (Silkoff, McClean et al. 1997; Rutgers, Meijer et al. 1998; Franklin, Turner et al. 2004). While a reasonable correlation has been found between the two (Silkoff, Stevens et al. 1999; Kissoon, Duckworth et al. 2000; Franklin, Turner et al. 2004), individual agreement can be poor (Rutgers, Meijer et al. 1998; Franklin, Turner et al. 2004). The lower results could be explained by increased inhaled air dilution from a low NO source and variable flow during tidal breathing, and the possibility of breath-holding and nasal leakage in single breaths (Hyde, Geigel et al. 1997; Silkoff, McClean et al. 1997; Rutgers, Meijer et al. 1998). This can be improved by controlling expiratory flow during tidal breathing – but when only passive co-operation is available and considerable operator experience appears necessary to manipulate the resistance correctly (Baraldi, Scollo et al. 2000; Buchvald and Bisgaard 2001). I would also be concerned that the rubber tubing itself, in one method described, may result in some NO loss following reaction with the sulphur hydryl groups. This online spontaneous breathing method still "requires passive cooperation as the child needs to breath slowly and regularly through a tight fitted mask, close the mouth around a mouth piece which is often the limiting factor, as well as being able to tolerate breathing against resistance" (Buchvald and Bisgaard 2001). Despite this, results have shown the same patterns as seen with the single online measurements in the cross-sectional population studies (Baraldi, Azzolin et al. 1997; Baraldi, Azzolin et al. 1998; Baraldi, Dario et al. 1999). Use of this in infants is discussed below in Section 9.16.
9.5 Off-line measurement

NO determinations can be measured from samples collected into a reservoir system and later fed through a chemiluminescence analyzer. Similar to early online experiments, while the absolute levels of NO measured by reservoir collection varied between investigators, the findings across disease entities remained consistent (Alving, Weitzberg et al. 1993; Persson, Wiklund et al. 1993; Kharitonov, Yates et al. 1994; Kharitonov, Yates et al. 1995; Massaro, Gaston et al. 1995; Deykin, Halpern et al. 1998).

A number of standardisation issues arose with this technique. Firstly, the concern regarding NO reactivity and that inert collection bags would be required. Other factors that could affect stability included temperature, light exposure, damage to bags, leaks and pressure changes. Mylar or tedlar bags were early reported as the most stable (Kharitonov, Alving et al. 1997) with NO levels consistent up to 12 hours post collection (Massaro, Gaston et al. 1995; Massaro, Mehta et al. 1996; Canady, Platts-Mills et al. 1999; Jobsis, Schellekens et al. 2001). Some investigators reported continued stability for 24-48 hours (Paredi, Loukides et al. 1998; Djupesland, Qian et al. 2001), while others found an increase in the NO concentration (Silkoff, Stevens et al. 1999). NO collected in a reservoir containing silica gel was stable for 24 hours (Paredi, Loukides et al. 1998). One group used a tube collection system showing better online and off-line agreement than bag collections for both exhaled and nasal levels (Djupesland, Qian et al. 2001). Reservoir stability also depends on temperature. NO results appeared to be stable for nine hours at 4°C to 37°C but increased between nine and 48 hours in samples with initially low concentrations. It was not stable at higher temperatures and the use of a drying agent did not improve the stability (Bodini, Pijnenburg et al. 2003). One study evaluated 185 breaths in bags that were re-used 10-20 times flushing three times with NO free air between uses and stored at 22°C. Over a four day period, the samples with low NO increased in a linear fashion, and those with high NO from asthmatic subjects showed a gradual decrease (Silkoff, Stevens et al. 1999; Kharitonov, Gonio et al. 2003). Higher temperatures made the changes more rapid and lower temperatures made it less likely to occur. The size of the balloon was not thought to be critical but was recommended to be similar to, or larger than, the subjects’ vital capacity (Linn, Avila et al. 2004).

Secondly, uncertainty existed as to whether the collection should be a single exhaled breath, several ‘single exhaled breaths’ or tidal breathing. The reproducibility with single breaths appeared to be fair or good with intra-subject coefficients of variation down to 5% (Gaston,

Thirdly, there was debate as to whether the whole exhaled breath should be collected. Early experiments discarded the first part of exhalation to reduce contamination by inhaled ambient NO, dead space and nasopharyngeal space with a good correlation demonstrated between the remaining exhalate and single online exhalation (Borland, Cox et al. 1993; Massaro, Mehta et al. 1996). Similarly, studies that discarded the first tidal volume when measuring during tidal breathing also achieved a closer approximation to online values (Paredi, Loukides et al. 1998; Jobsis, Raatgeep et al. 2001). The uncertainty was in deciding the absolute amount to discard and how to get consistency across subjects. While 150 to 200mls were used in the adult studies even greater uncertainty existed in the correct amount to discard in paediatric studies. The ‘discard’ was achieved employing spring loaded or manually activated valves, or low compliance reservoirs placed in series with the main collection vessel. Subsequently collecting the whole exhalation was shown to provide identical sensitivity and specificity as single breath online recordings, despite the fact that the absolute NO levels were not identical (Silkoff, Stevens et al. 1999; Djupesland, Qian et al. 2001; Jobsis, Raatgeep et al. 2001; Deykin, Massaro et al. 2002). The standardisation has therefore altered accordingly between 1997 and 2005 from discarding the first 0.75L exhaled (or 0.5L if the vital capacity was less than 2L) (European Respiratory Society 1993; Kharitonov, Alving et al. 1997) to collecting the entire breath (American Thoracic Society and Association. 1999; Baraldi, de Jongste et al. 2002).

Similar across all methods, higher flow rates result in decreased concentration of NO recovered from the exhaled reservoir sample (Hogman, Stromberg et al. 1997; Silkoff, McClean et al. 1997). Despite this, subject group differences can be seen at expiratory rates between 50 and 500mls/s as long as the flow remains identical for all subjects in any comparison (Jobsis, Raatgeep et al. 2001; Deykin, Massaro et al. 2002). With collection of tidal breathing, using standard expiratory flow reduces variability and improves comparison with online results (Kissoon, Duckworth et al. 2000). This can be controlled by the subject or by the operator (Massaro, Mehta et al. 1996; Baraldi, Azzolin et al. 1997; Baraldi, Carra et al. 1999; Baraldi, Dario et al. 1999; Jobsis, Schellekens et al. 2001). The taskforce ultimately proposed recommendations for 50mls/s exhalation for both off-line and online collections as showing good correlation in the paediatric arena (Baraldi, de Jongste et al. 2002). It is still possible to sample different compartments using a different flow even with this technique.
(Hyde, Geigel et al. 1997; Tsoukias and George 1998; Tsoukias, Tannous et al. 1998; Silkoff, Sylvester et al. 2000).

One of the reasons that higher levels were thought to be obtained using either single or tidal breathing into a reservoir was because of an open soft palate and upper nasopharyngeal contamination (Sharma, Traylor et al. 1987; Schilling, Holzer et al. 1994; Kimberly, Nejadnik et al. 1996; Kharitonov and Barnes 1997). A single exhalation against even low resistance into the reservoir gave much lower levels (Massaro, Gaston et al. 1995). A resistor is now included in most reservoir bags with a recommendation of standard expiratory flow and standard expiratory pressure of 5cmH₂O for both single breath (Massaro, Gaston et al. 1995; Deykin, Halpern et al. 1998) and tidal breathing reservoir collections (Canady, Platts-Mills et al. 1999; Jobsis, Schellekens et al. 2001). A dynamic flow restrictor is possible to aid the operator to standardise the exhaled breath with less able subjects by altering the resistance to give a standard flow during the exhalation (Pijnenburg, Lissenberg et al. 2002), although again I imagine this must introduce an element of complexity.

From 1999, inhaling via the mouth prior to exhalation into the reservoir bag was added to the protocol. By 2005 this recommendation had been revised to include the inhalation of NO free gas or via an NO scrubbing filter in the inspiratory limb. High concentrations of NO in the inspired air of greater than 20ppb significantly increased the off-line exhaled NO measurements (Gustafsson, Leone et al. 1991; Zayasu, Sekizawa et al. 1997). Again wearing a nose clip and breath holding are not recommended for similar reasons to the single breath online measurements (see section 9.3.1 (ii)) (Jobsis, Schellekens et al. 2001). Following these guidelines, subjects tested on multiple days had a variation between 1 and 5ppb, which is similar to that found in immediate online measurements, although in this study the collection was delayed for more than three seconds to discard the dead space (Borland, Cox et al. 1993; Massaro, Mehta et al. 1996). For the standard method to measure off-line collections as either single exhaled breaths or as tidal breathing collections see Table 9.2.
The current recommended technique for single exhalation collection into a reservoir is:

- inhale through the mouth from NO free air (<5ppb).
- a nose clip should not be worn.
- no partitioning of the sample and/or no discard of the initial expiratory.
- exhale into a reservoir bag with a steady exhalation rate of 0.35L/s not falling below 0.315L/s and not going above 0.385L/s.\(^5\)
- exhale against a pressure of at least 5cmH₂O.
- collection of the entire vital capacity.
- mylar or tedlar bags are recommended.
- storage of the bags should be at a stable temperature (around 22°C).
- the sample should be measured within 12 hours of collection.

The current recommended techniques for tidal breathing collection into a reservoir:

- inhaling from NO scrubbed or low NO concentration in air (<5ppb).
- a nose clip should not be worn.
- exhale at least three tidal breaths into a collection bag.
- exhale against a resistance of at least 5cmH₂O.
- a set expiratory flow remains necessary.
- expiratory flow can be controlled by either the subject or by the operator.

Table 9.2: The recommended standards for single breath and tidal breathing off-line NO measurement

<table>
<thead>
<tr>
<th>The current recommended standards for single breath and tidal breathing off-line NO measurement</th>
</tr>
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<tbody>
<tr>
<td>The current recommended technique for single exhalation collection into a reservoir is:</td>
</tr>
<tr>
<td>• inhale through the mouth from NO free air (&lt;5ppb).</td>
</tr>
<tr>
<td>• a nose clip should not be worn.</td>
</tr>
<tr>
<td>• no partitioning of the sample and/or no discard of the initial expiratory.</td>
</tr>
<tr>
<td>• exhale into a reservoir bag with a steady exhalation rate of 0.35L/s not falling below</td>
</tr>
<tr>
<td>0.315L/s and not going above 0.385L/s.</td>
</tr>
<tr>
<td>• exhale against a pressure of at least 5cmH₂O.</td>
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<tr>
<td>• collection of the entire vital capacity.</td>
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<tr>
<td>• mylar or tedlar bags are recommended.</td>
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<tr>
<td>• storage of the bags should be at a stable temperature (around 22°C).</td>
</tr>
<tr>
<td>• the sample should be measured within 12 hours of collection.</td>
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</tbody>
</table>

The current recommended techniques for tidal breathing collection into a reservoir:

- inhaling from NO scrubbed or low NO concentration in air (<5ppb).
- a nose clip should not be worn.
- exhale at least three tidal breaths into a collection bag.
- exhale against a resistance of at least 5cmH₂O.
- a set expiratory flow remains necessary.
- expiratory flow can be controlled by either the subject or by the operator.

Off-line collection clearly has its advantages. Sampling is simple, feasible, inexpensive and can be done in young age groups. It allows collection of samples at sites in the community or at a distance from the analyser and may allow a more efficient use of the analyser. The disadvantages are that a standard technique is more difficult to obtain, and a scatter of NO data due to variation and flow can occur (Baraldi, de Jongste et al. 2002). Sample storage is important, and if possible instantaneous feedback to assess technique for both operator and the subject should be available.

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9.6 Nasal nitric oxide measurement

For completion, I am going to briefly cover nasal NO although I did not measure this parameter as part of my research. Presentation of standard techniques for this measurement commenced in 1999 (American Thoracic Society and Association. 1999), with minor amendments in 2002 and 2005 (Baraldi, de Jongste et al. 2002; American Thoracic Society and European Respiratory Society 2005). The NO concentration is far greater in the nose than the lower respiratory tract and is measured in parts per million as opposed to parts per billion (Alving, Weitzberg et al. 1993; Gerlach, Rossaint et al. 1994; Lundberg, Farkas-Szallasi et al. 1995). NO concentration is even higher in the para-nasal sinuses (Lundberg, Rinder et al. 1994; Lundberg, Farkas-Szallasi et al. 1995; Haight, Qian et al. 2000).

Table 9.3: The recommended standard for nasal NO measurement

<table>
<thead>
<tr>
<th>The current recommended technique for measurement of nasal NO is:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• the subject is seated</td>
</tr>
<tr>
<td>• two nasal olives are placed with a central lumen in the nares.</td>
</tr>
<tr>
<td>• these must be of sufficient size to occlude the nostril.</td>
</tr>
<tr>
<td>• a sample is aspirated continuously at a constant rate from one narus.</td>
</tr>
<tr>
<td>• gas is entrained via the other narus giving a trans-nasal flow in series.</td>
</tr>
<tr>
<td>• a target sampling airflow of 0.25 to 0.3L/min is recommended.</td>
</tr>
<tr>
<td>• oropharyngeal and lower airway contamination is prevented by one of the methods suggested (see paragraph below).</td>
</tr>
<tr>
<td>• mean NO values are calculated for a stable plateau of greater than ten seconds or five breaths (Silkoff, Chatkin et al. 1999; Ratjen, Kavuk et al. 2000).</td>
</tr>
<tr>
<td>• the higher flow rate (compared to exhaled oral sampling) at 0.25-3L/min allows a steady plateau level of NO concentration in subjects within 20-30 seconds.</td>
</tr>
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</table>

This trans-nasal flow reflects the most common method of measurement. An alternative is to have continual aspiration of both nares; one to discard and one to measure. Velum closure can be achieved in a number of ways; the most common is to slowly exhale orally against a resistance similar to the exhaled NO recommendation (Arnal, Didier et al. 1997; Kharitonov, Rajakulasingam et al. 1997; Silkoff, McClean et al. 1997; Dubois, Douglas et al. 1998; Silkoff, Chatkin et al. 1999). Other options that have also been effective are purse-lip breathing via the mouth (Rodenstein and Stanescu 1983), breath-holding with the velum closed (Kimberly, Nejadnik et al. 1996) or voluntary elevation of the soft palate by a trained subject (Giraud, Nejadnik et al. 1998). Of note for nasal NO measurements, healthy adults have significantly better repeatability than healthy children (Kharitonov, Walker et al. 2005).
9.7 Physiological alterations that may affect measurement

In this next section, I review physiological variables that may alter NO results, recognising there are conflicting findings reported in most parameters.

9.7.1 Size and gender

Firstly, there have been a number of investigations across animal species, and the reasons I refer to this is with regarding the possible effect of size, and to describe an extraordinary study done in elephants! Exhaled NO results have been similar in rabbits, guinea pigs and rats (Gustafsson, Leone et al. 1991; Stewart, Valenza et al. 1995) but lower levels found in Antarctica seals (Stanek 1995). One heroic study measured direct exhalation with a one litre syringe and continuous online measurement in four elephants, finding no difference between these and human subjects. They also showed no difference of NO with height of the elephants which ranged from 240cm to 260cm (although how significant a 20cm difference in height is at these levels remains questionable), or weight which ranged from 2800kg to 4000kg (Lewandowski, Busch et al. 1996).

Secondly, with regard to gender, differences have been found in some studies (Tsang, Leung et al. 2002; van der Lee, van den Bosch et al. 2002; Olivieri, Talamini et al. 2006) but not in other adult (Jilma, Kastner et al. 1996; Morris, Sooranna et al. 1996; Bartley, Fergusson et al. 1999; Kharitonov, Gonio et al. 2003) or in paediatric studies (Baraldi, Dario et al. 1999). Measurements in over 100 (van der Lee, van den Bosch et al. 2002) and over 200 healthy adults (Olivieri, Talamini et al. 2006) found normal exhaled NO values to be significantly higher in men. One study showed these higher levels correlated with height and weight but not with age (Tsang, Ip et al. 2001). In another study, when corrected for body weight, men were calculated to exhale 50% more NO than women (Morris, Sooranna et al. 1996). Exhaled NO measured in nl/min/m² correlated with body surface area in one (Phillips, Giraud et al. 1996) but not a second study (Jilma, Kastner et al. 1996).

Paediatric studies have also looked for gender differences. In almost 1000 13-14 year olds, higher levels of exhaled NO were independently related to male gender, as well as to the presence of wheeze and rhinoconjunctivitis (Nordvall, Janson et al. 2005). In 258 children, exhaled NO did not correlate with height, weight, body mass index or body surface area but again demonstrated a higher result in boys (Wong, Liu et al. 2005). In over 100 healthy children using both online and off-line analyses, body area, age and FEF₂₅₋₇₅ were significant predictors of exhaled NO (Kissoon, Duckworth et al. 2002). In children aged 4-6 years
measured over two 24 hour periods, there was a gender difference (again higher in boys than girls) in the first assessment, but not in a second assessment (Napier and Turner 2005). However other paediatric studies have not confirmed these associations; showing no correlation observed between NO and height or spirometric data (Baraldi, Azzolin et al. 1999), and no correlation between nasal NO and body mass index (Struben, Wieringa et al. 2005). At the current time the reference ranges for ‘normal’ are given as one range, with no gender correction.

9.7.2 Age

Early in the research literature, it was suggested that NO increased with age correlating to pneumatisation of sinuses (see Figure 9.2) (Lundberg, Farkas-Szallasi et al. 1995). In over 650 healthy children aged 4-17 years in three studies, exhaled NO significantly increased with age in both off-line and online analyses (Franklin, Taplin et al. 1999; Kissoon, Duckworth et al. 2002; Buchvald, Baraldi et al. 2005). Nasal NO in 340 healthy children aged 6-17 years also increased with age which would appear appropriate if it does relate to sinus development (Struben, Wieringa et al. 2005). An increase of exhaled NO was confirmed in a younger age group of 2-5 year olds using tidal breathing (Avital, Uwyey et al. 2003). One study also demonstrated an increase between 1 and 24 hours of age in 13 healthy newborn infants (Schedin, Norman et al. 1996). However, again findings have been inconsistent with no correlation between age and exhaled NO seen in some groups (Baraldi, Azzolin et al. 1999; Bartley, Fergusson et al. 1999). Despite the findings above, the current practice is to use one range for normal values with no correction for the age of the subjects. It was however one of our reasons for choosing a tight age bracket when measuring the exhaled NO in children in the methodology study (see Chapter 8).

9.7.3 Circadian rhythm.

One study in children found the lowest exhaled NO and urinary EPX levels occurred at 7pm and the highest at 7am in 20 asthmatics compared to six healthy children (Mattes, Storm van's Gravesande et al. 2002). Otherwise no circadian differences have been demonstrated in adult subjects with exhaled NO (Kharitonov, Gonio et al. 2003) or nasal NO measurements (Bartley, Fergusson et al. 1999). NO was higher at night in asthmatics with nocturnal symptoms compared to asthmatics without nocturnal symptoms and higher at all times compared to healthy controls, but no circadian variation in any group was demonstrated (ten Hacken, van der Vaart et al. 1998; Georges, Bartelson et al. 1999; Palm, Graf et al. 2000). In children aged 4-6 years, exhaled NO was reproducible though a 24 hour period (Napier and Turner 2005) and in six children measured at different times on six consecutive days, no circadian rhythm was found (Latzin, Beck et al. 2002).

9.7.4 Menstrual cycle and pregnancy

There are few studies to review here. No significant changes were found with repeated measurements over weeks in healthy controls (Jilma, Kastner et al. 1996; Morris, Sooranna et al. 1996), while one study did seem to demonstrate high exhaled NO levels in 40 asthmatic
women who coincidentally experienced worsening of sputum scores, eosinophils and lung function pre-menstrually (Oguzulgen, Turktas et al. 2002). No difference has been shown in exhaled NO during pregnancy between 10 and 42 weeks (Morris, Carroll et al. 1995).

9.7.5 Food and beverages

In our own study, we found that drinking water prior to exhalation transiently reduced NO levels (Byrnes, Dinarevic et al. 1997). Caffeine also decreased exhaled NO in one paper (Bruce, Yates et al. 2002) but not in another (Taylor, Smith et al. 2004). Alcohol ingestion reduced exhaled NO in both asthmatic and healthy subjects (Persson, Cederqvist et al. 1994; Yates, Kharitonov et al. 1996) with a small decrease in exhaled NO observed up to 3-4 hours after drinking (Jones, Fransson et al. 2005). Smoking also significantly reduces exhaled NO and will this be discussed below with the studies on COPD subjects (see Section 9.15). The reduced NO levels subsequent to both cigarette smoking and alcohol consumption was suggested to be secondary to down regulation of iNOS (Steerenberg and van Amsterdam 2004). In contrast increased exhaled NO has been found after the ingestion of nitrite or nitrate containing foods (such as lettuce) with a maximum effect occurring two hours after ingestion (Zetterquist, Pedroletti et al. 1999; Olin, Aldenbratt et al. 2001). The gastric lumen has been noted to have high levels of NO, therefore contamination with gastric air results in very high exhaled NO (Lundberg, Weitzberg et al. 1994).

9.7.6 Summary of physiological factors that could alter nitric oxide levels

In conclusion, exhaled NO is likely to be higher in males than females, and relates to age through childhood but not adulthood, possibly in relation to pneumatisation of the sinuses. It is reduced with current smoking, recent ingestion of water, alcohol or caffeine and increased with accidental gut contamination or after nitrogen-compound rich meals. There may be differences throughout the 24 hour day in asthmatic subjects with nocturnal asthma, and through the menstrual cycle in those who experience premenstrual exacerbations of asthma. However, relationships with height, weight, surface area, menstrual cycle in non-asthmatic women, or a circadian rhythm in non-asthmatic individuals has not been demonstrated. Despite some of the findings above, there is only one range of ‘normal’ values given for the results of measurement for oral or nasal results.

9.8 Nitric oxide levels in asthma and atopy

Since commencement of the studies on exhaled NO, more research has been conducted in asthmatic populations than any other group; in particular assessing the use of NO for
screening, diagnostic and monitoring purposes. Initially cross sectional studies were carried out with comparisons to the traditional inflammatory and clinical markers used in asthma (see Chapter 1). Research then graduated to the use of longitudinal studies to assess the predictive value of NO for presence, severity and to denote exacerbations of asthma. Longitudinal studies have also been used to assess treatment effects in standard and new therapies. The relationships between NO and asthmatic inflammatory markers, lung function testing, bronchodilator responsiveness and airway challenges have all been studied. I have grouped the studies presented here under headings of the hypotheses that the studies were devised to address.

9.8.1 Does nitric oxide correlate with other asthmatic inflammatory markers?

Exhaled NO was found to correlate with sputum eosinophil numbers in asthmatics and healthy controls in adults (Jatakanon, Lim et al. 1998; Berlyne, Parameswaran et al. 2000; Tsujino, Nishimura et al. 2000; Reid, Johns et al. 2003; Silkoff, Lent et al. 2005; Fujimoto, Yamaguchi et al. 2006; Zietkowski, Bodzenta-Lukaszyk et al. 2006) and in children (Piacentini, Bodini et al. 1999; Little, Chalmers et al. 2000; Warke, Fitch et al. 2002; Sacco, Sale et al. 2003; Mahut, Delclaux et al. 2004; Thomas, Gibson et al. 2005; Li, Tsang et al. 2006). While these have been demonstrated in asthmatics whether on or off a range of medications, the correlations are strongest for steroid naive patients. These findings have been consistent across the studies that used variable expiratory flows, and were confirmed in one study that compared results in these subject groups across a range of flows (Berry, Shaw et al. 2005). A positive correlation has also been demonstrated between exhaled NO and serum eosinophil cell counts in adults and children (Tsujino, Nishimura et al. 2000; Reid, Johns et al. 2003; Silvestri, Sabatini et al. 2003; Strunk, Szeffler et al. 2003; Lehtimaki, Kankaanranta et al. 2005; Zietkowski, Bodzenta-Lukaszyk et al. 2006). Correlations have been described between NO and sputum ECP levels (Piacentini, Bodini et al. 1999; Warke, Fitch et al. 2002; Thomas, Gibson et al. 2005), as well as NO and serum ECP levels (Aziz, Wilson et al. 2000; Dal Negro, Micheletto et al. 2003; Strunk, Szeffler et al. 2003; Mahut, Delclaux et al. 2004; Zietkowski, Bodzenta-Lukaszyk et al. 2006). Perhaps unsurprisingly, in studies that compared both, it was sputum rather than serum markers that had stronger correlations with exhaled NO levels. A positive correlation was also shown with the leukotriene group of inflammatory markers – leukotriene E4, leukotriene B4 and 8-isoprostane (Mondino, Ciabattoni et al. 2004), and a negative correlation with the anti-inflammatory cytokines IL-4 and IL-13 (Shome, Starnes et al. 2006).
While these cross-sectional and the longitudinal studies described below (see Section 9.8.5) have confirmed these findings, it has not been universal. For example, in 58 asthmatic children no association between NO and eosinophils or ECP from sputum or serum was shown (Wilson, James et al. 2001). Other studies did not demonstrate a relation between one specific parameter such as NO with serum eosinophils (Turktas, Oguzulgen et al. 2003), with serum ECP (del Giudice, Brunese et al. 2004) or with sputum leukotriene levels (Strunk, Szefler et al. 2003), although they confirmed other associations. Looking at these studies, there is no apparent difference in methodology compared to others with positive findings, although they are more often heterogeneous groups of asthmatics on a wide range of medications, with smaller numbers of subjects and tended to be 'doctor diagnosed' asthmatics rather than those diagnosed with specific testing when in clinic which was also conducting the research.

Direct comparisons of NO with bronchial biopsy results have also been studied. One group found a correlation between exhaled NO and each of the eosinophil, lymphocyte and mast cell components (Silkoff, Lent et al. 2005), while another study found that NO was only related to the total number of inflammatory cells present (Turktas, Oguzulgen et al. 2003). In 31 children with difficult asthma, there was a relationship between exhaled NO and the eosinophil score in seven of 21 children for whom biopsies and NO were obtained. The strongest relationship was an exhaled NO >7ppb and raised eosinophils at baseline in children who had persistent symptoms despite high dose prednisolone for two weeks (Payne, McKenzie et al. 2001). Exhaled NO was also shown to correlate with the degree of airway remodeling on biopsy samples in a group of 28 children (Mahut, Delclaux et al. 2004) and the degree of bronchial wall thickening seen on a CT scan in nine asthmatic children (Ket i, Harkins et al. 2005).

9.8.2 Does nitric oxide correlate with lung function and bronchial hyper-responsiveness?

Comparisons of exhaled NO with lung function parameters have produced variable results. While many have shown a significant negative correlation, particularly to FEV1, in adults (de Gouw, Hendriks et al. 1998; Ho, Wood et al. 2000; Dal Negro, Micheletto et al. 2003; Nogami, Shoji et al. 2003) and children (Colon-Semidey, Marshik et al. 2000; Piacentini, Bodini et al. 2000; Spallarossa, Battistini et al. 2001; Beck-Ripp, Griese et al. 2002; Malmberg, Pelkonen et al. 2003; del Giudice, Brunese et al. 2004; Saito, Inoue et al. 2004) others were not able to demonstrate this (al-Ali, Eames et al. 1998; Silkoff, McClean et al. 1998; Piacentini, Bodini et al. 1999; Ho, Wood et al. 2000; Silvestri, Spallarossa et al. 2000;
Turktas, Oguzulgen et al. 2003; Mappa, Cardinale et al. 2005; Spergel, Fogg et al. 2005; Thomas, Gibson et al. 2005; Zietkowski, Bodzenta-Lukaszyk et al. 2006). Again some of these studies described correlations with certain parameters only such as $\text{FEF}_{25-75}^2$ (Mahut, Delacourt et al. 2004; Battaglia, den Hertog et al. 2005; Lehtimaki, Kankaanranta et al. 2005), $\text{FEV}_1/\text{FVC}$ ratio (Strunk, Szefler et al. 2003), residual volume (Mappa, Cardinale et al. 2005), or peak flow lability (al-Ali, Eames et al. 1998; Lim, Jatakanon et al. 2000). In other studies the correlations were only significant in certain groups such as atopic, rather than non-atopic, asthmatics (Franklin, Stick et al. 2004).

Positive correlations have been demonstrated between NO levels and airway reactivity as measured by methacholine challenges (Jatakanon, Lim et al. 1998; Henriksen, Lingsas-Holmen et al. 2000; Wilson, Dempsey et al. 2001; Prieto, Gutierrez et al. 2002; Prieto, Gutierrez et al. 2002; Buchvald, Eiberg et al. 2003; Langley, Goldthorpe et al. 2003; Malmberg, Pelkonen et al. 2003; Nogami, Shoji et al. 2003; Berkman, Avital et al. 2005; Ehrs, Sundblad et al. 2006), histamine challenges (al-Ali, Eames et al. 1998; de Gouw, Hendriks et al. 1998; Dupont, Rochette et al. 1998; Dupont, Remedts et al. 2003; Zietkowski, Bodzenta-Lukaszyk et al. 2006), or adenosine 5-monophosphate challenges (de Gouw, Hendriks et al. 1998; Aziz, Wilson et al. 2000; Prieto, Gutierrez et al. 2002; Prieto, Uixera et al. 2002; Prieto, Bruno et al. 2003; Berkman, Avital et al. 2005). Again some studies found correlations only in certain groups of subjects such as atopic asthmatics (Franklin, Stick et al. 2004) or those on IHCS (Reid, Johns et al. 2003) rather than all asthmatic and control subjects.

9.8.3 Can nitric oxide be used for diagnosis of asthma?

Studies have assessed whether measurement of exhaled NO can be used to diagnose asthma, either to screen a general population or to screen new clinic referrals. One group evaluated 47 consecutive adult patients referred with symptoms suggestive of asthma. The individual sensitivities for each of the conventional tests (peak flow, spirometry, serum eosinophils, bronchodilator responsiveness and airway challenge) were up to 47%; much lower than for exhaled NO at 88% and sputum eosinophilia at 86%. The results for conventional tests were not improved when using a trial of oral steroid as a diagnostic test. The researchers concluded that exhaled NO and induced sputum analysis, particularly when combined, were superior to the other conventional approaches in diagnosing asthma (Smith, Cowan et al. 2004). The same group looked at the predictive accuracy of exhaled NO to identify steroid responsiveness to IHCS for four weeks in 52 adults presenting with undiagnosed respiratory
symptoms. Steroid response was significantly greater in those with the higher exhaled NO levels (> 47ppb) independent of the original diagnostic label (asthma, COPD or chronic bronchitis) (Smith, Cowan et al. 2005). A similar study evaluated 95 patients presenting with respiratory symptoms in which 40 were subsequently diagnosed as asthmatic. A baseline value of NO at >7ppb best differentiated between asthmatics and non-asthmatics with a sensitivity of 82.5% and a specificity of 88.9%. The generated receiver-operated curves (ROC) gave a value for exhaled NO of 0.89, similar to both methacholine and adenosine-5 monophosphate challenges, better than lung function or exercise testing (Berkman, Avital et al. 2005). In 50 consecutive subjects being screened for exercise induced bronchospasm, exhaled NO at <12ppb gave a sensitivity of 1.0, a specificity 0.31, a negative predictive value 0.19 and a positive predictive value 1.0 of not having symptoms. Therefore no patient with a baseline NO <12ppb demonstrated exercise induced bronchospasm with the ROC curve giving a value of 0.63 (ElHalawani, Ly et al. 2003). In 160 patients diagnosed with asthma of the 240 adults referred with symptoms, a cut off value of 16ppb for exhaled NO had a specificity of 90% and a positive predictive value of greater than 90% (Dupont, Demedts et al. 2003). A cross sectional study of respiratory exacerbations in 42 adults showed a higher exhaled NO in those with asthma compared to those with COPD or acute pneumonia (Al-Ali and Howarth 2001).

Similar studies have been conducted in children. In almost 1000 13-14 year olds, exhaled NO levels had a similar value to the ISAAC questionnaire for detecting asthma, but was also found to be high in those with hay fever and/or eczema (Nordvall, Janson et al. 2005). In a cohort of over 100 Australian school children with a mean age of 14.7 years, exhaled NO had a negative predictive value for asthma of 3% and a positive predictive value of 54%. This was comparable to other tests evaluated, which included asthma symptoms, skin prick tests, sputum eosinophils and sputum ECP (Thomas, Gibson et al. 2005). Fifty-two 12-16 year olds with mild intermittent asthma not on treatment had higher levels of NO than 22 controls, in whom 43 (83%) had NO levels more than two standard deviations above normal values. This led the researchers to suggest that adolescents with mild asthma may need more accurate evaluation rather than assume that they were 'growing out of it' (Spallarossa, Battistini et al. 2003). A study assessed exhaled NO in 69 younger children aged 2-7 years using tidal breathing measurements and found the points of sensitivity differentiating the mild asthmatics from non-asthmatics (including subjects with chronic cough, recurrent pneumonia or who were healthy with no symptoms) were 77% and 88% for exhaled NO levels of 3.8ppb and 2.9ppb respectively (Avital, Uwyyed et al. 2001). Furthermore, in 158 preschool children
exhaled NO was the best predictor, when compared to lung function testing and bronchodilator response, to discriminate those who subsequently developed asthma with the sensitivity of 86% and a specificity of 92% at a cut off level chosen 1.5 standard deviations above the average for controls (Malmberg 2004).

9.8.4 **Is nitric oxide associated with symptoms and severity of asthma?**

Positive correlations with exhaled NO have been shown with bronchodilator use and symptom scores (Lanz, Leung et al. 1997; Tsujino, Nishimura et al. 2000; Roberts, Hurley et al. 2004; Warke, Mairs et al. 2004; Pijnenburg, Bakker et al. 2005; Spargel, Fogg et al. 2005; Prasad, Langford et al. 2006), cough (Li, Lex et al. 2003) and bronchodilator responsiveness (Colon-Semidey, Marshik et al. 2000; Little, Chalmers et al. 2000; Dupont, Demedts et al. 2003; Malmberg, Pelkonen et al. 2003; Silvestri, Sabatini et al. 2003; Pijnenburg, Bakker et al. 2005; Fujimoto, Yamaguchi et al. 2006; Zietkowsk!, Bodzenta-Lukaszyk et al. 2006). In many of the studies, the NO levels and the sputum eosinophil levels had stronger correlations to symptoms than other parameters measured. Again the findings were not universal. For example, in some studies no correlation was seen between NO and bronchial hyper-responsiveness (al-Ali, Eames et al. 1998; Silkoff, McClean et al. 1998; Chan-Yeung, Obata et al. 1999; van Rensen, Straathof et al. 1999; Ho, Wood et al. 2000; Silvestri, Spallarossa et al. 2000; del Giudice, Brunese et al. 2004; Thomas, Gibson et al. 2005; Jentzsch, le Bourgeois et al. 2006), NO and symptoms (al-Ali, Eames et al. 1998; Griese, Koch et al. 2000; Sippel, Holden et al. 2000) or NO and medication use (al-Ali, Eames et al. 1998; Sippel, Holden et al. 2000). In addition, no relationship was determined in 77 adult asthmatics between exhaled NO and quality of life (Ehri, Sundblad et al. 2006).

There are a number of possible explanations for the inconsistencies. Firstly, the correlation with severity is difficult depending on how ‘severity’ is defined. We know that exhaled NO is higher in steroid naive asthmatics, so those on increased amounts of treatment may have reduced levels of NO but their requirement for higher IHCS doses or additional medications to control symptoms deems them more ‘severe’. Several studies using either the GINA guidelines or the ATS asthma guidelines to categorize asthmatic patients into gradations of severity have been unable to show a relationship to exhaled NO levels in adults (Stirling, Kharitonov et al. 1998; Chan-Yeung, Obata et al. 1999; Lim, Jatakanon et al. 2000; Sippel, Holden et al. 2000) or in children (Griese, Koch et al. 2000). In one study of 30 children divided into mild, moderate and severe groups, the exhaled NO did appear to correlate with asthma severity (Delgado-Corcoran, Kissoon et al. 2004), and those on oral steroids with
'difficult asthma' also had higher exhaled NO suggesting ongoing inflammation (Stirling, Kharitonov et al. 1998; Payne, Adcock et al. 2001). Secondly, studies in asthma have commonly considered it as a single diagnostic entity when we increasingly have come to consider it as a more heterogeneous disease. Differing factors within the asthma 'syndrome' have been emphasized such as atopic versus non-atopic (or allergic versus intrinsic), and, more recently, defined by the cell most prominent in the airway inflammation; eosinophilic versus neutrophilic versus both or neither. In the cross sectional studies, most have consider asthmatics as a single group without the nicety of these divisions. Thirdly, other factors may influence asthma development, such as genetics. One group studied the genotype of the NOS enzyme in adult asthmatics and COPD patients, and showed that if there were high numbers of "AAT" sequences (greater than 12 repeats) in the specific region of "intron 20" then much lower levels of exhaled NO were obtained (Wechsler, Grasemann et al. 2000). Fourthly, we are comparing exhaled NO as a predictor against other single measurements such as sputum eosinophils or FEV₁ when it is unlikely that these are all isolated parameters. In a study of 92 children with 59% receiving IHCS, factor analysis selected four factors explaining 55.5% of the total variance. These factors were exhaled NO, plasma total IgE, plasma specific IgE and peripheral blood eosinophil percentage. The researchers suggested that these parameters operate as a group and should not be viewed as having non-overlapping characteristics (Leung, Wong et al. 2005).

Since cross sectional studies have not demonstrated a good correlation between asthma severity and NO, perhaps it is more appropriate to look at NO levels in controlled versus uncontrolled asthmatics. In 32 adults about to commence on a comparison of treatments, baseline exhaled NO was higher in those presenting with symptoms (Gratziou, Rovini et al. 2001). In 45 asthmatic children higher NO levels were obtained in those that were recently symptomatic, although all had levels above normals (Mahut, Delacourt et al. 2004), and in 14 of 22 atopic asthmatic children with current symptoms the exhaled NO was two standard deviations above the normal control group (Silvestri, Spallarossa et al. 1999). In studies looking at NO levels in a total of 625 asthmatic patients with allergy as a trigger, the NO levels were higher in those recently exposed (Artlich, Busch et al. 1999; Simpson, Custovic et al. 1999; Langley, Goldthorpe et al. 2003), in those with perennial exposure compared to seasonal allergies (Olin, Alving et al. 2004) and in those sensitised with common indoor allergens such as dust mite, cat and dog.

Is this pertinent to clinical practice? In the following studies, the physicians attending the children were blinded to the exhaled NO readings when making their concurrent assessment
and treatment decisions. Exhaled NO correctly predicted 21 asthmatic children deemed to be 'uncontrolled' versus 31 who were 'well controlled' (Meyts, Proesmans et al. 2003). Fifteen asthmatic children followed over 3-4 visits showed that exhaled NO was significantly correlated with the degree of control (Delgado-Corcoran, Kissoon et al. 2004). In 74 asthmatics and 31 healthy control children, the exhaled NO level when raised above normal (designated as > 13ppb in this study) had a sensitivity of 0.67 and a specificity of 0.65 to predicted the requirement for a step-up in therapy (Griese, Koch et al. 2000).

9.8.5 Can nitric oxide predict deterioration in asthma control?

The studies above looked at concurrent exhaled NO and symptoms. In one study, cited above, the cross sectional analysis of 100 asthmatic patients from 7 to 80 years of age showed that exhaled NO correlated better with markers of control such as lung function and sputum eosinophils rather than markers of severity such as history of respiratory failure, healthcare use, fixed air flow obstruction or an asthma severity score (Sippel, Holden et al. 2000). However, it would be a more useful tool if it could predict loss of control when following asthmatic individuals' longitudinally.

In 22 moderate to severe asthmatics seen in a routine clinic visit, those who subsequently had an exacerbation within two weeks had significantly higher mean exhaled NO (29ppb versus 13ppb) compared to those who remained stable (Harkins, Fiato et al. 2004). Levels of exhaled NO were followed prospectively in 44 asthmatics on daily ICS and LABA therapy over 18 months, with 22 having one or more exacerbations. While the baseline FEV₁ remained the best predictor, the ROC curve for exhaled NO to predict exacerbation was 0.71. An exhaled NO of 28ppb gave a sensitivity of 0.59, a specificity of 0.82, a positive predictive value of 0.77 and a negative predictive value of 0.87 for having increased symptoms. So, an exhaled NO >28ppb gave a relative risk of 3.4 for an exacerbation, although authors concluded that combining exhaled NO and FEV₁ was the most useful (Gelb, Flynn Taylor et al. 2006). In 44 sensitized children prospectively followed though one grass pollen season, NO was best associated with the mean pollen count the week before the NO measurement and was significantly associated with asthma control (Roberts, Hurley et al. 2004). In 105 asthmatic and healthy children, exhaled NO did not depict asthma severity but correlated with changes in asthma therapy. Levels >13ppb, had a sensitivity of 0.67 and a specificity of 0.65 to predict step-up in therapy (Griese, Koch et al. 2000). In 21 asthmatic children with seasonal allergic asthma compared to 21 healthy children that were followed throughout a grass pollen season, their NO levels were higher, increased through the pollen
season and then reduced down to baseline. However, their exhaled NO did not necessarily correlate with FEV\textsubscript{1} (Baraldi, Carra et al. 1999). Exhaled NO did relate to recent symptoms versus no symptoms when dividing 133 children aged 5-14 years attending a hospital clinic into ‘uncontrolled’ and ‘controlled’ asthma groups. The researchers also felt that NO predicted subgroups as to whether IHCS or medication was increased, decreased or remained unchanged (Warke, Mairs et al. 2004).

There have also been studies where exhaled NO was measured prospectively while IHCS doses were reduced. After three months of good control on moderate doses of IHCS (500-1000\textmu g/day beclomethasone), 37 asthmatics had their individual doses halved for twelve weeks. Parameters which detected the ten that developed an exacerbation were; a baseline exhaled NO level > 20ppb, or a baseline exhaled NO level > 15ppb plus a bronchoconstriction response to the adenosine 5-monophosphate challenge, or a rapid increase in exhaled NO over the first weeks of the assessments (Prieto, Bruno et al. 2003). In 78 asthmatic adults, IHCS treatment was withdrawn over six weeks with 60 subjects developing symptoms. Both the baseline measurement of exhaled NO and an increase of 60% over the baseline reading had positive predictive values between 80-90% for an exacerbation and was similar to the predictive values using sputum eosinophils or hypertonic saline airway responsiveness (Jones, Kittelson et al. 2001). Similarly, nine of 40 children with a mean age of 12.2 years relapsed 2-4 weeks after withdrawal of steroids. The exhaled NO was higher at a mean of 35.3ppb versus 15.7ppb at two weeks and 40.8ppb versus 15.9ppb at four weeks. A value of 49ppb at four weeks after steroid discontinuation had the best sensitivity at 71% and specificity at 93% for an asthma relapse (Pijnenburg, Hofhuis et al. 2005). One study was less conclusive. Seventeen moderate asthmatic adults had their daily dose of IHCS halved at 20 day intervals until loss of control or replacement with placebo. Of the parameters which were measured every ten days, the sputum eosinophil percentage altered 20 days ahead of deterioration while changes in exhaled NO, FEV\textsubscript{1} and methacholine PC\textsubscript{20} were only observed once symptoms had already developed (Belda, Parameswaran et al. 2006).

Studies have also compared changes in exhaled NO with traditional clinical and lung function assessments to dictate changes in treatment. In 97 adult asthmatics, future treatment decisions regarding steroid adjustment were assigned randomly to be based either on exhaled NO levels or on an algorithm based on the conventional GINA 2002 guideline (GINA 2002). The optimal IHCS dose per individual was determined in the first phase and patients were then followed for 12 months. The mean final doses of inhaled fluticasone used were 370\textmu g/day for the 46 patients in the exhaled NO group and 641\textmu g/day for the 48 patients in the guideline
Rates of exacerbation were 0.49 episodes/patient/year in the exhaled NO managed group and 0.90 in the guideline group, representing a 45.6% exacerbation reduction in the NO group, although this did not reach significance. There were no significant differences in pulmonary function changes, sputum eosinophils or the use of oral prednisone (Smith, Cowan et al. 2005). Similar to the adult study, children on IHCS were allocated to groups where treatment decisions were made by symptomatology in 46 or with the addition of exhaled NO in 39. Over the next year, those monitored using the NO readings had improved airway hyperresponsiveness from 2.5 to 1.1 doubling dose, less severe exacerbations at eight versus 18, and a trend to improved FEV₁ (Pijnenburg, Bakker et al. 2005). These studies suggest that NO can successfully be used longitudinally for individual patients to predict exacerbations and to aid management decisions regarding treatment.

9.8.6 What happens to nitric oxide during an acute asthma attack?

Most studies have examined NO levels during chronic asthma disease over time. So what happens to NO levels in the acute phase? Studies have used allergen challenges to simulate an acute asthmatic exacerbation. It appears that even when the NO is similar in the asthmatic and control groups at baseline, NO increases in the asthmatic groups during the late asthmatic reaction (Obata, Dittrick et al. 1999; Paredi, Leckie et al. 1999; Khatri, Hammel et al. 2003; Ihre, Gyllfors et al. 2006). It was found to increase three fold from baseline 24 hours post challenge (Khatri, Hammel et al. 2003). Repeated low dose allergen inhalation in 8 mild asthmatic subjects demonstrated an increased exhaled NO despite no change seen in asthma symptoms or lung function (Ihre, Gyllfors et al. 2006). These findings suggest that in acute asthma an increased production requiring synthesis of the NOS enzymes occurred and therefore some time was needed to appreciate the full insult (Khatri, Hammel et al. 2003). This may also explain why the use of NO to dictate treatment acutely has been less successful. One study used the exhaled NO levels to dictate the degree of treatment given to adult patients presenting to the emergency department with an asthma exacerbation. The study was discontinued after the first 53 enrolled subjects as those in whom the management offered was decided on their exhaled NO levels did less well when followed up than those in whom the management was decided by the more traditional measures of clinical scores or spirometry (Gill, Walker et al. 2005).

9.8.7 What is the effect of atopy alone on nitric oxide?

In assessing the validity of exhaled NO to assist in screening, diagnosis and longitudinal monitoring of asthma control, the effect of atopy alone must be taken into account. A number
of cross sectional studies with a total of 949 subjects have shown that there is a higher mean exhaled NO which decreases through the groups of atopic asthmatics, non-atopic asthmatics, atopic subjects and non-atopic controls. However, considerable overlap between groups exist so that a single cut off may not discern exactly into which group an individual belongs (Frank, Adisesh et al. 1998; Barreto, Villa et al. 2001; Henriksen, Holmen et al. 2001; Prieto, Gutierrez et al. 2002; Jouaville, Annesi-Maesano et al. 2003; Cardinale, de Benedictis et al. 2005; Prasad, Langford et al. 2006). In these studies, asthmatics divided into those who were atopic and non-atopic showed those with atopy had higher levels of exhaled NO. In 28 asthmatics on IHCS treatment, NO levels were positively correlated with total IgE and positive skin prick testing more than bronchial hyper-reactivity, lung function or asthma severity (Ho, Wood et al. 2000). In 92 steroid naive asthmatics, the NO was high in those that were atopic with no correlation to any other parameters (Silvestri, Sabatini et al. 2003). In 213 children, exhaled NO was higher in children with atopy alone compared to those with non-atopic asthma and was correlated to the wheal size of skin prick tests (Barreto, Villa et al. 2001). In 53 steroid naive asthmatic and 96 non-asthmatic children who had skin prick testing to 12 common allergens, the exhaled NO was higher in the asthmatic versus non-asthmatic individuals but was also higher in atopic (including those with allergic rhinitis alone) versus non-atopic children (Jouaville, Annesi-Maesano et al. 2003). In 53 subjects, no significant difference was found between non-atopic asthmatics and controls (Ludviksdottir, Janson et al. 1999). Similarly of 140 subjects, 15% of those with asthma but not deemed allergic had NO levels within the normal range (Zietkowski, Bodzenta-Lukaszyk et al. 2006).

Looking at the effect of atopy alone, 38 subjects with allergic rhinitis but not asthma had a raised exhaled NO (Prieto, Gutierrez et al. 2002) and in 64 atopic Pacific Island subjects, NO levels correlated with house dust mite wheal reaction and clinical atopy (Moody, Fergusson et al. 2000). More studies have been conducted evaluating exhaled NO with atopic status in children – mostly within community school cohorts. In 356 children from an unselected population, exhaled NO was two fold higher in atopic individuals (noted as having positive skin prick tests and a high IgE) with high eosinophil counts compared to atopic individuals with low eosinophil counts. However the exhaled NO in both these groups was higher than the non-atopic subjects regardless of the eosinophil count, suggesting it was a link with atopy not just eosinophilic inflammation (Barreto, Villa et al. 2005). Exhaled NO related to both eosinophil levels and atopy but not necessarily to asthma and/or wheeze in the last 12 months in 155 children (Franklin, Turner et al. 2003). NO also correlated with skin prick testing and sputum eosinophils but not airway responsiveness in 107 children (Thomas, Gibson et al. 2005).
Exhaled NO showed strongly positive correlations to non-specific IgE and mite specific IgE with weaker positive correlations to specific IgE to cat and cedar in 278 children (Saito, Inoue et al. 2004). Finally in 374 school children high exhaled NO levels were associated with pet sensitisation in atopic children, and respiratory infections and home window pane condensation in non-atopic children rather than other factors (Janson, Kalm-Stephens et al. 2005). In a hospital clinic setting in 45 children, there was a correlation between eczema and exhaled NO but not asthma, allergic rhinitis, food allergy, the use of inhaled corticosteroids, anti leukotriene therapy, antihistamine therapy or lung function (Jentzsch, le Bourgeois et al. 2006).

Thus atopy alone is an important confounding factor when using exhaled NO to assess asthmatic and healthy individuals.

9.8.8 Can nitric oxide be an outcome measure for assessing treatment?

9.8.8 (i) Corticosteroids

Similar to my own study, there have now been many publications confirming that levels of exhaled NO is high in asthmatics on bronchodilator therapy only and is reduced in asthmatics on steroid therapy often to the equivalent levels found in healthy controls using adult (Alving, Weitzberg et al. 1993; Kharitonov, Yates et al. 1994; Persson, Zetterstrom et al. 1994; Kharitonov, Chung et al. 1996; Massaro, Mehta et al. 1996; al-Ali, Eames et al. 1998; Stirling, Kharitonov et al. 1998; Berlyne, Parameswaran et al. 2000; Lim, Jatakanon et al. 2000; Tsujino, Nishimura et al. 2000; Reid, Johns et al. 2003; Shin, Rose-Gotttron et al. 2004) and paediatric subjects (Byrnes, Dinarevic et al. 1997; Nelson, Sears et al. 1997; Silvestri, Spallarossa et al. 1999; Colon-Semidey, Marshik et al. 2000; Little, Chalmers et al. 2000; Piacentini, Bodini et al. 2000; Silvestri, Spallarossa et al. 2000; Visser, de Wit et al. 2000; Avital, Uwyyed et al. 2001). Many of these studies also determined that the raised exhaled NO levels in asthmatics reduced when they were given inhaled or oral steroids from five days to several weeks in adults (Silkoff, McClean et al. 1998; Jatakanon, Kharitonov et al. 1999; Lim, Jatakanon et al. 1999; van Rensen, Straathof et al. 1999; Wilson and Lipworth 2000; Silkoff, McClean et al. 2001; Jones, Herbison et al. 2002; Kharitonov, Donnelly et al. 2002; Currie, Syme-Grant et al. 2003; Dal Negro, Micheletto et al. 2003; Zeidler, Kleerup et al. 2004; Brightling, Green et al. 2005; Silkoff 2005; Smith, Cowan et al. 2005; Zietkowskii, Bodzenta-Lukaszyk et al. 2006) and children (Byrnes, Dinarevic et al. 1997; Artlich, Busch et al. 1999; Lanz, Leung et al. 1999; Colon-Semidey, Marshik et al. 2000; Piacentini, Bodini et al. 2000; Spallarossa, Battistini et al. 2001; Beck-Ripp, Griese et al. 2002; Mondino.
Ciabattoni et al. 2004; Petersen, Agertoft et al. 2004; Cardinale, de Benedictis et al. 2005; Prasad, Langford et al. 2006; Zeiger, Szefer et al. 2006). In addition to commencing IHCS in steroid naive patients, some studies also showed a dose response reduction of exhaled NO (Stirling, Kharitonov et al. 1998; Colon-Semidey, Marshik et al. 2000; Piacentini, Bodini et al. 2000; Silkoff, McClean et al. 2001; Jones, Herbison et al. 2002; Kharitonov, Donnelly et al. 2002). Two studies demonstrated a plateau in the reduction of exhaled NO in asthmatic children at 400μg beclomethasone or equivalent (Jatakanon, Kharitonov et al. 1999; Wilson and Lipworth 2000) but was not seen between 50 and 100μg hydrofluoroalkane-beclomethasone (Petersen, Agertoft et al. 2004). One study showed that at a lower dose of 200μg beclomethasone there was a longer time required for the reduction of exhaled NO to the lowest levels measured compared to commencing at double this dose (Dal Negro, Micheletto et al. 2003), although another study found no additional reduction in exhaled NO after ten days of treatment even with low doses (Spallarossa, Battistini et al. 2001). The reduction of NO levels has even been seen with single nebulised steroid doses (Baraldi, Azzolin et al. 1997; Lanz, Leung et al. 1999; Tsai, Lee et al. 2001). In addition, these findings have been confirmed using tidal breathing techniques (Visser, de Wit et al. 2000) and reservoir samples (Baraldi, Dario et al. 1999; Colon-Semidey, Marshik et al. 2000). Many of these individual papers have been already been discussed in more detail in Chapters 6, 7 and 8. Only one cross sectional hospital based study in 392 adult patients with a range of asthma severity showed no difference in the exhaled NO between those on or not on IHCS, although the researchers did show a relationship between exhaled NO and airway reactivity (measured by methacholine challenge) and atopic status (Langley, Goldthorpe et al. 2003).

In addition to using NO as an outcome measure to follow asthmatics treated with conventional inhaled steroids (considered here to be fluticasone, budesonide and beclomethasone), NO has also been used to assess whether novel steroids have a similar effect. Significant improvements in exhaled NO levels and induced sputum eosinophil counts were seen in three separate studies of ciclosonide – one using it alone (Wilson, Duong et al. 2006), one comparing it to budesonide (Kanniess, Richter et al. 2001) and one comparing it to fluticasone (Lee, Fardon et al. 2005). Four week treatment periods in 25 children with the newer beclomethasone derivative, hydrofluoroalcane-beclomethasone dipropionate at doses of 50μg and 100μg daily also resulted in reducing exhaled NO, associated with improvements in FEV1 and exercise challenges (Petersen, Agertoft et al. 2004).
In this class of drugs, the two predominantly studied in conjunction with NO have been montelukast and pranlukast. Firstly, NO has been monitored when these have been used as an addition to regular treatment. An open label six week trial of oral montelukast in 20 adult asthmatics already on IHCs resulted in significant improvements for levels of exhaled NO, coinciding with an improvement in exercise tolerance (Berkman, Avital et al. 2003). Similarly, in 20 stable asthmatics, a crossover trial showed that montelukast, as opposed to placebo, reduced exhaled NO as well as reducing peak flow variability and symptom score over a two week treatment period (Sandrini, Ferreira et al. 2003). When montelucast was added to treatment of 22 adults with mild to moderate asthma using fluticasone 250μg and salmeterol 50μg twice a day, montelukast 10mg a day, compared to placebo, reduced levels of exhaled NO and blood eosinophilia (Currie, Lee et al. 2003). In 35 children treated with IHCs in whom the exhaled NO was greater than 20ppb, those that had montelukast added to their treatment had a significant reduction of exhaled NO which increased back to baseline level after withdrawal of the drug (Ghiro, Zanconato et al. 2002). While the reduction of NO coincided with some clinical improvements, there was no improvement in others such as bronchial responsiveness (Berkman, Avital et al. 2003), FEV₁ (Ghiro, Zanconato et al. 2002; Currie, Lee et al. 2003; Sandrini, Ferreira et al. 2003) or in measured exhaled H₂O₂ (Sandrini, Ferreira et al. 2003). Only one study conducted in 25 children did not show a difference in exhaled NO with the addition of montelukast to IHCs treatment (Strauch, Moske et al. 2003). Pranlukast added to a six week reduction of inhaled budesonide did prevent asthma deterioration compared to placebo, but did not show a difference in levels of exhaled NO, although NO levels in the placebo group increased (Tamaoki, Kondo et al. 1997).

Secondly, NO has also been used as an outcome measure when these medication have been used alone. In steroid naïve children (twelve to 21 subjects in each study), treatment with montelukast (5 or 10mgs per day) for between two and eight weeks resulted in a reduction in exhaled NO in two studies (Bratton, Lanz et al. 1999; Lee, Lai et al. 2005) but not in a third (Spahn, Covar et al. 2006) with the individual researchers also showing improvements in FEV₁ (Bratton, Lanz et al. 1999), salbutamol use (Bratton, Lanz et al. 1999), residual volume (Spahn, Covar et al. 2006) and serum ECP levels (Spahn, Covar et al. 2006). They did not demonstrate changes in symptom scores (Spahn, Covar et al. 2006) and the exhaled NO returned to pre treatment levels two weeks after withdrawal of the medication (Lee, Lai et al. 2005). In younger steroid naïve children aged ten months to five years (54 in total) with an early diagnosis of asthma, significant improvements were seen in levels of exhaled NO,
FEV<sub>0.5</sub>, symptom score and airways resistance but not bronchodilator responsiveness after four weeks treatment with 4mg daily of montelukast (Straub, Minocchieri et al. 2005; Straub, Moeller et al. 2005). In a study of twelve asthmatic children where montelukast reduced levels of exhaled NO, the four individuals that were heterogeneous at the gene locus of the leukotriene C4 synthase had a far better response than those who were homozygotes (Whelan, Blake et al. 2003).

Thirdly, NO has been measured as one factor in comparison studies between montelukast and IHCS which have all resulted in a greater reduction of levels of NO in the IHCS arm (Kanniess, Richter et al. 2002; Peroni, Bodini et al. 2005; Zeiger, Szefler et al. 2006). This coincided with improvements of hyperreactivity (Kanniess, Richter et al. 2002; Peroni, Bodini et al. 2005), FEV<sub>1</sub> (Kanniess, Richter et al. 2002; Zeiger, Szefler et al. 2006), sputum eosinophils (Kanniess, Richter et al. 2002), asthma control questionnaire and salbutamol use (Zeiger, Szefler et al. 2006) with both treatments, again greater in the IHCS groups. However, the other antagonist pranlukast at 450mg per day did not change levels of exhaled NO in 30 adults compared to IHCS but did show improvement in all other parameters measured (Yamauchi, Tanifuji et al. 2001). Comparisons between this drug class and the long acting β2 agonists (LABAs) are described below.

9.8.8 (iii) Long acting β2 agonists

Exhaled NO has been used as an outcome measure when looking at the addition of long acting β2 agonists (LABAs) to IHCS. Neither eformoterol nor salmeterol have shown any effect on the exhaled NO when used alone in comparison to either IHCS therapy alone (Prieto, Gutierrez et al. 2002) or to the combination of IHCS and LABA (Aziz, Wilson et al. 2000; Currie, Syme-Grant et al. 2003), and no difference where they have been added to IHCS (Yates, Kharitonov et al. 1997; Lee, Jackson et al. 2003). This is despite improvements in other parameters such as FEV<sub>1</sub>, adenosine 5-monophosphate challenges, sputum and blood eosinophil counts and serum ECP (Aziz, Wilson et al. 2000; Prieto, Gutierrez et al. 2002; Currie, Syme-Grant et al. 2003; Lee, Jackson et al. 2003).

In comparisons between montelukast and LABAs, neither of two studies showed an effect on the level of exhaled NO (Aziz, Wilson et al. 2000; Wilson, Dempsey et al. 2001). This is interesting given that in most of the montelukast studies, this medication did result in a reduction in exhaled NO. The studies here really just confirm that the LABAs do not have anti-inflammatory properties.
9.8.8 (iv) Nedocromil sodium

In two paediatric studies, this medication had no effect on levels of exhaled NO when compared to IHCS in 32 sensitised patients followed through a pollen season (Gratziou, Rovina et al. 2001) or in 118 four to six year olds studied as part of the childhood asthma management program (CAMP) studies (Covar, Szefler et al. 2003).

9.8.8 (v) Theophylline

Despite theophylline being a previously commonly used drug, there has only been one study looking at its effect on levels of exhaled NO. In a double blind crossover study 15 patients with mild asthma on theophylline of 250mg twice per day had a reduction in eosinophil counts in sputum, BAL, and biopsy samples but no change in levels of exhaled NO and no improvements in lung function or bronchial hyper-responsiveness (Lim, Tomita et al. 2001).

9.8.8 (vi) Novel medications

L-NAME – Nebulised pretreatment with this non-selective NOS inhibitor reduced levels of exhaled NO from baseline in 22 asthmatics with either an early asthmatic response only or both an early and late asthmatic response to allergen challenge by 77 and 71% respectively. Despite this result in NO, there was no change on the magnitude of the other acute responses in either group (Taylor, McGrath et al. 1998).

L-Arginine – L-arginine (the substrate for the NOS enzymes) was nebulised as single doses of 0.75g, 1.5g and 3g to healthy subjects and asthmatics with 6-7 in each group. This increased levels of exhaled NO in a dose dependent fashion at 60 minutes showing a negative correlation to the fall in FEV₁ (Sapienza, Kharitonov et al. 1998). Nebulisation of 2.5g of L-arginine and D-arginine on separate days to eight steroid naive asthmatics both resulted in bronchoconstriction. There was an initial proportional decline of NO then, with resolution of the constriction, the NO increased so by three hours it was higher than baseline (Chambers and Ayres 2001). It is hard to know whether L-arginine is acting as a substrate in these studies or just as an airway irritant.

Omalizumab – This humanised monoclonal antibody to Ig E was used in a steroid reduction study in adults with allergic asthma over one year. In the 18 that received active medication compared to the 11 receiving placebo, the variability of the exhaled NO was significantly less and remained similar to the levels obtained when the children were on higher doses of IHCS prior to the reduction phase (Silkoff, Romero et al. 2004).
Indomethacin – This medication alters the cyclo-oxygenase products of the arachidonic acid pathway which play a role in bronchoconstriction and airway inflammation. Indomethacin 50mg daily or placebo was given to 38 asthmatics on beclomethasone > 1500μg daily for six weeks while the IHCS was reduced to half at week two and to a third at week four. While levels of exhaled NO increased in both groups, the effects were less pronounced in the indomethacin group and corresponded to fewer exacerbations (Tamaoki, Nakata et al. 2000).

PGE2/PGF2α – These prostaglandins also provide negative feedback in the cyclo-oxygenase pathway and a trial of inhaled PGE2 and PGF2α significantly reduced levels of exhaled NO in both normal and asthmatic subjects. This was not associated with any alteration of lung function in the normal subjects, but PGE2 caused an increase in FEV₁ in the asthmatic group (Kharitonov, Sapienza et al. 1998).

9.8.9 Summary of nitric oxide in asthma and atopy

How do we make sense of all this information regarding NO and asthma/atopy? NO does correlate with other clinical and inflammatory parameters of asthma – most particularly in the steroid naïve asthmatic and most particularly with sensitivity and specificity seen with eosinophil counts. The utilization of NO to contribute to diagnosis seems hopeful. It performed as well as eosinophil counts in induced sputum (Smith, Cowan et al. 2004) and methacholine or adenosine 5-monophosphate challenges (Berkman, Avital et al. 2005). However this again is best used in steroid naïve patients and the effect of atopy alone in elevating NO levels must be taken into account. It is easier to show NO differences in groups of subjects such ‘asthma’, ‘atopy’, ‘other respiratory disease’ or ‘controls’, rather than being able to place one individual into the correct category based on the exhaled NO reading. NO does appear to determine current or recent symptoms and is more likely to be useful in the individual when followed longitudinally for loss of control or response to treatment. Studies using NO to guide treatment have shown beneficial effects. However, again eosinophils were more sensitive and of the most benefit when these two parameters are combined (Brightling, Green et al. 2005; Pijnenburg, Bakker et al. 2005; Smith, Cowan et al. 2005; Belda, Parameswaran et al. 2006). In medications that have anti-inflammatory effects such as the different IHCS, leukotriene receptor antagonists, indomethacin and prostaglandins, it appears the result is to decrease NO levels. NO remains unchanged in those without such properties as in the LABAs. In these studies, when NO did show a reduction, this often coincided with other clinical benefits.
However, the major advantage of using measurements exhaled NO, particularly over sputum
eosinophil counts, blood tests or airway challenges is obvious – particularly in children. It is a
non-invasive test, analogous to lung function testing to which patients are very familiar. It is
also likely to be available in more clinics and can be performed more quickly and more
cheaply with immediate results.

9.9 Nitric oxide levels in primary ciliary dyskinesia

This is an area where NO measurement was found early to be of particular value. Initially NO
was found to be absent in nasal measurements in four children with Kartagener’s syndrome
(Lundberg, Weitzberg et al. 1994). This led to the evaluation of 21 children with primary
ciliary dyskinesia (PCD) in whom both nasal and oral NO was found to be significantly lower
when compared to 60 healthy children. While there was some overlap with regard to
individual lower airway results, only one child was an outlier from each group in the nasal
NO ranges (Karadag, James et al. 1999). This has been confirmed in a recent study where the
impairment of NO output was less pronounced in lower than the upper nasal respiratory tract
after sampling at several sites in 17 PCD and 28 healthy subjects (Mahut, Escudier et al.
2006). In another large cross-sectional study involving 102 subjects, the concentration of
exhaled NO was significantly lower in both PCD and CF patients than bronchiectasis and
healthy subjects. The nasal NO, however, was markedly reduced only in the PCD subjects
(Horvath, Loukides et al. 2003). This change appears to occur early in life as seen in two
infants with PCD aged four and six months having significantly lower levels of NO compared
to five healthy controls (Baraldi, Pasquale et al. 2004). These low NO levels found are despite
levels of nitrite, nitrate and S-nitrosothiol when measured in exhaled breath condensate being
no different from normals (Csoma, Bush et al. 2003).

Use of NO as a screening tool for this condition has been investigated. From the study above
reviewing children already diagnosed with PCD, the authors determined a cut off of 250ppb
for nasal NO as having both a positive and a negative predictive value of 0.95. This correctly
detected 20 of 21 subjects as having PCD and correctly detected 19 of 20 as not having PCD
(Karadag, James et al. 1999). Measuring 31 children with PCD, 21 with non-CF
bronchiectasis, 17 with CF, 35 with asthma and 53 healthy controls, a nasal NO of 250ppb
gave a sensitivity of 97% and a specificity of 90% for a diagnosis of PCD (Narang, Ersu et al.
2002). Of 34 children referred for investigation for possible PCD to a tertiary clinic, the 17
that proved positive on biopsy again had significantly lower level of nasal NO. This group
determined a nasal NO of 105ppb gave a specificity of 88% and a positive predictor value of

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89% for PCD, and above that excluded PCD with 100% certainty (Corbelli, Bringolf-Isler et al. 2004). Reviews now recommend the use of nasal NO to screen for PCD, usually with the 250ppb cut-off (Silkoff 2004; Stehling, Roll et al. 2006).

Nebulised L-arginine given to ten patients with PCD and ten healthy individuals increased ciliary beat frequency (Loukides, Kharitonov et al. 1998). L-arginine given intravenously resulted in an increase of nasal and exhaled NO in both PCD and CF subjects, although the mean concentrations were still significantly lower than normals (Grasemann, Gartig et al. 1999). Neither study showed that the increase in NO translated into other clinical effects or improvements.

The mechanism behind the low nasal NO in PCD is not yet determined. In other diseases where low levels are obtained such as CF (Balfour-Lynn, Laverty et al. 1996; Dotsch, Demirakca et al. 1996; Lundberg, Nordvall et al. 1996), diffuse panbronchiolitis (Nakano, Ide et al. 2000), paranasal sinus inflammatory disease (Arnal, Flores et al. 1999), chronic sinusitis (Lindberg, Cervin et al. 1997; Deja, Busch et al. 2003) (described in sections below), it is thought to be secondary to thickened secretions and/or obstruction of the ostea preventing NO diffusion. However, in clinically stable PCD patients with low NO, their ostea were noted to be open (Wodehouse, Kharitonov et al. 2003) and sinuses clear (Arnal, Flores et al. 1999). NO has been implicated in control of ciliary function. In our own study (conducted after the data presented in this thesis), we found no correlation between exhaled NO levels and ciliary beat frequency in 135 children of European, Maori and Pacific ethnicities. However, we did find an increase in the percentage of ciliary structural defects, three times that reported in previous control groups (Edwards, Douglas et al. 2005). Endothelial NOS is located at the basal micro-tubular membrane in ciliated epithelium (Xue, Botkin et al. 1996) with a suggestion that NO acts as an intermediate messenger (Runer and Lindberg 1999) and TNFα or IL1 can up-regulate ciliary motility seeming to act through NO via induction of NOS (Jain, Rubinstein et al. 1993). Further research is needed in this area for an adequate explanation of this phenomenon of low NO levels in this condition.

9.10 Nitric oxide levels in cystic fibrosis

Studies of NO in subjects with cystic fibrosis (CF) have resulted in conflicting data. Contributing factors are likely to be the different techniques used for measurement when these studies began in the late 1990s, as well as the variation in age of the patients, co-morbidities and treatments, not always specified. Cross-sectional studies in children have shown no difference in levels of oral exhaled NO across CF, asthmatics on IHCS and healthy groups,
but those with CF did have significantly lower nasal NO levels (Balfour-Lynn, Laverty et al. 1996; Dotsch, Demirakca et al. 1996; Lundberg, Nordvall et al. 1996). However, three studies in adult patients with CF showed lower NO levels from both the oral and nasal sampling (Grasemann, Michler et al. 1997; Jones, Hegab et al. 2000; Thomas, Kharitonov et al. 2000). There has been no relationship demonstrated between the oral and nasal NO concentrations, although one study demonstrated a positive correlation between ambient and measured NO levels in all subject groups (Dotsch, Demirakca et al. 1996). In infants, one study showed reduced levels of exhaled NO in five infants with CF compared to 11 healthy infant controls at a mean age of 48.6 days (Elphick, Demoncheaux et al. 2001), but a more recent study showed no difference in exhaled NO levels in 18 infants with CF and 23 healthy infants at a mean age of 64.9 weeks (Franklin, Hall et al. 2006). Franklin’s study also demonstrated an inverse relationship between age and exhaled NO levels in the CF but not the healthy infant group (Franklin, Hall et al. 2006).

Studies of correlations of NO level to lung function have also been variable in the CF population. No correlation was demonstrated with levels of either nasal or oral NO in three studies with 135 subjects (Dotsch, Demirakca et al. 1996; Thomas, Kharitonov et al. 2000; Jaffe, Slade et al. 2003), while another two studies showed a weak but statistically significant positive relationship with FEV₁ in 63 subjects (Grasemann, Michler et al. 1997; Ho, Innes et al. 1998). It should be noted that this is a different response than seen in asthmatics as the CF patients with better lung function appeared to have higher exhaled NO levels. No association was observed between the CF genotype and NO values (Thomas, Kharitonov et al. 2000; Texereau, Fajac et al. 2005) but an inverse correlation between NO and the transepithelial baseline potential difference measured has been described (Texereau, Fajac et al. 2005).

Whether infection and type of organism has had an effect on the exhaled NO levels has also been examined. There was no difference in nasal or exhaled NO levels in children chronically infected with staphylococcal aureus (22 infected versus 11 non-infected) (Balfour-Lynn, Laverty et al. 1996). Nasal NO was significantly lower in 17 of 33 who were chronically infected with pseudomonas aeruginosa in one study (Balfour-Lynn, Laverty et al. 1996) but not confirmed in two others with 49 of the 68 subjects who had been chronically infected (Thomas, Kharitonov et al. 2000; Jaffe, Slade et al. 2003). Exhaled NO was lower in nine patients considered high risk of developing allergic bronchopulmonary aspergillosis (ABPA) compared to a low risk group (Lim, Chambers et al. 2003) but, as the high risk patients were on corticosteroids, this may reflect steroid use as opposed to ABPA risk per se.
This low NO occurs despite higher than normal levels of NO metabolites of nitrite and nitrate (Francoeur and Denis 1995; Grasemann, Ioannidis et al. 1998; Jones, Hegab et al. 2000) and nitrotyrosine (Jones, Hegab et al. 2000) which have been demonstrated in sputum and saliva samples. These metabolite levels have been correlated with sputum IL8 (Francoeur and Denis 1995) and lung function (Grasemann, Ioannidis et al. 1998). No correlation was seen when levels of exhaled NO were compared with markers of inflammation obtained from bronchoalveolar lavage in 18 CF infants (Franklin, Hall et al. 2006). Immunostaining studies of iNOS showed an inverse correlation to neutrophil counts and IL8 levels suggesting the iNOS expression decreases in the CF children as airway inflammation increases (Wooldridge, Deutsch et al. 2004). Similarly, in tracheobronchial brushings from 40 children with CF, the expression of mRNA for iNOS was found to be lower than seen in two healthy children, which the authors suggested may have consequences for local antimicrobial defense (Moeller, Horak et al. 2006).

Regarding NO as a possible outcome measure for treatment, no change in levels were seen from the onset of an acute infective exacerbation in eight patients followed for seven days during treatment (Ho, Innes et al. 1998). In contrast, increased exhaled NO levels, associated with a significant improvement in lung function, was seen in fourteen children after a course of IV antibiotics, although the NO level did not alter in six of the children that had chronic pseudomonas infection (Jaffe, Slade et al. 2003). In six children that were commenced on dornase alpha (Rh DNAse, ‘pulmozyme’), five had exhaled NO changes in parallel to the changes of pulmonary function tests, with no change in those receiving placebo (Grasemann, Lax et al. 2004). With single inhalations of L-arginine, there were increased levels of NO, FEV\textsubscript{1} and oxygen saturation (Grasemann, Kurtz et al. 2006). Oral L-arginine compared to placebo showed that a single dose of 200mg/kg also resulted in increasing levels of exhaled NO which continued for six weeks with continued supplementation but there was no change of lung function (Grasemann, Grasemann et al. 2005). Another study administered oral L-arginine daily for four weeks getting an increase in plasma L-arginine levels but there was no change in either exhaled NO levels or FEV\textsubscript{1} (Everard and Donnelly 2005).

On the face of it, it is hard to believe that the level of NO in such an inflammatory condition is low, especially when the NO metabolites are higher than that found in asthmatic and control groups. There are a number of possible explanations:

- Mucosal swelling with thickened mucus may result in mechanical obstruction of the sinus ossea which could impede the flow of NO into the nasal cavity. Consistent with this is that almost all patients with CF develop nasal and sinus disease.
• Some of these studies, particularly the early studies, may have included subjects on inhaled or oral corticosteroids and not differentiated them within the study groups.

• NO may degrade within the mucus giving low levels reaching the airway lumen for measurement.

• Pseudomonas aeruginosa produces a pigment pyocinen which in vitro has been shown to inactivate NO (Warren, Loi et al. 1990).

While the true reason is likely to be a combination of these factors, the obstruction to diffusion of NO into the airway and trapping of NO within the mucus, probably plays the major role. The low nasal NO level seems a consistent finding in this population, and in adults oral NO also appears to be low, with a possible correlation between higher lung function and higher NO levels.

9.11 Nitric oxide levels in bronchiectasis

An initial paper suggested both elevated levels exhaled NO and a correlation between this and the chest CT scan score in 20 patients with bronchiectasis, but neither were seen in another 19 on IHCS treatment (Kharitonov, Wells et al. 1995). Subsequent papers have not confirmed these findings in 16 (Ho, Innes et al. 1998), 31 (Horvath, Loukides et al. 2003) and 109 (Tsang, Leung et al. 2002) patients with bronchiectasis measured at a time of disease stability. Those with pseudomonas aeruginosa infection (25 of 109) did have significantly lower levels of exhaled NO although their sputum NO concentration was the same. There was no correlation between the levels of exhaled NO and FEV₁, FVC or the number of bronchiectatic lobes involved (Tsang, Leung et al. 2002). Similar to findings in CF, it is surprising not to find elevated NO in such an inflammatory and infective disease. The mucus layer may well be a barrier to detection of the production of NO, and in an inflamed environment the presence of reactive oxygen species may result in interaction with this highly reactive molecule resulting in effective removal of NO to form other compounds, such as peroxynitrite. The use of NO here is likely to be as a screen in bronchiectasis – if low nasal NO levels are obtained, then CF and PCD testing must be undertaken.

9.12 Nitric oxide levels in upper respiratory tract infections

The effect that upper respiratory tract infections (URTI) have on exhaled NO levels has also been studied. In 18 non-asthmatic adults during an URTI, increased exhaled NO levels to 315ppb were noted, decreasing to 87ppb during recovery at two to three weeks which became similar to aged matched healthy controls (Kharitonov, Yates et al. 1995). When 79 COPD
patients were followed through one winter, there were increases in levels of exhaled NO when they had a ‘cold’, ‘a sore throat’, or ‘dyspnoea combined with a cold’ compared to times of stability (Bhowmik, Seemungal et al. 2005). Two studies actually infected adult subjects with respiratory viruses. In the first, seven subjects infected with human rhinovirus 16 had significant increases in NO levels by days two and three compared to seven placebo ‘infections’ (de Gouw, Grunberg et al. 1998). In the second, six subjects infected with the same virus had increases in both nasal and lower airway NO levels with positive correlations to the severity of infection as measured by organism count, inflammatory markers from nasal lavages and nasal scrapings, and day four symptom scores (Sanders, Proud et al. 2004). However in 16 children with acute maxillary sinusitis, the NO nasal measurements dropped and then increased following antibiotic treatment over a two week period (Baraldi, Azzolin et al. 1997). This suggests that NO does increase with upper respiratory tract infections, but may be obstructed from measurement if there is significant sinus obstruction.

9.13 Nitric oxide levels in chronic obstructive pulmonary disease (COPD)

In cross-sectional studies of chronic obstructive pulmonary disease (COPD) subjects, levels of exhaled NO has been similar to, or higher than, healthy controls though not to levels seen in steroid naïve asthmatics (Clini, Bianchi et al. 1998; Kanazawa, Shoji et al. 1998; Rutgers, Meijer et al. 1998; Corradi, Majori et al. 1999; Zietkowski, Kucharewicz et al. 2005). This has been confirmed using single breath (Clini, Bianchi et al. 1998; Kanazawa, Shoji et al. 1998; Maziak, Loukides et al. 1998; Rutgers, Meijer et al. 1998; Zietkowski, Kucharewicz et al. 2005), tidal breathing (Rutgers, Meijer et al. 1998) and reservoir techniques (Corradi, Majori et al. 1999). A significant negative correlation has been shown with FEV₁ at times of disease stability (Clini, Bianchi et al. 1998; Maziak, Loukides et al. 1998; Ansarin, Chatkin et al. 2001). A correlation has also been found between baseline FEV₁ and the change in the level of exhaled NO following an eight week rehabilitation exercise program in mild, moderate and severe COPD patients but not those with cor pulmonale (Clini, Bianchi et al. 2000; Clini, Bianchi et al. 2001; Clini, Bianchi et al. 2002). With regard to other physiological parameters, exhaled NO was found to be inversely correlated with oxygen saturation and diffusion transfer factor (D₅₅CO) (Ansarin, Chatkin et al. 2001) and positively correlated with the residual lung volume, total lung capacity (Ansarin, Chatkin et al. 2001), and degree of airflow obstruction (Clini, Bianchi et al. 1998). These all suggest that the worse the disease is, as determined by these parameters, the higher the NO level obtained (Ansarin, Chatkin et al. 2001). More recently, multiple flows to assess the different airway compartments have been employed in this population (see Section 9.3.1 (i)). In all studies to date NO was found to be
high in the alveolar compartment which correlated with severity and progression regardless of the patient's smoking habit or current treatment (Tsoukas and George 1998; Kharitonov and Barnes 2004; Brindicci, Ito et al. 2005). As well, NO metabolites of nitrite and nitrate have been higher in induced sputum in COPD patients compared to normals (Kanazawa, Shoji et al. 1998). Lung biopsies from patients with severe COPD had higher iNOS seen than those that had more mild disease and normal controls (Maestrelli, Paska et al. 2003).

COPD patients having frequent exacerbations had higher levels of exhaled NO than stable current smokers and ex-smokers with COPD or chronic bronchitis (Agusti, Villaverde et al. 1999; Silkoff, Martin et al. 2001). Similarly during admission, the level of exhaled NO was high and remained elevated at discharge despite intravenous steroid therapy, but when the patients were once again clinically stable it had reduced to levels similar to control subjects (Agusti, Villaverde et al. 1999). The NO levels correlated to the drop in FEV1 during exacerbation in one study (Silkoff, Martin et al. 2001), but did not relate to FEV1 or other variables such as FVC, lung volumes, diffusing capacity or pulmonary gas exchange at the time of discharge in another study (Agusti, Villaverde et al. 1999). Assessing the effect of pollution; an association between exhaled NO and particulate matter when measured in a cross section of the population was significantly greater in subjects who had a doctor diagnosis of COPD (Adamkiewicz, Ebelt et al. 2004). Exhaled NO levels measured seasonally and during exacerbations in 79 COPD outpatients showed higher levels during winter months, as well as being higher during the 68 exacerbations seen in 38 of these patients. The degree of elevation, however, was noted to be lower in those more severely affected (Bhowmik, Seemungal et al. 2005). Elevated NO levels decreased significantly in 47 patients commencing IHCS therapy but there were no changes in FEV1 (Zietkowski, Kucharewicz et al. 2005). Two studies showed higher exhaled NO levels that positively correlated with post-bronchodilator FEV1 suggesting NO may be a marker for inflammation, responsive to bronchodilator treatment similar to asthmatics, although these COPD patients were not known to be atopic (Papi, Romagnoli et al. 2000; Zietkowski, Kucharewicz et al. 2005). Both exhaled NO and CO levels were low in 19 individuals with alpha-1 antitrypsin deficiency compared to both controls and COPD patients (Machado, Stoller et al. 2002).

9.14 Nitric oxide levels in smokers

NO is known to be contained within cigarettes as previously discussed (see Section 2.2.3). Exact concentrations between brands differ, but cigarettes produced in France and the United States exceeded the NO content of those produced in the UK up to 3-5 fold with no other
differences noted based on filter, design, tar, nicotine or CO yield. This research was done in the 1980s and the relationship to what is made and sold now here in New Zealand has not been studied. Many studies have demonstrated that both peak and plateau NO is lower in smokers than non-smokers who are otherwise ‘healthy’ with normal lung function (Persson, Zetterstrom et al. 1994; Schilling, Holzer et al. 1994; Kharitonov, Robbins et al. 1995; Robbins, Millatmal et al. 1997; Verleden, Dupont et al. 1999; Balint, Donnelly et al. 2001; Mazzone, Cusa et al. 2001; Hogman, Holmkvist et al. 2002; Horvath, Donnelly et al. 2004).

In mild asthmatics, smokers also had a lower level of exhaled NO when compared to non-smokers (Persson, Zetterstrom et al. 1994; Verleden, Dupont et al. 1999; Horvath, Donnelly et al. 2004; McSharry, McKay et al. 2005). Nasal NO measurements in ‘healthy’ normal and asthmatic smokers compared with their non-smoking counterparts were also reduced (Robbins, Millatmal et al. 1997; Olin, Hellgren et al. 1998). This reduction of NO was correlated with ‘pack years’ in current (McSharry, McKay et al. 2005) and ex-smokers (Corradi, Majori et al. 1999). Two studies also found significant reductions in levels of exhaled NO and NO output in a total of 118 healthy infants exposed to pre-natal cigarette smoke compared to the non-exposed (Hall, Reinmann et al. 2002; Frey, Kuehni et al. 2004).

The effects of a single cigarette have also been studied. A transient reduction in peak exhaled NO occurs immediately (Kharitonov, Robbins et al. 1995) and at 50 minutes with a co-incident rise in H2O2 (Horvath, Donnelly et al. 2004). However another study found an increase level of NO at one and ten minutes (Chambers, Tunnicliffe et al. 1998) while a fourth found no change in the levels of exhaled NO, S-nitrosothiol or nitrotyrosine at 30 and 90 minutes, although nitrite and nitrate levels were significantly increased (Balint, Donnelly et al. 2001). A decrease in plasma nitrate and nitrite concentrations were seen after smoking a single ‘real’ cigarette compared to a sham cigarette (Tsuchiya, Asada et al. 2002). Passive exposure and active smoking in usual non-smokers resulted in a reduction in the level of exhaled NO when measured every 15 minutes to one hour (Yates, Breen et al. 2001). Recently a murine model showed that cigarette smoke caused a reduction of NO by decreasing iNOS mRNA transcription but did not alter the iNOS mRNA half life once already developed (Hoyt, Robbins et al. 2003). It may be that the NO in the cigarettes operates as a negative feedback mechanism, resulting in reduction of activity of the NOS enzymes.

The effect of quitting smoking on the subsequent levels of NO has also been examined. In 20 smokers, nine that were able to refrain from smoking for four weeks resulted in increased levels of exhaled NO, which was no longer significantly lower than controls (Hogman, Holmkvist et al. 2002). Fourteen smokers had an increase in their exhaled NO compared to
their baseline NO when they gave up for one week. In the ten who were able to continue, their level of exhaled NO had increased to normal non-smoking levels when re-measured at eight weeks, while in those that were unable to sustain quitting, the NO had dropped to the previous low levels (Hogman, Holmkvist et al. 2002). This effect of smoking is unfortunate as it renders the measurement of NO unhelpful, particularly in the asthmatic population where it could be a helpful longitudinal indicator of airway inflammation and treatment effects.

9.15 Nitric oxide levels in interstitial lung diseases

Interstitial lung diseases have been particularly helpful in proving the value of using differing flows to measure NO from different areas within the lung (see Section 9.3.1 (i)). In 20 patients with scleroderma including five with additional pulmonary hypertension (Girgis, Gugnani et al. 2002) and in 24 patients with scleroderma including twelve with and twelve without clinical or radiological evidence of lung disease (Moodley and Lalloo 2001), significant differences in exhaled NO levels from control subjects were only seen when the alveolar rather than airway concentrations were measured. There was also a negative correlation demonstrated between the alveolar concentration of NO and the diffusion coefficient in the patient groups (Moodley and Lalloo 2001; Girgis, Gugnani et al. 2002). In active fibrosing alveolitis, an alveolar NO increase was associated with increasing lymphocyte cell counts and an alveolar decrease was associated with corticosteroid treatment (Paredi, Kharitonov et al. 1999). This is in contrast to studies using one standard expiratory flow for measurement, where no difference was found in 34 patients with systemic sclerosis (Sud, Khullar et al. 2000), or in 52 patients with sarcoidosis (Wilsher, Fergusson et al. 2005). One study did find higher exhaled NO levels using the standard flow in 47 patients with systemic sclerosis, particularly those with interstitial lung disease, and demonstrated an inverse correlation with pulmonary artery pressure but no correlation to age, disease duration, current therapy or the type of disease be it limited or diffuse (Rolla, Colagrande et al. 2000). One early paper also measured exhaled NO continuously in expired air at baseline and during an exercise test and calculated the output of NO, which was low in six subjects with pulmonary fibrosis compared to normal subjects. The authors suggested that that this patient group may fail to increase NO output by failing to recruit the capillary bed during exercise (Riley, Porszasz et al. 1997). Nasal NO was 88% lower than normals in diffuse panbronchiolitis, a pulmonary disease of unknown origin confined usually to individuals of Japanese, Korean or Chinese descent (Nakano, Ide et al. 2000). All current and future work in this area of interstitial lung disease should use the ability to measure NO in the alveolar compartment for meaningful results.
Nitric oxide levels in exercise

The effects of exercise on levels of exhaled NO in healthy, athletic adults, and those with respiratory and/or cardiac disease has been investigated, with a few studies also done in children. In healthy adults, the measurement of exhaled NO with exercise (treadmill or bicycle) showed a decrease when measuring the absolute levels in single breaths, but there was a two to three fold increase in NO output taking into account the increased ventilatory minute volume (Persson, Wiklund et al. 1993; Iwamoto, Pendergast et al. 1994; Trolin, Anden et al. 1994; Phillips, Giraud et al. 1996; Yasuda, Itoh et al. 1997). The increased output more closely related to increased ventilation than increased blood flow (Phillips, Giraud et al. 1996) and significantly correlated with CO₂ production (Iwamoto, Pendergast et al. 1994). Two studies assessed the contribution from nasal or oral exhalations showing that most of the NO coming from the lower airways (Lundberg, Rinder et al. 1997; Yasuda, Itoh et al. 1997) and from the airway rather than alveolar compartment (Persson, Wiklund et al. 1993). The nasal cavity NO reduced by 47% after one minute and 76% after five minutes of exercise (Lundberg, Rinder et al. 1997).

There have been many studies done in elite athletes. When compared to those with lower levels of fitness, the athletes had a significantly more linear increase in NO output (Maroun, Mehta et al. 1995; Chirpaz-Oddou, Favre-Juvin et al. 1997; Sheel, Edwards et al. 2000; Verges, Flore et al. 2005). This output correlated well with oxygen consumption, CO₂ production, minute ventilation and heart rate (Chirpaz-Oddou, Favre-Juvin et al. 1997). In one study, athletes that developed exercise induced hypoxemia had lower levels of NO compared to those who did not seen (Kippelen, Caillaud et al. 2002), but this was not confirmed in another (Sheel, Edwards et al. 2000). Exercise in cold challenge conditions resulted in lower levels of exhaled NO output being achieved and a slower time to recovery with lower FEV₁ (Therminarias, Flore et al. 1998; Pendergast, Krasney et al. 1999). These results suggested that physical conditioning increases expiratory NO output during exercise and this may be due to an increased vascular and/or epithelial production of NO. The enhanced vascular NO production may be the result of increased share stress or of an up regulation of the endothelial NOS gene expression (Maroun, Mehta et al. 1995).

Can NO predict development of exercise induced bronchospasm? Higher baseline levels were significantly associated with bronchoconstriction and histamine responsiveness in atopic rather than non-atopic steroid naïve healthy conscripts (Rouhos, Ekroos et al. 2005). In 50 consecutive subjects, a ROC curve yielded a value of 0.636 when comparing those who did...
and did not develop exercise-induced bronchospasm. A result of <12ppb gave a sensitivity of 1.0, a specificity of 0.31, a negative predictive value of 0.19 and a positive predictive value of 1.0 for bronchospasm. As no-one with a baseline exhaled NO <12ppb was affected, potentially a result in this range could render an exercise test unnecessary. Both stable COPD patients and healthy controls increased NO output significantly during exercise, but the peak NO output was significantly lower in COPD patients (Clini, Bianchi et al. 2000). Levels of exhaled NO rose significantly during an eight week multi-disciplinary pulmonary rehabilitation program in 14 (Clini, Bianchi et al. 2001) and 47 COPD patients (Clini, Bianchi et al. 2002) correlating to baseline FEV₁ and peak work rate in all patients except those with established cor pulmonale.

Exercise testing has long been a feature of cardiac assessment. There appears to be impairment to increasing NO output in those with cardiac disease including chronic heart failure (Funakoshi, Yamabe et al. 1999; Lovell, Stevenson et al. 2000; Agostoni and Bussotti 2003; Bussotti, Andreini et al. 2004), mitral stenosis (Omede, Bucca et al. 2004), primary pulmonary hypertension and pulmonary fibrosis (Riley, Porszasz et al. 1997). The exhaled NO output during exercise significantly correlated with oxygen deficit in 16 patients with congestive heart failure (Funakoshi, Yamabe et al. 1999). In 43 patients with mitral stenosis, the ability to increase NO output was associated with a functional capacity of greater than 75% (Omede, Bucca et al. 2004). The inability to increase NO may be associated with a reduced ability to dilate and recruit pulmonary capillaries during exercise (Riley, Porszasz et al. 1997).

In asthmatic and healthy children during treadmill exercises, NO has been measured by single exhalations online (similar to adult studies above) or as reservoir collections. As was found in adult studies, the pattern of exhaled NO was similar for both groups with a reduction in the absolute NO level but not output (Scollo, Zanconato et al. 2000; Terada, Fujisawa et al. 2001). In normal and asthmatic children, NO remained low following completion of the exercise, rebounded to above baseline at five minutes and returned to pre-exercise levels by 30 minutes (Terada, Fujisawa et al. 2001; Gabriele, Pijnenburg et al. 2005). In those with exercise induced bronchospasm, the exhaled NO level remained low post exercise only gradually returning to baseline with a faster recovery in children on steroid treatment. Interestingly, their NO recovery was not assisted by B₂ agonist rescue therapy even when their FEV₁ improved. (Terada, Fujisawa et al. 2001). In ten of 24 asthmatic children who developed bronchospasm during the exercise, their baseline NO levels were significantly higher and positively correlated with their fall in FEV₁ (Scollo, Zanconato et al. 2000). In
another study of 111 school children with asthma, exercise induced bronchospasm could be excluded with a probability of 90% in those with baseline NO levels <20ppb if not on IHCS therapy or <12ppb in children on current IHCS treatment (Buchvald, Hermansen et al. 2005).

9.17 Nitric oxide measurements in infants

Finally, an area of burgeoning interest is the measurement of NO in infants. This is potentially useful as assessments for respiratory disease in infants, including lung function testing, is difficult and remains far from routine.

9.17.1 Methodology

Clearly the measurement of exhaled NO by nasal or oral methods requires adjustment when no active (and usually no passive!) co-operation can be expected. Measurement of infant lung function was first described in 1977 (Motoyama 1977) and has been continuing to develop since, with ATS criteria published in the last decade regarding method, equipment and nomenclature with single breath and tidal breathing techniques described (Anonymous 1995; Anonymous 1995; Gaultier, Fletcher et al. 1995; Quanjer, Sly et al. 1995; Anonymous 2000; Frey, Stocks et al. 2000; Frey, Stocks et al. 2000; Sly, Tepper et al. 2000). These techniques have been further adapted to measure exhaled NO.

Reviewing the single breath technique; the infant lies supine at a time of quiet breathing (asleep or sedated) with a face mask which covers both mouth and nose. Prior to the forced exhalation to be measured, a fan pump connected to the face mask delivers increased inhalation during three consecutive cycles to an inflation pressure of 20cmH2O using a computer controlled circuit to approximate total lung capacity. At end inspiration of the third inflation cycle, a forced expiration is achieved by compressing the chest and abdomen with an inflatable jacket to transmit a pressure of 20cmH2O above the inflation pressure at end inspiration to the airway. This is deemed the 'raised volume rapid thoracic compression technique'. To measure NO with this circuit, one of the first groups to do so added a constant pressure of 15cmH2O to the expiratory limb aiming to deliver a constant expiratory flow at 50ml/s (see Figure 9.3) (Wildhaber, Hall et al. 1999).
Using this technique there was successful measurement of FEV\textsubscript{0.5} in 27 of 30 sedated infants aged 3-24 months and successful measurement of NO in all. The end tidal CO\textsubscript{2} level at the nose was not changed during expiration, indicating that the velum was closed during the manoeuvre (Wildhaber, Hall et al. 1999). Studies have now been carried out using variations on this technique reporting 95%+ success in sedated or quiet infants (Buchvald and Bisgaard 2001; Martinez, Weist et al. 2003; Franklin, Turner et al. 2004; Straub, Moeller et al. 2005).

The other method is by using tidal breathing where NO can be measured online or by reservoir collection. Again the infant is in a supine position asleep following sedation, with direct online measurement recorded during 20 seconds of quiet breathing (Franklin, Turner et al. 2004), or using a mean of five or ten consecutive breaths (Hall, Reinmann et al. 2002; Colnaghi, Condo et al. 2003; Leipala, Williams et al. 2004). For off-line measurements, tidal breathing can be collected into a gas sampling balloon which can then be attached to the sampler inlet of the NO analyser – collecting over a set time period such as 15-45 seconds (Dinakar, Craff et al. 2006), or during a set number of breaths (most use three or five) (Artlich, Busch et al. 1998; Baraldi, Dario et al. 1999; Ratjen, Kavuk et al. 2000; Artlich, Jonsson et al. 2001; Franklin, Turner et al. 2004; Franklin, Turner et al. 2004; Moeller, Franklin et al. 2004; Gabriele, Nieuwhof et al. 2006). Results have been reported as peak, average levels and the average calculated from the area under the curve during expiration.
Almost all studies used sedation, with only a couple stating that they had not (Artlich, Busch et al. 1998; Hall, Reinmann et al. 2002) and there was one study that had infants and young children awake and in their parents' lap (Baraldi, Dario et al. 1999). One group investigated whether sedation itself made a difference to the level of exhaled NO comparing results of 29 infants prior and subsequent to administering 60-100mg/kg of chloral hydrate. The mean exhaled NO of 21.4ppb when awake dropped significantly to 14.6ppb when asleep, associated with an intra-subject co-efficient of variation of 20% when awake versus 10% while sedated. The authors suggested this may be explained by variation in expiratory flow, contamination with ambient NO, breath-holding and crying, and often difficulty maintaining an appropriate seal of the mask around an agitated infant. They were ultimately successful in getting readings from 29 of 39 infants when they were awake (but only 11 were 'co-operative'), compared to achieving measurements in all the infants when sedated (Franklin, Turner et al. 2004). Another study in 20 healthy non-sedated infants showed the intra-subject variability from different phases of the exhalation ranged from 9.3 to 15.1% with the inter-subject variation much greater, ranging from 34.6 to 44.6% (Hall, Reinmann et al. 2002).

Infants were shown to have a similar pattern of levels of exhaled NO to that seen in older children and adults; rising to a peak with a rapid plateau. A linear relationship between NO output and plateau was also demonstrated (Martinez, Weist et al. 2003). One study investigated whether there was a difference between nasal and oral NO levels in 23 infants. Most studies use a mask covering both mouth and nose and it is likely, given that infants tend to preferentially breathe through their nose, that nasal NO may contribute significantly to overall readings. The nasal tidal expiratory breaths were measured by continuing to place a mask over both mouth and nose but ensuring that the mouth was firmly shut (not stated how) and the oral tidal breaths were collected by blocking the nose with the rim of the mask. This resulted in no significant difference between nose and mouth levels, which is different from all other ages (Franklin, Turner et al. 2004). It may be that the method of exclusion of either the oral or nasal exhalate was inadequate and/or that there was no effect from the presence of sinuses in this age group to add to the usual higher nasal measurements.

There have been comparisons between the single breath and tidal breathing NO results. In 32 infants with recurrent wheeze, 16 with recurrent cough and 23 healthy infants a moderate correlation of 0.6 was demonstrated between the two methods but with poor agreement (Franklin, Turner et al. 2004). This group also noted that the single breath technique appeared to be more sensitive to detect differences between infants with and without respiratory symptoms (Franklin, Turner et al. 2004). In another study of 20 infants, the tidal exhaled NO
washout characteristics were noted to vary from breath to breath (Hall, Reinmann et al. 2002). This group then divided the exhalation to assess whether one part of the expiratory signal exhibited less intra-subject variability and therefore could potentially be the most discriminatory. Firstly, based on the exhalation pattern of CO₂, they looked at the NO levels which corresponded to phase one (from the convective airways), phase two (progressive washout of the airways with alveolar gas) and phase three (alveolar compartment when exhaled CO₂ is high). Secondly, they divided the tidal volume into four quarters of exhalation time. The most variable periods were phase one, and the first and fourth quarters. Correspondingly, the second and third quarter time periods had about the same variability as phase two, which was the best, followed by phase three. The group concluded that in the absence of CO₂ measurement as a guide, the reported result should come from the second and third quarter of the exhalation (Hall, Reinmann et al. 2002).

Similar to studies in other age groups, flow dependency of exhaled NO levels have been demonstrated in infants by both methods. This was demonstrated in the original single breath study comparing a second flow of 100mls/s as well as the standard of 50mls/s in three subjects showing a significant difference in NO results (Wildhaber, Hall et al. 1999). Similarly, low to higher NO levels were obtained at expiratory flows of 50, 25 and 15mls/s in five full term healthy infants (Martinez, Weist et al. 2003). Breath-by-breath analysis for tidal breathing also showed that higher expiratory flow rates were associated with lower exhaled NO levels (Franklin, Turner et al. 2004). In a large prospective healthy birth cohort of 98 infants the tidal breathing parameters of flow, breathing rate and expiratory time, measured at age one month, all led to varying exhaled NO results. This group also came to the conclusion that the third expiratory quartile of exhaled NO during tidal breathing was the most reproducible (Frey, Kuehni et al. 2004). In a direct comparison in 71 infants, the peak flow was 128mls/s during tidal breathing and 11mls/s during single breath measurements (Franklin, Turner et al. 2004). This may also explain why single breath results versus tidal breathing results usually have higher absolute exhaled NO levels.

Other parameters have been seen to affect NO levels in infancy. Measurements in twelve infants showed that ambient NO concentrations as low as 5-10 ppb compared to the inhalation of NO free air resulted in an increase exhaled level (Franklin, Turner et al. 2004). In the 98 infants at one month, a gender difference, with boys having higher levels at 17.7ppb than girls at 14.6ppb, was noted. This remained significant after adjusting for weight and minute ventilation (Frey, Kuehni et al. 2004). In thirteen preterm infants the exhaled NO was almost absent and gradually increased over the first 48 hours, while in eleven term infants there was a
peak exhaled NO production in the first hours of life (Colnaghi, Condo et al. 2003). In a series of measurements of three premature infants while the absolute values were considerably lower than older children, when corrected for the body weight it appeared comparable (Artlich, Busch et al. 1998).

9.17.2 Levels of nitric oxide in infants with different respiratory diseases

Levels of exhaled NO were higher in wheezy infants compared with healthy controls (Baraldi, Dario et al. 1999; Wildhaber, Hall et al. 1999; Franklin, Turner et al. 2004), as well as compared to infants with a current URTI (Baraldi, Dario et al. 1999) and compared to infants with cough (Franklin, Turner et al. 2004). Having eczema also resulted in higher exhaled NO levels in a group 88 infants in one study (Franklin, Turner et al. 2004) and 43 in the second study (Dinakar, Craff et al. 2006). One study in six infants with URTI found higher levels of NO than healthy infants (Baraldi, Dario et al. 1999). However, two others found lower levels; one measuring 17 infants with an URTI by nasal and oral and end tidal oral measurements (Ratjen, Kavuk et al. 2000) and the second measuring 24 infants presenting with rhinorrhea with or without cough but not wheeze (Franklin, Turner et al. 2005). In eight of the infants who had rhinorrhea, repeated tests showed an increase from 7.5ppb to 34.1ppb when they were symptom free (Franklin, Turner et al. 2005). Recently a large cross sectional study of 218 infants, including a number with different respiratory diseases and healthy controls, had NO measured between 4.6 and 25.2 months of age. Higher levels were seen in 74 infants with recurrent wheeze at 18.6ppb compared to 100 controls at 10.4ppb and 20 infants with bronchopulmonary dysplasia at similar levels to controls with a mean of 11.8ppb, while 20 infants with CF had significantly lower levels at 5.9ppb (Gabriele, Nieuwhof et al. 2006). Different results were found in infants with chronic lung disease in another study (Leipala, Williams et al. 2004). Ten infants born at a median gestational age of 26 weeks and who had developed chronic lung disease had higher peak nasal and face mask NO levels than ten term infants and ten premature infants born at a median gestational age of 32 weeks without chronic lung disease. Those with chronic lung disease were not receiving diuretics, inhaled or systemic corticosteroids and bronchodilators or antibiotics at the time of measurement. All infants were measured at a post conceptual age between 36 and 45 weeks. This meant the infants with chronic lung disease were studied at an older post natal age at 85 days compared to two days for the term group and 32 days for the non-chronic lung disease premature group. There was, however, a wide range of NO levels in each group (Leipala, Williams et al. 2004).
9.17.3 Prenatal and maternal effects on nitric oxide levels

In one already previously mentioned study which measured 98 infants at age one month, exhaled NO levels were no different between infants from mothers with either asthma, other atopic diseases or without atopic disease (Frey, Kuehni et al. 2004). These results differ from another study where a family history of atopy gave a mean NO level of 41.1ppb if it was both parents, 24.2ppb if it was either parent and was lowest at 10.8ppb if it was neither parent (Wildhaber, Hall et al. 1999). What did have an effect in the Frey study was maternal smoking or coffee consumption during pregnancy, both resulting in significantly decreasing exhaled NO levels (Frey, Kuehni et al. 2004). Similarly, seven infants exposed to prenatal cigarette smoke had lower exhaled NO than thirteen unexposed infants when measured between 25 and 58 days of age (Hall, Reinmann et al. 2002).

9.17.4 Effects of treatment on nitric oxide levels

Few studies have looked at levels of NO in response to treatment in this age group. Thirty one infants aged 6-19 months with recurrent wheeze and raised exhaled NO determined by offline tidal breathing were treated with inhaled fluticasone 50μg twice per day or placebo for four weeks. There was substantial reduction in the level of exhaled NO from 35ppb to 16.5ppb with only a small increase in FEV0.5 and no difference in the symptom scores between groups (Moeller, Franklin et al. 2004). Similarly in 15 infants with recurrent wheeze and high levels of exhaled NO, this reduced by 52% after five days of prednisone becoming no different to NO levels of the control children (Baraldi, Dario et al. 1999). In 24 children aged 10-26 months with wheeze, allergy and a positive family history of asthma, montelukast 4mg per day for four weeks resulted in a significant decrease in levels of exhaled NO from 29.8ppb to 19.0ppb and this did correspond to improvements in FEV0.5 and symptom scores with no changes in the placebo group (Straub, Moeller et al. 2005). Finally in eight ventilated infants with a mean gestational age of 25.8 weeks and postnatal age of 55 days, exhaled NO levels reduced from a mean of 6.5ppb to 4.2ppb coinciding with a reduction 62 to 45% in supplemental oxygen requirements with three days of dexamethasone treatment (Williams, Bhat et al. 2004).

9.17.5 Summary of nitric oxide findings in infants

In summary, exhaled NO can be measured in infants by both single and tidal breathing methods incorporating online and reservoir techniques. The advantages of the single breath technique are, being able to use an expiratory pressure to standardise the desired expiratory
flow and to minimize nasal contamination. The tidal breathing method is less invasive and a simpler collection method but it is not always possible to control flow or nasal NO contamination. The most reproducible recordings have come from the second and third exhalation phases, and with use of sedation in the infants. The results so far have demonstrated a difference with gender, as suggested in some studies of older age groups, and confirmed the effect of expiratory flow on NO. Antenatal effects include a decrease in infants of smoking or caffeine ingesting mothers, though the effect of maternal (and paternal) atopy has been either nil or resulted in an increased levels of NO. Higher levels of NO were demonstrated in wheezy infants although the relation between this and those who develop asthma versus those who have viral induced wheeze of infancy which is likely to disappear between three and six years is not yet determined. The effect of a viral upper respiratory tract infection is less consistent on NO levels. As with infant lung function, the measurement of NO in this age group remains the premise of research groups and is not widely used in the clinical arena.

9.18 Chapter summary

Following early experiments throughout the 1990s, it was recognised that standardisation of the method of measuring NO was required to allow better interpretation of results and comparison between research groups. These began with a meeting in Stockholm in 1996 and from 1997 to 2005 four documents were generated involving the ERS and the ATS detailing standard procedures and then updating and refining these procedures as more data became available (Kharitonov, Alving et al. 1997; American Thoracic Society and Association. 1999; Baraldi, de Jongste et al. 2002; American Thoracic Society and European Respiratory Society 2005). What became increasingly apparent from very early in the research was the importance of getting consistent and reproducible results. The most important factors to control were expiratory flow and nasal contamination. Standard practices for single, tidal breathing, reservoir collections and nasal measurements have been developed for both adults and children. In addition, the use of different expiratory flows to assess NO from different lung compartments has also been explored. This is important when wanting to demonstrate inflammation in certain compartments in certain diseases, for example the airway compartment in asthma and the alveolar compartment in interstitial lung diseases. In the latter type of diseases, this technique should always be employed in the future to enable meaningful results to be obtained.
Physiological variables also affect the results. Exhaled NO levels have been shown to be higher in males than females, are reduced with current smoking, recent ingestion of water, alcohol or caffeine and increased with accidental gut contamination or after nitrogen-compound rich meals. Levels of NO also increase with age throughout childhood, but not adulthood, possibly in relation to pneumatisation of the sinuses. Relationships with height, weight, surface area, menstrual cycle in non-asthmatic women, or a circadian rhythm has not been demonstrated. However, there remains only one range of 'normal' values given for oral or nasal NO.

By far the most work in exhaled and nasal NO has been undertaken in atopic and/or asthmatic subjects with comparison to healthy controls. NO appears to correlate well with the inflammatory cells, lung function and bronchial hyper-responsiveness in steroid naive asthmatics and less well in those on IHCS or oral steroids. It does not correlate well with ‘severity’ of asthma as the NO result here is attenuated by treatment, and it is the requirement for the higher levels of treatment with possible breakthrough symptoms that determine the severity category in asthma guidelines(Anonymous 1997; GINA 2002; GINA 2005; SIGN 2005). There is the potential for NO measurements to contribute to diagnosis, management when measured longitudinally in individuals (on any medication), and to warn of loss of control. NO measurement can also be used as an outcome measure for anti-inflammatory agents used in asthma treatment. However there are some cautions to be made. Firstly, it does not appear useful to guide acute asthma treatment. Secondly, measurement of sputum eosinophils appear as, or more, accurate than NO itself, and the changes in the eosinophil counts may be a more accurate predictor of loss of control and a better target to tailor treatment. It is increasingly apparent that, like most parameters we measure, it is likely to be the pattern from a number of investigations along with an individual’s history that best describes their clinical disease rather than a single marker. The effect of atopy alone on exhaled NO levels needs to be kept in mind with overlapping results between asthmatic and atopic subjects. Unfortunately, smoking renders the test inaccurate and unhelpful. In addition, the presence of high NO levels may be a marker for respiratory diseases that are steroid sensitive no matter what the underlying disease is labelled.

In exercise, NO drops during single breath measurements, but the NO output is increased when taking into account the increased rate of breathing. An inability to increase the NO output during exercise was associated with more severe cardiac disease and, in elite athletes, worse performance suggesting that the increased NO output may depend on the ability to
recruit blood vessels in the lung. Studies have also suggested that a normal NO reading at the beginning of exercise predicted that no exercise-induced bronchospasm occurred.

Low nasal NO appears to be helpful in screening for PCD and possibly CF. It is particularly important to measure this in children with established bronchiectasis and detecting a low NO level early in assessment for recurrent respiratory illnesses would be additionally beneficial and would indicate that these diseases must be investigated and excluded. The low NO levels seen in PCD remain unexplained, while those in CF and other conditions such as sinusitis would appear to be secondary to sinus obstruction. Oral NO levels are also low in PCD, but the nasal NO measurements (using a cut point of 250ppb) have minimal overlap with normal children. Oral NO levels become low in CF with age and disease progression, probably with entrapment of NO within thick mucus and therefore likely to interact with other compounds, so the NO does not reach the air to be exhaled. NO levels are increased in acute infections in both normal subjects and those with COPD, and increase during times of higher pollution and also associated with respiratory symptoms. However ‘smoking’ remains a confounding factor here – again rendering the measurement of NO as useless. In interstitial lung disease, levels of NO co-relate with severity, in particular that assessed by the diffusion co-efficient, but only when measured in the alveolar compartment. The measurement of NO has been undertaken in infants and here while the parameters are still being worked through, it appears that the results are already low in those with PCD and CF, and high in children with wheeze, though it is still to be clarified whether this will result in detecting asthma versus infant viral associated wheeze, and whether it matters, as it may just detect disease that will respond to steroids.

The advantages of using NO measurement rather than (or in addition to) other parameters is obvious. It is much easier to perform, especially in children, than lung function testing or induced sputum for eosinophils or any of the blood measurements such as total IgE. In addition it is quicker, with immediate results, and potentially can be done away off hospital site. While it still needs an understanding of the test and qualified personnel, it can be taught and undertaken more easily than the other investigations.

So where do I see it as currently being MOST useful:

1. Nasal (and oral) measurement as a screen for PCD. If nasal levels are low – ensure investigation for PCD and CF are carried out. While we do have a newborn screening for CF here in New Zealand, 8% of children are not detected.

2. As confirmation of asthma in steroid naïve patients – when NO levels should be high.
3. As a marker to follow when commencing treatment, in essence IFCS in steroid naïve asthmatics (in non-smokers).

4. As a marker to follow longitudinally, particularly in poorly controlled asthmatics contributing to assessing disease control, and response to changes in treatment (in non-smokers).

5. It may become a method to assess the need for an exercise test in patients presenting with a complaint of possible exercise induced bronchospasm – if NO levels are normal then an exercise test may not be required and investigations should proceed down another avenue (in non-smokers).

The final, and brief, chapter will review where the research that I undertook contributed to both the clarity and confusion of early knowledge in this area. I will review what I learnt from the process of doing this research, writing up this thesis, and how this information has ultimately altered my research and clinical practice.
Chapter 10: Reflections

_The outcome of any serious research can only be to make two questions grow where only one grew before._ (Torstein Veblin 1857-1929)

By the time I had finished the research presented in this thesis, I felt that in attempting to answer a few questions - about the exact nature of exhaled NO and what made a difference to the levels obtained - I now had far more questions than when I began. It is hard to comprehend the rapid explosion regarding NO from the first discovery of its physiological role in 1987. Now the vital roles it occupies as a widespread mediator to maintain homeostasis and in host defence seems to be a long-known fact. The Medline searches reveal the rapid growth of research in this area in the 1990s and 2000s with studies conducted involving every organ system in every conceivable subject population (see Figure 10.1). When I started with the investigations presented in these chapters, very little was known about its role. Having acknowledged this, the measurement of exhaled NO is still not part of routine clinical practice and the exact use of this technology and its place in clinical medicine continues to be investigated today. In the thesis I have not touched on the measures of gases in exhaled breath condensate. This is an even more recent rapidly expanding area of research worthy of its own chapters by those involved in investigating its use.

Figure 10.1: Medline publications with a focus on nitric oxide research per year 1980-2006

When I first commenced research, it was as a means to an end. By the end of the 1990s, I knew that I wanted to train as a paediatric respiratory specialist given the burden of disease seen in children in the UK where I was currently working, and, perhaps more particularly, at
home in New Zealand. In order to do that I aimed to get a senior registrar / clinical lecturer post at a centre of excellence which for me, working in London at time, was the Royal Brompton Hospital. I decided that research would definitely be a pre-requisite to this appointment and so successfully obtained a research fellow job at the same hospital. However, having started down a research path, I can no longer envisage not having research as a major part of my role. Research involves the development of different skills to those required in clinical medicine. At the time, I enjoyed considering in depth and reading widely around one aspect of medicine. But, when I was asked by one scientist whether I used ‘positive’ or ‘negative’ pipetting when I did enzyme-linked immunosorbent assays (ELISAs) studies, I knew that I was in a different world. It teaches you attention to detail, the need to be very methodical, the need to be sceptical and the need to question every factor. It also allows an opportunity to work with people who are experts in different areas. From the scientists I learnt patience, information technology skills, statistics and careful laboratory bench-work. I also appreciated the very different knowledge and skills that Carolyn Busst as a medical and electrical engineer brought to the research. The topics of research and critical reviews were not previously emphasised but now occupy a central theme in current medical school training, and I consider this important in developing questioning practitioners.

Of course, now I would do everything very differently. One problem with research, as the quotation above suggests, is that no matter what topic you start with, it can expand exponentially. If commencing this research now, I would restrict the work to investigating exhaled NO and the long-acting β2 agonist (‘salmeterol’) trial that I was also conducting during this time. In addition, I was undertaking clinical training with on-call commitment in a paediatric cardiorespiratory intensive care setting. I would not, for example, have also embarked on learning induced sputum techniques and ELISAs for processing IL8 and TNFα. This latter research ultimately suffered for which I feel responsible. From the beginning I found the children, particularly healthy controls, did not like the use of hypertonic saline for sputum induction. This needed much greater attention and a full time researcher on this alone to determine a satisfactory technique. However, by this time I had established the machines, connections and protocols and was running with the exhaled NO research, and I was enjoying it. Also, in comparison, the adult respiratory service had four research fellows dedicated to investigating these areas of medicine which was a great deal more realistic. On the other hand, I would now also make the most of any overlapping area of research to combine investigations. For example, at the time I was the principal investigator in a cross-over trial involving two dosage regimes of salmeterol compared to one dosage regime of salbutamol
and I enrolled 52 asthmatic children. At the time there was a question as to whether the long acting β2 agonist therapy had any anti-inflammatory effects. I was well placed to answer this if I had measured the exhaled NO in these children, and/or connected them with an ‘induced sputum’ researcher.

My experience in research has, I hope, assisted me in the role of supervisor of other researchers. I have learnt from my supervisors and from my own successes and failures. I ensure regular contact no matter what is happening, and I limit the focus of the research. Early enthusiasm often encourages taking on too big a project and then feeling overwhelmed or that justice is not being done to every possible investigative avenue. I also returned to a one in two on-call roster providing paediatric respiratory cover regionally and nationally. Again my ‘take home’ message quite literally is to be certain to factor in time for writing. I believe important contributors to success in research are attention to detail, regular review, maintaining focus, and a realistic appreciation of the time required.

Since returning to New Zealand my own clinical and research focus has shifted to the area I am concerned which is a main burden of respiratory disease in New Zealand children – infection and in particular the development of chronic suppurative lung disease resulting in early morbidity and ultimately early adult mortality. My next endeavours are to continue to develop a good management programme and research into aetiology, appropriate intervention and strategies for prevention in this area of lung disease. While I also steadfastly remain a clinician, I could not stop doing research now. I thank the Royal Brompton Hospital and Dr Andy Bush in particular for opening up this path for me.
Appendices

Appendix I: Publications

Published manuscripts related to this thesis:


  **C A Byrnes:** Chapter 9: The Burden of Bronchiolitis in New Zealand (40-45).
  **C A Byrnes:** Chapter 11: The Burden of Bronchiectasis in New Zealand (51-55).


Publications based on research that has progressed from this thesis:


Presentations and published abstracts from this research and related to this research:


Other Publications:

• Byrnes C. Non cystic fibrosis bronchiectasis. Paediatric Respiratory Reviews 2006; 7 suppl 1: S255-S257.


Other presentations and published abstracts:

International Meetings:

• **Byrnes CA.** Pearls: Non-Cystic Fibrosis Bronchiectasis – 7th International Congress on Pediatric Pulmonology, Montreal, Canada July 2006 – invited presentation.

• **Byrnes CA.** Lung function accreditation guidelines - Australasian Paediatric Respiratory Group Annual Scientific Meeting 2004 – invited presentation.

• **Byrnes CA.** Lung function and lung function accreditation guidelines - Australasian Paediatric Respiratory Group Annual Scientific Meeting 2003 – invited presentation.

• **Byrnes CA.** Bronchiectasis – Australasian Paediatric Respiratory Group Annual Scientific Meeting 2002 – invited presentation.


National Meetings:

• **Byrnes CA.** Australia and New Zealand Cystic Fibrosis Bronchoalveolar Lavage - National Respiratory Meeting 2007 – invited presentation.

• **Byrnes CA.** Paediatric workshop – Standards of Care in Cystic Fibrosis in New Zealand - National Respiratory Meeting 2007 – invited presentation.

• **Byrnes CA.** Paediatric cases workshop - National Respiratory Meeting 1999-2006 – invited presentation.


• **Byrnes CA.** Pseudomonas aeruginosa – management of 1st infection in children with cystic fibrosis - National Respiratory Meeting 2003 – invited presentation.


• Edwards EA, Byrnes CA. Temperature control is vital for the measurement of cilia beat frequency when establishing a hospital protocol. European Respiratory Meeting & European Respiratory Journal 2001; 18: 300s.

• Byrnes CA. Screening for respiratory ciliary dysfunction – New Zealand Paediatric Society Annual Meeting 2001.


• Byrnes CA. Obstructive sleep apnea – New Zealand Paediatric Update 2000 – invited presentation.


Abstracts and poster presentations:


• **Edwards EA, Byrnes CA. Temperature *control is vital* for the measurement of cilia beat frequency when establishing a hospital protocol. European Respiratory Meeting & European Respiratory Journal 2001; 18: 300s.**


Current PhD supervision:

• 'Bronchiectasis in children and young people in New Zealand.' Dr Jacob Twiss.

• 'Effects of humidification on the airway in children with tracheostomies.' Dr David McNamara.
Appendix 2: Questionnaire for enrolling the children

NITRIC OXIDE QUESTIONNAIRE
Please answer the following questions:
ALL ANSWERS WILL BE TREATED IN STRICT CONFIDENCE.

NAME of child.............................. BOY/GIRL
Date of birth of child......................
Nationality.................................

Has he/she had a cold or chest infection in the last month? YES/NO
Has he/she ever had asthma? if yes, write age first diagnosed... YES/NO
Does he/she have asthma now? YES/NO
Has he/she ever wheezed? (made a whistling sound on breathing out) YES/NO
Does he/she cough...NEVER...SOMETIMES...OFTEN...CONSTANTLY.
Has he/she ever been prescribed Ventolin or Bricanyl? YES/NO

Has he/she ever had eczema? YES/NO
Has he/she ever had hay fever? YES/NO
Does he/she have eczema/hay fever now? YES/NO
Does he/she have any other chest complaint? please name...... YES/NO

Does anyone else in the family have any of the following:

asthma YES/NO if yes who?........
eczema YES/NO if yes who?........
hayfever YES/NO if yes who?........

Please give relationship to the child

Do you have any pets? if yes please name type........................

Does anyone in the family smoke?.....

Note: the spelling error noted in the heading of these sheets was only noted after we had delivered most of the questionnaires to the participating school - in view of that and as this how most of the collected forms remain - I have left it in.
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