Biophysical, Biochemical and Cellular Markers of Airway Inflammation in Asthma

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ABSTRACT

Current management of asthma is based on assessment of symptoms and spirometry to guide treatment. Measures of airway inflammation (AI) may lead to more appropriately targeted anti-inflammatory therapy. However, there is debate as to which markers are most useful and this is perpetuated by the lack of a gold standard measure of bronchial inflammation, the lack of direct comparisons for multiple markers in the same individuals, and the relative paucity of longitudinal studies. The aim to this thesis was to investigate the clinical utility of contrasting biophysical, biochemical and cellular markers of AI in stable persistent and acute asthma.

Respiratory heat and moisture loss (RHML) is proposed as a novel biophysical marker of AI. In a cross-sectional study of 32 patients with stable asthma, 25 patients with acute asthma and 25 controls, RHML was measured and compared against other proposed inflammatory markers [the exhaled gases nitric oxide (NO) and carbon monoxide (CO), exhaled breath condensate (EBC) pH and nitrite and sputum eosinophil percentage]. RHML was significantly elevated in stable asthma ($p = < 0.01$) and correlated with sputum eosinophil percentage ($r = 0.73$; $p = < 0.01$). Paradoxically, RHML was not elevated in acute asthma, and a number of possible explanations are discussed. Exhaled NO was significantly higher in stable asthma compared with controls ($p = < 0.01$) and EBC pH was significantly lower in stable asthma than controls ($p = < 0.05$). There was a further decrease in EBC pH in acute asthma ($p = < 0.01$). Sputum eosinophil percentage was elevated in acute asthma, compared with stable asthma ($p = < 0.05$). Exhaled CO and EBC nitrite were not elevated in stable or acute asthma.

To investigate the sensitivity of these markers to short term changes in AI, serial measurements were made during the resolution of an acute exacerbation of asthma. Exhaled NO decreased and EBC pH increased by day 7-9 of an exacerbation. In addition RHML decreased between day 3-5 and day 7-9 of an exacerbation. The changes in these markers lagged behind changes in FEV_1, suggesting that AI persisted beyond the principal period of bronchoconstriction.

The day-to-day repeatability of RHML, exhaled NO, EBC pH and nitrite was tested with repeat measurements in patients with stable asthma. Repeated measurements of RHML, exhaled NO and EBC nitrite were reproducible. However, EBC pH measurements were highly variable.

In summary: 1) RHML is elevated in stable asthma and correlates with sputum eosinophil percentage; 2) Exhaled NO is elevated in stable and acute asthma and decreased during the resolution of an asthma exacerbation; 3) EBC pH is decreased in stable, to a greater extent in acute asthma and increased as an exacerbation resolved; however its intra-subject repeatability is poor; and, 4) In sputum, eosinophil percentage is elevated in acute asthma compared with stable asthma.

DECLARATION

This thesis is a presentation of my own original research work. The work was undertaken under the guidance of Dr J. Alastair Innes, Consultant Physician and Part-time Reader at the University of Edinburgh. Every effort is made to acknowledge other persons that have contributed to any aspect of this research.

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CHAPTER I

NON-INVASIVE ASSESSMENT OF AIRWAY INFLAMMATION IN ASTHMA

INTRODUCTION

Despite the range of effective available treatments, asthma remains a significant health burden, accounting for around 71,000 hospital admissions and 1,400 deaths per year in the UK [1]. Approximately 8% of the UK adult population have a diagnosis of asthma, a figure which may be an underestimate as the prevalence of asthma symptoms is far greater (up to 25% of the population [2]). This indicates that there is considerable room for improvement in the assessment of asthma activity in guiding the appropriate use of the available treatments.

The aims of asthma treatment are to:

- Achieve and maintain control of symptoms
- Prevent asthma exacerbations

- Maintain pulmonary function as close to normal levels as possible
- Maintain normal activity levels, including exercise
- Avoid adverse effects from asthma medications
- Prevent development of irreversible airflow limitation
- Prevent asthma mortality [2]

Using measurements of airway inflammation to rationalise treatment decisions is proposed as a way of helping to achieve these goals. A key component of such an approach is the facility to detect and quantify airway inflammation easily and harmlessly, allowing informed decisions about treatment to be made.

Asthma is usually described as recurrent episodes of wheezing, coughing, chest tightness and shortness of breath particularly at night or early in the morning. Modern definitions of asthma make clear reference to the underlying pathology that causes these symptoms which is characterised by chronic airway inflammation [3]. The evidence that airway inflammation is pathological hallmark in asthma comes from studies of bronchial biopsies [4], bronchoalveolar lavage [5], induced sputum [6] and post-mortem studies of patients who have died as a result of acute asthma [7].

Indices of airway inflammation are not measured routinely in asthmatic populations. Peak expiratory flow rate (PEFR) and sometimes spirometry are used as objective measures to assist decisions regarding treatment. These are measures of airway calibre, which change in relation to airway inflammation, but these changes may also result from smooth muscle contraction or airway remodelling. The most direct assessment of mucosal airways inflammation can be made with bronchoscopy and

biopsy or broncho-alveolar lavage (BAL). However, this is invasive and clearly not suitable for repeated measures in an individual patient. Out with a research setting, this technique has limited clinical utility. The clinical need for non-invasive markers of airway inflammation is the driving force behind this rapidly expanding area of research.

The nature of the underlying inflammation in asthma is complex, with a large number of inflammatory pathways involved [8]. Asthma is also increasingly recognised as a heterogeneous inflammatory disorder where the type and degree of inflammation vary [9-11]. Examples of proposed asthma phenotypes that vary in their response to treatment and natural history are those with early onset of symptoms and atopy, who behave differently from asthmatic patients that are obese and have predominantly non-eosinophilic inflammation [12]. The former group tend to respond readily to treatment with inhaled corticosteroids, whereas the latter have a more limited response to treatment. The variability within our current clinical definition of asthma is very relevant in the context of developing an inflammatory marker or combination of markers that provide useful information to guide treatment. This is one reason why assessment of airway inflammation remains one of the major challenges in asthma research.

This thesis examines contrasting biophysical, biochemical and cellular approaches to the non-invasive assessment of airway inflammation in acute and stable asthma. Novel methods for assessing airway inflammation such as respiratory heat loss measurement and breath condensate markers have been studied, in addition to other more established markers, including exhaled nitric oxide and induced sputum cell

counts. This chapter will review the background and current knowledge of the markers of airway inflammation that are investigated in subsequent chapters of this manuscript, namely:

- Respiratory heat loss
- Exhaled nitric oxide
- Exhaled carbon monoxide
- Exhaled breath condensate pH
- Exhaled breath condensate nitrite
- Induced sputum differential inflammatory cell counts

RESPIRATORY HEAT LOSS

Inflammation is a complex process. However the gross changes that occur as a result of inflammation have long been recognised in the classic description of inflammation as rubor, calor, dolor and tumor. Measurement of respiratory heat loss is a novel application of this established definition that may provide a quantitative measure of airway inflammation. It is based on the hypothesis that increased vascularity and vasodilatation associated with inflammation will lead to a measurable increase in heat loss.

During normal respiration, a counter current mechanism for respiratory heat transfer operates [13]. As air is inspired, it is heated and humidified, resulting in cooling and

drying of the airway mucosa. By the time air reaches the alveoli it is at body temperature and fully saturated with water. During expiration, a variable proportion of this heat energy is regained by the conditioned mucosa as air exits the lung. The result is net heat loss. Conditioning of inspired air is dependent on a source of heat and water that comes from airway mucosal blood flow. In asthma, it is proposed that changes in airway mucosal blood flow associated with airway inflammation will result in altered conditioning of inspired air and lead to changes in net heat loss.

Factors That Affect Measurement of Respiratory Heat Loss

Measurement of respiratory heat loss is challenging. Heat is lost from the respiratory tract not only by convection, but also by evaporation. The latter is the dominant means of heat transfer in the respiratory tract [14]. To quantify total respiratory heat loss accurately, both the temperature and water content of expired air must be taken into account. Furthermore, respiratory pattern and inspired air conditions affect respiratory heat loss and must be controlled.

During normal tidal breathing in ambient room air conditions, the majority of heat transfer takes place in the upper respiratory tract [15]. When ventilation rises or inspired air temperature is lowered, unconditioned air passes deeper into the thoracic airways such that deeper generations of bronchi become involved in air conditioning [16]. This results in greater net respiratory heat loss. Therefore, to detect an alteration in respiratory heat exchange as a result of sub-glottic airway inflammation it is necessary to either increase the respiratory rate or tidal volume, or to reduce the temperature and water content of the inspirate. Unfortunately, these manoeuvres may

also alter the airway environment, by changing airway calibre or altering mucosal blood flow. In fact, the main focus of research into respiratory heat exchange in asthma has been the effects of extreme ventilatory conditions on the airway as a model for exercise or cold air induced asthma [17]. In designing apparatus to measure respiratory heat flux in the lower airway, a ventilatory condition must be found that engages the lower airways in heat exchange without itself altering the airway structure or function.

Out with the airway itself, the core body temperature of an individual should be taken into account. An increase in core body temperature is reported to result in an increase in respiratory heat loss [18]. Although this may be predominantly due to an increase in minute ventilation, it may be of relevance in studying patients with acute disease, where there may be associated pyrexia in the presence of infection.

Relationship between Respiratory Heat Loss and Airway Inflammation

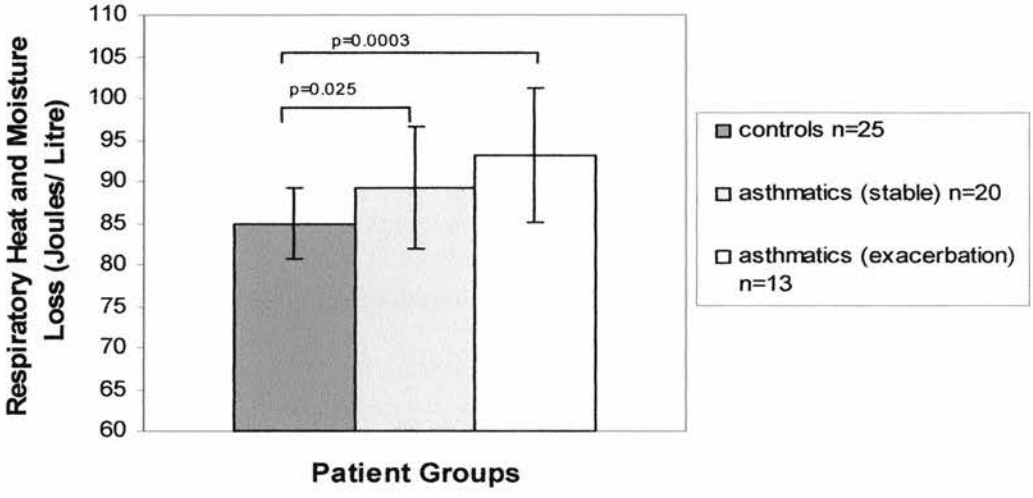
A hallmark of airway inflammation is increased mucosal vascularity. In asthma, this has been demonstrated in bronchial biopsy studies and using non-invasive gas diffusion techniques. Biopsy studies have demonstrated greater vascularity in asthmatic bronchial mucosa [19-21]. This has also been demonstrated visually using high magnification videobronchoscopy [22]. Using a soluble gas uptake method to measure airway mucosal blood flow, greater magnitudes of blood flow have been estimated in steroid naïve and steroid treated asthmatic patients compared with controls [23]. Using the same method, mucosal blood flow is reported to decrease

following treatment with an inhaled corticosteroid in individual asthmatic patients [24].

These structural changes in the vascularity of the airway mucosa associated with airway inflammation may lead to changes in respiratory heat flux. Paredi et al have reported a faster rise in breath temperature during expiration in asthmatics compared with controls [25]. Although only convective heat losses were measured in this study and subjects were breathing ambient room air, it suggests that changes in inflammation may alter heat transfer in the asthmatic airway. However, measurements of convective heat loss by breath temperature alone do not provide an accurate indication of total heat loss as they do not take heat loss via evaporation into consideration. This has been demonstrated in intubated patients where measurements of tracheal temperature do not accurately predict total respiratory heat losses [26].

McCafferty et al have reported an increase in total respiratory heat loss in asthmatic patients compared with controls [(27); figure 1], using a device that incorporates measurements of both convective and evaporative heat loss, and uses a conditioned cool, dry air to engage the lower airway in heat exchange. The temperature of inspired air used in the protocol was 7 °C, and a target minute ventilation of 15 L/min, with a tidal volume of 1.5 L was used. This represents a thermal burden of approximately 25 Watts. This is around 10% of the values used in most studies to induce a change in FEV_1, and equivalent to those found not to change bronchial blood flow in normal subjects [28]. In the study by McCafferty et al, breathing cold air did not appear to cause bronchoconstriction; no changes in FEV_1 were observed using the protocol described.

Figure 1: RHML in Joules / L of ventilation in asthmatics and healthy controls. Means ± 95% confidence intervals are denoted by error bars. P-values based on unpaired t-tests between groups [27].



These preliminary results raise the tantalising prospect that calor, one of the classical signs of inflammation may be measured in exhaled breath and provide a non-invasive means of assessing airway inflammation in asthma. In this thesis, further investigations were undertaken to seek confirmation of these observations and extend them to investigate longitudinal changes in heat and moisture loss.

EXHALED NITRIC OXIDE

Exhaled nitric oxide has been promoted widely as a non-invasive marker of airway inflammation in asthma. Nitric oxide was first identified in exhaled breath of animals

and humans using chemiluminescence by Gustavsson et al in 1991 [29]. Soon after, it was reported to be elevated in patients with asthma [30]. This finding has since been confirmed many times in different centres across the world. At the time of writing this review, there were over 700 citations for 'exhaled nitric oxide + asthma' in a Medline search. The scale of research into exhaled NO reflects not only great interest in its potential, but also problems that may limit its clinical application.

Nitric oxide is produced by nitric oxide synthase (NOS) which exists in two isoforms: constitutive NOS (cNOS) and inducible NOS (iNOS). Both forms are present in human airway tissue. However, the inducible form is capable of producing much greater quantities of nitric oxide. Expression of iNOS is markedly increased in asthma and is likely to be responsible for the elevated levels of exhaled NO seen in asthma [31, 32]. NOS expression can be induced by a variety of stimuli, in particular inflammatory cytokines although the exact mechanism in asthmatic airway inflammation remains unclear.

Measurement of Exhaled Nitric Oxide

Efforts to standardise measurement of exhaled NO has led to the publication of international guidelines that enable NO values to be compared across different centres [33]. The guidelines cover both online measurement, where real-time recordings of NO are made using a chemiluminescent technique, and offline measurement, where exhaled air is collected in gas impermeable bags and measured later. The on-line method, which we have used, has significant advantages including instant results and greater control over measurement error.

Factors that Affect Exhaled Nitric Oxide Concentration

Nitric oxide in exhaled breath is present in very low concentrations [up to 100 parts per billion (ppb)]. There is therefore significant potential for sample contamination and it is important to exclude other sources of NO where possible. It is produced in high quantities in the nose, which can effectively be excluded by using nose clips or asking the subject to exhale against resistance such that mouth pressure exceeds 5 cmH_2O, which is adequate to keep the soft palate closed [34]. The mouth is also a potential source of contamination. Exhaled NO from tracheostomised patients is significantly higher during oral exhalation compared with tracheal exhalation [35]. This is more difficult to control and highlights the potential for contamination when measuring very small concentrations of a volatile gas.

The effect of ambient NO on exhaled NO values is controversial. NO is present in the atmosphere and concentrations fluctuate. Jobsis et el reported that high ambient levels of NO correlate positively with exhaled NO, although an offline method was used [36]. Ho et al examined peak and plateau NO levels on-line using high and low levels of inspired NO and found that peak NO levels increased with high inspired NO [37]. Plateau levels were however unaffected, which suggests that any influence of exogenous NO is small and may only affect the NO peak. To minimise any possible effects of atmospheric NO, commercially available equipment allows a baseline of ambient NO to be established or provides a supply of NO-free air.

Exhaled NO shows marked dependence on expiratory flow rate [38, 39]. At higher flow rates, there is less time for diffusion of NO into the airway lumen and a relative reduction in exhaled NO. It is therefore important that expiratory flow rate is controlled and when exhaled nitric oxide levels are reported, the expiratory flow rate must also be expressed. In practice as long as the flow rate is clearly reported and controlled between subjects using a suitable targeting system, the results can be interpreted sensibly.

Despite controlling for exhalation flow rate, there is still great variability in both control values and values for patients with asthma whether treated with inhaled corticosteroids or not [(34, 39, 40, 42-47); table 1]. Some of this may be due to differences in disease activity in the cohorts of patients with asthma that were studied, but there are also numerous other factors that may affect exhaled NO concentration and further explain the variability seen.

Exhaled NO is not a disease-specific marker for asthma. It has been reported in other diseases or conditions that may co-exist with asthma. These include viral infections, allergic rhinitis, atopy (without asthma), COPD, chronic cough, and pneumonia [48]. The presence of these conditions in asthmatic subjects can make the interpretation of exhaled NO values very complex.

Table 1. Normal adult values of exhaled nitric oxide at exhalation flow rates of 50 ml/s and 250 ml/s (ICS = inhaled corticosteroid treatment). Values are median (interquartile range) or mean ± SD.

Author	Subjects (n)	Flow rate (ml/s)	Nitric oxide ppb	Reference
Dweik et al	Control (21)	50	6 (5-8)	[40]
	Asthma (14)	50	14 (8-25)	
Kharitonov et al	Control (10)	50	17.8 ± 6.8	[34]
	Asthma (10)	50	48.8 ± 27.2	
Delen et al	Control (18)	50	11.1 ± 1.6	[42]
	Asthma (42)	50	16.4 ± 1.3	
Deykin et al	Control (18)	250	8.4 ± 0.7	[39]
	Asthma (17)	250	16.0 ± 2.0	
Ojoo et al	Control (15)	250	9 ± 4	[43]
	Asthma (12)	250	35 ± 19	
Corradi et al	Control (10)	250	6.8 ± 0.3	[44]
	Mild asthma (9)	250	17.9 ± 1.8	
Lim et al	Asthma (no ICS; 16)	250	9.9 ± 3.5	[45]
	Asthma (ICS; 16)	250	13.6 ± 2.0	
Gratziou et al	Control (100)	250	4.8 ± 0.3	[46]
	Asthma (131)	250	13.3 ± 1.2	
Stirling et al	Control	250	7.4	[47]
	Asthma (no ICS; 9)	250	36.9	
	Severe persistent Asthma (ICS; 26)	250	13.9	

Lifestyle factors that may affect exhaled NO are diet and smoking status. Nitrate rich diets increase NO [35]. Caffeine may also have some effect although results are conflicting; it has been reported to both increase and decrease NO by modest amounts [49, 50]. Smoking is reported to reduce exhaled NO [51]. Most studies in asthma

have excluded smokers, however in current clinical practice, a significant proportion of asthmatic patients smoke.

Bronchoconstriction can decrease exhaled NO levels [52]. This is probably a result of increased air flow rate within the conducting airways to achieve the target exhalation flow rate and a consequent reduction in NO diffusion time. This may be of relevance in longitudinal studies where fluctuations in the airway calibre of individuals may have an effect on measurements of exhaled NO.

Finally, in many studies, other markers of airway inflammation are measured consecutively and it is worth noting that both spirometric manoeuvres and sputum induction have been reported to decrease exhaled NO [53]. Therefore, these tests should not be done before measuring exhaled NO.

Relationship between Exhaled Nitric Oxide and Airway Inflammation

Exhaled NO was originally proposed as a marker of airway inflammation following the observation that elevated levels are present in asthma and decrease after anti-inflammatory treatment with inhaled corticosteroids [54, 55]. Further evidence of a relationship between exhaled NO and mucosal inflammation has been sought in bronchoscopic biopsy studies. However, studies of this nature have been limited due to their invasiveness. Payne et al, have reported a correlation between an endobronchial biopsy score of inflammatory components and exhaled NO in a small cohort of children with difficult asthma who were treated with oral corticosteroids

[56]. In adults, there are conflicting findings. Van der Toorn et al reported a correlation between airway eosinophilia and exhaled NO [57], whereas, Lim et al found no relationship between the two [45]. Rather surprisingly, the former study examined subjects with a history of asthma who were in clinical remission. However both studies had small sample sizes, which highlights one of the disadvantages of such an invasive technique. A further limitation of biopsy studies is that a small sample from a discrete area of the bronchial tree is obtained, which may not reflect the global state of airway inflammation within the bronchial tree. In contrast, most non-invasive markers diffusely sample the airway, although the exact site of production of the measured substance is sometimes unclear.

Exhaled NO has been reported to correlate with blood eosinophilia, sputum eosinophilia, BAL eosinophilia and bronchial hyper-responsiveness in steroid naïve patients with asthma [58, 59]. This relationship appears particularly pronounced in atopic asthma [46]. In steroid-treated patients, the relationship is less clear [47, 60]. This may be a result of variable response to anti-inflammatory therapy with each marker. In a double blind placebo controlled trial of inhaled steroid treatment in mild asthma, van Rensen et al reported a decease in NO, a decrease in sputum eosinophilia and an increase in the concentration of histamine provoking a fall in FEV_1 of 20% or more [55]. There was no relationship to the changes in each of the three markers in the groups at any time. Thus, although correlations in cross-sectional untreated asthmatics may be apparent, the added complication of introducing disease modifying treatment can disturb the relationship between different inflammatory markers as they have a variable response to treatment.

The effect of an increase in airway inflammation on exhaled NO has also been studied. As steroid treatment suppresses airway inflammation, it is reasonable to assume that stopping steroid treatment will lead to an increase in airway inflammation. Jones et al have reported a significant increase in exhaled NO following withdrawal of inhaled corticosteroid that is associated with loss of control of asthma, defined as deterioration in symptoms or morning PEFR [61]. It may be difficult to differentiate whether the increase in exhaled NO is due to an increase inflammation or loss of a direct inhibitory effect of steroids on NO production. An association with worsening asthma symptoms and a reported concomitant increase in sputum eosinophils supports the notion the airway inflammation is increased [61]. In addition, a proportion of asthmatics from the same study, that did not experience worsening symptoms, had no increase in exhaled NO.

In the context of an asthma exacerbation, it is also reasonable to assume that airway inflammation will be increased. An allergen-induced asthma exacerbation can be simulated using allergen challenge testing in a controlled environment. Exhaled NO has been shown to increase in the late response phase following allergen challenge [62, 63]. However this finding was only present in asthmatics that were not on regular inhaled or oral corticosteroids. In spontaneous exacerbations of asthma exhaled NO is elevated and decreases following treatment with oral glucocorticoids [64].

An increase in exhaled NO has been proposed as a predictor of future exacerbations of asthma [61, 65]. In a survey of moderate-severe asthmatics attending an outpatient clinic, Harkins et al reported that those who had an exacerbation within two weeks of

attending clinic had higher exhaled nitric oxide levels in clinic [65]. However, the elevated NO levels may simply reflect severity of asthma and thereby a tendency to exacerbate. In another controlled steroid reduction trial by Leuppi et al, exhaled NO levels did not predict exacerbations, in contrast to sputum eosinophils and bronchial hyper-responsiveness which did [66].

Smith et al have reported that exhaled NO is superior to conventional methods for safely reducing the dose of inhaled corticosteroids [67]. In a single blind randomised controlled study comparing the used of exhaled NO measurement against symptoms and spirometry to adjust steroid dose, the exhaled NO group were on a significantly lower doses of inhaled corticosteroid by the end of the year long study. There was no difference in exacerbation rates between the two groups. However, more recently *Shaw et al* have reported no benefit of a similar strategy using exhaled NO measurements to guide reduction in corticosteroid dose [68].

Spirometry remains the standard objective test of asthma control. In general, exhaled NO does not correlate strongly with spirometry. This should not detract from the utility of exhaled NO as a marker of AI. The two tests could be regarded to be examining different components of asthma pathophysiology. If markers of airway inflammation all closely correlated with a straightforward test such as spirometry, then there would be little point in measuring them.

The evidence that supports the use of exhaled NO as a marker of airway inflammation is greatest in steroid naïve asthmatic patients. Exhaled NO may be elevated by conditions other than asthma, is greatly suppressed by inhaled steroids, and its

potential role in management of steroid-treated patients still needs further clarification. In this thesis exhaled NO will be measured in patients with stable asthma and acute exacerbations and compared with other non-invasive markers

EXHALED CARBON MONOXIDE

The notion that exhaled carbon monoxide levels may relate to airway inflammation assumes that oxidative stress plays an important role in airway inflammation in asthma. Carbon monoxide is produced by heme-oxygenase-1 (HO-1), an enzyme that is up-regulated by inflammatory cytokines. HO-1 catabolises heme to bilirubin, free iron, and carbon monoxide. It has been suggested that HO-1 serves a protective role in response to stress [69]. CO stimulates guanylate cyclase, which is thought to have a role in regulating inflammation, and another product bilirubin is an antioxidant.

Measurement of Exhaled Carbon Monoxide

Carbon monoxide in exhaled breath is measurable in real-time using an electrochemical analyser or a non-dispersive infrared technique. One of the attractions of exhaled CO is that it is present in much greater concentrations than nitric oxide. Subjects are asked to perform a slow vital capacity manoeuvre at a predetermined expiratory flow rate. Mean or end-tidal CO concentration is then measured.

Factors that Affect Exhaled Carbon Monoxide Concentration

The major confounding factor that limits use of exhaled CO is cigarette smoking, including passive smoking. Smoking can greatly elevate exhaled CO concentrations to several fold above the levels observed in non-smokers [70, 71]. Indeed exhaled CO is sometimes used as a marker of smoking status. It could also be speculated that environmental pollution would also lead to high exhaled CO levels, although evidence to support this is lacking [72].

There does not appear to be the same dependence on exhalation flow rate as there is with exhaled NO concentration [73]. However, levels are reported to increase following a breath hold of ten seconds [71]. This is an important issue as it implies that a significant proportion of exhaled CO may be from an alveolar source. Several studies of exhaled CO have used a breath-hold technique and this should be taken into account when interpreting the results.

Exhaled CO is not a specific marker of AI in asthma. It may be elevated in other conditions, including upper respiratory tract infections, COPD and cystic fibrosis [74-76].

Relationship between Exhaled Carbon Monoxide and Airway Inflammation

There are several reports of elevated breath CO levels in cross-sectional studies of asthmatics and controls in children and adults. In adults, Horvath et al and Zayasu et al, have reported that exhaled CO is elevated in mild untreated asthma but not in

steroid treated asthma [77, 78]. Yamaya et al have reported elevated exhaled CO levels in severe asthma, but not in mild asthma [79]. However, Zetterquist et al reported no difference in exhaled CO concentration between patients with asthma and controls [71]. In children, there is a similar conflicting picture in the two published studies examining exhaled CO. One study reports elevated CO in steroid naïve asthma [73] and the other reports no elevation in mild untreated asthma, but a significant increase in exhaled CO in persistent asthma on inhaled corticosteroids [71].

The reasons for the different findings may be due to differences in the spectrum of 'mild' asthma. Definitions of asthma severity are based on symptoms, level of treatment and spirometry, and they may not necessarily reflect the underlying state of AI. Another possible explanation is that patients with more severe or persistent asthma have more distal airway inflammation, and alterations in exhaled CO become apparent only when a breath hold manoeuvre is used. In the studies that have reported an increase in exhaled CO in steroid-treated asthma, a breath hold protocol was used [74, 79].

Longitudinal changes in exhaled CO following treatment with corticosteroids have been investigated. In mild asthma, a randomised double blind placebo controlled trial of inhaled corticosteroid treatment failed to demonstrate any change in exhaled CO in the treatment or placebo arms [80]. However in acute asthma exhaled CO has been reported to increase from baseline levels and then decrease following treatment with oral corticosteroids [81]. Different methods used in these two studies make it difficult to draw firm conclusions. In the study of mild asthmatics, a slow vital capacity

manoeuvre was used to measure exhaled CO. However, in the acute asthma study, a twenty second breath hold was followed by a rapid exhalation. The longitudinal changes observed in acute asthma appear convincing, although the baseline values of exhaled CO are low in comparison with other studies using a breath hold manoeuvre.

The response of exhaled CO to controlled allergen challenge has been studied. Paredi et al, have reported that exhaled CO increased in both the early and the late phase response to allergen challenge [81]. However, Khatri et al, have reported that exhaled CO decreased in the early phase and returned to baseline in the late phase [82]. The latter study used a 15 second breath hold and an off-line technique, and failed to demonstrate any difference in exhaled CO between the asthmatic patients studied and control subjects at baseline measurements. The study populations may be different as Paredi et al reported a significant elevation of baseline exhaled CO in their asthmatic group.

Correlations between exhaled CO and other markers of airway inflammation or pulmonary function are inconsistent. As with other non-invasive markers of inflammation, this may be due to different pathways of inflammation involved and variable response to anti-inflammatory treatment.

Investigators have attempted to demonstrate alterations in levels of HO-1, the likely source of CO production. Horvath et al have reported that respiratory macrophages from induced sputum of steroid naïve asthmatics have an up-regulation of HO-1 in association with an increase in exhaled CO [77]. However, Lim et al reported no difference in expression of HO-1 in bronchial biopsies between asthmatic and control

subjects and no change after four weeks of inhaled steroid treatment, although airway eosinophils and exhaled NO decreased [45].

There is no clear consensus as to the utility of exhaled carbon monoxide in assessing airway inflammation in asthma. Although it is present in greater concentrations than NO, the proportional fluctuations in levels seen are significantly less. The data available regarding exhaled CO are difficult to interpret due to different protocols for measurement and inadequate characterisation of asthmatic populations in some studies. Guidelines for measurement of exhaled NO have greatly assisted cross-study comparisons and similar guidelines for the measurement of exhaled CO would help progress research in this area. The key factors in measuring exhaled CO are that it is only of value in non-smokers and a breath-hold is not be recommended as this is likely to increase exhaled CO due to increased time for diffusion of CO in the alveoli. The exhalation flow rate does not appear to be important in determining exhaled CO, in contrast with exhaled NO. In this thesis exhaled CO is examined in stable asthma and during an acute exacerbation of asthma, in addition to other inflammatory markers.

EXHALED BREATH CONDENSATE

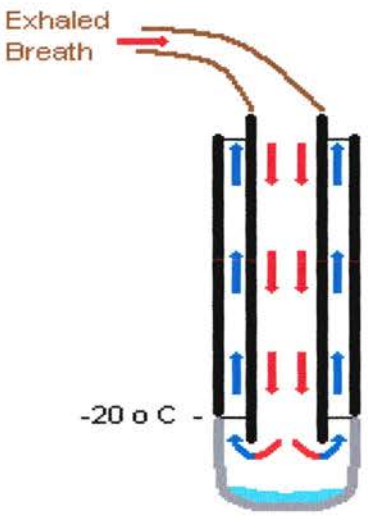
Exhaled breath condensate collection has been proposed as a convenient and non-invasive method of sampling airway lining fluid, which contains molecules that may be used as biomarkers of airway inflammation. Although a wide range of molecules

have been measured in EBC, none have yet been clinically validated. There is significant variability of results due to difficulty in standardising methods of collection and analysis, and also possibly intrinsic sample-to-sample variability.

Collection Methods

Breath condensate is collected by cooling expired gas in a cold air trap, allowing moisture to condense and collect in a reservoir (figure 2). Typically, 1-3 ml of breath condensate can be collected from ten minutes of tidal breathing, using a cold trap at -20 °C. Prevention of saliva contamination can be achieved by using a saliva trap and asking subjects to maintain a "dry" mouth and swallow any saliva that accumulates. Amylase assays may be used to confirm the absence of salivary contamination. Nose-clips are worn during EBC collection to prevent nasal contamination from nasal inhalation and to ensure that all exhaled air exits through the mouth.

Figure 2: Counter current method of breath condensate collection.



The efficiency of current methods of collecting breath condensate is approximately 30-40%, depending on the ventilatory pattern. An increase in minute ventilation, reducing the inspired air temperature will yield a greater volume of breath condensate, while the concentration of solutes appears to be unaffected in healthy control subjects [83].

Factors that Affect Exhaled Breath Condensate Markers

Measurement of substances in EBC has problems with poor reproducibility and inconsistency between different research centres [84]. The most likely explanation for this is a combination of extremely dilute samples [> 99% water vapour; (85)] and poor sensitivity of many of the commercially available assays that have been used to measure molecules in EBC. Many of the substances that have been measured in EBC are near the bottom of the standard curve for commercially available assays and therefore results must be interpreted with caution.

Effros et al have suggested that the use of an internal standard as a dilution marker can lead to greater reproducibility [86]. This proposal is based on the assumption that concentrations of certain substances in airway lining fluid have a steady relationship with plasma levels, such as urea, sodium and chloride. However, the assumption that airway lining fluid and plasma have the same osmolality should not be taken for granted, particularly in disease states. In exercise induced asthma, an increase in mucosal osmolality due to dehydration may be the trigger for mast cell degranulation and mediator release [87]. A further complication is that a large sample (several millilitres) is required to measure potassium and sodium levels, which is time

consuming to collect. Conductance measurements correlate well with sodium and potassium concentrations of lyophilized (freeze-dried) EBC and may provide an alternative internal standard for estimating dilution [86]. However, lyophilization removes volatile substances that may be of interest in EBC, such as ammonia.

There is also debate as to the exact site of origin of droplets in EBC from the respiratory tract [35]. There is little doubt that EBC contains compounds that have originated in the lower respiratory tract, and the characteristics of EBC are markedly different from saliva [88]. However air must pass in and out through the upper respiratory tract which will also contribute to the make-up of the condensate. Differences in concentrations of nitrite have been reported in tracheostomised patients during mouth and tracheal breathing [35].

The American Thoracic Society/ European Respiratory Society Taskforce on Exhaled Breath Condensate have published recommendations for collection, storage and assays of EBC [89]. However, it is recognised that there is great diversity in EBC (numerous different molecules that can be measured and variable assay techniques) and therefore the recommendations were largely limited to general guidance on standardising collection methods. EBC should be collected during tidal breathing using a nose-clip and the collection time and cooling temperature should be recorded.

Exhaled Breath Condensate Nitrite

Under physiological conditions, nitric oxide is unstable and reacts readily with oxygen to form the relatively stable soluble metabolites, nitrite (NO_2^-) and nitrate (NO_3^-). Nitrite can be measured in EBC using a colorimetric assay based on the

Greiss reaction [90]. However in EBC, nitrite is found in the 1μM range which is close to the detection limit for most assays.

As exhaled NO is elevated in asthma, one would expect that one of its metabolites, nitrite, would also be elevated in EBC. Indeed, nitrite and nitrate have been reported to be elevated in asthma in adults and children [91-93]. The levels may also decrease after treatment with corticosteroids. In a randomised controlled trial, Kharitonov et al reported that total nitrite and nitrate decreased following treatment with inhaled corticosteroids in mild asthma [80]. However, a study by Ojoo et al found no difference in EBC nitrite or nitrate in asthma compared with controls [43]. Further studies in cystic fibrosis (CF) suggest that the relationship between exhaled NO and EBC nitrite is not straightforward. Ho et al have reported that EBC nitrite levels are elevated in CF breath condensate, in contrast to exhaled NO, which is not different from controls [32]. Thus, elevated NO levels are not necessarily associated with elevated metabolites of NO in breath condensate.

Exhaled breath condensate pH

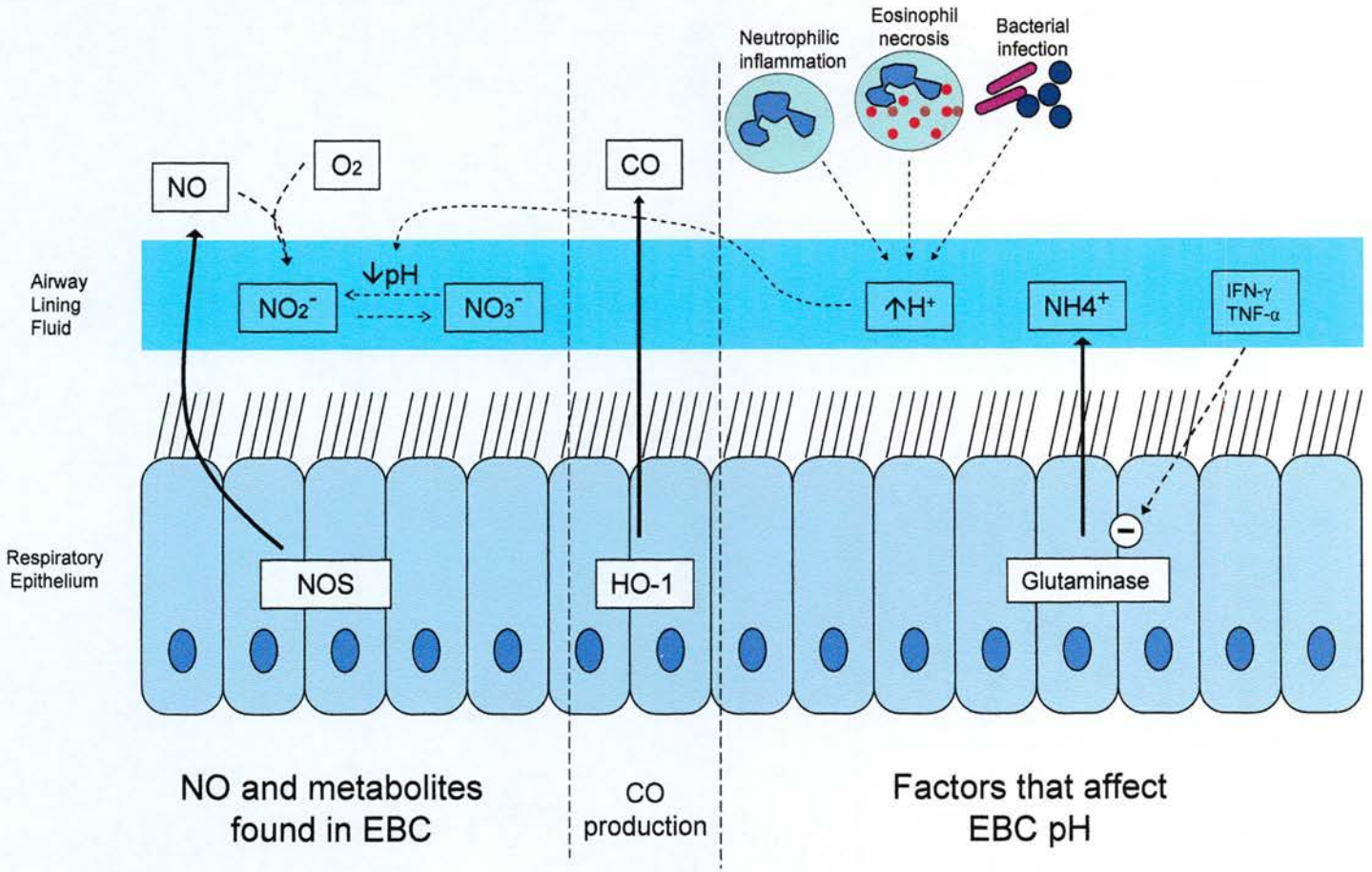
Hunt et al have reported that EBC from patients with acute asthma is acidic and the pH increases after treatment with steroids [94]. Potential implications of reduced airway pH in acute asthma are bronchoconstriction and decreased ciliary activity leading to mucous plugging and accelerated eosinophil necrosis [94]. In stable asthma, Kostikas et al have found a reduced pH, although only in patients with moderate persistent asthma and not in patients with mild asthma [95]. This study also reported more marked reductions in pH levels in COPD and cystic fibrosis, and

correlations between sputum neutrophil percentage and EBC pH, suggesting that neutrophilic inflammation may contribute to a reduced airway pH. In asthma, neutrophils are felt to be important in exacerbations [96, 97] and severe disease [98].

Another factor facilitating airway acidification in asthma may be a reduction in expression of glutaminase, which has the ability to buffer changes in extracellular pH by releasing ammonia. Hunt et al have demonstrated attenuated activity of glutaminase in epithelial cells following treatment with pro-inflammatory cytokines, interferon gamma (IFN-γ) or tumour necrosis factor alpha [TNF-α; (99)].

There is debate about the source of EBC acidification. Vaughn et al have reported that in control subjects, 30/32 subjects had minimal or no difference in the EBC pH obtained from oral collections and isolated lower airway collections [100]. However, airway acidification has not been confirmed invasively with bronchoscopy in airway disease. In acute lung injury, bronchial lining fluid pH and EBC pH does not correlate well [101]. EBC ammonia may be relevant as it forms an alkaline solution which may act as a buffer to acidic EBC. There is a significant decrease in EBC ammonia after intubation and after mouth rinsing, which suggests that a significant proportion of ammonia comes from the upper respiratory tract [35]. Interestingly EBC ammonium is reported to be lower in children with asthma than control subjects [102]. One study that examined the effect of ammonia removal found no effect on EBC pH, although this was in healthy subjects and not in disease states [103].

Figure 3. Summary diagram of the origin of biomarkers of asthmatic airway inflammation in exhaled breath condensate and exhaled air.



Breath condensate markers have provided great interest as potential markers of airway inflammation. However they have also been subject to debate as the source of breath condensate markers is unclear and the samples that are collected are extremely dilute, and may be variably dilute. The proposed origin of the EBC markers discussed in this thesis is summarised in figure 3. There are now guidelines for the methods of EBC collection, although the use of an 'internal standard' to correct for dilution remains controversial and further evidence is required before this will become standard practice. Further studies are required to validate EBC pH and nitrite as markers of airway inflammation in asthma. In this thesis, the behaviour of breath condensate markers will be studied in parallel with other markers of AI in stable asthma and acute disease.

INDUCED SPUTUM DIFFERENTIAL EOSINOPHIL COUNTS

Induced sputum collection using nebulised hypertonic saline was first advocated by Pin et al in 1992 [104]. Prior to this spontaneously expectorated sputum had been investigated, but was felt to be unreliable as it was not always available, and cells were difficult to identify in the unprocessed, whole sputum. The use of hypertonic saline to 'induce' sputum production made samples collected more reliable, with increased viability of cells [105], and subsequent advances in processing sputum has established its key role in research into airway inflammation. In contrast to EBC, sputum provides a concentrated sample of cells, proteins and other metabolites.

Method of Collection

The methods used to induce sputum vary between centres, but all share important characteristics. There is always pre-treatment with a bronchodilator, usually a beta-2-agonist is used to guard against bronchoconstriction, and inhalations of hypertonic saline are delivered via an ultrasonic nebuliser, usually in incremental concentrations that range from 3-5% [106]. Bronchodilation is generally effective although some breakthrough bronchoconstriction may still occur. Nevertheless, sputum induction is widely regarded as a safe procedure. Hypertonic saline is used as it is reported to enhance mucociliary clearance [107] and stimulate a cough response [108]. It results in greater quantities of sputum compared with normal saline, although samples are qualitatively the same [109]. Successful sputum collection also depends on intrinsic factors such as the degree of mucous secretion in an individual.

After collection sputum can be separated from saliva and divided into a cellular compartment to make cytospins for calculation of differential cell counts and supernatant for examination of extracellular proteins [110]. Cell differentials and supernatant protein markers are reported to be acceptably reproducible using this technique [111].

Factors that Affect Induced Sputum Markers

Induced sputum is presumed to originate from the lower airway. However Keating et al reported that eosinophil and neutrophil percentages are higher in sputum than in bronchial washings or BAL fluid [112]. In the same study, the proportions of

inflammatory cells in sputum correlated with bronchial washings, but not with BAL fluid and the authors therefore concluded that induced sputum reflects inflammation within the proximal airway.

There will be a proportion of patients (20-30%) in whom an adequate sample is not obtained. The quality of a sample is determined by the volume of sputum obtained and the proportion of squamous cells in the sample (usually < 20%). Using a selected portion of the sputum sample, that is felt to originate from the lower airway reduces contamination with saliva and squamous cells to a variable extent [113]. This is usually done using forceps to extract the solid components of a sputum sample. The variable contamination and dilution of sputum samples means that inflammatory cells are usually reported as a percentage rather than an absolute number. However this approach could potentially lead to an under or over-estimation of the degree of inflammation, depending on the total number of inflammatory cells present. For example, in the presence a large number of neutrophils in sputum, eosinophils may also be elevated, but the percentage may appear low due to an abundance of neutrophils.

The quality of the sample may also be affected by the time between sputum collection and processing. A sample processing time of < 2 hours is recommended to maintain the viability of cells within the sample. However one study suggests that cell viability is acceptable up to 9 hours after collection in refrigerated samples [114].

Despite the potential variability of the induced sputum samples, differential inflammatory cell percentage appears to be a reproducible measurement. There is

good between observer repeatability of cell counts [115], Furthermore, the within sample repeatability and day-to-day repeatability of cell percentages from induced sputum samples in healthy controls and mild asthma is reported to be good [116], as is the day-to-day repeatability in moderate-severe asthma [116, 117]. However within a 24 hour period, Nightingale et al have reported that serial induced sputum samples from healthy controls are not reproducible. The percentage neutrophils increases and percentage macrophages decreases after the first sample [118]. This finding has been confirmed by other authors and it is proposed that the process of sputum induction stimulates some mild AI [119].

Induced Sputum Eosinophil Percentages and Airway Inflammation

Sputum, due to its abundance of cells, proteins and other metabolites has widespread applications for investigating airway inflammation. The protein rich supernatant can be used to study extracellular markers of inflammation. However, the most widely used biological marker from induced sputum in asthma is the differential eosinophil cell count.

The proportion of eosinophils in sputum from asthmatics is elevated compared with controls. This is in keeping with the presence of BAL eosinophilia in asthmatics [112, 120]. Sputum eosinophilia is present in most cases of untreated or uncontrolled asthma and is suppressed by treatment with corticosteroids, which is associated with an amelioration of symptoms and an improvement in spirometry [121]. The presence of sputum eosinophilia can predict response to corticosteroid treatment [122], and the absence of sputum eosinophilia can identify a subgroup of patients who will not

respond well to steroid treatment. Pavord et al have reported no change in bronchial hyper-responsiveness in non–eosinophilic asthma (sputum eosinophils < 3%) in contrast with an eosinophilic asthma group who had an increase in PC20 following treatment with inhaled corticosteroids [budesonide 800 mcg/ day; (123)].

At odds with this is the observation that in severe asthmatics with persistent airflow limitation, there is still significant sputum eosinophilia, despite high doses of inhaled corticosteroids [124]. However, a subsequent study by the same group has found that this apparent resistance to the effects of high dose inhaled steroids or oral corticosteroids may be overcome with high dose systemic corticosteroid delivered by intramuscular injection [125]. This may represent a relative resistance to steroid treatment in severe persistent asthma, although delivering steroids by a long-acting injection also overcomes any problems that there may be with compliance with inhaled/ oral steroids.

Eosinophils are likely to play an important role in the development of asthma exacerbations. In a steroid reduction trial, sputum eosinophilia was reported to be a good predictor for loss of control of asthma [126]. However, the number of patients in this study was small and only seven subjects developed a loss of control of their asthma symptoms. Furthermore, the study did not use a blinded protocol.

The most compelling evidence for the use of induced sputum eosinophil cell counts in asthma is from a randomised controlled trial comparing treatment based on sputum eosinophil cell counts alone against treatment decisions based upon BTS guidelines for asthma management [127]. The patients studied all had moderate or severe

persistent asthma. The primary outcome was exacerbation rate. Patients in the induced sputum management group had a highly significant reduction in the number of exacerbations. There was no difference the dose of corticosteroids used in each group. In support of this study, a reduction in asthma exacerbation rate using strategies base on controlling sputum eosinophils percentage has subsequently been reported in two further randomised controlled trials [96, 128].

Sputum eosinophilia is useful for selecting patients that are likely to benefit from an increase in corticosteroid treatment and is likely to have a useful role in making treatment decisions in moderate to severe asthmatics. However, collection and processing of induced sputum samples is time consuming, and requires on-site laboratory facilities that can process samples within the required time frame. Also, although the technique is safe, it does induce bronchoconstriction in 10-20% of asthmatic patients and coughing, and therefore may not be popular with patients. Therefore, availability of resources and patient acceptability are likely to be the factors that limit routine use of induced sputum.

INDIRECT MEASURES OF AIRWAY INFLAMMATION

In addition to RHML, exhaled gases, breath condensate and induced sputum cell counts, there are measures of airway hyper-reactivity and patchiness of ventilation that can provide information on the degree of severity of asthma

Airway hyper-responsiveness is recognised as a key feature of asthma which correlates with symptoms, severity of disease [129]. It is usually assessed by giving incremental doses of methacholine or histamine and determining the concentration required to cause a fall in FEV1 of >20%. Airway hyper-responsiveness appears to be closely associated with airway inflammation [130, 131]. However the suppression of symptoms and airway inflammation following treatment with inhaled corticosteroids does not necessarily lead to resolution of bronchial hyper-responsiveness [132]. Although bronchial hyper-responsiveness can provide some indirect evidence of the state of airway inflammation in asthma, it may also be regarded as an additional component of the assessment of disease activity.

Ventilation heterogeneity is described as a feature of asthma in association with bronchial hyper-responsiveness and airway inflammation. The reasons for ventilation heterogeneity in asthma are proposed to be a result of airway wall thickening and mucous secretion due to inflammation. This is often variable throughout the lungs in asthma reflecting the lack of uniformity in asthmatic airway inflammation [133]. Measurement of ventilation heterogeneity using multiple breath washout tests of inert gases such as SF_6 or nitrogen could provide an indirect assessment of airway inflammation, although it is impossible to determine whether areas are poorly ventilated due to mucosal inflammation, transient bronchoconstriction, or airway remodelling. Heterogeneity could also theoretically magnify the degree of airway obstruction during airway challenge testing due to higher concentration of the inhaled broncho-constrictor in well ventilated areas of the lung, rather than areas that are already constricted [134]. In fact, there appears to be a clearer relationship between

ventilation heterogeneity and airway hyper-responsiveness, rather than airway inflammation [measured by exhaled NO; [135].

Although airway hyper-responsiveness and ventilation heterogeneity are associated with airway inflammation, discordance in changes of these parameters in response to treatment means that they should be regarded as measures of asthma disease activity that are complementary to measures of airway inflammation, but cannot be used to accurately assess inflammation. They may however provide an alternative target for treatment of asthma.

SUMMARY

There is a clear need for markers of airway inflammation in asthma and numerous candidates have been proposed. However, judging the clinical utility of a given marker is hampered by the lack of a gold standard measure of AI, which can be used for comparison purposes. Bronchoscopy and bronchial washings, lavage, or biopsy is not feasible on large numbers of patients, and although it provides direct access to the airways there is no validated bronchoscopic measure for assessing AI in asthma. The markers that are studied in this thesis have been reviewed in this chapter all have their advantages and disadvantages, which are summarized in Table 2.

Widely investigated markers such as induced sputum differential eosinophil counts and exhaled NO appear to be the most promising candidates at present. However

sputum induction may not always be successful or acceptable with patients and lack of resources outside of a large teaching hospital setting may limit its widespread application. Exhaled NO is a simpler measurement to make, but it may be overly sensitive to steroids making it less useful for monitoring AI in the majority of asthmatic patients that are established on inhaled steroids. The current data for exhaled CO is limited due to differences in techniques between studies. Breath condensate is an appealing method to non-invasively study substances in airway lining fluid, although none have been clinically validated due to problems with standardisation and reproducibility of measurements. While most attention has focused on cellular, biochemical and molecular markers, it is possible that fundamental changes occurring as a result of AI may have been overlooked, such as altered respiratory heat loss. The studies that follow are based on the following hypotheses:

1. RHML will be altered in asthma in association with other markers of airway inflammation.
2. Longitudinal changes in RHML will occur as an exacerbation of asthma resolves.

Table 2: Advantages and disadvantages of methods for estimating airway inflammation in asthma.

	Advantages	Disadvantages
Spirometry	• Well established normal range • Most patients can perform test	• Cannot distinguish between airway narrowing due to structural change or inflammation
Respiratory Heat and Moisture Loss	• Non-invasive • Most patients can perform test	• Needs careful control of confounding factors, e.g. temperature and humidity of inspirate, breathing pattern
Exhaled NO	• Sensitive • Easy test to perform	• Not specific for asthma • Potential for contamination/ measurement error) due to low concentrations on NO
Exhaled CO	• Present in concentrations greater than NO • Easy test to perform	• Environmental factors, smoking and possibly air pollution can have a marked effect on CO levels
EBC markers	• Easy to collect • Wide range of molecules can be measured in each sample	• Extremely dilute samples • Assays for some markers are near the limits of detection • Marked sample-to-sample variability • Site of origin of EBC molecules not clear (potential contamination from oropharynx)
Sputum differential eosinophil percentage	• Studies have shown clinical benefits in reducing asthma exacerbations • Provides information on the type of inflammation present	• Successful samples in approximately 80% of individuals • Samples require rapid processing and on-site laboratory facilities
Bronchial biopsy	• Direct visualisation of the airway and site of sample	• Invasive, and therefore not suitable for repeated sampling • Usually samples only a small part of the airway

PLAN OF STUDIES

The work reported in this thesis was designed to compare contrasting biophysical, biochemical and cellular approaches for estimating airway inflammation in asthma in a single research centre. The markers investigated are:

1. Respiratory Heat and Moisture Loss
2. Exhaled Nitric Oxide
3. Exhaled Carbon Monoxide
4. EBC pH
5. EBC nitrite
6. Induced sputum differential inflammatory cell counts

In particular the characteristics of RHML, which is proposed as a novel marker of airway inflammation, are subject to detailed study in stable and acute asthma. A series of cross-sectional and longitudinal studies were undertaken to address the following key research questions:

1. Are there cross-sectional differences in markers in stable asthma, acute asthma and control groups?
2. How do these different methods of assessing airway inflammation relate to one another?
3. How do these markers change as an exacerbation of asthma resolves?
4. How reproducible are these measurements over time in individuals?

1. Cross-sectional Study of Respiratory Heat and Moisture Loss and Other Markers of Airway Inflammation in Stable Asthma and Acute Exacerbations

The aim of this study was to determine whether RHML and other markers are altered in asthma in association with airway inflammation. RHML measurements in stable asthmatics, patients with an acute exacerbation of asthma, and control subjects were made in a cross-sectional comparison. In the asthmatic groups, parallel measurements of exhaled NO, exhaled CO, EBC pH, EBC NO_2^- and sputum differential inflammatory cell percentages were made to examine how these markers relate to RHML and each other. It was hypothesized that an increase in airway mucosal blood flow associated with AI in asthmatics would lead to a detectable change in RHML.

2. Longitudinal Study of Respiratory Heat and Moisture Loss and Other Inflammatory Markers during the Resolution of an Exacerbation of Asthma

The behaviour of RHML, the exhaled gases, NO and CO, and breath condensate pH and nitrite are studied in individuals recovering from an acute exacerbation of asthma. Measurements were made on day 1 of their exacerbation (within 24 hours of presentation) and between days 3-5 and days 7-9 following treatment as their exacerbation resolved. The aim was to evaluate the utility of these markers at a time when one would expect intense airway inflammation to be present. Examining longitudinal changes in these markers at this time demonstrates the kinetics of change in these markers as an exacerbation resolves.

3. Repeatability of RHML and Other Markers of Airway Inflammation

The day-to-day repeatability of RHML and the other inflammatory markers was studied. RHML, Exhaled NO, and EBC pH and nitrite are measured in patients with clinically stable asthma on two different days within a one week period. This has great importance in helping to resolve whether alterations in inflammatory markers in the previous studies significant or not.

CHAPTER II

METHODS

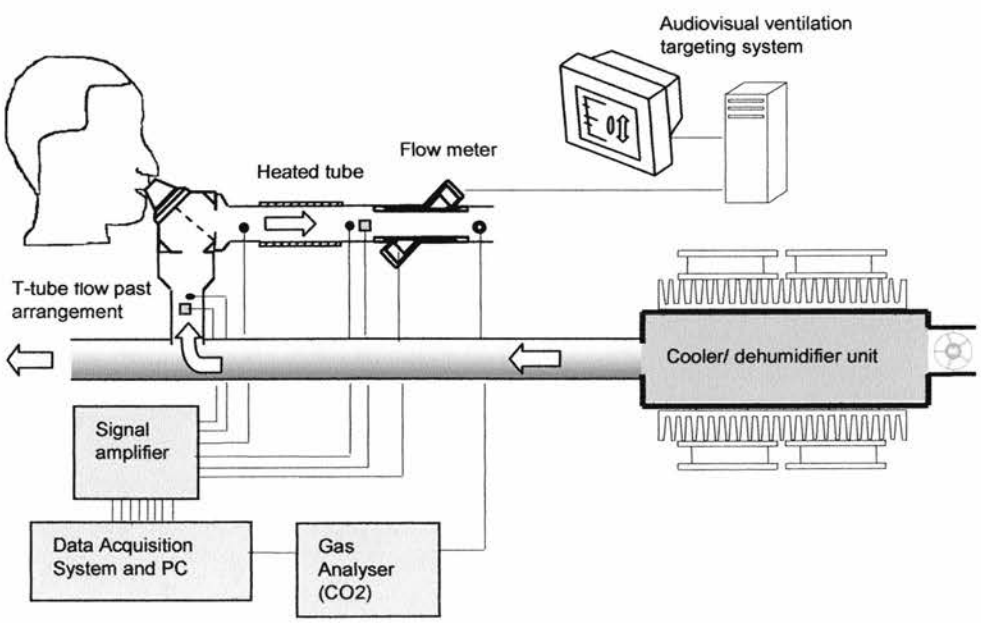
MEASUREMENT OF RESPIRATORY HEAT LOSS

Measurement of respiratory heat loss is not as straight forward as it may seem. To assess total respiratory heat transfer, both convective and evaporative heat losses must be measured. Furthermore, as the majority of heat transfer occurs in the upper airway in resting conditions, the inspirate conditions must be altered to engage the conducting airways in respiratory heat exchange. This can be done by increasing minute ventilation and/or lowering the temperature of the inspired air. To allow comparison between measurements the ventilatory pattern and the inspirate conditions need to be controlled between tests. In the work presented in this thesis a purpose built device that incorporates temperature and humidity measurement of inspirate and expirate, and allows precise control over inspiratory conditions and ventilatory pattern is used. This allows calculation of total convective and evaporative heat losses which we have termed Respiratory Heat and Moisture Loss (RHML). The apparatus was developed by Dr J. McCafferty as part of a separate research project [130].

Equipment

The equipment consists of an air conditioning unit that produces a cool dry inspirate of known temperature and humidity at a rate of up to 1.5 L/ s (figure 4). The subject inhales from the conditioned airstream via a flow-past arrangement (T-Tube; see Fig. 3). Multiple temperature and humidity sensors are located in the inspiratory and expiratory limbs of the apparatus to allow accurate measurement of the heat energy content of the inspirate and the expirate. Temperature sensors used are K-type thermocouples (chromel-alumel bead type), with a 90% response time of 50 ms. They were calibrated against a mercury standard prior to testing. Humidity sensors are of thermoset polymer capacitance construction (*model HIH-3602-A, Honeywell, USA*) supplied factory calibrated giving relative humidity with an accuracy of ± 2% and an estimated 95% response time of 5 seconds.

Figure 4. Equipment for measuring RHML : • Temperature sensor; □ Humidity sensor; ° End-tidal CO_2 sampling port.



On exhalation expired breath will immediately cool and as expired air is often near saturation with water, condensation will occur. The temperature sensors used were rapidly adapting and were therefore able to accurately measure expired breath temperature before significant cooling occurred and to track intra-breath changes in air temperature. However, the humidity sensors have a much slower response time of 5s and therefore would not be able to accurately assess rapid changes in the humidity.

To address this problem, inspiratory and expiratory air flows were separated using a valve and a time-weighted average of expired air humidity was measured. We have calculated that the potential error from time-weighted values is an underestimate of < 5% [136]. There is no reason to expect this error to be systematically different between patients with asthma and controls. In addition, a valve-isolated section of heated tubing was attached to the expiratory limb of the apparatus, downstream from the expired breath temperature thermocouple. The temperature of air inside this tube was controlled at 35 - 40 °C, which was greater than the temperature of exhaled air and therefore prevented any condensation occurring. The temperature and humidity of the expired air inside the heated tubing allowed calculation of the water content of expired air in each breath. Our moisture measurements have been compared against the gold standard "freeze out" technique over a range of minute ventilations from historical data and found to correlate closely [15, 136].

Expiratory air flow was measured using an ultrasonic phase-shift flow meter (model FR-413, BRDL, Birmingham, UK), which was calibrated for volume (L) using standard volume syringes (vitalograph, UK). The sensor's 100% response time was 12ms; linearity was < 2% and the residual error due to temperature variation <1% in

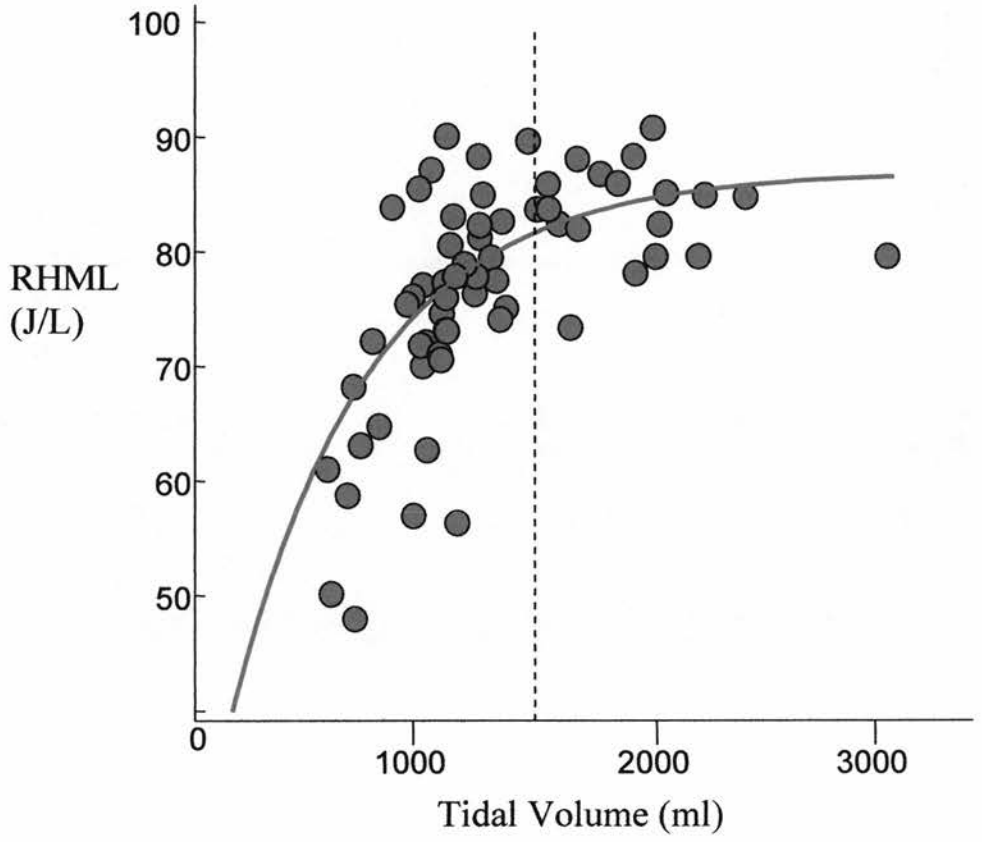
the temperature range 0 – 40 °C. An audiovisual feedback system was used to guide subjects to achieve a set tidal volume and respiratory rate. End-tidal CO_2 was monitored and maintained at the normal physiological level throughout testing by adding small quantities of CO_2 where required.

Protocol for Measuring Respiratory Heat and Moisture Loss

The inspired air conditions and respiratory patterns used were chosen to try and maximise heat exchange in the conducting airways without changing airway calibre. The temperature and humidity of inspired air were controlled at 10 °C and 50% relative humidity, giving an absolute humidity of 4.72 g/ m^3. The thermal energy content of air is calculated from these variables using standard formulae and is termed enthalpy. The enthalpy of the conditioned inspired air was calculated to be 20 J/ L.

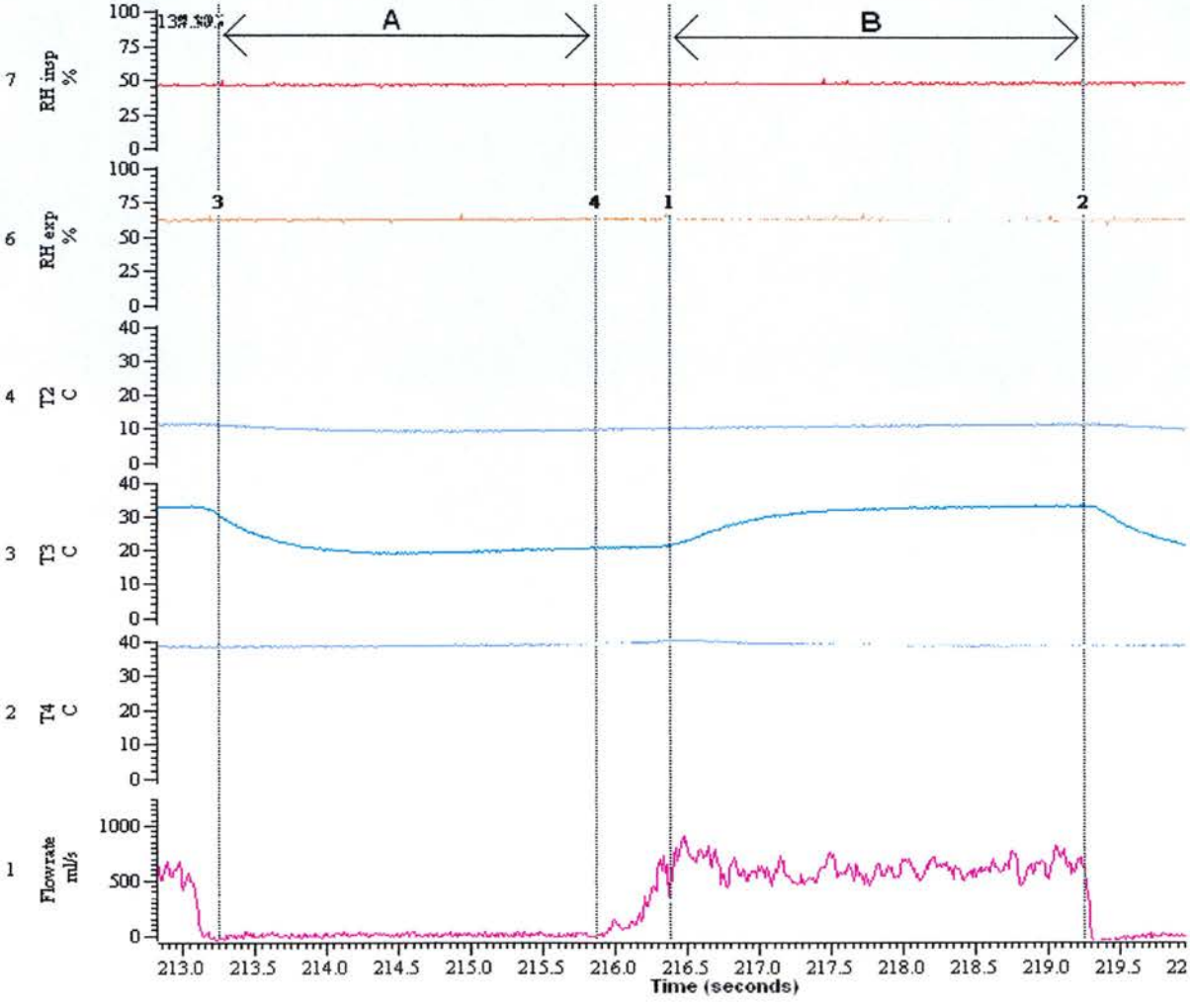
During the development of the apparatus for measuring RHML, Dr J McCafferty demonstrated that RHML appeared to be dependent on tidal volume up to a threshold value (1.5 L) and thereafter directly proportional to minute ventilation (figure 5, [27]). Therefore, the audiovisual feedback system was used to guide subjects to achieve a tidal volume of 1.5 L (expiratory flow rate of 500 ml/ s) and a respiratory rate of ten breaths per minute, to give a target minute ventilation of 15 L/ min. The thermal challenge of these conditions over five minutes is not sufficient to affect airway calibre from pilot data [27].

Figure 5. Respiratory heat and moisture loss (RHML) plotted against tidal volume in healthy control subjects, n=20. RHML is dependant upon tidal volume up to a threshold value of 1.5 L [27].



Measurements were made using real-time recording of data. All values used were averages during either inspiration or expiration (figure 6).

Figure 6. Visual display of raw data used to calculate RHML. During expiration, measurements are taken from the beginning of the expiratory breath temperature curve to eliminate equipment dead space (approximately 100 mls). A = inspiration; B = expiration; RH_{insp} = relative humidity of inspirate (%); RH_{exp} = relative humidity of expirate at T_4 (%); T_2 = temperature of inspirate (°C); T_3 = temperature of exhaled breath (°C); T_4 = Temperature of warmed expirate (°C; flowrate = expiratory flow rate (ml/ s).



Calculation of Respiratory Heat and Moisture Loss

RHML was calculated as the net heat energy loss per unit volume of expired air. The total heat or energy content of an air-water mixture is given the term enthalpy. Enthalpy values were calculated using a psychrometric calculator (PsyCalc 98, Linric Company, Bedford, NH, USA). The enthalpy of the inspirate and expirate are derived from mean air temperature and water content during inspiration and expiration respectively. Net enthalpy loss is then calculated as:

$h = he - hi$

Where: h = net enthalpy loss (J/g dry air), he = enthalpy of expirate, and hi = enthalpy of inspirate.

Total heat energy losses can be calculated by incorporating the mass flow rate into an equation. The mass flow rate air is the product of air density and flow rate. The density of a mixture of dry air molecules and water vapour molecules may be expressed as:

$D = [P_d / (R_d \times T)] + [P_v / (R_v \times T)]$

Where: D = *density* (kg/m^3), P_d = *pressure of dry air (Pa),* P_v = *pressure of water vapour (Pa),* R_d = *gas constant for dry air [J/(kg x degK) = 287.0],* R_v = *gas constant for water vapor [J/(kg x degK) = 461.5],* T = *temperature, (degK).*

Total respiratory heat loss is then calculated using the formula:

$$TRHL = DV \times h$$

Where: TRHL = Total Respiratory Heat Loss (Watts, J/s), D = Density of air (g/L), V= flowrate (L/s), h = net enthalpy loss (J/g)

In practice, despite using an audiovisual targeting system, there is some variation between individuals in expiratory flow rate. Therefore a more useful way of expressing respiratory heat loss is by heat energy loss per unit volume of expired air. RHML is calculated using the formula:

$$RHML = D \times h$$

Where: RHML = Respiratory Heat and Moisture Loss [Joules per litre (J/L)], D = Density of air/ water vapour mixture (g/L), h = net enthalpy loss (J/g).

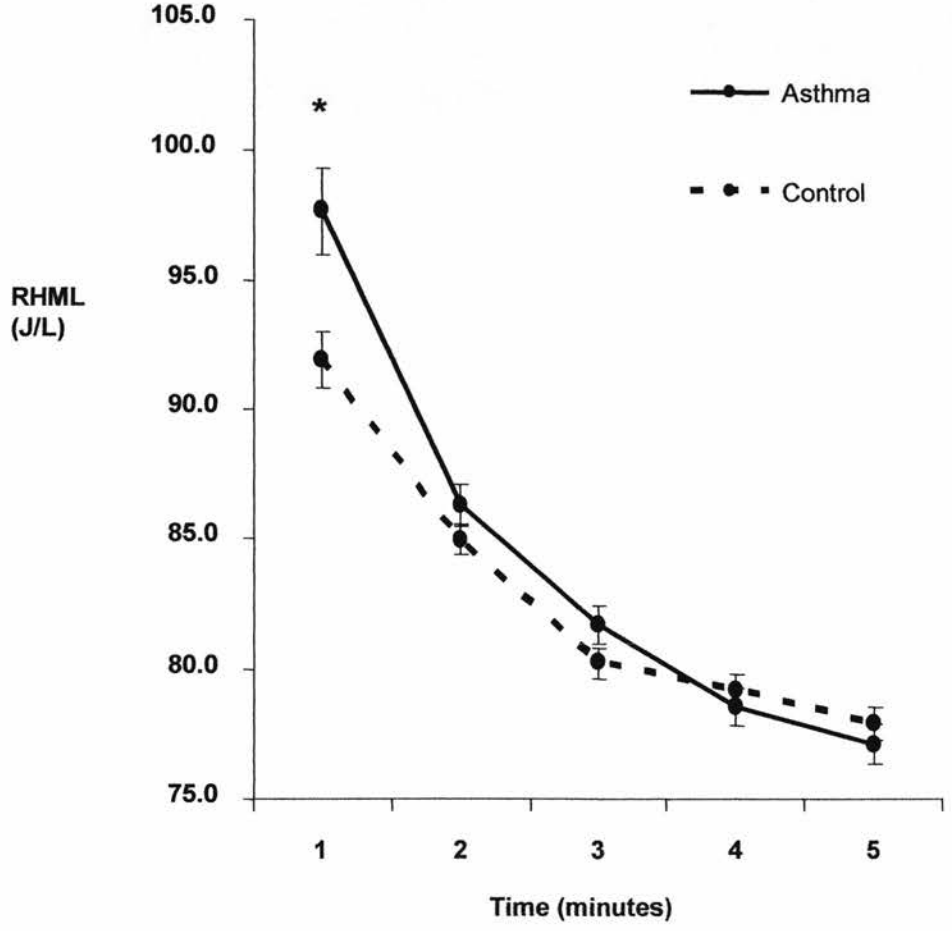
The data from the temperature and humidity sensors in the RHML equipment allows accurate measurement of net RHML in Joules per Litre of expired air using the formulae described.

Application of Existing Equipment to Current Studies

A preliminary study examined the time course of changes in RHML in individuals while breathing into the RHML apparatus for five minutes. At the inspirate and ventilatory settings used, RHML decreased over time, possibly due to airway drying

(figure 7; [137]). Thus, measurements should be taken at the same time point during each test. In addition, there appeared to be greater RHML in patients with stable asthma compared with controls in the first two minutes of breathing into the apparatus. Therefore, in the studies that follow, temperature and humidity measurements were taken from between 60 and 80 seconds of continuous breathing into the apparatus. This allows adequate time for the humidity sensors to adjust and the mean exhaled air temperature to reach a steady state. This is in contrast to a study by McCafferty et al that reported elevated RHML in asthma once respiratory heat loss had reached a steady state under similar conditions [136].

Figure 7. RHML during 5 minute test in subjects with asthma (n=21) and control subjects (n=18; [137]).



EXHALED NITRIC OXIDE MEASUREMENT

Fractional exhaled NO was measured in single breath using a target expiratory flow rate of 250 ml/s (F_ENO_{250}) with a modified chemiluminescence analyser (LR2000, Logan Research Limited, Kent, UK). The NO analyser was calibrated daily with N_2/NO calibration gas containing 93 ppb NO (BOC gases, Guilford, UK). A subject inspires to total lung capacity and with no breath hold, exhales into the sampling tube. Nasal clips were worn and a visual feedback system was used to maintain a mouth pressure of 5 cmH_2O, sufficient to keep the soft palate closed and prevent nasal contamination [34]. The exhalation tube had a resistor inserted for which a target mouth pressure of 5 cmH_2O was equivalent to an expiratory flow rate of 250 ml/s. This flow rate was selected as it was more comfortable than the ATS recommended flow rate of 50 ml/s for subjects with acute or severe asthma, who cannot tolerate very slow exhalation. There is a literature using an exhalation flow rate of 250 ml/s and normal ranges in patients with asthma and healthy controls have been widely reported (see Table 1, Chapter 1).

To measure exhaled NO concentration, expired air was drawn from a sampling tube at a standard rate of 4 ml/s. NO measurements were taken from the plateau phase at the end of expiration, in accordance with joint ATS/ ERS guidelines [33]. NO values were recorded as an average of three measurements. The 95% confidence interval of for repeat measurements in the same individual was determined by paired measurements in 32 subjects and was 3.3 ppb.

EXHALED CARBON MONOXIDE MEASUREMENT

Exhaled CO was measured by an electrochemical CO sensor, sensitive to CO from 0 to 500 ppm by volume, adapted for online recording of CO concentrations and integrated with the chemiluminescence analyser (LR2000, Logan Research, Rochester, UK) to control exhalation parameters. The CO sensor was calibrated daily using 50 ppm CO calibration gas. End-tidal CO (ETCO) levels were taken as an average from 3 exhalation manoeuvres with a target expiratory flow rate of 250ml/s, controlled using visual feedback as described above.

EXHALED BREATH CONDENSATE COLLECTION AND ASSAYS

Breath condensate was collected on a commercial breath condenser (EcoScreen, Jaeger, Germany). Subjects were asked to breathe through a non-rebreathing two-way valve for 5 minutes. Nose-clips were worn during the collection. The yield of breath condensate was approximately 1-2 mls. Samples were collected in interchangeable sampling tubes (one per sample). All sampling tubes were disinfected for 30 minutes using 1% potassium monopersulphate solution (Virkon, Antec International Ltd, UK), rinsed for 2 hours by flushing with tap water, then rinsed with ultrapure water (ELGA Labwater, UK) and air dried prior to use. After collection and pH recording, samples were stored in polypropylene containers at –80 °C and analysed within 2 weeks to minimise contamination and problems of instability.

The pH of the EBC was measured immediately after collection (without deaeration) using a calibrated pH meter incorporating an ISFET sensor with temperature compensation (model KS723, Camlab, Cambridge, UK) that has an accuracy of ±0.1 pH. A two-point pH calibration was performed before each breath condensate pH measurement.

The nitrite concentration in EBC samples was determined by a colorimetric assay based on the Griess reaction [90] in which triplicates of 100 µl EBC were reacted with 25 µl Griess reagent and measured at absorbance of 570 nm with a microplate reader (MR 710, Dynatec). This assay has been previously validated [37]. Assay sensitivity was 0.5 µmol/l.

INDUCED SPUTUM COLLECTION AND ANALYSIS

Sputum was induced using incremental concentrations of 3, 4 and 5% hypertonic saline each delivered over 4 minutes, via an ultrasonic nebuliser (DeVilbiss Ultraneb 99; DeVilbiss Healthcare, Somerset, PA, USA), set at an output of approximately 2.4 ml per minute. Subjects were pre-treated with 200 mcg of inhaled salbutamol via a metered dose inhaler, or salbutamol 2.5 mg via a nebuliser. FEV_1 was closely monitored throughout the test and the procedure was abandoned if the FEV_1 decreased by > 20%. Subjects were instructed to rinse mouth with water and blow nose prior to expectorating sputum to minimise contamination with saliva or post nasal drip.

Sputum processing was performed using the methods previously described by Pavord et al [138]. Sputum plugs were separated from the whole sputum sample using forceps to minimise salivary contamination. The plugs were weighed and four times the selected sputum volume of 0.1 % dithiothreitol solution was added. The sample was vortexed for 15 s and then gently mixed for 15 minutes. An equal volume of phosphate buffered saline was added to the sample. The sample was then filtered through 53 μm gauze (Lockertex; Warrington, England). The filtered sample was then centrifuged at 1200 rpm for 10 minutes. The cell free supernatant was removed and stored for later analysis. The cell pellet was re-suspended in phosphate buffered saline and cytospins slides were prepared. A haematoxylin and eosin stain was used for the cytospin.

Sputum differential cell counts were calculated from manual counting of four hundred inflammatory cells and expressed as percentages of the total inflammatory cell count. For quality control sputum samples needed squamous contamination of $< 20\%$. When inflammatory cells were counted on two separate occasions in twelve sputum samples from subjects with asthma, the correlation coefficient for differential eosinophil percentage was $r = 0.98$, $p = < 0.001$. The intra-observer 95% confidence interval for sputum eosinophils was $\pm$ 2.46%.

STATISTICAL ANALYSIS

The size of the study groups in the cross-sectional study was based on a realistic achievable number of patients estimated to allow the detection of a significant difference between groups. Although there was some pilot data available for

measurement of RHML, the measurement technique used in the studies that follow were time weighted and therefore differed significantly from previous measurement by Dr J. McCafferty (see page 54). As a consequence it was not possible to perform a formal power calculation as part of the study design. The statistical tests used to analyse data are described within individual chapters.

CHAPTER III

CROSS-SECTIONAL DATA COMPARING RHML AND OTHER MARKERS OF AIRWAY INFLAMMATION

INTRODUCTION

The aim of this study was to determine whether RHML is altered in asthma in association with airway inflammation and whether it bears any relation to the state of inflammation in the airways measured by alternative non-invasive methods. RHML measurements in stable asthmatics, patients with an acute exacerbation of asthma, and control subjects were made in a cross-sectional comparison. In the asthmatic groups, parallel measurements of exhaled nitric oxide and CO, breath condensate pH and nitrite, and sputum eosinophils and were made. We hypothesized that an increase in airway mucosal blood flow associated with airway inflammation in asthmatics would lead to a detectable rise in RHML.

METHODS

Study Design

Thirty-two patients with stable asthma and 25 patients with acute asthma were recruited to have parallel measurements of RHML, exhaled NO, exhaled CO, EBC pH and nitrite, induced sputum cell counts and spirometry. In addition, blood samples were taken from patients with acute asthma to measure peripheral blood eosinophilia. For comparison with healthy controls, 18 subjects had RHML and spirometry tested. To establish control values for exhaled NO, exhaled CO, EBC pH and nitrite, a further control group had these measurements in addition to spirometry.

Subjects

Stable asthmatics were recruited from a hospital outpatient population. Asthma was defined according to Global Initiative for Asthma (GINA) Guidelines [2]. Sixteen patients had severe persistent asthma, 12 had moderate persistent asthma and 4 had mild persistent asthma. All patients were on regular inhaled corticosteroids (mean daily dose 833 ± 561 micrograms of beclomethasone dipropionate (BDP) or BDP equivalent), and had not experienced any exacerbations of asthma in the two months prior to the study. Sixteen of the asthmatic subjects were taking a regular long acting beta-2 agonist (LABA). Inhaled medications were withheld for 12 hours prior to testing.

Patients with acute asthma were recruited from a hospital acute medical assessment unit, and inflammatory markers were measured within 24 hours of presentation. Acute asthma was defined as deterioration in symptoms with a concomitant reduction in peak expiratory flow rate (PEFR) from baseline, warranting treatment with a course of oral prednisolone therapy. This decision was made by the receiving clinician. Seventeen patients were admitted to hospital for in-patient treatment. All patients had their body temperature measured. RHML was only tested when core temperature was < 37.4 °C. Patients with acute exacerbations were treated according to national guidelines and therefore received nebulised bronchodilators and oral corticosteroids [3]. Twenty-two of the patients with acute asthma were taking regular inhaled corticosteroids prior to their exacerbation.

Healthy controls were recruited from hospital staff. All subjects were non-smokers, or ex-smokers (stopped > 6 months) with a smoking history of less than 10 pack years. This study was approved by the local ethics committee and all patients and control subjects gave written informed consent to take part.

Measurement of Inflammatory Markers

The equipment and protocols for collection and analysis of samples are discussed in detail in Chapter II. In order to minimise possible confounding effects of the test procedures, the tests were performed in the following order: 1) RHML measurement; 2) exhaled gas analysis; 3) EBC collection; 4) FEV_1; and 5) induced sputum collection. Not all measurements were possible in each individual. The number of data recordings collected in each group is detailed in figures 8 - 10. The main factor

that limited RHML data collection was equipment failure, including failure of the air conditioning unit and damage to the humidity sensors. End tidal CO measurements were not possible for a period due to a lack of calibration gas. Induced sputum samples were limited for a number of reasons that included unavailability of the laboratory processing facility, insufficient or inadequate samples and patient refusal.

Figure 8. Profile of recordings made in patients with stable persistent asthma

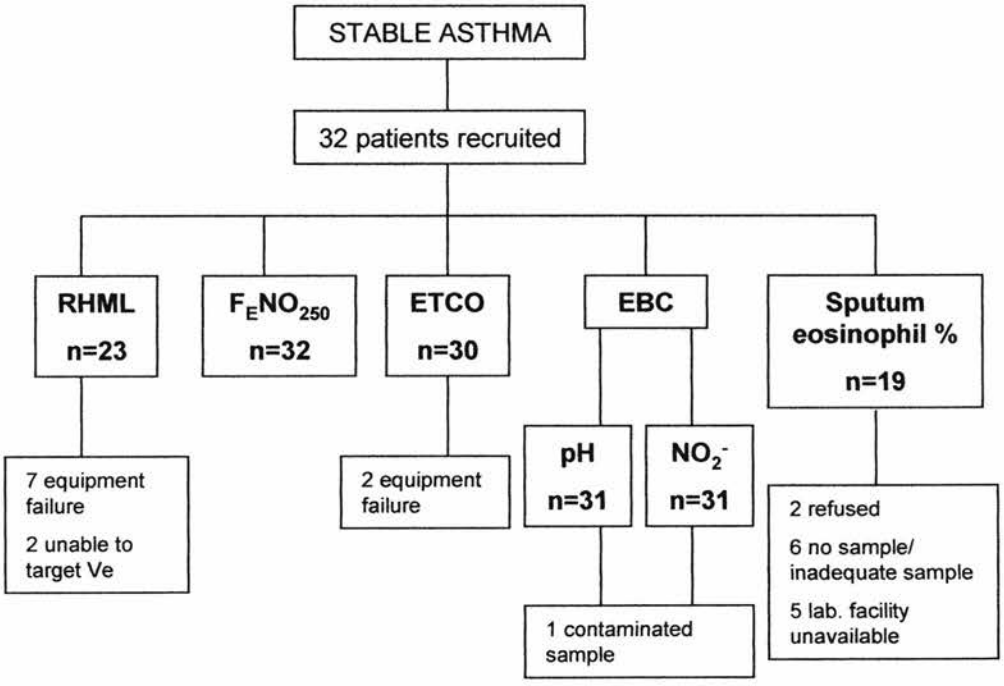


Figure 9. Profile of recordings made in patients with an acute exacerbation of asthma.

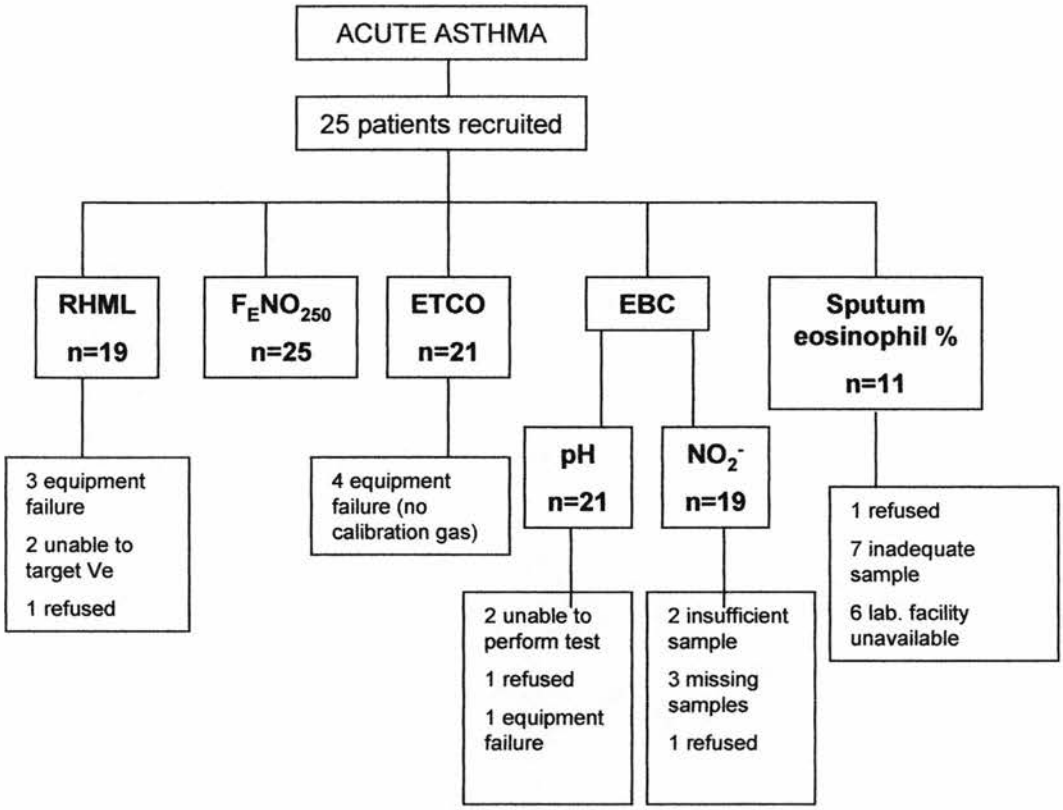
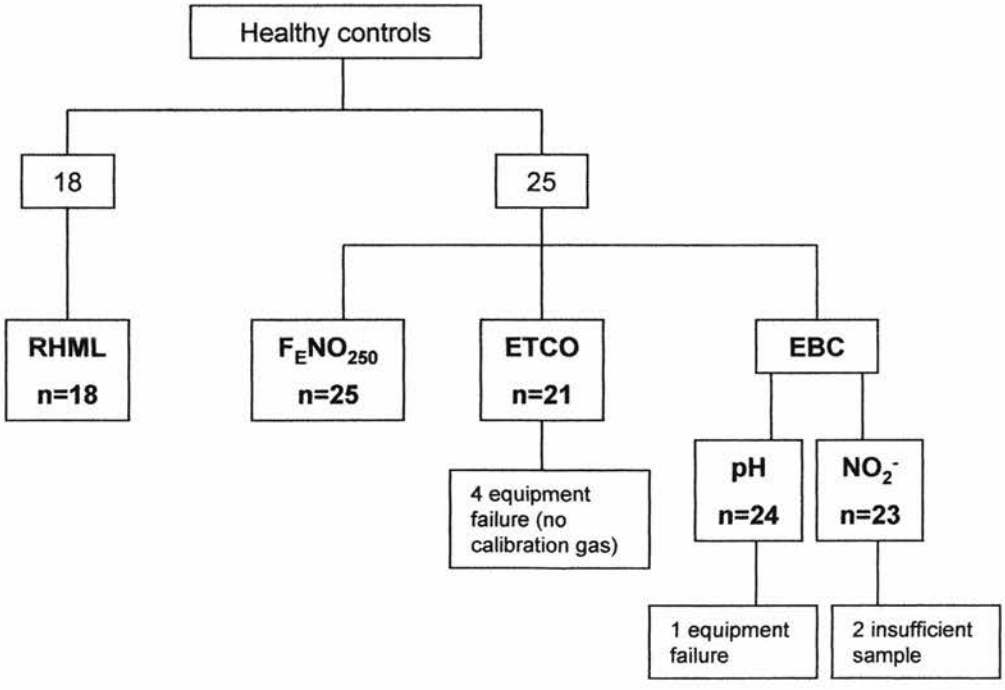


Figure 10. Profile of recordings made in healthy control subjects.



Statistical Analysis

Results were analysed using Sigmastat® statistical software (SPSS). For cross-sectional analysis between groups, a one way analysis of variance (ANOVA) was used determine whether there were any significant differences between groups and a Tukey post-test analysis was used to determine which groups were significantly different. For data that were not normally distributed an ANOVA on Ranks was used to test for differences between groups, and a Dunn's test was used to identify the groups that differed significantly from one another. Correlations between RHML and other inflammatory markers in the asthmatic group were determined using a Pearson Correlation coefficient. Data that were not normally distributed were log normalised prior to correlation analysis. Levels of significance were determined as $p = < 0.05$. Normally distributed data are expressed as mean ± standard deviation (SD) and non-normally distributed data are expressed as median (inter-quartile range). For each marker studies, only those patients with a dataset available were studies as opposed to an intention-to-protocal analysis.

RESULTS

Study groups were well matched in age, height and BMI (Table 3). Percentage predicted FEV_1 was significantly lower in stable asthma (83.5 ± 22.1 %) compared with controls (101.8 ± 7.5; $p = < 0.01$) and further reduced in acute asthma (55.1 ± 21.4 %) compared with stable asthma ($p = < 0.05$; Table 4).

Table 3. Study population demographics. Values are mean ± standard deviation.

	Stable Asthma	Acute Asthma	Healthy Control (RHML)	Healthy Control
Age	44.6 ± 14.0	44.9 ± 15.2	41.6 ± 13.1	41.6 ± 13.1
Number of subjects	32	25	18	25
Sex (F:M)	19:13	17:8	10:8	16:9
Height (m)	1.65 ± 0.08	1.65 ± 0.06	1.68 ± 0.07	1.67 ± 0.09
BMI	28.7 ± 9.3	25.8 ± 3.3	26.3 ± 3.9	26.1 ± 3.6
Inhaled steroid dose (*pre-admission)	833 ± 561	867 ± 633 *	-	-
Number of patients taking LABA (*pre-admission)	16	13 *	-	-

Respiratory Heat and Moisture Loss

RHML was significantly elevated in patients with stable asthma (98.1 ± 7.3 J/L) compared with control subjects (91.9 ± 4.5 J/L; $p = < 0.01$), but not in those with acute asthma (91.3 ± 5.9 J/L; table 4; figure 11). During RHML measurement, there was no significant difference between groups in the enthalpy of the inspired air ($p = 0.06$) or the ventilatory pattern ($p = 0.93$; table 5). The difference observed in stable asthma is therefore unlikely to be due to variation in test conditions.

Table 4. Results of RHML, FEV_1 and other inflammatory markers in study groups.

Values are mean ± standard deviation or median (inter-quartile range).

	Acute Asthma	Stable Asthma	Control	Control (RHML)
N	25	32	25	18
FEV1 (L)	1.7 ± 0.7	2.4 ± 0.9	3.3 ± 0.7	3.5 ± 0.8
	p = < 0.001		p = < 0.001	
FEV1 % Predicted	55.1 ± 21.4	83.5 ± 22.1	102.1 ± 6.8	101.8 ± 7.5
	p = < 0.001		p = < 0.001	
RHML (J/L)	91.3 ± 5.1 n=19	98.1 ± 7.3 n=23	-	91.9 ± 4.5 n=18
	p = < 0.01		p = < 0.01	
F_ENO_{250} (ppb)	22.5 (16.7-31.1) n=25	17.2 (10-28.8) n=32	6.0 (4.0-8.5) n=25	-
	p = 0.06		p = < 0.001	
ETCO (ppm)	1.78 ± 0.87 n=21	1.92 ± 0.54 n=30	2.4 ± 0.6 n=21	-
	p = 0.10			
EBC pH	6.1 ± 0.4 n=24	6.4 ± 0.3 n=31	6.6 ± 0.5 n=22	-
	p = < 0.01		p = < 0.05	
EBC NO_2^- (µmol)	5.3 ± 3.7 n=20	5.7 ± 3.4 n=31	5.3 ± 2.8 n=23	-
	p = 0.91			
Sputum Eosinophil %	18.7 (7.3-31.8) n=11	6.7 (3.1-13.5) n=19	-	-
	p = < 0.05			

Figure 11. RHML in control (n=18), stable asthma (n=23) and acute asthma (n=19) study groups (p = < 0.01).*

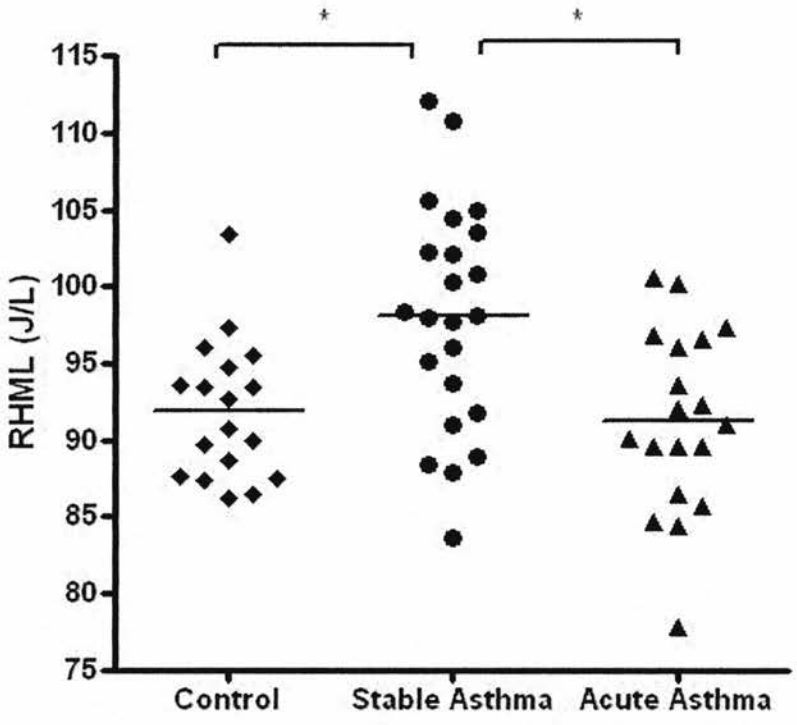


Table 5. Minute ventilation and enthalpy of conditioned inspired air in stable asthma, acute asthma and control groups during RHML testing. There was no significant difference between the enthalpy of inspired air (p=0.06) and the minute ventilation (p=0.93) between groups.

	Stable Asthma	Acute Asthma	Controls
n	23	19	18
Enthalpy Inspirate (J/L)	21.9 ± 1.6	21.5 ± 3.0	20.3 ± 1.6
Minute Ventilation (L/min)	15.9 ± 3.4	16.3 ± 3.2	16.1 ± 2.3

Due to technical problems with the air-conditioning system of the RHML apparatus, measurement of RHML was not possible in 7/ 32 patients in the stable asthma group and 3/ 25 patients in the acute asthma group. Four patients from all study groups were unable to follow the breath targeting system which requires a degree of co-ordination. Patients must be able to time their tidal breathing with an audio cue, and expire at a set expiratory flow rate, guided by a visual feedback display.

Subgroup analysis of the stable asthma group revealed that there was no significant difference in RHML between mild/ moderate persistent asthma and severe asthma (97.8 vs. 98.3 J/L; $p = 0.88$). In the stable asthma group, there was no significant difference in RHML between the 16 subjects who were on a regular LABA and subjects who were not (98.3 vs. 97.5 J/L; $p = 0.81$).

Exhaled Gases

Median F_ENO_{250} was elevated in stable asthma [17.2 (10.0-28.8) ppb; $p < 0.001$] compared with control values [6.0 (4.0-8.5) ppb; figure 12]. In acute asthma F_ENO_{250} showed a trend towards a further increase [22.5 (16.7-31.1) ppb; $p = 0.06$]. End tidal CO was lower in patients with asthma compared with control subjects across the study groups ($p = < 0.01$; figure 13). There was no difference in ETCO between patients with an acute exacerbation of asthma and patients with stable asthma ($p = 0.16$).

The protocols for exhaled NO and ETCO measurement were easy for study participants to perform. However, a number of ETCO measurements were not possible for a period during the study due to calibration gas not being available.

Figure 12. Exhaled NO in control subjects (n=25), stable asthma patients (n=32), and patients with an acute exacerbation of asthma (n=25; * p= < 0.01; Dunn's Test).

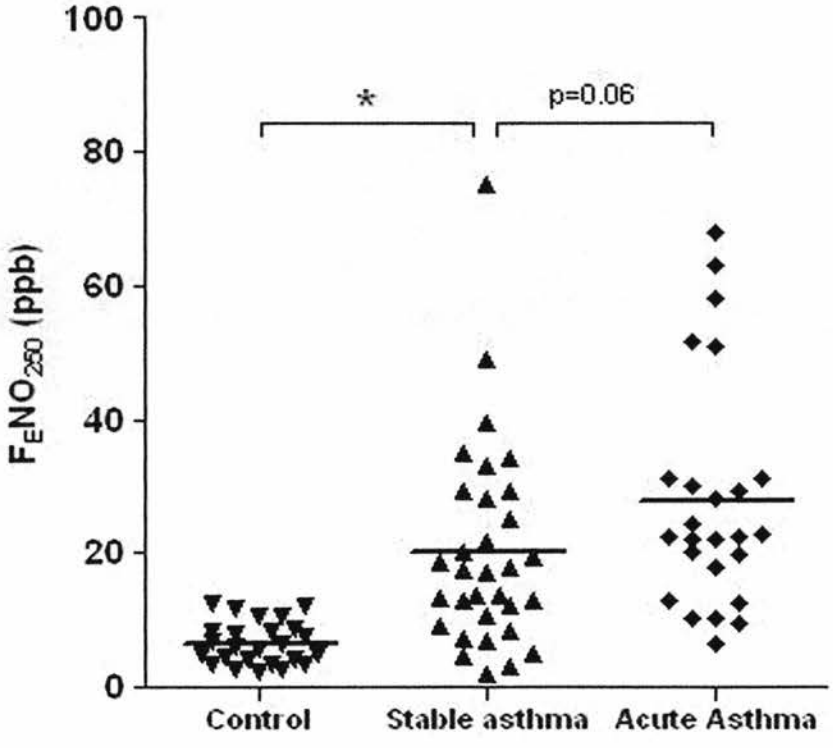
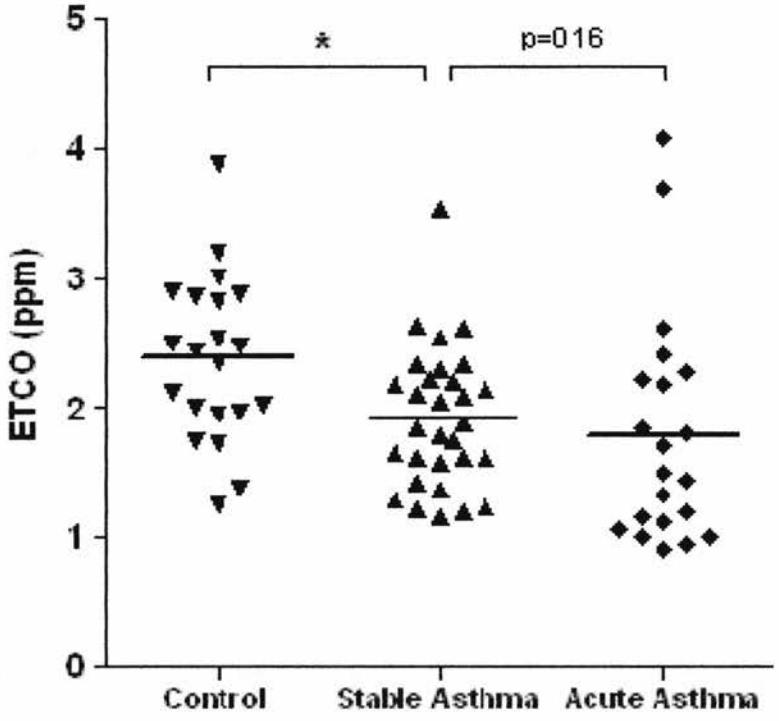


*Figure 13. Exhaled CO in control subjects (n=21), stable asthma patients (n=30), and patients with an acute exacerbation of asthma (n=21; * p= < 0.01; Tukey test).*



Breath Condensate Markers

EBC pH was significantly lower in stable asthma (6.4 ± 0.3; p = < 0.05) compared with controls (6.6 ± 0.5; table 4; figure 14). In acute asthma there was a further decrease in EBC pH (6.1; p = < 0.01) compared with stable asthma. EBC nitrite did not differ significantly in stable or acute asthma compared with control subjects (p = 0.91; figure 15).

Figure 14. Exhaled breath condensate pH in control subjects (n = 22), stable asthma patients (n = 31), and patients with an acute exacerbation of asthma (n = 24; # p = < 0.05; * p = < 0.01).

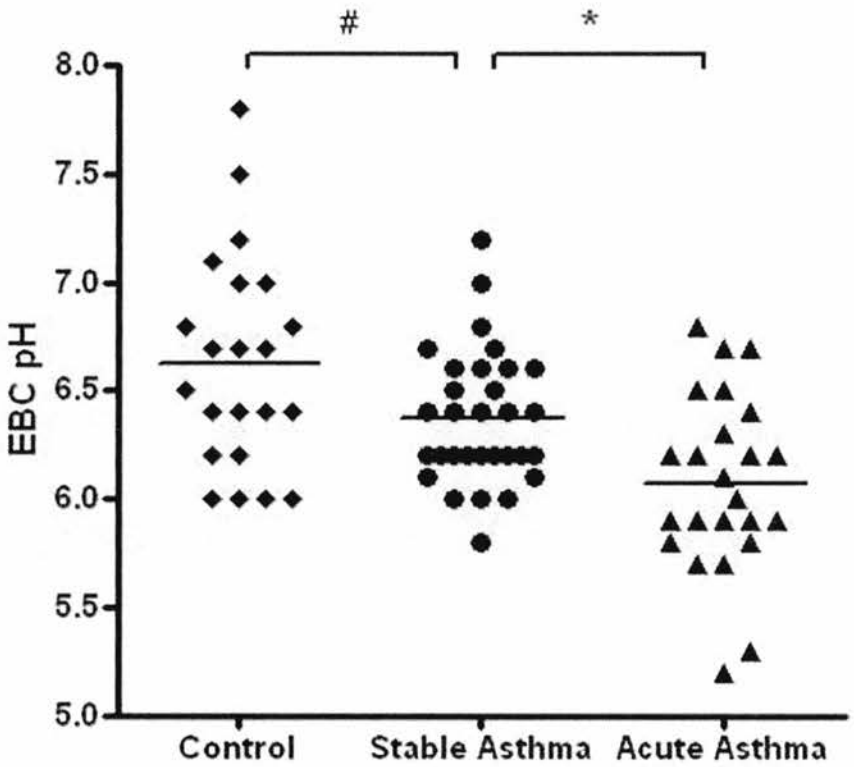
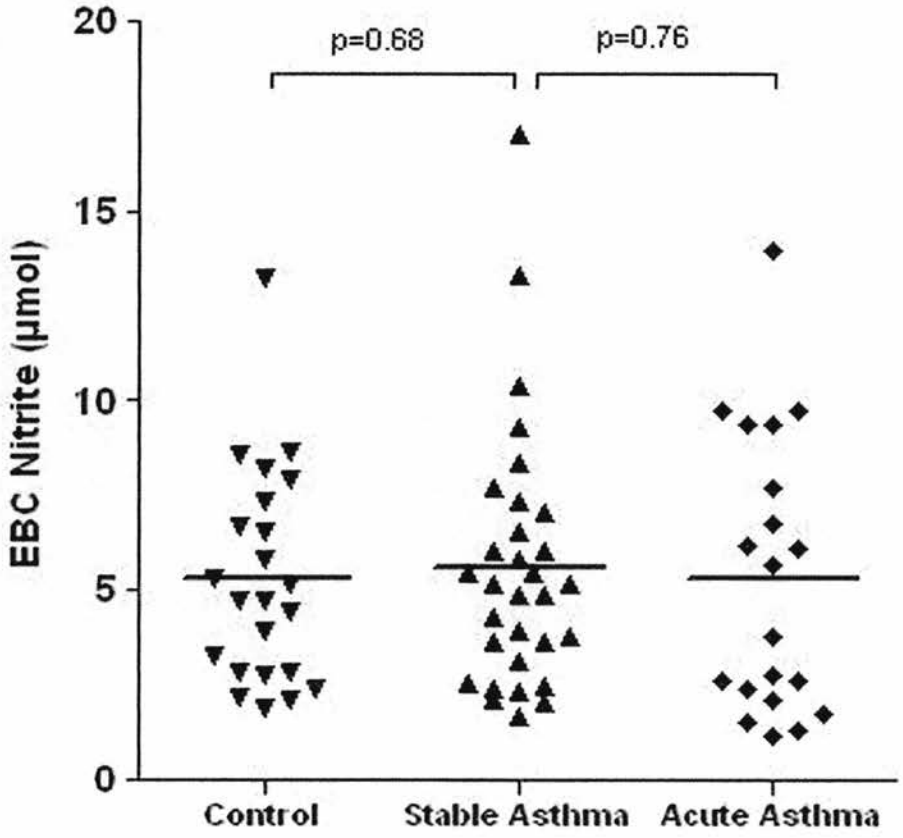


Figure 15. Exhaled breath condensate nitrite in control subjects (n=23), stable asthma patients (n=31), and patients with an acute exacerbation of asthma (n=20). p-values represent comparison between groups using a t-test.



Induced Sputum Eosinophil Percentage

Sputum collection was successful in 19/ 32 patients with stable asthma and 11/ 25 patients with acute, severe asthma. Induced sputum was the most difficult sample to collect and the reasons for failure to collect sputum are outlined in figures 8 and 9.

Sputum eosinophil counts were higher in acute asthma [18.7 % (7.3-31.8)] compared with stable asthma [6.7 % (3.1-13.5); p = <0.05; Figure 16]. Differential neutrophil, macrophage and lymphocyte counts were not significantly different in stable and acute asthma (table 6).

Figure 16. Sputum eosinophil percentage in patients with stable asthma (n=19) and acute exacerbations of asthma (n=11; # p < 0.05).

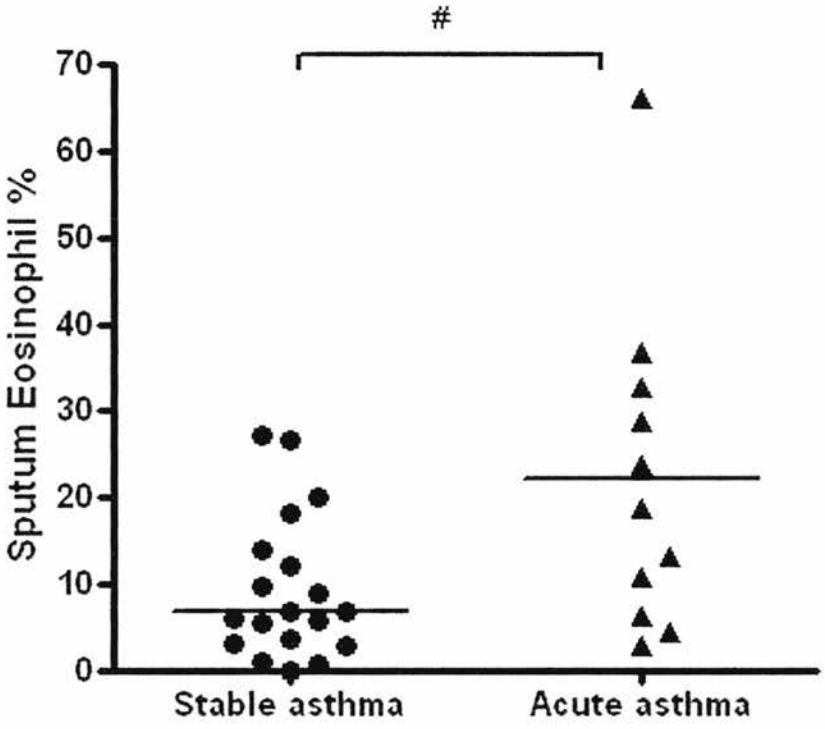


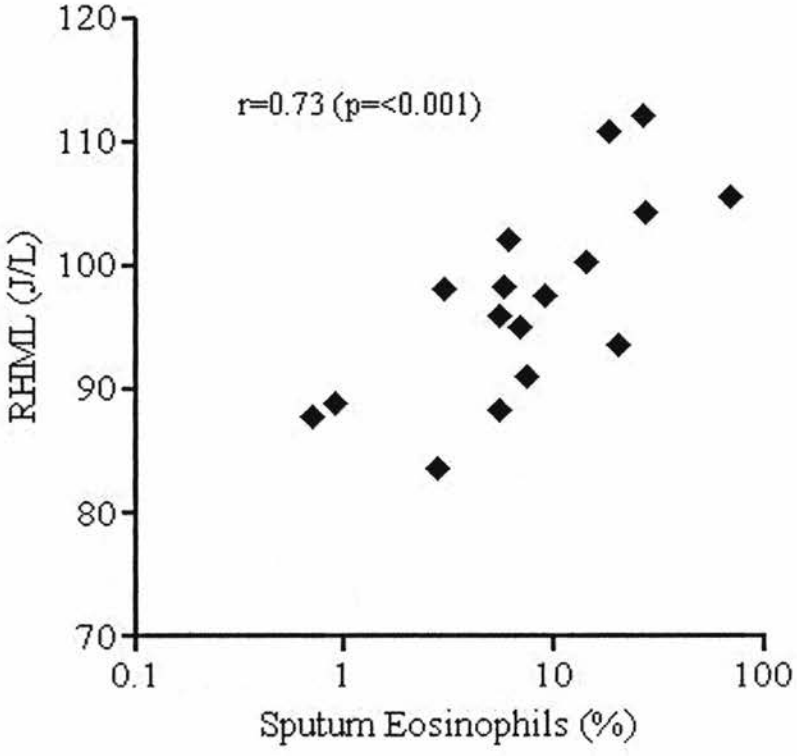
Table 6. Induced sputum differential inflammatory cell percentages in stable and acute asthma.

	Stable Asthma (n = 19)	**Acute Asthma (n = 11)**	**p value**
Eosinophil %	6.7 (3.1 - 13.5)	18.7 (7.3 - 31.8)	p = < 0.05
Neutrophil %	62.0 (33.8 - 79.3)	53.8 (37.8 - 74.6)	p = 0.93
Macrophage %	23.0 (14.5 - 38.8)	20.0 (6.4 - 26.9)	p = 0.18
Lymphocyte %	1.7 (0.9 - 2.8)	2.4 (1.6 - 4.2)	p = 0.18

Correlations between Markers

There was a close correlation between RHML and $_{log}10$ (sputum eosinophil percentage) in stable asthma (r = 0.73, p = < 0.001; figure 17) but no correlation with exhaled NO (r = 0.23), EBC pH (r = -0.09), FEV_1 (r = -0.21) or percentage predicted FEV_1 (r = -0.04). In stable asthma, there were no significant correlations between other non-invasive markers of airway inflammation measured.

Figure 17. Correlation between RHML and sputum eosinophil percentage in stable asthma (n=17).



In the context of an acute exacerbation of asthma, sputum eosinophils (%; $\log_{10}$) correlated inversely with percent predicted FEV_1 ($r = -0.83$; $p = < 0.01$; figure 18). Other markers had no correlation with FEV_1. F_ENO_{250} ($\log_{10}$) correlated with peripheral blood eosinophils ($r = 0.64$; $p = < 0.01$; figure 19). However, there was no significant correlation between exhaled NO and sputum eosinophils ($r = 0.48$, $p = 0.14$). There was a weak positive correlation between exhaled NO and EBC pH ($r = 0.46$; $p= < 0.05$).

Figure 18. Correlation between sputum eosinophil cell percentage and percentage predicted FEV_1 in acute asthma (Pearson; r = -0.83; p = < 0.01).

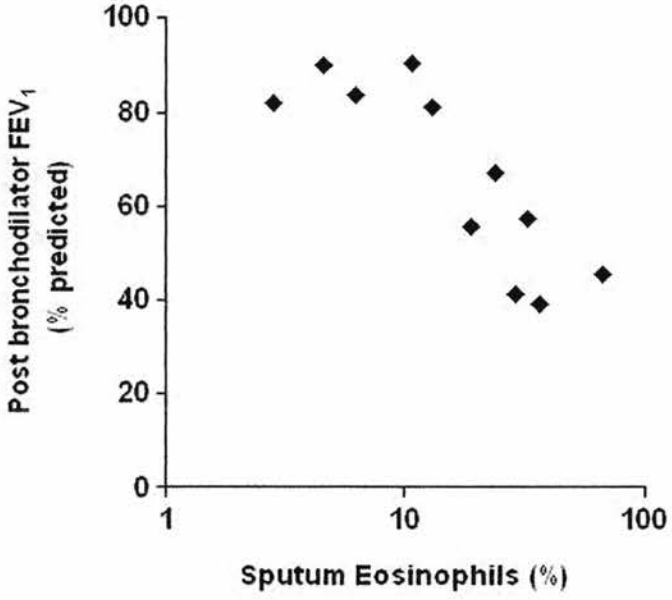
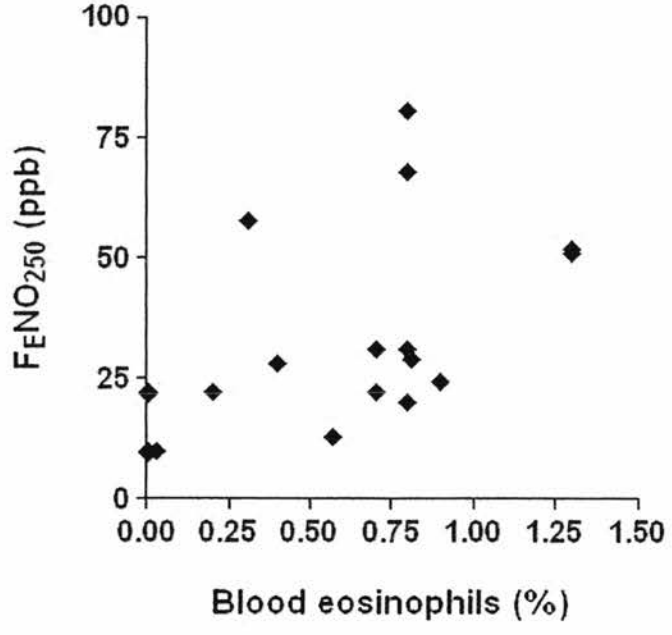


Figure 19. Correlation between exhaled NO and peripheral blood eosinophils in acute asthma. (Pearson; r = 0.64 ; p = < 0.01)



DISCUSSION

In this study a direct comparison was made between multiple markers of airway inflammation in a stable hospital outpatient asthmatic population, a group of patients with acute asthma and healthy controls. A significant increase in RHML can be detected in patients with stable but symptomatic asthma. In these patients, there is a strong correlation between RHML and sputum eosinophilia, a marker of asthmatic airway inflammation. This suggests that the elevation of RHML in asthma is due to increased airway inflammation. However, the signal appears to be absent in acute asthma, where one might expect abundant airway inflammation to cause a further increase in RHML. In the patients studied here, lower EBC pH levels, reduced FEV_1 and elevated sputum eosinophil percentages suggest that airway inflammation was indeed further increased in acute asthma. Exhaled NO also had a trend towards elevated levels during an acute exacerbation. In stable asthma, exhaled NO and EBC pH were able to distinguish asthmatic populations from controls. Exhaled CO and EBC nitrite did not appear to be valuable markers in the asthma patients studied.

Respiratory Heat and Moisture Loss

Original measurements of the thermal behaviour of airways in lung disease first appeared in the Russian literature in the 1970s using apparatus that integrated temperature changes against volume of exhaled air [139]. Improved apparatus was later used by Agarkov et al to demonstrate differences in caloric output between control subjects and several patient groups, including patients with bronchial asthma [140]. No attempt was made in these early studies to quantify evaporative heat loss. In the present study we have used cooler inspirate, targeted breathing to increase and

standardise minute ventilation (to engage the lower airway), and exhaled humidity measurement to quantify evaporative losses. These differences make direct comparison of the present results with the previous studies difficult.

In the current study, external factors that may influence respiratory heat loss have been controlled, as far as is possible in a clinical setting (Table 5). Inspiratory conditions were well controlled across groups and ventilatory pattern was well matched during each test. Minor differences in respiratory pattern are corrected by expressing RHML in J/L of expired air.

Drugs used in treatment of asthma may have conflicting effects on airway mucosal blood flow, potentially confounding this measurement. Inhaled corticosteroids are vasoconstrictors, whereas beta-2-agonists are vasodilators. Corticosteroids have been reported to reduce airway mucosal blood flow following two weeks treatment [24]. This is likely to reflect a reduction in inflammation-related vascularity. The acute effect of inhaled corticosteroids on bronchial blood flow is reported to be a more transient vasoconstriction that is maximal at 30 minutes and has disappeared by 90 minutes [141]. In contrast, salbutamol is reported to have a vasodilatory effect on the airway vasculature that may lead to an increase in respiratory heat loss [142]. Nebulised salbutamol can increase airway mucosal blood flow and breath temperature gradients in control subjects, although this effect is not apparent in asthmatic patients who already have a higher baseline of mucosal blood flow [141, 143].

In the present study, inhaler use was controlled in chronic asthmatics (inhalers were withheld for 12 hours), but ethical considerations precluded altering therapy in the

acute asthma group. In the chronic asthma group, 16 patients were taking LABAs and all were taking inhaled corticosteroids (mean dose 833 ± 561 mcg of BDP or equivalent). Despite the potential confounding effect of these treatments, significantly raised RHML was found in this group. In addition, there was no significant difference between patients taking a LABA and those not (98.3 vs 97.5 J/L, $p = 0.81$). It therefore seems unlikely that LABA exerted an effect on RHML values.

The possible effect of differences in work of breathing on RHML should be considered. However, there was no correlation between the severity of asthma measured by percent-predicted FEV_1 and RHML. Patients, who have a higher work of breathing, therefore do not appear to have higher RHML.

The link between RHML and sputum eosinophil percentage is an intriguing and novel correlation between a biophysical and a cellular marker. Monitoring sputum eosinophil percentage has been reported to have a positive impact on preventing asthma exacerbations when compared with conventional assessment of asthma in a randomised controlled trial [128]. A similar impact has not thus far been demonstrated with other non-invasive markers of airway inflammation.

RHML did not correlate with FEV_1, percent-predicted FEV_1, exhaled NO, or EBC pH in stable asthma. Spirometry does not always correlate consistently well with markers of airway inflammation [47, 144]. This is probably because inflammatory markers provide information on current airway inflammation, whereas spirometry cannot distinguish between background structural damage or remodelling and present

inflammation. Furthermore, the absence of a relationship between FEV_1 and RHML in the asthmatic group reduces the possibility of the increase in RHML being an airway calibre effect.

The paradoxical low RHML value observed in acute asthma has a number of possible explanations, including drug effects and altered pre-test ventilatory conditions. Drugs used in treatment of acute asthma may have conflicting effects on airway mucosal blood flow which have been described earlier. Standard practice in the department in which this study was based is to treat patients with acute asthma with systemic corticosteroids and nebulised salbutamol and ipratropium bromide. Ipratropium bromide has been used in allergic rhinitis for its mucosal drying effect [145], although it has been reported not to affect the ability of the nose to condition inspired air [146]. In contrast, salbutamol is reported to have a vasodilatory effect on the airway vasculature that may lead to an increase in respiratory heat loss [142]. Oxygen, which is stored in a dry form, may also have a dehydrating effect on the airway when delivered directly without humidification. It was not standard practice at the time of this study to humidify the high flow oxygen that patients receive on admission.

In acute asthma ventilatory pattern is altered. There is an increase in respiratory rate [147] and inspiratory flow rate is increased [148]. There may also be greater mouth breathing due to nasal congestion, which reduces upper airway conditioning of inspired air [149]. These factors in addition to airway narrowing, allow unconditioned air to penetrate deeper into the conducting airways, which may affect airway wall temperature and hydration. Moloney et al have reported lower humidity in exhaled air from patients with acute asthma compared with control subjects. The

temperature of exhaled air was not significantly different between groups [150]. Although ventilatory pattern was controlled during our tests, it is possible that an altered breathing pattern prior to testing had some pre-conditioning effect on airway mucosa that resulted in lower levels of RHML when compared against a stable asthmatic with a normal resting ventilatory pattern.

The potential clinical consequences of airway drying in acute asthma are worthwhile considering. This is likely to lead to thicker mucous secretions and may contribute to airway constriction. Changes in mucosal osmolality have been proposed as a mechanism for the bronchoconstriction that occurs in exercise induced asthma (151). At present humidified oxygen and intravenous fluids are recommended in guidelines for the management of acute asthma, although there is little evidence to support this recommendation [3]. A potential area for future research would be to study the possible benefits of strategies to minimise airway dehydration in acute asthma.

Exhaled Gases

In an earlier study of patients with mild asthma, Kharitonov et al reported that exhaled NO was elevated in steroid naïve asthma, but not in steroid treated asthma [54]. However, the elevated exhaled NO levels seen in our cohort of predominantly moderate-severe persistent asthmatics, who were all on inhaled corticosteroids is in keeping with a previous report in a similar group of steroid treated asthmatics with moderate or severe disease [47]. Exhaled NO appears less useful in distinguishing acute exacerbations from stable asthma according to the present study. This supports the findings of an emergency department study by Gill et al that reported exhaled NO

is a poor predictor of asthma severity in patients with asthma exacerbations [152]. However the design of the present study precluded observations as to the pre-exacerbation exhaled NO levels in those patients who developed acute severe asthma.

In mild untreated asthma, NO has been reported to correlate well with sputum eosinophils [58]. However, all patients in our stable asthma group were taking regular inhaled corticosteroids, and in steroid treated patients the relationship between NO and sputum eosinophils is much less pronounced [47, 144]. These two markers vary in their response to inhaled steroid treatment [56, 153].

Although our data suggest that exhaled NO may have some utility in monitoring airway inflammation, it is worth noting that 6/ 25 patients with acute asthma had an exhaled NO value of less than 15 ppb, which has been used as an effective cut-off level to predict poor asthma control [67]. The wide inter-individual variability precludes the diagnostic use of exhaled NO in individuals.

Previous reports of exhaled CO in asthma have produced conflicting results [71, 77, 78]. In the current study, there was an unexpected decrease in ETCO in stable asthma and acute asthma compared with controls. This is in marked contrast to two previous studies that have reported greatly elevated exhaled CO in patients with asthma compared with controls [78, 154]. However, there were significant differences in the technique used to measure exhaled CO compared with the current study. Zayasu et al and Yamaya et al both used a 20s breath-hold followed by rapid exhalation, whereas a slow exhalation was used with no breath-hold in the current study [78, 154]. In addition, the group of asthma patients that had elevated exhaled CO in the study by

Zayasu et al were steroid naive whereas all asthmatic patients in the current study were on regular inhaled steroids. Exhaled CO levels rise by approximately 80% following a 10 second breath-hold, but are unaffected by exhalation flow rate, which suggests a predominant alveolar source for exhaled CO [71]. Therefore any signal from airway CO production may be concealed by alterations in alveolar diffusion of CO.

It is possible that the ETCO levels in asthmatics were sub-maximal, because although all measured exhalations were greater than 10 seconds, control subjects were consistently able to exhale for longer than asthmatic patients (particularly those with an acute exacerbation of asthma). The absence of an elevation of exhaled CO in asthma may also be explained by uneven emptying of lung units in those patients with persistent asthma or exacerbations of asthma. Ventilation heterogeneity assessed using a nitrogen washout technique is associated with bronchial hyper-reactivity in asthma [133]. Late emptying of more severely affected parts of the lung may lead to a late rise in exhaled CO that may not have been captured in the current study.

Breath Condensate Markers

Hunt et al originally reported that EBC pH was decreased in acute exacerbations, but similar to control levels in treated asthma [94]. EBC pH has since been reported to be similar to control values in mild asthma, but decreased in moderate to severe asthma [95], and the results of the current study would be consistent with that observation.

EBC pH has previously been reported in one study to correlate with sputum eosinophils in asthma by Kostikas et al [95]. Important differences in the current study are that EBC samples were not deaerated prior to testing and the cohort of asthmatics in our study appear to have had more severe disease.

No significant difference in EBC nitrite levels between the three study groups was demonstrated. This is in contrast to earlier reports that suggested EBC nitrite is elevated in asthma [92, 93]. It is possible that measurement of nitrate in addition to nitrite may have led to a detectable difference in NO metabolites in asthmatic breath [91]. Indeed bronchoalveolar lavage fluid nitrate, but not nitrite is reported to increase following segmental allergen challenge in asthmatic subjects [155]. However, in support of the current data, a recent study has reported that neither EBC nitrite alone, or combined nitrite and nitrate are different from controls in asthma [43]. The same study did not demonstrate any additional value in incorporating nitrate measurement for distinguishing cystic fibrosis breath, which has consistently been shown to be rich in NO metabolites [37]. The discordant results reported are likely to owe much to the great inter-subject variability in EBC nitrite measurements. This may be due in part to measurement error as the concentrations of nitrite in many EBC samples were at the limits of detection for the assay used. Other factors such as variable dilution of samples [85] and intrinsic subject variability are also important.

The interaction between exhaled NO, EBC pH and EBC nitrite is highly complex. Although pH was reduced in acute asthma and exhaled NO tended to be high, there was a weak positive correlation between both markers rather than the negative correlation that might be expected. This suggests that they do not behave in a predictable fashion in individuals and raised exhaled NO may not be a result of

airway acidification as suggested by Hunt et al [94]. The present findings are supported by observations in cystic fibrosis, where exacerbations do not result in increased liberation of NO despite the presence of high levels of metabolites of NO and low airway pH [43]. Another possible explanation for this relationship between exhaled NO and EBC pH, is that those patients with a low pH had poorly controlled asthma and were therefore on high doses of inhaled corticosteroid, which are known to suppress exhaled NO.

Induced Sputum Differential Eosinophil Percentage

In this study, induced sputum eosinophil percentage was higher in acute asthma compared with stable asthma. In addition, an inverse correlation between percent predicted FEV_1 and sputum eosinophil percentage was observed. This suggests that in this setting, sputum eosinophil percentage may reflect the severity of an exacerbation. Other investigators have also reported predominant eosinophilic inflammation during an exacerbation of asthma [156, 157]. However, sputum from 2/11 patients with an exacerbation of asthma was characterised by low eosinophils and high neutrophils. Neutrophilic inflammation in asthma exacerbations has been reported elsewhere [158]. There appears to be heterogeneity in the pattern of inflammation in an exacerbation of asthma, which is likely to affect response to treatment.

The group of predominantly moderate-severe persistent asthmatics in the current study were all receiving inhaled corticosteroids. Despite the high mean dose of inhaled steroid, the median sputum eosinophil percentage was still elevated [5.9

(1.23-11.0) %], when compared with reported control values [117, 159], perhaps indicating potential for better control of airway inflammation. Previous studies have reported that sputum eosinophilia is associated with poor asthma control and persistent airflow obstruction in asthma [125, 160]. Furthermore, there are three randomised controlled trials that demonstrate a reduction in exacerbations of asthma when a strategy aimed at reducing sputum eosinophils is used as opposed to standard clinical assessment of asthma control [96, 127, 128].

Other studies that have examined cohorts of patients with severe persistent asthma, have reported elevated sputum eosinophils despite high doses of inhaled or oral corticosteroids [10, 124, 161]. This may reflect poor compliance with treatment or a relative resistance to steroid treatment. Compliance with steroid treatment has long been recognised as a problem in asthma management. In another study of patients with difficult asthma nine out of eighteen patients on oral prednisolone had undetectable levels of prednisolone in their blood [162]. An alternative explanation for the high levels of sputum eosinophils observed is a relative resistance to treatment. When a high dose long-acting steroid injection is given to patients with persistent asthma symptoms despite high dose inhaled steroid treatment, sputum eosinophil percentage is reported to decrease [125]. However such an approach would also provide effective treatment for individuals who do not comply with their usual asthma treatment, which may have influenced the results.

A significant problem with using induced sputum differential cell counts as a clinical tool may be difficulties with sample procurement on a regulator basis. In centres where induced sputum is used frequently as a research tool, adequate samples are

obtained in approximately 80-90 % of cases [116, 127, 163]. However the success in obtaining samples may be significantly less out with large research centres. Sputum analysis also requires adequate laboratory support and time which may limit its use to larger centres.

A further problem with induced sputum is that differential inflammatory cell percentages are most commonly reported. Although this may provide an internal reference within the sample, there is significant potential for error, particularly where one particular cell type is present in large numbers. For example, in patients with acute or chronic asthma, who have an abundance of neutrophils in their sputum, eosinophil percentage may appear low, although the total number of eosinophils may still be elevated.

LIMITATIONS OF THIS STUDY

Due to equipment availability, the control group for RHML was different form the control group for exhaled gases and breath condensate. In addition there was no control group for induced sputum. A single control group with all markers studied would have simplified this study, although it is unlikely to have altered the conclusions, as both control groups had similar demographics.

The inspired air conditions for measurement of RHML were not statistically different between groups, although there appears to have been trend towards lower inspired enthalpy in the control group. However, the numerical difference in enthalpy values between groups appears small. In addition, lower inspired enthalpy would be

predicted to lead to higher RHML due to a greater thermal challenge to the airways, and therefore reduce the difference observed between controls and patients with asthma.

There were missing datasets for individual inflammatory markers in each group and therefore data was analysed for the measurements that were available for each individual rather than an intention-to treat protocol. A further criticism of the statistical analysis is the analysis of multiple correlations between markers. This increases the possibility of an incidental correlation being found. A correction factor should perhaps have been incorporated into the analysis to account for the number of markers and possible correlations studied.

CONCLUSION

Respiratory Heat and Moisture Loss, a novel biophysical marker of AI is elevated in a hospital based outpatient asthmatic population. Furthermore, there is a close correlation between sputum eosinophil percentage and RHML, suggesting that RHML is elevated due to increased AI. Breath condensate pH and exhaled NO are also potentially useful markers in the context of asthmatic airway inflammation. However in the cross-sectional data presented here, their clinical utility appears to vary. Whereas exhaled NO can distinguish asthmatic subjects from control subjects, it is less able to differentiate acute asthma from stable asthma. Conversely, exhaled breath condensate pH can distinguish subjects with acute asthma from those with stable asthma, but is less able to discriminate between those with asthma and control subjects. Finally, in acute asthma, induced sputum eosinophil percentage is elevated

compared with stable asthma, although sample collection was limited in this study by a number of different factors.

CHAPTER IV

LONGITUDINAL CHANGES IN MARKERS OF AIRWAY INFLAMMATION IN ACUTE ASTHMA

INTRODUCTION

Cross-sectional analysis of RHML and the biochemical markers studied has demonstrated that they appear to vary in their utility. Exhaled NO, breath condensate pH and differential sputum eosinophil cell count were the only markers that were able to distinguish groups of patients with acute asthma from those with stable disease. There is a relative paucity of data for how these markers behave during an acute exacerbation of asthma. In the following longitudinal study, changes in inflammatory markers were observed during the resolution of an acute exacerbation of asthma. Following longitudinal changes in biomarkers in an individual addresses the problem of inter-individual variability. It also allows observation of the dynamics of change for biomarkers during a time when severity of airway inflammation is altering rapidly. The aim of this study was to determine whether RHML, F_ENO_{250}, ETCO, EBC pH, EBC NO_2^-, and sputum eosinophil percentage change as an exacerbation of asthma resolves.

METHODS

Eighteen of the 25 patients with acute asthma studied in a cross-sectional study of non-invasive inflammatory markers (Chapter III) had serial measurements of RHML, F_ENO_{250}, ETCO, EBC pH, EBC NO_2^-, and sputum eosinophil percentage. Measurements were made on day 1 of their exacerbation (within 24 hours of presentation) and between days 3-5 and days 7-9 following treatment as their exacerbation resolved. An exacerbation of asthma was defined as a deterioration in symptoms with a concomitant reduction in PEFR from baseline levels, that warranted treatment with oral corticosteroids. To avoid methodological interface between the tests, they were performed in the following order: 1) RHML; 2) exhaled gas analysis; 3) EBC collection; 4) FEV_1 and 4) induced sputum collection.

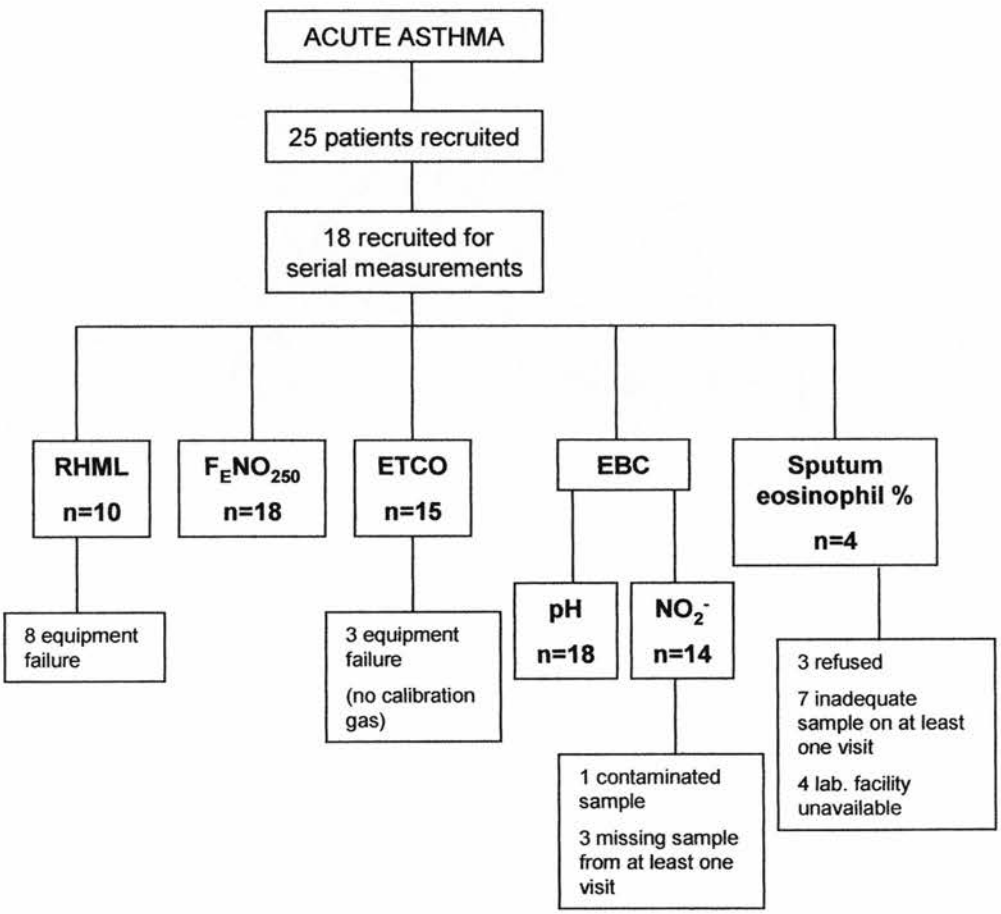
Fifteen of the 18 patients recruited were admitted to hospital for in-patient treatment of their asthma exacerbation. Patients with acute exacerbations were treated according to British Thoracic Society (BTS) guidelines and therefore received nebulised bronchodilators and oral corticosteroids [3].

Not all measurements were possible in all individuals at each visit (fig. 20). The reasons for incomplete data were very similar to those in the cross-sectional study. These reasons were randomly distributed in the data-set and therefore should not have caused any sampling bias in the study. Only complete data-sets for each marker were subsequently analysed.

Results were analysed using Sigmastat® statistical software. Repeated longitudinal measurements were analysed using a one way repeated measures analysis of variance (RM ANOVA) to determine whether significant changes occurred. A Tukey Test was

then used to determine when a significant change occured. For data that were not normally distributed, a repeated measures ANOVA on Ranks was used with a post-test Dunn's pairwise comparison to determine when a significant change occurred. Levels of significance were determined as $p = < 0.05$.

Figure 20. Profile of data collected from patients with an exacerbation of asthma that had serial measurements of inflammatory markers on day 1, between days 3-5 and days 7-9, following treatment.



RESULTS

Longitudinal changes in inflammatory markers are shown in table 7. Serial measurements following treatment of an exacerbation of asthma revealed a significant increase in FEV_1 (% predicted) from day 1 (55.7 ± 17.0) to day 3-5 (71.3 ± 16.5; p = < 0.001) and a further increase between day 3-5 and day 7-9 (80.0 ± 18.1; p = < 0.05).

Table 7. Summary of changes in FEV_1 and inflammatory markers as an exacerbation of asthma resolves. Figures are mean ± SD, or median (inter-quartile range).

	Day of Asthma Exacerbation		
	Day 1	**Day 3-5**	**Day 7-9**
FEV_1 (L) (n = 18)	1.9 ± 0.8	2.4 ± 0.9	2.7 ± 0.9
	p = < 0.001	p = 0.10	
RHML (J/L) (n = 10)	89.5 ± 6.3	95.4 ± 4.8	91.0 ± 5.8
	p = < 0.05	p = < 0.05	
F_ENO_{250} (ppb) (n = 18)	22.3 (13.0 - 31.3)	21.2 (9.9 - 34.0)	15.4 (8.3 – 22.6)
	p = 0.94	p = < 0.01	
ETCO (ppm) (n = 15)	1.55 ± 0.60	1.73 ± 0.40	1.62 ± 0.45
	p = 0.47		
EBC pH (n = 18)	6.0 ± 0.4	6.1 ± 0.3	6.6 ± 0.3
	p = 0.44	p = < 0.001	
EBC NO_2^- (mmol) (n = 16)	3.71 ± 2.26	5.86 ± 2.71	5.72 ± 2.34
	p = < 0.05	p = 0.87	

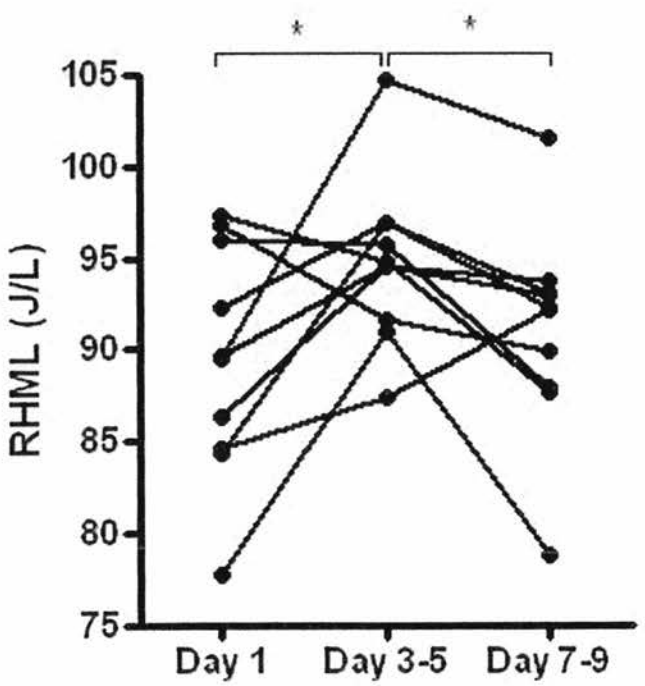
RHML increased between day 1 (89.5 ± 6.3 J/L) and day 3-5 (95.4 ± 4.8 J/L; p = < 0.05) and then decreased significantly by day 7-9 (91.0 ± 5.8 J/L; p = < 0.05; figure

21). Inspired air conditions and ventilatory pattern targeting were well matched between groups (table 8).

Table 8. Comparison of enthalpy of conditioned inspired air and minute ventilation between serial measurements of RHML.

	Day of Asthma Exacerbation		
	Day 1	**Day 3-5**	**Day 7-9**
Enthalpy Inspirate	21.6 ± 3.5	20.7 ± 3.6	19.7 ± 3.0
	p = 0.09		
Minute Ventilation	15.8 ± 3.3	16.3 ± 3.9	15.9 ± 3.7
	p = 0.60		

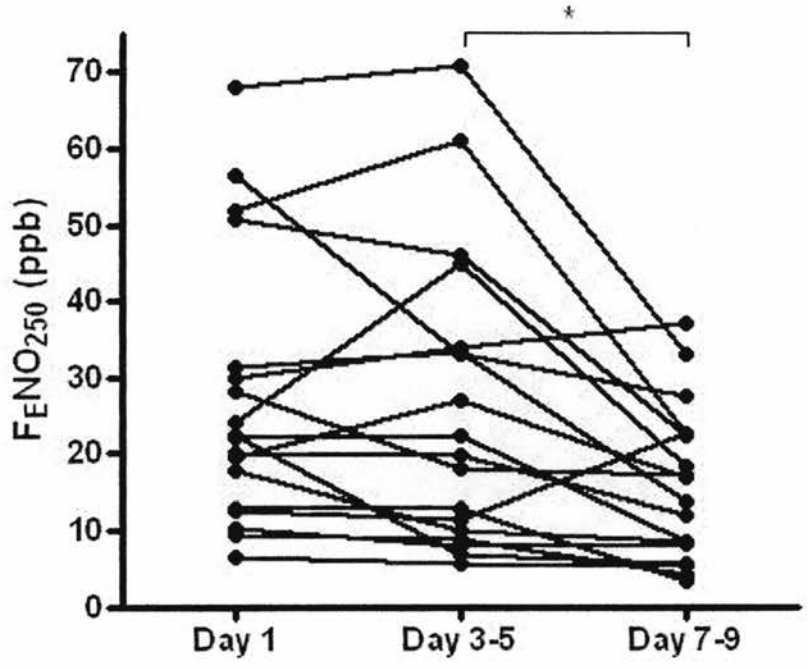
Figure 21. Longitudinal changes in RHML in patients with an exacerbation of asthma on day 1, day 3-5 and day 7-9 following treatment (* p = < 0.05).



Exhaled NO decreased significantly after treatment (p = < 0.001). In contrast with lung function changes, there was no significant difference between F_ENO_{250} on day 1 [22.3

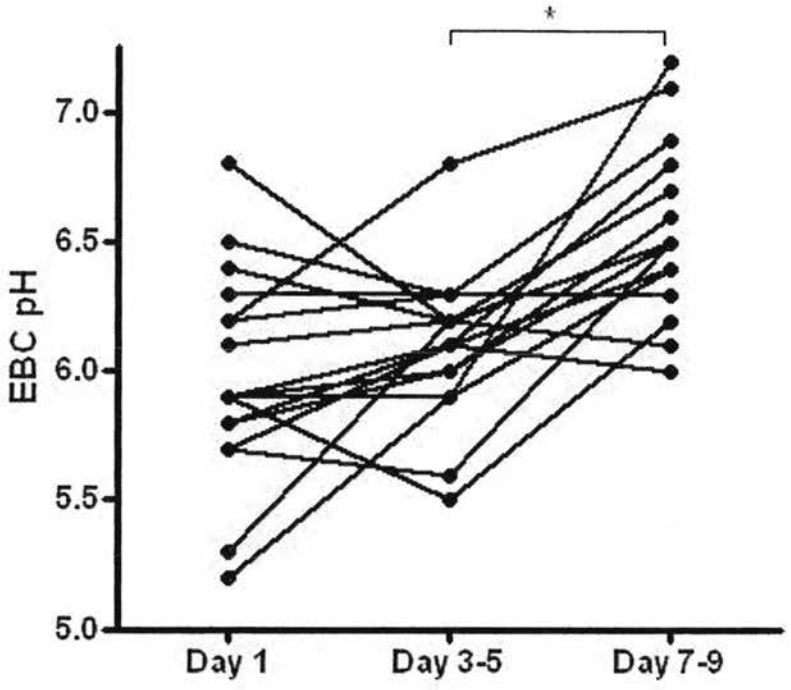
(13.0 - 31.3) ppb] and day 3-5 [21.2 (9.9 - 34) ppb; p = 0.94]. The decrease occurred between day 3-5 and day 7-9 [15.4 (8.3 - 22.6) ppb; p = < 0.01; figure 22].

Figure 22. Longitudinal changes in exhaled NO in acute asthma and its resolution. (* p = < 0.001)



A similar pattern was observed with EBC pH. There was no significant change between day 1 (6.0 ± 0.4) and day 3-5 (6.1 ± 0.3; p = 0.44), but a significant increase between day 3-5 and day 7-9 (6.6 ± 0.3; p = < 0.001; figure 23).

Figure 23. Longitudinal changes in exhaled breath condensate pH during the resolution of an exacerbation of asthma (* p = < 0.001).



Complete series of ETCO measurements and EBC nitrite assays were available in 15/18 and 16/18 subjects respectively. There was no significant change in serial measurements of exhaled CO ($p = 0.47$; figure 24), but EBC nitrite increased between day 1 and day 3-5 ($p = < 0.05$; figure 25). Sputum was not analysed as only 4 subjects had a complete set of samples from three visits.

Figure 24. Longitudinal changes in exhaled CO during the resolution of an exacerbation of asthma. No significant difference between visits.

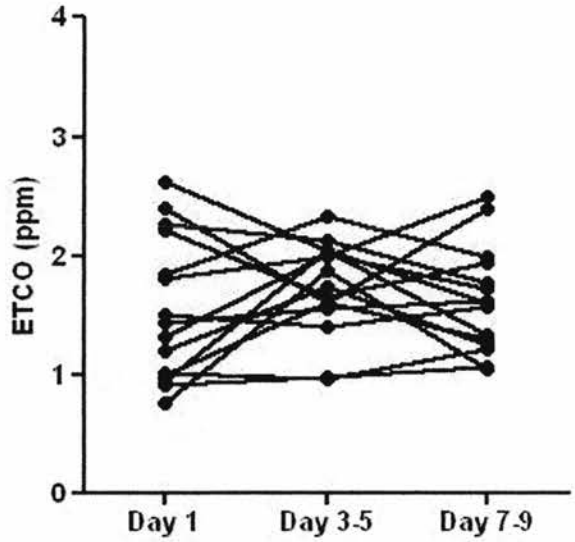
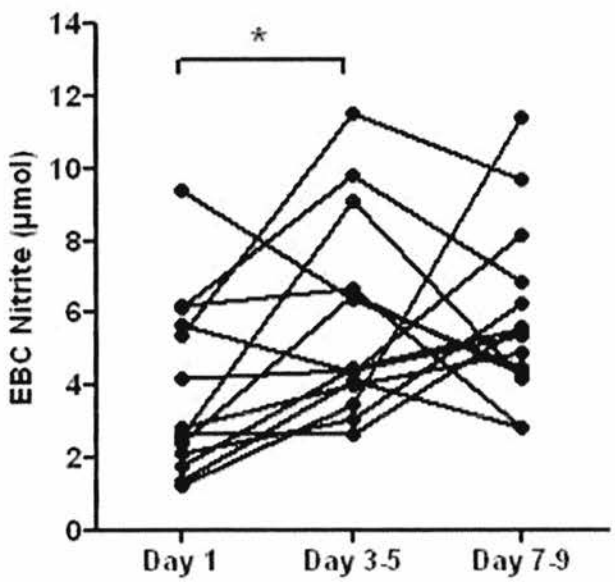


Figure 25. Longitudinal changes in EBC nitrite in an acute exacerbation of asthma (p = < 0.05).



DISCUSSION

Respiratory Heat and Moisture Loss

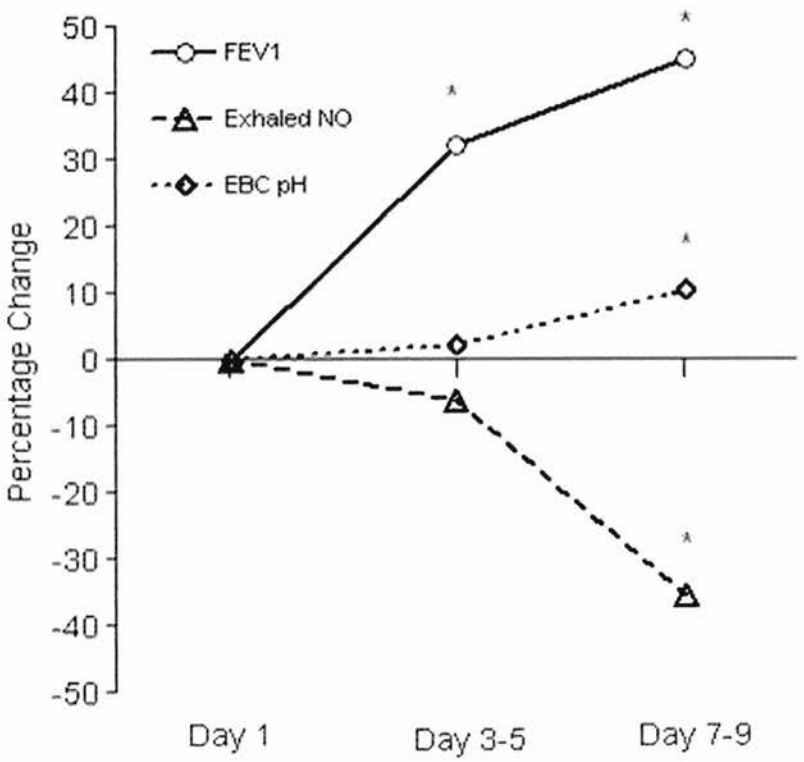
The earlier cross-sectional study demonstrated that RHML is not elevated in acute asthma (Chapter III). The possible reasons for this include drug effects and airway dehydration due to the altered ventilatory pattern in acute asthma. This longitudinal data demonstrates that RHML increases between day 1 and day 3-5 of an exacerbation of asthma. By the time of the second measurement, FEV_1 had increased greatly, and most subjects are likely to have had a near normal respiratory pattern. This supports the hypothesis that RHML measured in acute asthma may be suppressed due to external factors at the time of initial presentation, such as an elevated pre-test respiratory rate and drug-related airway drying effects.

A small decrease in RHML is witnessed between day 3-5 and day 7-9, which in the presence of relatively stable lung function may reflect reduced airway vascularity due to a reduction in airway inflammation following treatment with oral corticosteroids. Furthermore, the decrease in RHML was temporally related to changes in exhaled NO and EBC pH. Regardless of this, it appears that measurement of RHML in acute exacerbations of asthma will not be useful due the lack of a signal in this setting. It may have greater potential as a non-invasive marker of inflammation in patients with chronic persistent asthma.

Biochemical Markers

Following treatment of acute asthma, an increase in EBC pH and a decrease in exhaled NO lagged behind improvements in lung function, indicating that worsened airway inflammation persisted beyond the principal period of bronchoconstriction (figure 26).

Figure 26. The kinetics of change in inflammatory markers during the resolution of an exacerbation of asthma (* indicates a significant change from day 1, $p = < 0.01$).



The delayed changes in exhaled NO and EBC pH seen in the longitudinal exacerbation data are similar to the delay in resolution of sputum eosinophilia compared with lung function described by Pizzichini et al in acute asthma [157]. This supports worsened inflammation persisting beyond improvements in spirometry and indicates the latter is limited in guiding anti-inflammatory treatment.

Exhaled NO has previously been reported to respond very quickly to nebulised budesonide (in 6 hours) in paediatric exacerbations of asthma [164]. However, this study appeared to include mild "exacerbations" (i.e. transient symptomatic worsenings) of asthma. Patients were not treated with systemic corticosteroids and more severe patients who did need systemic treatment were excluded. Also, inhaled corticosteroids have a topical effect on airway vasculature that reduces blood flow and may result in a reduction in exhaled NO [141]. A very early reduction in exhaled NO may have been missed, as the first measurements were made up to 24 hours after presentation. Also a small reduction in exhaled NO between day 1 and day 3-5 may have been disguised by the concomitant changes in airway calibre [52]. However it seems more likely that the delayed fall in exhaled NO reflects persistent underlying inflammation in exacerbations of asthma. The observation that EBC pH follows the same pattern and the apparent elevation of RHML above baseline levels at 3-5 days support this theory.

There was no significant difference in EBC nitrite levels between the three study groups in the cross-sectional analysis of patients with acute asthma, stable asthma and control subjects (figure 15). However, in the cohort of acute asthmatics that were studied longitudinally as their exacerbation resolved, EBC nitrite was lower on day 1

compared with day 3-5. This result should be treated with caution. There are possible explanations for why nitrite may be lower in acute asthma. A significant proportion of the patients with acute asthma were treated with antibiotics in addition to oral steroid and nebulised bronchodilators. This may have altered normal flora of bacteria which convert nitrate to nitrite. Nitrate may have been elevated in this study, but was not measured. In addition a reduction in airway pH favours conversion from NO_2^- to NO [94], which may lead to depletion of nitrite levels in samples, although in the current study, no correlation was found between EBC NO_2^- and EBC pH or exhaled NO. There are also other technical factors that may have contributed to measurement error, including poor sensitivity of the assay used at the low concentrations of nitrite observed, possible variable dilution of breath condensate samples and uncertainty as to the site of origin of nitrite collected in breath condensate. Resolving these issues is a complex process and may be of limited benefit as if anything it appears to be lower in the acute phase of an asthma exacerbation, a time whether there should be abundant inflammation present.

CONCLUSION

At present anti-inflammatory treatment in exacerbations of asthma is largely empirical. Perhaps treatment could be tailored more specifically to patients' needs if measurements of inflammatory indices were incorporated into management of asthma exacerbations. In the current study, there was considerable heterogeneity in the response of each marker during the resolution of an exacerbation of asthma. This probably reflects variability in the severity of an exacerbation and possibly the nature

of the underlying inflammation or underlying asthma phenotype. However in subgroups of patients, breath condensate pH and exhaled NO are potentially useful markers in monitoring treatment response during acute exacerbations of asthma. Furthermore, exhaled NO, EBC pH (and RHML) lag behind changes in lung function as an exacerbation of asthma resolves, raising the possibility that they might provide information regarding the degree of inflammation present that is complementary to lung function measurements in assessing treatment response in acute asthma.

CHAPTER V

REPRODUCIBILITY OF MARKERS OF AIRWAY INFLAMMATION

One of the problems with the inflammatory markers studied and other proposed markers of AI, is that there is wide inter-individual variability in results. This may be due to variability in the severity of disease, or variability in the measurement techniques. In order to validate inflammatory markers and to interpret changes within individuals, it is important to have some understanding of the reproducibility of these measurements. The aim of the following study was to investigate the day-to-day repeatability of RHML and other inflammatory markers in individuals.

METHODS

The day-to-day repeatability of RHML, exhaled NO, breath condensate pH and EBC nitrite was assessed in eight stable asthmatics, who had two measurements on two separate days within a one week period at a time when their disease was clinically stable. FEV_1 was also recorded on each visit. The patients had a mean age of 42.1 ± 13.9; female: male ratio, 6:2. All patients were taking a regular inhaled corticosteroid

and 5/ 8 were taking a regular LABA. All were non-smokers. Inhaled medications were withheld for 12 hours prior to testing. The measurements were taken in the following order: RHML; Exhaled NO; EBC collection; and FEV_1. For assessment of inter-day variability, measurements were taken at the same time of day on each visit. In this study the repeatability of sputum eosinophils was not assessed (due to difficulties in previous studies with sample procurement) and it was not possible to measure exhaled CO (calibration gas not available at the time of the study).

The day-to-day repeatability of RHML was also assessed in nine healthy controls recruited from a hospital staff. Each subject had two measurements on different days within a one week period. In addition 12 subjects had back-to-back measurements of RHML, 10 minutes apart.

The repeatability of inflammatory markers was assessed by calculating the measurement error (intra-subject standard deviation) for repeated measurements, using methods described by Bland and Altman [164]. Confidence intervals for measurements were calculated as ± 1.96 x measurement error. The correlation coefficient of repeated measurements was calculated using a Pearson test. All data are expressed as mean ± SD. Inspired air conditions for RHML measurement were compared using a paired t-test.

RESULTS

Respiratory Heat and Moisture Loss

Mean values of RHML, F_ENO_{250}, EBC pH and EBC nitrite on visit 1 and visit 2 are detailed in table 9. The day-to-day repeatability of these inflammatory markers is presented as Bland and Altman plots in figure 27. Repeat measurements of RHML in eight stable asthmatics demonstrated a day-to-day measurement error for this test of 2.3 J/L, giving a 95% confidence interval of ± 4.6 J/L. In healthy controls, day-to-day repeat measurements of RHML had a measurement error of ± 1.6 J/L, giving a 95% confidence interval of ± 3.1 J/L. Overall, the correlation coefficient between day-to-day repeat measurements of RHML was r = 0.91; p = < 0.001 (Table 9; Figure 28). There was a tendency for greater variability in repeated measurements, as RHML increased (figure 27). For all subjects, inspired air enthalpy was similar on visit 1 (22.9 ± 1.3) and visit 2 (22.7 ± 1.0 J/L; p = 0.70), as was the minute ventilation (15.1 ± 1.8 vs 15.2 ± 2.2 L/min; p = 0.45). Back-to-back measurements of RHML had a 95% confidence interval of ± 3.0 J/L, and a correlation coefficient of r = 0.82 (p = < 0.01; figure 29).

Table 9. Day-to-day reproducibility of RHML, F_ENO_{250}, EBC pH and NO_2^- measurements in patients with stable asthma. Measurement values are mean ± SD.

		Visit 1	Visit 2	Correlation coefficient	p value
FEV_1		2.43 ± 0.54	2.40 ± 0.62	0.98	< 0.001
RHML	**Asthma**	96.6 ± 3.5	97.7 ± 3.3	0.91	< 0.001
	Control	88.0 ± 5.1	87.8 ± 6.2		
F_ENO_{250}		34.3 ± 26.4	32.5 ± 22.4	0.96	< 0.001
EBC pH		6.4 ± 0.3	6.2 ± 0.3	-	0.21
EBC NO_2^-		5.0 ± 2.6	4.2 ± 3.9	0.96	< 0.01

Figure 27. Bland and Altman plots of day-to-day repeat measurements of RHML, Exhaled NO, breath condensate pH and nitrite in individuals with asthma (*and control subjects).*

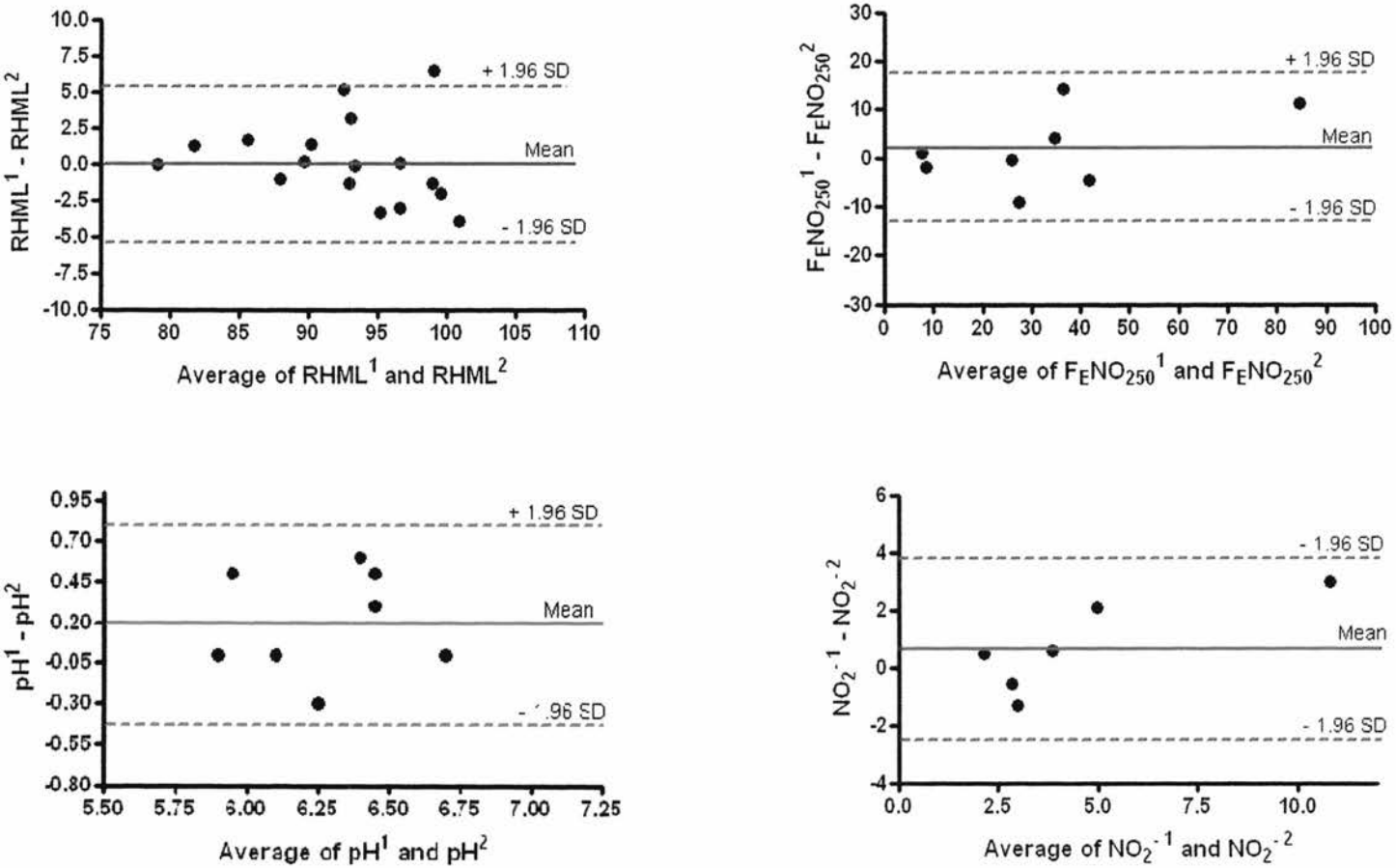


Figure 28. Measurement of RHML in patients with stable asthma (○) and healthy controls (●) in two tests within a one week period (r = 0.91; p = < 0.001).

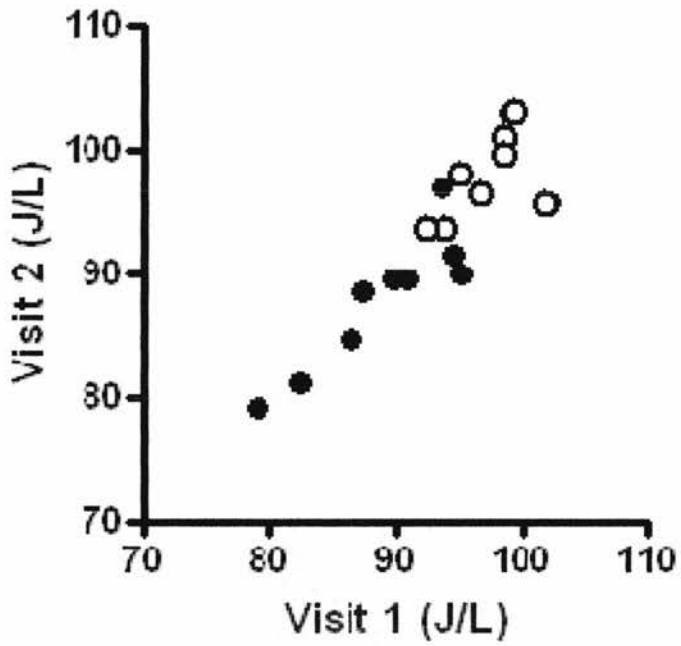
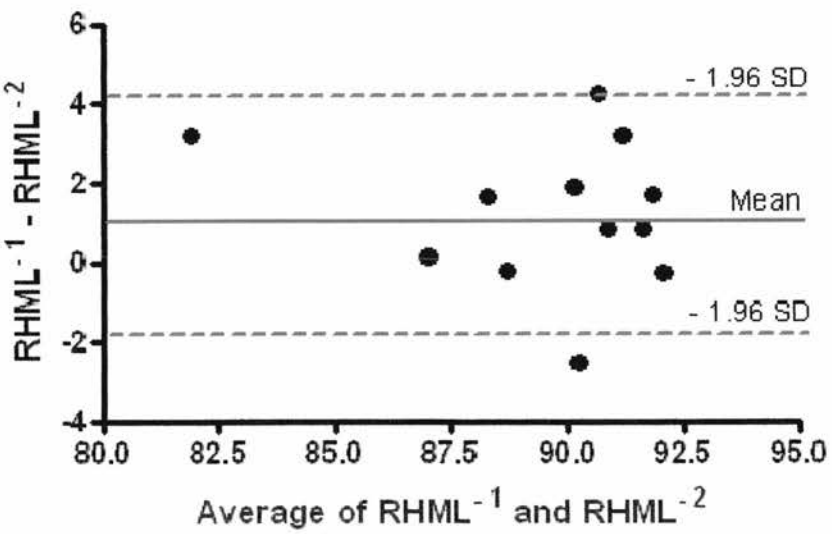


Figure 29. Bland and Altman plots of back-to-back measurements of RHML in healthy controls (n = 12).



Biochemical Markers

For day-to-day repeat measurements of F_ENO_{250}, the measurement error was 4.8 ppb, giving a 95% confidence interval of ± 9.5 ppb. The correlation co-efficient between the two measurements was r = 0.96 (p = < 0.001; Table 9). Repeated breath condensate pH measurements had a 95% confidence interval of ± 0.27. The correlation coefficient between repeated measurements was not significant (p = 0.21). Six paired EBC nitrite samples from different days within a one week period were analysed. The other samples did not have matching pairs due to one missing sample and one inadequate sample. The 95% confidence interval for repeated measurements was ± 2.26, and there was a significant correlation in EBC nitrite between visits (r = 0.96; p = < 0.01).

DISCUSSION

Respiratory Heat and Moisture Loss is a reproducible test in patients with stable asthma and control subjects. Exhaled NO and breath condensate nitrite were also reproducible in patients with stable asthma (although EBC nitrite was examined in only six subjects). In contrast, breath condensate pH was poorly reproducible in this cohort of stable asthmatics.

Respiratory Heat and Moisture Loss

RHML appeared to be reproducible and the changes that were observed in the exacerbation study were greater than the error margin of the test. From the current

data, a change in RHML of genuine significance is 4.6 J or a 5% difference between measurements. The variability in RHML may be due to problems with the measurement technique, which could be refined. Previous data has demonstrated that RHML is time sensitive and decreases during a five minute test, with the ventilatory settings used in this series of test (figure 7, Chapter II). To standardise measurements, a time-weighted measurement is used, in addition to controlled inspirate conditions and respiratory pattern. However the timing of measurements remains a potential source of measurement error. In addition, the pre-test respiratory pattern was not rigorously assessed. Greater mouth breathing or an altered respiratory rate or tidal volume before the test may affect measurements, as previously suggested in exacerbations of asthma.

There appears to be greater day-to-day variability as RHML increases. This is of particular relevance to the asthmatic patients studied, who had higher values of RHML. It is difficult to separate intrinsic disease variability from variability due to the measurement technique or equipment. However day-to-day repeatability appeared to be better in control subjects, who had a wide scatter of RHML recordings, suggesting that intrinsic variability in the state of the airway contributed partly to the increased variability in asthma.

The effect of medications (ICS, LABAs) was controlled to some extent, as inhaled medications were withheld for 12 hours prior to testing. It is unlikely that withholding these drugs for a short period of time had any effect on asthma control. FEV_1 was monitored on each visit and was similar.

Biochemical Markers

Measurement of exhaled NO is highly variable between individuals (figure 12, Chapter III). It is a sensitive technique and one of its problems is that it may be affected by many different factors, including atopy and viral respiratory tract infections [46, 48, 166]. Nevertheless, the day-to-day repeat measurements of exhaled NO in this study were reproducible. Kharitonov et al have previously reported that exhaled NO is a reproducible and also free from diurnal variation [41]. However, the longitudinal changes in median exhaled NO during the resolution of an exacerbation (Figure 22, Chapter IV) were within the 95% confidence interval for day-to-day repeated measurements in stable asthmatics. It is possible that determining individual 'normal' ranges could enhance the clinical utility of this test, or that a subgroup of patients with asthma could be identified, in whom the test is sensitive to changes in AI.

Breath condensate pH has poor day-to-day intra-subject repeatability. As with other markers of inflammation studied, it is difficult to know if variability in EBC stems from the measurement method or the assay technique. Leung et al have reported that EBC pH in non-deaerated samples was reproducible on consecutive days, although there was no correlation between samples collected through different devices [167]. This suggests that sampling technique may be relevant in explaining the variability in EBC analytes. However, in the current study, the method of collection of EBC was standardised (Chapter II), and in contrast to EBC pH, nitrite appeared to be reproducible in the 6 subjects with paired samples.

De-aeration (removal of CO_2) of EBC samples is proposed as a method of stabilising pH levels in breath condensate. Vaughn et al have reported that de-aerated EBC pH is a stable and reproducible assay in healthy control subjects [100]. Alternatively, standardisation of samples to a pre-determined CO_2 level is reported by Kullman et al to greatly enhance the reproducibility of EBC pH measurements [168]. However de-aeration increases the complexity of what is otherwise a simple measurement. In the current study, EBC samples were not de-aerated prior to pH measurement. This approach is supported by a previous study in the same lab, where Tate et al reported that EBC pH without de-aeration was reproducible in CF patients with stable disease and controls [169].

The inter-day reproducibility of RHML and exhaled NO in patients with stable asthma are described in this study. The fluctuations in RHML observed in the resolution of an exacerbation of asthma (Chapter IV), are likely to be significant as the magnitude of change is greater than the day-to-day repeatability. However, changes in exhaled NO during the resolution of an exacerbation of asthma are within the 95% confidence intervals for day-to-day measurements in stable asthma. Serial EBC pH measurement is less reproducible. This makes interpretation of small changes in exhaled NO and EBC pH difficult and raises questions over whether these markers are sensitive enough to detect significant changes in AI reliably.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Monitoring inflammation in asthma presents a number of challenges. A marker of inflammation must provide clinically useful information about the complex airway inflammation that causes asthma. As the airways are not directly accessible, there also needs to be reasonable confidence that non-invasive samples taken reflect disease processes in the airway mucosa. The inflammatory markers that we have examined in this manuscript vary considerably in their clinical utility.

Respiratory heat and moisture loss is elevated in stable persistent asthma and is reproducible in back-to-back measurements and in day-day measurements. Furthermore a correlation between RHML and sputum eosinophil percentage, and the elevated values at day 3-5 of an exacerbation, supports the hypothesis that the altered RHML in asthma is due to the presence of airway inflammation. However, it is not elevated at the onset of exacerbations of asthma. There are plausible explanations for what appears in the first instance to be a surprising result. In acute asthma, an altered ventilatory pattern, and the airway drying regimens that are part of the management of

exacerbations asthma are likely to affect the condition of airway mucosa in proximal conducting airways.

The attraction of RHML as a marker of airway inflammation is that is non-invasive, inexpensive, a global rather than a regional airway sample, and it measures inflammation non-specifically rather than relying on a specific inflammatory pathway. We believe that this technique is better suited to measure changes in heat exchange in the conducting airways compared with previous attempts because of the augmented ventilatory pattern and the cool inspirate used, which allows unconditioned air to engage the sub-glottic airway. The most significant challenge of this technique is that it is sensitive to extrinsic changes in the temperature and water content of inspired air. In addition, accurate measurement of real-time humidity changes presents a technical challenge. In this study, a time-weighted average of humidity changes was used to track inter-breath changes in humidity, due to the slow response time of the miniature humidity sensors that are commercially available. The technique for measuring RHML could be simplified by using rapidly adapting "chilled mirror" type humidity sensors. This would allow real-time tracking of changes in humidity. RHML measurements could then be made in a single breath test which would reduce the possibility of airway drying affecting the results. Improvements such as this to the equipment could reduce the error margin of RHML measurement, but are unlikely to alter the general conclusions from the cross-sectional and longitudinal exacerbation studies reported here.

Exhaled nitric oxide can distinguish asthmatic from non-asthmatic populations in contrast to the other exhaled gas examined, carbon monoxide, which was not elevated

in asthma. Furthermore changes in exhaled NO during exacerbations of asthma were able to track changes in the degree of inflammation as an exacerbation resolved. This may be a useful observation particularly as changes in exhaled NO were dissociated from changes in FEV_1, suggesting that airway inflammation persisted beyond the principal period of bronchoconstriction. Exhaled NO is of more limited use in asthmatic patients taking regular inhaled corticosteroids [47]. However, these data demonstrate that it remains elevated in moderate to severe persistent asthma, and during exacerbations of asthma where airway inflammation is uncontrolled. A distinct advantage of exhaled NO is that the measurement technique has been standardised. In addition there are commercially available handheld exhaled NO devices that may facilitate home monitoring of exhaled NO. Day-to-day measurements of exhaled NO were reproducible, but the fluctuations in this test between days was enough to cast doubt on whether the change in exhaled NO observed during the resolution of an exacerbation of asthma were clinically significant.

Exhaled CO measurements were also able to distinguish asthmatic patients from controls, although surprisingly end-tidal CO was lower in patients with asthma. Elevated levels of exhaled CO following a breath-hold and a continuous slow rise in exhaled CO during exhalation, suggest that a significant amount of it is from an alveolar source [71]. Therefore the signal from any change in CO released by airway inflammation may be obscured by high levels of alveolar CO. The reason why exhaled CO was lower in patients with asthma is unclear, although a shorter duration of exhalation, resulting in submaximal end-tidal CO is one possible explanation. The absence of any significant change in exhaled CO during the resolution of an

exacerbation of asthma supports our conclusion that exhaled CO measurements do not reflect changes in airway inflammation.

Exhaled breath condensate provides a non-invasive means of sampling airway lining fluid. However the samples are very dilute, and possibly variably dilute [85]. In addition, without intubating a patient it is difficult to avoid contamination from the upper respiratory tract [35]. It is therefore difficult to know what is being measured and where it is from. Fluctuations in a substance being measured may be due to variable dilution of the sample, or changes in concentrations of inflammatory markers in the airway lining fluid. Although exhaled NO is elevated in asthma, its metabolite nitrite is not elevated in EBC from individuals with asthma. On the other hand, exhaled breath condensate pH shows some potential as a measure of airway inflammation in acute asthma. EBC pH is low in acute asthma and rises as an exacerbation of asthma resolves. The changes in EBC pH have a temporal relationship with exhaled NO, in contrast with changes in FEV_1. However EBC pH had wide inter-individual variability and the intra-day reproducibility of measurements was poor. It is possible that the repeatability of EBC pH measurement could be enhanced by de-aeration or gas standardization of EBC samples [100].

Induced sputum eosinophil counts are widely reported as useful in assessing airway inflammation [127]. Induced sputum eosinophil percentage can distinguish patients with acute exacerbations of asthma from patients with stable asthma. In addition, sputum differential cell counts provide not only a quantitative measurement, but also information regarding the nature of the inflammation present. However, a significant disadvantage of induced sputum is the difficulty in obtaining adequate samples. In

centres which specialise in this measurement, adequate samples are obtained in approximately 80% of patients [127]. Out with a research setting, this figure may be less. This may make longitudinal assessment of patients difficult. This technique is also cumbersome, relies on the availability of adequate histopathological facilities and samples should be processed in under 2 hours [138]. This may limit the widespread clinical use of induced sputum cell counts.

All of the inflammatory markers examined in this thesis were highly variable between individuals in the same study groups, whether in stable asthma or acute asthma. Some of this will be measurement error due to the equipment or measurement techniques used. A further factor is that asthmatic airway inflammation is complex and it is increasingly being recognised that the spectrum of asthma includes some distinct disease phenotypes. It is possible that the utility of the various inflammatory markers studied here may become more apparent once there is a better understanding of the different subgroups of asthma. Further studies are required to understand how these inflammatory markers behave in particular subgroups of asthma.

To enhance the accuracy of the inflammatory markers proposed in this thesis, further work is required to refine the measurement techniques. This is particularly true for RHML and EBC markers. RHML is a novel inflammatory marker and there is scope to improve upon the measurement technique used in this thesis. For example using more accurate humidity sensors would allow a shorter test to be performed, making it less likely that the test itself will alter the airway environment. Further longitudinal studies are required to assess the clinical utility of these markers of airway inflammation. Longitudinal changes during the resolution of an exacerbation of

asthma were examined in the current study. However a phased reduction and withdrawal of inhaled steroids would be an alternative method of provoking an increase in airway inflammation.

The data presented in this thesis adds to the current literature that helps to reveal the clinical utility of measurement of exhaled nitric oxide, exhaled breath condensate pH and induced sputum differential cell counts in stable and acute asthma. From the current studies, exhaled CO and EBC nitrite appear to be less useful as markers of inflammation, as they do not appear to be elevated in stable asthma, or during an exacerbation. A novel method for measuring respiratory heat and moisture loss has been described. This may provide an alternative biophysical measure of airway inflammation, although it is likely to be limited to patients with stable persistent asthma, and not acute asthma. Non-invasive monitoring of airway inflammation remains a challenge, but it is possible that some of the inflammatory markers discussed here will aid physicians in their clinical management of difficult asthma cases in the future.

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Figure 29. Bland and Altman plots of back-to-back measurements or RHML in healthy controls ($n = 12$).

LIST OF ABBREVIATIONS

AI	Airway inflammation
BAL	Broncho-alveolar lavage
BDP	Beclomethasone diproprionate
CF	Cystic fibrosis
CO	Carbon monoxide
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
EBC	Exhaled breath condensate
ETCO	End-tidal CO
F_ENO_{50}	Fractional exhaled nitric oxide at an exhalation rate of 50 ml/s
F_ENO_{250}	Fractional exhaled nitric oxide at an exhalation rate of 250 ml/s
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
HO-1	Heme-oxygenase-1
ICS	Inhaled corticosteroid
IFN-γ	Interferon gamma
LABA	Long acting beta-2 agonist
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
PEFR	Peak expiratory flow rate
RHML	Respiratory heat and moisture loss
TNF-α	Tumour necrosis factor alpha

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APPENDIX 1

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Respiratory heat and moisture loss is associated with eosinophilic inflammation in asthma

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ABSTRACT: **Increased mucosal vascularity is a hallmark of airway inflammation in asthma. It was hypothesised that this would lead to a detectable increase in respiratory heat and moisture loss (RHML), which would reflect the degree of airway inflammation present.**

A total of 23 subjects with asthma and 18 healthy controls had RHML measured in a cross-sectional study. The measurements were made using a device that combines temperature and humidity measurement during inspiration and expiration and allows precise control over inspirate conditions and ventilatory pattern. The subjects with asthma underwent parallel measurements of exhaled nitric oxide, sputum eosinophil percentage and exhaled breath condensate pH.

Mean±SD RHML was elevated in patients with asthma (98.1±7.3 $J \cdot L^{-1}$) compared with control subjects (91.9±4.5 $J \cdot L^{-1}$). RHML measurement in asthma correlated with sputum eosinophil percentage.

This novel correlation between thermal and cellular measurements in asthma suggests that both of these noninvasive indices are sensitive to the degree of underlying chronic airway inflammation.

KEYWORDS: **Airway inflammation, asthma, exhaled breath condensate pH, exhaled nitric oxide, respiratory heat loss, sputum eosinophils**

Airway inflammation in asthma is characterised by increased mucosal vascularity. This has been demonstrated in bronchial biopsies [1–3] and using high-magnification videobronchoscopy [4]. Airway mucosal blood flow, estimated using a soluble gas uptake method, is also reported to be elevated in patients with asthma compared with control subjects [5]. It was hypothesised that an increase in airway mucosal blood flow associated with airway inflammation in asthmatics would lead to a detectable increase in respiratory heat and moisture loss (RHML).

During normal respiration, a counter-current mechanism for respiratory heat transfer operates [6]. As air is inspired, it is heated and humidified, resulting in cooling and drying of the airway mucosa. By the time air reaches the alveoli it is at body temperature and fully saturated with water vapour. During expiration, a variable fraction of the available heat energy is regained by the mucosa as air exits the lung, and the remainder is exhaled, resulting in net heat loss. Conditioning of inspired air is dependent on a source of heat and water. This comes from airway mucosal blood flow.

There is some evidence to suggest that respiratory heat flux may indeed be altered in asthma, and measurement of respiratory heat loss has been proposed as a marker of lung disease [7, 8]. PAREDI *et al.* [9] reported a faster rise in breath temperature during expiration in asthmatics compared with controls. However, in this study measurements were made with subjects breathing ambient room air and evaporative heat loss was not assessed. This is relevant because in resting conditions, the majority of heat exchange takes place in the upper airway and evaporative heat loss is a major component of total heat loss from the respiratory tract [10]. In intubated patients, measurements of tracheal temperature do not accurately predict total respiratory heat losses, but measurement of absolute humidity appears to be a better predictor [11].

The conducting airways become progressively more involved in respiratory heat exchange as either the inspired air temperature is lowered or the minute ventilation is augmented [12]. A potential hazard of these manoeuvres is that they may also alter the airway environment, by changing airway calibre or altering mucosal blood flow. In fact, the main focus of research

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into respiratory heat exchange in asthma has been the effects of extreme ventilatory conditions on the airway in the pathogenesis of exercise or cold air-induced asthma [13]. To engage the subglottic airway in heat exchange, with the purpose of measuring disease activity, conditions should be used that involve the lower airways in this process without altering their airway structure or function. In contrast to previous studies, the present authors thus used an inspirate of conditioned air with a lower temperature and water content than room air and moderately elevated minute ventilation. These conditions create a modest thermal challenge that does not affect airway calibre, but is still sufficient to engage the lower airway in heat exchange [14].

The aim of the current study was to determine whether RHML measured under these precisely controlled conditions is altered in asthma in association with airway inflammation and whether it bears any relation to the state of inflammation in the airways measured by alternative noninvasive methods. RHML measurements in asthmatic and control subjects were made in a cross-sectional comparison. In the asthmatic group, parallel measurements of exhaled nitric oxide (eNO), sputum eosinophil percentage and exhaled breath condensate (EBC) pH were made.

METHODS

Subjects

A total of 23 subjects with stable persistent asthma and 18 healthy controls were recruited. The subjects in the asthma group were recruited from respiratory outpatient clinics. They had had no exacerbations, nor any oral corticosteroid treatment, for $\geq$2 months prior to the study. Asthma was defined and classified according to Global Initiative for Asthma guidelines [15]. Out of the 23 asthmatic subjects, 11 had severe persistent asthma, eight had moderate persistent asthma and four had mild persistent asthma. Healthy controls were recruited from among hospital staff. All were nonsmokers or ex-smokers (stopped >6 months ago) with a smoking history of <10 pack-yrs. All asthmatic subjects were taking regular inhaled corticosteroids (mean dose 867$\pm$597 μg beclometasone diproprionate or equivalent). In total, 16 of the asthmatic subjects were taking a regular long-acting β_2-agonist (LABA). Inhaled medications were withheld for 12 h prior to testing. The study was approved by the local ethics committee and all subjects gave written informed consent.

Study design

RHML and forced expiratory volume in one second (FEV_1; Vitalograph, Buckingham, UK) were measured in the asthma and control groups. The asthmatic group underwent parallel measurements of other inflammatory markers after their RHML measurement. To minimise interaction between the measurement techniques, procedures were performed in the following order during a single visit: RHML measurement, eNO measurement, EBC collection, FEV_1 and induced sputum collection.

The day-to-day repeatability of RHML measurement was assessed in nine control subjects and eight stable asthmatics, who underwent two measurements on two separate days within a 1-week period.

RHML apparatus

RHML was measured using a purpose-built device (fig. 1) incorporating temperature and humidity measurement of inspirate and expirate and allowing precise control over inspiratory conditions and ventilatory pattern. Temperature sensors were K-type thermocouples (chromel-alumel bead type), with a 90% response time of 50 ms. They were calibrated against a mercury standard prior to testing. Humidity sensors were of thermoset polymer capacitance construction (HIH-3602-A; Honeywell, Morristown, NJ, USA), supplied as factory calibrated, giving relative humidity (RH) with an accuracy of $\pm$2% and an estimated 95% response time of 5 s. This response time, which is the fastest available from practical sensors, precluded intra-breath measurements, so inspiratory and expiratory flow were separated by a valve and RH and breath temperature were recorded downstream as time-weighted averages during expiration. It has been calculated that the potential error from using time-weighted values for humidity measurement is an underestimate of <5% [16]. Expiratory airflow was measured using an ultrasonic phase-shift flow-meter (FR-413; BRDL, Birmingham, UK), which was calibrated for volume (at ambient temperature and pressure and saturated with water vapour) using standard volume syringes (Vitalograph). The sensor's 100% response time was 12 ms; linearity was <2% and the residual error due to temperature variation <1% in the temperature range 0–40°C.

Target inspiratory air conditions of 10°C and 50% RH were created using a purpose built air conditioning device. The enthalpy of inspired air was therefore ~20 $J \cdot L^{-1}$. An audio-visual feedback system was used to guide subjects to achieve a tidal volume of 1.5 L (an expiratory flow rate of 500 $mL \cdot s^{-1}$ and a respiratory rate of 10 breaths·min^{-1}, to give a target minute ventilation of 15 $L \cdot min^{-1}$). This degree of elevated ventilation and cool inspirate was selected in order to engage the lower airway in heat exchange. Pilot studies have demonstrated that the thermal challenge of these conditions is not sufficient to affect airway calibre in a 5-min test [14]. Validation studies with this apparatus [16], have shown the calculated moisture

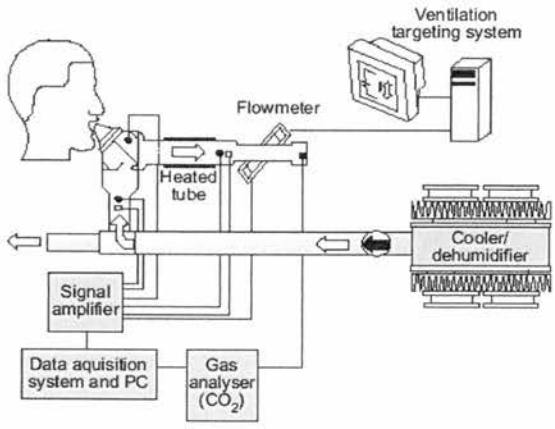

FIGURE 1. Apparatus for measurement of respiratory heat and moisture loss. ●: temperature sensor; □: moisture sensor; ■: end-tidal CO_2 sampling port.

loss for a range of minute ventilations agrees with published values using the gold standard-freeze out method [10]. Measurements were made after 1-min breathing into the apparatus. It was determined from pilot data that this is the optimum time for detecting alterations in RHML [17], allowing adequate time for the humidity sensors to adjust and the mean exhaled air temperature to reach a steady state.

RHML calculation

The total heat or energy content of an air/water mixture is given the term enthalpy. Enthalpy values were calculated using a psychrometric calculator (PsyCalc 98; Linric Company, Bedford, NH, USA). The enthalpy of the inspirate and expirate are derived from mean air temperature and RH during inspiration and expiration respectively. Net enthalpy loss is then calculated as:

$$h = h_e - h_i \quad (1)$$

Where h=net enthalpy loss ($J \cdot g$ dry air^{-1}), h_e=enthalpy of expirate and h_i=enthalpy of inspirate.

RHML is then calculated using the following formula:

$$RHML = D \times h \quad (2)$$

Where D=density of air/water vapour mixture ($g \cdot L^{-1}$).

eNO

Fractional exhaled nitric oxide (F_{ENO}) was measured in single breath using a target expiratory flow rate of 250 $mL \cdot s^{-1}$ ($F_{ENO_{250}}$) with a modified chemiluminescence analyser (LR2000; Logan Research Limited, Rochester, UK). The NO analyser was calibrated daily with N_2/NO calibration gas containing 93 ppb NO (BOC Gases, Guildford, UK). A visual feedback system was used to maintain a mouth pressure of >5 mmHg, sufficient to keep the soft palate closed and prevent nasal contamination. NO measurements were taken from the plateau phase at the end of expiration, in accordance with American Thoracic Society/European Respiratory Society (ERS) guidelines [18]. NO values were recorded as an average of three measurements. The measurement error in repeat measurements in the same individual was ±1.7 ppb, giving a 95% confidence interval (CI) of ±3.3 ppb.

EBC pH

EBC was collected using a purpose-built condenser (EcoScreen; Jaeger, Würzburg, Germany). Subjects, wearing a nose-clip, were asked to breathe through a nonrebreathing two-way valve into the apparatus for 5 min. Exhaled air was conducted through a lamellar condenser to an interchangeable sampling tube (one per sample) situated in a cooling cuff, cooled to -10°C. Condensate pH was measured immediately after collection (nondeaerated) using a calibrated pH-meter incorporating an ion-sensitive field-effect transistor sensor with temperature compensation (KS723; Camlab, Cambridge, UK) with an accuracy of ±0.1 pH units. The pH-meter underwent a two-point calibration prior to each measurement. The day-to-day repeatability of breath condensate pH in control subjects and patients with bronchiectasis due to cystic fibrosis in the unit has been reported as ±0.08 pH units [19].

Induced sputum processing and analysis

Sputum was induced using incremental concentrations of 3, 4 and 5% hypertonic saline each delivered over 4 min, *via* an ultrasonic nebuliser (DeVilbiss Ultraneb 99; DeVilbiss Healthcare, Somerset, PA, USA), set at an output of ~2.4$mL \cdot min^{-1}$. Subjects were pre-treated with 2.5 mg salbutamol *via* a nebuliser. FEV_1 was monitored closely throughout the test and the procedure was abandoned if FEV_1 decreased by >20%. Sputum processing was performed using the methods described by PAVORD *et al.* [20]. A haematoxylin and eosin stain was used for the cytospins. Sputum differential cell counts were calculated from counting 400 inflammatory cells and expressed as percentages of total inflammatory cell count. When sputum eosinophil cell percentage was counted on two separate occasions in 12 sputum samples from subjects with asthma, the correlation coefficient was r=0.98 ($p<0.001$). The intra-observer 95% CI was ±2.46%.

FEV_1 was measured to ERS standards using a standard Vitalograph wedge-bellows spirometer.

Statistical analysis

For cross-sectional analysis between groups, an unpaired t-test was used to determine whether differences observed were significant. Correlations between RHML and other inflammatory markers in the asthmatic group were determined using a Pearson correlation coefficient. Data that were not normally distributed were log-normalised prior to correlation analysis. The repeatability of RHML was assessed by calculating the measurement error (intra-subject SD), using a method described by BLAND and ALTMAN [21]. Levels of significance were determined as $p<0.05$. Normally distributed data are expressed as mean±SD and non-normally distributed data are expressed as median (interquartile range).

RESULTS

Study groups were well matched (table 1). FEV_1 (% predicted) was significantly lower in stable asthma (82.7±26.9%) compared with controls (101.8±7.5%; $p<0.01$). For RHML measurement, the enthalpy of the inspired air and the ventilatory pattern were closely matched between groups.

TABLE 1 Study demographics and respiratory heat exchange data

	Healthy control	Stable asthma
Age yrs	41.6±13.1	44.7±14.6
Subjects n	18	23
Sex F/M	10/8	17/6
Height m	1.68±0.07	1.65±0.08
FEV_1 % pred	101.8±7.5	82.7±26.9**
Enthalpy of inspirate $J \cdot g^{-1}$	20.3±1.6	21.9±1.6
Minute ventilation $L \cdot min^{-1}$	16.1±2.3	15.9±3.4
RHML $J \cdot L^{-1}$	91.9±4.5	98.1±7.3**

Data are presented as mean±SD or n. F: female; M: male; FEV_1: forced expiratory volume in one second; % pred: % predicted; RHML: respiratory heath and moisture loss. **: $p<0.01$.

RHML was significantly elevated in patients with stable asthma (98.1 ± 7.3 J·L^{-1}) compared with control subjects (91.9 ± 4.5 J·L^{-1}; $p<0.01$; fig. 2). Repeat measurements in nine control subjects demonstrated a measurement error of ± 1.6 J·L^{-1}, giving a 95% CI of ± 3.1 J·L^{-1}. Repeat measurements in eight stable asthmatics showed a measurement error for this test of 2.3 J·L^{-1}, giving a 95% CI of ± 4.6 J·L^{-1}. In the asthmatic group, induced sputum collection was successful in 17 out of 23 patients. Sputum eosinophils were 6.8% (4.9–18.6%), $Fe_{NO_{200}}$ 18.0 ppb (11.4–31.9 ppb) and EBC pH was 6.4 ± 0.3.

Subgroup analysis of the asthma group revealed that there was no significant difference in RHML between mild/moderate persistent asthma and severe asthma (97.8 *versus* 98.3 J·L^{-1}; $p=0.88$). There was also no significant difference in RHML between the 16 subjects who were on a regular LABA and subjects who were not (98.3 *versus* 97.5 J·L^{-1}; $p=0.81$).

Correlations between markers

There was a close correlation between RHML and $\log_{10}$ (sputum eosinophil percentage) in stable asthma ($r=0.73$, $p<0.0001$; fig. 3) but no correlation with eNO ($r=0.23$), EBC pH ($r=-0.09$), FEV_1 ($r=-0.21$) or FEV_1 % pred ($r=-0.04$). There were no significant correlations between other markers.

DISCUSSION

An increase in RHML can be detected in patients with stable but persistent symptomatic asthma. In these patients, there is a strong correlation between RHML and sputum eosinophilia, a robust marker of asthmatic airway inflammation. This suggests that the elevation in RHML in asthma is due to increased airway inflammation.

Original measurements of the thermal behaviour of airways in lung disease first appeared in the Russian literature in the 1970s, with apparatus that integrated temperature changes against volume of exhaled air [7]. Improved apparatus was later used by the same group to demonstrate differences in caloric output between control subjects and several patient groups, including patients with bronchial asthma [8]. However, in the present study, cooler inspirate, targeted breathing to increase and standardise minute ventilation (to engage the lower airway), and exhaled humidity measurement to quantify evaporative losses were used. These differences make a direct comparison of the present results with the previous study difficult.

As far as possible in a clinical setting, external factors that may influence respiratory heat loss were controlled. Inspiratory conditions were well controlled across groups and ventilatory pattern was well matched during each test. Minor differences in respiratory pattern were corrected for by expressing RHML per volume of expired air.

Drugs used in the treatment of asthma may have conflicting effects on airway mucosal blood flow, potentially confounding this measurement. Inhaled corticosteroids are vasoconstrictors, whereas β_2-agonists are vasodilators. Corticosteroids have been reported to reduce airway mucosal blood flow following 2 weeks' treatment [22]. This is likely to reflect a reduction in inflammation-related vascularity. The acute effect of inhaled corticosteroids on bronchial blood flow is reported to be a more transient vasoconstriction that is maximal at 30 min and has disappeared by 90 min [23]. In the context of the present study, the observed increase in RHML was seen despite potential confounding background vasoconstrictor effects of inhaled steroids. In contrast, salbutamol is reported to have a vasodilatory effect on the airway vasculature that may lead to an increase in respiratory heat loss [24]. Nebulised salbutamol can increase airway mucosal blood flow and breath-temperature gradients in control subjects, although this effect is not apparent in asthmatic patients who already have a higher baseline mucosal blood flow [23, 25]. There was no significant difference between patients taking a LABA and those not (98.3 *versus* 97.5 J·L^{-1}; $p=0.81$). It is unlikely that LABA exerted an effect on RHML values. Patients withheld inhalers for >12 h prior to testing, so it seems unlikely that the observed changes were due to asthma medication.

The possible effect of differences in work of breathing on RHML should be considered. However, there was no correlation between the severity of asthma, measured by FEV_1 % pred, and RHML. Patients who have a higher work of breathing, therefore, do not appear to have higher RHML.

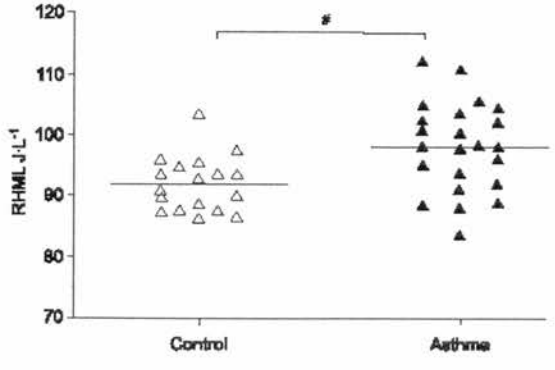

FIGURE 2. Respiratory heat and moisture loss (RHML) in healthy control subjects (n=18) and patients with stable asthma (n=23). #: p=0.007.

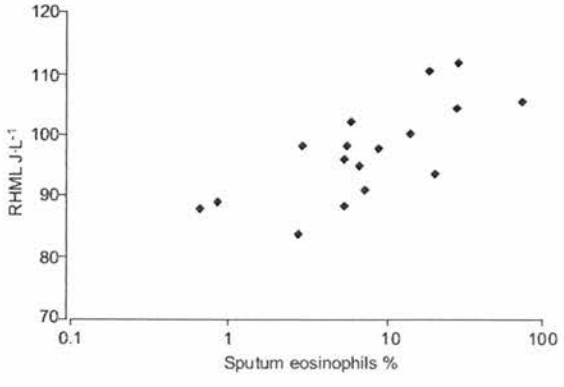

FIGURE 3. Correlation between respiratory heat and moisture loss (RHML) and sputum eosinophil percentage in stable asthma (n=17; r=0.73; p<0.001).

The link between RHML and sputum eosinophil percentage is an intriguing and novel correlation between a biophysical and a cellular marker. Monitoring sputum eosinophil percentage has been reported to have a positive impact on preventing asthma exacerbations when compared with conventional assessment of asthma in a randomised controlled trial [26]. A similar impact has not been demonstrated with other non-invasive markers of airway inflammation so far.

RHML did not correlate with FEV_1, FEV_1% pred, eNO, or EBC pH in stable asthma. Spirometry does not always correlate consistently with markers of airway inflammation [27, 28]. This is probably because inflammatory markers provide information on current airway inflammation, whereas spirometry cannot distinguish between background structural damage or remodelling and present inflammation. Furthermore, the absence of a relationship between FEV_1 and RHML in the asthmatic group reduces the possibility of the increase in RHML being an airway calibre effect.

In mild untreated asthma, eNO has been reported to correlate well with sputum eosinophils [29]. However, all patients in the current stable asthma group were taking regular inhaled corticosteroids, and in steroid-treated patients the relationship between NO and sputum eosinophils is much less pronounced [27, 28]. These two markers vary in their response to inhaled steroid treatment [30, 31]. EBC pH has previously been reported to correlate with sputum eosinophils in asthma by KOSTIKAS *et al.* [32]. Important differences in the present study are that EBC samples were not deaerated prior to testing and the subjects had more severe asthma.

The results of the present study demonstrate that respiratory heat and moisture loss is elevated in a hospital-based outpatient asthmatic population. Furthermore, a close correlation has been shown between sputum eosinophil percentage and respiratory heat and moisture loss. Further investigations may help to establish the effects of asthma medications on respiratory heat and moisture loss and its utility in the longitudinal assessment of patients with persistent asthma.

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