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Exhaled Nitric Oxide and the Clinical Control of Airway Inflammation

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Abstract

Measurements of exhaled nitric oxide (eNO) are non-invasive and easily measured. Levels have been postulated as providing an indication of underlying airway inflammation. This series of studies was conducted to investigate the role of eNO in measuring airway inflammation in patients with asthma and COPD (chronic obstructive pulmonary disease).

78 asthma patients controlled on maintenance inhaled corticosteroid were assessed for 2-4 weeks. Measurements of eNO demonstrated good repeatability both within-sitting (coefficient of variation (c.v.) 4.1%) and between weekly visits (c.v. 10.5%; 95% reference range of −38 to +61% of individual patient means). Patients then underwent cessation of inhaled corticosteroid therapy until loss of control occurred or for a maximum of six weeks. Comparisons were made between eNO, symptoms, lung function, sputum eosinophils and airway hyperresponsiveness to hypertonic saline (4.5%) in predicting and diagnosing loss of control. Sixty patients (77.9%) developed loss of control. Exhaled NO increased to a greater degree in patients who developed loss of control (2.16 versus 1.44-fold increase, p=0.004). Both single measurements and changes in eNO (10ppb, 15ppb or an increase of >60% over baseline) had positive predictive values that ranged from 80-90% for predicting and diagnosing loss of control. This compared well with other more invasive markers. In a further extension of this design but with a total of 87 patients participating, 65 developed loss of control and were entered into a randomised placebo-controlled, double-blind dose-response study of 50, 100, 200 and 500μg of beclomethasone/day for eight weeks. Linear dose-response relationships between dose and changes in eNO and FEV₁ existed at one-week (p=0.002 and p=0.043 respectively) and at the end of treatment (p=0.015 and p=0.006 respectively). A similar linear dose-response relationship was seen with sputum eosinophils (p=0.037) but not with airway hyperresponsiveness. In differentiating between treatment groups eNO was superior to both FEV₁ and eosinophils. Throughout all of these study phases eNO was shown to correlate significantly with other markers of asthma control. This correlation was
particularly strong with sputum eosinophils, where significant correlations were seen whether assessed as point-in-time measurements (range of $r=0.46$ to 0.62, $p<0.002$) or as changes-over-time during both deteriorating ($r=0.44$, $p<0.001$) and improving asthma control ($r=0.40$, $p=0.002$). Finally, the effect of a course of oral prednisone on eNO, lung function and 6-minute walk in 30 COPD patients was assessed. A significant decrease in eNO (-3.31ppb; 95%CI: -1.45 to -5.16) was seen in association with an improvement in 6-minute walk (34.36m; 95%CI: 22.64 to 46.08). An eNO of ≤10ppb at baseline was associated with a negative predictive value of 82.2% in predicting a significant improvement in 6-minute walk, but the positive predictive value was only 43.7%. No improvement was seen in lung function.

These results indicate that eNO measurements are reliable and provide important clinical information in the control of asthma, correlating to the degree of sputum eosinophilia. Levels can be used to predict and diagnose poor asthma control as well as demonstrating a dose-response to inhaled corticosteroid therapy. However its usefulness is limited in assessing steroid responsiveness in COPD, perhaps a reflection of the different inflammatory processes occurring. Further longitudinal studies are required to investigate whether adding serial eNO measurements into treatment algorithms leads to improved clinical outcome in both asthma and COPD.
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<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BDP</td>
<td>beclomethasone dipropionate</td>
</tr>
<tr>
<td>BUD</td>
<td>budesonide</td>
</tr>
<tr>
<td>cNOS</td>
<td>constitutive NOS</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophilic cationic protein</td>
</tr>
<tr>
<td>EGP</td>
<td>eosinophil granule peroxidase</td>
</tr>
<tr>
<td>eNO</td>
<td>exhaled nitric oxide</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>forced expiratory volume in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>HSC</td>
<td>hypertonic saline challenge</td>
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<tr>
<td>ICS</td>
<td>inhaled corticosteroid</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon γ</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible NOS</td>
</tr>
<tr>
<td>IS</td>
<td>induced sputum</td>
</tr>
<tr>
<td>MBP</td>
<td>major base protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PEFR</td>
<td>peak expiratory flow rate</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin-E&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>RV16</td>
<td>rhinovirus 16</td>
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<td>TNFα</td>
<td>tumour necrosis factor α</td>
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CHAPTER ONE:
INTRODUCTION: ASTHMA, INFLAMMATION & ASSESSMENT

1.1. Asthma and airway inflammation

Asthma is a complex disease of the airways characterised clinically by reversible airway obstruction and airway hyperresponsiveness (AHR). Histologically asthma consists of airway wall hypertrophy and oedema, excess mucous secretion, epithelial cell damage and the presence of inflammatory cells (Djukanovic et al. 1990). It is this inflammation which is thought to play the pivotal role in the whole disease process in asthma, and when left untreated can lead to potentially irreversible airway obstruction via a process of airway remodelling (Fabbri et al. 1998b).

The inflammatory process in asthma involves inflammatory cells such as eosinophils, neutrophils, macrophages, T-lymphocytes and mast cells (Djukanovic et al. 1990; Dirnberger et al. 1998). These cells produce their effect through the release of a vast number of inflammatory mediators, such as inter-leukin-4, inter-leukin-5, tumour necrosis factor-α, histamine and nitric oxide (NO), as well other substances causing oxidative damage to the airways. Such inflammatory infiltrates are present in the airways of patients with clinically mild disease (Foresi et al. 1990; Haley and Drazen 1998) as well as those with fatal asthma (Houston et al. 1953; Saetta et al. 1991).

For over a century the eosinophil has been documented as having an important role in the inflammatory process of asthma (Gollasch 1889; Ellis 1908), and despite there being an assortment of inflammatory cells involved, the eosinophil still appears to be central in its role (Bousquet et al. 1990). The level of eosinophilic inflammation as assessed both by bronchoalveolar lavage (BAL) fluid and bronchial biopsies correlate directly with the severity of asthma (Bousquet et al. 1990). Eosinophil numbers also change with changes in asthma...
control, increasing following allergen challenge (Metzger et al. 1986) and decreasing following treatment with corticosteroids (Bentley et al. 1996).

For example, Djukanovic et al (1992) obtained bronchial biopsies in 10 asthma patients prior to and following a 6-week course of anti-inflammatory treatment with inhaled beclomethasone dipropionate. Following treatment there was a significant reduction in median eosinophil numbers in both epithelial basement membrane (from 1.7 to 0 cells/mm, \( p=0.04 \)) and submucosal tissues (from 34.5 to 3.5 cell/mm\(^2\), \( p=0.005 \)). This was seen in conjunction with marked improvements in forced expiratory volume in one second (FEV\(_1\)) (FEV\(_1\) % predicted increased from a mean (SD) 99.8 (13.2)% to 107.5 (15.0)%, \( p=0.02 \)), morning peak expiratory flow rates (\( p<0.05 \)), use of reliever medications (\( p<0.05 \)) and a decrease in asthma symptoms (such as wheeze, cough, chest tightness and exercise limitation) (\( p<0.01 \)). There was also a sevenfold decrease in airway hyperresponsiveness to methacholine inhalation (\( p=0.001 \)), indicating reduced levels of airway inflammation and emphasising the important role that controlling eosinophilic inflammation plays in the clinical control of asthma.

1.2. Treatment with corticosteroids

Gluocorticoids are the most effective therapy currently available for the treatment of airway inflammation in asthma (Barnes 1998c; Barnes 1998b). They produce their effect at the molecular level where they have been shown to alter the transcription of various cell genes, as well as having a direct effect on recruitment and activation of inflammatory cells (Barnes 1998b; Barnes et al. 1998). For example corticosteroids cause an increase in the expression of anti-inflammatory proteins, such as interleukin-1 receptor antagonist, interleukin-10, and neutral endopeptidase. They also cause repression of the genes producing the pro-inflammatory cytokines interleukin-4 and interleukin-5, and chemokines involved in eosinophil recruitment and activation.

Since 1972 (Morrowbrown et al. 1972), when inhaled corticosteroid therapy was first used, the use of inhaled steroids has revolutionised the way that asthma is
Several biopsy studies in asthma have confirmed that inhaled steroids reduce the number and activation of inflammatory cells, leading to a reversal of the inflammatory changes in the airways (Djukanovic et al. 1992; Jeffery et al. 1992; Trigg et al. 1994). As a result, in the hope of preventing both short-term and long-term complications of asthma, inhaled corticosteroid therapy is now indicated in all but the mildest form of asthma (Sheffer 1992; NHLBI/WHO 1995; BTS 1997b).

Inhaled steroid treatment is effective in helping to control asthma in patients of all ages (Barnes 1998b). Their use results in improvements in symptoms, quality of life, lung function and exacerbation rates (Barnes 1998b). For example, a double-blind four-week crossover study comparing the effects of regular budesonide (an inhaled corticosteroid) versus regular terbutaline (a beta-agonist, 'reliever' medication) in mild-moderate asthma demonstrated that treatment with budesonide resulted in a marked improvement in lung function (Kraan et al. 1985). Budesonide was given at a dose of 400 μg/d and resulted in an improvement in FEV₁ % predicted from 85.3 to 96.2% occurring in a step-wise fashion over time. Additionally budesonide treatment resulted in a substantial increase in the dose of histamine required to induce a 20% fall in FEV₁ (PD₂₀ histamine); PD₂₀ rose from 4.0 to 9.5mg/ml. Over this same time period regular terbutaline was associated with no change in FEV₁ % predicted, and if anything a worsening in airway hyperresponsiveness. Similar results have been seen in children with asthma who were randomised to receive the inhaled steroid beclomethasone or salmeterol (a long-acting beta-agonist) for a year (Verberne et al. 1997).

Once maximal improvement in asthma is obtained in response to corticosteroid therapy, current guidelines suggest back titration of inhaled steroid dose to the minimum dose required for maintenance of asthma control (NHLBI/WHO 1995; BTS 1997b). The dose of inhaled therapy should be titrated using a combination of symptoms and lung function to indirectly gauge the level of control of inflammation. However the role of both symptoms and lung function in asthma
management has recently been questioned. Part of the difficulty lies in the fact that the dose-response curve for changes in lung function is relatively flat (Kamada et al. 1996; Lipworth 1996; Pederson and O’Byrne 1997). A marked effect is seen at low doses and only minor and insignificant differences are obtained using further adjacent doses, suggesting that lung function may be insensitive to changes occurring in airway inflammation. This insensitivity is demonstrated in a recent study by van Rensen et al (1999). They studied the effect of fluticasone propionate at a dose of 1000μg/d, on steroid naïve asthma over a four-week period. At baseline patients had a mean FEV₁ of 96.2% predicted and there was no significant improvement in this following treatment. However at baseline these patients had a high level of sputum eosinophils, which decreased significantly following treatment (mean (SD) of 2.85 (2.46)% to 0.44 (0.56)%, p<0.01). This change in eosinophils was seen in association with a decrease in airway hyperresponsiveness to methacholine (mean PD₂₀ (SD) 0.91 (1.62) mg/ml to 3.67 (1.05)mg/ml, p<0.01 respectively) and thus demonstrates a significant improvement in airway inflammation, an improvement undetected by measurements of lung function.

Further incongruities have been found in the relationship between both symptoms and lung function and the more direct markers of airway inflammation such as airway hyperresponsiveness and sputum eosinophils (Sont et al. 1996; Haley and Drazen 1998; Jatakanon et al. 1998a). A number of studies have yielded evidence of ongoing airway inflammation in patients who were clinically thought to be in remission (Foresi et al. 1990; Boulet et al. 1994; Sont et al. 1996; van der Thoorn et al. 2000). In contrast ongoing symptoms may occur despite adequate control of airway inflammation (Sont et al. 1996). These findings are consistent with a poor correlation between clinical judgement of asthma control and the actual degree of airway inflammation present (Parameswaran et al. 1998). Sont et al (1999) have recently demonstrated that by adding airway hyperresponsiveness into the normal treatment logarithm both clinical and histopathological outcomes are improved. These results again raise the question as to whether the optimal dose titration of inhaled corticosteroid therapy should be through the use of a specific marker of
airway inflammation, rather than the traditionally used symptoms and lung function.

1.3. Assessment of inflammation

1.3.1. Induced sputum

Since the last half of the 19th century clinicians have been interested in the macroscopic and microscopic characteristics of sputum in asthma (Pavord et al. 1997). In particular, interest has centred on the presence of sputum eosinophilia (Marrow-Brown 1958; Gibson et al. 1989; Hargreave 1998). Historically analysis has been limited to samples obtained from either spontaneously expectorated sputum or from fluid obtained at bronchoscopy. However both of these approaches are limited in their clinical applicability. Not every patient with asthma can spontaneously produce sputum, and bronchoscopy is a relatively invasive procedure - which limits its use both in poorly controlled asthma and for obtaining serial specimens.

Over the last decade the use of hypertonic saline nebulisation to induce sputum for cell analysis has been developed (Pin et al. 1992b; Hansel 1994; Pavord et al. 1997). Inhalation of hypertonic saline has been shown to increase mucociliary clearance in both asthmatic and healthy subjects therefore aiding the process of sputum production and expectoration (Daviskas et al. 1996). This method has resulted in sputum samples which show the same cell differential as spontaneous sputum but with the advantages of an increased yield, better cell viability, less squamous cell contamination, and a better quality of cytopsin (Pizzichini et al. 1996b; Bhowmik et al. 1998). Furthermore induced sputum analyses have been shown to be valid (Keatings and Barnes 1997), reliable (Pizzichini et al. 1996a), and produce highly reproducible results (Gibson et al. 1989; Pizzichini et al. 1996a).
The concentration of nebulised saline used to induce sputum has been shown not to affect the cell count in the sputum sample (Popov et al. 1995). However cell counts have been shown to change with increasing length of induction, samples collected later demonstrating a higher percentage of neutrophils (Holz et al. 1998a). Therefore in order to reduce any bias from the length of time of induction, it has been suggested that the first adequate sample produced by the patient should be used for analysis (or induction time standardised) (Magnussen and Holz 1999).

The rise in neutrophils seen during sputum induction suggests that the procedure itself may be pro-inflammatory. Consistent with this is the finding of a relative neutrophilia persisting for at least twenty-four-hours following sputum induction (Holz et al. 1998b; Nightingale et al. 1998). However any pro-inflammatory consequence is short lived, as repeated samples obtained within a six-day period demonstrate good reproducibility, with a high intraclass correlation coefficient and no evidence of ongoing neutrophilia (Pizzichini et al. 1996a). This therefore permits the use of serial measurements of induced sputum cell counts in analysing changes in airway inflammation providing a period of one week separates the procedures.

**Processing of sputum samples**

There are two methods of processing induced sputum for cell counts: analysis of the whole expectorate and plug selection (Kips et al. 1998a; Kips et al. 1998b; Spanevello et al. 1998). The former involves processing sputum plus saliva, whereas the latter requires the use of an inverted microscope to select sputum plugs. Initially it was thought that plug selection would provide a more reliable result, as it would decrease the level of contamination by other cells present in saliva. However 99.8% of cells in saliva are squamous cells and therefore do not affect the non-squamous cell differential (Fahy et al. 1993). As a consequence both methods have been shown to produce viable results (Kips et al. 1998a; Kips et al. 1998b; Spanevello et al. 1998). Furthermore minimising saliva contamination by incorporating a process of simple mouth rinsing prior to sputum
Expectoration, has been shown to decrease squamous cell content and improve slide quality of the ‘whole expectorate’ (Gershman et al. 1996).

But is the analysis of induced sputum a valid marker of airway inflammation? Are the cell counts obtained of clinical importance? These questions may be addressed first by comparing induced sputum to the established ‘gold standard’ (i.e. samples obtained from bronchoscopy) (criterion validity), then by demonstrating that it responds as expected to different states of asthma control (content validity).

**Criterion Validity**

Several studies have compared induced sputum cell counts with samples of bronchoalveolar lavage fluid obtained at bronchoscopy. Lensmar et al (1998) compared leucocyte counts obtained from induced sputum and lavage fluid in sixteen healthy volunteers (nine smokers, seven non-smokers) and found that the eosinophil count was consistent using both sampling methods. However sputum samples did have a lower proportion of macrophages (median (interquartile range) 52.0 (41.2-76.0)\% versus 95.4 (89.6-97.2)\%, p<0.001) and a higher proportion of neutrophils (47.0 (18.2-53.6) \% versus 1.4 (0.4-2.4) \%, p<0.001). These differences may have occurred because the sputum sample analysed was the second sample produced, and as discussed previously, sputum induction may in itself cause an increase in the number of neutrophils (Holz et al. 1998a). However this is unlikely to solely explain the large differences seen. Indeed similar differences have also been found in other studies of both healthy controls (Fahy et al. 1995) and asthmatic subjects (Fahy et al. 1995; Maestrelli et al. 1995; Grootendorst et al. 1997; Keatings et al. 1997a). Induced sputum cell counts consistently demonstrate the same proportion of eosinophils, increased levels of neutrophils, and lower levels of macrophages compared to counts obtained from lavage fluid. However do these differences mean that induced sputum is invalid as a marker of airway inflammation, or do they simply reflect different sampling sites in the airways?
The distribution of cell types has been shown to vary throughout the different lung compartments; in the alveoli the macrophage is the predominant cell type, whereas the neutrophil becomes more predominant in the proximal airways (Rankin et al. 1992). It could therefore be postulated that induced sputum comprises a sample from the airways whereas bronchoalveolar lavage may be more representative of the alveolar spaces. Consistent with this hypothesis is the finding that induced sputum cell differentials correlate to a greater degree with those obtained from bronchial washings (Keatings et al. 1997a). Bronchial washings are thought to sample the airways, whereas bronchoalveolar lavage samples more distal sites (Aalbers et al. 1993). Therefore, because asthma is primarily an inflammatory disease of the airways and this is where induced sputum samples are thought originate, the use of sputum may be more valid than the previous 'gold standard' of bronchoalveolar lavage.

Content Validity
There is a large body of evidence to confirm the content validity of induced sputum techniques. Induced sputum from patients with asthma has been shown to have a significantly higher percentage of eosinophils when compared to the normal population (Gibson et al. 1989; Pin et al. 1992b; Fahy et al. 1993; Maestrelli et al. 1995; Pizzichini et al. 1996a; Keatings and Barnes 1997; Pavord et al. 1997), the degree of eosinophilia correlating well with parameters of airway obstruction (Pin et al. 1992b). Furthermore sputum obtained from asthmatics has higher concentrations of eosinophil degranulation products, namely eosinophilic cationic protein (Fahy et al. 1993; Pizzichini et al. 1996a; Keatings and Barnes 1997; Pavord et al. 1997), eosinophil granule peroxidase (Keatings and Barnes 1997) and major base protein (Pizzichini et al. 1996a), indicating that induced sputum provides samples that reflect the eosinophil inflammation present in asthmatic airways.

a. Sputum Following Allergen Challenge
Fahy et al (1994) and Pin et al (1992a) have both demonstrated an increase in sputum eosinophil numbers in patients with asthma following allergen challenge,
consistent with the increase in inflammation occurring in this setting (Metzger et al. 1986). The increase in sputum eosinophil numbers occurred within four hours of the challenge (Fahy et al. 1994) and was still present at thirty-six hours post-challenge (Pin et al. 1992a). Furthermore, the increase in eosinophils correlated directly with the change in airway hyperresponsiveness occurring as a result of the allergen exposure (Pin et al. 1992a).

The link between sputum eosinophils and allergen exposure in asthma is further supported by the results of studies in occupational asthma (Lemiere et al. 1999; Obata et al. 1999). Patients with occupational asthma re-exposed to the occupational setting have been shown to increase the number of eosinophils and their degradation products in induced sputum samples. Median eosinophil differential increasing from 0.8 to 10.0% (p=0.007), and eosinophil cationic protein increasing from 166 to 3,840μg/l (p=0.01) on re-exposure (Lemiere et al. 1999). Likewise Obata et al (1999) demonstrated a significant increase in sputum eosinophils six and twenty-four hours following an allergen challenge in patients with suspected western red cedar induced asthma. The increase in eosinophils reflecting both impairment of lung function (Chan-Yeung et al. 1999; Obata et al. 1999) and the severity of symptoms (Chan-Yeung et al. 1999). Thus greater eosinophil numbers in induced sputum is associated with more severe disease.

b. Sputum During Exacerbations of Airway Inflammation

Induced sputum samples obtained during an acute exacerbation of asthma is characterised by marked eosinophilia, with median levels reaching 20% of the total cell differential (Pizzichini et al. 1997). Likewise during exacerbations resulting from the withdrawal of both oral and inhaled steroids sputum eosinophil numbers have been shown to increase greatly (Pizzichini et al. 1997; in’t Veen et al. 1999; Jatakanon et al. 2000). For example in’t Veen et al (1999) demonstrated an increase in sputum eosinophils from (mean ± SEM) 3.2 ± 1.1% to 12.5 ± 4.4% following a period of inhaled steroid tapering. Over the same time those who remained on a stable dose showed no change (3.1 ± 1.6% to 2.1 ± 0.7%) (p<0.05 for the comparison between the two changes).
Perhaps more importantly, increases in sputum eosinophils have been shown to occur prior to the onset of a symptomatic asthma exacerbation. For example, following tapering of oral prednisone increases in sputum eosinophils were seen four weeks prior to increases in blood eosinophils, and six weeks prior to worsening of symptoms and FEV₁ (Pizzichini et al. 1999). Similarly during stepwise withdrawal of inhaled corticosteroid therapy increasing sputum eosinophilia has been shown to be useful in predicting an up-coming exacerbation (Jatakanon et al. 2000). Again emphasising the importance of inflammation in asthma exacerbations, and highlighting the fact that airway inflammation may be present for some time before the development of symptoms.

c. Sputum in asymptomatic patients

As mentioned previously asthma symptoms are often an insensitive measure of ongoing airway inflammation. The role of induced sputum in detecting airway inflammation in asymptomatic patients is well demonstrated in a study of patients with allergic rhinitis by Gutiérrez et al (Gutiérrez et al. 1998). In this study all patients were asymptomatic as far as lower respiratory tract disease was concerned and yet these patients were found to have a significantly higher proportion of eosinophils than healthy controls. Moreover this increase in eosinophils was directly related to airway hyperresponsiveness to methacholine. Patients with allergic rhinitis and methacholine hyperresponsiveness (PC₂₀ <8mg/ml) had a higher eosinophil count than patients without hyperresponsiveness and the healthy controls (median eosinophil differentials of 7.3% versus 2.5% and 1.0% respectively, p=0.03 and p=0.02 respectively). This group with higher numbers of eosinophils and hyperresponsiveness also demonstrated significantly higher peak flow variability, suggesting underlying mild asthma despite being asymptomatic on study entry.

Similarly children with symptomatically controlled asthma receiving maintenance inhaled steroid treatment have also been shown to have elevated levels of eosinophils in induced sputum samples (Cai et al. 1998). These results indicate
that sputum eosinophilia may be able to identify mild airway inflammation even in the absence of clinical asthma, further supporting the use of induced sputum analysis in evaluating asthma control.

c. Sputum and Response to Steroids
Glucocorticoids provide the mainstay of treatment in asthmatic inflammation. Consistent with this, decreases in sputum eosinophil numbers occur during oral corticosteroid therapy in both previously steroid-naive asthma (Claman et al. 1994; Keatings et al. 1997b) and following an acute exacerbation of asthma (Pizzichini et al. 1997). This decrease occurs with corresponding improvements in lung function and symptom score, and decreases in the concentration of eosinophil degradation products (Claman et al. 1994; Pizzichini et al. 1997).

Likewise sputum eosinophil counts have also been shown to be response to treatment with inhaled corticosteroid. Eosinophil numbers decrease within three hours of inhaled corticosteroid administration (Oh et al. 1999) and result in markedly reduced levels when compared to placebo (Jatakanon et al. 1998a) (mean ±SEM, 1.4 ±0.8% and 4.0 ±1.1% respectively, p<0.005). These changes were accompanied by improvements in lung function and airway hyperresponsiveness, again demonstrating the responsiveness of induced sputum cell analysis in assessing treatment of asthma.

Summary
Induced sputum analyses results in reproducible cell differentials that appear to be indicative of cell counts in the airways. Asthma is associated with an increase in sputum eosinophils that correlates with disease severity. The eosinophil differential is high during exacerbations and in untreated asthma, and levels decrease following treatment with corticosteroids. Thus analysis of induced sputum provides a valid assessment of airway inflammation, and therefore provides a good marker against which future markers of airway inflammation can be compared.
1.3.2. **Hypertonic saline challenge**

Measurement of airway hyperresponsiveness by means of a bronchial challenge has been widely used in the assessment of asthma for many years (Sterk et al. 1993). Methods include the inhalation of both direct and indirect stimulants of smooth muscle contraction, resulting in airway narrowing. Quantification of the degree of airway hyperresponsiveness is based on obtaining a dose-response curve from which the provocative dose/concentration of the agent causing a fall in FEV$_1$ of either 15 or 20% is calculated. i.e. PC$_{20}$/PD$_{20}$ are the provocative concentration/dose of the inhaled agent producing a fall in FEV$_1$ of 20%.

Methacholine and histamine are two commonly used direct bronchial challenges, both of which cause bronchial smooth muscle contraction and thus airway narrowing via binding to specific receptors. These challenges are very sensitive for detecting airway hyperresponsiveness in asthma (Cockcroft 1997), the degree of responsiveness being related to the level of airway inflammation (Chetta et al. 1996) as well as to the severity of asthma (Juniper et al. 1981; Ryan et al. 1982). Following allergen exposure airway hyperresponsiveness increases (Cockcroft et al. 1977), and in contrast treatment with inhaled steroid decreases responsiveness (van Grunsven et al. 1999) - reflecting the changes in airway inflammation occurring in these settings. However the usefulness of both methacholine and histamine challenges in the population setting is limited because the airway hyperresponsiveness is normally rather than bimodally distributed (Salome et al. 1987), thus making the differentiation between asthma and non-asthma difficult. For example, Enarson et al (1987) found that a positive methacholine challenge (PC$_{20}$ ≤ 8 mg/ml) in the general population had a positive predictive value of only 12% in identifying symptomatic asthma. Likewise a similar population study of 876 adults in Western Australia has shown that approximately 30% of subjects with a positive histamine challenge had no history of any asthma symptoms (Woolcock et al. 1987).

Recently the use of the indirect bronchial challenges, adenosine-5-monophosphate, hypertonic saline, and exercise have been developed as more
specific markers of asthma inflammation. These challenges do not act directly on the airway smooth muscle cells themselves, but rather have their effect via the release of mediators from the inflammatory cells present in the airway. Responsiveness to these indirect challenges has been found to be a better indicator of changes in airway inflammation occurring with inhaled corticosteroid therapy than both methacholine and histamine (O'Connor et al. 1992; Sterk et al. 1993; duToit et al. 1997; Hofstra et al. 2000). Furthermore, in a number of population studies it has been found that a positive hypertonic saline challenge has greater specificity in diagnosing current asthma than previously found with methacholine and histamine (Riedler et al. 1994a; Rabone et al. 1996; Riedler et al. 1998). A positive hypertonic saline challenge has a specificity of between 92-97% and a positive predictive value of between 71-81% in diagnosing patients with 'current wheeze'.

The use of nebulised hypertonic saline to induce bronchoconstriction in asthma (Smith and Anderson 1989; Sterk et al. 1993; Anderson et al. 1995; Anderson 1996) is thought to occur indirectly via a variety of mechanisms (Smith and Anderson 1989), one of which is thought to be a change in airway osmolality. The volume of fluid lining the airways is very small (Anderson 1984), and so inhalation of hypertonic saline can easily change the osmolality of this airway lining fluid (Smith and Anderson 1989). In turn this provokes the release of endogenous mediators from cells (e.g. mast cells) located in the airway epithelium and submucosa (Eschenbacher and Gravelyn 1988; Gravelyn et al. 1988; Maxwell et al. 1990), causing airway narrowing through a combination of bronchial smooth muscle contraction and airway oedema. Thus airway hyperresponsiveness to hypertonic saline is greatly dependent on the presence of inflammatory cells in the airways, making it a potentially better indicator of underlying airway inflammation than the previously mentioned direct stimulants. Moreover hypertonic saline challenges have the advantages of requiring readily accessible equipment, are relatively cheap to perform and are easier to conduct than previous challenges (the methodology will be described in Chapter Four).
Reproducibility

Results obtained from repeated hypertonic saline challenges have shown the test to be reproducible in both adults (Smith and Anderson 1990) and children (Riedler et al. 1994b). In eight patients undergoing repeated challenges within thirty-seven days, Smith et al (1990) were able to calculate a coefficient of variation of 14%, with a correlation coefficient of 0.92 between the results. Similarly Riedler et al (1994b), in a study of seventeen children with asthma to whom hypertonic saline challenges were performed twice within ten days, have demonstrated good repeatability in the results to within 0.85 doubling doses.

Hypertonic Saline Challenges and Asthma Inflammation

The relationship between responsiveness to hypertonic saline and the presence of inflammatory cells within the airway makes it a useful tool in the assessment of the airway inflammation in asthma. Hypertonic saline challenges are less sensitive than direct stimulants at detecting airway hyperresponsiveness (Smith and Anderson 1990), however a positive challenge has a high predictive value for diagnosing patients with moderate to severe asthma (Smith and Anderson 1989; Smith and Anderson 1990). Indeed, subjects without asthma demonstrate very little change in lung function in response to hypertonic saline (Anderson 1985), with no false-positive results reported in healthy volunteers (Anderson 1996; duToit et al. 1997). Using nebulised potassium chloride (10% KCl) Magyar et al (1984) compared the increases in airway resistance occurring in asthma (n=97), chronic bronchitis (n=56), and normal healthy subjects (n=32). They found a significant increase in airway resistance in all patients with asthma following inhalation of hypertonic saline, but no increase was seen in either patients with chronic bronchitis or the healthy controls.

Responsiveness to hypertonic saline has also been shown to relate to the degree of airway inflammation in asthma. In an epidemiological study of 170 children, airway hyperresponsiveness to a hypertonic saline challenge was strongly associated with higher levels of sputum eosinophils (eosinophils of >2.5% of the cell differential) (odds ratio of 4.36, 95% C.I.: 1.89-11.86) (Gibson et al. 1998).
Likewise a statistically significant but weak correlation has been found between sputum eosinophils and airway hyperresponsiveness to hypertonic saline in patients with either occupational (n=24) or non-occupational asthma (n=24) (r=-0.4, p=0.03) (Di Franco et al. 1998).

**Hypertonic Saline Challenge and Corticosteroid Treatment**

Airway hyperresponsiveness to hypertonic saline decreases following the administration of corticosteroids (Rodwell et al. 1992b; duToit et al. 1997), nedocromil sodium (Rodwell et al. 1992a) and sodium cromoglycate (Anderson et al. 1994). For example, Rodwell et al (1992b) demonstrated an increase in saline PD$_{20}$ of 5.6 fold following eight weeks of treatment with inhaled beclomethasone 600-1,500 µg/day. Similarly, du Toit et al (1997) demonstrated progressive increases in hypertonic saline PD$_{20}$ after 1, 4-5, and 6-10 weeks of treatment with budesonide 1000 µg/day, culminating in an increase of 9.7 fold (95% C.I.: 4.2 to 22) at two months. Conversely steroid withdrawal results in an increase in hypertonic saline responsiveness, seen in association with a decline in lung function and an increase in airway inflammation (in't Veen et al. 1999).

However the relationship between responsiveness to hypertonic saline and the degree of underlying airway inflammation is less definite in the presence of corticosteroid use. In contrast to the above study by Di Franco et al (1998), Iredale et al (1994) found no correlation between hypertonic saline responsiveness and sputum eosinophils in mild to moderate asthma (range of % predicted FEV$_1$ 43.3-111.5%). The reason for this apparent discrepancy is most likely a result of the difference in the use of steroid therapy within the study populations. In the Di Franco study patients had been off their inhaled steroid therapy for a period of four weeks prior to investigation, whereas in the Iredale study 75% of the patients were currently on either inhaled or oral corticosteroids. This finding raises the possibility that induced sputum analyses and hypertonic saline challenges may measure different aspects of airway inflammation in steroid treated asthma. However this requires further investigation.
Summary
Hypertonic saline inhalation is thought to cause airway narrowing indirectly via a change in airway osmolality and mediator release. Resultant bronchoconstriction appears to be specific to those patients with moderate to severe asthma, and may be directly related to the level of underlying airway inflammation. Response to hypertonic saline has been shown to decrease with treatment, suggesting a potential role in steroid dose titration and management of asthma.
CHAPTER TWO:
EXHALED NITRIC OXIDE

2.1 Background

The discovery in 1987 (Palmer et al. 1987) that endothelium derived nitric oxide (NO) has an important role as a mediator of vascular smooth muscle relaxation was an important advance in our knowledge about the actions and interactions of cells. Since then, there has been an explosion in the roles identified for NO. Nitric oxides now appear to be involved in diverse physiological actions including neurotransmission, platelet function, smooth muscle function, cytotoxicity and as a mediator of immune responses (Barnes and Belvisi 1993; Gaston et al. 1994; Barnes and Liew 1995; Lundberg et al. 1996).

Recently both Gaston et al (1994) and Zapol et al (1994) have reviewed the physiology of NO in humans. NO is produced endogenously from a variety sources, the most important of which is L-arginine. Arginine is oxidised by the enzyme NO synthase to produce citrulline and NO. NO synthase (NOS) exist in at least three isoforms, two of which are constitutive isoforms and the other one is an inducible isoform. The constitutive NO synthase are found mainly in vascular endothelium and the nervous system, and are calcium and calmodulin dependent enzymes. When activated they produce a small amount of NO which has its effect locally, to produce endothelium-dependent vasodilatation, or in peripheral neurones to produce non-adrenergic non-cholinergic neural responses, such as bronchodilatation. The inducible NO synthase isoform is induced by pro-inflammatory cytokines such as interleukin-1β, tumour necrosis factor-α, and interferon-γ, as well as various endotoxins. Activation of inducible NO synthase results in the production of large amounts of NO and is postulated to be involved in several inflammatory processes. In the airway these high levels of NO can cause increased microvascular blood flow and oedema which may potentially offset any benefit from smooth muscle relaxation (Gaston et al. 1994).
2.2 Source of eNO

NO was first discovered in the expired air of humans in 1991 by Gustafsson et al (1991). Soon after this it was reported that expired NO (eNO) was elevated in asthma (Alving et al. 1993; Kharitonov et al. 1994b; Persson et al. 1994), and the plausible link between eNO and inflammation in asthma was made (Barnes and Liew 1995; Barnes and Kharitonov 1996). Studies have demonstrated numerous cells potentially capable of generating NO in the lung. These include macrophages, neutrophils, mast cells, nonadrenergic noncholinergic inhibitory neurones, fibroblasts, vascular smooth muscle cells, pulmonary arterial and venous endothelial cells, and pulmonary epithelial cells (Gaston et al. 1994).

Immunohistochemical studies from bronchial biopsies indicate that there is an enhancement of inducible NO synthase expression in asthma, and that this expression is further stimulated by the cytokine tumour necrosis factor α (Hamid et al. 1993). This is thought to result in large amounts of NO being produced, which then plays a central role in the mediation of the inflammatory process (Barnes and Belvisi 1993; Barnes and Liew 1995; Lundberg et al. 1996). Furthermore inhalation of a relatively selective inhibitor of inducible NO synthase, aminoguanide, decreases eNO in patients with asthma but not in normal subjects (Yates et al. 1996b), implying that the high eNO levels seen in asthma are largely derived from the inducible NO synthase isoform.

2.3 eNO and Asthma Inflammation

NO concentration in exhaled breath is elevated in steroid-naïve asthma (Kharitonov et al. 1994b; Persson et al. 1994; Rutgers et al. 1998; Sapienza et al. 1998) where levels correlate well with other markers of airway inflammation such as sputum eosinophils and airway hyperresponsiveness (Jatakanon et al. 1998). Exhaled NO has further been shown to be raised during both asthma exacerbations (Massaro et al. 1995; Crater et al. 1999) and following an allergen challenge (Kharitonov et al. 1995a). Importantly, the increase following allergen exposure does not occur during acute phase bronchoconstriction but rather during late phase events – consistent with worsening of airway inflammation during the
latter response (Pin et al. 1992a; Fahy et al. 1994). Moreover, this rise in eNO correlates with the fall in FEV₁ over the same time period.

**Response to Steroids**

Exhaled NO concentrations are not only elevated in asthma but they respond to treatment with corticosteroids. Concentrations decrease following anti-inflammatory treatment with both oral prednisone (Massaro et al. 1995; Yates et al. 1995) and inhaled corticosteroid (Kharitonov et al. 1996b; Jatakanon et al. 1998a; Lim et al. 1999; van Rensen et al. 1999), consistent with the decrease in inflammation observed in the airways as a result of steroid treatment (Djukanovic et al. 1992; Bentley et al. 1996). Furthermore, stable asthma treated with maintenance inhaled steroid is associated with eNO levels equivalent to healthy controls (Kharitonov et al. 1994b), again consistent with the relative absence of inflammation in these individuals. Conversely, following a reduction in inhaled steroid dose, levels of eNO increase, reflecting the recurrence of airway inflammation (Kharitonov et al. 1996c).

The response of eNO to corticosteroid therapy is rapid, reflecting the fast decline in sputum eosinophils seen following treatment initiation (Claman et al. 1994; Oh et al. 1999). For example, during an exacerbation of asthma, eNO levels decrease within forty-eight hours of prednisone administration (mean ±SEM eNO decreasing from 20.0 ±3.1ppb to 11.6 ±3.0ppb, p = 0.002) (Massaro et al. 1995). Similarly, eNO has been shown to fall within seventy-two hours of prednisone treatment in steroid-naïve asthma (Yates et al. 1995). In addition, the decrease in eNO with steroid therapy occurs in a step-wise fashion over time, seen in association with a decrease in airway hyperresponsiveness during this same period (Kharitonov et al. 1996b).

At the molecular level corticosteroid therapy results in a reduction in the expression of inducible NO synthase (Springall et al. 1995). It is this reduction in the inducible isoform expression that is thought to be largely responsible for the reduction in eNO seen with anti-inflammatory treatment, rather than any effect on
constitutive NO synthase function. In healthy controls, in which constitutive NO synthase is present in the airways but with little of the inducible isoform, there is no demonstrable decrease in eNO following corticosteroid treatment (Yates et al. 1995), suggesting that the main cause of the decreasing eNO in corticosteroid treated asthma is via changes in inducible NO synthase activity.

A potential role for eNO in assessing the dose-response to inhaled steroid therapy is suggested in a small study by Alving et al (1995). These authors reported a dose-response relationship between inhaled steroid dose and eNO in children with asthma. Children taking 200μg/day beclomethasone had a mean eNO level of 30ppb, whereas patients taking 400 and 800μg/day had eNO levels of 12 and 7ppb respectively. The groups were small (n = 11, 8, and 5 subjects respectively), but the results point to the possibility that eNO may be useful tool for monitoring responses to inhaled steroid therapy. This question will be addressed in Chapter 7.

Summary

Patients with asthma and active airway inflammation have been shown to exhale a higher concentration of NO. This level of eNO has been shown to decrease following anti-inflammatory treatment with both oral and inhaled steroids. These findings suggest that eNO may be a useful non-invasive indicator of airway inflammation, something that would be clinically very useful in the long-term treatment and control of asthma. This is the basis upon which the studies described in this thesis were developed.

2.4 Measuring eNO

The levels of NO in exhaled breath are very small and are measured in parts per billion (ppb). Such small quantities can only be accurately measured by means of a chemiluminescence analyser. The process of chemiluminescence is based on a reaction between NO and ozone in a cooled reaction chamber, during which NO is converted to nitrogen dioxide (NO₂). This reaction is a photochemical reaction that releases infrared light. A photomultiplier tube subsequently measures this emission of light, whose amount is linearly proportional to the concentration of
NO (Fontijn et al. 1970). Measurements of eNO can be made using samples of mixed expired air collected in a balloon, or by on-line measurement of single or tidal breaths (Robbins et al. 1996). In the subsequent investigations repeated in this thesis on-line measurements of single breaths were used and therefore further description of technique will be limited to this method.

The process of chemiluminescence analysis has been developed to enable online breath analysis using rapid response analysers (response time < 2 sec) linked to a computer module and a biofeedback monitor (fig. 2.1). The patient exhales at a constant flow rate through the mouthpiece tubing against a known flow restrictor. Patients observe a biofeedback monitor indicating exhalation flow/pressure; thus enabling them to exhale at a constant rate. The reason for the flow restrictor is to provide a small amount of resistance, thus increasing the mouth pressure and closing off the nasopharynx to ensure that no nasal contamination occurs (as will be discussed later). The mouth-piece and all tubing is made of the inert material Teflon/polytetrafluoroethylene (PTFE) to avoid reaction with the very reactive NO molecules.

Exhaled breath passing through the mouthpiece is then sampled via side arms for measurement of NO, carbon dioxide (CO₂) and pressure/flow. Recordings of NO, CO₂, pressure and lung volume are then displayed on the computer monitor (fig. 2.2) from where recordings can be either read instantly or alternatively saved for reading at a later date.
Figure 2.1. A schematic diagram of single breath exhaled nitric oxide (eNO) measurements. Patients exhaled through the mouthpiece against a flow restrictor creating in-built resistance (A). Nitric oxide measurements were made using a chemiluminescence analyser, sampling the breath via a side arm (B). Patients watched a biofeedback monitor (C) to ensure a constant exhalation pressure/flow was obtained.
Figure 2.2. A schematic illustration of the various recordings as displayed on the computer monitor. For any time point values for eNO, CO₂, mouth pressure, exhalation flow rate, and lung volume can be read.
Nitric oxide measurements (fig. 2.2) typically consist of an initial flat portion prior to the patient exhaling, during which ambient NO concentrations can be recorded. There is initially a peak in the exhaled NO seen during the early stages of exhalation, this is then followed by a plateau corresponding with the end of expiration. The reason for the peak followed by plateau is the difference between measuring air initially present in the dead space of the airways (peak) and air originating from the alveolar spaces (plateau). Recordings of eNO must always be made at the same stage of exhalation and can be read at either the peak or plateau values. However the plateau level is now the value most widely read and accepted, this is in order to reduce the risk of interference from nasal and ambient NO contamination which affects the peak levels.

2.5 Factors affecting eNO levels

Many factors are known to effect the measurement of eNO, so recently attempts have been made recently to standardise the procedure (Kharitonov et al. 1997a; Silkoff and Zamel 1998; ATS 1999). The following section is a summary the different factors causing variation in eNO levels, factors that need to be taken into account when using eNO in both the clinical and research settings.

**Exhalation flow rate**

One of the greatest influences on measuring eNO levels is the breath flow rate via the airways. Slower rates are associated with higher eNO recordings (Högman et al. 1997; Silkoff et al. 1997; Corradi et al. 1998). NO rapidly binds to haemoglobin (Sharma et al. 1987; Cremona et al. 1995). As a result NO is quickly taken up by haemoglobin in the capillaries adjoining the alveolar space, creating alveolar air in which the NO concentration is relatively low. It is postulated that as the ‘NO low’ air is exhaled through the airways it takes up NO which has diffused into the lumen, driven by the gradient between airway wall mucosal concentration and the concentration in the lumen (Silkoff and Zamel 1998). A slower flow will increase the transit time of the breath through the airways thus allowing more NO to diffuse into the lumen and an increase in eNO.
concentration. This is highlighted in a paper by Silkoff et al (1997) who, by measuring eNO during 9 separate exhalation flow rates (ranging from 1550 to 4.2 ml/sec), observed a rise in eNO of almost 35-fold over the range of decreasing flow rates.

The hypothesis of eNO measurements being flow rate dependent is also indirectly supported by studies demonstrating that eNO levels decrease during increasing degrees of bronchoconstriction (de Gouw et al. 1998b; Ho et al. 2000). During these studies different degrees of bronchoconstriction were induced and eNO measurements were performed at a constant exhalation flow rate. At greater bronchoconstriction there is an increase in velocity of air through the airways, resulting in a relative decrease in transit time, and a fall in measured eNO. This hypothesis is further supported by the finding that breath-holding results in an increase in the initial peak eNO, but no change in end-expiration values (Kharitonov et al. 1996a; Tunnicliffe et al. 1996). Stasis of breath in the airway results in increased diffusion and therefore increased concentration of NO in the initial portion of exhaled air, but not during end-exhalation.

*Nasal contamination*

High NO concentrations are found in the nasal passages of humans (Kharitonov et al. 1996a; Robbins et al. 1996; Silkoff et al. 1997) and initially there were concerns that these high levels might contaminate exhaled air measured at the mouth (Lundberg et al. 1994; Kimberly et al. 1996). Indeed studies have shown that contamination by nasal NO does occur with breath-holding (Kimberly et al. 1996) and tidal breathing (Kharitonov and Barnes 1997). For this reason the use of nose-clips was introduced. However, their use has since been shown to potentially increase the level of nasal contamination because intranasal airway pressures are raised when they are worn (Silkoff et al. 1997), thus the vellum does not close to separate oropharynx from nasopharynx and air from the nasal passages can contaminate the sample. Instead it has been found that exhaling against a small resistance causes the vellum to close tightly and thus the
nasopharynx is effectively closed off preventing nasal contamination (Kharitonov et al. 1996a; Kharitonov and Barnes 1997; Silkoff et al. 1997).

In order to examine the validity of exhalation against resistance, eNO levels at the mouth have been compared with those measured in the lower airways (Kharitonov et al. 1996a; Silkoff et al. 1997; Gabbay et al. 1998). These studies have shown that eNO measured at the mouth correlates well with levels obtained from the bronchi, trachea and glottis, and demonstrate a lack of nasal contamination.

**Ambient NO levels**

There has been conflicting reports about the effect of ambient NO concentrations on eNO recordings, although much of this variation can be explained by differences in techniques used. As mentioned previously (see fig 2.2) the first part of exhalation consists of air originating from the dead-space, a mixture of ambient NO and nasal NO contamination. Because of this, single breath recordings read at the peak level and NO concentrations taking from bags of mixed expiratory air will be greatly affected by ambient NO concentration. However, the use of a standardised single-breath on-line measurement of eNO, read during the end-expiration plateau (corresponding to air from the alveolar spaces), has been shown to be unaffected by ambient NO levels (Kharitonov et al. 1994b; Robbins et al. 1996; Piacentini et al. 1998; Sippel et al. 2000). This may be explained by the fact that NO is quickly bound to haemoglobin (Sharma et al. 1987; Cremona et al. 1995) therefore creating alveolar air that is relatively low in NO, independent of ambient levels. For example, Piacentini et al (1998) measured single-breath on-line eNO following inhalation of ambient levels ranging from 0 –150ppb. Values were read at the eNO plateau and no relationship was found between ambient and eNO. Furthermore, inhaling artificially high concentrations of NO (up to 1000ppb – much higher than those found in ambient levels) has been shown to have no effect on end-expiratory eNO values, although increases in the initial peak eNO were seen (Silkoff et al. 1997; Ho et al. 1998).
**Diurnal rhythm**

Nocturnal asthma is characterised by a circadian rhythm, with the greatest airway obstruction occurring at 0400 hours, the least at 1600 hours (Hetzel and Clark 1980). It has been postulated that eNO levels may follow a similar circadian rhythm, particularly in patients experiencing nocturnal symptoms. In a study by ten Hacken et al (1998), eNO was measured four-hourly from mid-day for a period of twenty-four hours. The mean eNO concentration was significantly higher in those patients with nocturnal asthma when compared to both healthy controls ($p<0.01$) and patients with asthma but no nocturnal symptoms ($p<0.05$) (mean ±SD of 46.0 ±18.7ppb, 9.9 ±2.1ppb, and 26.6 ±12.6ppb respectively). There was a circadian rhythm found for FEV$_1$ in asthma patients with and without nocturnal symptoms, but eNO levels did not vary with this circadian rhythm. However patients with nocturnal asthma did have significantly higher eNO recordings at 0400 hours compared to 1600 hours (mean ±SD of 50 ±20ppb and 42 ±15ppb respectively, $p<0.05$). There was no significant difference in eNO values at any other time point. In contrast to these findings, Georges et al (1999) found a decrease in eNO at 0400 hours in patients with nocturnal asthma as compared to values taken at 1600 hours and 2200 hours (mean ±SEM of 66.0 ±8.5ppb, 77.2 ±8.2ppb and 68.4 ±8.7ppb respectively). In this study no differences were found in the values of eNO recorded at these times in asthmatics with no nocturnal symptoms. The reason for the apparent discrepancy between these two studies is unclear. Both studies used similar methods for the analysis of eNO and followed a similar study design. Unfortunately both studies used small numbers of patients with nocturnal symptoms ($n=8$ and $n=5$ respectively), and so their results may have been skewed by data from just one or two individuals. The conclusion from these studies is that while there may be a change in eNO during night-time in patients with nocturnal asthma, the direction of change is inconsistent and the cause for it is unknown. However, in order to avoid any possible variation in eNO with time of day it is suggested that recordings be obtained as close as possible to the same time of day.
Cigarette smoke

Cigarette smoke is known to contain a high amount of NO (Norman and Keith 1965). Many studies have shown that current chronic cigarette smoking is associated with a decrease in eNO (Persson et al. 1994; Schilling et al. 1994; Kharitonov et al. 1995b; Robbins et al. 1996; Robbins et al. 1997; Rutgers et al. 1998; Verleden et al. 1999), the degree of reduction correlating with the number of cigarettes smoked per day (Kharitonov et al. 1995b). The mechanism for this reduction is as yet unknown, but may be related to the negative feedback inhibition exerted by high concentrations of airway NO on NO synthase (Assreuy et al. 1993). This is consistent with the observation of Robbins et al (1997) that within one week of cessation of smoking eNO levels increase, with further increases in eNO seen over the subsequent eight weeks.

Changes occurring in eNO following acute exposure to cigarette smoke are not so clear-cut. Initially, Kharitonov et al (1995b) demonstrated a decrease in peak eNO concentrations five minutes after smoking a single cigarette. This decrease returned to baseline levels within fifteen minutes. In contrast to this, Chambers et al (1998) observed an increase in plateau phase eNO following a single cigarette. This increase was greatest at one minute, but also present ten minutes later. The differences in these results may be a reflection of differences in the reading of eNO between the studies; the Kharitonov study measuring peak levels while the Chambers study measured plateau. Chambers et al (1998) have postulated that the increases they observed may be the result of NO being retained in the airway epithelial lining fluid. In turn this would result in high local NO concentrations causing an initial increase in eNO, this could also lead to the chronic down-regulation of NO synthase in the airways of smokers.

Alcohol

Ingestion of alcohol has been shown to decrease the level of eNO in both humans (Yates et al. 1996a) and in rabbits (Persson and Gustafsson 1992). Interestingly, this decrease in eNO was not reversed by the addition of the NO substrate L-
arginine, suggesting that alcohol may affect NO production via interaction with one of the NO synthase in the lung. As yet the precise mechanism is not known.

**Menstrual cycle**

Initially it was thought that eNO levels fluctuated with the female menstrual cycle. This was first reported by Kharitonov et al (1994a) who found that during midcycle female subjects exhaled almost three times the level of eNO measured during menses (mean (SD) 150 (39) v 59 (25)ppb). It was proposed that the increase in eNO might be due to the increases in oestradiol, oestrone, luteinising hormone and follicle stimulating hormone occurring at the luteal phase of the menstrual cycle. However, eNO levels in the study were read at peak rather than the plateau phase of exhalation, and patients were inhaling ambient air – which is known to affect the peak eNO, and the exhalation flow rate was not standardised.

In contrast to these findings, others have found no difference in eNO between male and female subjects (Massaro et al. 1995; Morris et al. 1996). Furthermore, Morris et al (1996) investigated the relationship between eNO and menstruation in 5 healthy premenopausal women who had eNO measured daily throughout the entire menstrual cycle. Levels were obtained on each occasion after inhaling both ambient and NO free air, exhaling at a constant rate. They found no relationship between female sex hormone production and eNO levels. Likewise the hormonal changes associated with pregnancy have been shown to have no effect on eNO levels (Morris et al. 1995), suggesting that eNO is unrelated to female hormone levels and that the results of the previous study may have been spurious.

**Exercise**

Exercise results in a decrease in the concentration of eNO, largely as a result of an increased flow rate of air through the airways (Iwamoto et al. 1994; Trolin et al. 1994; Phillips et al. 1996). Exercise also results in an increase in total NO excretion by means of an increased per minute ventilation rate (Iwamoto et al. 1994; Trolin et al. 1994). Similar findings affecting both eNO concentration and total excretion are also found with resting hyperventilation (Iwamoto et al. 1994;
Phillips et al. 1996), although there is some suggestion that hyperventilation only explains part of the changes seen, and that other factors such as pulmonary blood flow may also be involved (Phillips et al. 1996).

**Other Medication**

Apart from medications designed to treat inflammation in asthma there are a number of other medications that could possibly interfere with eNO readings.

*a) NO donors*

NO donors are commonly used in the treatment of cardiovascular disorders. A study investigating the effect of intravenous infusions of the NO donors glycerin trinitrate (GTN) and sodium nitroprusside on eNO has been performed. (Dirnberger et al. 1998). The rates of the infusions used were great enough to result in a significantly reduced diastolic blood pressure and increased heart rate, but they were found to have no effect on eNO levels. Interestingly, both GTN and sodium nitroprusside were found to increase FEV1, suggesting smooth muscle relaxation in the airways as a result of the infusions.

In contrast to this Marczin et al (1997), in a study of anaesthetised patients, was able to demonstrate an increase in eNO during both an infusion of GTN and following GTN boluses. The increase was small during the infusion, 12.6 (0.2)ppb to 14.1 (0.1)ppb, and the dose of GTN used was extremely high (45 mg/hr) (20-40 fold that of Dirnberger et al). This dose is far above what would be tolerated by a non-anaesthetised patient and much greater than would be used clinically. Likewise the boluses of 125, 250 and 500µg of GTN resulted in increases in eNO of 3.1, 6.0, and 8.1ppb respectively. However no statistical analysis was performed and small patient numbers (n = 7) would mean that these data need to be interpreted with caution.
b) Beta-agonists

Both short-acting and long-acting beta-agonists are widely used in the treatment of asthma. Their use results in increased levels of eNO (Yates et al. 1997; Fuglsang et al. 1998; Silkoff et al. 1999), although the increase is mild and often fails to reach statistical significance (Yates et al. 1997; Fuglsang et al. 1998). The cause of this mild increase is uncertain, but may be explained by changes in airway calibre. Beta-agonists bind to beta-receptors on smooth muscle causing relaxation and hence bronchodilation. As a result their use leads to increased airway calibre and improvements in FEV$_1$. This increase in airway calibre will result in a relative slowing of air through the airways when exhalation at the mouth is kept at a constant rate. Thus these drugs may result in a relative increase in the concentration of eNO as measured at the mouth.

c) Prostaglandins

Cyclo-oxygenase products are known to play a part in the pathophysiology of asthma. In particular prostaglandin-E$_2$ has been shown to inhibit exercise-induced bronchoconstriction and allergen-induced early- and late-phase responses, as well as modulate the expression of NO synthase in certain cell types. Recently a study by Kharitonov et al (1998) has demonstrated a decrease in eNO in asthma and healthy subjects following inhalation of the prostaglandins E$_2$ and F$_{2a}$. Study participants were given increasing doses of both prostaglandins, both of which caused a decrease in eNO, however the greatest response was seen following prostaglandin E$_2$ in the asthma patients. This decrease in eNO was independent of change in airway calibre, suggesting an effect at the molecular level.

Other medical conditions

A number of medical conditions other than asthma have been postulated to affect the concentration of eNO. Although some of these do alter eNO levels, generally the degree to which they do so is much less than that seen with asthma.
a) Systemic sclerosis
This systemic connective tissue disorder is associated with various lung complications. Exhaled NO levels are increased in patients with systemic sclerosis regardless of known lung pathology when compared to healthy controls (Kharitonov et al. 1997b). Interestingly, given the vasodilatory effect of NO on vascular smooth muscle, the presence of pulmonary hypertension in systemic sclerosis is associated with lower eNO values.

b) COPD (Chronic Obstructive Pulmonary Disease)
Studies investigating eNO in COPD have demonstrated varying results; some finding increased eNO (Kanazawa et al. 1998; Maziak et al. 1998; Corradi et al. 1999), while others finding normal or even low levels (Clini et al. 1998; Rutgers et al. 1999; Delen et al. 2000). Despite this, eNO has been found to be elevated in COPD patients with active inflammation (Maziak et al. 1998; Agusti et al. 1999) and levels have been shown to correlate with other markers of airway inflammation (Kanazawa et al. 1998; Rutgers et al. 1999; Silkoff et al. 2000). Furthermore, COPD patients demonstrating partial reversibility to bronchodilator also have increased levels of eNO (Papi et al. 2000), suggesting that eNO may be a good marker for inflammation and reversibility in COPD. The relationship between eNO and COPD will be discussed more fully in Chapters three and eight.

c) Cystic Fibrosis and Bronchiectasis
In the only study to date in which eNO was measured using a standardised technique, no differences were observed in eNO levels obtained from patients with cystic fibrosis or bronchiectasis compared to healthy controls (Ho et al. 1998). Moreover during an infective exacerbation of cystic fibrosis no increase in eNO was observed. The cause of this lack of increase in eNO with these inflammatory diseases is unknown. It may be the result of a lack of expression of inducible NO synthase in the airways, or alternatively NO molecules may somehow become “trapped” within the viscous airway
excretions that characterise these conditions (although given the small size of NO molecules and its rapid diffusion this would seem unlikely).

d) Upper respiratory tract infections

Upper respiratory tract infections are associated with an increase in peak eNO in normally healthy subjects (Kharitonov et al. 1995c). Levels then return to normal within 3 weeks. The cause for this increase is thought to be due to the induction of NO synthase expression. Induction of NO synthase is regulated by transcription factors of which the most important is nuclear factor-κB. Viral infections are thought to induce nuclear factor-κB thus increasing the expression of NO synthase and the production of NO.

De Gouw et al (1998a) have further studied the effect of viral infection in asthma. In a blinded study asthmatic patients underwent inoculation with either rhinovirus 16 or placebo. Following rhinovirus 16 infection there was a significant increase in eNO at days two and three compared to baseline (median 7.7 and 6.2ppb compared with 1.9ppb respectively), whereas there was no change seen in the placebo treated group. There was no change in FEV₁ in either group throughout the study, so this change in eNO appears to occur irrespective of any change in airway calibre that may be associated with a viral infection in asthma. An increase in airway hyperresponsiveness to histamine was also seen in the virus-inoculated group, the decrease in PC₂₀ histamine correlating inversely to the increase in eNO. Suggesting that the changes in eNO are a reflection of changes in underlying airway inflammation.

e) Allergic rhinitis

Allergic rhinitis is associated with inflammation of the nasal mucosa. Non-asthmatic patients with seasonal rhinitis have been shown to exhale a higher concentration of NO compared to nonatopic volunteers (Martin et al. 1996; Henriksen et al. 1999), even outside of the pollen season (Baraldi et al. 1999; Henriksen et al. 1999). These levels are higher both with nasal (Martin et al.
1996) and oral exhalation (Martin et al. 1996; Henriksen et al. 1999), and are associated with an increase in airway hyperresponsiveness (Henriksen et al. 1999). These findings suggest that patients with allergic rhinitis may also have generalised airway inflammation, independent of a previous diagnosis of asthma.
CHAPTER THREE: COPD

3.1. Introduction
Chronic obstructive pulmonary disease (COPD) is defined as a disorder characterised by abnormal tests of expiratory flow that do not change markedly over periods of several months observation (ATS 1987). It occurs in approximately 10-15% of smokers and the obstruction is thought to result from airway remodelling (Saetta et al. 1994b; Lambert and Pare 1997). The exact mechanism leading to airway remodelling is uncertain, however there is an increasing body of evidence to suggest inflammation may play an important role in this process.

3.2. COPD and airway inflammation
Cigarette smoking in itself causes both acute and chronic inflammatory processes affecting the central airways, peripheral airways and lung parenchyma - even in smokers with normal lung function (Saetta 1999). The inflammatory cells in the lungs consist mainly of macrophages, T lymphocytes and neutrophils, the presence of which is related to the degree of parenchymal destruction (Saetta 1999). In those patients who go on to develop COPD this inflammatory response is thought to be pivotal to disease progression.

During acute exacerbations of COPD there is a marked increase in the number of inflammatory cells and cytokines in the airways (Saetta et al. 1994a; Bhowmik et al. 2000). However even during stable COPD those patients who have more frequent exacerbations have greater amounts of inflammatory markers in their sputum (Bhowmik et al. 2000). Bronchial biopsies obtained from stable, corticosteroid free, patients with COPD have been shown to have increased numbers of inflammatory cells, the degree of which correlates directly with severity of lung function impairment (Di Stefano et al. 1998). Likewise, sputum samples, which contain cells from more peripheral airways, have also been shown
to have increased numbers of neutrophils and to lesser degree eosinophils in COPD, regardless of current smoking status (Stanescu et al. 1996; Balzano et al. 1999; Peleman et al. 1999; Rutgers et al. 2000). Furthermore, the degree of inflammation present in induced sputum correlates not only with the severity of airway obstruction (Balzano et al. 1999; Peleman et al. 1999; Rutgers et al. 2000) but also with a clinical history of a more rapid decline in lung function over time (Stanescu et al. 1996). Again emphasising the importance of inflammation in COPD progression.

3.3. Response to corticosteroids

Inhaled corticosteroid therapy is widely used in the treatment of airway inflammation associated with asthma and results in improvements in both symptoms and lung function (Barnes 1998b). Inhaled steroids have also been widely used in the treatment of COPD, although their role in long-term COPD management has often been debated (van Schayck et al. 1996; Barnes 1998a; Anthonisen 1999). It has been postulated that there may only be a subset of COPD patients (10-15%) who respond to corticosteroid therapy (Callahan et al. 1991; Nishimura et al. 1999). This view is reflected in current guidelines (BTS 1997a) which suggest that ongoing inhaled steroid therapy is recommended for those patients who show a clear objective response to a formal trial of either oral or high dose inhaled corticosteroid. A positive trial defined as a 15% increase in baseline FEV1, with an absolute increase of greater than 200ml.

It has been suggested that COPD patients who respond to steroids may actually belong a pathologically distinct subgroup with inflammatory features similar to asthma (Hargreave and Leigh 1999). Both the presence of increased amounts of eosinophils and their degradation product eosinophil cationic protein in bronchoalveolar lavage, and a thickened reticular basement membrane have been found to characterise COPD patients whose lung function improves following a course of prednisone (Chanez et al. 1997). Similarly, sputum eosinophilia has also been shown to correlate with the degree of reversibility of airway obstruction following corticosteroid treatment (Pizzichini et al. 1998; Fujimoto et al. 1999).
Interestingly these studies have demonstrated significant reductions in both eosinophil count and levels of eosinophil cationic protein following treatment, whereas neutrophil count and the various measures of neutrophil activity remained unchanged (Pizzichini et al. 1998; Fujimoto et al. 1999). In fact those patients who did not have sputum eosinophilia at study entry showed no change in lung function, symptoms or inflammatory markers following treatment. Suggesting that the reversible component of airway inflammation in COPD may be eosinophilic in nature (Hargreave and Leigh 1999).

3.4. Nitric oxide and COPD

Exhaled NO has recently been developed as a marker of airway inflammation in asthma (see Chapter 2.3). If those patients with COPD responsive to corticosteroids have underlying pathology similar to asthma then it follows that these patients may also have elevated levels of eNO. In turn eNO could then be used to identify those patients in whom corticosteroid therapy would be appropriate.

Recent studies analysing eNO in COPD have demonstrated varying results; some finding increased eNO (Kanazawa et al. 1998; Maziak et al. 1998; Corradi et al. 1999), while others finding normal or even low levels (Clini et al. 1998; Rutgers et al. 1999; Delen et al. 2000). These differences may be explained partly by differences in methodology used, such as single breath versus tidal breathing, concentrations versus excretion rates, and variability in exhalation flow rates (Sterk et al. 1999). Also differences may partly be explained because COPD is not a homogeneous disease, and that both the degree and type of inflammation present may vary widely. For example, in the study by Delen et al. (2000), in which low levels of eNO in COPD were reported, chronic bronchitis (COPD with a history of a productive cough) was associated with elevated levels of eNO when compared to other forms of COPD (non-productive) and to normal controls. Furthermore, the levels seen in chronic bronchitis were comparable to those obtained in asthma (17.0 ppb and 16.4 ppb, respectively).
During times of increased inflammation, such as with unstable COPD or during an acute exacerbation (Saetta et al. 1994a), it has been shown that eNO levels are elevated (Maziak et al. 1998; Agusti et al. 1999). Following treatment of an exacerbation and the return to clinical stability eNO levels then return to normal (Agusti et al. 1999). Exhaled NO levels have also been shown to correlate with other markers of airway inflammation (Kanazawa et al. 1998; Rutgers et al. 1999; Silkoff et al. 2000), suggesting that eNO may be a useful indicator of airway inflammation in COPD patients.

Another common characteristic of asthma is the presence of reversible airway obstruction, and just as asthma-like inflammation is associated with elevated levels of eNO in COPD, so elevated eNO has also been found in COPD patients demonstrating partial reversibility to bronchodilators (Papi et al. 2000). Papi et al divided COPD patients into two groups based on their response to inhalation of 200µg of salbutamol. Partial reversibility was defined as those who had an increase in FEV₁ of <12% of predicted but ≥200ml and non-responders as those with a response of <12% and <200ml. Partial reversibility was associated with a significantly higher eNO (24ppb) when compared to both non-responders (8.9ppb) (p<0.01). Alternatively, when divided on the basis of baseline eNO, those with a level of ≥15ppb had a significantly larger increase in FEV₁ post-salbutamol (median (interquartile range) 220 (200-230)mls versus 65 (10-180)mls respectively; p<0.01).

In conclusion, inflammation plays an important role in COPD, both in dictating disease progression and also in providing the basis for a reversible component against which the use of corticosteroids may be effective. Exhaled NO appears to be elevated in COPD characterised by increased inflammation and the presence of some reversibility, however the role of eNO in predicting those patients in whom corticosteroids will result in significant improvements in lung function has yet to be investigated. This will be discussed in Chapter eight, in which a study to investigate the usefulness of eNO as a predictor for the response to oral prednisone in COPD will be described.
CHAPTER FOUR: METHODS

In this chapter, the methods used in each of the subsequent studies are outlined in detail. Only minor variations in methodology will be repeated elsewhere.

4.1 Daily diary data

All participating patients completed a daily record card (fig 4.1) of morning and evening peak expiratory flow rates, bronchodilator use, and asthma symptoms. In addition during the dose-response study (Chapter Seven) diaries also included a section to record medication compliance.

PEFRs were measured prior to medication use using a mini-Wright peak flow meter (Clement Clarke Int Ltd, Harlow, UK). The highest of three measurements was recorded.

Daily peak flow variability was calculated by:

\[
\frac{|\text{Evening PEFR} - \text{Morning PEFR} (l/sec)| \times 100}{(\text{Morning PEFR} + \text{Evening PEFR})/2}
\]

For assessment of symptoms patients were asked to score their symptoms based on a scale of 0-3, 0 being no symptoms and 3 being severe. Scores were obtained for:

- **Nocturnal:** cough and wheeze
- **Daytime:** cough, wheeze, shortness of breath and sputum.

The daily total symptom score was the sum of the individual scores (maximum score of 18).
Fig 4.1  A sample page from the daily record card.

<table>
<thead>
<tr>
<th>Mon</th>
<th>Tues</th>
<th>Wed</th>
<th>Thurs</th>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

Date

**Complete this section in the morning**

- Best Peak Flow
- Awoke? Y/N
- Wheeze 0 - 3
- Cough 0 - 3

No. of puffs of reliever

Reliever in the last 6 hours? Y/N

**Complete this section in the evening**

- Best Peak Flow
- Wheeze 0 - 3
- Cough 0 - 3
- Activity 0 - 3
- Sputum 0 - 3
- No. of puffs of reliever
- Reliever in the last 6 hours? Y/N

Stuffy/runny nose? Y/N

Sore throat? Y/N

**Emergency medication**

- Prednisone? Y/N
- Additional inhaled steroids? Y/N
4.2 Spirometry

Spirometry was performed at each clinic visit in all of the studies. Spirometry was performed according to ATS standards and using the ‘Morris Normal Set’ for calculation of normal predictive values. Forced expiratory volume in one second (FEV$_1$) and forced vital capacity (FVC) were measured using a rolling seal spirometer (Spirotech, Graseby, Georgia, USA). The highest of three reproducible measurements was recorded. The highest FEV$_1$ and FVC from technically acceptable “blows” were used, even if not from the same “blow”.

4.3 Exhaled nitric oxide

Exhaled NO measurements were obtained using a chemiluminescence analyser designed for on-line measurement of eNO concentration (LR2000, Logan Research, Rochester, Kent, UK) (see fig. 2.1). The analyser had a response time of <2 seconds and was calibrated using a gas mixture containing 103ppb NO, thus permitting a signal within the range 0-200ppb to be accurately measured. Measurements were obtained using a standardised protocol (Kharitonov et al. 1997a; ATS 1999) with the exception of flow rate. The flow rate used was 250mls/sec, much higher than the now recommended 50mls/sec. This was in keeping with the manufacturer’s instructions, and was adopted prior to the publication of international guidelines (Kharitonov et al. 1997a; ATS 1999). At lower flow rates it has been shown that the spread of eNO measurements between individual patients is greater (Silkoff et al. 1997). Thus allowing for better differentiation between individual patients and improving both the sensitivity and specificity of eNO in distinguishing between asthma and non-asthma subjects (Silkoff et al. 1997; Pedroletti et al. 2000). This has implications for the following studies because using the higher flow rate may bias against finding significant differences between the patient groups.

When possible each visit was conducted at the same time of day. Patients were asked to refrain from doing strenuous exercise or having any alcohol for four hours prior to the clinic visit. The asthma studies were performed on subjects who
were non-smokers or who had stopped smoking for at least one year, with a total smoking history of <5 pack years (one pack year equals twenty cigarettes/day for a period of one year). In the COPD study, patients were asked to refrain from smoking on the day of the clinic visit. At each visit patients were asked regarding recent symptoms associated with either allergic rhinitis or an upper respiratory tract infection.

Nitric oxide measurements were made prior to any other study procedures. Patients sat quietly for five minutes before measurements were made. No nose clips were used. Measurements of eNO were made by expiring from total lung capacity without breath holding. Patients exhaled their whole vital capacity slowly through a mouthpiece against a known resistance (see Figure 2.1). Throughout exhalation the patient viewed a biofeedback monitor that enabled them to keep their exhalation constant at a flow rate of 250ml/sec and a mouth pressure of 50 mmH₂O. Tests in which a steady flow was not achieved were discarded. Exhaled NO was sampled at a rate of 250mls/sec via a side arm in the mouthpiece. A total of at least three measurements were made at each visit and stored for later analysis.

Nitric oxide measurements were read at a later date by an independent observer who was blinded to the patient’s current clinical status. The eNO plateau level at 70-80% of the exhaled volume was recorded (Chambers et al. 1996) (see Figure 2.2).

4.4 Hypertonic saline challenge

Hypertonic saline challenges were performed using a modification of a standardised protocol (Sterk et al. 1993; Iredale et al. 1994). Hypertonic (4.5%) saline solution was nebulised via an ultrasonic nebuliser, (De Vilbiss Ultraneb 2000; DeVilbiss, Somerset, PA., USA.), connected to 90 cm of corrugated aerosol tubing (smooth interior surface) and a large Hans-Rudolf two-way nonrebreathing valve (No. 2700 Hans Rudolph, Inc., Kansas City, Mo., USA.). During the challenge the nebuliser output was kept constant and the dose of saline increased
by increasing the inhalation time. The initial inhalation of saline was for a period of 30 seconds, followed by a repeat of 30 sec, then 1-min, 2-min, and repeated 4-min nebulisations. Spirometry was performed at one minute following each nebulisation and the better of two measurements was recorded.

The hypertonic saline challenge was completed when there had been a 20% drop in FEV₁, or a cumulative time of twenty minutes had been achieved. The nebuliser canister plus tubing was weighed prior to the challenge, and again after completion of the challenge, on a top pan Sartorius balance (Sartorius, Goettingen, Germany). From this the total amount of saline nebulised was measured and the nebuliser output per minute calculated. There is no need to weigh the amount of nebulised saline used after each inhalation because results obtained from calculating the saline delivered at each time point do not differ from those obtained by calculating the difference between initial and final weight (Riedler et al. 1994b). From these data both the cumulative dose of saline causing a 15% fall in FEV₁ (PD₁₅) and the dose-response slope (% drop in FEV₁ following the final dose divided by the total dose of saline administered) were calculated (Peat et al. 1992).

Following completion of the hypertonic saline challenge patients were then administered two puffs of 100μg of salbutamol (Ventolin, Glaxo-Welcome) via a spacer, these puffs were repeated every five minutes until lung function returned to at least 90% of baseline.

4.5 Sputum induction and cell counts

During the saline challenge patients were encouraged to produce sputum in the intervals between saline inhalation. At the end of each dose of saline, patients rinsed out their mouth with water, discarding the water and saliva in order to produce better quality sputum samples (Gershman et al. 1996). Patients were then asked to expectorate any sputum into a sterile container. A forced expiration "huff" technique was employed to aid the movement of airway secretions proximally (Pryor and Webber 1979).
If insufficient sputum had been produced at the end of the hypertonic saline challenge then sputum induction was continued. As long as the patient’s FEV$_1$ had returned to at least 90% of baseline following two puffs of salbutamol, they then inhaled further doses of saline as required to obtain a sufficient sample of sputum, up to a maximum cumulative dose of twenty minutes. This further nebulisation occurred after the hypertonic saline challenge had finished and the saline weighed, and therefore had no bearing on the challenge results. If the FEV$_1$ again dropped to <80% of baseline during these further periods of nebulisation then the patient was given further salbutamol and sputum induction discontinued. Patients remained under observation until such time that their FEV$_1$ had returned to at least 90% of their baseline value.

The first adequate sample of sputum produced was used for analysis, reducing the bias that may occur due to changes in sputum during the induction process (Holz et al. 1998a). Sputum was analysed using a standardised method (Fahy et al. 1993). Sputum was weighed and processed within two hours of the sample being obtained, during this time it was stored at 4°C. The whole specimen (sputum plus saliva) was combined with a solution of 10% dithiothreitol (Sputolysin, Calbiochem, La Jolla, California, USA) at a ratio of 1:2, gently mixed, and incubated in a shaking waterbath at 37°C for thirty minutes in order to ensure a homogenised solution. This was then filtered through a 48μm nylon mesh (B&SH Thompson, Mississauga, Ontario, Canada). Cell viability was assessed by the trypan blue exclusion test, and total cell count performed using a haemocytometer. Cytospin slides were prepared and stained with May-Grunwald-Giemsa stain. Each slide was coded and a total of 400 nonsquamous cells were counted on two separate occasions. The mean differential of these two counts was then recorded. If the difference between the two counts was greater than 10% for any cell type then the counts were repeated on a further two occasions and the mean differential of the four counts was recorded. The remaining sputum mixture was centrifuged at 1500rpm (1000G) for five minutes, the supernatant decanted and both the pellet and supernatant frozen at -80°C for future analysis.
4.6 Six-minute walk tests

Due to limitations in respiratory function, COPD patients may have markedly reduced functional capacity. "Functional exercise capacity" is defined as a patient's ability to undertake physically taxing activities encountered in everyday life. These are not always reflected by conventional methods of assessing disease activity. Over the last two of decades walking tests have been developed as a measure of "functional exercise capacity" and have been found to be particularly useful in patients suffering from moderate to severe exercise limitation due to pulmonary causes (Steele 1996). In particular the '6-minute walk' test has been developed as a test of functional capacity which can easily be repeated on the same day (Steele 1996) and results correlate well with those of longer walks (Butland et al. 1982).

Reproducibility

Walking tests have been shown to have good reproducibility, with an intra-subject coefficient of variation of 9.0% when measured at weekly intervals (Noseda et al. 1989). Learning effect appears to play an important role when measuring multiple tests, particularly so in the first 1-3 walks (Knox et al. 1988; Noseda et al. 1989). Thus the establishment of a stable baseline walking distance is of critical importance. The effects of coaching and encouragement have also been shown to significantly improve the distance covered during a walking test. Performance during a 6-minute walk has been shown to improve by as much as 30.5 metres when compared to distances covered while receiving no encouragement (Guyatt et al. 1984). As a result it is recommended that the level of coaching/encouragement should be recorded and standardised to reduce any variance from this.

Response to treatment

The 6-minute walking test has been used to assess the response to both pharmacological treatment (O'Reilly et al. 1980; Leitch et al. 1981) and pulmonary rehabilitation (Lacasse et al. 1996; Troosters et al. 2000). It has been calculated that the "minimally important clinical difference", which is the smallest
difference perceived by the average patient, for the 6-minute walk is 54m (95% CI: 37 to 71m) (Redelmeier et al. 1997). As a result, when assessing response to treatment in the COPD study “reversibility” was defined not only using spirometric criteria but also on the basis of improvement in 6-minute walk of greater than 50m.

**Method**

At each visit during the “eNO in COPD” study (Chapter Eight) patients were asked to perform a 6-minute walk using a standardised procedure (Steele 1996). Prior to the first walk, dyspnoea was assessed using the 10-point Borg Scale. Pulse and pulse oximetry was recorded. In addition, the patient took their medications they normally require prior to exercise (e.g. ipratropium, β2-agonist or GTN).

Patients were asked to walk from end to end of a marked walking track (of thirty metres in length), covering as much ground as possible in six minutes.

Each assessment of ‘6-minute walk’ consisted of two repeated walks with a rest period of at least fifteen minutes in between.

The following instructions were given to the patient:

“The purpose of this test is to find out how far you can walk in six minutes. You will start from this point (indicate marker at one end of the course) and follow the hallway to the marker at the other end, then turn around and walk back. When you arrive at the starting point, you will turn around and go back again. You will go back and forth as many times as possible in six minutes. If you need to, you may stop and rest. Just remain where you are until you can go again. However, the most important thing about this test is that you cover as much ground as you possibly can during the six minutes. I will let you know the time along the way, and when six minutes are up. When I say ‘stop’, please stand right where you are.”
During the test patients were told when two, four, and six minutes had elapsed. The following words of encouragement were also given:

1 minute  “keep going”
2 minutes  “keep going you’re doing well”
3 minutes  “halfway – keep going as far as possible”
4 minutes  “keep going you’re doing well”
5 minutes  “one minute to go – go as far as you can”
6 minutes  “30 seconds to go”

A staff member walked behind the patient so as not to influence the patient’s pace, and faced the patient only when giving encouragement.

During the walks, staff members completed a standardised 6-minute walk observation sheet recording pulse and O₂ saturation every minute throughout the walk and for three minutes post-walk. The longer distance walked during two identical tests was recorded, although both distances were noted. Duration of time spent resting was also recorded.

Immediately following completion of each walking test, patients were asked to rate their level of breathlessness on the Borg scale.
CHAPTER FIVE:
EXHALED NITRIC OXIDE – NORMAL VARIATION AND CORRELATION WITH OTHER MARKERS OF AIRWAY INFLAMMATION DURING STABLE ASTHMA

5.1. Abstract

Aim: To assess within-patient variation in exhaled nitric oxide (eNO) concentration, and to correlate eNO with other markers of inflammation in stable asthma.

Methods: 78 patients (69 atopic) on inhaled steroids were studied. On-line single breath eNO measurements at 70-80% of total lung capacity were obtained weekly for 2-4 weeks. Patients completed a twice-daily record of symptoms and peak flows. Following this patients underwent a hypertonic saline challenge with sputum induction.

Results: Geometric mean eNO was 10.28 and 9.38 ppb at the first and last visits. The within-visit and between-visit coefficients of variation were 4.1 and 10.5% respectively. The week-to-week 95% reference range was -38% to +61% of individual patient means. Higher eNO was associated with nocturnal waking and increased bronchodilator use (p=0.008 and p=0.026, respectively). Exhaled NO increased by 11% for each doubling of sputum eosinophils (p=0.002). There was no significant correlation between eNO and hypertonic saline challenge PD_{15} or slope of hypertonic saline challenge dose-response curve.

Conclusion: Exhaled NO demonstrates good repeatability within a sitting but shows greater variability week-to-week. Measurements are elevated in patients with recent breakthrough of asthma symptoms (nocturnal waking and reliever use) and in the presence of eosinophil airway inflammation.

5.2. Introduction

Asthma is an obstructive airways disease consisting of an interaction between inflammation and airway remodelling and hyperresponsiveness (Brusasco et al. 1998; Crimi et al. 1998; Haley and Drazen 1998). Treatment of asthma has largely
been based on the control of inflammation using medications such as inhaled corticosteroids (Djukanovic et al. 1992; Barnes 1998c). Until now monitoring asthma has largely depended on symptom control and measurements of lung function (ATS 1987; NHLBI/WHO 1995; BTS 1997b). Yet these measurements provide an incomplete picture of asthma control. Ongoing inflammation has been found in asthma patients thought to be clinically controlled (Foresi et al. 1990; Laitinen et al. 1993; Cai et al. 1998; Haley and Drazen 1998; Parameswaran et al. 1998). As a result over the last decade attempts have been made to develop a repeatable marker of airway inflammation that may be of use in longitudinal asthma control.

The gold standard for measuring airway inflammation is by bronchial biopsy obtained during bronchoscopy, but this procedure is not amenable to day-to-day use due to its invasive nature. Recently less invasive techniques of induced sputum (Pin et al. 1992b) and hypertonic saline challenges (Smith and Anderson 1989) have been developed to assess airway inflammation. However, these procedures are both time consuming and labour intensive limiting their clinical usefulness.

Exhaled NO has been developed as a marker of airway inflammation in asthma. Exhaled NO is higher in asthma (Alving et al. 1993; Kharitonov et al. 1994b; Persson et al. 1994) and in atopic individuals (Martin et al. 1996; Henriksen et al. 1999) when compared to normal controls. In steroid-naive asthma elevated levels of eNO correlate strongly with eosinophils in sputum (Jatakanon et al. 1998), but in corticosteroid treated asthma this relationship is less certain (Mattes et al. 1999; Piacentini et al. 1999; Berlyne et al. 2000; Lim et al. 2000).

If eNO is to be of use longitudinally in assessing asthma control then it needs to be a reproducible test that correlates well with the degree of underlying airway inflammation. It is also necessary to assess variation with time in stable asthma. This will then permit a valid assessment of what is “abnormal”. This study was performed for two reasons. Firstly to report the within-sitting repeatability and
week-to-week variability of eNO in a population of stable asthma patients taking regular inhaled steroids, and secondly to correlate eNO with other markers of disease control in this setting.

5.3. Methods

Subjects
Patients aged between eighteen and sixty-eight years with a diagnosis of mild to moderate stable asthma (ATS 1987) requiring maintenance inhaled corticosteroid therapy were invited to take part. Patients were required to have been on inhaled steroid therapy for at least six months with no change in dose over the preceding six weeks. Recruitment was made through a combination of newspaper advertisements, flyers in public places, and patients whose names were in the Respiratory Research database.

Exclusion criteria
1. Patients with a history of acute severe asthma requiring hospital admission within the previous twelve months or with asthma characterised by sudden, catastrophic changes in symptoms and peak flow rates.
2. Pre-bronchodilator FEV₁ at screening less than 50% predicted.
3. Patients whose asthma was seasonal or who experienced symptoms only after a viral respiratory tract infection.
4. Current or ex-smokers of greater than five pack years (one pack year equal to 20 cigarette/day for one year) who had smoked within the last year.
5. Unstable asthma requiring the use of oral prednisone during the previous three months.
6. Respiratory infection in last four weeks
7. Co-existing lung disease – for example bronchiectasis / cystic fibrosis

Design
Patients were maintained on a fixed dose of inhaled corticosteroid for a period of two (n=22) or four (n=56) weeks. Two times each day patients completed a record card recording peak expiratory flow rates, symptoms and bronchodilator use.
Patients attended weekly clinic visits at which eNO measurements and spirometry were performed. Exhaled NO measurements were obtained prior to any other study procedure. At the last of the series of visits patients also underwent a hypertonic saline challenge with associated sputum induction and cell counts.

For a further description of methods used please refer to Chapter Four (Methods).

Ethics
Written informed consent was obtained from all study participants before entry into the study. Ethical approval was obtained from the Otago Ethics Committee.

Statistical analysis
Sources of variability in eNO measurements were estimated using random effects regression models and variance components methods (Searle 1992). Associations between eNO and other measurements were analysed using the rank correlation coefficient since it does not depend on the measurement scale. Exhaled nitric oxide and PD_{15} saline measurements were analysed using logarithmic transformations to remove the skew in these data; other measurements were analysed without transformation. The results of these analyses did not change following the transformations.

5.4. Results
Seventy-eight patients entered the study, one withdrew following the baseline visit due to personal reasons. Demographic data are given in table 5.1. Twelve patients were ex-smokers and the remainder were life-long non-smokers. All patients remained on a fixed dose of inhaled corticosteroid (mean 630.8μg/day, range 100-1600μg/day, of beclomethasone or equivalent) throughout the study.
Table 5.1. Demographic data of the study participants. Unless stated results are given as mean (95% C.I.).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients, n</td>
<td>78</td>
</tr>
<tr>
<td>Male : Female</td>
<td>30:48</td>
</tr>
<tr>
<td>Age, yrs (range)</td>
<td>42.9 (19-64)</td>
</tr>
<tr>
<td>Height, m (range)</td>
<td>1.69 (1.49-1.85)</td>
</tr>
<tr>
<td>Duration of asthma, yrs</td>
<td>25.9 (22.4-28.4)</td>
</tr>
<tr>
<td>Skin test positive, n (%)</td>
<td>69 (88%)</td>
</tr>
<tr>
<td>History of rhinitis, n (%)</td>
<td>56 (72%)</td>
</tr>
<tr>
<td>History of eczema, n (%)</td>
<td>38 (49%)</td>
</tr>
<tr>
<td>FEV₁, litres</td>
<td>2.88 (2.70-3.06)</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>92.0 (87.9-96.1)</td>
</tr>
<tr>
<td>FEV₁ /FVC, %</td>
<td>71.0 (68.3-73.7)</td>
</tr>
</tbody>
</table>

FEV₁ = forced expiratory volume in one second.
FVC = forced vital capacity.

Variation in eNO

The mean eNO concentrations at each of the study visits are given in Table 5.2. The within-patient, within-sitting coefficient of variation for eNO was 4.1%, whereas the within-patient between-sitting coefficient of variation was 10.5%. The 95% reference range for variation in eNO between weekly visits was −38% to +61% of the individual patients mean eNO. The coefficient of variation for eNO between patients was 25.5% and accounted for 83.7% of the total variation seen in eNO measurements.
Table 5.2. Geometric mean eNO values at each of the study visits. No significant differences were found between the study visits.

<table>
<thead>
<tr>
<th>Visit</th>
<th>n</th>
<th>Mean eNO</th>
<th>± 2x SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>78</td>
<td>10.28</td>
<td>(2.75, 38.45)</td>
</tr>
<tr>
<td>R2</td>
<td>56</td>
<td>10.21</td>
<td>(2.82, 36.93)</td>
</tr>
<tr>
<td>R3</td>
<td>56</td>
<td>10.62</td>
<td>(2.91, 38.68)</td>
</tr>
<tr>
<td>R4</td>
<td>56</td>
<td>10.58</td>
<td>(2.70, 41.43)</td>
</tr>
<tr>
<td>V1</td>
<td>78</td>
<td>9.38</td>
<td>(2.72, 32.35)</td>
</tr>
</tbody>
</table>

R = run-in visit, V = visit at study completion.

Ambient NO recordings throughout the study ranged from 0 to 234.7ppb. The median ambient NO was 5.25ppb. By using random effects regression models no correlation was found between ambient NO and eNO (p=0.23).

There was no significant correlation between eNO and baseline patient characteristics except for height (Table 5.3.). For every increase in height of 10 cm, eNO was found to increase by 19% (p<0.03). The range of heights in the study population was 1.49m to 1.85m. None of the baseline patient characteristics were associated with significant differences in variability of eNO between visits (results not shown).
Table 5.3. The proportional change (ratio) in eNO associated with the baseline characteristics of the study population.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Proportional change in eNO</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male:female)</td>
<td>1.020</td>
<td>0.771, 1.350</td>
<td>0.89</td>
</tr>
<tr>
<td>Skin test positive (Y:N)</td>
<td>1.363</td>
<td>0.895, 2.074</td>
<td>0.15</td>
</tr>
<tr>
<td>Ex-smoker (Y:N)</td>
<td>1.305</td>
<td>0.900, 1.894</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Height (each increase of 10cm)</strong></td>
<td><strong>1.191</strong></td>
<td><strong>1.017, 1.394</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Weight (each increase of 10kg)</td>
<td>1.041</td>
<td>0.959, 1.131</td>
<td>0.34</td>
</tr>
<tr>
<td>Steroid dose (each increase of 100 µg/day)</td>
<td>0.965</td>
<td>0.925, 1.006</td>
<td>0.10</td>
</tr>
<tr>
<td>FEV₁ % predicted (each increase of 10%)</td>
<td>0.948</td>
<td>0.889, 1.010</td>
<td>0.102</td>
</tr>
<tr>
<td>FEV₁/FVC (each increase of 10%)</td>
<td>0.939</td>
<td>0.843, 1.046</td>
<td>0.257</td>
</tr>
</tbody>
</table>

FEV₁ = forced expiratory volume in one second.
FVC = forced vital capacity.

a = categorical variable. Proportional change is expressed as a ratio of the difference between the two groups.
b = Continuous variable. Proportional change is expressed as a ratio of increasing increments.
Exhaled NO and clinical control

Changes in eNO associated with data from the daily record cards as assessed over the one, three and five days prior to the clinic visits are given in Table 5.4. Exhaled NO was significantly higher in patients who had been woken from sleep by asthma symptoms and those who had used greater amounts of reliever in the one to three days/ nights before the study visit. Those who had been woken by their asthma the night immediately prior to the clinic visit had an eNO that was 28.6% higher than those who had not woken, and those who had woken sometime during the three nights prior to the visit had an eNO that was 19.8% higher than those who had not woken. No association was found between eNO and PEFR variation assessed either as diurnal differences or changes in morning PEFR over the preceding 5 days before the visit (p=0.053 and p=0.60, respectively).

Exhaled NO and sputum eosinophils

Adequate sputum samples were obtained from 72 of the 76 patients (94.7% success rate). Exhaled NO was directly correlated with the percentage of sputum eosinophils (r=0.46, p<0.002) and inversely correlated with neutrophils (r=-0.24, p<0.016) (Figures 5.1 and 5.2). There was no relationship between eNO and any of the other cell types. Changes in eNO associated with the respective increases in the proportion of sputum eosinophils and neutrophils are shown in Table 5.5. For each doubling in the percentage of sputum eosinophils there was an associated increase in eNO of 10.9%.

Exhaled NO and hypertonic saline challenge

A hypertonic saline challenge was performed in 68 of the 76 patients. Of these, 49 (72.1%) demonstrated a fall in FEV1 of 15% or greater. No significant relationships between eNO and either PD15 saline or the slope of the dose-response curve for hypertonic saline were found (Table 5.5).
Table 5.4. The proportional change (ratio) of eNO associated with measurements of asthma control recorded during the 1, 3 and 5 days prior to clinic visits.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of days prior to visit</th>
<th>Proportional change in eNO</th>
<th>95% C.I.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning PEFR (l/min) (^a)</td>
<td>1</td>
<td>1.001</td>
<td>(1.000, 1.002)</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.001</td>
<td>(1.000, 1.002)</td>
<td>0.264</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.000</td>
<td>(0.999, 1.002)</td>
<td>0.414</td>
</tr>
<tr>
<td>Awake any nights (Y:N) (^b)</td>
<td>1</td>
<td>1.286</td>
<td>(1.070, 1.545)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.198</td>
<td>(1.032, 1.392)</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.090</td>
<td>(0.948, 1.253)</td>
<td>0.226</td>
</tr>
<tr>
<td>Average daily reliever use (puffs/day) (^c)</td>
<td>1</td>
<td>1.045</td>
<td>(1.006, 1.085)</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.049</td>
<td>(1.001, 1.099)</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.052</td>
<td>(1.003, 1.103)</td>
<td>0.039</td>
</tr>
<tr>
<td>Average daily symptom score (^d)</td>
<td>1</td>
<td>1.014</td>
<td>(0.987, 1.042)</td>
<td>0.305</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.009</td>
<td>(0.978, 1.042)</td>
<td>0.583</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.004</td>
<td>(0.971, 1.037)</td>
<td>0.820</td>
</tr>
</tbody>
</table>

Significant results are highlighted in bold.

PEFR = peak expiratory flow rate.

\(^a\) = change in eNO for each increase in l/min

\(^b\) = change in eNO between those who had awoken during any of the nights versus those who had not.

\(^c\) = change for each increase in puffs/day.

\(^d\) = change in eNO for each increase in score of one (maximum daily score of 18)
Figure 5.1. Scatter plot demonstrating the correlation between eNO and percentage of eosinophils in sputum at the end of the run-in phase, $r=0.46$, $p<0.002$. Both eNO and percentage of sputum eosinophils are expressed on a log scale.
Figure 5.2. Scatter plot demonstrating the correlation between eNO and percentage of neutrophils in sputum, $r = -0.24$, $p < 0.016$. Both eNO and percentage of sputum neutrophils are expressed on a log scale.
Table 5.5. The proportional change (ratio) in eNO associated with every doubling in sputum cell count and airway hyperresponsiveness.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Proportional change</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% eosinophils a</td>
<td>1.109</td>
<td>1.042-1.181</td>
<td>0.002</td>
</tr>
<tr>
<td>% neutrophils a</td>
<td>0.846</td>
<td>0.742-0.966</td>
<td>0.016</td>
</tr>
<tr>
<td>PD_{15} saline</td>
<td>0.955</td>
<td>0.819-1.113</td>
<td>0.319</td>
</tr>
<tr>
<td>Slope of the hypertonic saline dose-response curve</td>
<td>1.106</td>
<td>0.957-1.278</td>
<td>0.242</td>
</tr>
</tbody>
</table>

a = eosinophils and neutrophils are expressed as a percentage of the total sputum cell count.
PD_{15} saline = the cumulative dose of saline required to induce a 15% fall in FEV₁.
5.5. Discussion

Variation in eNO

The results of this present study confirm that measurements of eNO are reproducible (Kharitonov et al. 1997a; Gabbay et al. 1998) and that these measurements provide a good marker of airway inflammation despite the use of maintenance inhaled steroid therapy. There was very little variation in eNO recordings within a sitting (a coefficient of variation of only 4.1%) and no difference between weekly mean eNO levels obtained (range 9.38 – 10.32 ppb). However, there was greater variation between the weekly measurements in individual patients (coefficient of variation 10.5%). The 95% reference range of weekly variation was −38% to +61% of the mean eNO. This larger variation may be a reflection of weekly changes in airway inflammation occurring in patients with less well-controlled asthma.

The pattern of variation in eNO recordings also provides evidence for the reliability of eNO measurements. The coefficient of variation was lowest within a sitting (the coefficient of variation of 4.1% accounting for only 2.2% of the total variation in eNO seen), was greater between visits, and was even greater still between patients (with the between-patient variation accounting for 83.7% of the total variation in eNO recordings). This makes biological sense as the degree of airway inflammation is unlikely to differ within the time frame of a single visit (consistent with the low coefficient of variation within a sitting), but may occur between weekly visits, and is most likely to occur when comparing patients with one another.

A possible problem with the study design when attempting to assess normal variation in eNO recordings lies in the definition of stable asthma. The entry criteria into the study were ‘asthma patients controlled on a stable dose of inhaled corticosteroid therapy’. This criterion was designed to exclude unstable asthma, in which the dose of inhaled therapy may have been altered during recent weeks. This however assumes that patient compliance with an appropriate ‘asthma self-
management plan' is satisfactory. It has been reported previously that patients with severe asthma may have poor insight into disease control (in't Veen et al. 1998; Jatakanon et al. 1998; Sont et al. 1999). This poor insight may mean that some of the patients entered into the study with unstable asthma. There is some evidence that this may have been the case. For example, on twenty-one occasions (out of a total of 249 visits) patients reported nocturnal waking during the night prior to the clinic visit, and on thirty-seven occasions patients had used more than three puffs of reliever during the preceding twenty-four hours. Likewise 25% (18/72) of patients had greater than 3.5% eosinophils in their sputum, indicating the presence of significant ongoing inflammation. Demonstrating that some of our patients may have had poorly controlled asthma at study entry. This does not invalidate the results of the study, but rather suggests that the weekly variation in eNO in fully controlled asthma may in fact be less than the variation recorded here. However, for the purposes of the subsequent study, in which eNO was evaluated as a predictor of poorly controlled asthma, the 95% reference range of −38% to +61% has been used.

Exhaled NO and baseline characteristics
On the whole, baseline patient characteristics did not affect eNO levels. No difference was seen when comparing male and female patients in either absolute eNO or in variation in eNO over several weeks. This is consistent with the findings of Morris et al (1994) who found no change in eNO throughout the menstrual cycle but is in contrast to the earlier findings of Kharitonov et al (1994a) who suggested that menstrual cycle may lead to changes in eNO. Some technical issues may have influenced the results of the Kharitonov study adversely including an unstandardised exhalation flow rate and the use of peak rather than plateau eNO recordings.

Previous studies have found that eNO levels are elevated with atopy (Martin et al. 1996; Moody et al. 2000). By contrast, no significant difference was seen between the eNO levels of atopic and non-atopic patients in our current study. The median
(interquartile range) of eNO in atopic compared with non-atopic patients in the current study was 11.0 ppb (6-16) and 5.1 ppb (4-8) respectively (Fig. 5.3).

**Figure 5.3.** Box-and-whisker plots of eNO, comparing atopic patients with non-atopic patients.

There are two possible explanations for the lack of significance in this comparison. Firstly all of our patients were on maintenance inhaled corticosteroid therapy, therefore suppressing underlying airway inflammation. Whereas the earlier studies had examined the relationship between atopy and eNO in patients not receiving inhaled corticosteroid therapy, thus measuring uncontrolled levels of
eNO. Further assessment of the effects of atopy on eNO in our asthma patients will be made in Chapter Six following cessation of inhaled corticosteroid therapy. The second reason for a lack of significance is that there were only a small number of non-atopic individuals in our study population (9/78), making a valid comparison between these groups difficult.

Higher levels of eNO were seen with increasing patient height in the current study population. A 19% increase in eNO was seen for every 10cm increase in height (p=0.03) (fig. 5.4).

Figure 5.4. Mean eNO levels from patients grouped into height quartiles.

Biologically the finding is plausible given that eNO concentration is dependent on the transit time of the breath through the airways; the greater the transit time through the airways and the greater the recorded eNO (Silkoff et al. 1997). Consistent with this, previous studies have demonstrated that acute bronchoconstriction decreases eNO (Ho et al. 2000), whereas bronchodilatation increases eNO (Yates et al. 1997; Silkoff et al. 1999; Ho et al. 2000). In a similar way tall people have both longer and wider airways than shorter people, this
would increase the overall transit time of air through the airways and in association with increased surface area would allow a greater concentration of eNO to diffuse into the breath. However, previously two studies have found no correlation between eNO and height (Ho et al. 2000; Sippel et al. 2000). The Ho et al study (2000) was limited by small numbers (n=12 asthmatics, n=17 normal controls), and in both studies neither the range of heights nor the eNO levels obtained were reported.

**Exhaled NO and ambient NO**

An important finding of the current study was the absence of any relationship between eNO and ambient NO concentrations (over a range of ambient levels of 0 to 234 ppb, p=0.23). This is important because it indicates that patients do not require to inhale 'NO-free' air in order to obtain valid measurements of eNO, thus improving the ease with which eNO can be measured in the clinical setting. It confirms previous reports that eNO levels, when read at end-expiration, are not affected by the concentration of NO in inhaled air (Silkoff et al. 1997; Corradi et al. 1998; Ho et al. 1998; Piacentini et al. 1998; Sippel et al. 2000) even when patients inhale NO concentrations up to 1000ppb (Silkoff et al. 1997). This is thought to be due to the high binding affinity that NO has with haemoglobin (Sharma et al. 1987; Cremona et al. 1995). This high binding affinity creates 'NO-free' air in the alveoli, and therefore allows the use of end-expiratory eNO (corresponding to air originating from the alveoli) as a measurement of the airway NO independent of ambient concentrations.

**Exhaled NO and asthma control**

An indication that eNO reflects the extent of underlying airway inflammation is gained from assessing its relationship with patient symptoms over the preceding twenty-four hours. Exhaled NO was elevated in patients with nocturnal waking; 13.47ppb for those who had woken during the night with asthma symptoms in the preceding twenty-four hours, compared to 9.92ppb for those who had not (p=0.008) (Fig. 5.5).
Figure 5.5. Mean eNO of patients who had woken with asthma symptoms during the night prior to the clinic visit compared to those who had not woken.

Exhaled NO was also elevated in those requiring greater use of β-agonist in the preceding twenty-four hours; 0 puff/day: 9.50ppb; 1 puff/day: 10.99ppb; 2 puffs/day: 9.68ppb; and greater than 3 puffs/day: 12.97ppb (p=0.026 for difference across these values) (Fig. 5.6). Thus patients with increasing severity of symptoms had increasing levels of eNO.
Figure 5.6. Mean eNO measured in patients grouped by the number of puffs of β-agonist used in the twenty-four hours preceding the clinic visit.

Furthermore, the occurrence of either nocturnal waking or increased bronchodilator use in the twenty-four hours prior to the clinic visit was associated with a higher eNO than if they had occurred during the three to five days earlier. Suggesting that eNO may be sensitive to acute changes in the amount of underlying inflammation. For example, patients who had woken due to asthma symptoms in the 24-hours prior to the clinic visit had eNO levels that were increased by 28.6% (95% CI: 7.0 to 54.5%) when compared to those who had not woken. Likewise, waking during the preceding three days was associated with an increase in eNO of 19.8% (95% CI: 3.2 to 39.2%). Whereas patients waking sometime during the preceding five days demonstrated no significant increase in eNO when compared to those who had not woken (mean difference of 9.0%, 95% CI: -5.4 to 25.3%).

Previously, elevated levels of eNO have been found in asthma characterised by nocturnal symptoms (ten Hacken et al. 1998). Similarly, elevated eNO has been found in poorly controlled asthma despite the use of inhaled corticosteroid therapy.
(Stirling et al. 1998; Sippel et al. 2000). In a cross-sectional study of 100 asthma patients, Sippel et al (2000) found elevated levels of eNO in patients who had been troubled by recent symptoms (within the last two weeks) despite the use of maintenance inhaled steroid therapy. They found that patients who had symptoms during the two weeks had an eNO of 74.1 ±8ppb, whereas those who had no symptoms had levels of 49.2 ±7ppb (p=0.02 for the comparison). However, these authors found no increase in eNO levels in the patients reporting symptoms occurring in the proceeding one and six months (p=0.99 and p=0.22 respectively). Furthermore, they found no difference in eNO levels when patients were divided solely in terms of previous history of asthma severity further indicating that eNO provides a good assessment of acute airway inflammation rather than chronic airway changes.

**Exhaled NO and airway inflammation**

It has been suggested that eNO may not a good indicator of airway inflammation in patients taking regular inhaled corticosteroid therapy, as previous studies have found no correlation between eNO levels and the amount of underlying airway eosinophilia in such circumstances (Berlyne et al. 2000; Lim et al. 2000). Interpretation of these previous studies is limited as they have involved patients with very low levels of ongoing airway inflammation. For example, in the study by Berlyne et al (2000) inhaled steroid treated patients had a median (interquartile range) sputum eosinophil count of only 0.6% (0-3.8%). In contrast, in our current study nearly one quarter of the patients had greater than 3.5% eosinophils in their sputum, and the group mean was 4.0% (95% C.I.: ±2.1%) indicating the presence of significant ongoing airway inflammation in our current study. In this setting of increased levels of airway inflammation eNO was shown to correlate directly with the degree of sputum eosinophilia despite inhaled corticosteroid use (r=0.46, p<0.002) (see Fig 5.1). Moreover, eNO was shown to increase by 10.9% (95% CI: 4.2 to 18.1%) for every doubling of sputum eosinophils observed, supporting the use of eNO as a marker of airway inflammation even when patients are taking inhaled corticosteroid therapy.
There was no relationship between eNO and the degree of airway hyperresponsiveness to hypertonic saline in our current study. Previously other investigators have found a relationship between eNO and airway hyperresponsiveness in steroid-naive asthma (Dupont et al. 1998; Jatakanon et al. 1998; Verleden et al. 1999; van der Thoorn et al. 2000) however this relationship appears to be lost in the presence of inhaled corticosteroid therapy (Verleden et al. 1999; Ichinose et al. 2000). The cause for this lack of consistent relationship is unknown but suggests that these two assessments of asthma severity are measuring different aspects of asthma pathophysiology.

A more in-depth look at the usefulness of eNO in assessing airway inflammation during inhaled corticosteroid therapy will be undertaken in Chapter Seven.

Summary

Measurements of eNO are easily performed and demonstrate good intra-patient reproducibility. Elevated levels of eNO are seen in patients with symptoms of unstable asthma and correlate directly with the degree of sputum eosinophilia despite maintenance inhaled corticosteroid therapy. These findings imply that serial eNO may be a useful tool in the clinical assessment of asthma control, both in identifying poorly controlled asthma and monitoring response to treatment. This will be analysed further in Chapters Six and Seven.
CHAPTER SIX:
THE PREDICTIVE VALUE OF EXHALED NITRIC OXIDE
MEASUREMENTS IN ASSESSING CHANGES IN ASTHMA
CONTROL

6.1 Abstract
Exhaled NO levels are increased in untreated or unstable asthma and measurements can be made easily.

Aim: To assess the usefulness of eNO for diagnosing and predicting loss of control in asthma following steroid withdrawal.

Method: Seventy-eight patients with mild/moderate asthma had their inhaled steroid therapy withdrawn until loss of control occurred or for a maximum of six weeks. Comparisons were made between eNO, symptoms, lung function, sputum eosinophils and airway hyperresponsiveness to hypertonic saline (4.5%) in both predicting and diagnosing loss of control.

Results: Sixty patients (77.9%) developed loss of control. There were highly significant correlations between the changes in eNO and symptoms (p<0.0001), FEV₁ (p<0.002), sputum eosinophils (p<0.0002) and PD₁₅ saline (p<0.0002), and there were significant differences between loss of control and no loss of control groups. Both single measurements and changes of eNO (10ppb, 15ppb or an increase of >60% over baseline) had positive predictive values that ranged from 80-90% for predicting and diagnosing loss of control. These values were similar to those obtained using sputum eosinophils and PD₁₅ saline measurements.

Conclusion: Exhaled NO measurements can be used to predict and diagnose poorly controlled asthma, and are as useful as induced sputum analysis and airway hyperresponsiveness in assessing airway inflammation, with the advantage that they are easy to perform.
6.2 Introduction

Nitric oxide is a key messenger for cell to cell signalling, and has an important role in the biochemistry of inflammation (Gaston et al. 1994; Barnes and Liew 1995). Exhaled NO has been confirmed as a marker of airway inflammation and is present in higher concentrations in steroid-naive asthma compared to normal controls (Alving et al. 1993). Higher levels of eNO are seen during asthma exacerbations (Crater et al. 1999), and decreases occur following treatment with both inhaled (Kharitonov et al. 1996b) and systemic corticosteroids (Massaro et al. 1995). Furthermore, eNO appears to be sensitive to changes in anti-inflammatory treatment, even in the absence of changes in lung function (Kharitonov et al. 1996c). These findings suggest that eNO may be a useful indicator in the longitudinal assessment of asthma control.

Both induced sputum cell counts (Pin et al. 1992b) and responsiveness to hypertonic saline (Smith and Anderson 1989) have also been investigated as markers of airway inflammation in asthma. Sputum eosinophil numbers increase during asthma exacerbations (Pizzichini et al. 1999) and decrease following both oral prednisone (Claman et al. 1994) and inhaled corticosteroid therapy (Jatakanon et al. 1998a). Likewise, Du Toit and co-workers (1997) have demonstrated a progressive reduction in airway responsiveness to hypertonic saline following initiation of inhaled corticosteroid treatment. However, the scope for using these techniques to monitor asthma control in clinical practice is limited by the resources required for repeated measurements. In contrast, measuring eNO is quick and easy to perform and lends itself to repeated measurements over time. Although single measurements of eNO have been used to assess airway inflammation in asthma (Jatakanon et al. 1998; Stirling et al. 1998; Piacentini et al. 1999; Lim et al. 2000), the usefulness of eNO in the longitudinal assessment of asthma control has not been extensively investigated. To be clinically useful eNO would need to correlate with known markers of asthma control as well as airway inflammation, and be responsive to changes in these parameters over time. If this were the case, then eNO measurements could be used to confirm poorly controlled asthma and to predict imminent deterioration. There might also be a
role for eNO in optimising anti-inflammatory therapy such as has been done using measurements of airway hyperresponsiveness (Sont et al. 1999).

The aim of this study was to evaluate the predictive and diagnostic value of eNO in unstable asthma and to correlate this with sputum eosinophils and airway hyperresponsiveness to hypertonic saline. A model of steroid withdrawal described by Gibson et al (1992) was used in order to induce a deterioration in asthma control in the majority of patients thus enabling the assessment of these parameters in the context of increasing degrees of airway inflammation.

6.3 Methods

Subjects
Patients with mild to moderate asthma, confirmed at our research screening clinic using ATS criteria (ATS 1987), and who had been taking inhaled corticosteroid therapy for at least six months were recruited. Their dose of inhaled steroid was unchanged for at least six weeks prior to study entry.

Exclusion criteria were made to exclude severe asthma (as the study involved the withdrawal of inhaled corticosteroid therapy) and also to exclude factors that may have interfered with eNO recordings. The exclusion criteria are as given in Chapter Five.

Study Design
Patients initially had a two to four week run-in period during which the maintenance dose of inhaled corticosteroid therapy remained unchanged. This run-in period provided the basis on which individual baseline data were derived. Following this run-in inhaled corticosteroid therapy was withdrawn. Patients were then reviewed weekly, or sooner if required, until loss of control developed OR for a maximum of six weeks. When loss of control occurred, patients were seen within twenty-four hours. The visit at which the inhaled steroids were stopped was designated visit 1; the final visit of the study, visit F; and the visit immediately prior to the final visit, visit P (penultimate).
Criteria for loss of control were:

1. A fall in the mean (over last 7 days) morning peak expiratory flow rate of greater than 10% from baseline; OR a fall in either morning or evening peak expiratory flow rate on two consecutive days to 80% of baseline or less; OR

2. Mean daily bronchodilator use (over the last 7 days) of greater than 3 puffs more than during run-in; OR

3. Nocturnal wakening with asthma symptoms on three nights or more per week greater than during the run-in; OR

4. Asthma symptoms which were disagreeable or distressing.

Study procedures
Diurnal peak expiratory flow rates, bronchodilator use, and symptom scores were recorded by the patient on a daily record card. Measurements obtained at each study visit are shown in Table 6.1. Exhaled NO was measured prior to all other study procedures using a calibrated chemiluminescence analyser with on-line measurement of single exhalations according to a standard protocol (Kharitonov et al. 1997a; ATS 1999), with the exception of flow rate (250 mls/sec). Exhaled NO levels were read at the plateau corresponding to 70-80% of the CO\textsubscript{2} curve. Full descriptions of the methods used for the various study procedures are given in Chapter Four.
Table 6.1. Study protocol for measurements at each visit.

<table>
<thead>
<tr>
<th>Visit No.</th>
<th>V 1</th>
<th>V 2</th>
<th>V 3</th>
<th>V 4</th>
<th>V 5</th>
<th>V P</th>
<th>V F</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNO</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>Spirometry</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>HSC</td>
<td>✗</td>
<td></td>
<td>✗</td>
<td></td>
<td></td>
<td></td>
<td>✗</td>
</tr>
<tr>
<td>Sputum analysis</td>
<td>✗</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✗</td>
</tr>
</tbody>
</table>

Visits at which measurements of exhaled nitric oxide (eNO), spirometry, hypertonic saline challenge (HSC), and sputum analysis were made are indicated by (x). The number of visits between V1 and VF was variable.

LOC = loss of control.
V = visit; P = penultimate; F = final.

Ethical considerations and safety

Each patient's asthma control was monitored closely throughout the study. All patients were provided with an individualised self-management plan, an emergency card, and a supply of prednisone tablets. Patients had 24-hour access to one of the study investigators via the hospital paging system. For ethical reasons, loss of control criteria included symptoms that were disagreeable or distressing irrespective of peak expiratory flow rate changes. Ethical approval was obtained from the Otago Ethics Committee and written informed consent was obtained from all study participants.
Statistical analysis

Associations between eNO and other measurements were analysed using the rank correlation coefficient since it does not depend on the measurement scale. Regression methods were used to compare airway inflammation parameters in patients who lost control with those who did not, and to adjust the comparisons for baseline differences. The prognostic and diagnostic utility of eNO was evaluated and compared to other measures using methods for constructing receiver-operator characteristic (ROC) curves (Swets and Picket 1982). Exhaled nitric oxide and PD_{15} saline measurements were analysed using logarithmic transformations to remove the skew in these data; other measurements were analysed without transformation. The results of these analyses did not change following the transformations.

6.4 Results

Seventy-eight patients entered the study. Demographic data are given in Table 6.2. The mean eNO during run-in was 9.38 ppb (95% reference range: 2.72-32.35). Over the run-in period eNO was not related to ambient NO (range 0-234 ppb, p=0.23) and so no corrections were made for ambient NO measurements. Patients with atopy had a significantly higher eNO at visit F than those non-atopic patients, regardless of asthma control status. Atopic patients had a mean (95% CI) eNO of 24.53 ppb (20.3 to 28.77 ppb) compared to 12.49 ppb (3.97 to 21.01 ppb) in non-atopic patients (p=0.009).

Loss of control

Sixty patients (77.9%) developed loss of control according to the pre-determined study criteria (the 'loss of control' (LOC) group) and seventeen did not (the no-LOC group). Figure 6.1 is a Kaplan-Meier survival plot demonstrating the proportion of patients at each time point remaining with controlled asthma. The median time to loss of control was seventeen days (95% C.I: 14 to 28). Twenty-two patients developed 'loss of control' within one week of corticosteroid
withdrawal. The frequencies with which 'loss of control' criteria were met were: fall in PEFR, 40; increased bronchodilator use, 15; increased nocturnal waking, 10; distressing symptoms, 39. Nine patients had 'loss of control' on the basis of distressing symptoms alone, 2 on the basis of increased reliever use alone. Thirty-two patients fulfilled two or more criteria at the time of 'loss of control'.

Table 6.2. Demographic data for study population.

<table>
<thead>
<tr>
<th>Number of patients (n)</th>
<th>78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male : Female</td>
<td>30:48</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>42.9 (range 18-74)</td>
</tr>
<tr>
<td>Duration of asthma (yrs)</td>
<td>25.9 (range 3-60)</td>
</tr>
<tr>
<td>Skin test positive (n)</td>
<td>69 (88%)</td>
</tr>
<tr>
<td>Ex-smokers: non-smokers</td>
<td>12:66</td>
</tr>
<tr>
<td>ICS dose (µg/day)</td>
<td>630 (beclomethasone equivalent) (range 100-1600)</td>
</tr>
<tr>
<td>FEV₁ (litres)</td>
<td>2.88 (2.70, 3.06)</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>92.0 (87.9, 96.1)</td>
</tr>
<tr>
<td>FEV₁ /FVC (%)</td>
<td>71.0 (68.3, 73.7)</td>
</tr>
</tbody>
</table>

Unless stated otherwise, figures are means with 95% C.I. in parenthesis.

Ex-smokers had not smoked for more than one year and had a smoking history of <5 pack years (one pack year = 20 cigarette/day for one year).

ICS = inhaled corticosteroid.

FEV₁ = forced expiratory volume in one second.

FVC = forced vital capacity.
Figure 6.1. Kaplan-Meier survival plot demonstrating the proportion of patients with controlled asthma at each time point. Dashed lines represent the 95% C.I.
Sputum induction was performed in all patients at visits 1 and F. Adequate sputum samples were obtained in 71/77 patients at visit 1 (92%), and in 54 (90%) and 15 (88%) of the LOC and no-LOC groups at visit F respectively.

Comparisons between the LOC and no-LOC groups at visit 1 and F and change between these visits are given in Table 6.3. The LOC group experienced a 2.16-fold increase in eNO between visits 1 and F, which was significantly greater than the 1.44-fold increase for the no-LOC group (p=0.004). There were also significant differences between LOC and no-LOC groups for: the fall in mean morning peak expiratory flow rate (13% versus 1%, p<0.0001); the decrease in FEV1 (mean fall of 11.9% predicted compared to 2.6% predicted, p=0.001); the increase in sputum eosinophils (4.73-fold increase compared to 2.05-fold, p=0.044); and the decrease in PD15 saline (0.8 doubling doses compared to 0.03 doubling doses, p=0.001).

**Correlations between study end-points**

There were highly significant correlations between the changes in eNO that occurred between visits 1 and F and the changes in symptoms, lung function, sputum eosinophils and airway hyperresponsiveness to hypertonic saline challenge that occurred over the same period (Table 6.4). In general, the correlations between single measurements of eNO and these same parameters were lower than those seen with changes over time and were not consistently significant (Table 6.4). Figure 6.2 shows a scatter-plot representation of the correlation between eNO and sputum eosinophils.
Table 6.3. Comparisons between loss of control and no loss of control groups in variables measured at visit 1 and visit F.

<table>
<thead>
<tr>
<th>Variable</th>
<th>LOC (n=60)</th>
<th>No LOC (n=17)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁ (% predicted) a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1</td>
<td>89.6</td>
<td>102.1</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>(84.7, 94.5)</td>
<td>(94.4, 109.8)</td>
<td></td>
</tr>
<tr>
<td>Visit F</td>
<td>77.7</td>
<td>101.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>(72.5, 82.9)</td>
<td>(92.2, 109.9)</td>
<td></td>
</tr>
<tr>
<td>Change (VF-V1)</td>
<td>-11.9</td>
<td>-1.1</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>(-15.2, -8.7)</td>
<td>(-3.3, 1.2)</td>
<td></td>
</tr>
<tr>
<td>eNO (ppb) b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1</td>
<td>9.67</td>
<td>8.34</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>(8.18, 11.43)</td>
<td>(6.36, 10.94)</td>
<td></td>
</tr>
<tr>
<td>Visit F</td>
<td>20.85</td>
<td>11.98</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>(17.15, 25.34)</td>
<td>(8.48, 16.91)</td>
<td></td>
</tr>
<tr>
<td>Change (VF-V1)</td>
<td>2.16</td>
<td>1.44</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>(1.88, 2.48)</td>
<td>(1.13, 1.82)</td>
<td></td>
</tr>
<tr>
<td>Sputum eosinophils (%) a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1</td>
<td>4.6</td>
<td>3.9</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>(1.8, 7.3)</td>
<td>(0.2, 7.7)</td>
<td></td>
</tr>
<tr>
<td>Visit F</td>
<td>18.9</td>
<td>6.8</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>(12.6, 25.1)</td>
<td>(1.6, 12.1)</td>
<td></td>
</tr>
<tr>
<td>Change (VF-V1)</td>
<td>14.3</td>
<td>3.3</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>(8.0, 20.6)</td>
<td>(-1.5, 8.0)</td>
<td></td>
</tr>
<tr>
<td>PD₁₅ saline (mls) b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit 1</td>
<td>10.9</td>
<td>13.8</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>(8.40, 14.23)</td>
<td>(9.30, 20.35)</td>
<td></td>
</tr>
<tr>
<td>Visit F</td>
<td>6.2</td>
<td>14.0</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>(4.53, 8.47)</td>
<td>(9.12, 21.56)</td>
<td></td>
</tr>
<tr>
<td>Change (d.d.)*</td>
<td>-0.80</td>
<td>-0.03</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>(-1.18, -0.42)</td>
<td>(-0.33, 0.28)</td>
<td></td>
</tr>
</tbody>
</table>

*a = arithmetic mean.

b = geometric mean.

LOC = loss of control.

FEV₁ = forced expiratory volume in one second.

PD₁₅ saline = the cumulative dose of hypertonic saline required to induce a 15% fall in FEV₁.

* d.d. = doubling dose change in PD₁₅ saline from visit 1 to visit F.
Table 6.4  Rank correlations between eNO and other measures of asthma control and airway inflammation measured at visit F (left hand column) and (right hand column) between the changes in eNO and the changes in each of the other parameters (visit 1 to visit F).

<table>
<thead>
<tr>
<th></th>
<th>Visit F</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rank correlation</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>FEV\textsubscript{1} % predicted</td>
<td>-0.20</td>
<td>-0.41, 0.02</td>
<td>0.079</td>
</tr>
<tr>
<td>Morning peak flow rate</td>
<td>0.14</td>
<td>-0.09, 0.35</td>
<td>0.23</td>
</tr>
<tr>
<td>Bronchodilator use</td>
<td>0.19</td>
<td>-0.04, 0.39</td>
<td>0.1</td>
</tr>
<tr>
<td>Symptom score</td>
<td>0.33</td>
<td>0.11, 0.51</td>
<td>0.0039</td>
</tr>
<tr>
<td>Sputum eosinophils</td>
<td>0.62</td>
<td>0.44, 0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PD\textsubscript{15} saline</td>
<td>-0.41</td>
<td>-0.6, -0.18</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

Significant correlations are shown in bold.

FEV\textsubscript{1} = forced expiratory volume in one second.

PD\textsubscript{15} saline = the cumulative dose of hypertonic saline required to induce a 15% fall in FEV\textsubscript{1}. 
Figure 6.2. Scatter plot of correlation between eNO and % sputum eosinophils at visit F, \( r = 0.62, p<0.0001 \).
Both eNO and % eosinophils are expressed on a logarithmic scale.
\( \times \) = patients who had loss of control.
\( \bullet \) = patients who did not have loss of control.
Predictive and diagnostic values

The ability of eNO measurements to predict up-coming ‘loss of control’ was assessed in three ways. Firstly by using the baseline eNO measurement (visit 1). Secondly by using the measurement of eNO at the visit immediately prior to loss of control (the penultimate visit, visit P). And thirdly by using the change in eNO which occurred between visit 1 and visit P. Analysis of receiver-operator curves demonstrated parity between these different approaches (Figure 6.3). The curves were similar whether absolute or proportional changes in eNO were used. The prognostic utility of changes in eNO between visit 1 and visit P is demonstrated by a receiver-operator curve in Figure 6.4 (top panel). In figure 6.4 (bottom panel) the receiver-operator curve for changes in eNO is then compared to changes in FEV₁% predicted, daily peak expiratory flow rate variation, symptom scores and bronchodilator use between visit 1 and visit P. None of these clinical parameters was found to be superior to eNO in predicting loss of control despite being a part of the definition of loss of control. The sensitivities, specificities, positive and negative predictive values at relevant cut points also showed that no one prognostic indicator was clearly superior (Table 6.5). Specific eNO cut points evaluated included 10ppb, 15ppb and a 60% increase over the baseline mean (the upper limit of the 95% reference range for weekly variation in eNO over the run-in period).

The prognostic value of eNO was also compared with other indices of airway inflammation, specifically single measurements of responsiveness to hypertonic saline (PD₁₅ less than 12mls) and sputum eosinophils (greater than 4%) obtained at visit 1 (Figure 6.5, Table 6.5). Again compared to eNO, no measurement was clearly superior.

The ability of eNO to diagnose ‘loss of control’ was also assessed using eNO measurements at visit F (Table 6.6). Both the single measurements of eNO at visit F and the change between visit 1 and visit F were evaluated. As for the assessment of prognostic utility, there was no clearly superior eNO measurement, and the
performance of eNO was comparable to that based on sputum eosinophil counts and PD$_{15}$ saline measurements.

**Figure 6.3.** Receiver-operator curves assessing the various methods of monitoring eNO in predicting up-coming loss of control. Point-in-time measurements at visits 1 and P and change in eNO assessed both as absolute change and proportional change from visit 1 to visit P are shown.
Figure 6.4. Receiver-operator curve for changes in eNO (proportional change between visit 1 and visit P) in predicting up-coming loss of control (Top panel). The lower panel shows this same receiver-operator curve for changes in eNO superimposed on the receiver-operator curves of lung function and daily record card data for predicting up-coming loss of control.
Table 6.5. Predicting loss of control. The predictive values of measurements of eNO, % eosinophils and PD_{15} saline measured at visit 1, and for eNO at the visit immediately prior to loss of control (visit P) in distinguishing those who went on to develop loss of control from those who did not. Figures in parenthesis are 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>eNO at visit 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 ppb.</td>
<td>0.50</td>
<td>0.53</td>
<td>0.79</td>
<td>0.23</td>
</tr>
<tr>
<td>(0.37, 0.63)</td>
<td>(0.28, 0.77)</td>
<td>(0.63, 0.90)</td>
<td>(0.11, 0.39)</td>
<td></td>
</tr>
<tr>
<td>&gt;15 ppb.</td>
<td>0.25</td>
<td>0.88</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>(0.15, 0.38)</td>
<td>(0.64, 0.99)</td>
<td>(0.28, 0.77)</td>
<td>(0.15, 0.38)</td>
<td></td>
</tr>
<tr>
<td><strong>eNO at visit P</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10 ppb.</td>
<td>0.65</td>
<td>0.41</td>
<td>0.80</td>
<td>0.25</td>
</tr>
<tr>
<td>(0.52, 0.77)</td>
<td>(0.18, 0.67)</td>
<td>(0.66, 0.90)</td>
<td>(0.11, 0.45)</td>
<td></td>
</tr>
<tr>
<td>&gt;15 ppb.</td>
<td>0.50</td>
<td>0.65</td>
<td>0.83</td>
<td>0.27</td>
</tr>
<tr>
<td>(0.37, 0.63)</td>
<td>(0.38, 0.86)</td>
<td>(0.67, 0.94)</td>
<td>(0.14, 0.43)</td>
<td></td>
</tr>
<tr>
<td><strong>Change in eNO from visit 1 to visit P</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ&gt;10 ppb.</td>
<td>0.27</td>
<td>0.76</td>
<td>0.80</td>
<td>0.23</td>
</tr>
<tr>
<td>(0.16, 0.40)</td>
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<td>(0.56, 0.94)</td>
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</tr>
<tr>
<td>Δ&gt;60%</td>
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<td>0.65</td>
<td>0.83</td>
<td>0.27</td>
</tr>
<tr>
<td>(0.37, 0.63)</td>
<td>(0.38, 0.86)</td>
<td>(0.67, 0.94)</td>
<td>(0.14, 0.43)</td>
<td></td>
</tr>
<tr>
<td><strong>% eosinophils at visit 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;4%</td>
<td>0.21</td>
<td>0.80</td>
<td>0.80</td>
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</tr>
<tr>
<td>(0.12, 0.34)</td>
<td>(0.52, 0.96)</td>
<td>(0.52, 0.96)</td>
<td>(0.12, 0.34)</td>
<td></td>
</tr>
<tr>
<td><strong>PD_{15} saline at visit 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12ml</td>
<td>0.53</td>
<td>0.50</td>
<td>0.77</td>
<td>0.25</td>
</tr>
<tr>
<td>(0.38, 0.67)</td>
<td>(0.25, 0.75)</td>
<td>(0.60, 0.90)</td>
<td>(0.11, 0.43)</td>
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</tr>
</tbody>
</table>

PD_{15} saline = the cumulative dose of hypertonic saline required to induce a 15% fall in FEV\textsubscript{1}. % eosinophils = the percentage of eosinophils in induced sputum. Δ = change.
Figure 6.5. Receiver-operator curves for eNO (proportional change between visit 1 and P), percentage sputum eosinophils at visit 1, and PD$_{15}$ saline at visit 1, for predicting up-coming loss of control.

PD$_{15}$ = the cumulative dose of hypertonic saline required to induce a 15% fall in FEV$_1$. 

![Figure 6.5](image.png)
Table 6.6.  
*Diagnosing loss of control.* The predictive values of eNO, sputum eosinophils, and PD$_{15}$ saline measured at visit F for diagnosing loss of control. Figures in parenthesis are 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
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<tr>
<td>eNO at visit F</td>
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<tr>
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<td>0.81</td>
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<td></td>
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<td>&gt;15 ppb.</td>
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<td>0.65</td>
<td>0.86</td>
<td>0.31</td>
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<td>(0.17, 0.49)</td>
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<td>Δ &gt;10 ppb.</td>
<td>0.48</td>
<td>0.82</td>
<td>0.91</td>
<td>0.31</td>
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<tr>
<td></td>
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<td>(0.57, 0.96)</td>
<td>(0.75, 0.98)</td>
<td>(0.18, 0.47)</td>
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<tr>
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<td>0.87</td>
<td>0.37</td>
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<td>Sputum eosinophils at visit F</td>
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<tr>
<td>&gt;4 %</td>
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<td>0.60</td>
<td>0.84</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>(0.45, 0.72)</td>
<td>(0.32, 0.84)</td>
<td>(0.69, 0.94)</td>
<td>(0.14, 0.48)</td>
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<tr>
<td>Change in sputum eosinophils from visit 1 to visit F</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ &gt;4 %</td>
<td>0.51</td>
<td>0.64</td>
<td>0.84</td>
<td>0.26</td>
</tr>
<tr>
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<td>(0.67, 0.95)</td>
<td>(0.12, 0.43)</td>
</tr>
<tr>
<td>PD$_{15}$ saline at visit F</td>
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<tr>
<td>&lt;12 mls.</td>
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<td>0.82</td>
<td>0.87</td>
<td>0.35</td>
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<td>(0.29, 0.61)</td>
<td>(0.57, 0.96)</td>
<td>(0.66, 0.97)</td>
<td>(0.21, 0.52)</td>
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<tr>
<td>Change in PD$_{15}$ saline from visit 1 to visit F</td>
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<td></td>
</tr>
<tr>
<td>Δ &gt;1 d.d. increase</td>
<td>0.41</td>
<td>0.94</td>
<td>0.95</td>
<td>0.36</td>
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<tr>
<td></td>
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<td>(0.70, 1.00)</td>
<td>(0.75, 1.00)</td>
<td>(0.22, 0.52)</td>
</tr>
</tbody>
</table>

PD$_{15}$ saline = the cumulative dose of hypertonic saline required to induce a 15% fall in FEV$_1$. % eosinophils = the percentage of eosinophils in induced sputum. Δ = change. d.d. = doubling dose increase.
6.5 Discussion

The results of the current study indicate that eNO is not only easily measured but can provide valuable information regarding the longitudinal control of asthma. In particular, both absolute values of eNO as well as changes over time provide useful clinical information in predicting and diagnosing poorly controlled asthma.

Loss of Control

Approximately 80% of the patients developed loss of control following cessation of their maintenance inhaled corticosteroid therapy, the median (95% CI) time to loss of control being seventeen (14 to 28) days (fig. 6.1). Gibson et al (1992) have previously demonstrated that withdrawal of inhaled corticosteroids results in an exacerbation of asthma in the majority of patients, which occurs on average sixteen days (range 7 to 26 days) following steroid reduction. Likewise, Leuppi et al (2001) found that exacerbations of asthma occurred in 85% of patients following a step-wise withdrawal of inhaled corticosteroid, and that a minority of patients remained well despite removal of inhaled corticosteroid therapy during a two month follow-up. These results confirm the importance of inhaled corticosteroid therapy in maintaining asthma control, but also suggest that there may be a few asthma patients in whom the need for continued inhaled corticosteroid therapy appears unnecessary, at least in the short-term.

The current study was designed to assess the usefulness of eNO in a clinical context. For this reason, loss of control criteria were prospectively based on a combination of peak flow and symptom changes as used in clinical practice. Evidence that these criteria were relevant and appropriate is provided by the significantly greater changes in FEV₁, sputum eosinophils and PD₁₅ saline seen in the LOC group (table 6.3 and fig. 6.6). However, it should also be noted that even in those patients in whom loss of control did not occur there was a significant increase in the levels of eNO. In this group there was a mean (95% CI) increase in eNO of 1.44-fold (1.13 to 1.82 fold). This increase was seen in association with a
Figure 6.6. Changes in FEV₁, airway hyperresponsiveness and sputum eosinophils occurring between visits 1 and F in the LOC and no-LOC groups:

a) Change in FEV₁ % predicted.

b) Change in PD₁₅ saline

c) Change in percentage of sputum eosinophils.
non-significant increase in sputum eosinophils of 3.3% (95% CI: -1.5 to 8.0%). These results suggest that even in the no-LOC group there may have been a small increase in airway inflammation that was undetected by the clinical parameters used in our diagnosis of loss of control but detected by changes in eNO. Such airway inflammation may play a role in the development of long-term irreversible damage to the airway via remodelling (Fabbri et al. 1998b).

**Exhaled NO and atopy**

At visit F atopic asthma was associated with eNO levels that were twice that of non-atopic asthma, eNO was 24.53ppb (20.3 to 28.77ppb) compared to 12.49ppb (3.97 to 21.01ppb) respectively (p=0.009). This is in contrast to the findings of Chapter Five where atopic status was not associated with increased levels of eNO. This discrepancy is most likely due to differences in inhaled corticosteroid use and the degree of airway inflammation. The finding of elevated levels of eNO in association with atopy in patients not receiving corticosteroid therapy is consistent with previous reports (Martin et al. 1996; Moody et al. 2000) and may reflect two different phenotypes of airway inflammation in these subsets of asthma. Moreover, it suggests that eNO may be of more clinical value in atopic asthma, in whom higher levels of eNO are seen during uncontrolled asthma. However, the current study was not designed to differentiate the relative merits of measuring eNO in atopic and non-atopic asthma, and because only nine patients were non-atopic no formal comparison of the usefulness of eNO in this subgroup was possible.

**Correlations between inflammatory markers**

The results of our current study confirm earlier findings that eNO levels are elevated in unstable asthma (Massaro et al. 1995; Crater et al. 1999), and point to eNO being a useful marker of airway inflammation. Previous studies have yielded inconsistent data regarding the correlation between eNO and sputum eosinophils because of the confounding effect of inhaled steroid use (Mattes et al. 1999; Piacentini et al. 1999; Lim et al. 2000). This was not the case in our present study in which data were obtained from patients in whom maintenance inhaled steroid
therapy had been temporarily withdrawn. Highly significant correlations were obtained at Visit F between eNO and the direct markers of airway inflammation, namely sputum eosinophils (rank correlation 0.62, \( p<0.0001 \)) and \( \text{PD}_{15} \) saline (rank correlation \(-0.41, p<0.0008 \)). However, apart from symptom score (\( r=0.33, \ p=0.004 \)) the point-in-time correlations at Visit F between eNO and both \( \text{FEV}_1 \) and the clinical markers of control were non-significant (Table 6.4). More importantly, when measured longitudinally, the \textit{changes} in eNO correlated significantly with changes in the direct markers of airway inflammation as well as with the changes occurring in \( \text{FEV}_1 \) and the clinical markers (see Table 6.4). These findings provide additional support for the use of eNO measurements as a tool in the assessment of airway inflammation and long-term asthma control.

\textit{Prognostic and diagnostic utility}

The results of the current study document the usefulness of eNO for predicting and diagnosing poorly controlled asthma compared to other currently used markers of airway inflammation and clinical parameters. Regardless of the way in which eNO measurements were analysed (absolute values, absolute changes, or proportional changes from baseline) the results were similar. Exhaled NO was associated with a positive predictive value of between 80 and 90\% for predicting and diagnosing poor asthma control. On the whole, \textit{changes} in eNO over time had higher positive predictive values, sensitivities and specificities both for predicting and diagnosing loss of control than did single measurements. For example, an increase in eNO between visit 1 and the penultimate visit of more than 60\% over baseline had a positive predictive value for \textit{predicting} up-coming loss of control of 83\% (sensitivity 50\%, specificity 65\%). A similar increase between visit 1 and visit F had a positive predictive value for \textit{diagnosing} poor asthma control of 87\% (sensitivity 68\%, specificity 65\%). In comparison, a single measurement of greater than 15ppb obtained at visit 1, when patients were still taking inhaled steroids, had a positive predictive value of 88\% (sensitivity 25\%, specificity 88\%) for \textit{predicting} loss of asthma control within one week.
Overall, using the cut points selected, these outcomes reflect poor sensitivity but good specificity for eNO measurements. Nevertheless, they compare favourably with the usefulness of the other, more elaborate techniques of sputum induction and hypertonic saline challenge used in this study to assess deteriorating asthma. For example, we found that a doubling dose increase in PD$_{15}$ saline was marginally better than the other measurements for diagnosing loss of control, with a positive predictive value of 95% (sensitivity 41%, specificity 94%). This compared to a positive predictive value of 84% for a 4% change in eosinophils (sensitivity 51%, specificity 64%) and with a positive predictive value of 91% for an increase in eNO of 10ppb (sensitivity 48%, specificity 82%). However, measuring eNO has the advantage of being quick and easy to perform, making it a more suitable test for use in the clinical rather than the research setting. This becomes all the more important given the finding that changes in eNO have higher sensitivity and specificity for changes in clinical status than do single measurements, implying the need for repeated tests. Interestingly, eNO proved to be comparable to other more conventional measurements such as FEV$_1$, peak flows, symptom score and daily reliever use in predicting up-coming loss of control (see Fig. 6.4) despite the fact that many of these parameters were used in the definition of loss of control.

A number of investigators have sought to evaluate the role of eNO in assessing long-term asthma control (Kharitonov et al. 1996c; Baraldi et al. 1999; Jatakanon et al. 2000; Leuppi et al. 2001). In the present study, withdrawal of inhaled corticosteroid therapy was performed in an attempt to induce deterioration in asthma control and mimic an exacerbation. This implies that the findings of the current study may not strictly apply to patients receiving maintenance inhaled corticosteroid. However, the usefulness of eNO in the presence of inhaled steroid use is supported by Stirling et al (1998) who found higher levels of eNO in patients with greater asthma severity irrespective of steroid use. Further, Kharitonov et al (1996c) have demonstrated that a simple reduction in the dose of inhaled corticosteroid increases eNO even in the absence of significant change in peak flow variability or spirometry.
It has been previously reported that eNO is not as reliable as either responsiveness to hypertonic saline or sputum eosinophils in predicting up-coming poor asthma control (Leuppi et al. 2001). In that study Leuppi et al halved the dose of inhaled corticosteroid therapy every eight weeks until patients developed an exacerbation of asthma or had been off inhaled corticosteroid therapy for at least two months. Measurements of eNO, FEV₁, hypertonic saline challenge and sputum eosinophils were made on a monthly basis. Exhaled NO was reported to have no predictive value at any of the time points in predicting poor asthma control. Part of the reason for this negative finding may have been due to technical factors involving the measurement of eNO. Measurements were performed on samples of mixed expiratory air collected in a polyethylene bag following inhalation of ambient air, therefore making these measurements affected by ambient NO. Also the authors found no significant increase in eNO during asthma exacerbations which is in contrast with both our present study and previous studies which have found raised eNO levels during exacerbations (Crater et al. 1999; Jatakanon et al. 2000). This finding highlights the limitations in comparing results involving eNO when different measurement techniques are used (Silkoff and Zamel 1998; ATS 1999).

Using a similar study design to our own, but with incomplete withdrawal of inhaled corticosteroid, Jatakanon et al (2000) reduced the dose of inhaled budesonide to 200μg/day (less than one quarter of the patients usual maintenance dose) in fifteen asthmatics. Patients were followed for a maximum of eight weeks. Just under half developed an exacerbation. There was a parallel increase in eNO and sputum eosinophils, which correlated over time with changes in FEV₁ and β-agonist use. The authors suggested that changes in sputum eosinophil numbers were superior to eNO in predicting loss of asthma control. However, owing to the small study numbers a quantitative assessment of the predictive values of the changes in eNO and sputum eosinophils was not possible.

In another longitudinal study, Baraldi et al (1999) measured eNO in asthmatic children before, during and after the pollen season. A rise in eNO was seen during
the pollen season consistent with the presumed increase in airway inflammation that occurred with allergen exposure, although no measurements of sputum eosinophils were made to confirm the presence of this inflammation. This rise in eNO occurred despite the fact that over one third of the children were taking inhaled steroids, and it occurred in the absence of any changes in FEV1. The authors concluded that eNO may be useful in the longitudinal assessment of asthma.

During our current study measurements of eNO were made online using single exhalations at an exhalation flow rate of 250mls/sec. This flow rate is significantly more than current guidelines suggest (ATS 1999), however the study was commenced before these guidelines were published. Exhaled NO has been shown to be flow dependent (Silkoff et al. 1997), and at lower flow rates the differences between healthy and asthmatic individuals are increased (Pedroletti et al. 2000). Thus it is possible that at a lower expiratory flow rate (e.g. 50mls/sec) differences between patients who did and did not experience ‘loss of control’ might have been greater. If this were the case, then the sensitivities, specificities and predictive values of eNO as a diagnostic test may be better than what is reported in the current study.

**Summary**

Following cessation of corticosteroid therapy an exacerbation occurred in the majority of asthma patients. Deterioration of asthma control was associated with significant worsening of lung function, increase in sputum eosinophils and increase airway hyperresponsiveness. Steroid cessation also lead to a significant increase in the levels of eNO. This increase in eNO occurred to a greater degree in patients who developed clinical ‘loss of control’ and in those with atopic asthma. Serial measurements of eNO proved useful in both the diagnosis of ‘loss of control’ and the prediction of up-coming ‘loss of control’ when used both as a point-in-time and as a change-over-time measurement. The data indicate that an absolute value for eNO of 15ppb or greater, or an increase of more than 10ppb or
60% over baseline, are useful thresholds for the detection of ongoing airway inflammation, and they also positively predict the occurrence of breakthrough symptoms (positive predictive values of 80-90%). However, although specific for poorly controlled asthma, eNO often lacked good sensitivity and thus the absence of these changes does not preclude the possibility of deteriorating asthma. It is possible that this may have been improved by the use of lower exhalation flow rates, however further investigations will need to be done to confirm this. Also further long-term studies are required to confirm that the clinical application of serial eNO measurements is worthwhile in optimising asthma management. In particular, the usefulness of eNO in detecting airway inflammation in the presence of inhaled corticosteroid therapy has not been fully investigated; this is the subject for Chapter Seven.
CHAPTER SEVEN:
ASSESSING THE ANTI-INFLAMMATORY EFFECTS OF INHALED CORTICOSTEROIDS.

Dose-response relationships for exhaled nitric oxide, sputum eosinophils, hypertonic saline challenge and lung function.

7.1. Abstract

**Background:** Dose-response relationships for the anti-inflammatory effects of inhaled corticosteroid have not been extensively investigated. This study was undertaken to evaluate the usefulness of serial measurements of exhaled nitric oxide (eNO) for this, and to compare eNO with other markers of airway inflammation.

**Methods:** Following deterioration in asthma control, 65 patients entered a double-blind, parallel-group, placebo-controlled trial of 50, 100, 200, or 500μg/day beclomethasone for eight weeks. Exhaled NO and spirometry were performed weekly and a hypertonic saline challenge with sputum induction was performed at the beginning and end of treatment.

**Results:** Linear relationships between the dose of inhaled corticosteroid and changes in eNO and FEV\textsubscript{1} existed at one-week (p=0.022 and p=0.043, respectively) and at end of treatment (p=0.015 and p=0.006, respectively). A linear dose-response relationship was also seen for sputum eosinophils (p=0.037). Changes in eNO were able to differentiate between treatment groups to a greater degree than FEV\textsubscript{1} or eosinophils. Changes in eNO correlated significantly with changes in sputum eosinophils (r=0.403; p=0.002). Changes in PD\textsubscript{15} did not differ across the treatment groups nor correlate with changes in other measurements.

**Conclusions:** Over the range 0-500μg/day beclomethasone there was a linear dose-response relationship for its anti-inflammatory effects. Exhaled NO provides useful information regarding this relationship and corresponds significantly to the degree of ongoing airway inflammation as measured by sputum eosinophils.
7.2. Introduction

Inhaled corticosteroids are the most effective treatment for airway inflammation in asthma (Barnes 1998b; Barnes et al. 1998). Their use results in improvement in symptoms and lung function, as well as reductions in inflammatory cells in bronchial biopsies (Djukanovic et al. 1992) and induced sputum (Jatakanon et al. 1998a; van Rensen et al. 1999). As a result airway hyperresponsiveness is also reduced (Rodwell et al. 1992b; duToit et al. 1997; van Grunsven et al. 1999). Current guidelines recommend the use of inhaled corticosteroid therapy in all but the mildest asthma (NHLBI/WHO 1995; BTS 1997b). The guidelines are based on the assumption that treating airway inflammation leads to improvement in asthma control. However, several studies have demonstrated incongruities in the relationship between both symptoms and lung function and airway inflammation (Sont et al. 1996; Haley and Drazen 1998; Jatakanon et al. 1998a). Evidence for ongoing airway inflammation has been found in asthmatic patients during clinical remission (Foresi et al. 1990; Boulet et al. 1994; Sont et al. 1996; van der Thoorn et al. 2000). Conversely ongoing symptoms may occur despite the fact that airway inflammation is adequately controlled (Sont et al. 1996). This raises questions as to whether the primary goal when using inhaled corticosteroid is to achieve symptom control, optimise lung function, minimise airway inflammation or reduce airway hyperresponsiveness.

Dose-response relationships for inhaled corticosteroid have been described in the past using symptoms and lung function as the main outcome variables. Although a dose-response relationship exists for these endpoints, it may plateau at a relatively low dose (Lipworth 1996; Pederson and O'Byrne 1997; Barnes 1998b; Barnes et al. 1998). Thus, there may be difficulty in differentiating between the effects of adjacent doses. Other studies have shown that dose-response relationships vary when other end-points are used (Toogood et al. 1977). For example, much higher doses of inhaled corticosteroid are required to control airway hyperresponsiveness than symptoms (Toogood et al. 1977; Kraan et al. 1988). There are fewer data to
clarify these relationships for airway inflammation. Such information might be clinically helpful in assessing the relative efficacy of different inhaled corticosteroid doses and as a guide to optimising long-term therapy.

Exhaled nitric oxide (eNO) is a repeatable easily measured marker of airway inflammation (Kharitonov et al. 1997a; Silkoff and Zamel 1998; ATS 1999). High levels are seen in both steroid-naive asthma (Alving et al. 1993) and during acute exacerbations (Crater et al. 1999). Furthermore, levels decrease following treatment with both inhaled (Kharitonov et al. 1996b; Jatakanon et al. 1998a; Lim et al. 1999; van Rensen et al. 1999) and oral steroids (Massaro et al. 1995). These results suggest that eNO may be a useful end-point in measuring the dose-response relationship for the effect of inhaled corticosteroid therapy on airway inflammation.

In this study, we aimed to evaluate the usefulness of serial eNO measurements in measuring the anti-inflammatory effects of inhaled beclomethasone, and to make comparisons with other end-points including lung function, sputum eosinophils and airway hyperresponsiveness to hypertonic saline.

7.3. Methods

Subjects
Patients with mild to moderate asthma (ATS 1987) and who had been taking inhaled corticosteroid therapy for at least six months were recruited.

Inclusion and exclusion criteria were the same as those given in Chapter Five.

Study Design
Inhaled corticosteroid therapy was discontinued after a run-in period of 2-4 weeks during which inhaled corticosteroid dose was held constant. Thereafter patients were reviewed weekly until 'loss of control' developed OR for a maximum of six weeks. 'Loss of control' was defined by pre-determined criteria based on changes
from baseline in peak flow rates and symptoms (see Chapter Six) (Jones et al. 2001). Those who did not develop loss of control were withdrawn from further participation.

Following loss of control, patients received 20mg prednisone orally for two days in order to alleviate their deteriorating symptoms. This strategy was necessary for reasons of safety, although this may theoretically have influenced interpretation of subsequent inhaled corticosteroid effects, especially at one week. Simultaneously, at loss of control, patients were randomised to receive double-blind inhaled corticosteroid treatment for eight weeks, taking one puff daily from each of two identical metered dose inhalers (CFC delivery system) (Autohaler, 3M Pharmaceuticals) labelled “morning” and “evening”. The treatments were:

- Placebo (1 puff of placebo twice daily)
- 50µg/day (1 puff of 50µg beclomethasone and 1 puff of placebo daily)
- 100µg/day (1 puff of 100µg beclomethasone and one puff of placebo daily)
- 200µg/day (1 puff of 100µg beclomethasone twice daily)
- 500µg/day (1 puff of 250µg beclomethasone twice daily).

Following randomisation (visit 1), patients were reviewed weekly for the first four weeks (visits 2-5) and then at the end of eight weeks treatment (visit 6). Those patients who experienced significant worsening of their asthma while taking the randomisation medication were withdrawn from that treatment group, given 20mg prednisone orally for a further two days, and entered into an open label treatment arm of 1000µg/day (2 puffs of 250µg beclomethasone twice daily). These patients were also reviewed weekly for four weeks and again at the end of eight weeks treatment with 1000µg/day.

Study procedures
Diurnal peak expiratory flow measurements, bronchodilator use, and symptom scores were recorded in a daily record card. Measurements obtained at each study visit are shown in Table 7.1. Exhaled NO was measured prior to all other study procedures using a calibrated chemiluminescence analyser with on-line
measurement of single exhalations according to a standard protocol (Kharitonov et al. 1997a; ATS 1999), with the exception of flow rate (250 mls/sec) (the study was commenced prior to the publication of internationally agreed guidelines). Exhaled NO levels were read at the plateau corresponding to 70-80% of the CO₂ curve. For a full description of study procedures refer to Chapter Four.

**Table 7.1.** Study design and measurements obtained at each of the study visits.

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</tr>
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<td>✗</td>
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<td></td>
</tr>
<tr>
<td>PD₁₅ saline</td>
<td>✗</td>
<td>✗</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Withdrawal of ICS  LOC/Randomisation  End of treatment

V = visit, ICS = inhaled corticosteroid, LOC = loss of control, PD₁₅ saline = provocative dose of hypertonic saline required to induce a 15% fall in FEV₁. Visit 0 = withdrawal of ICS. Visit 1 = randomisation. Visit 6 = end of treatment.
Ethical considerations and safety

Each patient's asthma control was monitored closely throughout the study. For ethical reasons, loss of control criteria included symptoms that were "disagreeable or distressing" irrespective of changes in peak expiratory flow rate. All patients were provided with an individualised self-management plan, an emergency card, and a supply of prednisone tablets. Patients had 24-hour access to one of the study investigators via the hospital paging system. Ethical approval was obtained from the Otago Ethics Committee and written informed consent was obtained from all study participants.

Statistics

Pearson correlations were calculated to assess the relationships between variables at randomisation and the end of the study. All data was analysed on an intention-to-treat basis. Differences between groups were analysed by analysis of covariance (ANCOVA), using group and entry into the open 1000µg/day treatment subgroup as factors, and with baseline values as covariates. PD₁₅ saline values were analysed as doubling dose change from baseline. Polynomial contrasts were fitted across the randomisation groups to look at the dose-response relationship. Estimated marginal means were calculated and compared, with Bonferroni adjustments for multiple comparisons. Post-hoc analyses were also performed excluding treatment withdrawals from the data. In all of the figures bars represent the 95% CI.
7.4. Results

Randomisation

Eighty-seven patients entered the study. Three patients withdrew consent prior to inhaled corticosteroid withdrawal. Sixty-five patients (77%) developed loss of control and were randomised into one of the five treatment groups. Demographic data are given in Table 7.2. Data for study end-points measured at baseline before withdrawal of inhaled corticosteroid, visit 0, and at randomisation following ‘loss of control’, visit 1, are shown in Table 7.3. The only measurement demonstrating a statistically significant difference across the groups was sputum eosinophils at visit 1 (p = 0.029), where the 100μg/day group had a mean of 31.9% whereas the placebo group had only 6% eosinophils. However baseline levels in all the variables at randomisation were included as covariates in the further analyses therefore correcting for any differences between the groups.
Table 7.2. Demographic data (at entry to the run-in phase) for the randomised study participants.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Placebo</th>
<th>50µg/day</th>
<th>100µg/day</th>
<th>200µg/day</th>
<th>500µg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (n)</td>
<td>65</td>
<td>13</td>
<td>14</td>
<td>14</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Male: Female</td>
<td>22:43</td>
<td>4:9</td>
<td>6:8</td>
<td>7:7</td>
<td>2:10</td>
<td>3:9</td>
</tr>
<tr>
<td>Age, yrs (range)</td>
<td>42.4</td>
<td>42.5</td>
<td>43.3</td>
<td>44.7</td>
<td>41.2</td>
<td>39.6</td>
</tr>
<tr>
<td>Duration of asthma, yrs (range)</td>
<td>27.0</td>
<td>30.3</td>
<td>30.6</td>
<td>22.5</td>
<td>31.2</td>
<td>20.2</td>
</tr>
<tr>
<td>Skin test positive, n (%)</td>
<td>60</td>
<td>12</td>
<td>14 (100%)</td>
<td>12 (85.7%)</td>
<td>12 (100%)</td>
<td>10 (83.3%)</td>
</tr>
<tr>
<td>ICS dose, µg/day (range)</td>
<td>658</td>
<td>731</td>
<td>550</td>
<td>700</td>
<td>671</td>
<td>642</td>
</tr>
<tr>
<td>FEV₁, litres (range)</td>
<td>2.61</td>
<td>2.07</td>
<td>2.39</td>
<td>2.42</td>
<td>2.15</td>
<td>2.60</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>90.8</td>
<td>85.4</td>
<td>87.5</td>
<td>91.4</td>
<td>89.0</td>
<td>100.2</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>68.1</td>
<td>59.5</td>
<td>64.4</td>
<td>62.2</td>
<td>63.2</td>
<td>72.2</td>
</tr>
</tbody>
</table>

Unless stated results are expressed as mean (95% CI).

ICS = inhaled corticosteroid, expressed as beclomethasone equivalent dose.

FEV₁ = forced expiratory volume in one second.

FVC = forced vital capacity.
Table 7.3. Comparison of randomisation groups at withdrawal of maintenance inhaled corticosteroid (VO) and at loss of control/randomisation (V1). Comparisons are also made between those who completed treatment with randomised medication and those who withdrew from the randomised treatment group and subsequently received 1000µg/day beclomethasone.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>eNO (ppb)</th>
<th>FEV₁ % predicted</th>
<th>% eosinophils</th>
<th>PD₁₅ saline (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>V0</td>
<td>V1</td>
<td>V0</td>
<td>V1</td>
</tr>
<tr>
<td>Placebo</td>
<td>13</td>
<td>9.55 (6.4,12.7)</td>
<td>20.91 (12.2,29.6)</td>
<td>85.42 (72.1,98.8)</td>
<td>82.01 (67.8,96.3)</td>
</tr>
<tr>
<td>50µg/day</td>
<td>14</td>
<td>11.55 (6.8,16.3)</td>
<td>22.11 (14.0,30.2)</td>
<td>87.45 (76.2,98.8)</td>
<td>77.68 (66.1,89.3)</td>
</tr>
<tr>
<td>100µg/d</td>
<td>14</td>
<td>13.79 (7.3,20.3)</td>
<td>32.06 (18.8,45.4)</td>
<td>91.44 (81.4,101.5)</td>
<td>75.10 (65.7,84.5)</td>
</tr>
<tr>
<td>200µg/d</td>
<td>12</td>
<td>11.29 (7.9,14.7)</td>
<td>29.93 (13.3,46.6)</td>
<td>88.99 (76.1,101.9)</td>
<td>73.91 (59.7,88.1)</td>
</tr>
<tr>
<td>500µg/d</td>
<td>12</td>
<td>11.36 (4.4,18.3)</td>
<td>25.19 (12.6,37.8)</td>
<td>100.18 (92.2,108.2)</td>
<td>87.87 (75.2,100.5)</td>
</tr>
<tr>
<td>Completed</td>
<td>40</td>
<td>24.66 (18.5,30.8)</td>
<td>82.48 (76.4,88.6)</td>
<td>17.84 (10.4,25.3)</td>
<td>12.05 (8.0,16.1)</td>
</tr>
<tr>
<td>Withdrew</td>
<td>25</td>
<td>28.21 (19.6,36.8)</td>
<td>73.89 (64.9,82.9)</td>
<td>17.34 (7.5,27.2)</td>
<td>11.79 (5.8,16.8)</td>
</tr>
</tbody>
</table>

Results are expressed as mean (95% CI). FEV₁ = forced expiratory volume in one second. PD₁₅ saline = provocative dose of hypertonic saline required to induce a 15% fall in FEV₁.
Treatment withdrawals
One patient randomised to receive 500μg/day was withdrawn at visit 1 due to the severity of their asthma, and was not considered further in the analysis. Twenty-five of the remaining 64 patients developed worsening of their asthma while receiving the randomised study medication, and were entered into the open-label arm of the study in which they were treated with 1000μg of beclomethasone/day. The number (%) of patients withdrawn from the placebo, 50μg/day, 100μg/day, 200μg/day, and 500μg/day groups was 6 (46%), 5 (38%), 8 (57%), 4 (31%), and 2 (18%) respectively. There was no statistically significant difference in the rate of treatment withdrawals between these doses. Comparisons between those who withdrew from the randomised treatment arm and those who completed treatment are shown in Table 7.3. There were no significant differences between those who completed treatment and those who withdrew for eNO, sputum eosinophils or PD_{15} saline at the time of randomisation. However, those who withdrew had reduced lung function at randomisation, as demonstrated by a FEV_{1} % predicted of 73.89% compared to 82.48% in those who remained on the randomised medications (p=0.005). Patients withdrawing also had a significantly higher dose of maintenance inhaled corticosteroid therapy at entry into the study. Those who withdrew had a mean (95% C.I.) of 776 (635 to 917)μg of BDP/day compared to 614 (536 to 694)μg of BDP/day in those who remained on randomised medication (2-tailed t-test p=0.042).

Dose-Responses
Changes from baseline in eNO and FEV_{1} following one week and at the end of treatment are shown in Figures 7.1 and 7.2 respectively. Likewise changes in sputum eosinophils and PD_{15} saline from baseline to the end of treatment are shown in Figures 7.3 and 7.4 respectively. Data shown are derived from all patients, but adjusted for baseline measurements and treatment withdrawals.
Figure 7.1. Percentage change in eNO with increasing doses of inhaled beclomethasone. Mean changes after one week and at the end of the eight weeks treatment are shown (error bars represent 95% CI).

There was a significant difference in the change in eNO across treatment groups at one week (p=0.005) and at the end of treatment (p=0.015). The difference across the groups was linear at both one week and at the end of treatment (p=0.022 and p=0.003 respectively).
Figure 7.2. Percent change in FEV\(_1\) with increasing doses of inhaled beclomethasone. Mean changes occurring after one week and at the end of treatment are shown (error bars represent 95% CI).

There was a significant difference in the change in FEV\(_1\) across treatment groups at one week (p=0.014) and at the end of treatment (p=0.036). The difference across the groups was linear at both one week and at the end of treatment (p=0.043 and p=0.006, respectively).
Figure 7.3. Percentage change in sputum eosinophils at the end of treatment with increasing doses of inhaled beclomethasone. Mean changes occurring following eight weeks treatment are shown (error bars represent 95% CI).

There was a significant linear relationship across the treatment groups (p=0.037).
**Figure 7.4.** Mean doubling dose changes in PD$_{15}$ saline at the end of treatment occurring with increasing doses of inhaled beclomethasone. Results shown are taken from the end of eight weeks treatment (error bars represent 95% CI).

![Graph showing mean doubling dose changes in PD$_{15}$ saline at the end of treatment occurring with increasing doses of inhaled beclomethasone.](image)

No significant differences were seen across the treatment groups (p=0.837).
Pair-wise comparisons

Pair-wise comparisons of the changes in eNO, FEV₁, sputum eosinophils and PD₁₅ saline occurring with each of the treatment groups were also made. These are shown for values obtained following one week and at the end of treatment for both eNO and FEV₁ are shown in Figures 7.5 and 7.6 respectively. Likewise results at the end of treatment for both sputum eosinophils and PD₁₅ saline are shown in Figure 7.7. In addition these graphs contain an insert showing the changes seen in the subset of patients receiving the open-label 1000µg/day treatment arm are also shown in the figures. These patients were included in the analysis of the randomisation groups (on an intention-to-treat basis), but on the graph are in addition shown as a separate group. The 1000µg/day subset have not been formally analysed due to selection bias: patients in this group were those who had failed treatment on a lower dose of inhaled corticosteroid.
**Figure 7.5.** Pair-wise comparisons of the percentage change in eNO after one week and at the end of the eight weeks treatment with inhaled beclomethasone. Mean ± 95% CI is shown.

![Graph showing percentage change in eNO with different doses of beclomethasone](image)

There were significant differences found between 100µg/day and both 200 and 500µg/day at one week (*; p<0.05), and between 500µg/day and both placebo and 100µg/day at the end of treatment (†; p=0.01 and p=0.023 respectively). Data shown are derived from all patients, but adjusted for baseline measurements and treatment withdrawals.
Figure 7.6. Pair-wise comparisons of the percent change in FEV₁ after one week and at the end of eight weeks treatment with beclomethasone. Mean ± 95% CI is shown.

There were only significant differences found between 100µg/day and placebo at one week (*; p=0.005) and 500µg/day and placebo at the end of treatment (†; p=0.040). Data are derived from all patients, but adjusted for baseline measurements and treatment withdrawals.
Figure 7.7.

A. Pair-wise comparisons of the percentage change in sputum eosinophils at the end of treatment. There was only a significant difference found between 500 μg/day and 100 μg/day (*; p=0.049). Mean ± 95% CI is shown.

B. Pair-wise comparisons of the doubling dose changes in PD₁₅ saline at the end of treatment. No significant differences were found on pair-wise comparisons.
Post-hoc analysis
When those entering the 1000μg/day arm were excluded from the analysis the linear relationship seen across the groups for changes in eNO at the end of treatment was still seen (p=0.003). In those who remained on randomised medication until study completion the mean (±95% C.I.) change in eNO at the end of treatment with placebo, 50, 100, 200, and 500μg/day was 7.0 (-1.6, 15.63), -1.0 (-9.1, 7.1), -2.3 (-11.6, 7.0), -9.6 (-17.4, -1.77), and -8.6 (-16.4, -0.8), respectively. These patients was also demonstrated a significant difference in change in sputum eosinophils across the treatment groups (p=0.033), however this relationship was no longer linear (p=0.75). Similar analyses using changes in FEV$_1$ and PD$_{15}$ saline found no difference across treatments (p=0.327 and p=0.202, respectively).

Correlations between inflammatory markers
Correlations between eNO, sputum eosinophils, PD$_{15}$ saline and FEV$_1$ % predicted at baseline and following treatment (expressed as absolute values and as changes from baseline) are shown in Table 7.4. Significant correlations were found between eNO and sputum eosinophils throughout the study. Changes in eNO correlated significantly with changes in sputum eosinophils. Changes in PD$_{15}$ saline did not correlate with changes in any of the other markers.
Table 7.4. Correlations between markers of airway inflammation and lung function at randomisation (visit 1) and following treatment (visit 6).

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Visit 1</th>
<th>Visit 6</th>
<th>Δ Visit (6-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNO (ppb) and % eosinophils</td>
<td>( r = 0.519 )</td>
<td>( r = 0.548 )</td>
<td>( r = 0.403 )</td>
</tr>
<tr>
<td></td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p = 0.002 )</td>
</tr>
<tr>
<td>eNO (ppb) and PD(_{15}) saline (ml)</td>
<td>( r = -0.352 )</td>
<td>( r = -0.270 )</td>
<td>( r = -0.192 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.003 )</td>
<td>( p = 0.051 )</td>
<td>( p = 0.187 )</td>
</tr>
<tr>
<td>eNO (ppb) and FEV(_1)% predicted</td>
<td>( r = -0.169 )</td>
<td>( r = 0.021 )</td>
<td>( r = -0.252 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.125 )</td>
<td>( p = 0.870 )</td>
<td>( p = 0.045 )</td>
</tr>
<tr>
<td>% eosinophils and PD(_{15}) saline (ml)</td>
<td>( r = -0.254 )</td>
<td>( r = -0.185 )</td>
<td>( r = 0.045 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.040 )</td>
<td>( p = 0.204 )</td>
<td>( p = 0.771 )</td>
</tr>
<tr>
<td>% eosinophils and FEV(_1)% predicted</td>
<td>( r = -0.262 )</td>
<td>( r = 0.015 )</td>
<td>( r = -0.585 )</td>
</tr>
<tr>
<td></td>
<td>( p = 0.023 )</td>
<td>( p = 0.913 )</td>
<td>( p &lt; 0.001 )</td>
</tr>
<tr>
<td>PD(_{15}) saline (ml) and FEV(_1)% predicted</td>
<td>( r = 0.420 )</td>
<td>( r = 0.533 )</td>
<td>( r = -0.092 )</td>
</tr>
<tr>
<td></td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p = 0.529 )</td>
</tr>
</tbody>
</table>

Figures in bold represent statistically significant results.

Δ Visit (6-1) = the change in the variables between visit 1 and 6.

FEV\(_1\) = the forced expiratory volume in one second and is expressed as a percentage of the predicted FEV\(_1\). PD\(_{15}\) saline = the provocative dose of hypertonic saline required to induce a 15% fall in FEV\(_1\). % eosinophils = the percentage of eosinophils in induced sputum cell count.
7.5. Discussion

Dose-response relationships for eNO

This study demonstrates that eNO measurements provide useful information regarding the dose-response relationship for the anti-inflammatory effects of inhaled corticosteroids. This has potential practical importance for the monitoring of anti-inflammatory treatment in individual patients, as well as assessing the efficacy of different inhaled corticosteroids at different doses.

There was a significant linear relationship between the change in eNO and the dose of inhaled corticosteroid used (over a range 0-500μg/day of beclomethasone) in patients with moderate bronchial asthma in whom inhaled corticosteroid had been withdrawn and "loss of control" occurred. This ensured that airway inflammation was sufficiently present for anti-inflammatory effects to be measured. The linear dose-response relationship was evident as early as one week after commencing therapy, and continued to eight weeks, indicating that eNO measurements respond rapidly to changes in airway inflammation and reflect the anti-inflammatory actions of inhaled corticosteroid therapy with time. Similar linear dose-response relationships were seen with FEV₁ at one and eight weeks and with sputum eosinophils at the end of eight weeks. Silkoff et al (2001) has also recently demonstrated that in patients with elevated levels of eNO at baseline there is a strong relationship between inhaled corticosteroid dose and changes in eNO over the dose range 0-1600μg beclomethasone/day. However, further to the results of that investigation, the highly significant correlation between changes in eNO and changes in sputum eosinophils in the present study confirms that decreases in eNO correspond to reductions in airway inflammation.

It has previously been shown that changes in eNO are seen in association with changes in other markers of airway inflammation following treatment with inhaled beclomethasone (Ichinose et al. 2000), budesonide (Lim et al. 1999) and fluticasone (van Rensen et al. 1999). Furthermore, eNO appears to decrease in a dose-dependent manner following low dose inhaled corticosteroid therapy (Alving et al. 1995; Kharitonov et al. 2000).
It has been reported that the effect of inhaled corticosteroid therapy on eNO may plateau at a dose of 400μg/day (Jatakanon et al. 1999; Wilson and Lipworth 2000), with no further decreases in eNO seen using larger doses despite further reductions in other markers. Wilson and Lipworth (2000) studied twenty-six asthma patients before and after three weeks treatment with 400, 800 and 1600μg/day of budesonide. These treatment doses were given successively to all patients with no washout period between treatments. Changes occurring in eNO were then compared to changes found in airway hyperresponsiveness, serum levels of eosinophil cationic protein and lung function following the treatments. They found that above 400μg/day (the first step in their dose-ranging study), no further reduction in eNO occurred despite further dose-related improvements in airway hyperresponsiveness to adenosine-5-monophosphate and serum eosinophil cationic protein. These authors concluded that the decrease in airway hyperresponsiveness seen with the higher doses demonstrated further reductions in airway inflammation undetected by eNO. However, this conclusion firstly assumes that airway hyperresponsiveness directly relates to airway inflammation, a point currently being debated (Brusasco et al. 1998; Haley and Drazen 1998). Secondly, it assumes that the reductions in airway hyperresponsiveness seen with the larger doses were not due to carry-over time-dependent effects. Airway hyperresponsiveness is known to decrease over a period of months in response to inhaled corticosteroid therapy (Kraan et al. 1988; Vathenen et al. 1991; van Essen-Zandvliet et al. 1992; Hofstra et al. 2000). During the Wilson and Lipworth study treatment regimens were given successively without a washout period, therefore the results may potentially have been complicated by these time-dependent changes. Recent data suggest that these time-dependent decreases may not be so noticeable using indirect bronchial challenges for assessing airway hyperresponsiveness (such as adenosine-5-monophosphate, hypertonic saline, and exercise) (Hofstra et al. 2000), however, the potential confounding influence of time in the Wilson and Lipworth study still exists.

Importantly in the Wilson and Lipworth study maximal suppression of airway hyperresponsiveness with the highest corticosteroid dose came at the expense of
increased systemic effects as demonstrated by adrenal suppression. Thus illustrating the need to balance beneficial and adverse effects of inhaled corticosteroid in the clinical setting (Kamada et al. 1996; Lipworth and Wiilson 1998; O'Byrne and Pederson 1998).

A plateau in eNO has also been found in a smaller study by Jatakanon et al (1999). They assessed the effects of placebo, 100, 400 and 1600μg of beclomethasone/day on eNO, lung function, sputum eosinophils, and airway hyperresponsiveness to methacholine in patients with mild asthma. These authors reported a plateau in the eNO response at 400μg/day, despite finding continuing reductions in sputum eosinophils with the higher dose (1600μg/day). However, this study is also limited by its methodology. It consisted of combining two studies together, one investigating placebo, 100 and 400μg/day, while the other investigated placebo and 1600μg/day, thus making a direct comparison between the treatments difficult. Notably, during the second study no significant difference was found in the change in eNO when comparing the effects of placebo and 1600μg/day. This is a reflection of the small number of patients involved (n=10) and the mild degree of asthma in the patients investigated.

By contrast, in our current study the dose-response relationship between eNO and inhaled corticosteroid was linear and there was no evidence of a plateau throughout the range of doses studied (0-500μg/day). The current study was designed specifically to explore the dose-response relationship at relatively low doses of inhaled corticosteroid, and although the relationship was linear across the doses studied no extrapolation of these results to higher doses is possible. The contrast between our results and those of Jatakanon et al (1999) may be due to differences in the degree of asthma inflammation at the time of the investigation. In our study treatment was withdrawn until "loss of control" occurred and those who did not exhibit loss of control were excluded. A similar study design has been used by other investigators when evaluating the dose-response for inhaled corticosteroid (Welch et al. 1997; Busse et al. 1999). The rationale for this approach is that the therapeutic effect of inhaled corticosteroid may be influenced by asthma severity: patients with milder asthma having little room for improvement. As a result, the degree of airway inflammation at commencement of treatment in our study was significantly
greater than that of the Jatakanon et al study. Our patients had a mean sputum eosinophil differential of 17% compared to 4% in the Jatakanon et al study. This therefore would have allowed for a greater improvement following inhaled corticosteroid treatment in our current study.

Recently, Silkoff et al (2001) have demonstrated that eNO follows a dose-response relationship with higher doses of inhaled corticosteroid therapy in patients in whom baseline eNO is greatly elevated. In that study serial eNO levels were recorded from fifteen previously steroid-naïve asthma patients after successive weekly treatments with placebo, 200, 800, and 1600μg beclomethasone/day. They found that eNO levels fell progressively over this dose range from 103.5 (95% CI: 78.5, 136.7)ppb with placebo to 37.4 (95% CI: 29.1, 48.0)ppb following 1600μg/day (p=0.001). Furthermore, following dicotomization of the patients into two groups based on baseline eNO (into either mildly or greatly elevated eNO) these authors were able to show that those with higher baseline eNO had a significantly greater dose-response with increasing doses. In comparison, the dose-response relationship seen in those with mildly elevated eNO appeared to plateau following administration of 200μg/day. This again highlights that patients with higher levels of baseline inflammation will demonstrate dose-response relationships over a greater range of inhaled corticosteroid doses, whereas those with less inflammation will demonstrate a plateau in effect following low doses of corticosteroid therapy.

Dose-response relationships – airway hyperresponsiveness

The role of airway hyperresponsiveness as a measure of airway inflammation is still difficult to define. Non-specific airway responsiveness appears to reflect airway inflammation even in the absence of ongoing symptoms and abnormal lung function (Sont et al. 1996). This would suggest that changes in airway hyperresponsiveness might provide a useful measure by which the anti-inflammatory effects of inhaled corticosteroid could be evaluated. However, in our current study there were no significant changes in airway hyperresponsiveness to hypertonic saline following treatment with beclomethasone. This may be the result of the low doses of inhaled corticosteroid used (maximum 500μg/day) or the relatively short duration of treatment (eight weeks),
although a change in airway hyperresponsiveness to hypertonic saline challenge should be visible within this time frame (du'Toit et al. 1997).

Other authors have reported contrasting results using alternative methodologies for assessing airway hyperresponsiveness (Kraan et al. 1988; Dahl et al. 1993; Jatakanon et al. 1999; Taylor et al. 1999; Hofstra et al. 2000; Wilson and Lipworth 2000). Jatakanon et al (1999) found no improvement in airway hyperresponsiveness to methacholine following four weeks of treatment with 100 or 400μg budesonide/day despite significant improvements in sputum eosinophils. However, the same authors found a marked decrease in methacholine induced airway responsiveness following treatment with 1600μg/day, despite only mild further reductions in sputum eosinophils. This illustrates the inconsistencies in the relationship between eosinophils and airway hyperresponsiveness. Part of the reason for this apparent discrepancy may lie in the different time effects of these two measurements. Previously it has been shown that changes in airway hyperresponsiveness to methacholine and histamine are time-dependent, decreasing progressively over a period of at least eight weeks (Kraan et al. 1988; Vathenen et al. 1991; Hofstra et al. 2000) with some suggestion that it may take many months for full effect (van Essen-Zandvliet et al. 1992). In contrast, eosinophil numbers have been shown to decrease rapidly following corticosteroid treatment, with decreases noted within three hours of inhaled steroid administration (Oh et al. 1999).

Airway hyperresponsiveness to methacholine and histamine generally appears to be less sensitive than indirect bronchial challenges in detecting changes occurring with corticosteroid treatment (O'Connor et al. 1992; Hofstra et al. 2000; Wilson and Lipworth 2000). By measuring airway hyperresponsiveness using adenosine-5-monophosphate Taylor et al (1999) have reported that this measurement is more sensitive than either sputum eosinophils or concentrations of the eosinophil degradation product, eosinophil cationic protein, in detecting changes in airway inflammation. This conclusion was based on the finding of a dose-dependent reduction in adenosine-5-monophosphate airway hyperresponsiveness following 100, 400, and 1600μg/day of circlesonide, whereas sputum eosinophils decreased with 400μg/day and no further dose-dependent decrease
was seen with the higher dosing. In contrast to the author's conclusion, it could be argued that sputum eosinophils are more sensitive to the effects of inhaled corticosteroid than adenosine-5-monophosphate, and that at doses above 400μg/day there is little further effect on airway inflammation. If this was the case, then use of airway hyperresponsiveness to guide inhaled corticosteroid dose titration may lead to many patients being over treated, with the increased risk of systemic side effects (Wilson and Lipworth 2000).

**Dose-response relationships – lung function**

In the present study we also found that there was a dose-response relationship for changes in FEV₁ following treatment. As for eNO, this relationship was seen as early as one week into treatment. Previous studies have been conflicting in their findings of a dose-response relationship in lung function. Some have demonstrated a lack of dose-response, although these studies were often performed in mild asthma and thus patients were tested at the plateau of the dose-response relationship (Kamada et al. 1996; Lipworth 1996; Pederson and O’Byrne 1997). By contrast, in other studies in which patients were treated following induced deterioration of their asthma, similar outcomes to our study have been observed (Dahl et al. 1993; Welch et al. 1997; Busse et al. 1999).

The finding of a dose-response relationship for FEV₁ raises the question of whether eNO provides any further information than serial lung function in assessing the response to inhaled corticosteroid? Ultimately this question will only be answered in large longitudinal studies incorporating these measurements into clinical practice and comparing long-term outcomes. However, a suggestion that eNO provides different data to that of lung function is given in the close relationship seen between eNO and sputum eosinophils throughout the study, a relationship which was considerably weaker with both lung function and airway hyperresponsiveness.

**Correlations between eNO and eosinophils / airway responsiveness**

In our study changes in FEV₁ (% predicted) correlated well with changes in sputum eosinophils following inhaled corticosteroid treatment (r= -0.585, p=<0.001). However,
FEV₁ (% predicted) could not be used directly as a marker of airway inflammation as it only weakly correlated with sputum eosinophils prior to treatment ($r = -0.262, p=0.023$) and there was no relationship with eosinophils following treatment ($r=0.015, p=0.913$). In contrast, highly significant correlations were found between eNO and sputum eosinophils at baseline ($r=0.52, p<0.001$), at the end of eight weeks treatment ($r=0.55, p<0.001$), and as changes in these parameters with treatment ($r=0.40, p=0.002$). These findings further validate the use of eNO as a means of measuring ongoing airway inflammation in asthma.

The relationship between eNO and airway inflammation has been previously reported as being less reliable during the use of inhaled corticosteroid therapy (Berlyne et al. 2000; Lim et al. 2000). However, in contrast to our present study, these studies have been cross-sectional rather than longitudinal and the study patients had lower levels of airway inflammation. The only previous longitudinal study to report on the correlation between changes in eNO and eosinophils with inhaled steroid therapy had found no correlation between the two (Pearson's correlation, $r<0.56, p>0.15$) (van Rensen et al. 1999). However, again, this study is limited by both small numbers ($n=10$ in the steroid treated group) and the mildness of asthma treated (mean eosinophil differential of 2.85%, compared with 17% in our current study), thus limiting the range over which changes in inflammation can be measured. Little et al (2000) have shown that an elevated level of eNO ($>10$ ppb) in patients treated with inhaled steroids has a positive predictive value of 83% in distinguishing those patients in whom an improvement in lung function will occur following oral prednisone. This gives indirect evidence that eNO levels reflect reversible airway inflammation despite the concomitant use of inhaled corticosteroid therapy and is consistent with our findings.

In our current study the relationship between eNO and PD₁₅ saline was less well defined. When assessed as point-in-time measurements before treatment there was a weak but highly significant correlation between them ($r = -0.352, p=0.003$). Following inhaled steroid treatment this correlation was even weaker ($r = -0.270, p=0.051$). Previous reports have found similar results, with a small correlation between eNO and airway
hyperresponsiveness found in corticosteroid-naïve asthma (Dupont et al. 1998; Jatakanon et al. 1998; Verleden et al. 1999; van der Thoorn et al. 2000) and an absence of a correlation following inhaled corticosteroid therapy (Verleden et al. 1999; Ichinose et al. 2000).

The absence of a relationship between eNO and airway hyperresponsiveness following inhaled corticosteroid therapy is further highlighted in the present study by our failure to find any relationship between changes in these measurements following inhaled steroid use. This apparent discrepancy may be partly explained by differing response times for eNO and airway hyperresponsiveness to inhaled corticosteroid therapy. Importantly, changes in PD\(_{15}\) saline did not correlate with changes in any of the other markers measured. In particular, the relationship between PD\(_{15}\) saline and sputum eosinophils remained minimal throughout the study, again suggesting that these two indices may be measuring differing aspects of the inflammatory process (Brusasco et al. 1998; Crimi et al. 1998; Haley and Drazen 1998). This does not invalidate the use of hypertonic saline to induce airway hyperresponsiveness in assessing asthma control, but merely highlights the clinical difficulty in deciding which marker of asthma control should be measured in order to best assess long term outcomes.

**Study criticisms**

There are a number of potential criticisms of the current study. Firstly, the range of doses of beclomethasone to which patients were randomised did not include 1000\(\mu\)g/day. This was because of earlier suggestions that the clinical benefits of inhaled corticosteroid may be limited at higher doses (Kamada et al. 1996; O'Byrne and Pederson 1998) even in patients with severe asthma (Hummel and Lehtonen 1992). Because of this, our study was specifically designed to address the possibility that there is a threshold for the anti-inflammatory effects of inhaled corticosteroid within the lower dose range. However, our results demonstrate that this is not the case.

Secondly, the study model employed, i.e. withdrawal of inhaled corticosteroid prior to beginning the randomised phase of the study (Gibson et al. 1992), resulted in an
unexpectedly high number of patients whose asthma remained uncontrolled after being randomised. A total of 25/64 (39%) of patients remained uncontrolled and for ethical reasons these patients were re-allocated to receive 1000µg/day. The patients who entered the 1000µg/day arm had both a lower FEV₁ % predicted (73.9% versus 82.5% respectively, p=0.005) and a higher dose of maintenance inhaled corticosteroid at study entry (776µg/day versus 614µg/day beclomethasone respectively, p=0.042) when compared to those who remained on randomised medication, suggesting that these individuals had more severe asthma. The analyses of the dose-response effects were on an intention-to-treat basis, and took account of those patients who were changed to the higher dose. Because of selection bias no formal comparisons were made with the subset who received 1000µg/day. However, this group did provide useful information: eNO levels at week eight were higher following 1000µg/day than was seen in the 500µg/day group. The 1000µg/day group had a mean eNO of 17.3ppb (95% C.I.: 11.08, 23.52) compared to 10.4ppb (95% C.I.: 4.41, 16.39) in those receiving 500µg/day. This finding confirms that despite receiving higher doses of inhaled corticosteroid eNO levels may remain elevated in a subset of patients with more severe asthma (Stirling et al. 1998; Mattes et al. 1999), reflecting ongoing airway inflammation (Louis et al. 2000).

Finally, despite finding a linear relationship between eNO and dose of inhaled corticosteroid, eNO measurements were not able to distinguish between adjacent inhaled corticosteroid doses consistently when assessed in pair-wise comparisons, although eNO was better in this regard than all other measured parameters. This appears to be a difficulty pertaining to almost all dose-response studies for inhaled corticosteroid, irrespective of the end-point used, and with even larger numbers of patients (Dahl et al. 1993; Welch et al. 1997; Busse et al. 1999).

**Summary**

A dose-response relationship between eNO and inhaled corticosteroid therapy existed over a dose range of 0-500µg/day beclomethasone in patients treated following a recent deterioration in asthma control. This dose-response relationship was seen as early as one week into treatment and increased over the eight weeks of treatment. Similar dose-
response relationships were seen with FEV$_1$ and sputum eosinophils at the end of treatment and with FEV$_1$ at one week. In contrast to FEV$_1$, eNO was strongly correlated to sputum eosinophil numbers at baseline and after treatment, as well as when assessed as change occurring with treatment. These findings suggest that eNO is a good marker of airway inflammation during inhaled corticosteroid therapy, being both easily measured and rapidly responsive to changes in inflammation.
CHAPTER EIGHT:
EXHALED NITRIC OXIDE AND RESPONSE TO CORTICOSTEROID ADMINISTRATION IN COPD

8.1 Abstract

**Background:** The appropriate use of corticosteroids in the management of stable COPD is still uncertain. This study was conducted to investigate whether eNO measurements could be helpful in identifying patients in whom benefit is gained from corticosteroid therapy.

**Method:** Twenty-seven ‘corticosteroid-free’ patients with COPD were given a 10-14 day course of oral prednisone (30mg/day). Measurements of eNO, spirometry and 6-minute walk were performed before (visit 2) and after (visit 3) prednisone. The value of eNO in predicting a response to treatment defined as an improvement in 6-minute walk of ≥50m was calculated.

**Results:** A significant decrease in eNO of −3.31ppb (-1.46 to −5.16) and an increase in 6-minute walk of 34.4m (22.6 to 46.1) was seen following prednisone. No significant change occurred in lung function (ΔFEV₁ 0.04l (-0.02 to 0.09)). Exhaled NO at visit 2 correlated marginally with the change in 6-minute walk (r=0.38, p=0.06) and FVC (r=0.53, p=0.01) following prednisone, but not with change in FEV₁ (r=0.12, p=0.58). An eNO at visit 2 of ≤10ppb had a negative predictive value of 82.2% and positive predictive value 43.7% for a significant improvement in 6-minute walk following prednisone.

**Conclusion.** Low eNO at baseline predicts a poor functional improvement following corticosteroid in stable COPD. Measurements of eNO may be helpful in identifying ‘irreversible’ COPD patients.
8.2 Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by abnormal expiratory airflow that does not change significantly over short to medium time intervals or with treatment (1987). Despite this, inhaled corticosteroids are often used in the treatment of COPD, although recent evidence has called their role in long-term management of COPD into question (van Schayck et al. 1996; Barnes 1998a; Anthonisen 1999; McEvoy and Niewoehner 2000). Further, current guidelines recommend inhaled corticosteroid therapy for those patients who show a clear objective response to a formal trial of either oral prednisone or high dose inhaled steroid. A positive response is defined as an improvement in FEV₁ of ≥15% and ≥200ml, although this only occurs in a small minority of patients (BTS 1997a).

One of the challenges in the treatment of COPD is identifying potential “responders” to corticosteroid therapy. Recent studies have shown that such patients are likely to be characterised by the presence of eosinophilic airway inflammation (Chanez et al. 1997; Pizzichini et al. 1998; Fujimoto et al. 1999; Brightling et al. 2000). Exhaled nitric oxide (eNO) is raised in asthma, a disease characterised by eosinophilic inflammation. Some studies have also demonstrated elevated levels of eNO and NO derivatives in the sputum of patients with COPD (Kanazawa et al. 1998; Corradi et al. 1999), especially in patients with unstable and partially reversible disease (Maziak et al. 1998; Agusti et al. 1999; Papi et al. 2000). These findings suggest that eNO measurements may be beneficial as a surrogate for assessing airway inflammation in patients with COPD, and therefore identify those patients who have the potential to benefit from anti-inflammatory treatment. In this study we aimed to evaluate eNO measurements in predicting changes in lung function and exercise capacity resulting from a 10-14 day course of oral corticosteroid in patients with COPD.
8.3 Methods

Patients
Patients aged 45-75 years with a clinical diagnosis of stable COPD were recruited (ATS 1987; Fabbri et al. 1998a). Inclusion criteria for entry were FEV₁/FVC <65%, FEV₁ <70% predicted and a smoking history of ≥30 pack years. Exclusion criteria included a history of asthma, onset of symptoms before age 35, a respiratory tract infection in the previous four weeks, and those currently participating in a COPD rehabilitation programme. Patients were not selected on the basis of reversibility to β-agonist or their atopic status.

Study design
At study entry all current inhaled corticosteroid therapy was discontinued (Visit 1). Patients were assessed 2 weeks later (Visit 2) and commenced on a 10-14 day course of prednisone 30mg/day, followed by a further clinic visit at the end of the treatment period (Visit 3). At each clinic visit measurements of eNO, spirometry with reversibility to beta-agonist, and 6-minute walk were performed. Bronchodilator therapy was avoided prior to each visit (β-agonist for 4 hours, ipratropium for 6 hours). Those patients who were current smokers were asked to refrain from smoking on the morning of testing.

Ethical approval was obtained from the Otago Ethics Committee and written informed consent was obtained from all study participants.

Nitric oxide
Measurements of eNO were obtained prior to all other study procedures using a chemiluminescence analyser (Logan LR 2000) according to a standardised technique for single breath on-line measurements (Kharitonov et al. 1997a; 1999), with the exception of flow rate which was 250 ml/sec. A previous study in patients with asthma (Jones et al. 2001) had been conducted using a flow rate of 250ml/sec and this was the basis upon which the current study was designed. A total of three measurements was obtained at each visit. Exhaled NO levels were read at a later date by a technician who was blinded to
the patient's clinical status. The plateau NO at end-expiration was read and the mean of the three measurements was recorded.

**Spirometry**

Spirometry was performed with the patient sitting, using a rolling seal spirometer. Reference values were obtained from the report of the working party of the European Community for Coal and Steel: "Standardisation of Lung Function Tests" (Quanjer et al. 1993). Measurements were taken before and 15 minutes after two puffs of terbutaline administered via Turbuhaler (Bricanyl, 250µg/puff).

**6-Minute Walk**

Functional exercise performance was assessed by means of a standardised 6-minute walk (Steele 1996). Patients performed two 6-minute walk tests, with a rest period of at least 15 minutes in between. Patients were allowed to use their usual inhaled bronchodilator as needed during the test. Standardised instructions and encouragement were given (Guyatt et al. 1984) and the furthest distance walked recorded. An improvement of greater than 50 metres was considered clinically important (Redelmeier et al. 1997). Patients undertook 6-minute walk tests at each of the three study visits. The tests performed at visit 1 were deemed 'practice' walks, and only the distances measured during visits 2 and 3 were analysed.

**Statistical analysis**

A clinically significant improvement in FEV$_1$ was defined as an increase of ≥15% and at least 200ml from the baseline value. Reversibility was assessed as both change in FEV$_1$ from baseline and as an increase of ≥15% of the predicted FEV$_1$ (Brand et al. 1992). Correlations between eNO and FEV$_1$, FVC and 6-minute walk tests were calculated at visit 2, and for the change between visits 2 and 3 using Spearman rank correlation coefficients. The predictive values for eNO were determined using 2x2 contingency tables, permitting calculation of sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV). Cut points for eNO of ≥10ppb and ≥15ppb
were used as these have previously been shown to be clinically useful in asthma (Little et al. 2000; Jones et al. 2001).

8.4 Results

Thirty patients entered the study. Three patients developed an exacerbation of their COPD between visits 1 and 2 (while off their maintenance inhaled steroid) and were withdrawn. Demographic data for the remaining 27 obtained at visit 2 are given in Table 8.1. Three patients demonstrated FEV\textsubscript{1} reversibility to β-agonist of ≥15% and ≥200ml of baseline at visit 2. However, none of these patients had reversibility to β-agonist of ≥15% of predicted FEV\textsubscript{1} (Brand et al. 1992). A further two patients developed chest infections between visits 2 and 3 (while on prednisone) and were also withdrawn. A total of 25 patients completed the study.

Mean eNO, 6-minute walk distances, and spirometric measurements obtained before and after prednisone are shown in Table 8.2. A significant change in eNO of -3.31ppb (95% CI: -1.46 to -5.16ppb) was seen following treatment with prednisone. A plot of individual patients’ eNO recordings at visit 2 and 3 are shown in Figure 8.1 along with the group means. The mean 6-minute walk distance increased by 34.4m (95% CI: 22.6 to 46.1m) between the two visits. A total of 8 patients had an increase in 6-minute walk distance of ≥50m. Both FEV\textsubscript{1} and FVC demonstrated non-significant changes (0.04 l, 95% CI: -0.02 to 0.09; and 0.06 l, 95% CI: -0.06 to 0.18 l respectively). Only two patients had an improvement in FEV\textsubscript{1} of ≥200ml following prednisone.
Table 8.1. Demographic and baseline data at visit 2.

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>27</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>23/4</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td>(range 53-75)</td>
</tr>
<tr>
<td>Current ICS use, n (%)</td>
<td>15 (56%)</td>
</tr>
<tr>
<td>Current/ex-smoker</td>
<td>13/14</td>
</tr>
<tr>
<td>Smoking history, pack years (^a)</td>
<td>52.6</td>
</tr>
<tr>
<td></td>
<td>(range 30-80)</td>
</tr>
<tr>
<td>FEV(_1) % predicted</td>
<td>41.8%</td>
</tr>
<tr>
<td></td>
<td>(95% CI: 35.8-47.9)</td>
</tr>
<tr>
<td>FEV(_1)/FVC, %</td>
<td>42.9%</td>
</tr>
<tr>
<td></td>
<td>(95% CI: 39.2-46.6)</td>
</tr>
<tr>
<td>FEV(_1) reversibility, % absolute</td>
<td>13.8%</td>
</tr>
<tr>
<td></td>
<td>(95% CI: 9.7-17.9)</td>
</tr>
<tr>
<td>FEV(_1) reversibility, % of predicted</td>
<td>4.9%</td>
</tr>
<tr>
<td></td>
<td>(95% CI: 3.7-6.1)</td>
</tr>
</tbody>
</table>

FEV\(_1\) = forced expiratory volume in one second.  
FVC = forced vital capacity.  
FEV\(_1\) reversibility is calculated following beta-agonist administration (two puffs of 250µg/puff terbutaline via a Turbuhaler) and results expressed both as a percentage of baseline FEV\(_1\) (% absolute) and as a percentage of predicted FEV\(_1\).  
ICS = inhaled corticosteroid.  
\(^a\) One pack year is equal to 20 cigarettes/day for one year.
Table 8.2. Study measurements obtained before (visit 2) and after (visit 3) treatment with 30mg prednisone daily for 10-14 days. n = 25.

<table>
<thead>
<tr>
<th></th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>ΔVisit (3-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNO (ppb)</td>
<td>10.60</td>
<td>7.30</td>
<td>-3.31</td>
</tr>
<tr>
<td></td>
<td>(7.95, 13.26)</td>
<td>(5.36, 9.23)</td>
<td>(-5.16, -1.46)</td>
</tr>
<tr>
<td>6-minute walk (m)</td>
<td>474.6</td>
<td>508.9</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>(443.0, 506.1)</td>
<td>(477.5, 540.3)</td>
<td>(22.6, 46.1)</td>
</tr>
<tr>
<td>FEV₁ (l)</td>
<td>1.25</td>
<td>1.28</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(1.02, 1.47)</td>
<td>(1.06, 1.50)</td>
<td>(-0.02, 0.09)</td>
</tr>
<tr>
<td>FVC (l)</td>
<td>2.79</td>
<td>2.85</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(2.41, 3.16)</td>
<td>(2.48, 3.21)</td>
<td>(-0.06, 0.18)</td>
</tr>
</tbody>
</table>

ΔVisit (3-2) = the difference in measurements between visit 2 and 3.

Results are expressed as mean (95% CI).

FEV₁ = forced expiratory volume in one second.

FVC = forced vital capacity.
Figure 8.1. Exhaled NO measurements before (visit 2) and after (visit 3) treatment with prednisone. Solid line and ♦ representing those patients having an increase of greater than 50m in 6-minute walk, and dashed line and ● representing those who did not. The group means are also shown, error bars signifying the 95% C.I. (p=0.001 for these comparisons).
At visit 2 eleven patients (44%) had an eNO of greater than 10ppb, and seven (28%) had a level higher than 15ppb. Exhaled NO inversely correlated with the 6-minute walk distance at visit 2 ($r = -0.42$, $p=0.028$). However the correlation between eNO at visit 2 and the subsequent change in 6-minute walk following prednisone showed only marginal significance ($r=0.38$, $p=0.06$). There was no correlation between eNO and FEV$_1$ reversibility to $\beta$-agonist at visit 2 ($r = -0.03$, $p=0.88$). Likewise, eNO at visit 2 did not correlate with the subsequent change in FEV$_1$ following treatment with prednisone ($r=0.12$, $p=0.58$), however eNO at visit 2 did correlate with post-steroid improvement in FVC ($r=0.53$, $p=0.01$).

Patients were dichotomised into two groups based on the cut-off of 10ppb eNO measured at visit 2. The changes occurring within these two groups of patients are shown in Figure 8.3. Those with an eNO of ≥10ppb had a significantly larger decrease eNO ($p=0.03$) and increase in FVC ($p=0.04$) following prednisone treatment. These patients also had a larger increase in six minute walk distance following prednisone, although this difference was not statistically significant ($p=0.07$).

An attempt was made to calculate the sensitivities, specificities, positive and negative predictive values of the ability of eNO measurements at visit 2 to predict an improvement in 6-minute walk. However due to the small study population these resulted in very large 95% confidence intervals, limiting our ability to draw many conclusions from this data. These analyses are shown in a series of two-by-two contingency tables in Table 8.4. Bearing in mind the limitation of large confidence intervals, an eNO at visit 2 of <10ppb had a negative predictive value of 86% in predicting a failure to improve the 6-minute walk by ≥50m. However, at that cut-point the positive predictive value was only 55%. On the whole, a cut-point for eNO of ≥10ppb was associated with stronger predictive values than ≥15ppb. Analysis of the predictive value of eNO for predicting an improvement in FEV$_1$ following prednisone was not possible because only two patients had an improvement in FEV$_1$ of ≥200ml.
Table 8.3. Comparison of patients based on eNO level at visit 2. Patients were dichotomised into two groups, those with an eNO of greater than 10ppb and those who had a level less than 10ppb.

<table>
<thead>
<tr>
<th></th>
<th>eNO at V2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10ppb (n=14)</td>
<td>≥10ppb (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (95% CI)</td>
<td>Mean (95% CI)</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>ΔeNO(V3-V2) (ppb)</td>
<td>-1.4 (-0.1, -2.8)</td>
<td>-5.7 (-2.5, -8.9)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>ΔFEV₁(V3-V2) (ml)</td>
<td>29 (106, -48)</td>
<td>44 (113, -25)</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Δ% predicted FEV₁(V3-V2) (%)</td>
<td>1.4 (4.2, -1.5)</td>
<td>1.5 (3.7, -0.7)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>ΔFVC(V3-V2) (ml)</td>
<td>-42 (82, -165)</td>
<td>191 (371, 10)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Δ6-minute walk (V3-V2) (m)</td>
<td>25 (39, 12)</td>
<td>46 (64, 28)</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

ΔeNO(V3-V2) = change in eNO between visits 2 and 3.
ΔFEV₁(V3-V2) = change in forced expiratory volume in one second (FEV₁) between visits 2 and 3.
ΔFVC(V3-V2) = change in forced vital capacity (FVC) between visits 2 and 3.
Δ6-minute walk (V3-V2) = change in six minute walk distance between visits 2 and 3.
Table 8.4. Two-by-two contingency tables assessing the ability of eNO at visit 2 (V2) in predicting improvement in 6-minute walk (6-MW) following prednisone. The respective sensitivities, specificities, positive and negative predictive values (PPV and NPV, respectively) for the various cut points used are also given.

<table>
<thead>
<tr>
<th>Cut Point</th>
<th>Increase in 6-MW</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>50m</td>
<td>75% (35-97)</td>
<td>71% (44-90)</td>
<td>55% (23-83)</td>
<td>86% (57-98)</td>
</tr>
<tr>
<td>eNO ≥10ppb</td>
<td>Yes</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8</td>
<td>17</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>b)</td>
<td>50m</td>
<td>38% (9-76)</td>
<td>76% (50-93)</td>
<td>43% (10-82)</td>
<td>72% (47-90)</td>
</tr>
<tr>
<td>eNO ≥15ppb</td>
<td>Yes</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>5</td>
<td>13</td>
<td>18</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td>8</td>
<td>17</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td>10%</td>
<td>63% (24-91)</td>
<td>65% (38-86)</td>
<td>46% (17-77)</td>
<td>79% (49-95)</td>
</tr>
<tr>
<td>eNO ≥10ppb</td>
<td>Yes</td>
<td>5</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3</td>
<td>11</td>
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<td></td>
<td>Total</td>
<td>8</td>
<td>17</td>
<td>25</td>
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</tr>
<tr>
<td>d)</td>
<td>10%</td>
<td>38% (9-76)</td>
<td>76% (50-93)</td>
<td>43% (10-82)</td>
<td>72% (47-90)</td>
</tr>
<tr>
<td>eNO ≥15ppb</td>
<td>Yes</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>5</td>
<td>13</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8</td>
<td>17</td>
<td>25</td>
<td></td>
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</tbody>
</table>
8.5 Discussion

This study was conducted in order to assess whether measurements of eNO could be used to identify patients with COPD in whom improvement with corticosteroid therapy might occur. The results demonstrate that elevated levels of eNO (≥10ppb) are seen in a proportion of COPD patients and that these patients show a greater decrease in eNO and increases in FVC following prednisone treatment. Patients with eNO ≥10ppb also demonstrated twice as much improvement in 6-minute walk distance following prednisone compared to those with an eNO of <10ppb (46 metres versus 25), however the difference between the two groups was non-significant (p=0.07).

In our study treatment with oral prednisone resulted in a significant decrease in the mean eNO level of -3.31ppb. It has been previously shown that eNO levels in COPD decrease with corticosteroid therapy following an acute exacerbation (Agusti et al. 1999) but no other study has assessed the effect of prednisone therapy on eNO in clinically stable COPD. Other studies have reported no difference in eNO levels in COPD patients on inhaled corticosteroid therapy when compared to those not on inhaled steroid therapy (Robbins et al. 1996; Delen et al. 2000). However, these investigations were cross-sectional and did not allow for possible differences in baseline severity, or that eNO levels may differ between the groups while ‘corticosteroid free’. In our study eleven patients (44%) had an eNO of ≥10ppb and seven (28%) had a level of ≥15ppb indicating that there is a subset of COPD patients in whom elevated levels of eNO are present. Furthermore the patients with elevated eNO had a significantly greater decrease in eNO following prednisone treatment (-5.7ppb compared to -1.4ppb, p=0.03). Despite this, the decrease in eNO that occurred with prednisone was small, and although there was also a significant improvement in 6-minute walk the relationship between the changes occurring in these markers was non-significant.

We found no significant improvement in either FEV$_1$ or FVC following prednisone. Neither did changes in these measurements correlate with improvements in 6-minute
walk. This highlights the limitation that measurements of lung function have in assessing response to treatment in COPD.

Although calculation of sensitivities and specificities from our current study is limited by the small study number, the results suggest that eNO may be of benefit in distinguishing those patients in whom no functional benefit is seen following prednisone treatment. Patients who did not have an elevated eNO (≥10ppb) while ‘corticosteroid-free’ were unlikely to obtain any benefit following treatment with prednisone. An eNO of less than 10ppb had a negative predictive value of 86% (95%CI: 57-98%) in predicting an improvement in 6-minute walk distance of ≥50m. However we were unable to demonstrate that those with a high level of eNO would necessarily improve. The positive predictive value of eNO was only 55% (95%CI: 23-83%), much smaller than the results of a similar trial in asthma where an eNO of >10ppb was associated with a positive predictive value of 83% for predicting an improvement in FEV₁ of >15% (Little et al. 2000). These findings are a reflection of the overall poor response to prednisone found in COPD. In this study only eight patients had an improvement in 6-minute walk of more than 50m and only two had an increase in FEV₁ of more than 200ml.

Others have shown that short-term response to corticosteroid therapy is limited to COPD characterised by eosinophilic inflammation (Chanez et al. 1997; Pizzichini et al. 1998; Fujimoto et al. 1999), not neutrophilic inflammation (Keatings et al. 1997b). It may be that eNO was associated with a low positive predictive value in our current study because it cannot be used to discriminate between these two types of inflammation, although this would appear to go against our findings in the asthma studies. Nitric oxide is released not only from eosinophils but also from a large number of other cell types found in airways, including neutrophils and macrophages (Gaston et al. 1994). For example, Kanazawa et al (1998) found that concentrations of sputum NO correlated with neutrophils and not eosinophils, whereas Rutgers et al (1999) found that eNO correlated with eosinophils and not neutrophils. Thus if short-term improvement is more likely in patients with sputum eosinophilia and yet eNO measurements cannot be used to discriminate between the types of inflammation in COPD, it follows that eNO is unlikely to be helpful in distinguishing
patients in whom short-term clinical improvement will be obtained. Unfortunately no sputum samples for cell analysis were obtained during our current study.

There are a number of potential limitations to the present study. Firstly, because a placebo-control arm was not included it is possible that eNO may have decreased spontaneously over time or that the decrease may have been a direct effect of prednisone on intrinsic NO production rather than on airway inflammation. Furthermore, five patients were withdrawn due to exacerbations and were not able to be included in the analysis. COPD characterised by frequent exacerbations is associated with greater amounts of airway inflammation (Bhowmik et al. 2000). Therefore patients experiencing exacerbations may potentially have demonstrated the greatest response to corticosteroid therapy. Their withdrawal may have weakened our chances of obtaining a “positive” result. Thirdly, no sputum samples were obtained so it remains unclear what role eNO has in identifying inflammation in COPD. Finally, the study included both current and ex-smokers. Previous studies have found that current smoking is associated with decreased levels of eNO when compared to ex-smokers (Maziak et al. 1998; Corradi et al. 1999; Delen et al. 2000). Unfortunately at the time of design for our current study these results were not available. The inclusion of smokers may have reduced our ability to detect a significant positive predictive value for eNO.

COPD is a heterogeneous disease for which treatment with corticosteroid results in improvement in only a small proportion of patients. Several large multi-centre studies have confirmed this overall view (Burge 1999; Pauwels et al. 1999; Vestbo et al. 1999; Burge et al. 2000) and the challenge still remains to easily identify COPD patients who will obtain a beneficial response to steroids. The results of this current study show that eNO is elevated in a subset of COPD patients and suggests that these patients may have an increased response to corticosteroids in the short-term. However on the whole the response was small and it’s clinical significance uncertain. Whether these patients have any long-term benefit from the use of inhaled steroids is still unknown. Further longitudinal studies assessing the utility of eNO in predicting responses to long-term therapy with inhaled corticosteroids are required.
CHAPTER NINE:
CONCLUSIONS AND FUTURE RESEARCH

9.1 Asthma

In the studies presented in this thesis the role of eNO as a marker of airway inflammation in both controlled and uncontrolled asthma has been considered. I have reported the variation occurring in eNO measurements during normal asthma control and have assessed the ability of serial eNO to predict and diagnose poorly controlled asthma. Finally, the dose-response relationship of eNO measurements to anti-inflammatory treatment with inhaled corticosteroid therapy was evaluated. From these studies it is concluded that eNO can provide accurate and clinically important information regarding the control of airway inflammation in asthma. These findings will have important implications for both the clinical assessment of asthma control in individual patients, and the comparison of differing doses of various anti-inflammatory treatments.

Asthma patients were initially followed for a period of two to four weeks while remaining on a stable dose of maintenance inhaled corticosteroid therapy. Here eNO was found to be highly reproducible within a sitting (coefficient of variation 4.1%). An important finding during this period was a lack of relationship between eNO measured at plateau and ambient NO levels. This aids the assessment of eNO in the clinical setting as patients are not required to inhale NO-free air in order to get reliable recordings. Additionally, in this sample eNO correlated positively with height but no other baseline patient demographic. This relationship with height has not been reported before, however it is biologically plausible given that concentration of eNO measured at the mouth is dependent on the velocity of air through the airways. The faster the velocity the higher the concentration of eNO. This possible relationship with height requires further investigation in order to help standardise eNO recordings.

Serial measurements of eNO were shown to be useful in both predicting and diagnosing poor asthma control. Data obtained following inhaled corticosteroid withdrawal indicated
that an absolute value of 15ppb, an increase of more than 10ppb or a increase of 60% over baseline, are useful thresholds for the detection of ongoing airway inflammation. These cut points also positively predicted the occurrence of breakthrough symptoms. This finding is clinically important as deteriorating asthma may be identified earlier than is currently possible. Thus treatment might be altered to avoid the occurrence of significant clinical symptoms based on eNO measurements. However these results need to be interpreted with a degree of caution, as complete cessation of inhaled corticosteroid therapy, as was used in our investigation, is not consistent with current clinical practice.

Exhaled NO demonstrated a dose-response relationship over a dose range of inhaled corticosteroid therapy of 0-500μg beclomethasone/day. This relationship was linear and was present both at one week and following eight weeks treatment. Similar dose-response relationships were seen with both sputum eosinophils and lung function. These results suggest that eNO is a good marker of airway inflammation during inhaled corticosteroid therapy, as it is easily measured and responds rapidly when airway inflammation is reduced by steroid administration. This finding demonstrates that eNO may be useful for dose titration of inhaled corticosteroid therapy in individual patients and it also suggests that eNO may be used to compare the doses of differing anti-inflammatory treatments, thus enabling optimum dosing with these agents. This is a question that is currently being investigated in our research unit as a follow-up study from the research presented in this thesis.

Throughout all of these studies eNO was shown to correlate well with markers of asthma control and airway inflammation. In particular the relationship was strongest between eNO and sputum eosinophils. This relationship was present when these measurements were compared as both point-in-time and change-over-time values in the setting of deteriorating and improving asthma control. The consistency of this relationship throughout all of the studies provides further supportive evidence for the use of eNO as a marker of eosinophilic airway inflammation, both in the absence and presence of inhaled corticosteroids. A relationship between eNO and the more traditional methods of assessment of asthma control, such as FEV₁ and symptoms, is present but not as strong.
When measured as point-in-time there were no correlation between these markers and eNO. However the rise in eNO was significantly higher in the loss of control group (where loss of control had been based on traditional markers), and changes in these markers over time correlated with changes in eNO, confirming the usefulness of measuring eNO in assessing the clinical control of asthma over time. Perhaps one of the most surprising results from the studies was the lack of a consistent relationship between eNO and airway hyperresponsiveness to hypertonic saline. Although at loss of control there was a correlation between eNO and PD$_{15}$ saline there was no consistent relationship elsewhere through the studies, suggesting that these measurements are assessing slightly different aspects of the asthma phenotype. For example there was no change in PD$_{15}$ saline in any of the randomised treatment groups (over 0-500$\mu$g/day BDP), despite significant linear changes in eNO. Thus demonstrating that PD$_{15}$ is less sensitive to the effects of inhaled steroid therapy than eNO. Further long-term longitudinal studies are needed to confirm that the clinical application of eNO measurement is worthwhile in optimising asthma management, and in particular its usefulness in the presence of inhaled corticosteroid therapy.

Another aspect that needs to be considered in future research is the effect of lower exhalation rates on the clinical usefulness of eNO. During my studies eNO measurements were made during an exhalation flow rate of 250ml/sec. At the time of study commencement this was the accepted methodology, however since that time international guidelines have been developed suggesting the use of a lower flow rate of 50ml/sec. At lower flow rates there is a greater spread in eNO recordings between subjects and in particular the difference is greater between asthma and non-asthma patients. Thus it appears that the results reported in this thesis are conservative, and it is possible that at lower flow rates there is better differentiation in eNO recordings between controlled and uncontrolled airway inflammation. This may therefore improve the sensitivity of eNO and increase its clinical applicability.

The usefulness of serial eNO measurements may be greatest in patients with atopic asthma. My studies were not designed to compare the relative merits of eNO in atopic
versus non-atopic asthma, and indeed only 9 of the 87 patients were non-atopic. However at 'loss of control' eNO values in the atopic group were double those of the non-atopic group, raising the possibility that eNO may be a more responsive marker in atopic asthma. This again needs to be the subject of further research.

In summary, the optimum approach to monitoring asthma patients on inhaled corticosteroid has still not been defined. Measuring lung function does not appear to give an adequate representation of airway inflammation. Whether improved clinical outcomes are possible when monitoring specific markers of airway inflammation is still unknown. Recently it has been shown that minimising airway hyperresponsiveness improves both histological and clinical outcomes (Sont et al. 1999). Unfortunately, repeated measurements of airway hyperresponsiveness are unsuited for routine clinical practice and their widespread application seems unlikely. The same is true for induced sputum analyses. In contrast, eNO measurements are easy to perform, repeatable, and may be used to diagnose ongoing airway inflammation, predict the advent of breakthrough symptoms, and demonstrate a dose-response relationship to anti-inflammatory treatment. These findings offer the possibility that repeated measurements of eNO may be used to adjust dose of inhaled corticosteroid in patients with asthma and thus improve clinical outcomes. However this requires further prospective investigation.

9.2 COPD

The role of eNO in the assessment of COPD is less clear-cut. Exhaled NO was shown to decrease following a course of oral prednisone and this decrease was seen in association with improvements in functional ability. Furthermore, a low eNO at baseline predicted lack of significant functional improvement following prednisone, indicating that those patients with low eNO are unlikely to benefit from corticosteroid therapy. This may have some clinical application as it could help prevent the unnecessary usage of steroids in this group of patients. However, eNO was poor in predicting a positive response to steroids in COPD. This may be due to heterogeneity in the underlying inflammatory process. As no sputum samples were obtained during this study assessment of the type of inflammation and relative responses was not possible. Alternatively the poor predictive value of eNO in
COPD may have been due to the relatively short duration of treatment. The role of eNO in COPD deserves fuller exploration; in particular long-term clinical benefits and the identification of the different types of inflammation need to be assessed.
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Appendix

This thesis is based on work performed while I was Respiratory Research Fellow in the Department of Respiratory Medicine, Dunedin School of Medicine, University of Otago. It represents two years of full time data collection and further two-and-a-half years of part-time writing up.

Current publications in peer-reviewed journals include:


Conference proceedings in which data has been presented are:

The Predictive Value of Exhaled Nitric Oxide Measurements in Assessing Changes in Asthma Control

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Exhaled nitric oxide (eNO) levels are increased in untreated or unstable asthma and measurements can be made easily. Our aim was to assess the usefulness of eNO for diagnosing and predicting loss of control (LOC) in asthma following steroid withdrawal. Comparisons were made against sputum eosinophils and airway hyperresponsiveness (AHR) to hypertonic saline (HSC) in 45 adults with asthma. Seventy-eight patients with mild to moderate asthma had their inhaled steroid therapy withdrawn until LOC occurred or for a maximum of 6 wk. Sixty (77.9%) developed LOC. There were highly significant correlations between the changes in eNO and symptoms (p < 0.0001), FEV1 (p < 0.0002), sputum eosinophils (p < 0.0002), and saline PD20 (p < 0.0002), and there were significant differences between LOC and no LOC groups. Both single measurements and changes of eNO (10 ppb, 15 ppb, or an increase of > 60% over baseline) had positive predictive values that ranged from 80 to 90% for predicting and diagnosing LOC. These values were similar to those obtained using sputum eosinophils and saline PD20 measurements. We conclude that eNO measurements are as useful as induced sputum analysis and AHR in assessing airway inflammation, with the advantage that they are easy to perform.

Keywords: asthma; exacerbation; nitric oxide; eosinophils; bronchial provocation tests

Nitric oxide is a key messenger for cell to cell signaling, and has an important role in the biochemistry of inflammation (1, 2). Exhaled nitric oxide (eNO) has been confirmed as a marker of airway inflammation and is present in higher concentrations in steroid-naïve asthma compared with normal control subjects (3). Higher levels of eNO are seen during asthma exacerbations (4), and decreases occur following treatment with both inhaled (5) and systemic corticosteroids (6). Furthermore eNO appears to be sensitive to changes in antiinflammatory treatment, even in the absence of changes in lung function (7). These findings suggest that eNO may be a useful indicator in the longitudinal assessment of asthma control.

Both induced sputum cell counts (8) and responsiveness to hypertonic saline challenge (HSC) (9) have also been investigated as markers of airway inflammation in asthma. Sputum eosinophil numbers increase during asthma exacerbations (10).

Conversely, a decrease in the percentage of sputum eosinophils occurs following prednisone treatment (11), and after commencing inhaled corticosteroid (ICS) (12). Du Toit and coworkers have demonstrated a progressive reduction in responsiveness to HSC following initiation of ICS treatment (13). However, the scope for using these techniques to monitor asthma control in clinical practice is limited by the resources required for repeated measurements. In contrast the measurement of eNO is quick and easy to perform, thus lending itself to repeated measurements over time. Although single measurements of eNO have been used to assess airway inflammation in asthma (14-17), the usefulness of eNO in the longitudinal assessment of asthma control has not been extensively investigated. To be clinically useful eNO would need to correlate with known markers of asthma control as well as airway inflammation, and be responsive to changes in these parameters over time. If this were the case, then eNO measurements could be used to confirm poorly controlled asthma and to predict imminent deterioration. There might also be a role for eNO in optimizing antiinflammatory therapy such as has been done using measurements of airway hyperresponsiveness (AHR) (18).

The aim of our study was to evaluate the predictive and diagnostic value of eNO in unstable asthma and to correlate this with sputum eosinophils and AHR to hypertonic saline. Using a model of steroid withdrawal described by Gibson and coworkers (19), we aimed to induce a deterioration in asthma control in the majority of patients thus enabling us to assess these parameters in the context of increasing degrees of airway inflammation.

METHODS

Subjects

Patients with mild to moderate asthma, confirmed at our research screening clinic using ATS criteria (20), and who had been taking ICS therapy for at least 6 mo were recruited. The dose of ICS was unchanged for at least 6 wk. Patients were excluded (because the study involved withdrawal of ICS treatment) if they had a history of acute asthma requiring hospital admission, asthma characterized by sudden attacks, or used oral prednisone during the previous 3 mo.

Study Design

ICS treatment was stopped following a 2- to 4-wk run-in during which the maintenance dose remained unchanged. Patients were then reviewed weekly until loss of control (LOC) developed or for a maximum of 6 wk. When LOC occurred, patients were seen within 24 h. The visit at which ICS therapy was stopped was designated visit 1, the final visit of the study was designated visit F, and the visit immediately prior to the final visit was designated visit P (penultimate).

Criteria for LOC were as follows:

1. A fall in the mean (over last 7 d) morning peak expiratory flow rate (PEFR) of greater than 10% from baseline, or a fall in either morning or evening PEFR on two consecutive days to 80% of baseline or less,
Jones, Kittelson, Cowan, et al.: Predictive Value of eNO

2. Mean daily bronchodilator use of greater than three puffs more than during run-in, or
3. Nocturnal waking with asthma symptoms on three nights or more per week greater than during the run-in, or
4. Asthma symptoms that were disagreeable or distressing.

Study Procedures

Diurnal PEFR, bronchodilator use, and symptom scores were recorded in a daily record card. Measurements obtained at each study visit are shown in Table 1. eNO was measured prior to all other study procedures using a calibrated chemiluminescence analyzer with on-line measurement of single exhalations according to a standard protocol (21, 22), with the exception of flow rate (250 ml/s). eNO levels were read at the plateau corresponding to 70-80% of the CO2 curve. Spirometry was measured using a rolling seal spirometer.

AHR to hypertonic saline (4.5%) was measured using a modified standardized protocol (23, 24). Spirometry was performed 1 min after saline nebulization, and patients were encouraged to produce sputum between inhalations. The challenge was discontinued when a 20% fall in FEV1 occurred or a cumulative inhalation time of 20 min was reached. The PD15 was calculated as the cumulative dose of saline causing a 15% fall in FEV1.

If a 20% fall in FEV1 occurred salbutamol was administered and sputum induction continued until an adequate sputum sample was obtained. The whole specimen (sputum plus saliva) was analyzed using a standardized method (25). Cell viability was assessed by the trypan blue exclusion test, and a cell count was performed by hemocytometer. Cytospin slides were stained with May-Grunwald-Giemsa stain and a total of 400 nonnucleus cells were counted on two occasions. Where the difference between the two counts was greater than 10% for any cell type then the count was repeated twice more and the mean for all four was recorded.

Ethical Considerations and Safety

Each patient's asthma control was monitored closely throughout the study. All patients were provided with an individualized self-management plan, an emergency card, and a supply of prednisone tablets. Patients had 24-h access to one of the study investigators via the hospital paging system. For ethical reasons, LOC criteria included symptoms that were disagreeable or distressing irrespective of PEFR changes.

Ethical approval was obtained from the Otago Ethics Committee and informed consent was obtained from all study participants.

Statistical Analysis

Sources of variability in eNO measurements during run-in were estimated using variance components methods (26). Associations between eNO and other measurements were analyzed using the rank correlation coefficient because it does not depend on the measurement scale. Regression methods were used to compare airway inflammation parameters in patients who lost control with those who did not, and to adjust the comparisons for baseline differences. The prognostic and diagnostic utility of eNO was evaluated and compared with other measures using methods for constructing receiver-operator characteristic (ROC) curves (27). Exhaled nitric oxide and PD15 measurements were analyzed using logarithmic transformations to remove the skew in these data; other measurements were analyzed without transformation. The results of these analyses did not change following the transformations.

RESULTS

Run-in Data

Seventy-eight patients entered the study. Demographic data are given in Table 2. The mean eNO during run-in was 9.38 ppb (95% reference range 2.72-32.35). The coefficients of variation for eNO were measured over four visits during the run-in phase of the study. The within-patient within-sitting coefficient of variation was 4.1% and the within-patient between-sitting variation was 16.5%. eNO was not related to ambient NO (range 0.0-234 ppb, p = 0.25) and so no corrections were made for ambient NO measurements.

Loss of Control

Sixty patients (77.9%) developed loss of control according to predetermined study criteria. The median time to LOC was 17 days (95% CI: 14, 28). Twenty-two patients developed LOC within 1 wk of corticosteroid withdrawal. The frequencies with which LOC criteria were met were fall in PEFR, 40; increased bronchodilator use, 15; increased nocturnal waking, 10; distressing symptoms, 39. Nineteen patients had LOC on the basis of distressing symptoms alone, two on the basis of increased reliever use alone. Thirty-two patients fulfilled two or more criteria at the time of LOC. Sputum induction was performed in all patients at visits 1 and F. Adequate sputum samples were obtained in 71 of 77 patients at visit 1 (92%), and in 54 (90%) and 15 (88%) of the LOC and no LOC groups at visit F, respectively.

The LOC group experienced a 2.16-fold increase in eNO between visits 1 and F, which was significantly greater than the 1.44-fold increase for the no LOC group (p = 0.001) (Table 3). There were also significant differences between LOC and no LOC groups for the fall in mean morning PEFR (13% versus 1%, p < 0.0001) the decrease in FEV1 (mean fall of 11.9% predicted compared to 2.6% predicted, p = 0.001), the increase in sputum eosinophils (4.73-fold increase compared with 2.05-fold, p = 0.044), and the decrease in saline PD15 (0.8 doubling doses compared with 0.03 doubling doses, p = 0.001).

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TABLE 1. MEASUREMENTS OF EXHALED NITRIC OXIDE, SPIROMETRY, HYPERTONIC SALINE CHALLENGE, AND SPUTUM ANALYSIS AT VARIOUS STUDY VISITS*  

<table>
<thead>
<tr>
<th>Visit Number</th>
<th>R1</th>
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<th>R3</th>
<th>R4</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>V4</th>
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<tr>
<td>eNO</td>
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<td>x</td>
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* Definition of abbreviations: eNO = exhaled nitric oxide; F = final; HSC  = hypertonic saline challenge; ICS = inhaled corticosteroid; LOC = loss of control; p = penumetane; R = run-in; V = visit.

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TABLE 2. DEMOGRAPHIC DATA FOR STUDY POPULATION MEANS*  

| Number of patients, n | 78 |
| Age, yr              | 30.48 |
| Duration of asthma, yr | 25.9 (range 18-74) |
| Skin test positive, n | 69 (88%) |
| Ex-smokers/nonsmokers | 12:86 |
| ICS dose (mg/d)       | 6.0 |
| FEV1, % predicted     | 2.88 (2.0, 3.06) |
| FEV1/FVC, %           | 92.0 (87.9, 96.1) |
| FEV1 (range 100-1600) | 71.0 (68.3, 73.7) |

* Definition of abbreviations: ICS = inhaled corticosteroid.

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* Unless stated otherwise, figures in parentheses are 95% confidence intervals. Ex-smokers had not smoked for more than 1 yr and had a smoking history of < 5 pack-years.
The ability of FEV₁ to predict upcoming LOC was assessed in three ways: first, using the baseline FEV₁ measurement (visit 1), second, using the measurement of eNO at the visit immediately prior to LOC (the penultimate visit, visit P), and third, using the change in eNO that occurred between visit 1 and visit P. Analysis of receiver-operator curves (ROCs) demonstrated parity between these different approaches. The curves were similar whether absolute or proportional changes in eNO were used. The sensitivities, specificities, and positive and negative predictive values at relevant cut points also showed that no one prognostic indicator was clearly superior (Table 5). Specific eNO cut points evaluated included 10 ppb, 15 ppb, and a 60% increase over the baseline mean (the upper limit of the 95% reference range for weekly variation in eNO over the run-in period).

The prognostic utility of eNO was also compared with other indices of airway inflammation, specifically single measurements of responsiveness to hypertonic saline (PD₉₀ less than 12 ml) and sputum eosinophils (greater than 4% obtained at visit 1) (Figure 1, top panel, and Table 3). Compared with eNO, no measurement was clearly superior. Similarly, the prognostic value of changes in FEV₁, % predicted, daily PEFR variation, symptom scores, and bronchodilator use between visit 1 and visit P was evaluated. None of these clinical parameters was found to be superior to eNO in predicting LOC (Figure 1, bottom panel). The ability of eNO to diagnose LOC was also assessed using eNO measurements at visit F. Both the single measurements of eNO at visit F and the change between visit 1 and visit F were evaluated. As for the assessment of prognostic utility, there was no clearly superior eNO measurement, and the performance of eNO was comparable to that based on sputum eosinophil counts and saline PD₉₀ measurements.

**DISCUSSION**

This is the largest longitudinal study to date in which the utility of eNO measurement in asthma management has been assessed. Our results document the usefulness of eNO for predicting and diagnosing poorly controlled asthma compared with other currently used markers of airway inflammation and clinical parameters. Regardless of the way in which eNO measurements were analyzed (absolute values, absolute changes, or proportional changes from baseline) the results were similar. eNO was associated with a positive predictive value (PPV) of between 80 and 90% for predicting and diagnosing LOC. On the whole, changes in eNO over time had higher PPV, sensitivities, and specificities both for predicting and diagnosing LOC than did single measurements. For example, an increase in eNO between visit 1 and visit P of more than 60% over baseline had a PPV for predicting LOC of 83% (sensitivity 50%, specificity 65%). A similar increase between visit 1 and visit F had a PPV for diagnosing LOC of 87% (sensitivity 68%, specificity 65%). In comparison, for a single measurement of greater than 15 ppb obtained at visit 1, when patients were still...
TABLE 5. PREDICTIVE VALUE OF eNO

<table>
<thead>
<tr>
<th>Change in eNO from visit 1 to visit 2</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10 ppb</td>
<td>0.50</td>
<td>0.53</td>
<td>0.79</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt; 10 ppb</td>
<td>(0.37, 0.63)</td>
<td>(0.28, 0.77)</td>
<td>(0.63, 0.90)</td>
<td>(0.11, 0.39)</td>
</tr>
<tr>
<td>&gt; 15 ppb</td>
<td>0.25</td>
<td>0.88</td>
<td>0.88</td>
<td>0.23</td>
</tr>
<tr>
<td>(0.13, 0.38)</td>
<td>(0.64, 0.99)</td>
<td>(0.64, 0.99)</td>
<td>(0.15, 0.38)</td>
<td></td>
</tr>
<tr>
<td>eNO at visit P</td>
<td>0.65</td>
<td>0.80</td>
<td>0.80</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt; 10 ppb</td>
<td>(0.52, 0.77)</td>
<td>(0.18, 0.67)</td>
<td>(0.66, 0.90)</td>
<td>(0.11, 0.45)</td>
</tr>
<tr>
<td>&gt; 15 ppb</td>
<td>0.65</td>
<td>0.83</td>
<td>0.83</td>
<td>0.27</td>
</tr>
<tr>
<td>(0.37, 0.63)</td>
<td>(0.38, 0.86)</td>
<td>(0.67, 0.94)</td>
<td>(0.14, 0.43)</td>
<td></td>
</tr>
<tr>
<td>Percentage eosinophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at visit 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 4%</td>
<td>0.21</td>
<td>0.80</td>
<td>0.80</td>
<td>0.21</td>
</tr>
<tr>
<td>(0.12, 0.34)</td>
<td>(0.52, 0.96)</td>
<td>(0.52, 0.96)</td>
<td>(0.12, 0.34)</td>
<td></td>
</tr>
<tr>
<td>Saline PDL at visit 1</td>
<td>0.53</td>
<td>0.77</td>
<td>0.77</td>
<td>0.25</td>
</tr>
<tr>
<td>&gt; 12 ml</td>
<td>(0.38, 0.67)</td>
<td>(0.25, 0.75)</td>
<td>(0.60, 0.90)</td>
<td>(0.11, 0.43)</td>
</tr>
</tbody>
</table>

*Definition of abbreviations: eNO = exhaled nitric oxide; PDL = dose of saline causing a 15% fall in FEV1.

significance of between eNO and sputum eosinophils (rank correlation 0.62, p < 0.0001) and saline PDL (rank correlation 0.41, p < 0.0008) at a single point in time (visit 1) were obtained, although the correlations for symptoms and lung function were nonsignificant (see Table 4). More importantly, when measured longitudinally the changes in eNO correlated significantly not only with changes in the other markers of airway inflammation but also with measurements of airway caliber and symptoms (see Table 4). These findings provide additional support for the use of eNO measurements as a tool in the assessment of airway inflammation and long-term asthma control.

Our study was designed to assess the usefulness of eNO in a clinical context. For this reason, loss of control criteria were prospectively based on a combination of peak flow and symptom changes as used in clinical practice. Evidence that these criteria were relevant and appropriate is provided by the significantly greater changes in FEV1, sputum eosinophils, and saline PDL, seen in the LOC group. Thus the subsequent comparisons between LOC and no LOC groups were valid. During our study eNO was measured using an exhalation flow rate of 250 mls, which is significantly more than current guidelines suggest. Our study was commenced before these guidelines were published. eNO has been shown to be flow dependent (29) and at lower flow rates the differences between healthy and asthmatic individuals are increased (31). Thus it is possible that at a lower expiratory flow rate (e.g., 50 mls) differences between patients who did and did not experience LOC might have been greater. If this were the case, then the sensitivities and specificities for eNO as a diagnostic test may be better than what we have reported.

A number of investigators have sought to evaluate the role of eNO in assessing long-term asthma control. In the present study we chose to withdraw ICS in an attempt to induce a deterioration in asthma control and mimic an exacerbation. This implies that our findings may not strictly apply to patients receiving maintenance ICS. However, the usefulness of eNO in the presence of ICS is supported by Stirling and coworkers who found higher levels of eNO in patients with greater asthma severity irrespective of steroid use (17). Further, Khartonov and coworkers have demonstrated that a simple reduction in the dose of ICS increases eNO (7) even in the absence of significant change in peak flow variability or spirometry. Using a study design similar to our own, but with incomplete withdrawal of ICS, Jatakanon and colleagues (31) reduced the dose of inhaled budesonide to 200 μg/day (less than one-fourth of the usual maintenance dose) in 15 patients with asthma.

Patients were followed for a maximum of 8 wk. Just under half developed an exacerbation. There was a parallel increase in eNO and sputum eosinophils, which correlated over time with changes in FEV1 and β-agonist use. The authors suggested that changes in sputum eosinophil numbers were superior to eNO in predicting loss of asthma control. However, because of the small study numbers a quantitative assessment of the predictive values of the changes in eNO and sputum eosinophils was not possible. In another longitudinal study, Baraldi and coworkers (32) measured eNO in children with asthma before, during, and after the pollen season. A rise in eNO was seen during the pollen season consistent with the presumed increase in airway inflammation that occurred with allergen exposure. This occurred despite the fact that over one third of the children were taking inhaled steroids and in the absence of changes in FEV1. These authors also concluded that eNO might be useful in the longitudinal assessment of asthma.

Asthma is a complex disease whose symptoms are dependent on the severity of airway inflammation, airway remodeling, and AHR. There is no firm agreement as to whether therapeutic...
intervention ought to be aimed at controlling symptoms alone, optimizing lung function, or minimizing airway inflammation, and hyperresponsiveness (33–35). A recent study has provided evidence that when antiinflammatory therapy is tailored to improve AHR, clinical outcomes are also improved (18). Our results suggest that a similar approach may be valid for eNO. Our data indicate that at an exhalation flow rate of 250 ml/s, an absolute value for eNO of 15 ppb or greater, or an increase of more than 10 ppb or 80% over baseline, is a useful threshold for the detection of ongoing airway inflammation, and that it also positively predicts the advent of breakthrough symptoms. Unfortunately, the absence of these changes does not preclude the possibility of deteriorating asthma. Further studies are needed to confirm that the clinical application of eNO measurement is worthwhile in optimizing asthma management.

**References**

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Does Exhaled Nitric Oxide Reflect Asthma Control?
Yes, It Does!

There has been an explosion of research into exhaled nitric oxide (eNO) since levels were found to be increased in patients with asthma. But what is the clinical value of eNO measurements in asthma? We are edging closer to the answer after the timely study by Jones and colleagues published in this issue (1).

The key findings of this article are that eNO measurements have a positive predictive value of between 80 and 90% for predicting and diagnosing loss of control in asthma, and are as useful as induced sputum eosinophils and airway hyperresponsiveness to hypertonic saline, but with the enormous advantage that they are easy to perform. In addition, it has been shown that the changes in eNO were strongly related to asthma symptoms and FEV₁, and that the levels of eNO were significantly different in patients with and without loss of control.

This is the largest longitudinal study (11 wk) to date in which the utility of repeated (once a week for 7 wk) eNO, symptoms, and spirometry measurements has been explored in 78 patients with predominantly atopic asthma. Patients maintained a good lung function (FEV₁ 92% predicted) on inhaled corticosteroids of 630 μg/d (range 100–1600 μg beclomethasone equivalent) during a 4-wk run-in period before their steroid treatment was stopped. Although a placebo-controlled study would be a better choice, the current design was simple and sufficient to pick up 78% of the patients with deterioration of their asthma within 6 wk after the cessation of steroid treatment. The median time to loss of control was 17 d, and the most frequent criteria of the loss were fall in peak expiratory flow and symptoms.

This study is a natural continuation of previous studies, which convincingly demonstrated that eNO is a useful marker of airway inflammation (2). It seems, however, that eNO reflects asthma control better than asthma severity (3). Thus, it is elevated in mild asthma, but it is almost normal in stable moderate asthma adequately treated with corticosteroids (4). However, eNO levels are often further elevated in patients with severe (5) and uncontrolled asthma (6).

An advantage of eNO as a “loss-of-control marker” (7) is that an increase in eNO and asthma symptoms may be seen before any significant deterioration in airway hyperresponsiveness, sputum eosinophils, or lung function during asthma exacerbation induced by steroid reduction (8, 9). Recently, it has been confirmed that eNO is closely related to several markers of asthma control, such as asthma symptoms, dyspnea score, daily use of rescue medication, and reversibility of airflow obstruction (6).

The results were less conclusive when single baseline eNO measurements instead of serial assessments were used. Although the high number of sputum eosinophils of patients who eventually develop exacerbations was a good predictor of asthma deterioration, the changes in eosinophils following steroid reduction were slow and insignificant (9). Similarly, a single baseline assessment of either exhaled NO (9, 10) or sputum eosinophils (10) had a low power to predict asthma deterioration during the reduction of steroid treatment.

Unfortunately, one of the main advantages of serial eNO measurements has not been fully exploited in the latter study. This is because monitoring of eNO was performed at 2, 4, and
6 mo after the dose of corticosteroids was halved (10). However, it is well known that changes in eNO can be seen within 3-5 d following either introduction (11) or discontinuation of steroids (3), and a different study design with more frequent measurements would have been more informative.

Jones and coworkers have realized the importance of assessment of the changes in eNO when measured longitudinally. Thus, eNO was measured every week for 6 wk after steroid treatment was stopped, in contrast to sputum eosinophils and airway hyperresponsiveness, which were measured twice only, at baseline and at the final visit. Importantly, the authors have calculated the differences and correlations between the changes in eNO that occurred between the first visit at which inhaled corticosteroids treatment was stopped, and the final visit at which loss of control developed, but no longer than 6 wk. In addition, the ability of eNO to predict upcoming loss of control was assessed in three ways: first, using the baseline eNO: second, using eNO measurement immediately before loss of control (the penultimate visit); and third, using the change in eNO that occurred between baseline and the penultimate visit.

The finding that changes in eNO measured over time have higher predictive values, sensitivities, and specificities both for predicting and diagnosing loss of control than did single measurements clearly indicates the need for repeated tests. When measured longitudinally the changes in eNO correlated significantly not only with changes in sputum eosinophils and hyperresponsiveness, but also with lung function and asthma symptoms.

The utility of eNO measurements in relation to sputum analysis or airway hyperresponsiveness is important. Methodologically, standardized eNO measurements (12) have obvious advantages over sputum induction and airway hyperresponsiveness or any other bronchial provocation tests due to their simplicity, reproducibility, and entirely noninvasive nature. It is vital that this technique can be used repeatedly in patients with severe disease and to assess disease in children, making it a suitable test for use in the clinical as well as the research practice. The scope for using sputum analysis to monitor asthma control in clinical practice is limited not only by the resources required, but also by the well-documented proinflammatory action of hypertonic saline.

In conclusion, the available evidence suggests that eNO, especially when repeated, as longitudinal measurements, reflects control of asthma. Because the technique is noninvasive, it is possible to make repeated measurements without disturbing the system, in contrast to the invasive or semiinvasive procedures currently used. Individual eNO values, like individual peak expiratory flows, should be established and monitored, and when the levels are above or below a certain reference level, steroid treatment should be either reduced or increased. We clearly need further clinical research on eNO to be able to tailor strategies for effective treatment and early intervention in asthma. As eNO analyzers become more widely available and miniaturized, it is likely that this measurement will become routine in monitoring asthma control, particularly in patients with unstable and difficult to control asthma.

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Exhaled NO and assessment of anti-inflammatory effects of inhaled steroid: dose-response relationship


ABSTRACT: Exhaled nitric oxide (eNO) is an easily measured marker of airway inflammation. This study was undertaken to evaluate the usefulness of serial eNO in investigating the dose-response relationship for inhaled beclomethasone (BDP), and to compare eNO with other markers of airway inflammation.

Following withdrawal of inhaled corticosteroid (ICS) therapy, 65 patients entered a double-blind, parallel-group, placebo-controlled trial of 50, 100, 200 or 500 μg BDP·day⁻¹ for eight weeks. eNO and spirometry were performed weekly and a hypertonic saline challenge with sputum induction was performed at the beginning and end of treatment.

The relationship between the dose of ICS and changes in eNO and forced expiratory volume in one second (FEV₁) was linear at 1 week and at the end of treatment. A linear dose-response relationship was also seen for sputum eosinophils. Changes in eNO correlated significantly with changes in sputum eosinophils. Changes in the provocative dose of saline causing a 15% fall in FEV₁ saline did not differ across the treatment groups nor did they correlate with changes in other measurements.

Exhaled nitric oxide may be used to assess the dose-response relationship for the anti-inflammatory effects of inhaled beclomethasone. The relationship found in this study was linear over the dose range 0–500 μg·day⁻¹ soon after commencing therapy and continued over time.


Inhaled corticosteroids (ICS) are the most effective treatment for airway inflammation in asthma [1]. Their use results in an improvement in symptoms and lung function, as well as in reductions in inflammatory cells in bronchial biopsies [2] and induced sputum [3]. As a result, airway hyperresponsiveness (AHR) is also reduced [4, 5]. Current guidelines recommend the use of ICS therapy in all but the mildest asthma [6]. The guidelines are based on the assumption that treating airway inflammation leads to improvement in asthma control. However, several studies have demonstrated incongruities in the relationship between both symptoms and lung function and airway inflammation [7–9]. This raises questions as to whether the primary goal when using ICS is to achieve symptom control, optimise lung function, minimise airway inflammation or reduce AHR.

In the past, dose-response relationships for ICS have been described using symptoms and lung function as the main outcome variables. Although a dose-response relationship exists for these end points, it may plateau at a relatively low dose. Thus, there may be some difficulty in differentiating between the effects of adjacent doses [1, 10]. Other studies have shown that dose-response relationships vary when other end points are used [11]. For example, much higher doses of ICS are required to control airway hyperresponsiveness than symptoms [11, 12]. There are fewer data to clarify these relationships for airway inflammation, even though control of airway inflammation is the primary reason for administering these drugs. Such information might be clinically helpful in assessing the relative efficacy of different ICS doses and as a guide to optimising long-term therapy.

Exhaled nitric oxide (eNO) is a repeatable, easily measured marker of airway inflammation [13, 14]. High levels are seen in both steroid-naïve asthma [15] and during acute exacerbations [16, 17]. Furthermore, levels decrease following treatment with both inhaled [3, 18, 19] and oral steroids [20]. These results suggest that eNO may be a useful end point in measuring the dose-response relationship for the effect of ICS therapy on airway inflammation.

Recently the current authors have confirmed the usefulness of eNO measurements in predicting deterioration in asthma control when ICS are withdrawn [17]. In this follow-up study the aim was to evaluate the usefulness of serial eNO measurements in measuring the anti-inflammatory effects of different doses of inhaled beclomethasone (BDP), and to make comparisons...
with other end points including lung function, sputum eosinophils and AHR to hypertonic saline.

Methods

Subjects

Patients with mild-to-moderate asthma [21] on maintenance ICS therapy were recruited. Patients were excluded (because the study involved withdrawal of ICS treatment) if they had a history of acute asthma requiring hospital admission, asthma characterised by sudden attacks, or had used oral prednisone during the previous 3 months.

Study design

Following run-in, ICS therapy was discontinued. Patients were reviewed weekly until “loss of control” developed, or for a maximum of 6 weeks. Those who did not develop loss of control were then withdrawn. Loss of control was defined by predetermined criteria [17] consisting of: 1) a fall in the mean (over the last 7 days) morning peak expiratory flow rate (PEFR) of >10% from baseline, or a fall in either morning or evening PEFR on two consecutive days to <80% of baseline; or 2) mean daily bronchodilator use of >3 puffs longer than during run-in; or 3) nocturnal wakening with asthma symptoms on >3 nights-week<sup>-1</sup> greater than during the run-in; or 4) asthma symptoms which were disagreeable or distressing.

Following loss of control, patients received 20 mg prednisone orally for 2 days in order to alleviate their deteriorating symptoms. Although this may theoretically have influenced interpretation of subsequent ICS effects at 1 week, this strategy was necessary for reasons of safety. Patients were randomised to receive double-blind ICS treatment for 8 weeks, taking one puff from two identical metered-dose inhalers (Autohaler; 3M Pharmaceuticals, St Paul, MN, USA) labelled “morning” and “evening”. The treatments were: placebo (placebo twice daily b.i.d); 50 µg·day<sup>-1</sup> (50 µg BDP in the morning, placebo at night); 100 µg·day<sup>-1</sup> (100 µg BDP in the morning, placebo at night); 200 µg·day<sup>-1</sup> (100 µg BDP b.i.d); 500 µg·day<sup>-1</sup> (250 µg BDP b.i.d).

Those patients who experienced significant worsening of their asthma while taking the randomised medication were withdrawn from that treatment group, given 20 mg prednisone orally for a further 2 days, and entered into an open label treatment arm of 1,000 µg·day<sup>-1</sup> (2 puffs of 250 µg BDP b.i.d).

Following randomisation (visit 1), patients were reviewed weekly for the first 4 weeks (visits 2–5) and then at the end of treatment (8 weeks, visit 6). Those patients whose ICS dose needed to be increased to 1,000 µg·day<sup>-1</sup> were also reviewed weekly for 4 weeks and again at the end of 8 week’s treatment.

Study procedures

Diurnal PEFR, bronchodilator use and symptom scores were recorded on a daily record card. Measurements of eNO and spirometry were made at each study visit and a hypertonic saline challenge with sputum induction was performed at the beginning and end of treatment.

Exhaled nitric oxide. eNO was measured prior to all other study procedures using a calibrated chemiluminescence analyser with on-line measurement of single exhalations according to a standard protocol [13, 14], with the exception of flow rate (250 mL·s<sup>-1</sup>) (the study was commenced prior to the publication of consensus guidelines). eNO levels were read at the plateau corresponding to 70–80% of the carbon dioxide curve. These readings were made at a later date by a person blinded to the patient’s clinical status.

Hypertonic saline challenge. AHR to hypertonic saline (4.5%) was measured using a modified standardised protocol [22, 23]. Spirometry was performed 1 min after each saline nebulisation. The challenge was discontinued when a 20% fall in forced expiratory volume in one second (FEV<sub>1</sub>) occurred or a cumulative inhalation time of 20 min was reached. The PD<sub>15</sub> was calculated as the cumulative provocation dose of saline causing a 15% fall in FEV<sub>1</sub>.

Sputum induction. During the hypertonic saline challenge patients were encouraged to produce sputum between nebulisations. If a 20% fall in FEV<sub>1</sub> occurred before an adequate sputum sample was obtained, inhaled salbutamol was administered and sputum induction was continued for a maximum cumulative time of 20 min. Once an adequate sputum sample was obtained the whole specimen (sputum plus saliva) was analysed using a standardised method [24]. Cytospin slides were stained with May–Grunwald–Giemsa stain and a total of 400 nonsquamous cells were counted on two occasions. Where the difference between the two counts was >10% for any cell type then the count was repeated twice and the mean for all four was recorded.

Ethical considerations and safety

Each patient’s asthma control was monitored closely throughout the study. For ethical reasons, loss of control criteria included symptoms that were “disagreeable or distressing” irrespective of PEFR changes. All patients were provided with an individualised self-management plan, an emergency card, and a supply of prednisone tablets. Patients had 24-h access to one of the study investigators via the hospital paging system. In addition, recognising that ICS withdrawal had the potential to result in poor asthma control, each patient was telephoned by a study investigator on days 1 and 3 following randomisation. Ethical approval was obtained from the Otago Ethics Committee and informed consent was obtained from all study participants.
Statistical analysis

The primary outcome of the study was to assess whether a dose-response relationship exists between inhaled corticosteroid dose in the range 0–500 μg·day⁻¹ and changes in eNO. Comparisons were made with sputum eosinophils, FEV₁ and PD₁₅ saline. Data were analysed on an intention-to-treat basis using analysis of covariance, adjusting for values at randomisation and for patients who for reasons of safety were later switched during the active treatment phase to receive 1000 μg·day⁻¹. Polynomial contrasts were fitted across the randomisation groups to establish the nature of the dose-response relationships. PD₁₅ saline values were analysed as doubling-dose change from baseline. Estimated marginal means were calculated, and post-hoc analysis of pairwise between-dose comparisons were made, with Bonferroni adjustments for multiple comparisons. Pearson’s correlations were calculated to assess the relationship between sputum eosinophils, FEV₁ and PD₁₅ saline at randomisation and at the end of the study.

At the time the study was designed there was no information on which to make power calculations. Based on anecdotal evidence it was calculated that 15 patients per group would offer sufficient power to detect a linear trend across the groups, this was not determined a priori.

Results

Randomisation

Eighty-seven patients entered the study. Three patients withdrew consent prior to ICS withdrawal. Sixty-five patients (77%) developed loss of control and were randomised into one of the five treatment groups. Demographic data are given in table 1. Data for study end points measured at baseline (before withdrawal of ICS, visit 0) and at randomisation (visit 1) are shown in table 2.

Treatment withdrawals

One patient randomised to receive 500 μg·day⁻¹ was withdrawn at visit 1 due to the severity of their asthma, and was not considered further in the analysis. Twenty-five of the remaining 64 patients developed worsening of their asthma after randomisation and were entered into the open-label, 1,000 μg·day⁻¹ arm of the study. The number of patients withdrawn from each of the treatment groups was six, five, eight, four, and two in the placebo, 50, 100, 200, and 500 μg·day⁻¹ BDP groups, respectively. Comparisons between those who withdrew from the randomised treatment arm and those who completed treatment are shown in table 2. There were no significant differences between these two groups for eNO, sputum eosinophils or PD₁₅ saline at the time of randomisation. Those who withdrew had a lower FEV₁ % predicted than those who did not withdraw (73.9 versus 82.5%, respectively, p=0.005). Those who withdrew also had a higher dose of maintenance ICS at study entry (mean (95% confidence interval (CI) 776 (635–917) μg·day⁻¹ and 614 (536–694) μg·day⁻¹ respectively, p=0.042).

Dose-responses

Changes in eNO and FEV₁ (at 1 week and at the end of treatment) and in sputum eosinophils, and

Table 1.—Demographic data for the randomised study participants

<table>
<thead>
<tr>
<th>Total</th>
<th>Placebo</th>
<th>50 μg·day⁻¹</th>
<th>100 μg·day⁻¹</th>
<th>200 μg·day⁻¹</th>
<th>500 μg·day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients n</td>
<td>65</td>
<td>13</td>
<td>14</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Number switched to 1000 μg·day⁻¹</td>
<td>25</td>
<td>6</td>
<td>5</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Age yrs (range)</td>
<td>42.4</td>
<td>42.5</td>
<td>43.3</td>
<td>44.7</td>
<td>41.2</td>
</tr>
<tr>
<td>Duration of asthma yrs (range)</td>
<td>27.0</td>
<td>30.3</td>
<td>30.6</td>
<td>22.5</td>
<td>31.2</td>
</tr>
<tr>
<td>Skin test positive n (%)</td>
<td>60 (92.3)</td>
<td>12 (92.3)</td>
<td>14 (100)</td>
<td>12 (85.7)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>ICS dose μg·day⁻¹ (BDP equivalent)</td>
<td>658</td>
<td>731</td>
<td>550</td>
<td>700</td>
<td>671</td>
</tr>
<tr>
<td>FEV₁ L</td>
<td>2.81</td>
<td>2.50</td>
<td>2.91</td>
<td>2.94</td>
<td>2.62</td>
</tr>
<tr>
<td>FEV₁ % pred</td>
<td>86.3–95.2</td>
<td>71.3–96.1</td>
<td>82.1–99.8</td>
<td>82.2–101.5</td>
<td>71.6–98.8</td>
</tr>
<tr>
<td>FEV₁/FVC %</td>
<td>70.8</td>
<td>68.3</td>
<td>70.6</td>
<td>69.1</td>
<td>69.7</td>
</tr>
</tbody>
</table>

Data are presented as mean (95% confidence intervals) unless otherwise stated. ICS: inhaled corticosteroids; BDP: beclomethasone; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity.
Table 2.—Data for study end-points measured at baseline (visit (V) 0) and at randomisation (V1)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>eNO ppb</th>
<th>FEV1 %pred</th>
<th>Sputum eosinophils %</th>
<th>PD15 saline mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V0</td>
<td>V1</td>
<td>V0</td>
<td>V1</td>
</tr>
<tr>
<td>Treatment group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>13</td>
<td>9.6</td>
<td>20.9</td>
<td>85.4</td>
</tr>
<tr>
<td>50 μg·day⁻¹</td>
<td>14</td>
<td>(6.4-12.7)</td>
<td>(12.2-29.6)</td>
<td>(72.1-98.8)</td>
</tr>
<tr>
<td>100 μg·day⁻¹</td>
<td>14</td>
<td>11.6</td>
<td>22.1</td>
<td>87.5</td>
</tr>
<tr>
<td>200 μg·day⁻¹</td>
<td>12</td>
<td>13.8</td>
<td>32.1</td>
<td>91.4</td>
</tr>
<tr>
<td>500 μg·day⁻¹</td>
<td>12</td>
<td>(7.3-20.3)</td>
<td>(18.8-45.4)</td>
<td>(81.4-101.5)</td>
</tr>
<tr>
<td>Completed</td>
<td>40</td>
<td>11.3</td>
<td>29.9</td>
<td>89.0</td>
</tr>
<tr>
<td>Withdrew</td>
<td>25</td>
<td>(4.4-18.3)</td>
<td>(12.6-37.8)</td>
<td>(76.1-101.9)</td>
</tr>
</tbody>
</table>

Data are presented as mean (95% confidence interval). eNO: exhaled nitric oxide; FEV1: forced expiratory volume in one second; PD15 saline: the cumulative provocative dose of saline causing a 15% fall in FEV1.

PD15 saline (at the end of treatment) are shown in figs. 1 and 2, respectively.

There was a significant linear relationship between ICS dose and each of the following: changes in eNO at 1 week and end of treatment (p=0.022 and p=0.015, respectively); changes in FEV1 at 1 week and end of treatment (p=0.043 and p=0.006, respectively); and changes in sputum eosinophils at the end of treatment (p=0.037). There was no significant relationship between ICS dose and the change in PD15 saline. All of these comparisons were adjusted for baseline measurements and changes in numbers due to withdrawals. When those entering the 1,000 μg·day⁻¹ arm were excluded from the analysis the significance of the linear relationship seen across the groups for changes in eNO persisted (p=0.003), however, the linear relationship seen with FEV1 and sputum eosinophils became nonsignificant.

In assessing the between-dose effects of ICS therapy, pairwise comparisons for each of the treatment groups were performed. There were significant differences in eNO between 100 μg·day⁻¹ and both 200 and 500 μg·day⁻¹ at 1 week (p<0.05 for each), and between 500 μg·day⁻¹ and both placebo and 100 μg·day⁻¹ and at the end of treatment (p=0.01 and p=0.023, respectively). For FEV1, there was a significant difference between 500 μg·day⁻¹ and placebo at the end of treatment (p=0.04) but not for any of the other between-dose comparisons. Likewise, the only significant difference in sputum eosinophils with treatment occurred between 100 μg·day⁻¹ and 500 μg·day⁻¹ (p=0.049). No other significant differences were found using pairwise comparisons. There were no significant differences in PD15 saline between treatments.

**Correlations between inflammatory markers**

Correlations between eNO, sputum eosinophils, PD15 saline and FEV1 % pred at baseline and following treatment (expressed as absolute values and as changes from baseline) are shown in table 3. Significant correlations were found between eNO and sputum eosinophils throughout the study. Also, changes in eNO correlated significantly with changes in sputum eosinophils. Changes in PD15 saline did not correlate with changes in any of the other markers.

**Discussion**

In this study it has been demonstrated that eNO measurements provide useful information regarding the dose-response relationship for the anti-inflammatory effects of ICS. This has potential practical importance not only to facilitate the monitoring of anti-inflammatory treatment in individual patients, but also in assessing the efficacy of different inhaled corticosteroids at different doses. A significant linear relationship between the change in eNO and the dose of ICS used (over a range 0–500 μg·day⁻¹ of BDP) was found in patients with moderate bronchial asthma in whom ICS had been withdrawn and loss of control occurred. This ensured that airway inflammation was sufficient for anti-inflammatory effects to be measured. The linear dose-response relationship was significant as early as 1 week after commencing therapy, and continued to 8 weeks, indicating that eNO measurements are not only rapidly responsive to changes in airway inflammation but that they also reflect the ongoing anti-inflammatory action of ICS therapy with time. A similar linear dose-response relationship was also found with FEV1 at 1- and 8-week’s treatment. In a recently reported investigation Silkoff et al. [25] have also demonstrated a stepwise decrease in eNO over a dose range of 0–800 μg·BDP·day⁻¹ in patients with elevated levels of eNO. The results of the present study go further. The highly significant correlation between changes in eNO and changes in sputum eosinophils, together with a linear dose-response relationship between ICS dose and changes in sputum eosinophils at the end of treatment confirm that the
ENNO AND ANTI-INFLAMMATORY DOSE-RESPONSE

Fig. 1. - Percentage change in a) exhaled nitric oxide (eNO) and b) forced expiratory volume in one second (FEV1) after 1 week of inhaled corticosteroid treatment. Data are presented as means±95% confidence intervals. Data were derived from all patients, but adjusted for differences in baseline measurements and the numbers of patients remaining in the group at each time point. a) There was a significant difference across treatments at 1 week (p=0.005) and at the end of treatment (p=0.015). These differences were linear (p=0.022 and p=0.003, respectively). b) There was a significant difference across treatment groups at 1 week (p=0.014) and at the end of treatment (p=0.036). These differences were linear (p=0.043 and p=0.006, respectively).

dose-related decreases occurring in eNO correspond to a reduction in airway inflammation and provide validation of the primary outcome of the study.

It has been shown previously that changes occurring in eNO are seen in association with changes in other markers of airway inflammation following treatment with inhaled budesonide [26] and fluticasone [3]. However, the present study is the largest to date in which dose-dependency for these anti-inflammatory effects has been investigated. Wilson and Lippworth [27] have reported that at doses of >400 µg budesonide daily (the first step in their dose-ranging study for budesonide), no further reduction in eNO occurred despite further dose-related improvements in FEV1 and sputum eosinophil cationic protein. In a smaller study, Jatakanon et al. [28] reported that despite continuing reductions in sputum eosinophils at doses up to 1,600 µg budesonide·day⁻¹, changes in eNO reached a plateau at 400 µg·day⁻¹. In contrast, in the present study the dose-response relationship between eNO and ICS was linear and there was no evidence of a plateau over the range of doses studied (0–500 µg·day⁻¹), with similar outcomes observed for both sputum eosinophils and FEV1. However, the current study is not able to offer comment on whether or not a plateau to the dose-response relationship occurs with doses >500 µg·day⁻¹.

The apparent conflict between these results and those of previous studies may be due to differences in asthma severity at the time of the investigation. In the current study, treatment was withdrawn until loss of control occurred and those who did not exhibit loss of control were excluded. A similar study design has been used by other investigators when evaluating the dose-response for ICS [29, 30]. The rationale for this approach is that the therapeutic ratio for ICS may be influenced by asthma severity: patients with milder asthma may have little room for improvement. This may have influenced the results of previous studies [27, 28]. This is supported further by the findings...
Table 3. Correlations between various markers of airway inflammation and lung function

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Visit 1</th>
<th>Visit 6</th>
<th>ΔVisit (6-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNO versus eNO</td>
<td>r=0.519</td>
<td>r=0.548</td>
<td>r=0.403</td>
</tr>
<tr>
<td>% eosinophils</td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
<td>p=0.002*</td>
</tr>
<tr>
<td>eNO versus eNO</td>
<td>r=0.352</td>
<td>r=0.270</td>
<td>r=0.192</td>
</tr>
<tr>
<td>PD15 saline</td>
<td>p=0.003*</td>
<td>p=0.051</td>
<td>p=0.187</td>
</tr>
<tr>
<td>eNO versus</td>
<td>r=0.169</td>
<td>r=0.021</td>
<td>r=0.252</td>
</tr>
<tr>
<td>FEV1 % pred eNO</td>
<td>p=0.125</td>
<td>p=0.870</td>
<td>p=0.045*</td>
</tr>
<tr>
<td>% eosinophils versus eNO</td>
<td>r=0.254</td>
<td>r=0.185</td>
<td>r=0.045</td>
</tr>
<tr>
<td>PD15 saline</td>
<td>p=0.040*</td>
<td>p=0.204</td>
<td>p=0.771</td>
</tr>
<tr>
<td>% eosinophils versus eNO</td>
<td>r=0.262</td>
<td>r=0.015</td>
<td>r=0.585</td>
</tr>
<tr>
<td>FEV1 % pred eNO</td>
<td>p=0.023*</td>
<td>p=0.913</td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>PD15 saline versus eNO</td>
<td>r=0.420</td>
<td>r=0.533</td>
<td>r=0.092</td>
</tr>
<tr>
<td>FEV1 % pred eNO</td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
<td>p=0.529</td>
</tr>
</tbody>
</table>

eNO: exhaled nitric oxide; PD15 saline: cumulative provocative dose of saline causing a 15% fall in forced expiratory volume in one second (FEV1).

of Silkoff et al. [25] who noted that patients with higher baseline eNO demonstrated dose-response reductions over a higher dose range (0-800 μg BDP·day⁻¹) than those with lower levels of eNO at baseline (0-200 μg BDP·day⁻¹).

There are a number of potential criticisms of the current study. Firstly, the range of doses of BDP to which patients were randomised did not include 1,000 μg·day⁻¹. This was because of earlier suggestions that not only were eNO measurements very sensitive to changes in ICS dose, but that the clinical benefits of ICS were limited at higher doses [31], even in patients with severe asthma [32]. Therefore, the current authors wished to address the possibility that there is a threshold for the anti-inflammatory effects of ICS within the lower dose range. Secondly, the study model which was employed i.e. withdrawal of ICS prior to beginning the randomised phase of the study [33], resulted in a significant number of patients whose asthma remained uncontrolled after randomisation. For ethical reasons these patients were re-allocated to receive 1,000 μg·day⁻¹. The analysis was on an intention-to-treat basis, and therefore included statistical adjustments for those patients who were changed to the higher dose. When analysed post-hoc as a separate treatment group, the patients receiving 1,000 μg·day⁻¹ did provide useful information: in this group eNO levels at week 8 were higher (mean eNO, 17.3 parts per billion (ppb) (95% CI, 11.3-23.5)) than patients receiving 500 μg·day⁻¹ (mean eNO, 10.4 ppb (95% CI, 4.4-16.4)). Because of selection bias the difference between the groups was not formally analysed. However, this suggests that despite higher doses of ICS, eNO levels remain elevated in a subset of patients with more severe asthma [34], reflecting ongoing airway inflammation in this subgroup, and confirming the need for higher doses of ICS. Finally, despite finding a linear relationship between eNO and the dose of ICS, eNO measurements were not able to distinguish between adjacent ICS doses consistently when assessed in pairwise comparisons, although eNO was better in this regard than all other measured parameters. This appears to be a feature of almost all dose-response studies for ICS, irrespective of the end point used, and with even larger numbers of patients [29, 30, 35].

Nonspecific airway responsiveness appears to reflect airway inflammation even in the absence of ongoing symptoms and abnormal lung function [7]. Changes in AHR might therefore provide a useful measure by which the anti-inflammatory effects of ICS could be evaluated. However in the current study, there were no significant changes in AHR to hypertonic saline. This may reflect the low doses of ICS used or the relatively short duration of treatment. Other authors have reported contrasting results using alternative methodologies [27, 28, 35-37]. In general AHR to the direct bronchoconstrictors methacholine and histamine appears to be less sensitive than indirect bronchoconstrictors in detecting changes occurring with corticosteroid treatment [27, 38]. Unfortunately, the interpretation of many studies is made difficult because they have included patients with mild asthma and have used high doses of ICS [4].

The optimal approach to establishing patients on an appropriate dose of ICS has still not been defined. There are two questions of relevance. Firstly, does increasing the dose of ICS above a certain threshold result in greater efficacy? In the majority of patients only small increments in lung function are achieved when the dose of ICS is increased progressively [29, 30, 35]. This calls into question either the effectiveness of higher doses of ICS, or more likely, the validity of simple lung function measurements in this setting. Secondly, is it necessary to suppress airway inflammation completely in order to obtain optimum control, and how is this best assessed? Minimising airway responsiveness to adenosine monophosphate or methacholine (as a surrogate for airway inflammation [7]) appears to be dose-related [27, 36, 37]. When anti-inflammatory therapy is tailored to reduce AHR, clinical outcomes are improved [39]. Unfortunately, repeated measurements of AHR are unsuited for routine clinical practice and it is unlikely that their widespread application will become commonplace. In contrast, eNO measurements are repeatable and easy to perform.

The current authors have recently shown that exhaled nitric oxide measurements may be used to predict deteriorating asthma control, and that they reflect changes in underlying airway inflammation [17]. These findings, together with the results of the present study, offer the possibility that repeated measurements of exhaled nitric oxide may be useful in guiding dose adjustments of inhaled corticosteroids in patients with persistent asthma. This requires further prospective investigation.

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