Airway Inflammation, Diagnosis, Perception of Asthma, and Sputum Zinc Levels in a Community Cohort.

Lata Jayaram

Department of Medicine

University of Adelaide

August 2009

Table of Contents

Abstract

Induced sputum examination (IS), an established research tool to measure airway inflammation (AI), is normally confined to specialised institutions and selected populations with airway disease, especially asthma. This thesis examines the role of IS in the diagnosis of asthma in a community.

The first study explores the accepted definitions of asthma, the utility of IS, and another marker of AI, exhaled nitric oxide (eNO), in establishing the diagnosis of asthma. The findings confirm that symptoms, variable airflow obstruction and airway hyper-responsiveness (AHR) are inter-linked in the definition of asthma. Bronchodilator reversibility (BDR), used traditionally, remains the most specific test to aid a diagnosis of asthma in the community. The results favour a tailored approach in the diagnosis of asthma using BDR initially, then selecting a test, either eNO or IS depending on the clinical scenario. The usefulness of AHR with hypertonic saline to diagnose asthma is equivocal given the moderate sensitivity and poor specificity of the test documented within. If a global assessment of AI is required, an eNO measurement is recommended initially, given its ease of use. Sputum examination is useful in delineating the subtype of AI present.

Dyspnoea is a cardinal symptom in asthma. Studies have shown a correlation between AI measured by IS and an altered perception of dyspnoea (POD) in selected subjects with asthma. The aim of the second and third studies was to determine if a similar relationship exists in subjects with and without AHR from a community sample. In both groups, increasing POD was related to worsening lung function and increased BMI. Increased POD was also associated with poorer psychosocial and economic outcomes in subjects with AHR. In the context of previous research, these results illustrate

that heightened POD itself, rather than asthma, is associated with these outcomes. Sputum eosinophilia was not associated with an altered POD in subjects with and without asthma.

There has been mounting research establishing the role of zinc as an immunomodulator in asthma. Mouse models have demonstrated that zinc deficiency is associated with airway eosinophilia. Two pools of zinc exist in the body: largely fixed, enzyme-bound zinc, and free or labile zinc, the biologically active component. With zinc deficiency, it is the latter pool that is preferentially depleted. Our laboratory has developed a novel method, Zinquin fluorometry, allowing measurement of labile zinc in body fluids. The final two studies demonstrate that IS lends itself to labile zinc measurements. Zinquin fluorometry was optimised to measure free pools of zinc in sputum. It was then used to quantify labile sputum zinc concentrations in subjects with and without asthma. Lower zinc concentrations were found in the sputum of subjects with asthma and a significant association noted between lower zinc concentrations and worsening asthma severity.

From a community perspective, these findings suggest that while IS has a limited role in diagnosing asthma, it lends itself to measurement of airway zinc. This work has been conducted in a cross-sectional community cohort where relationships were explored. Ongoing research is required to establish causal links conclusively.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Lata Jayaram and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program(ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Lata Jayaram

Acknowledgements

I would like to thank the following individuals for their contribution to this thesis:

The participants from the North Western Adelaide Health Cohort (NWAHS) who kindly undertook the studies. Mrs. Sandy Pickering, Project Manager of the NWAHS cohort for performing the hypertonic saline challenge tests and sputum concurrent sputum induction and her unflagging support for this work. Dr Sanjeev Chunilal, Haematologist, Queen Elizabeth Hospital, Adelaide for quantifying the differential sputum cell counts. Mr. Sean Homan for performing the eNO measurements. Professor Richard Ruffin, Dr Peter Zalewski and Associate Professor Robert Adams for their supervision, encouragement, and words of wisdom. Finally, my family for their immeasurable support in bringing this thesis to fruition.

Abbreviations

AHR: airway hyper-responsiveness

AE: airway epithelium

AI: airway inflammation

BAL: brochoalveolar lavage

BDI: baseline dyspnoea index

BDR: bronchodilator reversibility

BMI: body mass index measured as weight in kilograms(kg) divided by height in metres squared(m^2);

CDF: cation diffusion facilitator

COPD : chronic obstructive pulmonary disease

ECP: eosinophil cation protein

eNO: exhaled nitric oxide

Eo: eosinophil

ESAHR: episodic symptoms and airway hyper -responsiveness

ESDBDR: episodic symptoms and bronchodilator reversibility

FEV_1: forced expiratory volume in one second

FVC: forced vital capacity

GP : general practitioner

HS: hypertonic saline

IQR: interquartile range

IS: induced sputum examination

L: litres

µL: microlitres

NWAHS : North West Adelaide Health Study

OVA: ovalbumin

PC_{20}: provocation concentration causing 20 percent fall in FEV_1 from baseline

PEF: peak expiratory flow

POD: perception of dyspnoea

ppb: parts per billion

SABA: short acting beta 2 agonist

SD: standard deviation

SF-36: short form 36 quality of life questionnaire

SRDD: self reported doctor diagnosis

TCC: total cell count

TH1: T helper 1

TH2: T helper 2

Zn : zinc

ZIP: ZRT/IRT –related protein

CHAPTER 1

Introduction

1.0 Defining asthma: past, present, and future

Over the centuries, there have been many attempts to define asthma. The word asthma originates from the Greek " azo" or "aazein" meaning " gasping"(1). It was recognised as far back as Greek and Roman times as a disorder of respiration associated with dyspnoea, cough, and wheeze and treated by balancing the four humors: yellow and black bile, blood and phlegm(1). A more detailed description of asthma noting the episodic nature of symptoms was outlined by Floyer in 1698(2). By the 1900's the role of environmental allergens in the aetiology of asthma was established(2).The 20th century saw asthma defined primarily by its clinical presentation, as a disorder of variable airflow limitation triggered by a variety of stimuli with resultant symptoms(3-6). More recently, experts in the field of asthma have suggested that this remains the identifying feature of asthma(7). Variable airway obstruction is commonly measured in 2 ways: either by peak expiratory flow monitoring (PEF) over several (usually 2 to 4) weeks or by bronchodilator reversibility(BDR) which is the improvement in forced expiratory volume in one second (FEV_1) within 10 to 30 minutes after inhalation of a rapidly acting bronchodilator(8).

Over the last 20 years asthma has more frequently become defined by its inflammatory component and indeed is now considered to be first and foremost an inflammatory disorder. In 1997 the National Heart, Lung, and Blood Institute (NHLBI) described asthma as a heterogeneous disorder of the airways characterised by "chronic airway inflammation in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells", clinically resulting in "recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning" and "usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment"(9)

Epidemiologically, or for population based studies, asthma has been defined in several ways; by the presence of episodic symptoms, especially wheeze, over a pre-determined time period such as 3 to 12 months(10, 11). Secondly, by a self reported doctor diagnosis of asthma, and thirdly by the combination of current symptoms and a self reported doctor diagnosis of asthma(10, 11). Epidemiological literature suggests that an additional, objective measure of variable airway obstruction, either the presence of bronchodilator reversibility or airway hyper-responsiveness, increases the specificity for a diagnosis for asthma in the community(11, 12). Toelle and colleagues have suggested that for population based studies current asthma should be defined as appropriate symptoms in the last 12 months plus increased airway hyper-responsiveness(13). Other literature has contested this approach, finding airway hyper-responsiveness neither sensitive nor specific for a diagnosis of current asthma in epidemiological studies(14, 15). It has, therefore been suggested that given asthma is a clinical diagnosis, a physician adjudication of what is and what is not asthma provides a reasonable "gold standard" for these studies(16). Pekkanen and Pearce(17) critically examined the issues regarding defining asthma for epidemiological studies. They found that a single definition of asthma is not applicable to all studies. Secondly, in cohort and case controlled studies, severe symptoms, a previous diagnosis of asthma and symptomatic airway hyper-responsiveness were most discriminatory in detecting subjects with a current clinical diagnosis of asthma(17). Table 1.1 highlights the existing definitions of asthma used in the literature and their strengths and weaknesses. Definitions for specific subtypes of asthma such as cough variant asthma and exercise induced asthma have not been included in the table.

None of the definitions for asthma described above encompass the syndrome of asthma totally, highlighting the lack of a unifying definition for this disorder (18). This has led to ongoing debate regarding the characterisation, assessment of severity, and management of individuals with 'asthma'.

1.1 The burden of asthma

Over 2 million Australians suffer from asthma which is a frequent cause of disability and some deaths, making its management a public health issue(8) . The prevalence of asthma increased through the 1990's and has now plateaued(19). Mortality remains high both nationally and internationally(8). The documented prevalence of asthma both nationally and internationally is influenced by the definition used to describe the disorder(20). In South Australia the reported prevalence of doctor diagnosed asthma in adults greater than 16 years of age has increased from 7.6 % in 1990 to 12.0% in 2003(21), peaking at 13.6% in 2004(22). It appears to have fallen subsequently to 11.4% in 2007 (22). The direct treatment cost of adult asthma in Australia was estimated at 452 million dollars in 1991 (23). Despite increasing pharmaceutical options for asthma treatment and increased health care spending, it is estimated that 26% of young adults with asthma have ongoing symptoms (24) and 16% of the adult SA population with asthma waken weekly or more often with symptoms (21). Thus the burden of asthma remains considerable. Why then is there this discrepancy between improved available treatment and continued asthma morbidity?

One possible reason is that the assessment and treatment of asthma is currently mainly based on the presence or absence of symptoms. Both patients and doctors may under or over perceive symptoms, especially breathlessness, a cardinal symptom in both diagnosing and assessing asthma severity. Consequently an asthma episode maybe under or over diagnosed and therefore not managed optimally. Another explanation is that both patient and doctor education regarding asthma may be insufficient, also resulting in suboptimal management of this disorder. Lastly, airway inflammation (AI), considered to be fundamental to the aetiology and persistence of asthma (9), may be under diagnosed using the present tools available, leading to under treatment and poor asthma control.

1.2. Measuring Airway Inflammation

1.2.1 The importance of measuring AI in asthma

Ongoing airway inflammation, the predominant feature in asthma, can cause structural changes or airway remodelling, including thickening of the sub-epithelial reticular layer and hypertrophy of smooth muscle (25-28) These changes in turn are likely contribute to airway hyper-responsiveness (26-29) (or irritability of the airway) and to progressive, persistent airflow limitation(30)

Airway inflammation in asthma until recently has been measured indirectly and predominantly clinically, by assessing symptoms, the frequent use of reliever medications, and by measuring lung function using peak expiratory flows, spirometry and tests of airway hyper-responsiveness(8).

Over the last decade more direct tools to measure AI have evolved. These include induced sputum examination (IS) which will be discussed in detail in this thesis and exhaled nitric oxide (eNO), which will be discussed briefly in the following pages. Both these tools have been shown to assess AI in asthma reliably(31, 32). Atopic asthma is characterised by a raised sputum eosinophil count which responds to corticosteroid treatment(31). Hospital based studies have demonstrated that the prevalence of eosinophilic AI in asthma ranges from 40 to 80 percent(33, 34). A raised eNO level is also a feature of poorly controlled asthma which responds well to corticosteroid therapy(35-37). Sputum examination, and to a lesser degree eNO, have also demonstrated a clear role in monitoring asthma control and compliance with therapy in patients with asthma seen in hospital clinics (37-39). Their role in evaluating asthma in the community, has not been studied in great detail. Patients with asthma chosen for studies from hospital based respiratory clinics have usually been observed on several occasions and thoroughly investigated with objective tests. The results from studies which

include such patients may not be generalisable to the community where the definition of asthma is mainly symptom based and self reported. One of the aims of this thesis, therefore, is to ascertain the role of IS and eNO in diagnosing asthma in the community.

1.2.2 AI and the perception of dyspnoea (or breathlessness)

Breathlessness or dyspnoea, is a cardinal symptom of asthma. Breathlessness may result from worsening asthma or the distress and anxiety associated with it. Interestingly studies have demonstrated that patients with asthma may have a *reduced* or *heightened* awareness of their breathlessness. The reasons for this are multifactorial and include the blunted perception of dyspnoea with hypoxia(40), behavioural elements(41) and possibly an association with AI(42, 43). Early studies have demonstrated that *both* in severe asthma where breathlessness is often under-perceived(43), and mild asthma where this symptom may be over perceived(42), there is an increased eosinophil count in bronchial fluid and in sputum. There have been a paucity of studies, to our knowledge, examining the relationship between eNO and perception of dyspnoea in asthma(44). This thesis aims to explore the link between AI and the perception of breathlessness in asthma further.

1.3. Zinc and asthma

The therapeutic role of zinc in asthma has been documented as far back as the eighteenth century when William Cullen described the benefits of flowers of zinc in 50 detailed case histories(45). Over the last decade there has been substantive research confirming the role of zinc as an anti-inflammatory agent, maintaining the functional integrity of several organs and epithelial surfaces, promoting growth, development and tissue repair. Zinc deficiency has been associated with skin disorders, diarrohea, loss of appetite and taste, delayed sexual maturation, generalised inflammation and immunodeficiency(46). More recently research has also demonstrated that a zinc deficient state may exist in animals and humans with ongoing airway inflammation and asthma(46) and this aspect is further explored in this thesis.

Questionnaire based studies have reported significantly lower intake of zinc in those with asthma compared with controls(47, 48). Total zinc concentrations in blood and hair have also been measured in subjects with asthma and without asthma in several studies, with most demonstrating low zinc levels in asthma (46, 49). While plasma zinc concentrations have been measured in zinc deficiency, serum or plasma measurements may not accurately reflect zinc concentrations in the airway for the following reasons: firstly, plasma zinc, which is extracellular, may not reflect intracellular zinc levels(50); secondly, plasma zinc has traditionally been measured by atomic absorption spectrometry(AAS) which measures both fixed stores of zinc which are tightly bound to plasma proteins and labile or loosely bound stores of zinc which move freely between body compartments(51). Measuring zinc concentrations in sputum may provide a more direct assessment of airway zinc levels compared with blood or hair media. Additionally, as it is the labile or loose zinc which is reduced in zinc deficiency, measuring this directly by alternative methods such as Zinquin

fluorometry, a novel method of assessing labile zinc concentrations in body fluids, may be more appropriate.

Zinquin fluorometry is a technique developed in our laboratory by Dr Peter Zalewski amongst others(52). The technique has been developed and validated for measurement of zinc concentrations in blood. Through the work in this thesis, the methodology has been refined and for the first time, used to measure labile zinc concentrations in sputum in individuals with asthma and healthy controls.

1.4. Hypotheses

1. The prevalence of AI measured by sputum eosinophilia (defined as a raised sputum eosinophil in asthma in the community is similar to hospital based studies. Two cut off points, 1% and 2% are used to define sputum eosinophilia, and are further detailed in Chapter 2.

2. Sputum eosinophilia and eNO will discriminate subjects with asthma from those without asthma in a community cohort.

3. A zinc deficient state (total and labile) exists in airway secretions in adults with asthma compared with those without asthma. This will be manifested by lower sputum total and labile zinc concentrations in subjects with asthma compared with those without asthma.

4. Increasing AI, measured by sputum eosinophilia, in asthma is associated with lower zinc concentrations in sputum and in blood (plasma).

5. Increasing asthma severity measured by a combination of symptoms, medications and spirometry is correlated with lower zinc concentrations in sputum and in blood (plasma).

1.5. Aims

The aims of the studies undertaken in this thesis were:

1. To assess the prevalence of eosinophilic AI present using sputum examination in a community sample population with and without asthma.

2. To assess the clinical utility of IS and eNO in the diagnosis of asthma compared with spirometry with BDR, and AHR with hypertonic saline.

3. To correlate the eosinophilic AI demonstrated using sputum examination with:
   (a) asthma severity as measured by symptoms, medications and lung function.
   (b) perception of breathlessness in a community sample population.

4. To optimise the methodology to enable labile zinc to be measured in sputum supernatant (or extracellular component) with Zinquin fluorometry.

5. To measure total and labile zinc levels in sputum supernatant and in plasma in a community sample population and to correlate these levels with asthma severity.

1.6. Thesis contents: the subsequent chapters.

The following chapters examine the literature in the area of sputum examination and the role of zinc in asthma, and further explore the hypotheses and aims outlined above with original research.

Chapter 2: reviews the literature related to the topics in this thesis.

Chapter 3: outlines the materials and methods used in the research undertaken.

Chapter 4: describes the prevalence of, and the cellular profile of AI present (using IS) in a community sample population with and without asthma. It also assesses the sensitivity, specificity, positive and negative predictive value of bronchodilator reversibility, hypertonic saline challenge test of AHR, presence of sputum eosinophilia measured by induced sputum examination, and eNO in diagnosing asthma in the community defined in 4 ways;

1. self reported doctor diagnosis (SRDD)
2. presence or absence of wheeze
3. episodic symptoms and bronchodilator reversibility (ESBDR)
4. episodic symptoms and airway hyper- responsiveness (ESAHR)

Chapter 5: examines the perception of breathlessness (POD) in the same community sample of subjects with asthma and correlates these findings with clinical, psychological, socio-economic variables and the presence of AI as measured by IS and eNO. Subjects with asthma are defined for this chapter, as those individuals with episodic respiratory symptoms and airway hyper-responsiveness (ESAHR) based on a hypertonic saline challenge test. This definition allowed the degree of dyspnoea to be quantified against a measurable degree of airway bronchoconstriction, (or narrowing). Secondly, it standardised the methodology used to measure POD with previous studies(53), allowing comparison of results across studies to be made.

Chapter 6: examines POD in community based subjects without asthma (healthy controls) and correlates these findings with clinical, psychological, socio-economic variables, and AI assessed by sputum cell counts and eNO. Subjects are defined for this chapter, as those individuals without current respiratory symptoms and without airway hyper- responsiveness based on a hypertonic saline challenge test.

Chapter 7: describes the process of optimising the methodology for measuring labile zinc in sputum supernatant with Zinquin fluorometry.

Chapter 8: reports on the zinc concentrations in the sputum and plasma from a community sample population of subjects with asthma and healthy controls, and correlates these results with asthma severity.

Asthma is defined for this chapter by:

1. self reported doctor diagnosis of asthma or
2. episodic symptoms and BDR(ESBDR) or
3. episodic symptoms and airway hyper- responsiveness (ESAHR)

The reason for this is that the aim of this chapter is to determine if zinc levels are altered in asthma in real life. Asthma in real life is defined in all the ways described above depending on context and situation.

Chapter 9: concludes with a summary of the findings from the above original research and makes suggestions for future research based on the results of this thesis.

Table1.1. Commonly used definitions of asthma

Definition *	Strength	Weakness	Use
Self reported doctor diagnosis	Immediacy of diagnosis. Physician diagnosis of asthma considered by some authors to be the nearest to a gold standard (16, 54).	Possibly unreliable due to : (a) Wide variation in the information and tools available to GP in making the diagnosis. (b)Wide variation in the criteria used by GP's to make the diagnosis.(c) relies on patient recall which may be subject to errors.	In clinical practice, epidemiological studies
Episodic symptoms alone, predominantly wheeze	Immediacy of diagnosis.	Non specific for asthma. Wheeze may be due to other causes e.g. heart failure, smoking related COPD, bronchiectasis. Wheeze is a more specific symptom is children where other causes are less likely. Relies on patient recall which may be subject to errors.	In clinical practice, epidemiological studies involving children.
Symptoms and objective test of variable airflow obstruction measured either by peak expiratory flow (PEF) or bronchodilator reversibility (BDR) on spirometry	PEF: simple to use; provides serial measurements over time easily. Spirometry: single measurement.	PEF: poor reproducibility with measurements which are highly effort/technique dependent. Single measurement inadequate to make diagnosis, requires serial measurements over 2 to 4 weeks. May have issues with non compliance with repeated PEF recordings. Spirometry may not be available in GP practice; may require hospital laboratory /clinic visit to provide reliable data. BDR may be seen with asthma and COPD.	In clinical practice, epidemiological studies and clinical studies. Definition used in most international guidelines
Symptoms and objective test of airway hyper-responsiveness (AHR) *references: 12, 13, 16, 17, 18, , 53	Addition of AHR increases sensitivity /specificity for the diagnosis of asthma depending on substance used for challenge(13).Reliable , reproducible with two fold concentrations.	AHR needs to be measured in lung function laboratory(or mobile laboratory), therefore, not necessarily easily accessible and prevents an immediate diagnosis. AHR may be less sensitive than symptom questionnaires for a physician diagnosis of asthma in epidemiological studies(17, 54).	In clinical practice epidemiological studies and clinical studies. Useful in patients with equivocal symptoms or atypical presentation e.g. cough variant or exercise induced asthma

CHAPTER 2

Literature Review

2.0 Introduction

There are 2 approaches for measuring AI: *indirect* and *direct* methods. The *indirect* method uses surrogate measurements such as symptoms, variation in spirometry or peak expiratory flow (PEF) recordings, tests of airway responsiveness (histamine, methacholine or hypertonic saline challenge) and peripheral blood inflammatory markers (55) which include the eosinophil count and its by product eosinophilic cationic protein (ECP). The *direct* method employs more invasive techniques such as bronchoscopy with bronchial washings, biopsy or bronchoalveolar lavage (BAL)(56). Both methods have limitations. Indirect measures relate variably with each other and do not necessarily correlate with direct measurements of airway inflammation(55-57). While bronchial biopsy is considered to be the "gold standard" for the assessment of AI (58), it is limited by discomfort, risks to the patient, and expense. It is not a tool that can be used easily or regularly in patients. Hence, a relatively non-invasive method to directly examine inflammation is required. Over the last decade 2 techniques have been developed, refined, and validated to directly measure airway inflammation. The first is the measurement of exhaled nitric oxide (eNO) in the breath, and the second is the examination of induced sputum.

These *indirect* and *direct* methods of measuring airway inflammation are discussed in more detail in this chapter, along with the current evidence supporting the use of induced sputum examination as a clinical tool.

2.1 Measuring AI in asthma: Indirect Markers

2.1.1 Symptoms, peak expiratory flow (PEF) and spirometry

Symptoms, PEF monitoring and spirometry have traditionally been used as tools to gauge AI in asthma based on the postulate that worsening symptoms and lung function reflect the intensity of AI. It has been shown that self monitoring of symptoms or PEF with an action plan significantly reduces emergency department and unscheduled doctor visits, hospitalisations, and time lost from work due to asthma(59-61).There are several national and international guidelines and validated questionnaires that incorporate a symptom and PEF or spirometry based approach to optimising asthma control(8, 9, 62, 63) Studies have confirmed that adhering to these guidelines do indeed, improve asthma control over and above individual best practice (62). There are, however, limitations with this approach. Firstly, symptoms are a subjective, and patient based means of assessing asthma. Rubinfeld and Pain in an early study of perception of breathlessness in asthma demonstrated that up to 15% of subjects with asthma could not perceive marked airways obstruction with an FEV_1 of 50% or less of predicted(64). This discrepancy between the perception of symptoms and airway bronchoconstriction has been confirmed by other studies(65, 66). Secondly, asthma is associated with variable airflow limitation(7). Thus, the result of spirometric testing performed at one time point may not accurately reflect average asthma control over a period of time. For example, a chart review of 67 subjects with asthma demonstrated a poor correlation between asthma symptoms and FEV_1 (67), again substantiated by other studies(68). Lastly, symptoms, PEF and spirometry have a variable relationship with more direct markers of airway inflammation such as the eosinophil count in bronchial fluid and biopsies, sputum and exhaled nitric oxide measurements, suggesting that while regular monitoring of symptoms and simple measures of lung function may improve asthma control, they do not totally reflect airway inflammation(69-73).

2.1.2 Tests of Airway Hyper- responsiveness (AHR)

Airway hyper- responsiveness is defined as an "abnormal increase in airflow limitation following exposure to a nonallergic stimulus"(74) A test of AHR is considered to reflect the degree of smooth muscle irritability and inflammation involved in the airway. There are 2 types of airway challenges. Firstly *direct challenges* which use chemicals such as histamine and methacholine to cause bronchial smooth muscle contraction directly(75). Secondly*, indirect challenges* such as exercise, hypertonic saline and certain drugs (mannitol and adenosine monophosphate) which mimic the airway response by releasing endogenous mediators which then cause smooth muscle contraction (74). Most studies to date on asthma have used either methacholine or histamine challenge tests to characterise patients' asthma and to determine a dose response with corticosteroids, the mainstay treatment for persistent asthma. Methacholine and histamine are approximately equivalent on a microgram or micromolar basis (76, 77). The measurement of AHR is determined by an arbitrary, consensus defined cut-off point set at the higher end of the borderline range. Airway hyper- responsiveness is considered to be present when the provocation concentration of histamine or methacholine to induce a 20% fall in FEV_1(PC_{20}) is less than 8 -16 mg/mL(75) or the provocation dose to induce a 20% fall in FEV_1 is less than 3.9 -7.8 umol (78). These cut-off points result in a histamine or methacholine test that is highly sensitive with a high negative predictive value, but with low specificity and positive predictive value for current asthma symptoms(75-77). Airway hyper- responsiveness is also seen in subjects without asthma and in those with bronchitis or emphysema (79),(80). When these tests are conducted in epidemiological settings in a community sample, as opposed to patients recruited from hospital clinics, their sensitivity and specificity is even lower (81).

Indirect challenges have been considered to be more specific but less sensitive for disease activity in asthma than direct stimuli (74), (82-84). Hypertonic saline and other indirect challenges are

increasingly used in children as they are natural stimuli, not pharmacological agents (82). Hypertonic saline challenge has been shown to be an effective and robust surrogate marker for exercise induced asthma (82),(85, 86) and in identifying those who may be at risk of developing underlying airway narrowing while scuba diving(82).

More recently there has been a body of literature suggesting that indirect challenges are as, or more, sensitive than direct challenges, although the sensitivity of both appears to be low in the community. In an epidemiological study, a histamine challenge in 2,363 Australian school children aged between 8-11 years resulted in sensitivity of 53% and specificity of 90% to detect subjects with a diagnosis of asthma based on a self-administered questionnaire to parents(87). Similar sensitivities and specificities were found using indirect challenges, in a community based, cross-sectional study involving 393 schoolchildren aged 13- 15 years(88). The school children underwent a hypertonic saline challenge and a free running exercise challenge, and the sensitivity and specificity of these tests to identify current wheeze were 47% and 92% and 46 and 88% respectively(88). Mannitol is another osmotic indirect challenge test that has been developed and refined in recent years (82, 89). It is easier to use compared with hypertonic saline but is currently a research based tool. It is undergoing further studies to gain approval for clinical use (90).

The correlation between AHR and markers of airway inflammation remains variable. Some studies have demonstrated that AHR is independent of eNO and the number of inflammatory cells in the airway lumen or mucosa while others have shown that a relationship exists(91-94). Indirect challenges are considered to reflect the underlying AI in asthma more closely than direct challenges(95).

Despite this debate, there is evidence that normalising tests of airway hyper-responsiveness can improve asthma control over and above controlling symptoms and spirometry alone. In a prospective, randomised, management study over 2 years, 75 subjects with asthma were enrolled to one of 2 strategies; *Group1: reference strategy* where subjects were managed on clinical guidelines (symptoms and spirometry) alone, and *Group2: AHR strategy* where subjects were managed by optimising treatment based on methacholine airway hyper-responsiveness in addition to clinical guidelines(29). Bronchial biopsies were performed at entry into the study and at 2 years. Adjusting patients' corticosteroid dose three monthly, using a test of airway hyper-responsiveness resulted in significantly fewer exacerbations, improved the forced expiratory volume in one second (FEV_1), and reduced airway remodelling by decreasing the sub epithelial reticular layer than clinical outcomes alone. The changes in AHR correlated significantly with eosinophil counts in the biopsies: r= - 0.48, p=0.003. Thus, the greater the decrease in number of eosinophils, the greater the improvement in AHR (29).

The studies comparing direct and indirect challenges to monitor asthma during anti-inflammatory (predominantly inhaled corticosteroid) treatment, demonstrate that indirect challenges such as hypertonic saline are more responsive than direct challenges such as histamine to therapy(96, 97). An indirect challenge responds to treatment within hours to days while a direct challenge takes longer; months. This may reflect the fact that the challenges are likely measuring different aspects of the bronchial response: indirect challenges mirroring the inflammatory response and direct challenges the smooth muscle irritability(96).

Gronke and colleagues have suggested that the relationship between AHR and markers of airway inflammation may depend on the duration of asthma(98). The authors examined 66 subjects with steroid naive asthma and found that in those with a 16 year or less duration of asthma, AHR with methacholine, correlated well with sputum eosinophils and eNO. In those subjects with a greater than 16 year duration of asthma , AHR correlated with FEV_1 rather than inflammatory markers suggesting that remodelling of the airway had occurred(98).

Hypertonic saline, the most commonly used indirect challenge test, lends itself to use both in the laboratory and in the community. The hypertonic saline challenge procedure using 4.5 % which is now standard, was described in 1989 by Smith and Anderson(99). The saline challenge procedure can be concurrently used to induce a sputum sample (82, 100) with a greater than 90% success rate (101). The procedure is safe and well tolerated (102). Thus it is possible to obtain an assessment of airway responsiveness and airway inflammation simultaneously. While there are studies investigating and supporting the use of indirect challenges in epidemiological studies to diagnose asthma in children, there is little similar work in adults. One of the aims of this thesis is to explore the utility of hypertonic saline in assessing AI and asthma in the adult community.

2.1.3 Peripheral blood markers: the eosinophil count and eosinophil cationic protein (ECP).

It has been established that blood eosinophil counts reflect asthma control (69, 103). In a seminal study Horn and colleagues examined the clinical utility of measuring total eosinophil counts in the diagnosis and management of steroid dependent asthma(103). Subjects with asthma were noted to have significantly higher eosinophil counts than healthy controls, suggesting that eosinophilia was an

important discriminating feature in asthma. The counts also demonstrated a significant inverse correlation (r=-0.74, p<0.001) with specific airway conductance, a lung function measure similar to FEV_1 but which reflects small airway narrowing, suggesting that the total eosinophil count could reflect asthma control(103). More recently measurements of eosinophils in BAL fluid and sputum have offered alternative methods of assessing airway inflammation in asthma. The relationship between eosinophil counts in blood, BAL or sputum, and clinical and physiological makers in asthma remain variable. Ulrik and colleagues demonstrated a close correlation between blood eosinophils and histamine responsiveness(r=0.60, p=<0.001) and a weak but significant correlation between blood eosinophilia and reduced FEV_1(104). Based on these findings, the authors' postulated that blood eosinophilia in asthma reflects ongoing airway inflammation, which in turn may lead to chronic airflow obstruction (104). Taylor and colleagues also confirmed similar results(105). Further studies, however, demonstrated that the relationship between sputum eosinophilia and airway obstruction (FEV_1) was stronger than blood eosinophilia and FEV_1(69, 105).The percentage(%) of eosinophils in sputum was also shown to be more sensitive and specific for differentiating subjects with asthma from controls compared with blood eosinophils (Receiver operating curves: AUC(area under the curve) for % eosinophils in sputum vs blood : 0.90 vs 0.72, p=0.02 (69).

Eosinophil cationic protein (ECP) is an eosinophil by- product that is released when eosinophils are activated. Studies have shown that ECP concentrations are increased in the blood and sputum of subjects with asthma compared to subjects without asthma and is a useful marker of inflammation (69, 106, 107). Pizzichini and colleagues demonstrated that sputum eosinophils are a more sensitive and specific measure in discriminating between subjects with and without asthma compared with serum ECP: sputum eosinophilia sensitivity and specificity of 70% and 90% vs serum ECP sensitivity and specificity of 50% and 50% (69, 108). In other studies, the serum ECP concentrations did not

mirror sputum ECP concentrations(108, 109). Sorva and colleagues found that sputum ECP levels correlated well with improving clinical status following treatment with ICS in subjects with asthma ($r=0.69$, $p<0.05$ for symptoms; $r=-0.75$, $p<0.05$ for FEV_1 i.e. falling sputum ECP was associated with an increase in FEV_1(109).The measurement of ECP levels in blood and sputum is currently a time consuming test that is still research based. Recent studies have shown that ECP is also produced by neutrophils, making it a less specific marker of eosinophil activation than considered previously(110) . Measurement of the blood eosinophil count on the other hand, is an automated, well established clinical tool used as an adjunct in a variety of disorders.

In summary, blood eosinophilia is a surrogate marker for airway inflammation but sputum eosinophilia provides a direct measurement that is more sensitive and specific. The role of blood and sputum ECP as a marker of AI is controversial and not clearly established clinically.

2.2 Measuring AI in asthma: Direct Markers

There have been two major developments in the assessment of airway inflammation in asthma over the past decade: exhaled nitric oxide and induced sputum examination. These techniques complement each other, and current worldwide research is focusing on establishing their clinical relevance in a variety of airway disorders. This thesis examines the role of induced sputum, and secondarily examines the role of eNO in the diagnosis of asthma in the community.

2.2.1 Exhaled nitric oxide (eNO)

Exhaled nitric oxide is a molecule that is produced by airway epithelial cells and inflammatory cells in response to upregulation of the enzyme inducible NO synthase by pro inflammatory cytokines. It diffuses into airway lumen and is a confirmed marker of airway inflammation(111-115) Exhaled nitric oxide levels are raised in uncontrolled asthma, possibly bronchiectasis, and with viral infections. Exhaled nitric oxide levels are reduced by corticosteroids and smoking(111-115)

Exhaled nitric oxide is measured by rapid linear-response chemiluminescence i.e. by a chemical reaction that emits light(111, 112). It is a standardised measurement that is reproducible(111, 112). Normal values have been established, but are currently being redefined in the light of recent studies demonstrating higher than expected levels in normal subjects and in subjects with well controlled asthma (111-116) It is presently accepted that the interpretation of an eNO level between 30 to 50 parts per billion (ppb) depends on the clinical scenario. An eNO level of greater than 50 ppb in association with characteristic respiratory symptoms may suggest a diagnosis of asthma or the presence of uncontrolled asthma(111-117).

Exhaled nitric oxide measurements correlate well with sputum eosinophilia but less well with symptoms and spirometry (FEV_1) outcomes (70, 118). Exhaled nitric oxide levels are very sensitive to, and normalise rapidly, with corticosteroids (35, 36, 119).It has been shown that using eNO measurements in addition to standard clinical guidelines to monitor asthma control, reduces maintenance corticosteroid dose requirements in patients with asthma but does not reduce exacerbation rates (37).

Conversely, raised eNO levels, like raised sputum eosinophils, predict an asthma exacerbation better than change in symptoms or lung function with a positive predictive value of 88%(36). Advantages of eNO as a marker of airway inflammation include its ease of measurement. Disadvantages include its expense and the fact that it does not subtype the cellular components of the inflammatory response. Furthermore, a recent study has demonstrated discordance between eNO and simultaneously measured sputum eosinophil counts in patients with asthma (120).Current costs for an in laboratory eNO analyser range from Australian 30 to 50,000 dollars, while a small hand held analyser costs approximately Australian 10,000 dollars(sourced from companies producing eNO analysers).

The 2 cut –off points used for eNO in this thesis are 19 ppb and 30ppb respectively .These were based on the literature at the time of commencement of this thesis, and is supported by a recent publication reviewing normal values(115).

2.2.2 Induced sputum examination

Induced sputum examination is the other non invasive method for measuring AI directly.

2.2.2.1 History of sputum examination

Sputum for the purpose of this thesis is defined as secretions from the airways of the lungs, although some refer to it as the expectorate of sputum plus saliva. Its constituents include mucus and cells. The cell composition has been known for over one hundred years to be altered in asthma, showing an increase in eosinophils(31) Initially, sputum cells were examined on stained smears and there was a

brief period of their use in clinical practice in the 1950's and 1960's. However, the sputum could not always be expectorated and only one smear tended to be examined in which the cells were irregularly distributed and difficult to recognise within the mucus(31). The results therefore were not reliable and this use of sputum fell into disrepute. Over the last two decades the examination of sputum has been enhanced by the successful expectoration of sputum by induction with an aerosol of hypertonic saline(31). Thus the techniques of inducing and examining sputum have been refined, evaluated and applied to investigate the pathogenesis, pathophysiology, and treatment of asthma and other airway conditions, and are standardised in a European Task Force document(121).The following observations have been made with respect to induced sputum examination and are relevant to the studies in this thesis:

1. Sputum can be successfully and safely induced (101, 102, 122). An audit of 304 inductions in subjects with asthma has shown the procedure to be successful in 93 % of inductions(101). In a literature review, in subjects pre treated with salbutamol the mean fall in FEV_1 after sputum induction in subjects with mild stable asthma was 5.7%(95%CI: 4.1-7.2%), in those with moderate to severe stable asthma it was 5.6%(95%CI: not calculated), and in those with uncontrolled or exacerbated asthma it was7.2%(95%CI: 4.1-10.3%)(102).Furthermore, a modified procedure can be performed safely even in severe exacerbations of asthma and in those subjects with an FEV_1 below 1 litre (102, 123).

2. Induced sputum cell counts are the same as those obtained in spontaneous sputum(124), and in tracheal or proximal bronchial aspirates(125).They are similar to those in bronchial washings but differ quantitatively from those in bronchial biopsies and bronchoalveolar

lavage(126-131). These relationships are understandable since they reflect differences in different airway compartments; induced sputum and bronchial washings reflect secretions from the central airway lumen, bronchial biopsies from the more central airway bronchial mucosa and BAL from the peripheral airway lumen (Table 2.1).

1. The methodology of induced sputum examination is well established and is robust. Sputum cell counts are a precise, reliable, valid and responsive measurement of airway inflammation, all qualities accepted as indications for the clinical and routine applicability of a good laboratory test (69, 128, 132-138) (Table 2.2). The time required for sputum induction is approximately 20 minutes, and 60 to 90 minutes is needed to process the sample. Laboratory space and staff experienced in performing differential cell counts (usually haematology or cytology personnel) are required.

2. There are currently 2 established and validated methods of processing and measuring sputum cell differential counts(139).The selected procedure involves removing the sputum from the entire expectorate and thereby reducing contamination with saliva(133). The whole procedure uses the entire expectorate(134) . While the reproducibility and validity of these 2 methods is excellent, the methods cannot be used interchangeably. In this thesis the selected procedure has been used.

3. Sputum differential counts are expressed in 2 ways with respect to the total cell count: as proportions or percentages (with the differential cell examined as the numerator and the total cell count as the denominator), and as absolute cell counts. Studies have

demonstrated that there is good agreement between both measures (kappa statistic and 95% CI: 0.66 (0.50-0.82) (34).Normal values have been established for both absolute differential cell counts, and differential cell percentages(Tables 2.3) (137, 140). Percentage differential cell counts are clinically meaningful, responsive to change and generalisable across studies(121). Distinct inflammatory subtypes have been classified on the basis of differential cell percentages(34)(Table 2.4).Over the last decade expressing differential cell counts by percentages has also become increasingly used compared with absolute cell counts. It is for these reasons, that the work in this thesis expresses cell counts by percentage alone .

4. Sputum cell counts identify different aspects of airway inflammation which have different causes and can result in different clinical effects(31). Four major cellular subtypes of AI, based on sputum examination, have been identified in subjects with asthma: eosinophilic AI (raised eosinophil count) in 41% ; neutrophilic AI (raised neutrophil count) in 21%, paucigranulocytic (non neutrophilic, non eosinophilic) in 31% and mixed granulocytic (neutrophilic and eosinophilic) in 7% (34)(Table 2.4). Exacerbations of asthma can also be non-eosinophilic, being caused by respiratory viral or bacterial infections. The inflammatory response to these agents is indicated by an increase in the proportion of neutrophils and by a greater increase in total cell count compared with eosinophilic exacerbations of asthma(31). Other causes of sputum neutrophilia include cigarette smoking (especially when this is associated with chronic airflow limitation), pollutants such as ozone, and exposure to endotoxin(31). Differentiating the types of inflammation is helpful in guiding treatment(31).

5. Sputum eosinophilia is characteristic of atopic asthma. A sputum eosinophil count of 2.2 % or less has been considered to be within the normal range(137, 141)(Table 2.3). Recent studies have demonstrated that the normal range may lie between 1 and 2 % depending on the population examined. Simpson and colleagues, for example, found that amongst their population of 42 healthy individuals, an eosinophil count of 1.01% or less was considered normal based on the 95% percentile (34). For this thesis cut points of 1 and 2 % are examined.

6. Sputum eosinophilia usually precedes clinical exacerbations of asthma even when the subject is on treatment with inhaled steroid or prednisone(36, 123, 142-145). It will therefore detect under treatment before this is apparent clinically.

7. The degree of sputum eosinophilia correlates variably with symptoms, spirometry and tests of airway responsiveness(146) (Table 2.5). It is therefore not surprising that physicians are poor at recognising the presence or absence of sputum eosinophilia based on clinical presentation(147).

8. Sputum eosinophilia predicts a response to treatment with corticosteroid (142, 148-151) while lack of eosinophilia predicts a lack of benefit to treatment with corticosteroids(152, 153)

9. Various indices of inflammation can be measured in the supernatant or fluid phase of processed sputum. Acceptable repeatability and responsiveness of many of these has been shown, but investigation of their validity, and clinical utility is still limited(154-157)

In conclusion, these published observations provide support for the use of sputum cell counts in clinical practice, specifically for the diagnosis of the type of airway inflammation associated with asthma (eosinophilic or neutrophilic) and for a prediction of response to corticosteroid treatment. As clinical judgement of the presence or type of airway inflammation is often incorrect and as sputum eosinophilia precedes exacerbations, sputum examination may be of great value in monitoring asthma treatment in selected patients, as suggested in two recent longitudinal, randomised trials (158, 159).These studies demonstrated that using sputum examination in addition to standard methods of assessing asthma control, reduced the frequency of asthma exacerbations and hospitalisations due to asthma in patients recruited from hospital respiratory clinics. The role of sputum examination in the community (primary as opposed to secondary care) to diagnose and monitor asthma is less clear, and the former explored in this thesis.

2.3 The prevalence and type of airway inflammation in asthma in the community.

The prevalence of airway inflammation in asthma, using direct rather than indirect measurements, is not well characterised in patients seen in the community. Louis and colleagues, estimated that

sputum eosinophilia occurs in up to 80% of steroid naïve patients and 50% of corticosteroid treated patients in a respiratory clinic setting (146, 160). Lemiere and colleagues, in a small, community based Canadian pilot study (n=107), found that median sputum eosinophil counts were within the normal range and similar in adult subjects with and without asthma(161). Only 19% (13/67) of subjects with respiratory symptoms had sputum eosinophilia >1%(161). In larger studies in Australian children, marked differences in the sputum eosinophil counts were demonstrated between children with and without asthma(162-164). For example in one study, the median eosinophil count (interquartile range) was1.0(0.3-1.8) in healthy children and 3.8(1.7-14.0) in those with persistent asthma, $p<0.05$(163).

The frequency of eosinophilic versus non eosinophilic airway inflammation in asthma in the community is also unknown. Increasing research into this area demonstrates that the prevalence of non eosinophilic AI is higher than previously recognised. In selected subjects with asthma the prevalence of non eosinophilic versus eosinophilic airway inflammation was surprisingly high at over 50% (34, 165). This thesis examines the prevalence and type of AI in the community using IS.

2.4 Perception of dyspnoea (or breathlessness) in asthma and in healthy subjects and its relationship to airway inflammation.

Asthma severity, in current national and international guidelines for asthma management, is primarily defined by symptoms (8, 9, 166, 167). There are, however, people with asthma who have poor perception or a heightened perception of dyspnoea(POD) and their symptoms (64, 168). The severity

of asthma in such cases may be either underestimated and under treated (with a poor perception of symptoms) or overestimated and over- treated (with a heightened awareness of symptoms). A Japanese study found that when symptoms and current treatment of 861 well controlled subjects with asthma (726 adults and 135 children aged 6 years or older) were assessed according to Japanese Asthma Consensus Guidelines, 49% of adults and 35% of children were over treated, while 30% of adults and 40% of children were under treated. In addition, 50% of adults and 35% of these subjects misjudged the severity of their asthma(169). Rubinfeld and Pain interestingly found no correlation between current severity of asthma based on symptoms and bronchial reactivity measured by a methacholine challenge suggesting that the relationship between these 2 variables is complex(170). Several mechanisms for the occurrence of an altered POD are postulated. These include behavioural and temporal adaptation, an altered pattern of breathing, the presence of hypoxia and airway inflammation (40, 66, 171-173).

Burdon and colleagues studied subjects with asthma undergoing a histamine provocation test. They found that those with a baseline FEV_1 of less than 80% experienced the same degree of breathlessness as those subjects with a normal resting FEV_1 , but were significantly more obstructed at all degrees of breathlessness(65). The authors postulated that subjects with asthma and an abnormal baseline FEV_1 downgraded their sensory experience over time, a phenomenon called temporal adaptation. It is not known how long temporal adaptation takes to occur and if this phenomenon is reversible(66). Some studies have shown that it can occur, to some degree, over minutes (174, 175).

Eckert and colleagues have elegantly demonstrated that hypoxia (low oxygen levels) suppresses respiratory load sensation and ventilation. Sixteen individuals with stable asthma were exposed to three gas conditions (34 minutes each): isocapnic hypoxia (normal carbon dioxide (CO_2) levels and low oxygen levels with arterial blood oxygen (O_2) saturation of approximately 80%), hypercapnia (increase in end-tidal CO_2 of approximately 5-10 Torr), or isocapnic normoxia (normal CO_2 and O_2 levels) on 3 separate days. The perceived magnitude of externally applied resistive loads, measured during each gas condition, was reduced throughout hypoxia compared with normoxia, with a trend for a progressive decline during hypercapnia. Within the 15-minutes post gas inhalation period, methacholine-induced symptoms of difficult breathing, chest tightness, and breathlessness, measured using modified Borg scales, were 25-30% lower (i.e. POD was blunted) after hypoxia compared with normoxia. They were not reduced after hypercapnia. The altered perception of symptoms persisted for at least 10 minutes after returning to normoxia(176).

Airway inflammation may also contribute to the altered perception of breathlessness in asthma. Reduced dyspnoea perception in severe asthma is associated with a decrease in lung function and increase in eosinophils in bronchoalveolar lavage, biopsy and sputum samples (42, 127). Contrastingly, in mild asthma, *heightened* dyspnoea perception has been shown to correlate with an elevated sputum eosinophil count(172)

To date, to our knowledge, the prevalence of adults with asthma and an altered POD in the community, and the correlation of POD with objective measures of airway inflammation in a community sample has not been evaluated.

Even less is known regarding the POD in healthy subjects(177). Normal subjects demonstrate a wide variability in POD to the same ventilatory stimulus, hypoxia and hypercapnia (178-181). Normal subjects also appear to detect induced airflow obstruction more accurately than subjects with asthma(182). While an association between AI and POD has been demonstrated in subjects with asthma, it is unknown if a similar relationship exists in normal subjects. One can hypothesise that individuals without respiratory disorders should have no underlying AI. These individuals may, however, be exposed to agents or stimuli which precipitate AI and associated dyspnoea. This concept, along with other factors that may influence POD in a community sample of normal individuals (those without respiratory disorders) is explored further in Chapter 6.

2.5 Zinc and asthma

Zinc is an essential trace element in human beings. There is an increasing body of literature substantiating the role of zinc as an anti- oxidant and immunomodulatory agent. As discussed in Chapter 1, zinc has a key role in maintaining the functional integrity of several organs and epithelial surfaces, promoting growth, development and tissue repair. Recent animal and population based studies have suggested that a zinc deficient state exists in asthma which may promote airway inflammation. Normal zinc homeostasis and the evidence supporting a zinc deficient state in asthma are discussed below. Techniques for measuring zinc in the airway fluid (sputum) are described including the use of a novel method, Zinquin fluorometry, pioneered in our laboratory amongst others.

2.5.1 Zinc Metabolism and Homeostasis

There are no storage pools of zinc in the body and so an adequate amount of zinc needs to be taken in the diet. The body requires 2-3 grams of zinc to function and this translates (accounting for zinc losses) to a dietary intake of 5 mg/day for infants, 10 mg/day for children and 15 mg/day for adults and 16-19 mg/day for lactating women (49). High quantities of zinc are found in red meat, zinc fortified breakfast cereals (183), bread, nuts, dairy products and shellfish. Zinc homeostasis entails a balance between zinc absorption and zinc loss in the body (Figure 2.1). Approximately five milligrams /day of zinc is absorbed in the gastrointestinal tract and a large part of this is eventually excreted in the faeces, with minimal urinary excretion(49). Significant zinc losses can also occur by secretion of zinc across the epithelial surface into the airway and gut lumen or from the skin. Zinc absorption is aided across the gastrointestinal tract by zinc transporter proteins that reside in the epithelial membrane(49). Once across the epithelium, zinc is bound to albumin and transported to the liver transiently and then to the other tissues. Zinc concentrations are high in some tissues including parts of the brain (neocortex, hippocampus), retina, pancreas, prostate, sperm, and mast cells. When the body is under stress for example with acute infection or inflammation, burns or malignancy there is a redistribution of zinc from the plasma to the liver. This process is mediated by interleukins IL1 and IL6 (49).

2.5.2 Intracellular zinc homeostasis

Zinc within the cell exists in 2 forms: eighty to ninety percent is bound tightly to metalloenzymes, zinc finger transcription factors, metallothionein and other proteins(50, 184),and exists as a largely fixed pool with slow turnover. Binding to proteins is largely via cysteine and histidine residues(185). The remaining zinc exists as free or labile zinc, the exchangeable component, which moves easily within and outside the cell via the action of specific Zn transporters.

Based on their sequence homology and structural properties, the transporters have been assigned to two families: SLC39 or ZIP (ZRT/IRT-related protein) and SLC30 or CDF (Cation Diffusion Facilitator), also known as ZnTs(186). ZIPs mediate uptake of Zn across the plasma membrane; CDFs facilitate efflux from cells or mobilization of the metal in intracellular organelles. By mobilising zinc in intracellular organelles, these transporters not only contribute to zinc storage and detoxification but also supply the metal to zinc-dependent proteins and interact with cell-signalling pathways (187, 188). Mutations in Zn transporters can cause altered Zn homeostasis in disease(189).

The fixed pools of zinc are responsible for cellular metabolism and gene expression while the labile pools of zinc are involved in secretion, signal transduction and cytoprotection(46). Labile pools of zinc are present in high quantity in tissues that have a secretory role such as prostatic epithelium (secretion of seminal fluid), pancreatic islets (insulin and glucagon) ,mast cells (histamine) eosinophils and neutrophils(46). Recent research has demonstrated that zinc also exists in similar fixed and labile pools in the airway epithelium and may have a role in cytoprotection, signal transduction and regulating secretion of anti -inflammatory mediators(46).

2.5.3 Zinc and the normal airway.

Normal airway epithelium is a pseudo-stratified epithelium that lines the entire airway up to but not including the terminal bronchioles. The main cell in the airway epithelium is a ciliated columnar cell with a basal and an apical component(46) The apical aspect of the cell is ciliated and protrudes into the airway. In between the columnar cells reside a variety of mucin producing and secretory cells (goblet cells, Clara cells, serous cells, small mucous granule cells, brush and neuroendocrine cells)(46). The airway cells are responsible for maintaining airway epithelial integrity and protecting the epithelium from foreign bodies including bacteria via the secretion of antibacterial agents and mucociliary clearance(46). This is a process whereby mucin, a high molecular weight mucopolysaccharide, produced by airway cells is secreted into the epithelial lining fluid trapping foreign particles. This complex, called sputum, is then propelled up the airway by the beating cilia to the larger airways and then to the mouth by coughing where it is expectorated. Recent studies using Zinquin (see section 2.5.4) have demonstrated the presence of labile pools of zinc in the apical aspect of airway epithelial cells of animals and humans(49) . Based on this distribution and work on certain zinc transporters, Zinc T4 and ZIP1(unpublished data, personal correspondence Dr P Zalewski), zinc is believed to move from the basal surface of airway epithelium cells to the apical aspect. Zinc loss or movement across the airway epithelium, from blood to airway lumen, has not been demonstrated to date.

2.5.4 Methods of measuring zinc in body fluids and tissue

Zinc is a difficult trace element to measure in biological tissues. It is an ubiquitous metal ion that is present in significant concentrations in the environment compared to body fluids and cells. Zinc is found in the body in concentrations between 0.1 to 10 parts per million. Zinc is also present in a

variety of laboratory reagents including re distilled water, and is easily absorbed by glassware(190). Thus when zinc levels are measured, it is important that these factors are accounted for. Buffers, for example, can be depleted of contaminating zinc by treatment with Zn-binding resins prior to their use in zinc assays.

Several techniques exist for measuring total (bound and unbound) zinc in body fluids. These include atomic absorption spectrophotometry (AAS), X-ray fluoresence, emission spectrography, and radioisotope studies. Of these, AAS is the gold standard and the most frequently used method. It measures total bound and unbound (free) or labile zinc. The other techniques mentioned above are more specialised , only available in specific laboratories, and thus will not be discussed further . More recently Zinquin fluorometry, has been developed to measure free or labile zinc, the active component in tissues and body fluids.

2.5.4.1 Methods to measure total zinc.

Atomic absorption spectrophotometry (AAS) is a simple and reliable method to measure total zinc levels. The procedure is available in large analytical laboratories. The solution containing the unknown amount of zinc is sprayed into the flame of the spectrophotometer and atomised. Light of a suitable wavelength for zinc is then shone through the flame. This light is absorbed by the zinc atoms of the solution. The amount of light absorbed by these atoms is proportional to the concentration of zinc in the solution, as determined from a standard curve. This technique is very sensitive and specific for the measurement of total zinc(191, 192). Normal ranges have been determined for some body tissues and fluids including serum/plasma (Normal reference range 10.0-17.0 μmol/l (plasma) http://www.cdhb.govt.nz/chlabs/help/4227hlp.htm.

2.5.4.2 Measurement of labile zinc.

There is an increasing body of literature suggesting that it is free or labile zinc that is the active or biologically relevant component and of primary importance in the inflammatory response. Zinquin is a quinoline based compound that binds predominantly to labile zinc. It has been developed in our laboratory, at the University of Adelaide (Zalewski and colleagues), as a fluorophore to measure free or labile zinc concentrations intra and extracellularly in body fluids and in tissues.

2.5.4.3 The development of Zinquin to measure free /labile zinc

The compound quinolone sulfonamide, was found in early studies to demonstrate marked fluorescence when it bound to zinc (52). This compound was subsequently modified at our laboratory and others, to maximize stability, fluorescence and sensitivity and specificity for labile zinc; all characteristics of an ideal flurophore(52). This was achieved by the addition of a methyl group at position 2, an ester at position 1 and a sulphonylamido group at position 8 (Figure 2.2). This designer compound was labeled *Zinquin* (ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate). Zinquin functions by binding to labile zinc and emitting a blue light when exposed to ultra violet (UV) radiation; the reaction is detected by the UV fluorescence microscope for cells and tissues or UV fluorometry for Zn in body fluids. Zinquin has proven to date to be relatively specific for the measurement of labile zinc in a variety of cells and body fluids. Specificity for Zn in cells was confirmed by showing quenching of Zinquin fluorescence with the membrane permeable Zn chelator TPEN(N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine) which binds Zn more tightly. Copper and cadmium are the only other identified cations that interfere with the binding of zinc with Zinquin. Cadmium is an

insignificant ion in the body, and copper is mostly found in tight complex with Cu-containing enzymes and ceruloplasmin(193).

Zinquin is non-toxic to cells, readily taken up (30 min at 37^{o}C), and works on fresh, frozen or ethanol - fixed cells, as well as frozen sections of tissues. Problems with tissue UV autofluorescence have restricted its use in paraffin sections. Zinquin has been used to measure labile pools of zinc in secretory granules of a variety of tissues including pancreatic islet cells and mast cells, ciliated airway epithelial cells, spermatozoa, hepatocytes, proximal tubules of the kidney, and vesicular structures of cells undergoing programmed cell death(49). Zinquin is capable of detecting nanomolar (nM) concentrations of Zn in solutions. It detects between 40% and 60% of total zinc in plasma, largely the labile transport pool of plasma zinc associated loosely with albumin. It does not detect more tightly bound zinc in plasma alpha-2-macroglobulin (which constitutes most of the remaining plasma Zn)(52).

2.5.5 Zinc and airway inflammation

Zinc is a major player in the inflammatory response, undertaking many roles. Zinc is an **antioxidant** present in cells and their secretions(49). It prevents cell damage induced by hyperoxia (abnormally increased concentration of oxygen) by stabilising sulphydryls and membrane lipids and by inhibiting nitric oxide production. Zinc is also a component of copper /zinc superoxide dimutase (Cu/Zn SOD), a key anti-oxidant enzyme in the airway epithelium(49).

Zinc is an **anti-apoptotic** agent. Apoptosis, defined as programmed cell death occurs in every cell type in the body, and is increased in inflammatory states. Zinc has a role in inhibiting caspase -3, an

important effector enzyme in apoptosis (49, 194, 195). Zinc also **maintains the integrity of cellular membranes,** associated cytoskeleton and cilia(49).

Lastly, zinc is an **immunomodulatory agent** , essential in phagocytic function, cellular and humoral immunity(49) . Zinc is an essential element in the development and function of T lymphocytes and their cytokines, in addition to B lymphocytes and antibody production, especially immunoglobulin G. Zinc is integral to macrophage function, the cell which presents the antigen to the T cell which then initiates the immune response(196).

Zinc deficiency is considered to promote airway inflammation in several ways:

1. by **altering aspects of the immune response**.

Zinc deficiency results in decreased T and B lymphocyte concentrations and function, key players in the cellular immune response to an antigen(196, 197). Normally there is a balance between the T Helper 2 lymphocytes (TH2) which produce pro-inflammatory cytokines (substances that promote inflammation) and T Helper 1 lymphocytes which produce anti-inflammatory cytokines. Zinc deficiency is known to increase the production of TH2 cytokines involved in inflammation and in asthma and reduce the CD4 to CD 8 cell ratios altering the balance between TH2 and TH1 lymphocytes(197). Zinc deficiency blocks the development of B lymphocytes in the bone marrow and inhibits B cell antibody responses(197). Zinc deficiency also reduces the chemotactic response of the cells produced by the T cell response such as the natural killer cell, the polymorphonuclear leucocyte, and macrophage(197). Animal and human studies have demonstrated that repleting zinc deficient states with zinc supplements reverses these immunological changes (197).

2. by **increasing apoptosis** or programmed cell death of airway epithelial cells. This in turn results in rapid cell turnover and proliferation of airway epithelial cells which may have implications for later airway remodelling and chronic airflow limitation(194-196).

3. by **impairing cilial function**. Recent studies have shown that zinc deficiency in vitro may impair cilial function on airway epithelial cells which protect the airway from foreign agents such as allergens, viruses and bacteria (manuscript in preparation Grosser and colleagues).

4. by **increasing oxidative stress**. Oxidative stress involving free radicals, is a recognised pro-inflammatory trigger and zinc is a known anti-oxidant Given zinc's anti-oxidative role, zinc deficiency could promote airway inflammation by this mechanism(197).

5. by **increasing eosinophilic infiltration into the airway**. Zinc has been shown to directly reduce the infiltration of inflammatory cells, especially eosinophils, and neutrophils in the airway of mice.((198),(49)) In one study, the addition of zinc to drinking water effectively reduced airway eosinophilia in mice! (199).

2.5.6 Zinc deficiency and asthma

As asthma is predominantly an inflammatory disorder of the airways and zinc is known to have an anti-inflammatory role, a possible relationship between asthma and zinc deficiency has, and is being explored by several researchers. Questionnaire based studies (case-controlled and cohort studies) (47, 48) have reported significantly lower intake of zinc in those with seasonal and allergic symptoms

and in those with asthma compared with controls. Total zinc concentrations in blood and hair have also been measured in asthmatics and non asthmatics in several studies with varying results: some have demonstrated a significant reduction in blood or hair zinc concentrations in asthmatics(200-202), while others have shown no difference between groups(203-205) or an increase (206).

2.5.7 Zinc deficiency and zinc supplementation in a mouse model of asthma

When airway inflammation using nebulised ovalbumin (OVA) as the allergen was induced in zinc deficient mice and in zinc normal mice, zinc deficient mice had significantly greater airway inflammation measured by hyper-responsiveness of the airways, epithelial apoptosis, mucous hyperplasia and eosinophilia. Conversely,when mice with OVA induced airway inflammation were treated with zinc supplementation intraperitoneally, they had significantly less airway inflammation (evidenced by fewer eosinophils and eosinophil peroxidase activity in the bronchoalveolar lavage fluid compared with placebo treated mice(46)

This thesis extends and optimises the methodology for measuring labile zinc in blood and tissue to include sputum, with the aim of determining airway zinc levels in asthma. Novel work exploring the relationship between zinc and AI in asthma using induced sputum examination is also presented.

Table 2.1: Correlation(r) between cell counts examined by induced sputum and bronchoalveolar lavage, bronchial washing, and bronchial biopsy in asthma.

Author (ref)	Sputum cell type	BAL(r)	Bronchial washing(r)	Biopsy(r)
Pizzichini(131)	eosinophil	0.32	0.70*	
Keatings (129)		NS	0.70*	
Fahy (126)		0.50*	0.70*	
Maestrelli (130)		0.60*		NS
Grootendorst (127)		0.55*		NS
Pizzichini(131)	neutrophil	0.78*		
Keatings(129)		NS	0.60*	
Fahy (126)		0.30	0.40	
Grootendorst (127)			NS	NS
Fahy(126)	macrophage	0.10	0.10	
Grootendorst (127)				NS
Pizzichini (131)	lymphocyte	0.78*		
Fahy (126)		-0.10	0.10	
Grootendorst (127)			NS	NS

* significant correlation at $p \leq 0.05$; NS=non significant correlation ;BAL: bronchoalveolar lavage;

Table 2.2: Reliability of induced sputum cell counts expressed by the intraclass correlation coefficient (ICC).

Author (ref)	subject(s)	method	**eos**	neut	macro	lymph
Gibson (135)	asthma n=10 bronchitis n=8	day to day within sample	**0.96** **0.97**	0.90 0.92	0.94 0.90	0.68 0.86
Pin(133)	asthma n=17 normals n=17	day to day	**0.80**	0.73	0.71	
Pizzichini (132)	asthma n=19 bronchitis n=10 normals n= 10	day to day	**0.94**	0.81	0.71	0.25
In't Veen (128)	asthma n=21	day to day	**0.85**	0.57	0.76	0.76
Spanevello (138)	asthma n=53 normals n=19	day to day within sample	**0.84** **0.98**	0.75 0.85	0.76 0.86	0.39 0.77
Inter rater and intra rater reliability of sputum differential cell counts						
Popov (136)	within examiner count 1 vs 2 n=29		**0.99**	0.96	0.84	0.37
	between examiner reproducibility n=20		**0.98**	0.93	0.92	0.09
Ward(207)	between examiner reproducibility n=24		**0.97**	0.94	0.94	0.27

An ICC $\geq$ 0.8 is considered to be acceptable. Inter rater: between examiner reproducibility; Intra rater: within examiner reproducibility; eos:eosinophil;neut:neutrophil; macro:macrophage; lymph:lymphocyte

Table 2.3: Sputum total cell count and differential cell percentages in healthy adults

NOTE:
This table is included on page 56
of the print copy of the thesis held in
the University of Adelaide Library.

Modified from Belda and colleagues (137); TCC : total cell count; SD: standard deviation; IQR Inter quartile range;% : percent; mL: millilitre;

Table 2.4: Features of sputum inflammatory subtypes in asthma

	Eosinophilic	Neutrophilic	Paucigranulocytic	Mixed granulocytic
TCC,10^6/mL	1.9 (1–3.3)	14.9 (5.8–25.5)	2.8 (1.4–3.7)	13.5 (8.3–20)
Viability, %	70 (59–86)	98 (88–98)	76 (71–86)	87 (81–99)
Neutrophils,%	24.5 (14.3–36.4)	81 (70–93)	28.7 (15.7–44.1)	71.8 (64–87.8)
Eosinophils,%	3.4 (2.2–12.8)	0.3 (0–0.5)	0.3 (0–0.5)	4.8 (2.1–21)
Macrophages,%	59.1 (38–74)	18 (6.5–27.3)	64.6 (53.6–80.6)	18.5 (12–25.5)
Lymphocytes,%	0.5 (0–1.6)	0.3 (0–1)	0.5 (0–1)	0.5 (0.3–3)

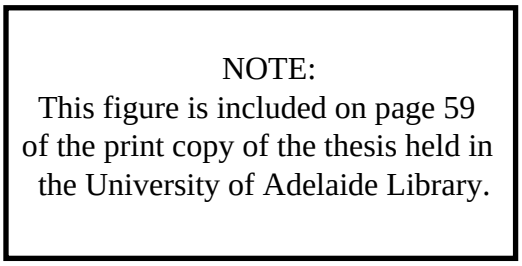
modified from Simpson and colleagues(34); median (IQR);TCC : total cell count %; percent; mL: millilitre

Table 2.5: Correlation (r) between clinical variables and inflammatory markers; symptoms, FEV_1, methacholine PC_{20} sputum and blood eosinophil count and eNO.

	Author (ref)	Symptoms	FEV_1 (L /% pred)	PC_{20} mg/ml	Blood eosinophil	Sputum eosinophil
sputum eosinophil	Pizzichini (132)	-0.68*	-0.68*	-0.66*		
	Lim (144)		-0.63*	-0.67*		
	Jatakanon (143)			-0.40*		
	Louis (146)	0.43*	-0.43*	-0.55*	0.61*	
	Jatakanon(70)	0.48	-0.71*			
	Duncan(208)	0.72*	-0.46*			
	Polosa(209)			-0.47*		
sputum neutrophil	Louis(146)	0.23	-0.20	-0.46*	-0.46*	
blood eosinophil	Pizzichini(69)		-0.57*	-0.52		
	Duncan(208)	0.57*	-0.39*			
	Taylor (105)			-0.71*		
eNO	Lim(71)	-0.02*				
	Jatakanon(70)		-0.12*	-0.64*		
	Salome(210),			0.39#*	0.35*	
	Mattes(211)					0.30
	Berry(212)					0.26*∞

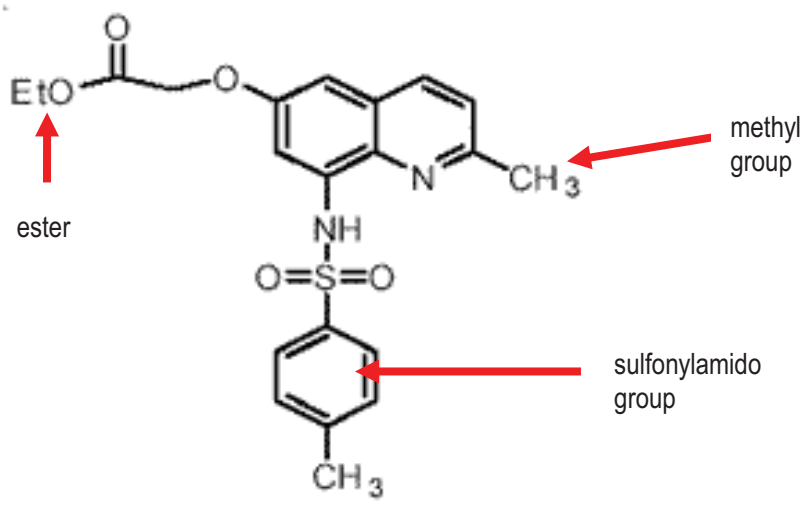
*p<0.05; NS=non significant; #histamine; ∞: r^2 (therefore correlation r=0.509);FEV_1: forced expiratory volume in 1 second; PC_{20}: provocation concentration resulting in 20% fall in FEV_1 from baseline; eNO :exhaled nitric oxide

Figure 2.1: Zinc homeostasis.

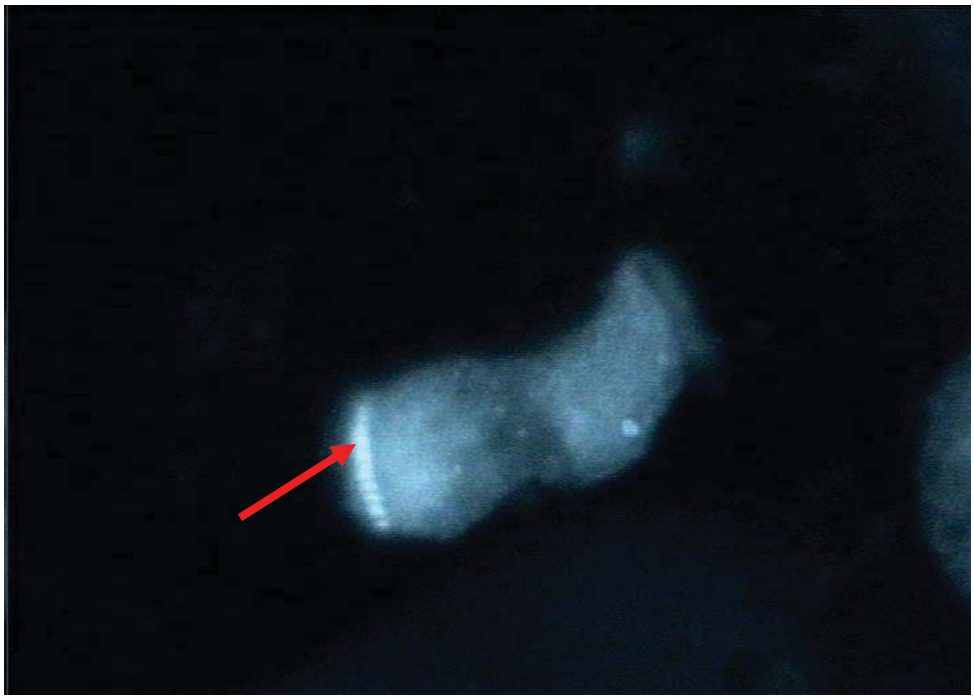


Reprinted with permission from A/Prof Peter Coyle, Hanson Institute, University of Adelaide

Figure 2.2 (a) Zinquin structure and (b) Labile Zinc fluorescing with Zinquin in the apical component of airway ciliated columnar cell.



(a)



(b) Labile zinc in the apical segment of an isolated, ciliated the columnar cell.

CHAPTER 3

Materials and Methods

This chapter describes the North West Adelaide Health Study (NWAHS) cohort from which participants were enrolled. It also outlines the materials and methods used in the experimental chapters of this thesis.

3.0 The North West Adelaide Health Study (NWAHS) Cohort.

The North West Adelaide Health Study is an ongoing 4060 population based study in South Australia which is examining the impact of chronic disease (asthma, chronic obstructive pulmonary disease, diabetes, and cardiovascular disease) on the individual and society. All participants have undergone a clinical assessment which has included a comprehensive questionnaire, standardised allergy skin prick tests for atopy, and baseline lung function measurement with spirometry and bronchodilator response. Full details of the cohort have been published (213) with a dedicated website providing details of methodology and results to date: http://www.nwadelaidehealthstudy.org.

Within this cohort, a community sample of 300 individuals with self reported-doctor diagnosed asthma, 80 subjects without symptoms of asthma but evidence of a bronchodilator response (BDR) according to reversibility have also been identified. All these subjects (n=380) were invited to participate in this study (thesis) of which 77 (20%), all with a self reported doctor diagnosis of **asthma** consented to participate.

Subjects without asthma(n= 91), defined by the absence of a self reported-doctor diagnosis of asthma and the absence of reversibility of airway obstruction based on American Thoracic Society criteria

were also voluntarily recruited from the larger NWAHS study, and formed the **control group** for this study.

All subjects were over the age of eighteen. Informed consent was obtained on all subjects. All aspects of the studies undertaken in this thesis conformed to National Health and Medical Research guidelines and were approved by The North West Adelaide Health Service Ethics of Human Research Committee.

3.1 Clinical Procedures

3.1.1 Questionnaires

3.1.1.1 The NWAHS questionnaire is a comprehensive questionnaire that seeks detailed feedback from participants on a variety of chronic disorders. The respiratory section of the questionnaire is shown in Appendix 1. Episodic symtoms were defined as the presence of cough, wheeze, shortness of breath or chest tightness during the past 12 months.

3.1.1.2. Quality of life was assessed by the generic SF (short form) 36 Quality of Life Questionnaire(214) The SF-36 is a multi-purpose, short-form health survey comprising of 36 questions. It provides information across 8 domains in an individual: Physical Functioning, Role-Physical (RP) Bodily Pain, General Health (GH), Vitality, Social Functioning, Role-Emotional (RE) and Mental Health (MH). Each domain is transformed into a 0-100 scale with zero indicating poorest

health and 100 indicating best health. The SF-36 is a generic measure, as opposed to one that targets a specific age, disease, or treatment group. Accordingly, the SF-36 allows quality of life comparisons to be made across a wide range of diseases and treatments.

3.1.1.3. The Visual Analogue Scale (VAS) is a 100millimetre (mm) horizontal scale on which subjects rate their magnitude of breathlessness. It ranges from a minimum of zero, defined as no respiratory sensation, to a maximum of 100mm, defined as the worst respiratory complaint imaginable. The scale is responsive to change in symptom perception following an intervention(215). It was used in this thesis to quantify the perception or sensation of dyspnoea (POD) to a stimulus, namely the hypertonic saline challenge, which is described in detail below (section 3.2.2).

(a) in asthma.

Perception of dyspnoea (POD) was measured before the saline challenge commenced, and when a 15% fall in FEV_1 from baseline occurred(216). Previous VAS scores were not shown to the subjects. Over perceivers of breathlessness (hyperperceivers) and under perceivers of breathlessness (hypoperceivers) were defined by a VAS score of more than or less than 1 standard deviation (SD) from the mean respectively, as data were normally distributed. This categorisation was based on that used by Boulet and colleagues who demonstrated that in a sample population with increased airway hyper -responsiveness, POD documented at a 20% fall in FEV_1 from baseline (PS_{20}),with the modified Borg scale , were normally distributed(53).

(b) in normal subjects or subjects without asthma.

VAS assessment in subjects without asthma was performed as described above. As our subjects did not achieve a 15% fall in FEV_1 ,in order to standardise or anchor the change (delta or Δ) in the VAS score to the change in FEV_1 with the saline challenge, POD was measured as Δ VAS/ Δ FEV_1.

Furthermore, as VAS results were not normally distributed in our study group of healthy subjects, percentile values were substituted for SD, based on the studies by Boulet and colleagues (53, 217). Low perception of breathlessness (hypoperceiver) was defined, a priori, as a VAS score below or equal to the 25th percentile and increased perception of breathlessness (hyperperceiver) was defined as a VAS score equal or greater than 75th percentile.

3.2. Lung Function assessment and assessment of asthma severity

3.2.1 Spirometry

Spirometry was performed with a Microlab 3300 (Micro Medical Ltd; Kent, United Kingdom) according to American Thoracic Society (ATS) criteria (218) using reference values from Crapo and colleagues (219). Reversibility of airway obstruction was defined as an increase of at least 12% and 200mls in forced expiratory volume in 1 second (FEV_1) 10 minutes after 400 μg of salbutamol via a spacer device (Aerochamber® or Volumatic®). Determination of bronchodilator response (BDR) according to reversibility was performed at a prior clinic visit to the hypertonic saline challenge (within the preceding 3 months) as part of the NWAHS study.

3.2.2. Hypertonic saline challenge

The test was performed using the method described by Anderson(82), Gibson and colleagues(162, 220) Briefly, the subject inhaled a solution of hypertonic saline (4.5 %) for increasing time periods for a total of 15.5 minutes (30 seconds, 1 minute, 2 minutes , 4 minutes, 4 minutes, and then 4 minutes again) via an ultrasonic nebuliser with a mouthpiece. After each period of inhalation, FEV_1 was

measured for safety. The procedure was terminated when a 15% fall in FEV_1 from baseline (PD_{15}) occurred or when 15.5 minutes of nebulisation was complete, whichever occurred first. Medications that interfere with the test were ceased temporarily for an appropriate time prior to the test (162). A positive test of AHR was described as a 15% fall in FEV_1 from baseline (PD_{15}) prior to, or at completion of the challenge. A negative hypertonic saline challenge test was documented when the 15% fall in FEV_1 from baseline (PD_{15}) did not occur within, or at completion of, the 15.5 minutes of nebulisation.

3.2.3. Asthma severity was assessed using modified Global Initiative of Asthma (GINA) criteria(9)**.** This classification of asthma severity is based on clinical features: symptoms and lung function (FEV_1 and Peak Expiratory Flow (PEF) monitoring). More recently this classification has been modified by the National Asthma Council of Australia to include the inhaled corticosteroid dose used(8) (Appendix 2), as reiterated recently in the literature(221).

3.3 Laboratory procedures

3.3.1 Allergy skin prick tests

Allergy skin prick tests were performed at the initial visit into the NWAHS study to determine if the participant reacted to six allergens: rye grass, cat, house dust mite, alternaria (mould), feather and cockroach. This involved putting drops of liquid allergens on the participant's slightly scratched skin on their forearm. After 15 minutes, the diameter (millimetres) of the wheal or bump of the skin was measured. A successful test required that a person's negative control wheal be less than 2 mm in diameter. A person was defined as having an allergic

reaction to the selected allergen if the difference between the negative control wheal and the allergen wheal was more than 2 mm in diameter, and the allergen wheal on the skin measured 2 mm or more in diameter (from http://www.nwadelaidehealthstudy.org).

3.3.2 Sputum induction and processing

Sputum was obtained by the hypertonic saline challenge test described above. At the end of the procedure subjects were asked to blow through the nose and rinse their mouth and swallow the water to minimise contamination with saliva and post nasal drip. Then, they were instructed to cough sputum into a sterile container. Sputum was processed within 2 hours of collection according to European Respiratory Society Task Force guidelines(139). Briefly, the expectorated specimen was poured into a Petri dish and the macroscopic appearance was recorded. A portion of sputum up to 500 mg was selected and placed in a 15 ml polystyrene tube. It was then treated with dithiothreitol (DTT) (Sputolysin10%, Calbiochem Corp, San Diego, CA), vortexed, and rocked for 15 minutes to break up the mucus and disperse cells. Phosphate buffered saline was added to the suspension which was filtered through 60 μm nylon mesh (Millipore, Nth Ryde, NSW, Australia). The filtrate was centrifuged for 10 min at 400g, the sputum supernatant was removed and stored at minus 20 degrees Celcius(^{o}C) and the cell pellet was resuspended in 0.5 ml of Hanks balanced salt solution (HBSS). The total cell count (TCC) and viability (V), as measured by exclusion of 0.2% trypan blue, were obtained simultaneously in a modified Neubauer chamber. Cytospins were prepared and stained with May Grunwald - Giemsa for a differential cell count (DCC) of 400 non squamous cells. All sputum cell and differential counts were performed independent to, and blinded to, clinical details and subject grouping.

3.3.2.1Quality Control

During the course of optimising the sputum processing methodology for the studies undertaken in thesis, we liaised with a central laboratory with expertise in this area. (Hunter Medical Research Institute, John Hunter Hospital, Newcastle, NSW) and sent stained slides for double reading. The between observer repeatability for the differential cell count expressed by the intraclass correlation coefficient (ICC) was as follows: eosinophil 0.96; neutrophil 0.99; macrophage 0.98; lymphocyte 0.35. These results are in keeping with previous studies(207) (see Table 2, Chapter 2). All differential cell counts were performed by a consultant haematologist. The overall mean (SD) viability for the differential cell counts was 84(17) %. The overall median (IQR) squamous cell contamination for the differential cell counts was 15(30) %.

3.3.3 Exhaled Nitric Oxide (eNO)

Exhaled nitric oxide was measured prior to any forced expiratory manoeuvres. Measurements were obtained online, using a calibrated chemiluminescence analyzer with an integrated flow transducer (ECO Physics AG, Switzerland, model CLD 77AM) according to current ATS guidelines (111). An exhalation flow rate of 50ml/second was used. Nose clips were not used. All subjects remained standing for the measurements. Subjects were instructed to inspire fully and then, without hesitation, expire via a disposable mouthpiece through a 1.5 mm orifice. Flow was recorded by an ultrasonic spirometer connected downstream of the orifice. A pressure gauge attached to the upstream side of the orifice was used to provide feedback to the subject to facilitate a constant expiratory flow. A pressure of 14 cmH2O was found to correspond to a flow of 50 ml/second. Expired gas was sampled continuously from a port between the orifice and the spirometer. Each subject performed one or more practice measurements, as necessary, until they were able to perform the required manoeuvres. Three readings were performed and the average was used for each subject. Each online recording of

eNO was inspected and the eNO recorded as the average reading over at least 10 seconds of the record after initial transient changes due to sampling of expirate from the upper airway and equipment dead space. The resistance formed by the orifice was sufficient to close the velum thereby preventing contamination of the expirate by nasal NO. Exhaled nitric oxide measurements were conducted on 80 consecutive subjects,as this procedure was introduced during the study and not at commencement. There were no equipment or subject failures. Readings were obtained by technical staff blinded to the clinical details of subjects.

3.3.4 Collection of blood and saliva.

A sample of blood and saliva was taken for zinc measurement. Five millilitres(mls) of blood was collected in a heparin tube and centrifuged immediately for 10 minutes at 1000g. The plasma was removed and frozen at -20°C for later measurements. Prior to commencing the hypertonic saline challenge, the subject was asked to expectorate a sample of saliva into a sterile container.

3.3.5. Measurement of total and labile zinc in induced sputum, plasma and labile zinc in saliva.

The materials and methods for measuring total and labile zinc in these body fluids are described in Chapter 7 as optimising the method to measure labile zinc in sputum, and comparing it with total zinc measurements in sputum and plasma constitute an experimental chapter in this thesis.

CHAPTER 4

The relationship between airway inflammation and asthma defined by self reported doctor diagnosis, wheeze, episodic symptoms with bronchodilator reversibility, or episodic symptoms with airway hyper-responsiveness in a South Australian adult community.

4.0 Introduction

Asthma is a disorder that is defined in many ways (Table1.1, Chapter 1) and which is currently the topic of much debate, primarily due to the lack of a gold standard definition. The traditional and most commonly used definition, which has stood the test of time, includes the presence of episodic symptoms with evidence of variable airflow obstruction(7). In community populations these symptoms are usually assessed by questionnaires, and often complemented with an objective measure of variable airflow obstruction such as PEF, spirometry with bronchodilator reversibility (BDR), or test of airway hyper-responsiveness(11, 13). The episodic nature of symptoms and variability of airflow obstruction encompassed in these definitions however, may underestimate the true prevalence of asthma.

More recently, with the realisation that asthma is primarily an inflammatory process, and with the advent of eNO and induced sputum examination, tools developed to assess AI accurately, researchers have been assessing the role of these techniques in the diagnosis and management of asthma(31, 113). Exhaled nitric oxide and IS, by determining the presence or absence of AI and the cellular subtype of AI (with IS), have a clearly defined role in diagnosing and monitoring AI in asthma, and in optimising asthma control through titration of the inhaled corticosteroid dose to AI measurements(37, 38). These findings, are however, based on studies where subjects were recruited from hospital clinics and their asthma was well characterised. The usefulness of these tools in the community, where patients present with self reported symptoms or a doctor diagnosis suggestive of asthma is less defined.

Lemiere and colleagues explored the premise that sputum eosinophil counts would be useful in validating self reported symptoms suggestive of asthma within an adult community(161). Subjects (n=107, 30% on ICS) were divided into the four groups; no respiratory symptoms, those with self reported diagnosis of asthma, subjects reporting recurrent wheeze but not diagnosed with asthma, and those reporting exposure to industrial irritants. The authors found that 'in a community setting, induced sputum eosinophil cell counts in subjects reporting asthma or wheezing were most often within the normal range and not sufficiently abnormal to be useful in validating a diagnosis of asthma in epidemiological studies'(161). In contrast, in another epidemiological study (n=170, 6% on ICS) examining the association between AI, asthma symptoms and airway hyper-responsiveness (AHR) in children, a significant relationship was found between sputum eosinophilia and asthma symptoms (odds ratio (OR) 2.25 (95%CI: 1.2-4.24; $p<0.01$) and between sputum eosinophilia and AHR (OR 4.36 (1.7-11.2, $p<0.001$)(162).

There are few studies evaluating the clinical utility of eNO in the community. Recently, a randomised, single blind trial examined the additional clinical benefit provided by eNO measurements compared with traditional management (symptom and lung function assessment) alone, in improving asthma control and exacerbations over 12 months(222). The authors found that, in subjects recruited from primary care with a doctor diagnosis of asthma, regular eNO measurements did not reduce the exacerbation rates significantly or result in a difference in the total amount of ICS used (222). In another cross sectional study of steroid naïve patients(n=51), the usefulness of eNO compared with spirometry measurements in influencing a General Practioner's working diagnosis of respiratory symptoms was assessed. Exhaled nitric oxide measurements improved diagnostic confidence in 48/51 (94%) cases in a primary care setting while spirometry was considered to be helpful in 27/51(53%) of cases(223). A comparable study specifically examining the clinical utility of eNO

measurements in supporting a doctor diagnosis of asthma has not been performed. In a recent community based study developing reference ranges for eNO, Travers and colleagues found that eNO was a poor discriminator between normal subjects and those with asthma (115).

Similarly, while histamine and methacholine challenges are well researched and established tests of AHR frequently used in the diagnosis of asthma in the hospital setting and in community based studies(224), the usefulness of a hypertonic saline challenge (HS) test to diagnose asthma in the adult community has not been studied comprehensively. Epidemiological studies have demonstrated a low sensitivity and high specificity for HS challenge to identify a doctor diagnosis of current asthma (54) and to identify asthma defined by questionnaire (84). Conversely, Leuppi and colleagues found in patients with a doctor diagnosis of asthma (n=604) referred for a HS challenge test, self recognition of asthma, and frequency of bronchodilator use in the 3 months prior to the challenge were predictive of a positive response (PD_{15})(225).

The aims of this study were therefore to firstly explore the frequency and type of AI present in an adult South Australian community; secondly to examine if the addition of a measure of AI, namely induced sputum eosinophil count and eNO measurements would aid the diagnosis of asthma compared with the traditional physiological measures of BDR and AHR; thirdly to assess the clinical utility of HS challenge in the diagnosis of asthma. Asthma for this study was defined by self reported doctor diagnosis (SRDD), presence or absence of wheeze, episodic symptoms and bronchodilator reversibility(ESBDR), and by episodic symptoms and airway hyper- responsiveness (ESAHR).

4.1 Materials and Methods

These are detailed in Chapter 3.

Subjects

Subjects were recruited from the North West Adelaide Health Study cohort for this cross-sectional study.

Procedures

Subjects undertook a single visit to the laboratory and had the following procedures:

Clinical assessment and symptom questionnaire, spirometry, hypertonic saline challenge test and sputum induction. Reversibility of airway obstruction was defined as an increase of at least 12% and 200mls in forced expiratory volume in 1 second (FEV_1) 10 minutes after 400 μg of salbutamol via a spacer device (Aerochamber® or Volumatic®). Determination of bronchodilator response (BDR) according to reversibility was performed at a prior clinic visit, within the preceding 3 months as part of the NWAHS study. Sputum was processed as per ERS Task Force guidelines using the selected method(139). Exhaled nitric oxide was measured prior to any forced expiratory manoeuvres, according to current guidelines(111). The study profile is outlined in Figure 4.1.

4.2 Statistical analysis

Descriptive statistics were used to summarise the clinical characteristics of the participants. Variables with non normal distribution (sputum total cell count and eosinophils) were log transformed before analysis if possible. Comparisons between asthma and no asthma groups were made using the t test for normally distributed variables and Mann-Whitney U test for non normal data. Sensitivities, specificities, positive and negative predictive values (accuracy analysis) for the diagnosis of asthma by self report, presence of wheeze, ESBDR, and ESAHR were calculated for each of the tests. For sputum eosinophils the cut points used were 1% and 2%. For eNO the cut points used were 19 ppb and 30ppb. Relationships amongst a series of dependent outcomes, asthma diagnosed by self report, wheeze, ESBDR or ESAHR and sputum eosinophils and eNO were explored by 2 methods: firstly, by logistic regression and expressed as the odds ratio (OR); and secondly with correlations. Results were calculated for the HS challenge test, sputum eosinophil and eNO in all subjects and with subjects on ICS removed from the analysis. This was done as these measurements are known to be very sensitive to ICS(31, 111). All statistical tests were two sided and significance was accepted at the 95% level. Data analysis was performed using statistical software SPSS for windows7 Version 10.0 (SPSS Inc., Chicago, Illinois).

4.3 Results

Baseline subject characteristics are illustrated in Table 4.1. Age, male gender, and FEV_1 (Litres and percent (%) predicted) were significantly lower in subjects with SRDD asthma compared with those without SRRD asthma. Atopy and use of inhaled medications were increased in subjects with SRDD

asthma compared to those without. Most subjects with SRDD had mild intermittent asthma based on the NAC (2006) criteria (8)

Frequency and type of airway inflammation

The prevalence of eosinophilic, neutrophilic, and pauci-granulocytic (non eosinophilic, non neutrophilic) AI in this South Australian community was similar in participants with and without SRDD asthma (Table 4.2). Sputum eosinophilia >1% was present in approximately 30% of subjects with and without SRDD asthma, with non eosinophilic AI present in the majority. Exhaled nitric oxide was measured in fewer (n = 80) subjects. A raised eNO of $\geq$ 19 parts per billion (ppb) was similarly present in subjects with (43.4%) and without asthma (40.1%) while eNO of $\geq$30 parts per billion was more frequent in subjects with asthma (Table 4.2). Comparable results were obtained when subjects on ICS were removed from the analysis (Table 4.2).

The relationship between asthma defined by SRDD, wheeze or ESAHR and bronchodilator reversibility(BDR) .

Wheeze was noted in 69% (53/77) of subjects with, and in 38% (35/91) of subjects without SRDD asthma, but most reported occasional wheeze in the last 3 months (Table 4.3). BDR was present in 20.7% of subjects with SRDD asthma and in no one without SRDD asthma (Table 4.4). The HSAHR test was positive in 73% of those subjects with, and in 55% without SRDD asthma (Table 4.5). Significant correlations were noted between asthma defined by self report and wheeze (r=0.30; p=0.00; n=168), asthma defined by self report and ESBDR (r=0.35; p=0.00, n=165) or ESAHR (r=0.48; p=0.00, n=144). Bronchodilator reversibility correlated significantly with all definitions of

asthma: BDR and asthma defined by self report (r=0.3; p=0.00, n=165), BDR and wheeze (0.20; p=0.01, n=165), and BDR and ESAHR(r=0.23; p=0.005, n=144).

Sensitivity, specificity, positive and negative predictive values of BDR, HSAHR, sputum eosinophilia and eNO measurements for asthma defined by self report, wheeze, ESBDR and ESAHR.

Bronchodilator reversibility had poor sensitivity but excellent specificity and a high positive predictive value for a diagnosis of asthma based on self report, wheeze and ESAHR(Table 4.4).Contrastingly, HSAHR had moderate sensitivity and poor specificity for a diagnosis of asthma defined by self report or wheeze in subjects on or off ICS (Table 4.5). An eNO measurement of greater than or equal to 30 ppb was associated with poor sensitivity but excellent specificity for asthma defined by self report, wheeze, ESBDR and ESAHR (Table 4.6). Additionally, an eNO $\geq$ 30 ppb was associated with a high specificity for a positive test of HSAHR alone (Table 4.7). An eosinophil count of greater than 2% was associated with poor sensitivity and high specificity for asthma defined by self report, wheeze, ESBDR and ESAHR (Table 4.8). Additionally, an eosinophil count > 2%, akin to eNO>30ppb, was associated with a high specificity for a positive test of HSAHR alone (Table 4.9).

Associations between physiological and inflammatory markers and asthma defined by self report, wheeze, ESBDR and ESAHR

Asthma defined by SRDD asthma was significantly associated with the presence of wheeze, BDR and a positive HSAHR (Table 4.10). A strong relationship was present between asthma defined by wheeze and BDR, while a significant association was noted between asthma defined by ESAHR and BDR(Table 4.10). Relationships were not noted between sputum eosinophilia and asthma defined by

SRDD, wheeze, ESBDR or ESAHR. Contrastingly, eNO ≥ 30 ppb was strongly associated with asthma defined by SRDD: OR 4.0 (95% CI: 0.9-18.6), p=0.08 subjects not on ICS, and by ESAHR: OR 6.6 (95% CI: 0.8-58),p=0.08 subjects not on ICS (Table 4.10). Exhaled nitric oxide levels ≥ 19 and 30 ppb were not significantly associated with a diagnosis of asthma defined by ESBDR.

Table 4.11 documents the additional numbers of subjects identified if asthma was defined by the presence of AI (a raised eosinophil count >2% and /or raised eNO≥30 ppb) over and above a positive self reported doctor diagnosis of asthma. An eNO ≥ 30ppb detected most cases, and a combination of a raised eNO ≥ 30ppb and presence of sputum eosinophilia >2% detected nearly all cases of asthma defined as such.

Table 4.12 demonstrates the number of subjects classified as having asthma depending on the definition used (SRDD, ESBDR or ESAHR) when compared to a "global definition" of asthma including all of the above , and which is similar to that used in real life.

4.4 Discussion

It is now unequivocally established that asthma is a disorder "heterogeneous in its clinical features, treatment response, prognosis and pathophysiological mechanisms"(226). Several definitions for asthma exist, and the most appropriate choice is often determined by the clinical situation and context. This study has shown that the prevalence of eosinophilic airway inflammation in a community cohort with asthma is approximately 30%, and that non-eosinophilic, paucigranulocytic (sputum eosinophils and neutrophils in the normal range) AI predominates. These findings have been confirmed in previous hospital based studies (34) and for the first time, also verified in this community based study involving adults. A recent epidemiological study in 381 adults with asthma found a similar prevalence of cellular subtypes in peripheral blood; 44 % of subjects had eosinophilic inflammation while 56% had non eosinophilic, predominantly paucigranulocytic inflammation(227). The pathological difference between paucigranulocytic AI and normal cell counts, and methods to differentiate these 2 states in asthma is currently unclear and open to debate.

Interestingly, 20 to 32% of participants without documented SRRD asthma were also noted to have sputum eosinophlilia (Table 4.2). The prevalence of sputum eosinophilia in a healthy community cohort is unknown. Previous published reports involving healthy individuals in experimental studies have estimated the prevalence of airway eosinophilia (defined by sputum eosinophils >2.5% or BAL eosinophils >1%) at 5.5% (95%CI: 1.5 to 9.6)% (228). The higher prevalence noted in this study may be authentic, or may include individuals with cough alone as a symptom with sputum eosinophilia in the absence of BDR or airway hyper-responsiveness (eosinophilic bronchitis), and those with undiagnosed asthma without evidence BDR or AHR on the day of testing. These findings may also reflect the presence of co- exisiting disorders in participants such as allergic rhinitis and gastro-oesophageal reflux (228) which were not objectively quantified at the clinic visit.

The relationship between asthma defined by self report (SRDD), presence of wheeze, and physiological (BDR and AHR) and inflammatory markers (sputum eosinophilia and ENO) was also explored. The sensitivity and specificity and predictive value of BDR, HSAHR, eNO and sputum eosinophil measurements were similar for asthma defined by self report, wheeze, ESBDR or symptomatic airway hyper- responsiveness (ESAHR). These findings suggest that symptoms, variable airflow obstruction, and airway hyper- responsiveness remain interrelated in the diagnosis of asthma.

Bronchodilator reversibility, traditionally used in the definition of asthma (8, 9), had excellent specificity (95 to 100%) and positive predictive value (75 to 100%) for asthma, defined either by self report, wheeze or ESAHR. The findings differ with current opinion suggesting that a single recording of reversibility is of limited value in the diagnosis of asthma(229). This may reflect the poor sensitivity of the test (13 to 20.7%) which restricts its use as a screening tool. Our findings suggest BDR is an extremely useful test in "ruling in" a diagnosis of asthma where the index of suspicion is high. Appleton and colleagues additionally found that BDR in the NWAHS cohort was associated with the presence of significant respiratory symptoms and impairment (230).

Hypertonic saline challenge testing was moderately sensitive (73%), with an equivocal positive predictive value of 49% and poor specificity (45%) in subjects with asthma defined by self report in the community. This result differs from previous community based and laboratory based studies where hypertonic saline has been shown to be very specific in the diagnosis of asthma defined by either current wheeze in children, or by the self reporting of symptoms or a doctor diagnosis of asthma in adults (54, 74, 84, 88, 231). In one epidemiological study (n=91 adults), the sensitivity and specificity

of HS (with 95 % confidence intervals(CI)) to identify a doctor diagnosis of current asthma was 39% (95% CI:21 -61)% and 90% (95%CI:80-96)% with a positive predictive value of 55% (95%CI:31-79)% (54). In another cross sectional, epidemiological survey of 99 adults from the timber industry, the sensitivity and specificity of HS challenge to diagnose asthma defined by the question " Have you ever had an attack of asthma" (IUAT Bronchial Symptoms Questionnaire,1986) was 57 % (95% CI: 20-94%)and 86% (95% CI: 79-94%) respectively with a positive predictive value of 71% (84). Why is there a difference between the results obtained in this study and those described previously? There are several possible explanations. Firstly, that the differences noted amongst the studies may reflect varying disease prevalence, which ranged from 4.9 to 6.4% for a diagnostic label of asthma in the above studies. The prevalence for doctor diagnosed asthma was 12% in our population during the study period (22). Secondly, the sample size in this study (n=168) compared with previous larger studies, may have affected the outcomes. Unlike other community studies this cohort included slightly older participants who may have other co-existing respiratory disorders such as chronic bronchitis resulting in symptoms contributing to the low specificity and higher sensitivity of the challenge test. Furthermore, a selection bias may have occurred due to the 20% response rate for participation in this study. Thirdly, while this study followed the definitions for asthma described in previous adult epidemiological studies, the subjectivity of these definitions may have contributed to the documented low specificity(11). Finally, most of the epidemiological studies assessing the clinical utility of hypertonic saline challenge have been performed in children who may have a different physiological and inflammatory airway profile to adults.

A positive hypertonic saline challenge test result was also noted in 48 (55%) of our subjects without SRRD asthma which may also have contributed to the poor discriminative properties of this test in our population (Table 4.5). Possible reasons for this include a high false positive rate (i.e. the occurrence

positive result when the disease state does not exist) due to the technical limitations of the test, and the cut off points used to define and interpret a positive result; the presence of asymptomatic airway hyper-responsiveness; under perception of symptoms; and the lack of a reference or gold standard definition for asthma. The saline challenge test is a well established clinical test performed at a variety of respiratory centres and with quality control and calibration measures in place, similar to spirometry, making high false positive rates unlikely. It is known that over 30 percent of the normal population have documented asymptomatic airway hyper-responsiveness on airway challenge tests, which may be a precursor of asthma (232, 233) and this may explain some of the positive HSAHR results. Some subjects may have under perceived and under reported their symptoms, potentially underestimating a diagnosis of asthma. This hypothesis is less plausible as only 3 out of the 48 subjects (6.3%) with a positive saline challenge test but no SRRD asthma under perceived their dyspnoea in this study (methodology detailed in Chapter 3). Ultimately, the definition of SRRD asthma used as the reference standard may not be sufficiently discriminatory for a 'true' diagnosis of asthma, again fuelling the debate as to how best to define this disorder.

In contrast, the measurement of eNO $\geq$ 30 ppb in a community setting appears to be highly specific for a diagnosis of asthma defined by SRDD, wheeze, ESBDR and ESAHR. Furthermore, eNO measurements $\geq$ 30 ppb demonstrate a potentially robust relationship (as reflected by the odds ratio, table 4.10) for asthma defined by SRDD and ESAHR. The findings are similar to another community based study where cut-off points of 50 ppb for eNO were associated with a high specificity (96%) but poor sensitivity for an inclusive diagnosis of asthma. An eNO cut-off levels of 20 ppb in the same study demonstrated moderate specificity (61%) and equivocal sensitivity (49%) (115)

These results, however, need to be interpreted with caution due to the limitations of small sample size and consequent borderline statistical significance, or non significant p values and the odds ratio values including 1. Sputum eosinophilia > 2% was also specific, but less so when compared with eNO, for asthma defined by SRDD, presence of wheeze, ESBDR or ESAHR. Sputum eosinophilia did not demonstrate an association (as reflected by the odds ratio) for SRDD, ESBDR or ESAHR, suggesting that it is not a useful marker in validating asthma defined by self report in community (Table 4.10)(161). It differs from other hospital clinic based studies which have clearly demonstrated the utility of induced sputum examination in predicting a diagnosis of asthma based on symptoms combined with evidence of variable airflow obstruction (234, 235). The low diagnostic yield in this study again most likely reflects the heterogeneous nature of asthma in a community population when compared with the well defined sample of subjects with asthma seen in a hospital clinic.

What do these findings mean? Given the number of variables assessed by logistic regression in this study, the significant associations may be due to chance alone. They may also reflect the variability encountered with patients' (and their health practioners') understanding of symptom definitions and perception of symptoms. Moreover, these results lend support to the argument that symptoms of asthma such as wheeze do not reflect airway inflammation (AI) directly and that additional measures of AI are required(31). For example, the use of eNO measurements and sputum eosinophil counts either alone or in combination in the diagnosis of asthma, over and above the traditionally used epidemiological definitions of self reported doctor diagnosis, detected up to a further 33% of cases in this study (Table 4.11).

Strengths of this study include the population based design, comprising of subjects with varying severity of asthma and those without asthma, a mixture of medications, and a range of investigations (HSAHR, sputum cell counts, and eNO) assessed both solely and in combination. This allows the results to be generalisable to the wider community and real life. Interestingly although 44% of our subjects with a diagnosis of asthma based on self report were on inhaled corticosteroids, compared with 15% in other studies such as Riedler and colleagues (88), the results obtained with HSAHR, eNO and sputum examination were similar with ICS use included. This finding is surprising given that these measures are known to be very responsive to ICS therapy (82, 236). One explanation is that only 34 subjects in this study were on ICS; too small a number to affect overall results. Another explanation, well supported by the literature, is that compliance with regular ICS therapy in the community is poor (237-239).

Possible limitations of this study which may have impacted on the results include the definition of asthma used to classify subjects, the age of the participants, the sample size and the measurement of BDR and HSAHR on different days. Asthma in this study was defined by self report of a doctor diagnosis and assessment of respiratory symptoms was made by questionnaire. While these methods are used routinely in epidemiological studies, they may have led to an under-reporting of associations between inflammatory markers and a diagnosis of asthma. Jenkins and colleagues, however, compared the validity of using questionnaire based definitions of asthma with or without an additional test of airway hyper-responsiveness against a gold standard of a respiratory physician diagnosis of asthma(54). They found that questionnaires showed excellent agreement (high sensitivity and specificity) with a physician diagnosis of asthma. Airway hyper-responsiveness alone or with questionnaire positivity resulted in high specificity but low sensitivity for a physician diagnosis of

asthma(54). Secondly, the average age of subjects with asthma in this population was 54 years which may confine interpretation of the study results to older adults with asthma.

Exhaled nitric oxide measurements were not conducted on all subjects as this procedure was introduced during the study. This may have resulted in an insufficient sample size to detect significant change with these measurements. The effects of limited sample size were also seen with the saline challenge test. While the NWAHS cohort comprises of 380 subjects with SRDD asthma, only 77 (20%) agreed to participate in this study as it involved a saline challenge, and 18 enrolled subjects (10.7%) did not complete the saline challenge test for the reasons outlined in Figure 4.1. The NWAHS cohort is an ongoing community based study where maintaining subject participation and goodwill over many years is important. A higher than usual baseline $FEV_{1,}$ (<70% predicted) (102) below which the procedure was not performed was, therefore instituted in order to minimise severe symptoms which may have affected ongoing participation. Lastly, the temporal interval in BDR ($\leq$ 3 months) and HSAHR measurements may have led to assumptions regarding the relationship between these 2 variables that might be different if they had been performed within 24 hours of each other. The data therefore needs to be interpreted with these caveats in mind.

In summary, this study has demonstrated for the first time that in the community: the prevalence of eosinophilic AI (>1%), measured by IS, approximates 30% in subjects with and without SRRD asthma with non eosinophilic (pauci-granulocytic) AI predominating. Secondly, that BDR is highly specific for a diagnosis of asthma defined by SRDD or ESAHR. Thirdly, that measurement of eNO holds promise as a clinically useful tool in confirming a diagnosis of asthma, more so than induced sputum examination in the community, and larger epidemiological and randomised controlled studies are

required to explore this further. Increasingly as more simplified and economical eNO analysers become available, they may provide a viable alternative to spirometry with BDR to validate a clinical diagnosis of asthma in the adult community.

How then should inflammatory markers and HSAHR be utilised in the community when the traditional definition of asthma based on episodic symptoms and variable airflow obstruction appears to be robust? This depends on the purpose of the test. The results from this study suggest that HSAHR, eNO and induced sputum examination cannot be used independently to screen for, or to diagnose, asthma. Rather, they should be used to confirm a clinical suspicion of asthma based on symptoms .Unfortunately, our current findings suggest that HSAHR may not have a discriminative role in the diagnosis of asthma in the adult community. If a global assessment of the underlying AI is required, an eNO measurement is recommended initially, given its ease of use and specificity for the diagnosis of asthma both by self report, ESBDR and ESAHR. The strength of sputum examination lies in delineating the subtype of AI present which may be important in predicting therapy(31). This study reinforces the heterogeneity of asthma and the absence of a gold standard definition or test for asthma. Taken together, and with the increasing body of evidence suggesting non eosinophilic asthma is not responsive to corticosteroids(152), these findings endorse a multi-faceted tailored approach to asthma management that includes both an assessment of the airway inflammatory subtype and the clinical and physiological features of an individual.

Figure 4.1: Study Profile

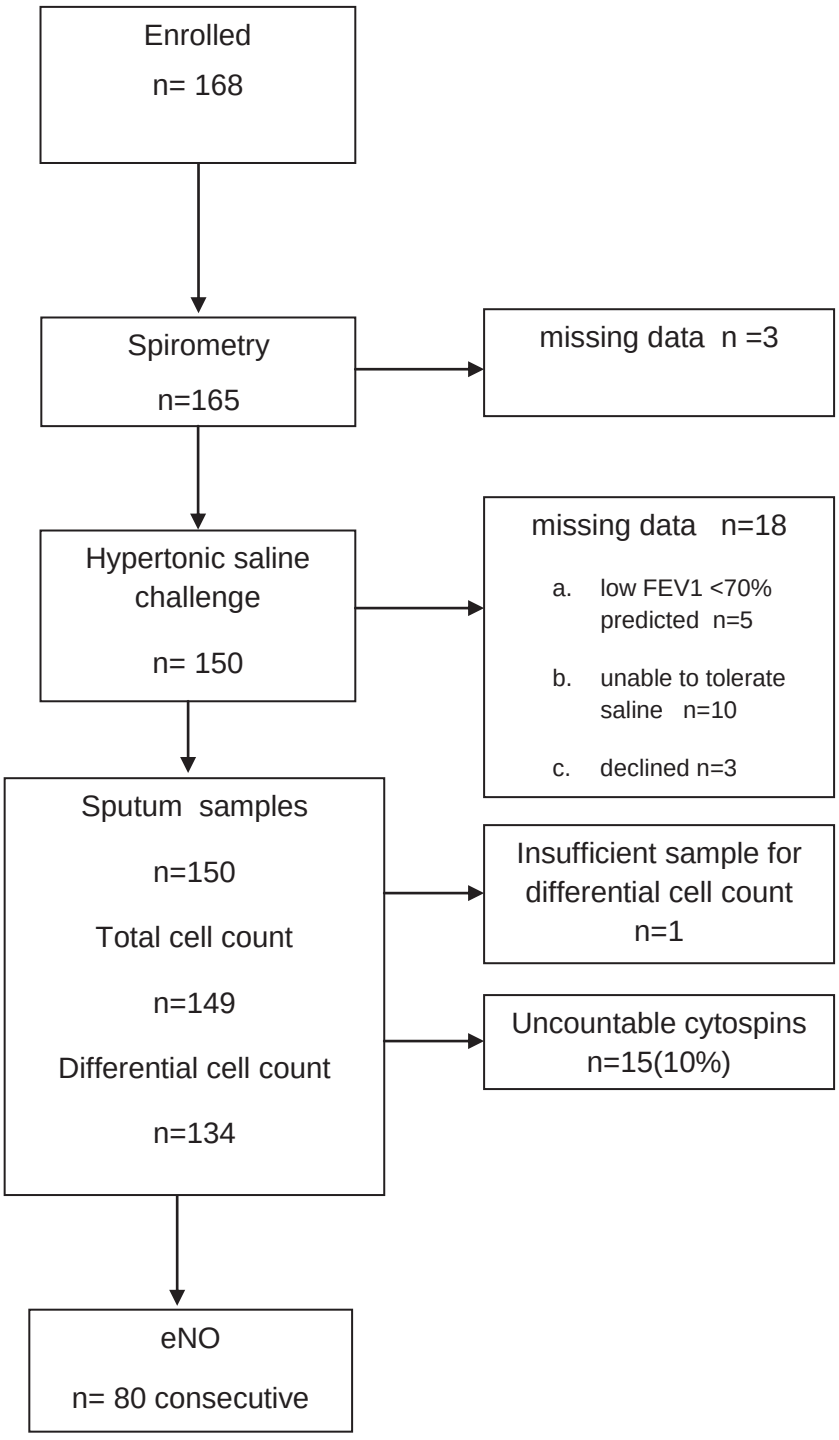


Table 4.1: Subject characteristics

	SRDD asthma	no SRDD asthma	p value
n= 168	77	91	
Male, n	27	47	0.02
Age, years	54 (14)	58 (13)	0.05
Atopy (one or more positive) ,n	53	49	0.04
Inhaled steroid, n	34	0	< 0.001
Long acting beta 2 agonist, n	18	0	<0.001
Other medication, n	3	0	0.27
Current smokers	12	12	0.7
Ex smokers	30	27	0.2
Asthma severity,* n			
Intermittent	40		
Mild persistent	2		
Moderate	21		
Severe	13		
Missing data	1		
Pre BDFEV_1, L	2.6 (0.8)	3.0 (0.9)	0.008
Pre BD FEV_1 % predicted	91(15)	102 (17)	< 0.001
Bronchodilator reversibility ,%	7.8 (8.3)	3.5 (3.2)	0.00
PD_{15} mls geometric mean (SD)	10 (4.4)	25 (3.1)	0.001

SRRD: self reported doctor diagnosis of asthma;n:number; mean (standard deviation (SD));* based on NAC(2006) criteria; FEV_1 : forced expiratory volume in 1 second; BD: bronchodilator; PD_{15}: provocation dose resulting in a 15% fall in FEV_1 from baseline;L : litres; mls: milliltres; %percent;< less than.

Table 4.2: Frequency of AI based on induced sputum examination (n=134) and eNO levels (n=80) in subjects with and without SRDD asthma. Subjects not on ICS*.

Test	SRDD asthma %, (n)	no SRDD asthma %, (n)	p value
Eosinophils>1%	28% (17/60) 20% (7/35)*	32% (24/74)	0.61 0.18*
Eosinophils>2%	18.3% (11/60) 14 % (5/35)*	20.1% (15/74)	0.78 0.45*
Neutrophils>61%	11.7% (7/60) 11.4% (4/35)*	5.4% (4/74)	0.19 0.26*
Paucigranulocytic Eo≤1 and Neuts≤61%	67% (40/60) 74% (26/35)*	63.5% (47/74)	0.70 0.26*
eNO≥19ppb	43.4% (10/23) 62% (8/13)*	40.1% (23/57)	0.80 0.16*
eNO≥ 30ppb	17.4%(4/23) 23% (3/13)*	7% (4/57)	0.16 0.08*

SRRD: self reported doctor diagnosis of asthma; Eo : eosinophil count ; Neuts ; neutrophil count; eNO:exhaled nitric oxide; ppb: parts per billion;%:percent; (n) number of subjects

Table 4.3: The relationship between the presence and frequency of wheeze in last 3 months and SRDD asthma.

Frequency of wheeze in last 3 months	SRDD asthma n=77	no SRDD asthma n=91
Nil	24	56
Occasionally	42	25
Most days	9	2
Other	2	8

SRRD: self reported doctor diagnosis of asthma; n:number

Table 4.4: Sensitivity, specificity, positive and negative predictive value for bronchodilator reversibility in SRDD asthma , presence or absence of wheeze in last 3 months and ESAHR.

	SRDD asthma n	no SRDD asthma n		
			Sensitivity	20.7% (12-32)
Reversibility +	16	0	Specificity	100% (96-100)
Reversibility -	61	88	PPV	100% (79-100)
	77	88	NPV	59% (51-67)

	wheeze n	no wheeze n		
			Sensitivity	14% (7-23)
Reversibility +	12	4	Specificity	95% (88-99)
Reversibility -	74	75	PPV	75% (48-93)
	86	79	NPV	51% (42-58)

	ESAHR asthma n	No ESAHR asthma n		
			Sensitivity	12% (6-21)
Reversibility +	11	1	Specificity	96% (90-100)
Reversibility -	78	54	PPV	92% (62-100)
	89	55	NPV	41% (33-49)

SRRD: self reported doctor diagnosis of asthma; ESAHR: episodic symptoms and airway hyper-responsiveness ; PPV: positive predictive value; NPV: negative predictive value; n: number of subjects; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

Table 4.5: Sensitivity, specificity, positive and negative predictive value for HSAHR in asthma defined by SRDD and presence or absence of wheeze in last 3 months. (Subjects not on ICS)

	SRDD asthma n	no SRDD asthma n		
HS AHR+	46 (22)	48 (48)	Sensitivity	73(63)% (60-83)
HS AHR -	17 (13)	39 (39)	Specificity	45% (34-56)
	63 (35)	87 (87)	PPV	49(31)% (39- 60)
			NPV	70(75)% (56-81)

	wheeze n	no wheeze n		
HS AHR+	51 (31)	43 (39)	Sensitivity	66(57)% (55-77)
HS AHR -	26 (23)	30 (29)	Specificity	41(43)% (47-70)
	77(54)	73 (68)	PPV	54(44)% (44-65)
			NPV	54(56)% (40-67)

SRRD: self reported doctor diagnosis of asthma; HSAHR: hypertonic saline airway hyper-responsiveness ; PPV: positive predictive value; NPV: negative predictive value; n: number of subjects ; ICS : inhaled corticosteroids. Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval). Confidence intervals calculated for **all subjects only**.

Table 4.6: Sensitivity, specificity, positive and negative predictive value for eNO for asthma defined by (a) SRDD, (b) presence or absence of wheeze in last 3 months, (c) ESBDR and (d) ESAHR.(Subjects not on ICS).

(a) SRDD asthma

	SRDD asthma n	no SRDD asthma n	
eNO ≥19ppb	10 (8)	23 (23)	Sensitivity 43.5(62)% (23-65)
eNO < 19 ppb	13 (5)	34 (34)	Specificity 60% (28-54)
	23 (13)	57 (57)	PPV 30(26)% (16-49)
			NPV 72(87)% (58-84)

	SRDD asthma n	no SRDD asthma n	
eNO ≥ 30ppb	4 (3)	4(4)	Sensitivity 17(23)% (5-39)
eNO < 30ppb	19 (10)	53(53)	Specificity 93% (83-98)
	23 (13)	57(57)	PPV 50(43)% (16-84)
			NPV 74(84)% (62-83)

Total number of subjects with exhaled nitric oxide readings performed in this thesis: n=80;eNO: exhaled nitric oxide; ppb: parts per billion; % : percent; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

(b) Presence or absence of wheeze in last 3 months (Subjects not on ICS).

	wheeze n	no wheeze n		
eNO ≥19ppb	14 (13)	19 (18)	Sensitivity	44(52)% (26-62)
eNO < 19 ppb	18 (12)	29 (27)	Specificity	60(69)% (45-74)
	32 (25)	48 (45)	PPV	42% (26-60)
			NPV	61(69)% (46-76)

	wheeze n	no wheeze n		
eNO ≥ 30ppb	1	7 (6)	Sensitivity	3(4)% (0.08-16)
eNO < 30 ppb	31 (24)	41 (39)	Specificity	85(87)% (72-94)
	32 (25)	48 (45)	PPV	13(14)% (0.3-53)
			NPV	57(62)% (15-69)

eNO: exhaled nitric oxide; ppb:parts per billion; % : percent; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

(c) ESBDR asthma (Subjects not on ICS).

	ESBDR asthma n	no ESBDR asthma n		
eNO ≥19ppb	3 (2)	30 (29)	Sensitivity	75(100)% (19-99)
eNO < 19 ppb	1(0)	44 (38)	Specificity	59(57)% (47-71)
	4(2)	74 (67)	PPV	9 (6)% (2-24)
			NPV	98(100)% 88-100)

	ESBDR asthma n	no ESBDR asthma n		
eNO ≥30ppb	1	7 (6)	Sensitivity	25(50)% (0.6-81)
eNO < 30 ppb	3(1)	67 (61)	Specificity	91(91)% (4-19)
	4(2)	74 (67)	PPV	13(14)% (0.3-53)
			NPV	96(98)% (82-96)

ESBDR: episodic symptoms and bronchodilator reversibility; eNO: exhaled nitric oxide; ppb:parts per billion; % : percent; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

(d) ESAHR asthma (Subjects not on ICS).

	ESAHR asthma n	no ESAHR asthma n		
eNO ≥19ppb	20 (19)	10 (10)	Sensitivity	45(53)% (30-61)
eNO < 19 ppb	24 (17)	18 (18)	Specificity	64% (19-56)
	44 (36)	28 (28)	PPV	66% (47-83)
			NPV	43(51)% (24-54)

	ESAHR asthma n	no ESAHR asthma n		
eNO ≥ 30ppb	7 (6)	1 (1)	Sensitivity	16(17)% (7-30)
eNO < 30 ppb	37 (30)	27 (27)	Specificity	96% (82-100)
	44 (36)	28 (28)	PPV	88(75)% (47-98)
			NPV	42% (30-55)

ESAHR: episodic symptoms and airway hyper-responsiveness; eNO: exhaled nitric oxide; ppb: parts per billion; % : percent; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

Table 4.7: Sensitivity, specificity, positive and negative predictive value for eNO for HSAHR alone.(Subjects not on ICS).

	HSAHR n	no HSAHR n		
eNO ≥19ppb	21 (20)	10 (10)	Sensitivity	45(51)% (30-60)
eNO < 19 ppb	26 (19)	17 (17)	Specificity	63% (42-81)
	47 (39)	27 (27)	PPV	68(67)% (49-83)
			NPV	40(47)% (25-56)

	HSAHR n	no HSAHR n		
eNO ≥ 30ppb	7 (6)	1(1)	Sensitivity	15(13)% (6-28)
eNO < 30 ppb	40 (33)	26 (26)	Specificity	96% (81-100)
	47 (39)	27 (27)	PPV	88(86)% (47-98)
			NPV	39(44)% (28-52)

HSAHR: hypertonic saline airway hyper-responsiveness ; eNO:exhaled nitric oxide; PPV: positive predictive value; NPV: negative predictive value ; n: number of subjects ; %: percent; ICS : inhaled corticosteroids. Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

Table 4.8: Sensitivity, specificity, positive and negative predictive value for sputum eosinophilia in asthma defined by (a) SRDD, (b) presence or absence of wheeze in last 3 months, (c) ESBDR and (d) ESAHR. (Subjects not on ICS).

(a) SRDD asthma

	SRDD asthma n	no SRDD asthma n	
Eo >1%	17 (7)	24 (24)	Sensitivity 28(20)% (17-41)
Eo ≤1%	43 (28)	50 (50)	Specificity 68(68)% (22-44)
	60 (35)	74 (74)	PPV 41(23)% (26-58)
			NPV 54(64)% (43-64)

	SRDD asthma n	no SRDD asthma n	
Eo>2%	11 (5)	15 (15)	Sensitivity 18(14)% (10-30)
Eo≤2%	49 (30)	59 (59)	Specificity 80% (69-88)
	60 (35)	74 (74)	PPV 42(25)% (23-63)
			NPV 55(66)% (45-64)

SRRD: self reported doctor diagnosis of asthma; Eo: eosinophil count; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

(b) presence or absence of wheeze in last 3 months (Subjects not on ICS).

	wheeze n	no wheeze n		
Eo >1%	18 (9)	23 (22)	Sensitivity	25(18)% (16-37)
Eo ≤1%	53 (40)	40 (38)	Specificity	63% (50-75)
	71 (49)	63 (60)	PPV	44(29)% (29-60)
			NPV	43(48)% (33-54)

	wheeze n	no wheeze n		
Eo >2%	12 (6)	14 (14)	Sensitivity	17 (12)% (10-28)
Eo ≤2%	59 (43)	49 (46)	Specificity	78 (77)% (70-91)
	71 (49)	63 (60)	PPV	46 (30)% (27-67)
			NPV	45(52)% (36-55)

Eo: eosinophil count; Sensitivity, specificity, PPV, NPV expressed as percentage

(95% confidence interval).

(c) ESBDR asthma. (Subjects not on ICS).

	ESBDR asthma n	no ESBDR asthma n		
Eo>1%	5(1)	36(30)	Sensitivity	36(14)% (13-65)
Eo≤1%	9(6)	82(71)	Specificity	70(70)% (62-78)
	14(7)	118(101)	PPV	12(3)% (4-26)
			NPV	90(92)% (82-95)

	ESBDR asthma n	no ESBDR asthma n		
Eo>2%	3(0)	23 (20)	Sensitivity	21(#)% (5-51)
Eo≤2%	11(7)	95 (81)	Specificity	81(80)% (73-88)
	14(7)	118 (101)	PPV	12(#)% (3-30)
			NPV	90(92)% (84-95)

ESBDR: episodic symptoms and bronchodilator reversibility; Eo: eosinophil count; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval); #: unable to calculate.

(d) ESAHR asthma. (Subjects not on ICS).

	ESAHR asthma n	no ESAHR asthma n		
Eo>1%	23(17)	17 (14)	Sensitivity	29% (20-41)
Eo≤1%	55 (41)	27 (27)	Specificity	61(66)% (46-76)
	78 (58)	44 (41)	PPV	58(55)% (41-73)
			NPV	33(40)% (23-44)

	ESAHR asthma n	no ESAHR asthma n		
Eo>2%	17 (13)	8 (7)	Sensitivity	22% (13-33)
Eo≤2%	61 (45)	36 (34)	Specificity	82(83)% (67-92)
	78 (58)	44 (41)	PPV	68(65)% (47-85)
			NPV	37(43)% (28-48)

ESAHR: episodic symptoms and airway hyper-responsiveness Eo: eosinophil count; Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

Table 4.9: Sensitivity, specificity, positive and negative predictive value for sputum eosinophilia for HSAHR alone. (Subjects not on ICS).

	HSAHR n	no HSAHR n		
Eo>1%	24 (17)	15 (13)	Sensitivity	29(28)% (20-40)
Eo≤1%	68 (44)	28 (27)	Specificity	65(68)% (49-79)
	82 (61)	43 (40)	PPV	62(57)% (45-77)
			NPV	33(38)% (20-39)

	HSAHR	no HSAHR		
Eo>2%	18 (13)	6 (6)	Sensitivity	22(21)% (14-33)
Eo≤2%	64 (48)	37 (34)	Specificity	86(85)% (77-98)
	82 (61)	43 (40)	PPV	75(68)% (53-90)
			NPV	37(41)% (27-46)

HSAHR: hypertonic saline airway hyper-responsiveness ; Eo: eosinophil count; PPV: positive predictive value; NPV: negative predictive value ; n: number of subjects; % : percent; ICS: inhaled corticosteroids. Sensitivity, specificity, PPV, NPV expressed as percentage (95% confidence interval).

Table 4.10: Associations between asthma defined by (a) SRDD and presence or absence of wheeze in last 3 months and physiological and inflammatory markers expressed as Odds ratio (OR) (95% Confidence Interval (CI)); Subjects not on ICS.

	SRDD asthma		p value	
	OR on ICS	OR not on ICS	ICS	no ICS
Wheeze	3.5 (1.9-6.7)	2.0 (0.97-4.2)	0.00	0.06
BDR	22 (3.9-119)	20 (2.4-166)	0.003	0.005
HSAHR	2.3 (1.1-4.6)	1.5 (0.66-3.2)	0.02	0.35
Eosinophilia>1%	0.8 (0.39-1.7)	0.52 (0.2-1.3)	0.6	0.2
Eosinophilia>2%	0.9(0.37-2.1)	0.7 (0.2-1.9)	0.8	0.5
eNO ≥19ppb	1.1(0.42-3.0)	2.4 (0.71-7.8)	0.8	0.16
eNO≥30ppb	2.8(0.6-12.3)	4.0 (0.9-18.6)	0.18	0.08
	Wheeze in last 3 months			
BDR	3.0 (0.94- 9.9)	1.7 (0.42-6.4)	0.06	0.47
HSAHR	1.4 (0.7-2.7)	1.0 (0.49-2.1)	0.35	0.99
Eosinophilia>1%	0.6(0.28-1.2)	0.38 (0.16-0.95)	0.16	0.04
Eosinophilia>2%	0.7 (0.3-1.7)	0.46 (0.2-1.3)	0.44	0.14
eNO ≥19ppb	1.2 (0.5-2.9)	1.6 (0.6- 4.3)	0.71	0.33
eNO≥30ppb	0.2(0.02-1.6)	0.3 (0.03- 2.4)	0.13	0.2

SRRD: self reported doctor diagnosis of asthma; BDR: bronchodilator reversibility; ESBDR: episodic symptoms and bronchodilator reversibility; ESAHR: episodic symptoms and airway hyper-responsiveness; HSAHR: hypertonic saline airway hyper-responsiveness ; Eo: eosinophil count; eNO:exhaled nitric oxide; ICS: inhaled corticosteroids.

Associations between asthma defined by (b) ESBDR and ESAHR and physiological and inflammatory makers expressed as Odds ratio (OR) (95% Confidence Interval(CI)); Subjects not on ICS .

	ESBDR		p value	
	OR on ICS	OR not on ICS	ICS	no ICS
Eosinophilia>1%	1.3(0.4-4.0)	0.4(0.05-3.4)	0.69	0.40
Eosinophilia>2%	1.1 (0.29-4.4)	#	0.86	#
eNO ≥19ppb	4.4 (0.44-4.4)	#	0.21	#
eNO≥30ppb	3.2(0.29-34.9)	10.7(0.56-184)	0.34	0.12
	ESAHR			
BDR	3.5 (1.0 -13)	2.1(0.5- 8.8)	0.05	0.30
Eosinophilia>1%	0.9(0.4-2.0)	1.1(0.5- 2.5)	0.86	0.83
Eosinophilia>2%	1.7 (0.7-4.2)	1.8 (0. 7- 5.0)	0.27	0.25
eNO ≥19ppb	1.5 (0.6-3.8)	2.2 (0.8- 5.4)	0.35	0.14
eNO≥30ppb	6.0 (0.7-50)	6.6 (0.8 -58)	0.09	0.08

SRRD: self reported doctor diagnosis of asthma; BDR: bronchodilator reversibility; ESBDR: episodic symptoms and bronchodilator reversibility; ESAHR: episodic symptoms and airway hyper-responsiveness; HSAHR: hypertonic saline airway hyper-responsiveness ; Eo: eosinophil count; eNO:exhaled nitric oxide;# unable to calculate due to small sample; ICS: inhaled corticosteroids.

Table 4.11. Demonstrates the additional number of subjects identified if asthma is defined by AI (raised eosinophil count and/or raised eNO) plus SRDD in the 168 particpants.

Additional number of subjects identified over and above SRDD asthma		
	on ICS	not on ICS
eNO≥19ppb	23/80 (29%)	23/70 (33%)
eNO≥ 30ppb	4/80 (5%)	4/70 (5.7%)
eosinophil>1%	24/134 (18%)	24/109 (22%)
eosinophil>2%	15/134 (11%)	15/109 (14%)
eNO≥19ppb + eosinophil>1%	3/119 (2.5%)	3/100 (3%)
eNO≥19ppb + eosinophil>2%	2/126 (1.6%)	2/104 (1.9%)
eNO≥ 30ppb + eosinophil>1%	1/118 (0.8%)	1/101 (1%)
eNO≥ 30ppb + eosinophil>2%	1/133 (0.8%)	1/111 (0.9%)

Eo: eosinophil count; eNO:exhaled nitric oxide; SRRD: self reported doctor diagnosis of asthma; HSAHR: hypertonic saline airway hyper-responsiveness ; ICS: inhaled corticosteroids; %;percent; ICS: inhaled corticosteroids.

Table 4.12. Demonstrates the number of subjects with and without asthma depending on the definition used (SRDD, ESBDR or ESAHR), when compared to the "global definition" used in real life which defines asthma in all of the above ways (n=163) .

Definition	Asthma	no asthma	Total
SRDD or ESBDR or ESAHR	**114**	**49**	**163**
SRDD	77	86	163
ESBDR	16	144	160*
ESAHR	89	55	144

Total enrolled =168: 6 subjects had insufficient data to classify them clearly into asthma or no asthma groups based on ESAHR result and were removed from the analysis, therefore total n=163; * BDR data unavailable n=3; SRRD: self reported doctor diagnosis of asthma; ESBDR: episodic symptoms and bronchodilator reversibility; ESAHR: episodic symptoms and airway hyper-responsiveness .

CHAPTER 5

Perception of breathlessness associated with airway hyper-responsiveness: its relationship with clinical, inflammatory, psychosocial and economic variables

5.0 Introduction

While asthma is characterised by episodic symptoms of breathlessness (or dyspnoea), chest tightness and wheeze, perception of these symptoms remains subjective. Perception of breathlessness (POD), varies widely amongst individuals with asthma and does not always correlate with objective tests of airway obstruction. Some experience significant breathlessness with mild airway obstruction while others complain of minimal or no breathlessness with severe airway obstruction. Rubinfeld and Pain have demonstrated that while worsening airway function is associated with an increased perception of dyspnoea as a protective mechanism(240), up to 15% of subjects may under perceive their asthma(170). The reasons for impaired POD in asthma are multi-factorial and not fully explained to date(173). They are discussed in Chapter 2. Poor perception of dyspnoea may lead to under treatment (53) and is a clear predictor of severe or near fatal asthma (216, 241, 242). Thus it is important to identify those individuals, or characteristics, that are associated with blunted POD and to ensure safety measures with objective testing such as regular peak expiratory flow monitoring or spirometry are in place.

Perception of breathlessness is assessed by scales that are reliable, repeatable and valid. These include the Visual Analogue Scale, modified BORG scale, Baseline Dyspnoea Index (BDI), Asthma related quality of life (HRQOL) and Medical Research Council (MRC) dyspnoea scale. These scales correlate with each other significantly ($r=0.77$ to 0.85; $p<0.05$)(243) and with physiological variables of lung function(243).

In this study we examine firstly, the factors that might affect POD in a community sample of subjects with asthma defined by episodic symptoms and AHR (ESAHR), and secondly, the relationship between POD and AI evaluated by induced sputum examination and eNO.

5.1 Materials and methods

Study Design

This was a cross-sectional population based study.

Subjects

Eighty nine subjects with asthma defined by episodic symptoms and a positive test of airway hyper-responsiveness(ESAHR), characterised by a fall in FEV_1 of $\geq$15% from baseline to hypertonic saline, were enrolled in this study. They were assessed at a single visit with a clinical questionnaire, a quality of life questionnaire (SF 36), height and weight measurements to calculate body mass index (BMI), spirometry and a hypertonic saline challenge test with a visual analogue scale at the beginning and at the end of the challenge to assess perception of dyspnoea (POD). A sample of sputum was induced concurrently with the hypertonic saline challenge test.

Methods

The materials and methods used in this study are described in Chapter 3.

5.2 Statistical analysis

Descriptive statistics were used to summarise the clinical characteristics of the participants. Normally distributed data were expressed using arithmetic mean and standard deviations (SD) and non-normal distributed data (sputum total cell count and eosinophil count) as median and interquartile range (IQR) or range. Correlations were expressed with the Pearson's correlation coefficient for normally distributed data, and the Spearman's correlation coefficient for data that was not normally distributed. Differences between the 3 groups (hypoperceivers, normal perceivers and hyperperceivers) were determined using an analysis of variance (ANOVA) for normally distributed and log transformed data where possible, or the Kruskal Wallis test for data that was not normally distributed. The relationship between body mass index (BMI) and inflammatory markers was explored by using BMI as a covariate in the ANOVA analysis. Categorical data were analysed using a Chi square test. Significance was accepted at a p of $\leq$ 0.05. Statistical analysis was performed with SPSS Windows ® Version 10.0 (SPSS Inc., Chicago, IL).

5.3 Results

Subject demographics are outlined in Table 5.1. Subjects had a mean(SD) age of 54(14) years and a mean(SD) FEV_1 of 91(13) % predicted. Most were atopic, and had mild to moderate severity asthma based on National Asthma Council of Australia guidelines(8). Twenty seven percent were on inhaled corticosteroids with a mean (SD) fluticasone dose of 574 (314) micrograms per day.

Visual analogue scale scores were normally distributed in this population (Figure 5.1). Perception of dyspnoea correlated positively with asthma severity, fall in FEV_1 and BMI and inversely with airway hyper- responsiveness as measured by PD_{15} (i.e. greater the airway responsiveness, greater the POD) (Table 5.2). It also correlated strongly with a doctor diagnosis of depression, reduced social contact and emotional health as measured by the SF 36 Quality of Life Questionnaire and poor income (Table 5.2). A significant relationship was not noted, in this cohort, between POD and markers of airway inflammation.

Subjects were then classified into hypoperceivers, hyperperceivers and normal perceivers. Subject characteristics are described in Table 5.3(a). Age, gender distribution and BMI were similar among hypo, hyper, and normal perceivers. All had similar baseline FEV_1, percent predicted. Normal perceivers and hyperperceivers had a similar degree of airway responsiveness (PD_{15}) with hypertonic saline. Hypoperceivers, in contrast, had significantly less airway responsiveness (PD_{15}) compared with the other 2 groups. Average daily inhaled corticosteroid dose was higher in hyperperceivers than normal or hypoperceivers ($p=0.07$). Hyperperceivers had significantly lower social functioning scores based on the SF-36 QOL questionnaire (mean(SD):68(32) versus 87(20) in normals and 86(21) hypoperceivers; $p=0.03$. They also had a higher than expected number of subjects diagnosed with depression compared with the 2 other groups(Table 5.3 (a)). Sputum cell counts, apart from columnar epithelial cells, and eNO measurements were similar amongst all 3 groups (Table 5.3(b)), and remained similar amongst groups when adjusted for BMI as a covariate. Columnar cells were significantly higher in hyperperceivers compared with normal perceivers.

5.4 Discussion

This study has demonstrated that in a community setting, factors associated with increased POD during induced bronchoconstriction in subjects with ESAHR asthma include underlying asthma severity, the magnitude of fall in FEV_1 during the challenge, degree of hyper responsiveness (PD_{15}), increased BMI, doctor diagnosed depression, reduced social functioning and emotional health and poor household income.

Furthermore, when subjects were divided into hypoperceivers, hyperperceivers and normal perceivers, all groups were similar for age, gender, and baseline FEV_1 similar to Boulet's study(53). Normal perceivers were the predominant group in this study, contributing to 77% of the study population. Fifteen percent overestimated the severity of bronchoconstriction (hyperperceivers) while eight percent of subjects underestimated the severity of bronchoconstriction (hypoperceivers).These findings are echoed in hospital based studies (53, 216) and in another community based study where a histamine challenge was performed in 412 randomly selected subjects (244) and most subjects with dyspnoea and airway hyper-responsiveness perceived increasing airway narrowing accurately. The results also confirm more worrying results from earlier studies suggesting that up to 15% of subjects from the community under- perceive dyspnoea associated with their asthma(170), and that 13% to 30%(216, 217) of subjects have reduced POD to induced bronchoconstriction in asthma. Hyperperceivers used higher doses of ICS compared with normal perceivers (mean (SD):780(303) vs 490(350) μg/day; p=0.07) for the same degree of fall in FEV_1 during the bronchial challenge. Hypoperceivers, disturbingly, were not on any ICS. This may, in part, reflect the milder nature of their asthma in addition to poor symptom perception, compared with hyperperceivers, of whom almost 50% had more severe asthma based on NAC (2006) guidelines (Table 5.3(a)). There is also a possibility

that poor perception of symptoms feeds into poor decision making with respect to medication use in the absence of other markers of asthma control such as spirometry or eNO.

This study has demonstrated that psychosocial and economic factors, namely lower household income, depression and conceivably reduced social contact are associated with an increased POD in subjects with ESAHR. The latter two associations are most strongly noted in the hyperperceiver group. Martinez-Moragon and colleagues demonstrated similar results in 153 subjects recruited from outpatient clinics with asthma defined by spirometric criteria (243). Subjects were asked to grade their functional level of dyspnoea using 3 different scales (modified Borg, BDI and MRC dypnoea scales), and to complete a demographic and clinical questionnaire. Multiple regression analysis demonstrated that increasing age, reduced FEV_1 and depression were independent factors in determining dyspnoea scores. In addition to these factors, poorer quality of life, lower economic status and lower educational levels also correlated significantly with dyspnoea(243). These studies extend previous information highlighting the importance of social and economic factors in the development and persistence of asthma (245). Postulated mechanisms for the association between psychosocial and economic factors and increased POD include anxiety, stress, and poorer compliance with optimum management and treatment plans for a variety of reasons; all of which require further evaluation.

A relationship between inflammatory markers measured by sputum cell counts and eNO and POD was not demonstrated in our subjects. This differs from previous studies(42, 43, 172), and may reflect subject selection from a heterogeneous community and the possibility of an insufficiently large sample size to detect significant associations in subjects with mild to moderate, stable asthma. They are, however, supported by the studies of Salome and colleagues who found no correlation between POD

and sputum or blood eosinophils, or eNO in subjects with asthma on or off budesonide, during a controlled steroid withdrawal trial and induced exacerbation(246). The significance of the increased columnar cells in hyperperceivers is of questionable clinical significance, and may be due to chance alone, as the remaining inflammatory variables were similar in all groups. A possible explanation could be the fragility of airway epithelium in undertreated asthma. This raises the possibility that sputum columnar cells could be a marker of poorly controlled asthma or undertreated asthma. This aspect of sputum cellular content needs to be further examined, and if verified, could be a useful marker in identifying a subset of patients with asthma.

It is uncertain if hypertonic saline, the agent chosen to induce bronchoconstriction in this study, may have affected the POD. Previous studies have demonstrated that other bronchoconstricting agents such as histamine, methacholine, sodium metabisulfite, bradykinin and adenosine phosphate can influence POD (172, 247, 248). For example, Tetzalf and colleagues found that significantly more symptoms and a higher degree of symptom intensity were reported with histamine compared with methacholine(248).

A correlation between BMI and POD was demonstrated in this study, similar to a previous study where BMI was a significant predictor of dyspnoea(41). A relationship was, however, not shown between BMI and eNO or sputum cell counts. These latter findings lend support to the studies by Todd and colleagues(249), van Veen and colleagues (250) Sutherland and colleagues (251) and others (252)demonstrating no association between BMI and airway inflammation assessed by sputum cell counts and eNO in subjects with asthma.

The strength of the relationship between the clinical and inflammatory markers and POD outlined in Table 5.2 is weaker than that noted in some studies (42, 43). Int'Veen and colleagues, for example, demonstrated a correlation(r) between POD (measured by VAS) and sputum eosinophils in severe asthma of r=-0.37, p=0.049, and in mild asthma r=0.67, p=0.05(42). Jang and colleagues demonstrated a correlation between POD and FEV_1/FVC ratio of -0.53, p<0.01, and POD and sputum eosinophils of r=0.7, p=0.01(43). The findings in our study are similar to others (41) (53) and again are likely reflect the unselected, heterogeneous nature of the study population.

Limitations of this study include the cross-sectional rather than longitudinal design, which if performed, would have added statistical power to our findings. A longitudinal study would have permitted an assessment of the constancy or repeatability of POD in asthmatics over time, an area which requires more research. Martinez- Moraagon and colleagues recently demonstrated that in 22 stable subjects with asthma, classified as hyperperceivers in a study 9 years earlier, repeating the histamine challenge, assessing their level of anxiety, and POD using the Borg scale, that most hyperperceivers (64%) remained unchanged in their POD(253). In addition POD was related to anxiety. The poor response rate, 20% for those diagnosed with asthma, from the NWAHS study, could have also resulted in selection bias, possibly limiting the generalisability of these results to the wider community. A larger sample size in future would allow a more comprehensive assessment of factors contributing to POD in subjects on and off corticosteroids, and in those subjects classified as hypo and hyper perceivers.

In conclusion, this study has demonstrated that in a community based sample of subjects with ESAHR, POD alone is related to physiological, socioeconomic and psychological variables. Airway

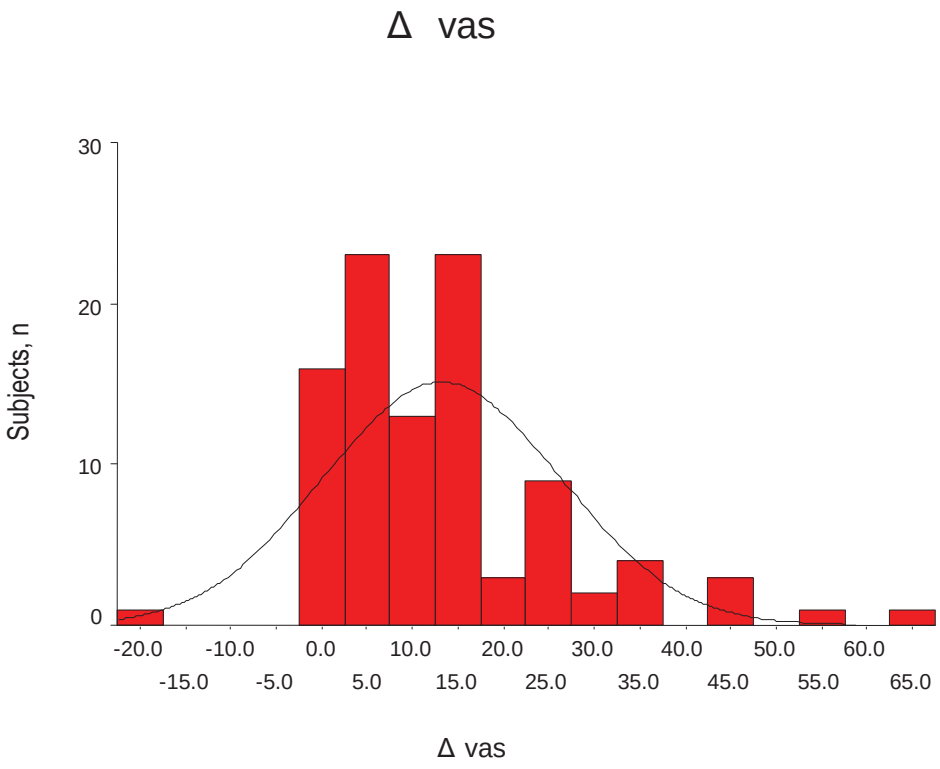
inflammation measured by eNO or sputum cell counts was not a contributing factor. Of concern, this study confirms that a percentage of subjects with asthma under perceive the severity of their airway hyper-responsiveness, suggesting that measures need to be in place in the community to detect and monitor poor perceivers. A significant proportion of subjects also over perceive their symptoms and may require special attention to minimise excessive medication use. From a practical perspective, raising the awareness of the characteristics associated with breathlessness in asthma and in under and over perceivers, may enable medical practitioners to better manage their patients' health.

Table 5.1: Baseline characteristics subjects with asthma defined by ESAHR .

Subjects , n	89
Male, n	41
Age, years	54(14)
Atopy (one or more positive),n	56
Inhaled steroid, n Inhaled steroid fluticasone equivalent, µg/day	24 574 (314)
Long acting beta 2 agonist, n	18
Current smokers Ex smokers	14 26
Pre BDFEV_1, L Pre BD FEV_1 % predicted	2.8 (0.75) 91 (13)
PD_{15} mls geometric mean (SD)	8.7 (3.0)
Asthma severity #, n Intermittent Mild persistent Moderate Severe	59 2 21 5 (2 unable to categorise)

Mean (SD);# National Australian Asthma Council (2006)guidelines. ESAHR: episodic symptoms and airway hyper-responsiveness; n: number of subjects; FEV_1: forced expiratory volume in 1 second; L litres; % : percentage;PD_{15}: provocation dose resulting in a 15% fall from baseline; SD: standard deviation; µg : micrograms

Figure 5.1: Distribution of Δ VAS readings for subjects (n=89) at 15% fall in FEV_1 (PD_{15}) with hypertonic saline challenge test.



Mean (SD) Δ VAS: 13.5(13.3)

n: number of subjects; Δ VAS: change in Visual Analogue Score from baseline ; FEV_1: forced expiratory volume in 1 second; PD_{15}: provocation dose resulting in a 15% fall in FEV_1 from baseline.

Table 5.2: Correlation(r) between VAS, clinical, psychosocial and economic and inflammatory markers

Clinical	Δ VAS	
	r	n
Asthma severity	0.21,	87*
Fall in FEV_1, %	0.34,	89**
PD_{15}	-0.21,	89*
BMI	0.21,	87*
Psychosocial and Economic		
Doctor diagnosed depression	0.21	85*
SF-36 social functioning	-0.24,	88*
SF-36 role -emotional	-0.22	88*
Annual gross household income	-0.21,	88*
Inflammatory		
Total Cell Count x 10^6/mL	0.05,	83
Eosinophil,%	0.13,	78
Neutrophil,%	-0.13	78
Macrophage,%	0.09	83
Lymphocyte,%	0.01	78
eNO	-0.07,	44

*p $\leq$ 0.05; ** p$\leq$ 0.01; r: correlation coefficient; n: number of subjects; Δ VAS : change in Visual Analogue Score from baseline ; FEV_1: forced expiratory volume in 1 second; PD_{15}: provocation dose resulting in a 15% fall in FEV_1 from baseline ;

BMI: body mass index; SF-36: generic Quality of Life Questionnaire ;

eNO: exhaled nitric oxide;%: percentage

Table 5.3: (a) Subject characteristics and (b) inflammatory markers in hypoperceivers, normal perceivers and hyperperceivers with ESAHR asthma.

***(a)* Subject characteristics**

	Hypoperceivers n=7	Normal perceivers n=69	Hyperperceivers n=13	p value
Age	50 (18)	56 (13)	49 (12)	0.12
Gender, male	15	44	5	0.34
BMI	27(5.6)	28 (5)	30 (6.1)	0.31
Atopy,n (≥1 positive)	2	43	11	0.35
Smoking history , pack years	13 (22)	9 (14)	10 (13)	0.78
Doctor diagnosed depression	1* (0.8)**	4* (7.6)**	5* (1.5)**	0.04
SF-36 social functioning SF-36 role -emotional	86 (21) 100 (0)	87(20) 87(30)	68(32) 74 (41)	0.03 ‡ 0.19
ICS , n	0	15	9	0.002
ICS, µcg/day fluticasone equivalent*	0 (0)	490(350)	780(303)	0.07
LABA , n	2	11	5	0.02
FEV_1,L	2.9 (0.8)	2.8 (0.75)	2.8(0.78)	0.81
FEV_1, % predicted	91(3.5)	91 (14)	89(11.5)	0.95
Δ VAS	-0.73(3.7)	9.4 (7.0)	37.8 (11)	0.000Ω
PD_{15} mls #	20 (1.3)	8.1 (3.2)	7.8 (2.3)	0.000∞
Asthma severity, n Intermittent Mild persistent Moderate Severe	 7	 47 16 4	 7 5 1	 0.32

(b) Sputum cell counts and eNO

Sputum cell counts	Hypoperceivers	Normal perceivers	Hyperperceivers	p value
TCC x 10^6/mL#	0.8 (2.0)	0.62 (1.2)	1.1 (1.0)	0.47
Viability,%	75(16.6)	84(18.7)	80.5(24.1)	0.49
Eosinophil,%#	0 (1.0)	0 (1.0)	0 (0.1)	0.69
Neutrophil,%	23 (26)	30 (22)	21 (17)	0.33
Macrophage,%	51 (30)	41 (26)	52 (19)	0.35
Lymphocyte,%#	0 (1.0)	0 (0)	0 (0)	0.29
Columnar epithelial cell,% †	0(6)	0(5)	0(20)	0.007*
eNO, ppb	24 (7.0)	20 (11)	24 (21)	0.73

Table (a): Mean (SD); *ICS (inhaled corticosteroid dose): expressed as fluticasone (F) equivalent with the following conversion ratios : F: budesonide ratio 2:1; F:beclomethasone ratio: 1:1. * observed count; ** expected count; ‡ significance noted between hyperperceivers vs normal/hypoperceivers; Ω significance noted amongst all 3 groups;∞ significance noted between hypoperceivers and normal/hyperperceivers; ESAHR: episodic symptoms and airway hyper-responsiveness; n: number of subjects ;BMI**:** body mass index**;** SF-36: generic Quality of Life Questionnaire(0 worst, 100 best) **;** LABA: long acting beta 2 agonist; FEV_1: forced expiratory volume in 1 second;PD_{15}: provocation dose resulting in a 15% fall in FEV_1 from baseline **;** # : geometric mean (SD)Δ VAS : change in Visual Analogue Score from baseline ; %: percentage; L; litres.

Table (b): Mean (SD); # median (IQR); †median(range);* post hoc analysis demonstrated significance between normal perceivers and hyperperceivers.TCC: total cell count; eNO: exhaled nitric oxide; mL: milliltres; % :percent; ppb: parts per billion.

CHAPTER 6

Perception of breathlessness in subjects without respiratory symptoms or airway hyper-responsiveness: relationship with clinical, inflammatory, psychosocial and economic variables.

6.0 Introduction

Few studies have examined POD and its variability in normal subjects or those without airway hyper-responsiveness. These subjects seem to demonstrate a wide variability in POD to the same ventilatory stimulus(179). They also may exhibit the same or similar magnitude of breathlessness to a range of stimuli(179). Anxiety, the degree of airway obstruction induced by the ventilatory stimulus, and distraction during the stimulus, all affect POD in normal subjects, similar to that noted in asthma. In one study, 15 normal or subjects without asthma, and 15 subjects with asthma (classified by symptoms and spirometry), underwent a methacholine challenge test to induce a 20 to 50% fall in FEV_1(217).At a given level of breathlessness, normal subjects perceived more airflow obstruction compared to subjects with asthma. Anxiety levels were low and did not correlate with POD. Contrastingly, in another study examining perceived dyspnoea during progressive arm ergometry in normal subjects, a significant positive correlation was noted between anxiety and POD which was more marked in women (254). Anxiety itself has been shown to both heighten and blunt POD in normal subjects (255). In a series of experiments in healthy volunteers, Von Leupoldt and colleagues demonstrated that POD is a multi-dimensional process affected by both sensory input and by non sensory factors such as attention. The authors found that distracting subjects reduced the unpleasant sensation of breathlessness(256-259)

While studies have demonstrated a variable association between AI (assessed by sputum eosinophilia and raised eNO measurements) and POD in subjects with asthma(42, 43),(44, 260) it is unknown if a similar relationship exists in normal subjects without respiratory disorders or AHR.

Perception of breathlessness, as described in Chapter 5, is usually assessed with a subjective scale, and in response to induced airway bronchoconstriction(243, 261) These scales are reproducible, and sensitive to change or responsive, in subjects with asthma. They are also robust in individuals without asthma(262, 263). Grant and colleagues, for example, compared the reproducibility and responsiveness of the visual analogue scale (VAS), Borg scale and Likert scale in 23 normal subjects during a sub maximal exercise challenge. They found that the VAS scale had the best reproducibility [reproducibility coefficient:78% (95%CI:66-89) vs 50% (95%CI:36-76) Borg scale; $p<0.05$] and responsiveness [sensitivity ratio: 2.7(95%CI: 2.4-3.0) vs 2.0(95% CI: 1.6-2.4) Borg scale; $p<0.05$] for breathlessness(262). In another study Boulet and colleagues found no significant difference between the VAS and Borg scale in subjects with and without asthma (217).

The aims of this study were therefore, firstly, to describe POD in subjects selected from the community without respiratory symptoms or AHR to a hypertonic saline challenge test; and secondly, to describe the relationship between POD and lung function, AI measured by sputum cell counts and eNO, psychosocial and economic factors in these subjects.

6.1 Materials and methods

Study Design

This was a cross-sectional population based study.

Subjects

Forty six subjects without respiratory symptoms based on the NWAHS questionnaire, on no respiratory medications and without evidence of AHR during a hypertonic saline challenge were enrolled. They were assessed at a single visit with a quality of life questionnaire (SF-36), height and weight measurements to calculate body mass index (BMI), spirometry and a hypertonic saline challenge test with a visual analogue scale at the beginning and at the end of the challenge to assess perception of dyspnoea (POD). A sample of sputum was induced concurrently with the hypertonic saline challenge test.

Methods

Materials and methods used in this study are detailed in Chapter 3. While both POD and FEV_1 in these subjects changed from baseline in response to the hypertonic saline challenge, FEV_1 did not reach the 15% fall that constitutes a positive challenge test. Therefore, in order to standardise, or anchor the change (delta or Δ) in the VAS or POD score to the change in FEV_1 with the saline challenge, POD was expressed as Δ VAS/ Δ FEV_1. In this study, as VAS results were not normally distributed, percentile values were substituted for SD. Low perception of breathlessness (hypoperceiver) was defined, a priori, as a VAS score below or equal to the 25^{th} percentile and increased perception of breathlessness (hyperperceiver) was defined as a VAS score equal or greater than 75th percentile. This premise was based on studies by Boulet and colleagues demonstrating that POD follows a normal distribution in subjects with airway hyper-responsiveness(53, 217), with hypoperceivers and hyperperceivers defined as subjects 1 standard deviation below and above mean respectively(53).

6.2 Statistical analysis

Descriptive statistics were used to summarise the clinical characteristics of the participants. Normally distributed data were expressed using arithmetic mean and standard deviations and data not normally distributed as median and interquartile range (IQR) or range. Correlations were expressed with the Pearson's correlation coefficient for normally distributed data and the Spearman's correlation coefficient for non- normal distributed data. Differences between the 3 groups (hypoperceivers, normal perceivers and hyperperceivers) were determined using an analysis of variance (ANOVA) for normally distributed and log transformed data where possible, or the Kruskal Wallis test for data not normally distributed. Categorical data were analysed using a chi squared test. Significance was accepted at a p of $\leq$ 0.05. Statistical analysis was performed with SPSS Windows ® Version 10.0 (SPSS Inc., Chicago, IL).

6.3 Results

Subjects had a mean age of 54.7(12.7) years and a mean FEV_1 of 101.2 (15.3) % predicted. Nearly 74% were atopic (Table 6.1). Completed VAS scores were available in 38 subjects. Visual analogue scale scores were not normally distributed in this study population (Figure 6.1). POD correlated significantly and inversely with baseline FEV_1 L and percent predicted, and positively with BMI (Table 6.2). A significant relationship was noted between BMI and baseline FEV_1 L (r=-0.23, p=0.05, n= 45). Perception of dyspnoea correlated significantly with eNO (r=0.47, p=0.04, n=35 Table 6.2) but did not demonstrate an association with the sputum total cell count, or the remainder of the differential cell count(TCC: r =-0.16,p=0.36, n=38,eosinophils: r=-0.31,p=0.11, n= 38;neutrophils:r=0.092,p=0.54,n=38; macrophages r=- 0.15, p=0.42, n= 38; lymphocytes r=- 0.01, p=0.96, n=38). Psychological and

socioeconomic factors of anxiety (r=-0.21, p=0.21, n=38), depression (r=0.08, p=0.66, n= 38), poor education (r= -0.08, p=0.64, n= 38) and low household income (r= -0.05, p=0.76, n= 38) did not demonstrate a correlation with POD. There was no correlation between quality of life assessed by the SF-36 and POD (Table 6.2).

Subjects were subsequently classified into normal perceivers, hypoperceivers and hyperperceivers. The majority(68%) were normal perceivers, and 31.5% were hyperperceivers. None were hypoperceivers. Subject characteristics were similar between groups (Table 5.3(a)) apart from a significantly increased BMI, and POD in hyperperceivers. Quality of life SF-36 physical function was significantly lower in hyperperceivers compared with normal perceivers (Table 6.3 (a)), and correlated positively with baseline FEV_1 (r=0.35, p=0.019, n=46; i.e. lower physical function score, lower baseline FEV_1) and negatively with BMI (-0.53, p=0.00, n=46; i.e. lower physical function score, higher BMI). The total cell count and differential counts were similar in both groups. Exhaled nitric oxide measurements were significantly raised in hyperperceivers compared with normal perceivers (Table6.3(b)).

6.4 Discussion

This study has demonstrated that in normal subjects without respiratory symptoms and no AHR there is a relationship between POD measured by Δ VAS/ Δ FEV_1, lung function, BMI, and possibly inflammatory markers. Increasing POD was associated with a falling baseline FEV_1 both litres and percent predicted, elevated BMI and eNO measurements. A raised BMI explained 33.6% (0.58 squared) of the variability in POD, while FEV_1 (both litres and percent predicted), and eNO explained 9% and 22% of POD variability respectively. No association was noted between POD and quality of life, psychological or socioeconomic variables.

Previous studies have demonstrated that, an association exists between POD and sputum eosinophilia in subjects with asthma (42, 43). This relationship was not seen our subjects without AHR. The participants in this study had total and differential sputum cell counts within the normal range (Table 6.3(b)) consistent with either paucigranulocytic AI, based on cell subtype classification by Simpson and colleagues(34), or no AI at all. The differentiation of these 2 scenarios is currently being debated. When subjects were divided into hypoperceivers, hyperperceivers and normal perceivers, significantly higher eNO levels were noted in hyperperceivers compared with normal perceivers. This conceivably supports the presence of paucigranulocytic AI rather than no AI in these individuals. Elevated eNO levels in the range of 16 to 26 ppb and higher have been considered to reflect eosinophilic AI(212). Emerging data suggests that much higher eNO levels, in the range of 30 to 45 ppb or more, are in fact present in eosinophilic asthma(114). The levels of eNO documented in this study are therefore likely to reflect the normal range of the test, or non -eosinophilic AI (114, 115). It also lends support to the concept that the association between POD and eNO levels may be similar to lung function; a continuum ranging from normality into pathological states such as asthma. Alternatively, these findings raise the possibility that

'normal' subjects without respiratory symptoms or AHR may have other non respiratory disorders associated with non eosinophilic AI as the source of their mildly raised eNO levels. Previously documented causes of a mildly raised eNO level and non eosinophilic AI include gastro-esophageal reflux, rhinosinusitis, viral infection (all usually associated with sputum neutrophilia), cardiac disease and vocal cord dysfunction (114) (223). These disorders were not actively investigated, and therefore, neither confirmed nor excluded in our population. In a recent community based study establishing reference ranges for eNO measurements, 10.6% of subjects classified as healthy, 'normal' individuals were found to have elevated eNO levels (115). Interestingly, anxiety and hyperventilation per se, are also associated with mildy raised eNO levels (114, 223). While an association between anxiety or depression (elicited by questionnaires) and eNO levels was not shown, a direct association between anxiety levels and increased breathlessness provoked by the hypertonic saline challenge test and eNO levels was not examined (and therefore cannot be discounted).

The relationship between increasing BMI and increased POD is partly explained by compromised lung function amongst other factors. This mechanism is supported by the correlation between BMI and FEV_1 (r=-0.23, p=0.05). Increased weight results in physiological impairment including a restrictive pattern of breathing and increased airways resistance (264-266). The expiratory reserve volume is reduced, and the work of breathing increased (265, 266). These adverse effects may result in symptoms such as wheeze and dyspnoea (265, 266). This in turn may lead to a heightened POD as evidenced in this study. The significantly lower score in the SF-36 physical function domain noted in hyperperceivers compared with normal perceivers and the negative correlation with BMI (lower SF-36 score, higher BMI) associated with the positive correlation with FEV_1 L and percent predicted (lower SF-36 score, lower FEV_1), lends support to this concept.

A raised BMI, moreover, is postulated to have a pro inflammatory role in asthma (264, 267-270) and this may extend to subjects without asthma, although not demonstrated in this study. The lack of an association between BMI and eNO levels has been demonstrated previously. McLachlan and colleagues found no significant relationship between BMI and eNO levels despite demonstrating increased eNO levels in asthma (252). In another study, weight loss following bariatric surgery resulted in improvements with asthma symptoms and lung function but not eNO levels(271).

It is noteworthy that none of the subjects under perceived dyspnoea for a given fall in FEV_1. This supports Boulet's earlier work demonstrating that at a given level of breathlessness, normal subjects perceived change in airflow obstruction more accurately than subjects with asthma(217). It may also reflect the lack of psychological determinants such as depression, temporal adaptation, and documented underlying lung pathology in this study population.

The classification of *no asthma* used in this study may be open to debate. This was defined by the absence of current respiratory symptoms associated with a fall in FEV_1 of less than 15% from baseline during the hypertonic saline challenge test. A negative airway challenge test was incorporated into the definition of *no asthma*, as it mirrored the methodology used to define asthma in the previous chapter. An alternative definition of "no asthma" in assessing POD, incorporates the absence of current symptoms, and the lack of bronchodilator reversibility on spirometry(217). Subjects defined in this manner for a previous study were, however, induced to achieve a fall in FEV_1 of at least 20% from baseline with large doses, indicative of airway hyper-responsiveness(217). The division of subjects into having asthma is still defined by a fall in FEV_1 of 20% from baseline with a dose of up to 16mg/ml of

methacholine. Thus it is unlikely that these subjects had asthma. It however does not exclude them from having increased airway hyper-responsiveness and possibly asthma at other times.

Atopy, a feature normally present in asthma, was prominent in our subjects. A recent longitudinal study following asymptomatic adolescents with AHR for 14 years found that while AHR was not a significant risk factor for developing asthma, the presence of allergy at baseline increased the risk of developing asthma considerably over time (Odds Ratio(OR) 4.45 (95 CI: 1.46-13.54, p=0.009, n=199)(272).

Caveats in interpreting the results include the descriptive nature of this study, the small sample size, and the arbitrary classification of POD into hypoperceivers, hyperperceivers and normal perceivers modified from Boulet and colleagues(53). The correlations noted in this study are based on a small sample size and larger studies are required to confirm these findings. Furthermore, subjects in this study had a small drop in FEV_1 from baseline (Table 6.3), much less than the 15% fall in FEV_1 required to define airway hyper-responsiveness with hypertonic saline. These small changes in FEV_1 may have theoretically lead to an over estimation of the POD measured by Δ VAS/ Δ FEV_1. Changes in VAS scores from baseline were, in the majority, also small or did not change at all. This raises the concern that the VAS scale may have been not have been sensitive enough to detect small changes despite a concurrent fall in FEV_1 from baseline, thereby underestimating the true POD. Thus the classification of subjects without airway hyper-responsiveness into hypo and hyperperceivers may not be robust on this basis. It is interesting to note, however, that not one subject under perceived his or her symptoms or to describe it differently, POD was appropriate to the fall in FEV_1 in normal subjects, a feature that has been demonstrated previously(217).

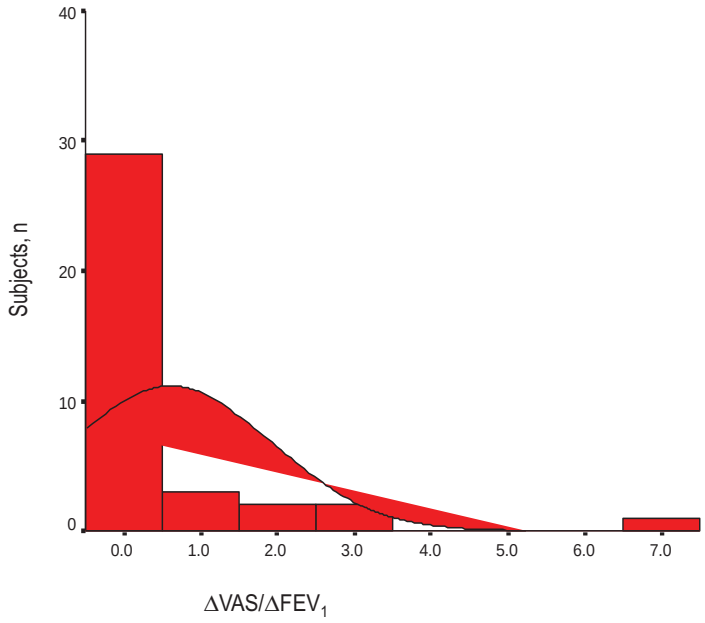
Despite these limitations, this community based study has highlighted an association between POD and reduced lung function and AI as measured by eNO in subjects without respiratory symptoms and AHR that requires further exploration. Studies with a larger sample size to assess if these findings are repeatable, and longitudinal studies assessing subjects over a period of time to determine if any develop asthma are warranted.

Table 6.1: Baseline characteristics of subjects without asthma: defined by absence of respiratory symptoms and a negative hypertonic saline challenge test.

Baseline characteristic	
n	46
Male, n	21
Age, years	54.7(12.7)
Atopy, n	34
Pre BD FEV_1, L Pre BD FEV_1 % predicted	3.1(0.77) 101.2(15.3)

Mean (SD); n: number of subjects; BD: bronchodilator; FEV_1: forced expiratory volume in one second; L: litre ; %: percent

Figure 6.1: Distribution of Δ VAS/ Δ FEV_1 readings (n=38) in subjects without respiratory symptoms and a negative hypertonic saline challenge test.



n: number of subjects;Median (IQR): 0.11(0.42); Δ VAS/ Δ FEV_1 : change in visual analogue scale from baseline divided by change in forced expiratory volume in 1 second from baseline; IQR: interquartile range.

Table 6.2: Correlation between Δ VAS/ Δ FEV1, clinical and inflammatory variables.

Clinical variables	r	p value	n
BMI	0.58	0.00	38
FEV_1 ,L	- 0.30	0.03	38
FEV_1 % predicted	- 0.29	0.04	38
SF-36 role physical	0.16	0.55	38
SF-36 physical function	- 0.13	0.49	38
SF-36 bodily pain	0.18	0.33	38
SF-36 general health	0.19	0.70	38
SF-36 vitality	0.07	0.49	38
SF-36 social function	0.16	0.41	38
SF-36 role emotional	0.14	0.46	38
SF-36 mental health	0.12	0.49	38
Inflammatory variable			
eNO ,ppb	0.47	0.04	35

r: correlation coefficient ; n: number of subjects; BMI: body mass index;

FEV_1: forced expiratory volume in 1 second; L: litres; %: percent;

SF-36: Short form (SF) generic Quality of Life Questionnaire;

eNO: exhaled nitric oxide; ppb: parts per billion

Table 6.3: Hyperperceivers and normal perceivers

***(a)*Subject characteristics**

	Normal perceivers n= 26	Hyperperceivers n= 12	p value
Age	54(12)	55(12)	0.82
Gender , male	14	3	0.2
Atopy,n (≥1 positive)	19	7	0.2
BMI	26(2.9)	31(7.4)	0.02
Smoking history , pack years	8.1(9.1)	13.1(18.1)	0.28
Current	3	2	0.51
Ex smokers, n	12	3	0.38
SF-36 role physical	76.2(39.1)	71.9(45.2)	0.80
SF-36 physical function	85.7(18.8)	66.9(28)	0.05
SF-36 bodily pain	69.3(21.8)	65.1	0.67
SF-36 general health	71.5(22.5)	72.8(20.9)	0.89
SF-36 vitality	65.5(20.9)	58.8(28.8)	0.49
SF-36 social function	85.7(25.1)	84.4(25.7)	0.90
SF-36 role emotional	87.3(31)	100(0)	0.26
SF-36 mental health	76.6(23.6)	79(15.5)	0.79
FEV_1, L	3.2(1.0)	2.6 (1.0)	0.07
FEV_1, % predicted	100(24)	87(15)	0.07
Δ VAS/ Δ FEV_1 #	0(0.22)	1.3(2.4)	0.000
Fall in $FEV_1$from baseline %	6.8(4.6)	7.5 (0.93)	0.56

Mean (SD); # median (IQR); no subjects were classified as hypoperceivers; n: number of subjects; BMI: body mass index ; SF-36: Short form (SF) generic Quality of Life Questionnaire; FEV_1: forced expiratory volume in 1 second; L : litres; % : percent; Δ VAS/ Δ FEV_1 : change in visual analogue scale from baseline divided by change in forced expiratory volume in 1 second from baseline; eNO: exhaled nitric oxide;

(b) Sputum cell counts and eNO

Sputum cell counts	Normal perceivers n= 26	Hyperperceivers n= 11	p value
TCC x 10^6 /mL#	0.82(1.2)	0.44(1.2)	0.32
Viability	89.3(11.3)	92.9(80)	0.45
Eosinophil,% #	0 (1.0)	0(0.75)	0.29
Neutrophil,%	25(26)	34(21)	0.33
Macrophage,%	53(25)	49(27)	0.68
Lymphocyte,% #	0(1.0)	0(0)	0.29
Columnar epithelial cells ,% #	0(0)	0(0)	0.55
eNO, ppb	14.5(5.2) n=18	22.6(13) n=6	0.03

Mean (SD); #median (IQR);n: number of subjects; TCC: total cell count; mL: milliltre;

%: percent; eNO:exhaled nitric oxide; ppb: parts per billion;

CHAPTER 7

Optimising the methodology for measuring labile zinc in induced sputum

7.0 Introduction

As discussed in Chapter 2, labile zinc is an essential antioxidant and anti-inflammatory agent in a variety of disorders including asthma. Thus measuring labile zinc concentrations in the appropriate body compartments accurately is important, but has proven difficult until recently.

Zinc (Zn) in body tissues and fluids has traditionally been measured by AAS, which detects both fixed and labile pools of zinc. These fixed pools of Zn are relatively unaffected in Zn deficiency and are poor indicators of body Zn status. In addition to these pools, there are more loosely-bound, dynamic pools of Zn within cells and tissues, and loosely bound to albumin in plasma , known as labile Zn. Labile Zn pools are readily measured in cells by measurement of specific Zn-dependent fluorescence with Zinquin (ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate) or other Zn fluorophores. These fluorophores bind Zn with dissociation constants in the nanomolar range (typical of intracellular concentrations of labile Zn), giving a stable signal in intact cells, frozen tissues and body fluids. They are specific for Zn (outlined in Chapter 2, section 5).

We have recently developed a Zinquin-based assay to measure labile Zn in plasma and possibly other extracellular body fluids(52) . With the advent of induced sputum to measure and monitor AI in airway disorders such as asthma, it was felt that this Zinquin assay could be modified and refined to measure labile or active zinc concentrations in sputum. Dithiothreitol (DTT) or Sputolysin (trade name), a mucolytic agent used to liquefy sputum prior to measuring total and differential cell counts, has been found to interfere with the assessment of mediators in the fluid phase or supernatant (273-277). It was unclear if DTT would interfere similarly with labile zinc measurements.

Here we describe the methodology to assess zinc levels in sputum, and optimisation of the procedure with respect to DTT, in order to subsequently measure labile or free zinc in the sputum of subjects with asthma. The measurements of labile zinc obtained in sputum were compared with saliva and plasma. In a subset of subjects, repeatability of labile zinc measurements and the effects of freezing the entire sputum sample on labile zinc measurements were explored.

7.1 Materials and methods

Study Design

This was a cross-sectional study.

Subjects

91 subjects without a doctor diagnosis of asthma and the absence of reversibility of airway obstruction based on American Thoracic Society criteria were randomly chosen from the NWAHS (North Western Adelaide Health Study), a larger population based study. They were assessed in a single visit with a clinical questionnaire, sputum induction with hypertonic saline challenge and blood sample. Details of the procedures are outlined in Chapter 3.

Materials

Major materials and their suppliers were: EGTA, TPEN, Chelex-100 resin (Sigma Chemicals, St Louis, MO); Sputolysin reagent (Calbiochem, San Diego, CA); Zinquin, ethyl-[2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy]acetate was obtained from Dr David Ward, Department of Chemistry, University of Adelaide, South Australia). It was dissolved at 2 mg/ml (5mM) in DMSO and stored in aliquots at –20^{o}C. All other reagents not listed were reagent-grade, unless indicated. All water used in these experiments was high purity (Milli-Q grade). A stock 10x Hanks balanced salt

solution (10xHBSS) was made up using reagent grade chemicals and Milli-Q water. Contaminating Zn was removed by two cycles of treatment with Chelex-100 resin. The resin was pre-washed with Milli-Q water (3 x 5 min, as per manufacturer's instructions) and added to the 10x HBSS in the ratio of 5g /50 ml. After rocking for 40 min at RT, the buffer was decanted and treated for a further 40 min with a second batch of Chelex-100 and stored at 4^{o}C in the presence of Chelex resin.

Sputum induction and processing

The procedure for sputum induction and processing is described in Chapter 3, Materials and Methods. Sputum was processed within 2 hours of collection. Sputum supernatant was frozen at minus 20°C for fluid phase measurements.

Collection of blood and saliva

A sample of blood and saliva was taken for zinc measurement. Five mls of blood was collected in a heparin tube and centrifuged immediately for 10 minutes at 1000g. The plasma was removed and frozen at minus 20°C for later measurements. Prior to commencing the hypertonic saline challenge, the subject was asked to expectorate a sample of saliva into a sterile container.

7.1.1 Measurement of labile zinc in induced sputum by Zinquin fluorometry

The concentration of labile Zn in the induced sputum supernatant (fluid phase) was determined by fluorescence with Zinquin. To allow for Zn (present as a contaminant) in the DTT reagent, a blank (DTT alone) was prepared for each batch of sputa processed on a given day. Zinquin fluorescence of blanks was subtracted from the Zinquin fluorescence of the sputum supernatant, as described below.

Preparation of Zn standards (Figure 7.1(a)): For each run, a set of Zn standards were set up to provide a standard curve for conversion of fluorescence units into Zn concentrations. Stock concentrations (0, 5, 10, 20 and 40 μM) of Zn nitrate (Merck, 1g/l) were made up in water and stored for up to 3 months at 4°C. On the day of assay, 50 μl of each stock Zn solution were added to 200 μl of diluted DTT reagent (1 volume to 9 volumes of water as per manufacturer's instructions). 50 μl of each were then added to each of 3 cuvettes (Greiner Halb-Mikro 1 ml disposable fluorometry cuvettes). This achieved a final Zn concentration range in the cuvettes of 50–400 nM; Zinquin fluorescence was linear over this range, but at concentrations > 500 nM it began to plateau .

Preparation of blanks and sputum supernatants (Figure 7.1(b)): 50 μl of sputum supernatant was placed into each of 6 cuvettes. To three of these cuvettes (designated + EGTA), 10μl of 30mM EGTA was added and allowed to mix with the sputum supernatant. These cuvettes were used to obtain the non specific or non-Zn-dependent fluorescence (if any). Since EGTA is a Zn chelator, it will eliminate fluorescence due to Zn, thereby allowing calculation of the non specific or non-Zn-dependent fluorescence such as autofluorescing elements in sputum. The other 3 cuvettes (- EGTA) received 10 μl of water instead of EGTA and were used to obtain the total fluorescence (specific + non-specific).

Similarly, 6 cuvettes, 3 with EGTA and 3 without, were set up for the blank (DTT solution used for processing the sample on that day).

Addition of Zinquin buffer and fluorometry: All cuvettes (standards, blanks and supernatants) then received 940 μl of Zinquin-containing buffer. This buffer consisted of Chelex-treated 1X HBSS containing 0.3 mg/ml OVA and 10 μM Zinquin. Since Zinquin is hydrophobic and will precipitate out of aqueous solutions with time, OVA was added as a stabilising protein. OVA is ideal since unlike serum albumin it lacks sulphydryls and does not bind or contain Zn(52). Zinquin was added to the buffer immediately before pipetting into the cuvettes. Cuvettes were allowed to incubate for 40 min at room temperature in the dark. Fluorescences were then read using a TBS-380 mini fluorometer equipped with a UV filter (Turner Biosystems). Specific Zn-dependent fluorescence for blanks and sputa were derived by subtracting mean fluorescence in presence of EGTA from that in the absence of EGTA. Fluorescence of blanks were then subtracted from fluorescence of sputa and the difference was converted to a concentration of Zn using the standard curve. Generally, fluorescence of blanks was very low compared with that of sputum supernatants; however, if the DTT was allowed to age for more than 2 weeks, high blank fluorescence's were obtained (see Results). Fluorescences of sputum supernatants in the presence of EGTA were nearly always negligible.

Measurement of labile Zn in saliva and plasma by Zinquin fluorometry

The method for labile Zn in saliva was similar to that for sputum except that standards were made up in water or saline rather than diluted DTT. The method for plasma labile Zn was similar to that for sputum except that there were no blanks, the standards were made up in water rather than diluted

DTT and the volumes of plasma added to cuvettes was 10µl. The latter was in order to achieve a final concentration of Zn in the cuvettes within the 50-400 nM range.

Labile zinc measurements in frozen sputum versus fresh sputum.

In 4 subjects, after the initial sputum sample was processed and a cell differential performed as described above, the remainder of the sample was frozen in its entirety. After at least 7 days of storage at minus 20^{o}C, the samples were thawed and processed in the same manner as the fresh sample, and zinc measurements were made.

7.1.2 Measurement of total zinc concentrations in plasma and sputum supernatant and sputum blanks by Atomic Absorption Spectrophotometry

Total Zn in plasma and sputum samples were measured by absorption at 214 nm in a PE 3030 Flame Atomic Absorption Spectrometer with a graphite furnace (278)(Figure 2). With sputum samples, the Zn contaminating the DTT was allowed for by subtracting the Zn in the blank (sputum processing reagent alone) from the Zn in the sputum supernatant.

7.2 Statistical Analysis

Normally distributed data were expressed using arithmetic mean and standard deviations and non-normal distributed data as median and interquartile range (IQR). Differences between the groups were determined using a paired t-test for normally distributed and log transformed data where possible, or the Wilcoxon Rank test for data not normally distributed. Correlations were expressed with the Pearson's correlation coefficient for normally distributed data or data with a linear relationship, and the Spearman's correlation coefficient for non- normal distributed data. Significance was accepted at a p of $\leq$ 0.05. Statistical analysis was performed with SPSS Windows ® Version 10.0 (SPSS Inc., Chicago, IL).

7.3 Results

Subjects had a mean (SD) age of 58 (13) years and FEV_1 of 102 (17) % predicted (Table 7.1).

7.3.1 Methodological issues

The method for measuring labile zinc in plasma using Zinquin, was adapted for sputum in the following ways to yield repeatable results:

1. The sample volume of sputum supernatant was increased from 20 microlitres(µL) used for plasma to 50µL. Secondly, the EGTA volume reduced from the 20 µL used for plasma to 10 µL for sputum

supernatant. In preliminary experiments, various ratios of sample volume to EGTA volume were tested, and the ratio described above allowed zinc fluoresence to be detected in sputum consistently.

2. Comparison of Zn standard curves performed in the presence and absence of DTT showed that, in the presence of DTT, Zinquin had almost twice the fluorescence with Zn as it did in the absence of this agent (Figure 2). It is therefore important when analysing sputum Zn concentrations to perform the standard curve in a medium containing the same concentration of DTT as used in the processing of the sample.

3. After opening of a new DTT (Sputolysin) bottle, it was observed that storage beyond 2 weeks at 4^{o}C, led to a dramatic increase in fluorescence of the blank with Zinquin (Figure 3). This fluorescence was entirely quenched by EGTA, suggesting that it was due to the release of zinc. Therefore, for labile Zn measurements with Zinquin, an opened vial of DTT should only be stored for a maximum of 1 week. Whether purging the contents of the vial with an inert gas prior to storage would prevent this change has not been studied.

Labile zinc measurements in saliva

Salivary labile zinc measurements were very low (Table 7.2).

Sputum and plasma labile and total zinc measurements

Sputum and plasma labile and total zinc measurements are shown in Table 7.2 and followed similar trends: total zinc measurements were higher than labile zinc measurements.

There were no correlations noted between age and total or labile zinc levels in sputum (total zinc r=0.10, p=0.42, n=70; labile zinc r=-0.05, p=0.70, n=91) and plasma (total zinc r= -0.16, p=0.12, n=91; labile zinc r=-0.03, p=0.81, n=73). There were no differences in zinc levels between male and female subjects (sputum: total zinc r=0.07, p=0.55, n=70; labile zinc r= 0.17, p=0.15, n=75; plasma: total zinc r=-0.13, p=0.21, n=91; labile zinc r=-0.07, p=0.56, n=73).

Repeatability of sputum labile zinc measurements

The repeatability of labile zinc measurements from the same sputum supernatant sample, expressed by the intraclass correlation coefficient (R), was excellent. Three sputum samples had labile zinc measurements performed at 2 different time points, at least two weeks apart*. Two out of the three samples had another zinc measurement taken at a third time point, again at least two weeks apart*. Repeatability (R) was 0.96 in all cases, with an R of $\geq$ 0.8 considered acceptable.

(*measured in supernatant samples which were frozen at the time of sputum processing, then thawed for further analysis)

Correlations between sputum labile zinc using Zinquin, and total zinc measurements using AAS.

Labile zinc measurements correlated well and significantly with total zinc measurements (r=0.51, p=0.000, n=70) and explained up to 26% percent of the variability in total sputum zinc measurements. This relationship was more robust than that found between total and labile plasma zinc concentrations in plasma (r=0.16, p=0.67, n=91), where labile zinc explained 2.5 % the variability in total zinc measurements.

Labile zinc measurements in frozen sputum versus fresh sputum.

Zinc measurements in frozen sputum samples (n=4) were considerably higher (median (IQR): 6.38 (11.58) versus 0.11(1.25) p= 0.068) compared with sputum samples processed within 2 hours (Table 7.3).

7.4 Discussion

This study confirms for the first time that induced sputum examination, a reliable, non invasive technique of assessing airway inflammation, lends itself to labile zinc measurements. Zinc concentrations have traditionally been assessed in a variety of tissues and body fluids by AAS, which measures both fixed and labile zinc stores. Our laboratory has developed a novel method using Zinquin fluorphore, to assess free or labile zinc concentrations, considered to be the biologically active component of zinc, in cells and, more recently, in plasma. The materials and methodology required for Zinquin fluorometry are available in most laboratories. It is, therefore, likely to be a more cost-effective and generalisable procedure compared with AAS. At the time of commencement of this thesis, the assay had not been used in induced sputum or saliva. An important aim of this thesis was to establish that labile Zn could be measured in induced sputum, and to optimise the technique, with the objective of measuring sputum zinc concentrations in asthma. This was achieved as described in this chapter, and included firstly, altering the concentrations of sample and EGTA buffer used to achieve maximum Zinquin fluoresence, and secondly and more importantly, correcting for the presence of DTT (Sputolysin).

While DTT is widely used as a mucolytic agent in examination of cell counts in sputum, and results in improved dispersal of cells for cell counts compared with saline alone(274, 279), this study has demonstrated that DTT in Sputolysin contains zinc which needs to be adjusted for in the calculation of sputum zinc concentrations. Limitations in using DTT have been raised previously in the measurement of other inflammatory makers in the fluid phase medium or supernatant of sputum. For example Simpson and colleagues have measured inflammatory mediators in induced sputum using standards prepared in the presence and absence of DTT and have noted that DTT interferes with the

assay and needs to be adjusted for(280). This has also been echoed in a recent study optimising the process for measuring chemokines and cytokines in sputum in the presence of DTT(275). The increase in Zinquin fluorescence with DTT noted over a period of 2 weeks is considered to be due to the oxidation of DTT releasing tightly-chelated zinc present in Sputolysin. This hypothesis is supported by the ability of EGTA, in our experiments, to fully quench the high blank fluorescence.

Experiments in optimising Zinquin fluorometry for sputum demonstrated that the technique is highly reproducible as measured by the intraclass correlation coefficient (R= 0.96). It was difficult to assess the validity of labile zinc measurements using Zinquin. While AAS is the currently accepted, gold standard method for measuring zinc levels in body fluids, it measures both total and labile zinc pools. Zinquin fluorometry is the only method currently available to assess free or labile pools of zinc. Thus these techniques measure different compartments of zinc homeostasis, and cannot be compared directly.

Interestingly, the correlation between total and labile zinc concentrations in sputum was reasonable and significant. It, however, explained only 26% of the variability in the total zinc measurement, likely reflecting the fact that most of the zinc measured by AAS is tightly fixed to protein. Contrastingly, the correlation between total and labile zinc in plasma was poor. There are several possible reasons for this. In plasma, zinc is largely bound to two different proteins, alpha 2 macroglobulin and albumin. Zinc binds tightly with most metalloenzymes including alpha 2 macroglobulin, using all 4 ligand binding sites, thereby not permitting Zinquin to attach and be detected. Zinc, however, binds weakly to albumin with only two of its 4 ligand binding sites interacting with albumin, leaving the other 2 to attach to Zinquin. Secondly, the proportion of albumin to alpha 2 macroglobulin can vary greatly

between healthy subjects. These two factors may result in a variable relationship between total and labile plasma levels. In sputum, it is postulated that zinc is bound to mucin, a glycosylated protein, but this remains unproven. Mucin levels may unlike plasma proteins, may not vary much between healthy subjects (low inter subject variability), thereby resulting in a better correlation between total and labile sputum zinc concentrations compared with plasma. Additionally, the proportion of protein bound zinc present in sputum may be considerably less compared with plasma.

Some subjects had undetectable labile sputum zinc measurements (Tables 7.2, 7.3). Possible explanations include a reduced dietary intake of zinc or an increased turnover of zinc in subjects with underlying airway or systemic inflammation due to other disorders besides asthma. Rapid turnover of zinc may occur as a result of increased cellular uptake and utilization of zinc with airway inflammation. Upregulation of zinc transporter proteins or the presence of specific zinc transporter polymorphisms may contribute to this process. Mouse models of allergic inflammation (281) have demonstrated that AI is associated with an altered expression of zinc transporter proteins, especially zinc transporter proteins ZIP 1 and ZIP 14 directed towards increasing zinc uptake and possibly retention of zinc, suggesting a similar process may occur in the human airway. Asthma is also associated with increased cellular apoptosis and airway epithelial shedding with loss of cellular zinc (49, 282). One may postulate from this that early in inflammation zinc levels may increase in sputum as zinc is lost from airway tissue but in a chronic inflammatory state, where airway inflammation is persistent and uncontrolled, airway tissue zinc is depleted resulting in lower sputum zinc concentrations. The low sputum zinc concentrations also raise the prospect that more sensitive assays to detect labile zinc may be required.

This study has shown that while sputum may be processed, and the supernatant frozen, for repeated, reliable zinc measurements, the original sputum sample cannot be frozen. Freezing the sputum sample, subsequently thawing it, processing it, and measuring zinc in the supernatant results in higher zinc concentrations compared with the fresh sample. This discrepancy is most likely explained by the fact that zinc leaches out of cells after the cell membranes are disrupted by freeze-thawing. There are no studies, to our knowledge, examining the effects of freezing sputum samples prior to homogenisation with DTT, and its effects on cell counts and mediators including zinc. Previous authors have found that freezing sputum samples in the presence of dimethylsulfoxide (DMSO), a cryoprotective agent (with or without bovine serum albumin), for up to 10 days either after homogenisation with DTT, or immediately before cytospin preparation, yielded reliable total and differential cells counts(283, 284).

Finally, this study has shown that saliva has negligible concentrations of labile zinc. Thus when a sputum plug is selected from the salivary component of the expectorated sample, there is likely to be little contamination from salivary labile zinc. Unfortunately, it was not possible to measure total salivary zinc levels due to restricted access to atomic absorption spectrophotometry, and this limits comparisons between free zinc alone and combined free and protein bound zinc levels in saliva. Historical data, however, suggest that total salivary zinc levels are variable ranging from mean (SD) 55(17) µg/L to 173(94) ng/ml in healthy volunteers (285, 286) . Salivary zinc levels in healthy volunteers and subjects with asthma are further studied in Chapter 8.

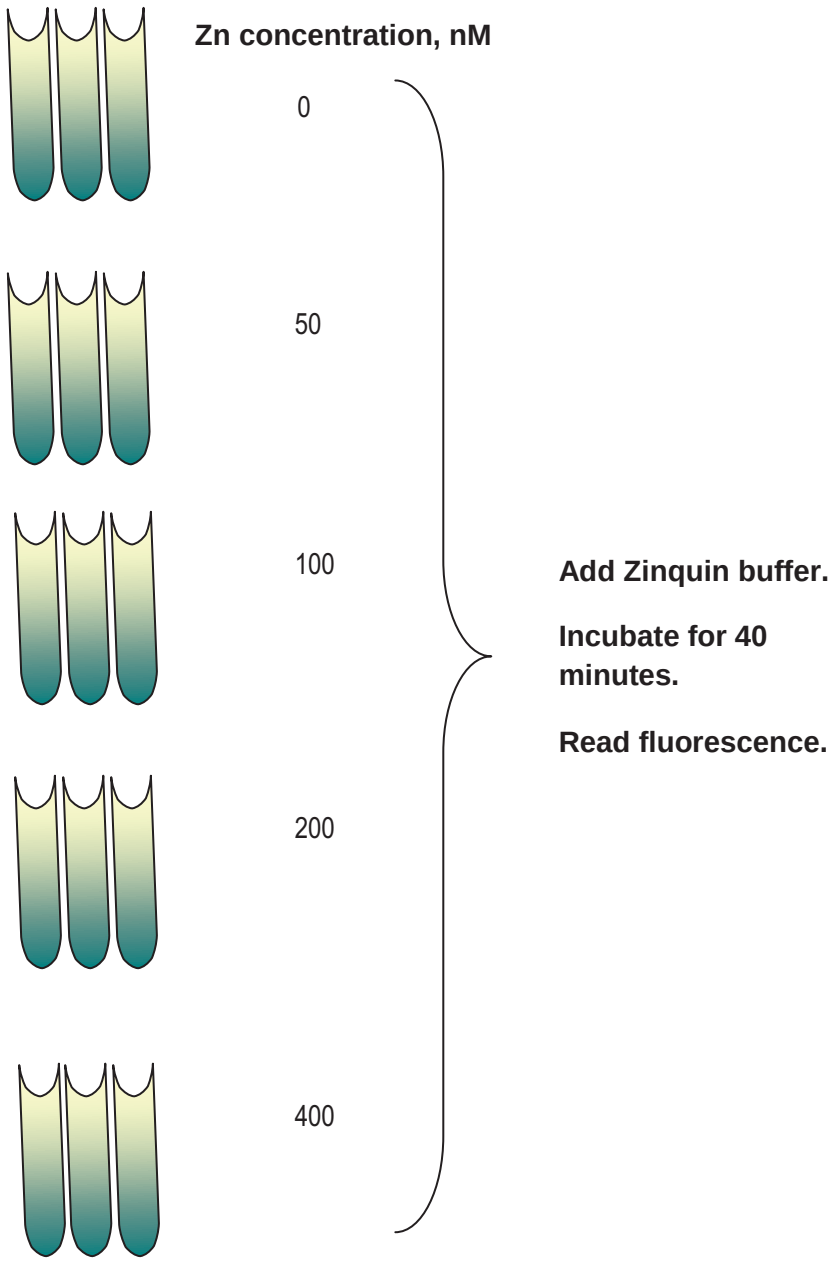
A variety of biological variables may influence sputum zinc measurements. These factors include, the amount of zinc consumed in the diet, age of the participant, inflammatory and other stressors. Further

studies are required to study the effects of these variables systematically, and to develop normal values and ranges for zinc, similar to cell counts and other mediators assessed in sputum(34, 137, 155-157)

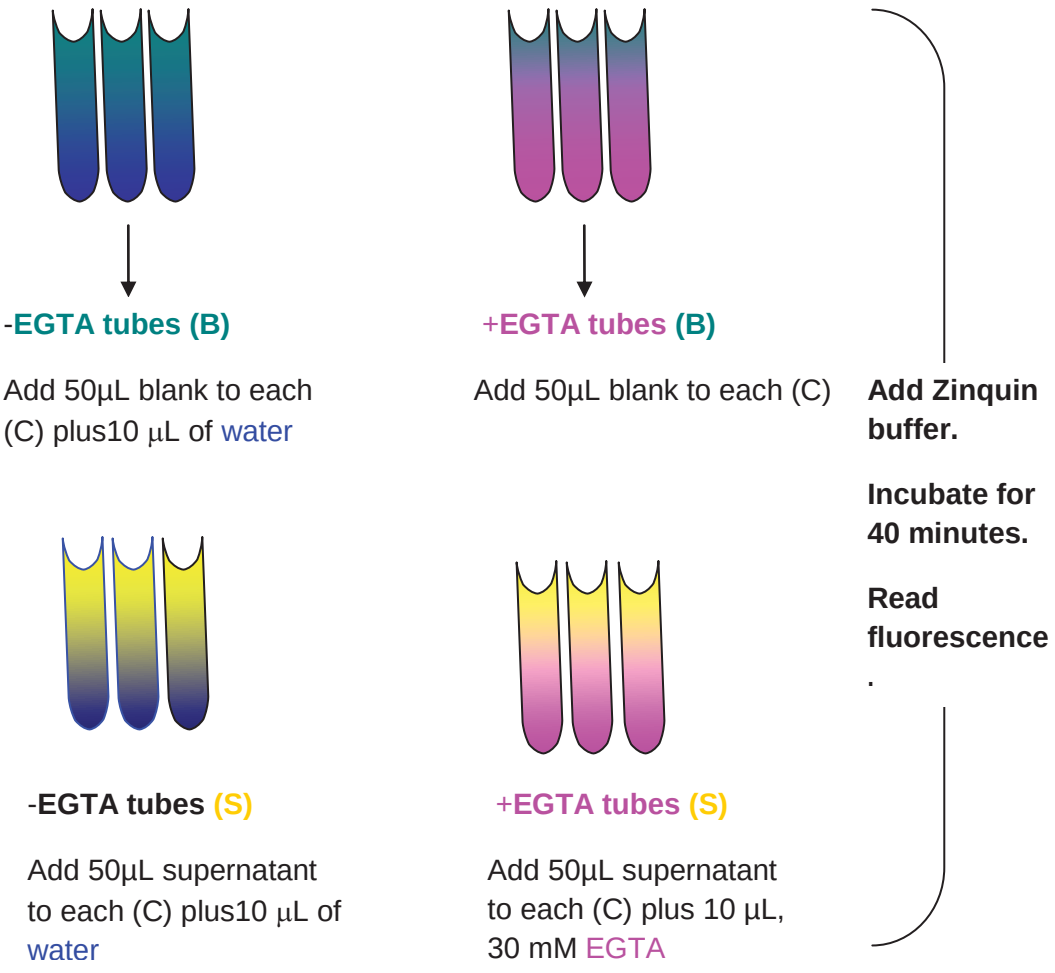
In conclusion, this study describes a novel method, Zinquin fluorometry, for measuring labile or free pools of zinc in sputum, and the steps taken in optimising this procedure. It also confirms that Zinquin fluorometry is a repeatable method for measuring free zinc in sputum. Some of these results with sputum Zn have now been published, as part of a larger study of optimising labile Zn measurement in various body fluids (52).

Figure 7.1: Labile zinc measurements with Zinquin, modified and optimised for sputum .

(a) Preparation of Zn standards. 50 uL of each stock Zn solution (0, 5, 10, 20, and 40 μM) respectively were added to 200 μL of diluted (1:9) sputolysin (DTT) reagent. 50 μL of each diluted Zn concentration was added to 3 cuvettes. Zinquin fluorescence was recorded.



(b) Preparation of samples.



Blank (B): is the stock sputolysin (DTT) solution; **(C)** cuvette ; **EGTA** is a zinc chelator;

(S)supernatant; Nm: nanomolar; µL : microlitre.

Figure 7.2: Total zinc measurements by AAS

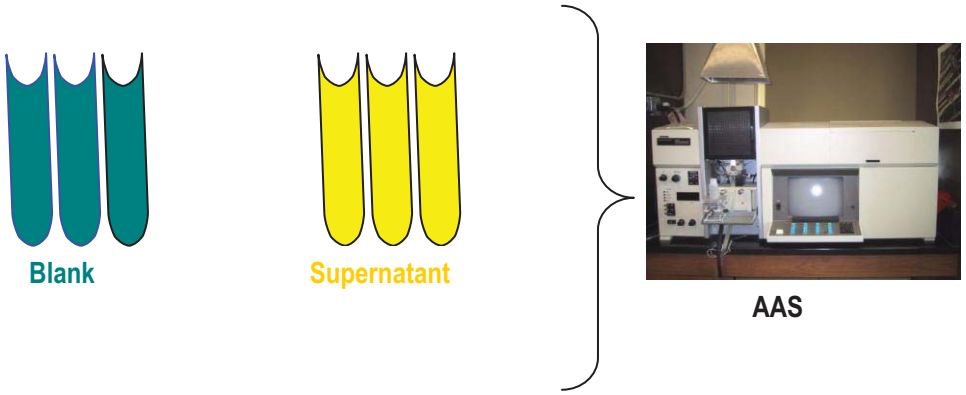


Table 7.1: Subject characteristics

Total subjects ,n	91
Male, n	47
Age, years	58(13)
Pre BDFEV_1, L	3.0(0.90)
Pre BD FEV_1 % predicted	102(17)

Mean (Standard deviation (SD)); n: number of subjects; BD: bronchodilator; FEV_1: forced expiratory volume in 1 second; L: litres; % percent

Table 7.2: Zinc concentrations in saliva, sputum and plasma in subjects

Specimen, n		Zinc concentration, µmol/L
Saliva	n=11	0.0 (1.7)#
Sputum		
Total zinc	n =70	0.57(1.7)#
Labile zinc	n =75	0. 29(2.07)#
Plasma		
Total zinc	n= 90	11.7 (2.4)
Labile zinc	n =72	5.4 (3.3)

Mean(SD); median(IQR)# ; n:number of subjects

Table 7.3: Labile zinc measurements in fresh versus frozen sputum samples.

Sputum sample	Fresh Labile zinc, µmol/L	Frozen Labile zinc, µmol/L
1	0	1.3
2	0	16.4
3	0	6.9
4	0.76	6.0

Wilcoxon Rank Test: difference between fresh and frozen samples p=0.068;

µmol/L: micromoles/litre

Figure 7.2: Comparison of standards, saline alone, sputolysin (DTT) alone and combination of DTT/saline used in processing sputum in standardised protocols.

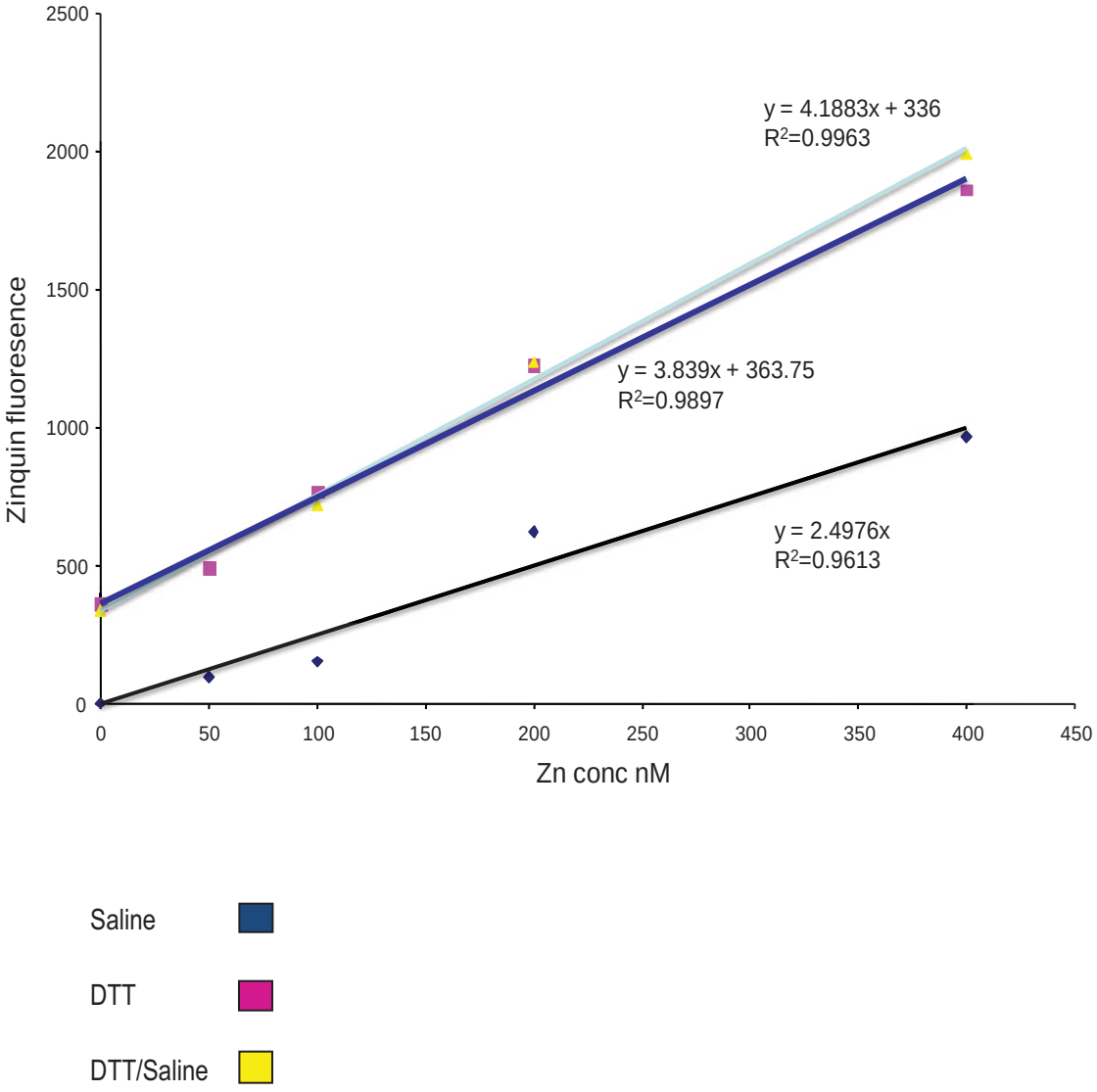
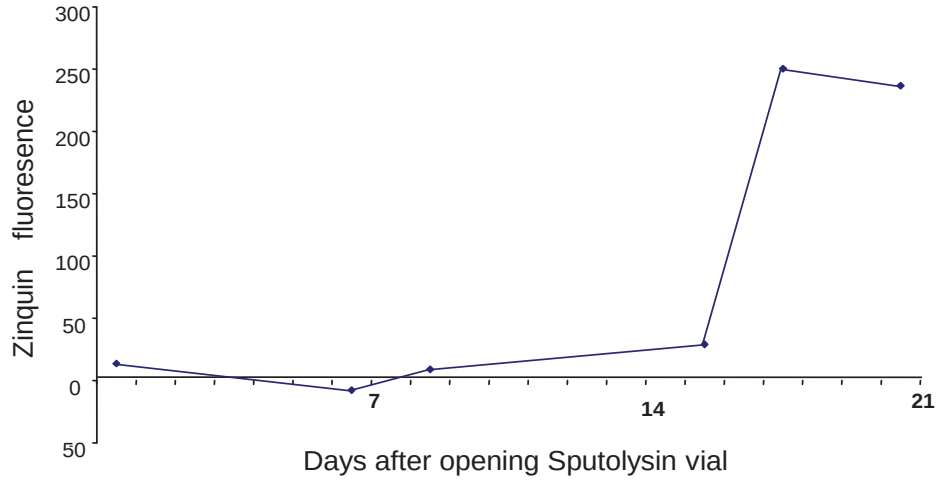


Figure 7.2 demonstrates higher Zinquin fluorescence in Zn standards prepared in the presence of DTT (Sputolysin).There are 2 possible explanations for this:(1) DTT contains some contaminating Zn resulting in higher baseline Zn concentrations in the cuvettes compared with saline alone; (2) the slope is greater in the presence of DTT, suggesting that Zinquin is more sensitive or more responsive to Zn in the presence of DTT; this may relate to the reducing effect of DTT; nM: nanomolar; R^2:The regression coefficient, expressing the 'goodness of fit' of the regression line with the actual data points: R^2 values range from 0 to 1, with 1 representing a perfect fit between the data and the line drawn through them, and 0 representing no statistical correlation.

Figure 7.3: Increasing fluorescence in blanks when bottle of Sputolysin had been opened for more than 2 weeks.



CHAPTER 8

Asthma is associated with reductions in labile and total zinc concentrations in induced sputum

8.0 Introduction

Although the situation in humans remains unclear, a number of studies have indicated associations between low body Zn and human asthma, reviewed in (49) . Zinc in the body exists in fixed and labile pools, with the latter being the exchangeable component.

Until recently, it has been difficult to measure labile zinc concentrations accurately. The traditional method of assessing zinc levels with AAS encompasses both fixed and labile zinc pools (total levels) Zinquin-based assay has recently been developed in our laboratory to measure only labile Zn in plasma(52). With the advent of induced sputum to diagnose, measure and monitor airway inflammation in airway disorders such as asthma, it was felt that this Zinquin assay could be optimised to measure labile or freely moving zinc concentrations in sputum (described in Chapter 7).

Here, we report a study of the concentrations of labile and total Zn in induced sputum as a surrogate measure of Zn in the AE lining fluid in an epidemiological study in subjects with and without asthma. Sputum Zn concentrations were compared with those in plasma, and correlations were sought with the severity of asthma based on frequency of symptoms and lung function. We postulated that sputum zinc concentrations would be lower in asthma. This hypothesis was based on the animal studies and the majority of studies in humans demonstrating lower zinc concentrations in the blood and hair of subjects with asthma than without asthma (detailed in Chapter 2).

While the previous chapter demonstrated that zinc levels in saliva are negligible, they were measured in this study to confirm this finding and to assess if salivary zinc levels were similar in subjects with and without asthma.

8.1 Materials and methods

Study Design

This was a cross-sectional, population based study.

Subjects

One hundred and fourteen subjects with *asthma*, defined by either a self reported doctor diagnosis, or the presence of episodic symptoms with bronchodilator reversibility noted on spirometry and/or a positive saline challenge test (characterised by a fall in FEV_1 of $\geq$15% from baseline (PD_{15}))(82), and 49 subjects *without asthma* defined by the absence of the above, were recruited from the NWAHS cohort (Table 8.1). They were assessed in a single visit with a clinical questionnaire sputum induction with hypertonic saline and a blood sample. Investigators were blinded to the grouping of the subjects. Salivary zinc was measured in a subset of these subjects (n=9 with asthma and n=9 without asthma).

Materials.

The materials used in this study are described in Chapters 3 and 7.

Methods

The procedures used are described in Chapter 3. All sputum cell counts and sputum zinc measurements were performed independent to and blinded to clinical details and subject grouping.

Measurement of labile zinc in induced sputum, plasma and saliva by Zinquin fluorometry

The concentration of labile Zn in the induced sputum supernatant (fluid phase), plasma and saliva was determined by fluorescence with Zinquin as described in Chapter 7.

Measurement of total zinc concentrations in plasma and sputum supernatant and sputum blanks by Atomic Absorption Spectrophotometry(AAS)

Total Zn in plasma and sputum samples were measured by absorption at 214 nm in a PE 3030 Flame Atomic Absorption Spectrometer with a graphite furnace as described in Chapter 7.

8.2 Statistical Analysis

Normally distributed data were expressed using arithmetic mean and standard deviations and non-normal distributed data as median and interquartile range (IQR). Differences between the groups were determined using a paired t-test for normally distributed and log transformed data where possible, or the Wilcoxon Rank test for data not normally distributed. Correlations were expressed with the Pearson's correlation coefficient for normally distributed data and the Spearman's correlation coefficient for data that was not normally distributed. Significance was accepted at a p of $\leq$ 0.05. Statistical analysis was performed with SPSS Windows ® Version 10.0 (SPSS Inc., Chicago, IL).

8.3 Results

Table 8.1 outlines the baseline characteristics for the subjects with and without asthma defined as described above. Subjects with asthma had a significantly lower baseline FEV_1 (both in litres and % predicted) and increased bronchial hyper-responsiveness (measured by PD_{15}) compared with subjects without asthma.

Zinc concentrations in saliva

Salivary zinc concentrations were very low and similar in both groups: asthma (n=9) vs no asthma (n=9) 0.0 (0.80) vs 0.0 (1.18) µmol/L, p=0.73.

Zinc concentrations in sputum

Total and labile sputum zinc

Total and labile sputum zinc concentrations were significantly lower in subjects with asthma compared to those without asthma: total zinc median (IQR): 0.49 (1.8) vs 0.77 (2.9), p=0.02; labile zinc: 0.0(0.74) vs 0.4(1.3), p=0.05 (Table 8.2). They were similar in subjects with asthma on inhaled corticosteroids and long acting beta 2 agonists compared with those on no medication (Table 8.3(a)). Undetectable labile sputum zinc measurements were noted in 41% subjects with asthma compared to 27% of subjects without asthma.

Relationship of sputum zinc concentrations with frequency of symptoms and lung function in asthma and asthma severity measured by NAC (2006) criteria

No association was seen between total sputum zinc concentrations and increasing frequency of symptoms or worsening lung function as measured by FEV_1 (Table 8.4). Significantly lower labile zinc concentrations were associated with increasing severity of FEV_1 and increasing severity of asthma measured by NAC (2006) criteria (Table 8.4, Figure 8.1). Significant correlations were noted between FEV_1 and labile sputum zinc (r=0.27, p=0.008, n=95) and between asthma severity and labile sputum zinc (r=-0.23, p=0.028, n=95).

Zinc concentrations in plasma

Total and labile plasma zinc

Total and labile plasma zinc concentrations were significantly higher in subjects with asthma compared with those without asthma (Table 8.2). They were similar in subjects with asthma on inhaled corticosteroids and long acting beta 2 agonists compared with those on no medication (Table 8.3(b).

Relationship of plasma zinc to frequency of symptoms and lung function in asthma and asthma severity measured by NAC (2006) criteria.

Increasing frequency of symptoms in asthma was associated with a strong trend towards increasing total plasma zinc concentrations (p=0.065) and was mirrored with worsening lung function in asthma (p=0.015) (Table 8.4(a)). No relationship was noted between increasing severity of asthma, and total (r=-0.16, n=111, p=0.09) or labile plasma zinc concentrations

(r= 0.02, n=87, p=0.89). (Table 8.4(b))

Sputum total and cell differential counts

Sputum total and differential cell counts were similar in subjects with asthma and those without apart from viability which was significantly lower in the former (Table 8.5). In subjects with asthma, worsening lung function (r=0.74, n=112, p=0.00) and increasing severity of asthma (r=0.28, n=95, p=0.007)were significantly associated with increasing neutrophil counts (Table 8.4,Figure 8.1). Significant correlations were not present between plasma or sputum zinc levels and sputum total and differential cell counts (Table 8.6(a)) in subjects with and without asthma (Table 8.6(a) and (b)).

8.4 Discussion

This is the first study, to our knowledge, to report markedly lower total and labile zinc concentrations in the sputum of subjects with asthma compared with those without asthma. Furthermore, increasing severity of asthma based on FEV_1 criteria or NAC (2006) criteria(8), was associated with significantly lower sputum labile zinc concentrations.

There is growing evidence that asthma is associated with a zinc deficient state (49). Zinc deficiency promotes airway inflammation by altering various aspects of the normal and innate immune response. Zinc deficiency is known to increase the production of T Helper 2 cytokines involved in inflammation and asthma(287), increase apoptosis or programmed cell death of airway epithelial cells which results in rapid cell shedding and turnover and compromised barrier function(196). Inflammatory cells, including eosinophils, neutrophils and mast cells, key players in AI, are zinc rich(196, 288) (289, 290), and the loss of these cells via the airway lumen in mucus , may result in significant zinc loss. It has yet to be determined whether a fall in zinc levels promotes inflammation in asthma or whether the fall in zinc levels is a secondary manifestation of ongoing airway inflammation. Both may co-exist.

Significantly higher total and labile plasma zinc concentrations were noted in subjects with asthma compared with those without asthma (Table 8.2). This result, while confirming the findings of 2 previous population based studies, differs from most studies that demonstrate lower plasma zinc concentrations in asthma (49). Taken together, the low sputum and comparatively high plasma zinc levels demonstrated in subjects with asthma in this study may reflect compartmental fluxes. The pathway of the flux is yet to be determined but the following are postulated: initially, with acute airway

inflammation, there may be a “block” to zinc loss from airway epithethial cell, reflected by low sputum zinc levels and concurrent high plasma zinc levels. Zinc loss from the airway epithelial cell into the airway lumen may be inhibited by zinc transporter proteins. These proteins may also impair uptake and compartmentalisation of zinc into the airway epithelial cell. Knoell and colleagues have recently demonstrated upregulation of the zinc SLC39A8 transporter in airway epithelial cells protects the cell from apoptosis in acute lung injury(291), and there may be similar mechanisms in asthma. We are currently looking at the expression of a range of ZIP and CDF zinc transporters in AE of normal and inflamed airways from mice and humans.

Over time, if the inflammatory process continues unabated, and airway epithelial cells are shed with the inflammatory process, we postulate that zinc levels may rise initially in sputum and then fall, as overall body zinc stores are depleted. In response to this, zinc may leach out of tissue cells into the plasma extracellular space in order to maintain homeostasis. It is not known what happens to sputum zinc concentrations in asthma during exacerbations or episodes of poor control. One may postulate that zinc concentrations may rise acutely during exacerbations, but return to baseline levels with the resolution of symptoms. They may also fall below baseline levels if body zinc stores are not repleted through the diet during and following periods of exacerbation, leading to an overall zinc deficient state. Further studies are required to examine these hypotheses. Measuring zinc in airway cells will be helpful in clarifying this process.

Increasing frequency of symptoms and declining FEV_1 were associated with increasing total plasma zinc concentrations, and a trend towards lower labile plasma zinc concentrations (Table 8.4). It may be postulated that this reciprocity reflects the maintenance of zinc homeostasis during progressive

airway inflammation, with labile zinc moving from plasma into the airway, and in response, zinc from body tissues mobilised into fixed stores in plasma (mirrored in the rising total plasma zinc measurements). Interestingly, unlike plasma zinc, while labile sputum zinc concentrations fell with worsening lung function and increasing severity of asthma, total sputum zinc concentrations did not demonstrate a clear relationship. Possible explanations for this discrepancy between plasma and sputum zinc measurements with respect to lung function include sample size, with sputum zinc measurements requiring a larger subject sample to detect change. Secondly, compartmental fluxes in zinc may equilibrate more rapidly in plasma than in sputum possibly providing an explanation for our results. Lastly, the measurements of total and labile zinc may be subject to less variability (smaller coefficient of variation with the test) in plasma than in sputum for reasons that are yet to be elucidated.

The similar total and differential cell counts in subjects with and without asthma (Table 8.5), lend support to a previous epidemiological study demonstrating that in the community, induced sputum eosinophil cell counts in subjects reporting asthma were most often within the normal range and not sufficiently abnormal to be useful in validating a diagnosis of asthma(161). A robust relationship was, however, observed between declining FEV_1 and increasing sputum neutrophil counts, and between increasing severity of asthma, based on NAC (2006) criteria, and increasing sputum neutrophil counts. These findings support previous studies where sputum neutrophilia was associated with more severe asthma and non steroid responsive asthma (152, 158, 159, 280, 292). Surprisingly unlike earlier studies, an association was not demonstrated between sputum eosinophils and asthma symptoms, lung function or asthma severity (158, 159, 162, 280).

Assessment of zinc status has included measuring concentrations in plasma, hair and bronchoalveolar lavage fluid (BAL)(46, 293).Plasma and hair measurements reflect total body zinc concentrations and do not necessarily reflect airway zinc levels. Bronchoscopy with bronchoalveolar fluid measurements, on the other hand, provides a direct, invasive method of examining the airway milieu and assessing zinc concentrations, but is not without risk to the patient. It is also a tool that is not readily amenable to daily use. This study confirms that induced sputum examination provides a reliable, non invasive method of measuring airway zinc levels in subjects with and without asthma.

A considerable proportion of subjects had undetectable labile sputum zinc measurements, more notable in those with asthma. This may reflect the sensitivity of the Zinquin assay itself or possibly abnormalities with zinc transporter proteins as discussed above and in Chapter 7. Airway inflammation in asthma results in increased cell death or apoptosis with clearance of the cells and consequent zinc loss via sputum. Most subjects in this study had mild to moderate clinically stable asthma, and therefore the proportion of negligible labile sputum zinc measurements obtained in this study may actually reflect the absence of significant AI in these subjects.

Strengths of this study include the assessor blind, population based study design, which allows the results to be generalisable to the wider community of individuals with asthma. The definition of asthma used in this study, namely, self reported doctor diagnosed asthma and asthma defined by episodic symptoms and the presence of BDR or airway hyper- responsiveness, is broad and reflects real life, again making the results generalisable. Possible limitations may include firstly, that the findings from this population with predominantly mild to moderate, clinically stable asthma may not necessarily reflect the zinc status of individuals with asthma during an exacerbation, with more severe

asthma. Furthermore, the average age of subjects with asthma in this population was 55 years which may perhaps limit interpretation of the study results to older individuals with asthma.

In conclusion, this study has shown that stable mild to moderate asthma, using a global definition of asthma based on a combination of symptoms, doctor diagnosis, and objective testing, is associated with low concentrations of labile and total sputum zinc. Increasing severity of asthma appears to be associated with lower sputum labile zinc concentrations and progressive sputum neutrophilia but not eosinophilia. In combination, these findings strongly support the existence of a zinc deficient state or defective homeostasis in asthma which appears to worsen with increasing severity of asthma. The cross-sectional, epidemiological design of this study, while demonstrating a strong relationship between asthma and reduced zinc concentrations in sputum, does not allow assumptions regarding causality to be made. Longitudinal studies and randomised controlled studies comparing both total and labile sputum zinc concentrations and cell counts in subjects with asthma of varying severity and during exacerbations are required to elucidate this association further.

Table 8.1: Baseline subject characteristics

	Asthma n=114	No asthma n=49	p value
Male, n	48	24	0.64
Age, years	55 (14)	57 (14)	0. 47
Atopy (one or more positive) ,n	73	28	0.33
Inhaled steroid, n	34	0	0.00*
Long acting beta 2 agonist, n	18	0	0.01*
Current smokers Ex smokers	7 41	17 13	0.88 0.21
Pre BDFEV1, L Pre BD FEV1 % predicted	2.8 (0.81) 94.2(16)	3.0(0.79) 103(16.6)	0.047* 0.002*
Bronchodilator reversibility ,%	6.5 (7.3)	3.8 (3.4)	0.015*
PD_{15} mls geometric mean (SD)	10 (3.5)	50.1 (2.2)	0.000*

Mean (SD); *$p \leq 0.05$; n: number of subjects; BD: bronchodilator; FEV_1: forced expiratory volume in 1 second;%: percent; L: litres; PD_{15}: provocation dose resulting in 15% fall in FEV_1 from baseline; mls: milllitres

Table 8.2: Sputum and Plasma zinc concentrations in subjects without and with asthma

Zinc concentration, µmol/L	Asthma	No asthma	p value
Sputum			
Total zinc	0.49(1.8) [#] n =90	0.77 (2.9)[#] n =39	0.02*
Labile zinc	0.0 (0.74)[#] n=95	0.4 (1.3)[#] n =39	0.05*
Plasma			
Total zinc	11.8 (2.8) n= 112	11.3 (2.0) n =49	0.03*
Labile zinc	6.1(3.1) n =87	4.8 (3.2) n =38	0.04*

Mean (SD); [#] median (IQR);*p≤0.05; n=number of subjects; µmol/L: micromoles/litre

Table 8.3(a): Sputum zinc concentrations on and off medication in subjects with asthma.(b) Plasma zinc concentrations on and off medication in subjects with asthma.

(a) Sputum

ICS	Yes	No	p value
Total zinc, µmol/L	0.67(3.0)	0.31(1.7)	0.29
Labile zinc, µmol/L	0 (1.0)	0.15(0.85)	0.22
LABA			
Total zinc, µmol/L	0.60 (2.7)	0. 36(1.8)	0.46
Labile zinc, µmol/L	0(0.86)	0(0.86)	0.84

(b) Plasma

ICS	Yes	No	p value
Total zinc, µmol/L	11.9 (3.3)	11.7(2.0)	0.45
Labile zinc, µmol/L	7.2(2.7)	5.6(3.1)	0.63
LABA			
Total zinc, µmol/L	10.6(2.0)	12.0(2.9)	0.18
Labile zinc, µmol/L	6.6(2.9)	6.0(3.1)	0.86

Median(IQR); µmol/L:micromoles/litre;ICS: inhaled corticosteroid, n=34;

LABA:long acting beta 2 agonist,n=18.

Table 8.4: The relationship between sputum cell counts, sputum and plasma zinc concentrations in asthma and (a) symptom frequency and FEV_1, (b)asthma severity.

Symptom frequency	Sputum (T) zinc µmol/L	Sputum (L) zinc µmol/L	Plasma (T) zinc µmol/L	Plasma (L) zinc µmol/L	TCC 10^6/mL	Neut %	Macro %	log Eos %
None n=17	0(1.5)#	0.15# (2.4)	12.3 (2.3)	4.7 (1.8)	0.66# (1.3)	31 (26)	43 (28)	0 (0.1)
Occasional n=82	0.5# (1.8)	0# (0.81)	11.4 (2.5)	6.5 (3. 2)	0.8# (1.2)	24 (19)	45 (25)	0.11 (0.47)
Most days n= 4	0.6# (3.4)	0# (1.2)	13.5 (4.4)	4.8 (2.5)	0.54# (2.1)	33 (22)	34 (25)	0.25 (0.47)
p value	0.80	0.13	0.065	0.04	0.50#	0.38	0.41	0.81
FEV_1 % predicted								
>80% n=84	0.52# (1.7)	0.17# (1.3)	11.8 (2.6)	6.0 (3.0)	0.65# (1.0)	24 (19)	48 (26)	0.11 (0.32)
60-80% n=23	0.15# (7.4)	0# (0.21)	11 (2.0)	6.7 (3.3)	0.7# (1.0)	29 (21)	40 (21)	0 (0.74)
<60% n=5	0.36# (0.72)	0# (0)	15 (6.3)	3.4 (2.8)	3.6# (1.8)	68 (25)	17.7 (10)	0.68 (0.96)
p value	0.73	0.02	0.015	0.14	0.01	0.001	0.09	0.18

(b) Asthma severity by NAC (2006) criteria.

Asthma severity	Sputum (T) zinc µmol/L	Sputum (L) zinc µmol/L	Plasma (T) zinc µmol/L	Plasma (L) zinc µmol/L	TCC 10^6/mL	Neut %	Macro %	Log Eos %
intermittent and mild persistent n= 73	0.54 (1.7)#	0.16 (1.6)#	12 (2.6)#	6.0 (3.0)	0.68# (1.3)	23 (18)	47 (26)	0.06 (0.45)
moderate persistent n= 27	0.15 (3.3)#	0 (0.35)#	11(2.0)	6.6 (3.1)	0.7# (0.9)	27 (20)	45 (25)	0.16 (0.31)
severe n= 13	0.36 (2.7)#	0 (0.41)#	12 (4.6)	5.4 (3.1)	1.2# (3.3)	46 (33)	33 (20)	0.49 (0.58)
p value	0.37	0.04	0.27	0.53	0.50	0.016	0.37	0.108

Table (a): mean(SD); # median (IQR); FEV_1: forced expiratory volume in 1 second ;(T): total; (L): labile; TCC: total cell count; neut: neutrophil; macro: macrophage; % : percent ; µmol/L: micromoles/litre; mL: millilitres .

Table (b): mean(SD); # median (IQR); n= number of subjects; NAC: National Asthma Council; (T): total; (L): labile; TCC: total cell count; neut: neutrophil; macro: macrophage; % : percent ; µmol/L: micromoles/litre; mL: millilitres .

Table 8.5: Sputum total and differential cell counts in no asthma and asthma

	Asthma n= 102	No asthma n= 43	p value
Total cell count, 10^6/mL #	0.71(1.3)#	0.75(1.3)#	0.51
Viability, %	82 (20)	90(11)	0.01
Log eosinophil count ,%*	0.12(0.44)	0.07(0.47)	0.62
Neutrophil count, %*	26(21)	34(23)	0.06
Macrophage count, %*	45.4(26)	45.2(25)	0.97
Lymphocyte count, % #*	0(0)#	0(0)#	0.82
Columnar epithelial cells , % #*	0(0) #	0 (0) #	0.21

*differential cell count: no asthma, n=39, asthma, n=95 ;Mean (SD);

#median (Interquartile Range); n= number of subjects: mL: millilitre;%: percent

Table 8.6: Correlations (r) between sputum and plasma zinc and sputum differential cell counts in subjects with (a) asthma and (b) no asthma

(a)

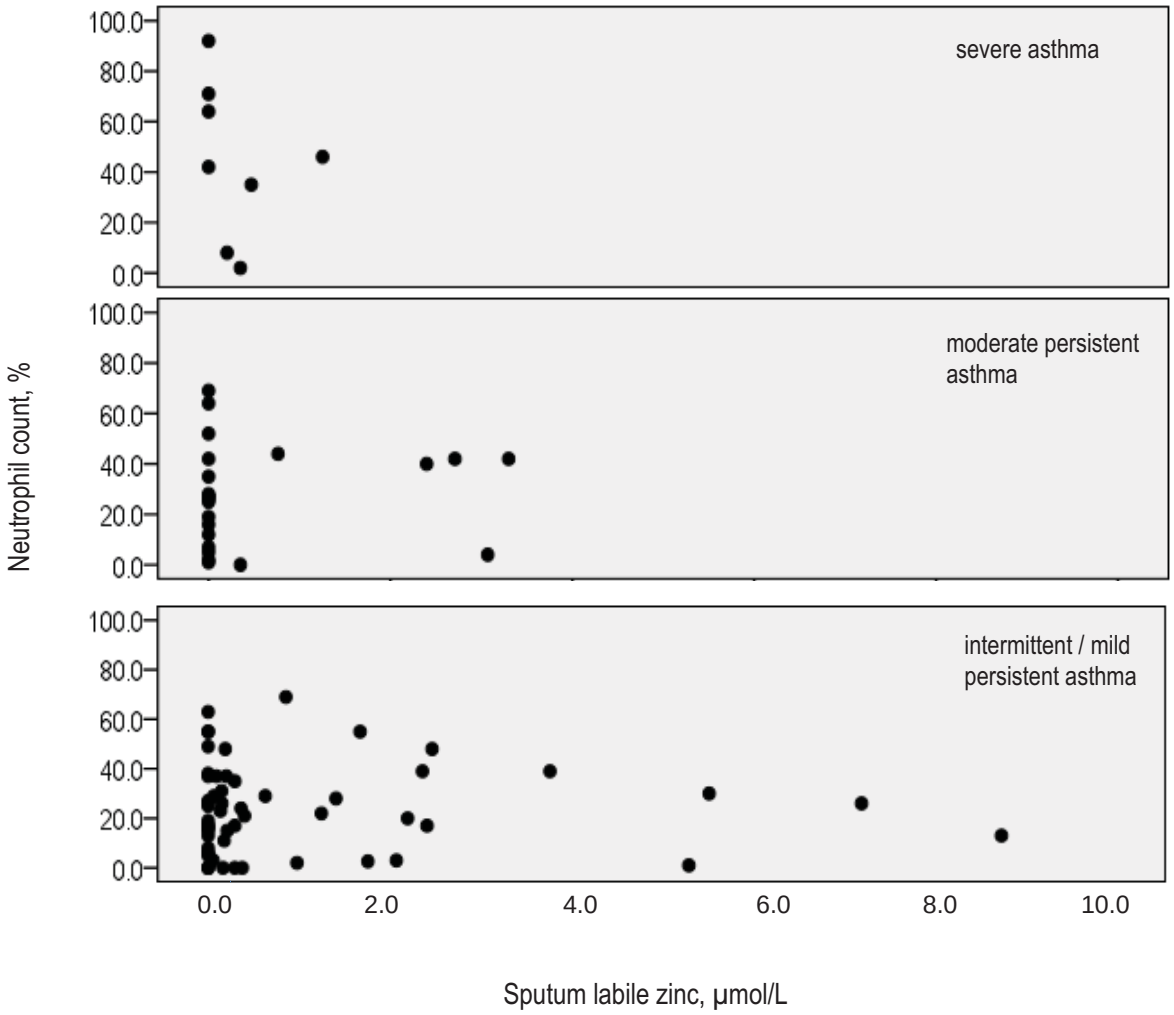
Asthma	TCC, 10^6/mL	Eos %	Neut %	Macro %	Lymph %	Columnar %
Total sputum zinc , µmol/L	0.18 p=0.09 n=90	0.030 p=0.79 n=88	0.014 p=0.90 n=87	0.02, p=0.98 n=87	0.16, p=0.14 n=88	0.03, p=0.80 n=76
Labile sputum zinc,µmol/L	0.06, p=0.57 n=95	0.038, p=0.73 n=83	-0.045, p=0.68 n=38	0.014 p=0.9 n=82	0.037, p=0.74 n=83	0.007, p=0.95 n=81
Total plasma Zinc,µmol/L	0.17, p=0.08 n=101	0.090, p=0.41 n=88	0.13 p=0.23 n=89	0.09, p=0.41, n=87	0.004, p=0.97 n=88	0.33, p=0.76 n=87
Labile plasma Zinc µmol/L	0.18 p=0.11 n=83	0.029 p=0.79 n=87	0.014 p=0.90 n=87	0.09 p=0.41 n=87	0.16, p=0.14 n=87	0.07, p=0.57 n=70

(b)

	TCC 10^6/mL	Eos %	Neut %	Macro %	Lymph %	Columnar %
Total sputum zinc , µmol/L	0.083 p=0.62 n=39	0.15 p=0.36 n=39	0.03 p=0.86 n=39	0.15 p=0.37 n=39	-0.009 p=0.96 n=33	-0.103 p=0.58 n=31
Labile sputum zinc,µmol/L	0.088 p=0.59 n=39	0.17 p=0.09 n=39	0.03 p=0.86 n=39	0.12 p=0.50 n=39	0.28 p=0.89 n=39	-0.104 p=0.58 n=30
Total plasma Zinc,µmol/L	0.23 p=0.48 n=39	0.14 p=0.64 n=39	0.30 p=0.07 n=39	-0.09 p=0.60 n=39	-0.009 p=0.96 n=39	0.012 p =0.95 n =33
Labile plasma Zinc µmol/L	0.16 p=0.37 n=35	0.14 p=0.39 n=39	0.22 p=0.23 n=39	-0.08 p=0.60 n=39	0.03 p=0.87 n=39	0.27 p=0.20 n=31

Tables (a) and (b) : r: correlation; n: number of subjects; TCC: total cell count; eos : eosinophil; neut: neutrophil; macro: macrophage; lymp: lymphocyte; µmol/L : micromoles/litre

Figure 8.1 : Labile zinc concentrations in sputum versus neutrophil counts grouped by asthma severity (NAC 2006) criteria



CHAPTER 9

Conclusions and future directions

The work in this thesis explored the accepted definitions of asthma and the role of objective measures of airway inflammation, namely induced sputum examination and eNO in the diagnosis of asthma in a community cohort. It also examined the perception of dyspnoea in subjects with and without airway hyper-responsiveness. Finally, methods to measure sputum zinc, increasingly considered to be an important inflammatory mediator in asthma, were optimised and applied to subjects with and without asthma from the same community cohort.

The findings confirm that asthma remains primarily a clinical diagnosis. Bronchodilator reversibility noted on spirometric criteria, airway hyper-responsiveness with a hypertonic saline challenge test, sputum eosinophilia on IS examination and a raised eNO measurement alone are insufficient to screen for, and to diagnose asthma in the community due to the poor sensitivity of these tests. Bronchodilator reversibility (BDR) is, nevertheless, the most specific test to aid a clinical diagnosis of asthma in the community, with the hypertonic saline challenge test having limited discriminative properties for the same purpose. The role of IS and eNO in the community relates to quantifying the underlying AI in asthma. An eNO measurement provides a global assessment relatively simply, while IS delineates the subtype of AI present to guide future investigations and management. While this thesis illustrates that a single IS and eNO measurement does not diagnose asthma, previous studies have demonstrated that the strength of these tools lie in monitoring AI longitudinally(37, 38). Sputum cell counts identify different aspects of AI which have different causes and can result in different clinical effects(31). Sputum eosinophilia is characteristic of atopic asthma and is steroid responsive. Non eosinophilic asthma, predominantly due to respiratory viral or bacterial infections, is generally not steroid responsive and alternative therapies may be required(31, 152, 294).

Exhaled nitric oxide, given its high specificity and ease of use, holds promise as a clinically useful tool in supporting a doctor diagnosis of asthma in the community, more so than induced sputum examination. As more simplified and economical eNO analysers become available, eNO may provide a viable alternative to spirometry with BDR to validate a clinical diagnosis of asthma in the adult community, especially in the General Practioner's surgery. Larger case controlled epidemiological and cross sectional studies are required to explore this. Contrastingly, in order for IS to be feasible in the community, research needs to be directed towards simplifying the induction process and the methodology, which currently requires time, laboratory space and staff. Future directions for sputum analysis include producing a kit which allows sputum sample processing to commence outside the laboratory, and efforts to automate the differential cell counts. Alternatively there may be chemical patterns found in sputum and in the exhaled breath condensate that can provide an estimate of cellular distributions.

A significant association was not demonstrated between perception of dyspnoea (POD) and underlying AI as measured by IS eosinophilia in subjects with and without AHR. It therefore cannot be used as a surrogate marker of AI in the community setting. In normal subjects without AHR, an unambiguous relationship was demonstrated between increased POD and eNO. The significance of this is uncertain, and needs to be interpreted with caution given the small sample size and the likelihood of this association being due to chance alone. It, however, raises the possibility that these subjects may have another source of inflammation apart from the airway, or may develop asthma in the future. Exhaled nitric oxide measurements may also reflect non eosinophilic AI. Longitudinal studies are required to firstly, determine if subjects without AHR and with increased POD and eNO levels develop AHR and overt asthma over time. Secondly, to determine if, clinical outcomes (frequency of respiratory symptoms, lung function, and quality of life) and sputum inflammatory

phenotypes vary in these 'healthy controls' with increased POD and eNO levels compared to those with normal POD and eNO levels. In extending this work, studies with a larger sample size are also required to clarify if a relationship does indeed exist between POD and eNO levels in subject with asthma. If present, eNO measurements may provide a viable method of assessing POD and possibly the underlying AI accurately.

This work has also highlighted the need for simple methods and tools with which to assess POD accurately in the community. A considerable proportion of subjects with asthma (20% in this study) over or under perceive their breathlessness which may lead to mis-diagnosis and subsequent over or under self- treatment and management by their medical practioners. It would be clinically relevant, for example, to determine whether POD measured by bronchoconstriction is equivalent to POD measured by bronchodilatation. If this is so, then spirometry with bronchodilator reversibility may be a useful tool with which to identify patients with altered POD (i.e.hypo-perceivers). These patients could then be monitored closely and encouraged to use Peak Expiratory Flow meters regularly as part of their asthma management plan. Education initiatives directed at both patients and their health providers in raising the awareness of this issue would be beneficial.

Induced sputum examination, as demonstrated by this thesis, lends itself to the measurement of airway zinc. Reduced sputum zinc levels were demonstrated in subjects with asthma compared with subjects without asthma. To date, the cause of low zinc levels in asthma remains unclear. Asthma may be associated with a low zinc state which may subsequently initiate and promote AI. Alternatively, the ongoing underlying AI in asthma may either trigger zinc loss or inhibit zinc loss from the airway as evidenced by low sputum zinc levels. If this hypothesis is correct then sputum zinc

levels may be proportional to the duration of asthma in an individual. Further studies are required to clarify and establish these causal links conclusively, both in animal models and in humans. More laboratory based studies delineating the zinc pathway and zinc homeostasis, especially with respect to loss of airway zinc are necessary. Key areas of research include studies elucidating the zinc transporter polymorphisms, of note ZIP 1 and ZIP14, and their role in the uptake of ingested zinc, the absorption of zinc into individual cells and the excretion of zinc from those cells. Studies correlating zinc transporter protein expression with plasma and sputum zinc levels in subjects with and without asthma are required. Subjects in the NWAHS cohort have recently given approval for their blood sample to be used for these genetic studies.

New techniques for measuring airway intracellular zinc have been developed in our laboratory and are currently being optimised. Previous work and the work from this thesis suggest that total and labile zinc measurements reflect different body compartments. Secondly, that labile zinc measurements are more sensitive to change in zinc status than total zinc measurements. It is however, important to measure both total and labile zinc in sputum and blood in any prospective studies of asthma to assess the role of total versus labile zinc measurements more fully. Studies measuring total and labile zinc levels in other body compartments simultaneously to the airway, both in animal models and in subjects with and without asthma, are also required to confirm the sputum and plasma zinc results obtained in this thesis and the postulated homeostatic mechanisms. These future directions in conjunction with genetic studies and the measurement of zinc in body fluids including sputum, should provide the tools with which to explicate the role of zinc in the airway and in asthma.

Finally, clinical studies are necessary clarify the function of zinc in asthma. Longitudinal studies would be useful to assess sputum and plasma zinc levels in subjects during periods of asthma stability and during exacerbations, and to correlate sputum zinc levels with clinical, physiological and inflammatory outcomes such as symptoms, quality of life, lung function, sputum cell counts and eNO measurements. Randomised, placebo controlled trials assessing the response of these same clinical and inflammatory outcomes to zinc supplementation in asthma would also provide insight.

Thus, asthma despite much debate is still best characterised by a combination of episodic symptoms, variable airflow limitation and underlying AI. Tools and methods which measure each of these components accurately are required to optimise management. Induced sputum examination and eNO have a niche role in the community at present. Research targeted at simplifying these techniques and their associated costs is likely to increase their availability and beneficial use in the community.

Appendices

NOTE:
The appendices are included on pages 187 - 195 of the print copy of the thesis held in the University of Adelaide Library.

Bibliography

1. Marketos S, Ballas C. Bronchial asthma in the medical literature of Greek Antiquity . J Asthma. 1982;19(4):263-9.

2. Chu E, Drazen J. Asthma: One Hundred Years of Treatment and Onward. Am J Respir Crit Care Med. 2005;171(11):1202-8.

3. American Thoracic Society. Definitions and classification of chronic bronchitis, asthma, and pulmonary emphysema. Am Rev Respir Dis. 1962;85:762-8.

4. Fletcher C, Gilson J, Hugh Jones P, Scadding J. Terminology, by the Committee definitions and classifications of chronic emphysema and related conditions : a report of the conclusions of a CIBA Guest Symposium. Thorax. 1959;14:286-99.

5. Porter R, Birch J, eds. Identification of Asthma. Ciba Foundation Study Group No 38. Edinburgh and London: Churchill Livingstone; 1971.

6. Pride N. Definitions of emphysema, chronic bronchitis, asthma and airflow obstruction: 25 years on from the Ciba symposium. Thorax. 1984;39:81-5.

7. Hargreave FE, Parameswaran K. Asthma, COPD and bronchitis are just components of airway disease. Eur Respir J. 2006 August 1, 2006;28(2):264-7.

8. National Asthma Council of Australia. Asthma Management Handbook 2006. Melbourne: National Asthma Council of Australia Ltd

9. National Heart Lung Blood Institute WHO. Proceedings of NHLBI/WHO workshop: Global Strategy for asthma management and prevention. 1997.

10. Burney P. Interpretation of epidemiological surveys of asthma. Ciba Found Symp. 1997;206:111-8.

11. Peat JK, Toelle BG, Marks GB, Mellis CM. Continuing the debate about measuring asthma in population studies. Thorax. 2001;56(5):406-11.

12. Toelle BG, Peat JK, van den Berg RH, Dermand J, Woolcock AJ. Comparison of three definitions of asthma: a longitudinal perspective. J Asthma. 1997;34:161-7.

13. Toelle BG, Peat JK, Salome CM, Mellis CM, Woolcock AJ. Toward a definition of asthma for epidemiology. Am Rev Respir Dis. 1992;146:633-7.

14. Enarson DA, Vedal S, Schulzer M, Dybuncio A, Chan-Yeung M. Asthma, asthma like symptoms, chronic bronchitis, and the degree of bronchial hyperresponsiveness in epidemiologic surveys. Am Rev Respir Dis. 1987;136(3):613-7.

15. Pattemore PK, Asher MI, Harrison AC, Mitchell EA, Rea HH, Stewart AW. The interrelationship among bronchial hyperresponsiveness, the diagnosis of asthma, and asthma symptoms. Am Rev Respir Dis. 1990;142(3):549-54.

16. Burr ML. Diagnosing asthma by questionnaire in epidemiological surveys. Clin Exp Allergy. 1992;22(5):509-10.

17. Pekkanen J, Pearce N. Defining asthma in epidemiological studies. Eur Respir J. 1999;14(4):951-7.

18. Taylor DR. Making the diagnosis of asthma. BMJ. 1997;315(7099):4-5.

19. Australian Centre for Asthma Monitoring. Asthma in Australia: Woolcock Institute of Medical Research and Australian Institute of Health and Welfare, Australian Government 2005 Contract No.: Document Number|.

20. Australian Centre for Asthma Monitoring. Asthma in Australia 2008. AIHW Asthma Series No 3. Canberra.

21. Wilson D, Adams RJ, Appleton SL, Hugo G, Wilkinson D, Hiller J, et al. Prevalence of asthma and asthma action plans in South Australia: population surveys from 1990 to 2001. Med J Aust. 2003;178(10):483-5.

22. Population Research and Outcome Studies Unit. Asthma Prevalence comparing SAMSS and HOS data sources 2008: South Australian Health, Contract No.: Document Number|.

23. Mellis C.M. PJK, Bauman A.E., Woolcock A.J.,. The cost of asthma in New South Wales. Med J Aust. 1991;155(8):522-8.

24. Woods RK, Walters EH, Wharton C, Watson N, Abramson M. The rising prevalence of asthma in young Melbourne adults is associated with improvement in treatment. Ann Allergy Asthma Immunol. 2001;87(2):89-90.

25. Boulet LP, Becker A, Berube D, Beveridge R, Ernst P. Canadian asthma consensus report. CMAJ. 1999;161(11suppl):1-61.

26. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, et al. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. Chest. 1997;112(1):45-52.

27. Bousquet J, Vignola AM, Chanez P, Campbell AM, Bonsingore G, Michel FB. Airways remodelling in asthma: no doubt, no more? Int Arch Allergy Immunol. 1995;107:211-4.

28. Redington AE, Howarth PH. Airway wall remodelling in asthma. Thorax. 1997; 52: 310-2.

29. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. Am J Respir Crit Care Med. 1999;159(4 Pt 1):1043-51.

30. Brown PJ GH, Finucane KE. Asthma and irreversible airflow obstruction. Thorax. 1984;39:131-6.

31. Jayaram L, Parameswaran K, Sears MR, Hargreave FE. Induced sputum cell counts: their usefulness in clinical practice. Eur Respir J. 2000;16(1):150-8.

32. Turner S. Exhaled nitric oxide in the diagnosis and management of asthma. Curr Opin Allergy Clin Immunol. 2008;8:70-6.

33. Louis R, Sele J, Henket M, Cataldo D, Bettiol J, Seiden L, et al. Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness. Allergy. 2002;57(10):907-12.

34. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology. 2006;11(1):54-61.

35. Jones SL, Herbison P, Cowan JO, Flannery EM, Hancox RJ, McLachlan CR, et al. Exhaled NO and assessment of anti-inflammatory effects of inhaled steroid: dose-response relationship. Eur Respir J. 2002;20(3):601-8.

36. Jones SL, Kittelson J, Cowan JO, Flannery EM, Hancox RJ, McLachlan CR, et al. The predictive value of exhaled nitric oxide measurements in assessing changes in asthma control. Am J Respir Crit Care Med. 2001;164(5):738-43.

37. Petsky H, Cates C, Li A, Kynaston J, Turner C, Chang A. Tailored interventions based on exhaled nitric oxide versus clinical symptoms for asthma in children and adults. Cochrane Database Syst Rev. 2008;(2):CD006340.

38. Petsky H, Kynaston J, Turner C, Li A, Cates C, Lasserson T, et al. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. Cochrane Database Syst Rev. 2007;(2):CD005603.

39. Parameswaran K, Leigh R, Hargreave FE. Sputum eosinophil count to assess compliance with corticosteroid therapy in asthma. J Allergy Clin Immunol. 1999;104(2 Pt 1):502-3.

40. Eckert DJ, Catcheside PG, Smith JH, Frith PA, McEvoy RD. Hypoxia Suppresses Symptom Perception in Asthma. Am J Respir Crit Care Med. 2004;169(11):1224-30.

41. Nowobilski R, Furgal M, Czyz P, De Barbaro B, Polczyk R, Bochenek G, et al. Psychopathology and personality factors modify the perception of dyspnea in asthmatics. J Asthma. 2007;44(3):203-7.

42. in't Veen J, Smits H, Ravsensberg A, Hiemstra P, Sterk P, Bel E. Impaired Perception of Dyspnea in Patients with Severe Asthma . Relation to Sputum Eosinophils. Am J Respir Crit Care Med. 1998;158(4):1134-41.

43. Jang A, Choi I. Relationship between the perception of dyspnoea and airway inflammatory markers. Respir Med. 2002;96(3):150-4.

44. Aronsson D, Tufvesson E, Bjermer L. Allergic rhinitis with or without concomitant asthma: difference in perception of dyspnoea and levels of fractional exhaled nitric oxide. Clin Exp Allergy. 2005;35(11):1457-61.

45. Jackson M. The art of medicine, Asthma, illness and identity. Lancet. 2008;372:1030-1.

46. Murgia C, Lang CJ, Truong-Tran AQ, Grosser D, Jayaram L, Ruffin RE, et al. Zinc and its specific transporters as potential targets in airway disease. Curr Drug Targets. 2006 May;7(5):607-27.

47. Soutar A, Seaton A, Brown K. Bronchial reactivity and dietary antioxidants. Thorax. 1997; 52:166-70.

48. de Luis D, Izaola O, Aller R, Armentia A, Cuellar L. Antioxidant and fat intake in patients with polinic asthma. Med Clin (Barc). 2003;121(17):653-4.

49. Zalewski PD, Truong-Tran AQ, Grosser D, Jayaram L, Murgia C, RE. R. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. A review. Pharmacol Ther 2005;105(2):127-49. .

50. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev. 1993;73:79-118.

51. Inagaki K, Mikuriya N, Morita S, Haraguchi H, Nakahara Y, Hattori M, et al. Speciation of protein-binding zinc and copper in human blood serum by chelating resin pre-treatment and inductively coupled plasma mass spectrometry. Analyst. 2000;125(1):197-203.

52. Zalewski P, Truong-Tran A, Lincoln S, Ward D, Shankar A, Coyle P, et al. Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media. Biotechniques. 2006; 40(4):509-20.

53. Boulet L, Leblanc P, Turcotte H. Perception scoring of induced bronchoconstriction as an index of awareness of asthma symptoms. Chest. 1994;105(5):1430-3.

54. Jenkins MA, Clarke JR, Carlin JB, Robertson CF, Hopper JL, Dalton MF, et al. Validation of questionnaire and bronchial hyperresponsiveness against respiratory physician assessment in the diagnosis of asthma. Int J Epidemiol. 1996;25(3):609-16.

55. Haley KJ, Drazen JM. Inflammation and airway function in asthma: what you see is not necessarily what you get. Am J Respir Crit Care Med. 1998;157:1-3.

56. Faul JL, Demers EA, Burke CM, Poulter LW. The reproducibility of repeat measures of airway inflammation in stable atopic asthma. Am J Respir Crit Care Med. 1999;160:1457-61.

57. Hargreave FE. The investigation of airway inflammation in asthma: sputum examination. Clin Exp Allergy. 1997;27 Suppl 1:36-40.

58. Kips JC, Fahy JV, Hargreave FE, Ind PW, in't Veen JC. Methods for sputum induction and analysis of induced sputum: a method for assessing airway inflammation in asthma. Eur Respir J Suppl. 1998;26:9S-12S.

59. Charlton I, Charlton G, Broomfiled J, Mullee MA. Evaluation of peak flow and symptoms only self management plans for control of asthma in general practice. BMJ. 1990;301(6765):1355–9.

60. Gibson PG. Monitoring the patient with asthma: an evidence-based approach. J Allergy Clin Immunol. 2000;106(1 Pt 1):17-26.

61. Turner MO, Taylor DR, Bennett R, Fitzgerald JM. A Randomized Trial Comparing Peak Expiratory Flow and Symptom Self-management Plans for Patients with Asthma Attending a Primary Care Clinic. Am J Respir Crit Care Med. 1998;157(2):540-6.

62. Bateman ED, Boushey HA, Bousquet J, Busse WW, Clark TJH, Pauwels RA, et al. Can Guideline-defined Asthma Control Be Achieved?: The Gaining Optimal Asthma ControL Study. Am J Respir Crit Care Med. 2004;170(8):836-44.

63. Juniper EF, Bousquet J, Abetz L, Bateman ED. Identifying 'well-controlled' and 'not well-controlled' asthma using the Asthma Control Questionnaire. Respir Med. 2006;100(4):616-21.

64. Rubinfeld AR, Pain MC. Perception of asthma. Lancet. 1976;1(7965):882-4.

65. Burdon JG, Juniper EF, Killian KJ, Hargreave FE, Campbell EJ. The perception of breathlessness in asthma. Am Rev Respir Dis. 1982;126(5):825-8.

66. Burdon J, Pain M, Rubinfeld A, Nana A. Chronic lung diseases and the perception of breathlessness: a clinical perspective. Eur Respir J. 1994;7(7):1342-9.

67. Teeter JG, Bleecker ER. Relationship between airway obstruction and respiratory symptoms in adult asthmatics. Chest. 1998;113:272-7.

68. Hanania N. Revisiting asthma control: how should it best be defined? Pulm Pharmacol Ther. 2007;20(5): 483-92.

69. Pizzichini E, Pizzichini MM, Efthimiadis A, Dolovich J, Hargreave FE. Measuring airway inflammation in asthma: eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. J Allergy Clin Immunol. 1997;99(4):539-44.

70. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. Thorax. 1998;53(2):91-5.

71. Lim S, Jatakanon A, Meah S, Oates T, Chung KF, Barnes PJ. Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in mild to moderately severe asthma. Thorax. 2000;55(3):184-8.

72. Gibson PG, Saltos N, Borgas T. Airway mast cells and eosinophils correlate with clinical severity and airway hyperresponsiveness in corticosteroid-treated asthma. J Allergy Clin Immunol. 2000;105(4):752-9.

73. van Rensen ELJ, Sont JK, Evertse CE, Willems LNA, Mauad T, Hiemstra PS, et al. Bronchial CD8 Cell Infiltrate and Lung Function Decline in Asthma
Am J Respir Crit Care Med. 2005;172(7):837-41.

74. Joos GF, O'Connor B, Anderson SD, Chung F, Cockcroft DW, Dahlen B, et al. Indirect airway challenges. Eur Respir J. 2003;21(6):1050-68.

75. Cockcroft D. Bronchoprovocation methods: direct challenges. Clin Rev Allergy Immunol. 2003;24(1):19-26.

76. Juniper EF, Frith PA, Dunnett C, Cockcroft DW, Hargreave FE. Reproducibility and comparison of responses to inhaled histamine and methacholine. Thorax. 1978;33(6):705-10.

77. Woolcock AJ. Expression of results of airway hyperresponsiveness. In: Hargreave F, Woolcock, AJ editors., editor. Airway Hyperresponsiveness Measurement and Interpretation: Astra Pharmaceuticals Canada Ltd,; 1985. p. 80–90.

78. Yan K, Salome C, Woolcock AJ. Rapid method for measurement of bronchial responsiveness. Thorax. 1983;38(10):760-5.

79. Verma VK, Cockcroft DW, Dosman JA. Airway responsiveness to inhaled histamine in chronic obstructive airways disease. Chronic bronchitis vs emphysema. Chest. 1988;94(3):457-61.

80. Ramsdale EH, Hargreave FE. Differences in airway responsiveness in asthma and chronic airflow obstruction. Med Clin North Am. 1990;74(3):741-51.

81. Cockcroft DW, Hargreave FE. Airway hyperresponsiveness. Relevance of random population data to clinical usefulness. Am Rev Respir Dis. 1990;142(3):497-500.

82. Anderson S, Brannan J. Methods for "indirect" challenge tests including exercise, eucapnic voluntary hyperpnea, and hypertonic aerosols. Clin Rev Allergy Immunol 2003;24(1):27-54.

83. Doull J, Sandall D, Smith S, Schreiber J, Freezer NJ, Holgate ST. Differential inhibitory effect of regular inhaled corticosteroid on airway responsiveness to adenosine 5' monophosphate, methacholine, and bradykinin in symptomatic children with recurrent wheeze. Pediatr Pulmonol. 1997;23(6):404-11.

84. Rabone S, Phoon W, Anderson S, Wan K, Seneviratne M, Gutierrez L, et al. Hypertonic saline challenge in an adult epidemiological survey. Occup Med (Lond). 1996;46(3):177-85.

85. Belcher NG, Lee TH, Rees PJ. Airway responses to hypertonic saline, exercise and histamine challenges in bronchial asthma. Eur Respir J. 1989;2(1):44-8.

86. Makker HK, Holgate ST. Relation of the hypertonic saline responsiveness of the airways to exercise induced asthma symptom severity and to histamine or methacholine reactivity. Thorax. 1993;48(2):142-7.

87. Salome CM, Peat JK, Britton WJ, Woolcock AJ. Bronchial hyperresponsiveness in two populations of Australian schoolchildren.Relation to respiratory symptoms and diagnosed asthma. Clin Allergy. 1987;17(4):271-8.

88. Riedler J, Reade T, Dalton M, Holst D, Robertson C. Hypertonic saline challenge in an epidemiologic survey of asthma in children. Am J Respir Crit Care Med 1994;150:1632-9.

89. Anderson SD, Brannan J, Spring J, Spalding N, Rodwell LT, Chan K, et al. A New Method For Bronchial-provocation Testing in Asthmatic Subjects Using a Dry Powder of Mannitol. Am J Respir Crit Care Med. 1997;156(3):758-65.

90. Brannan JD, Anderson SD, Perry CP, Freed-Martens R, Lassig AR, Charlton B, et al. The safety and efficacy of inhaled dry powder mannitol as a bronchial provocation test for airway hyperresponsiveness: a phase 3 comparison study with hypertonic (4.5%) saline. Respir Res. 2005;6(144):1-12.

91. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. American Journal of Respiratory & Critical Care Medicine. 1998;157(1):4-9.

92. Silvestri M, Spallarossa D, Battistini E, Brusasco V, Rossi GA. Dissociation between exhaled nitric oxide and hyperresponsiveness in children with mild intermittent asthma. Thorax. 2000;55(6):484-8.

93. Iredale MJ, Wanklyn SA, Phillips IP, Krausz T, Ind PW. Non-invasive assessment of bronchial inflammation in asthma: no correlation between eosinophilia of induced sputum and bronchial responsiveness to inhaled hypertonic saline. Clin Exp Allergy. 1994;24(10):940-5.

94. Pacini F, Filippelli M, Duranti R, Rosi E, Romagnoli I, Grazzini M, et al. Reduction in bronchodilation following a deep inhalation is poorly related to airway inflammation in asthma. Eur Respir J. 1999;14(5):1055-60.

95. Choi I, Hong S, Lee Y, Koh YI, Jang A, Lee H. Asthmatic airway inflammation is more closely related to airway hyperresponsiveness to hypertonic saline than to methacholine. Korean J Intern Med. 2003;18(2):83-8.

96. Joos GF. Bronchial hyperresponsiveness: too complex to be useful? Curr Opin Pharmacol. 2003;3(3):233-8.

97. Polosa R, Li Gotti F, Mangano G, Mastruzzo C, Pistorio MP, Crimi N. Monitoring of seasonal variability in bronchial hyper-responsiveness and sputum cell counts in non-asthmatic subjects with rhinitis and effect of specific immunotherapy. Clin Exp Allergy. 2003;33(7):873-81.

98. Gronke L, Kanniess F, Holz O, Jorres RA, Magnussen H. The relationship between airway hyper-responsiveness, markers of inflammation and lung function depends on the duration of the asthmatic disease. Clin Exp Allergy. 2002;32(1):57-63.

99. Smith CM, Anderson SD. Inhalation provocation tests using nonisotonic aerosols. J Allergy Clin Immunol. 1989;84(5 Pt 1):781-90.

100. Jones PD, Hankin R, Simpson J, Gibson PG, Henry RL. The tolerability, safety, and success of sputum induction and combined hypertonic saline challenge in children. Am J Respir Crit Care Med. 2001;164(7):1146-9.

101. Vlachos-Mayer H, Leigh R, Sharon RF, Hussack P, Hargreave FE. Success and safety of sputum induction in the clinical setting. Eur Respir J. 2000;16(5):997-1000.

102. Pizzichini E, Pizzichini MM, Leigh R, Djukanovic R, Sterk PJ. Safety of sputum induction. Eur Respir J Suppl. 2002;37:9s-18s.

103. Horn B, Robin ED, Theodore J, Van Kessel A. Total eosinophil counts in the management of bronchial asthma. N Engl J Med. 1975;292(22):1152-5.

104. Ulrik C. Peripheral eosinophil counts as a marker of disease activity in intrinsic and extrinsic asthma. Clin Exp Allergy. 1995;25(9):820-7.

105. Taylor KJ, Luksza AR. Peripheral blood eosinophil counts and bronchial responsiveness. Thorax. 1987;42(6):452-6.

106. Niimi A, Amitani R, Suzuki K, Tanaka E, Murayama T, Kuze F. Serum eosinophil cationic protein as a marker of eosinophilic inflammation in asthma. Clin Exp Allergy. 1998;28(2):233-40.

107. Venge P. Monitoring of asthma inflammation by serum measurements of eosinophil cationic protein (ECP). A new clinical approach to asthma management. Respir Med. 1995;89(1):1-2.

108. Piacentini GL, Bodini A, Costella S, Vicentini L, Mazzi P, Sperandio S, et al. Exhaled nitric oxide and sputum eosinophil markers of inflammation in asthmatic children. Eur Respir J. 1999;13(6):1386-90.

109. Sorva R, Metso T, Turpeinen M, Juntunen-Backman K, Bjorksten F, Haahtela T. Eosinophil cationic protein in induced sputum as a marker of inflammation in asthmatic children. Pediatr Allergy Immunol. 1997;8(1):45-50.

110. Leigh R, Belda J, Kelly MM, Cox G, Squillace DL, Gleich GJ, et al. Eosinophil cationic protein relates to sputum neutrophil counts in healthy subjects. J Allergy Clin Immunol. 2000;106(3):593-4.

111. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. Am J Respir Crit Care Med. 2005;171(8):912-30.

112. Silkoff PE, Erzurum SC, Lundberg JO, George SC, Marczin N, Hunt JF, et al. ATS workshop proceedings: exhaled nitric oxide and nitric oxide oxidative metabolism in exhaled breath condensate. Proc Am Thorac Soc. 2006;3(2):131-45.

113. Taylor DR. Exhaled NO: Forward, Backward, or Sideways? Am J Respir Crit Care Med. 2007;176(3):221-2.

114. Taylor DR, Pijnenburg MW, Smith AD, De Jongste JC. Exhaled nitric oxide measurements: clinical application and interpretation. Thorax. 2006;61(9):817-27.

115. Travers J, Marsh S, Aldington S, Williams M, Shirtcliffe P, Pritchard A, et al. Reference ranges for exhaled nitric oxide derived from a random community survey of adults. Am J Respir Crit Care Med. 2007;176(3):238-42.

116. Pavord ID, Shaw D. The use of exhaled nitric oxide in the management of asthma. J Asthma. 2008;45(7):523-31.

117. Bates CA, Silkoff PE. Exhaled nitric oxide in asthma: from bench to bedside. J Allergy Clin Immunol. 2003;111(2):256-62.

118. Van Den Toorn L, Overbeek S, De Jongste J, Leman K, Hoogstenden HC, Prins J. Airway Inflammation Is Present during Clinical Remission of Atopic Asthma. Am J Respir Crit Care Med. 2001 December 1, 2001;164(11):2107-13.

119. Silkoff PE, McClean P, Spino M, Erlich L, Slutsky AS, Zamel N. Dose-response relationship and reproducibility of the fall in exhaled nitric oxide after inhaled beclomethasone dipropionate therapy in asthma patients. Chest. 2001;119(5):1322-8.

120. Shaw DE, Berry MA, Thomas M, Green RH, Brightling CE, Wardlaw AJ, et al. The Use of Exhaled Nitric Oxide to Guide Asthma Management: A Randomized Controlled Trial. Am J Respir Crit Care Med. 2007 August 1, 2007;176(3):231-7.

121. Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. Standardised methodology of sputum induction and processing. Eur Respir J Suppl. 2002;37:1s-55s.

122. Wong HH, Fahy JV. Safety of one method of sputum induction in asthmatic subjects. Am J Respir Crit Care Med. 1997;156:299-303.

123. Pizzichini MM, Pizzichini E, Clelland L, Efthimiadis A, Mahony J, Dolovich J, et al. Sputum in severe exacerbations of asthma: kinetics of inflammatory indices after prednisone treatment. Am J Respir Crit Care Med. 1997;155(5):1501-8.

124. Pizzichini MM, Popov TA, Efthimiadis A, Hussack P, Evans S, Pizzichini E, et al. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. Am J Respir Crit Care Med. 1996;154(4 Pt 1):866-9.

125. Moodley YP, Krishnan V, Lalloo UG. Neutrophils in induced sputum airse from central airways. Eur Respir J. 2000;15:36-40.

126. Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. Am J Respir Crit Care Med. 1995;152:53-8.

127. Grootendorst DC, Sont JK, Willems LN, Kluin-Nelemans JC, Van Krieken JH, Veselic-Charvat M, et al. Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. Clin Exp Allergy. 1997;27(7):769-79.

128. in 't Veen JC, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, et al. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. Eur Respir J. 1996 Dec;9(12):2441-7.

129. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. Thorax. 1997;52(4):372-4.

130. Maestrelli P, Seatta M, di Stefano A, Calcangi PG, Turato G, Ruggieri MP et al. Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoaleveolar lavage. Am J Respir Crit Care Med. 1995;152:1926-31.

131. Pizzichini E, Pizzichini MM, Kidney JC, Efthimiadis A, Hussack P, Popov T, et al. Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subsets and soluble markers compared. Eur Respir J. 1998;11(4):828-34.

132. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. Am J Respir Crit Care Med. 1996;154(2 Pt 1):308-17.

133. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. Thorax. 1992;47(1):25-9.

134. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and healthy individuals. Am Rev Respir Dis. 1993;146:1126-31.

135. Gibson PG, Girgis-Gabardo A, Morris MM, Mattoli S, Kay JM, Dolovich J, et al. Cellular characteristics of sputum from patients with asthma and chronic bronchitis. Thorax. 1989;44(9):693-9.

136. Popov T, Gottschalk R, Kolendowicz R, Dolovich J, Powers P, Hargreave FE. The evaluation of a cell dispersion method of sputum examination. Clin Exp Allergy. 1994;24(8):778-83.

137. Belda J, Leigh R, Parameswaran K, O'Byrne PM, Sears MR, Hargreave FE. Induced sputum cell counts in healthy adults. Am J Respir Crit Care Med. 2000;161(2 Pt 1):475-8.

138. Spanevello A, Migliori GB, Sharara A, Ballardini L, Bridge P, Pisati P, et al. Induced sputum to assess airway inflammation: A study of reproducibility. Clinical & Experimental Allergy. 1997;27(10):1138-44.

139. Efthimiadis A, Spanevello A, Hamid Q, Kelly MM, Linden M, Louis R, et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. European Respiratory Journal - Supplement. 2002;20(37):19s-23s.

140. Spanevello A, Confalonieri M, Sulotto F, Romano F, Balzano G, Migliori GB, et al. Induced sputum cellularity: Reference values and distribution in normal volunteers. American Journal of Respiratory & Critical Care Medicine. 2000;162(3):1172-4.

141. Balbi B, Pignatti P, Corradi M, Baiardi P, Bianchi L, Brunetti G, et al. Bronchoalveolar lavage, sputum and exhaled clinically relevant inflammatory markers: values in healthy adults. Eur Respir J. 2007 October 1, 2007;30(4):769-81.

142. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. Thorax. 1999;54(2):108-14.

143. Jatakanon A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. Am J Respir Crit Care Med. 2000;161(1):64-72.

144. Lim S, Jatakanon A, John M, Gilbey T, O'Connor B J, Chung KF, et al. Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. Am J Respir Crit Care Med. 1999;159(1):22-30.

145. McIvor RA, Pizzichini E, Turner MO, Hussack P, Hargreave FE, Sears MR. Potential masking effects of salmeterol on airway inflammation in asthma. Am J Respir Crit Care Med. 1998;158(3):924-30.

146. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. Am J Respir Crit Care Med. 2000 Jan;161(1):9-16.

147. Parameswaran K, Pizzichini E, Pizzichini MM, Hussack P, Efthimiadis A, Hargreave FE. Clinical judgement of airway inflammation versus sputum cell counts in patients with asthma. Eur Respir J. 2000;15(3):486-90.

148. Fahy JV, Boushey HA. Effect of low-dose beclomethasone dipropionate on asthma control and airway inflammation. Eur Respir J. 1998;11(6):1240-7.

149. Kelly MM, Leigh R, Jayaram L, Goldsmith CH, Parameswaran K, Hargreave FE. Eosinophilic bronchitis in asthma: a model for establishing dose-response and relative potency of inhaled corticosteroids. J Allergy Clin Immunol. 2006;117(5):989-94.

150. Turner MO, Johnston PR, Pizzichini E, Pizzichini MM, Hussack PA, Hargreave FE. Anti-inflammatory effects of salmeterol compared with beclomethasone in eosinophilic mild exacerbations of asthma: a randomized, placebo controlled trial. Can Respir J. 1998;5(4):261-8.

151. Meijer RJ, Postma DS, Kauffman HF, Arends LR, Koëter GH, Kerstjens HAM. Accuracy of eosinophils and eosinophil cationic protein to predict steroid improvement in asthma. Clinical & Experimental Allergy. 2002;32(7):1096-103.

152. Berry M, Morgan A, Shaw DE, Parker D, Green R, Brightling C, et al. Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. Thorax. 2007;62(12):1043-9.

153. Pizzichini MM, Pizzichini E, Parameswaran K, Clelland L, Efthimiadis A, Dolovich J, et al. Nonasthmatic chronic cough: No effect of treatment with an inhaled corticosteroid in patients without sputum eosinophilia. Can Respir J. 1999;6(4):323-30.

154. Kelly M, Leigh R, Hargreave FE. Validation of assays for inflammatory mediators in sputum. Eur Respir J. 2000;16(6):1208-9.

155. Kelly MM, Keatings V, Leigh R, Peterson C, Shute J, Venge P, et al. Analysis of fluid-phase mediators. European Respiratory Journal - Supplement. 2002;20(37):24s-39s.

156. Simpson JL, Timmins NL, Fakes K, Talbot PI, Gibson PG. Effect of saliva contamination on induced sputum cell counts, IL-8 and eosinophil cationic protein levels. Eur Respir J. 2004;23(5):759-62.

157. Stockley RA, Bayley DL. Validation of assays for inflammatory mediators in sputum. Eur Respir J. 2000;15(4):778-81.

158. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet. 2002;360(9347):1715-21.

159. Jayaram L, Pizzichini MM, Cook RJ, Boulet LP, Lemiere C, Pizzichini E, et al. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. Eur Respir J. 2006;27(3):483-94.

160. Louis R, Sele J, Henket M, Cataldo D, Bettiol J, Seiden L, et al. Sputum eosinophil count in a large population of patients with mild to moderate steroid-naive asthma: distribution and relationship with methacholine bronchial hyperresponsiveness. Allergy. 2002 Oct;57(10):907-12.

161. Lemiere C, Walker C, O'Shaughnessy D, Efthimiadis A, Hargreave FE, Sears MR. Differential cell counts in sputum in respiratory epidemiology: a pilot study. Chest. 2001;120(4):1107-13.

162. Gibson PG, Wlodarczyk JW, Hensley MJ, Gleeson M, Henry RL, Cripps AW, et al. Epidemiological association of airway inflammation with asthma symptoms and airway hyperresponsiveness in childhood. Am J Respir Crit Care Med. 1998;158(1):36-41.

163. Gibson PG, Simpson JL, Hankin R, Powell H, Henry RL. Relationship between induced sputum eosinophils and the clinical pattern of childhood asthma. Thorax. 2003 Feb;58(2):116-21.

164. Cai Y, Carty K, Henry RL, Gibson PG. Persistence of sputum eosinophilia in children with controlled asthma when compared with healthy children. Eur Respir J. 1998;11(4):848-53.

165. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax. 2002;57(10):875-9.

166. Becker A, Lemiere C, Berube D, Boulet L-P, Ducharme FM, FitzGerald M, et al. Summary of recommendations from the Canadian Asthma Consensus Guidelines, 2003. CMAJ. 2005;173(6 suppl):S3-11.

167. British Thoracic Society Scottish Intercollegiate Guidelines Network. British Guideline on the Management of Asthma. Thorax. 2008;63(Suppl 4):iv1-121.

168. Wamboldt MZ, Bihun JT, Szefler S, Hewitt J. Perception of induced bronchoconstriction in a community sample of adolescents. J Allergy Clin Immunol. 2000;106(6):1102-7.

169. Tomita K, Hanaki K, Hasegawa Y, Watanabe M, Sano H, Igishi T, et al. Underrecognition of the severity of asthma and undertreatment of asthma in a rural area of Japan. J Asthma. 2005;42(8):689-96.

170. Rubinfeld AR, Pain MC. Relationship between bronchial reactivity, airway caliber, and severity of asthma. Am Rev Respir Dis 1977 115 (3):381-7.

171. Killian KJ, Watson R, Otis J, St. Amand TA, O'Byrne PM. Symptom Perception during Acute Bronchoconstriction. Am J Respir Crit Care Med. 2000;162(2):490-6.

172. Roisman GL, Peiffer C, Lacronique JG, Le Cae A, Dusser DJ. Perception of bronchial obstruction in asthmatic patients. Relationship with bronchial eosinophiic inflammation and epithelial damage and effect of corticosteroid treatment. J Clin Invest. 1995;1:12-21.

173. Scano G, Stendardi L. Dyspnea and asthma. Curr Opin Pulm Med. 2006;12(1):18-22.

174. McCloskey D. The effects of pre-existing loads upon detection of externally applied resistances to breathing in man. Clin Sci Mol Med. 1973;45(4):561-4.

175. Burdon J, Killian, KJ, Stubbing, DG, Campbell, EJ. Effect of background loads on the perception of added loads to breathing. J Appl Physiol 1983;54(5):1222-8.

176. Eckert DJ, Catcheside PG, McDonald R, Adams AM, Webster KE, Hlavac MC, et al. Sustained Hypoxia Depresses Sensory Processing of Respiratory Resistive Loads. Am J Respir Crit Care Med. 2005;172(8):1047-54.

177. Cockcroft A, Adams L. Measurement and mechanisms of breathlessness. Bull Eur Physiopathol Respir. 1986;22(1):85-92.

178. Wilson RC, Jones P. Differentiation between the intensity of breathlessness and the distress it evokes in normal subjects during exercise. Clin Sci (Lond)

1991;80 (1):65-70.

179. Adams L, Chronos N, Lane R, Guz A. The measurement of breathlessness induced in normal subjects: individual differences. Clin Sci (Lond). 1986;70(2):131-40.

180. Adams L, Lane R, Shea SA, Cockcroft A, Guz A. Breathlessness during different forms of ventilatory stimulation: a study of mechanisms in normal subjects and respiratory patients. Clin Sci (Lond). 1985;69(6):663-72.

181. Chronos N, Adams L, Guz A. Effect of hyperoxia and hypoxia on exercise-induced breathlessness in normal subjects. Clin Sci (Lond). 1988;74(5):531-7.

182. Boulet L, Cournoyer, I, Deschesnes, F, Leblanc, P, Nouwen, A. Perception of airflow obstruction and associated breathlessness in normal and asthmatic subjects: correlation with anxiety and bronchodilator needs. Thorax 1994 49 (10):965-70.

183. Arsenault JE, Brown KH. Zinc intake of US preschool children exceeds new dietary reference intakes. Am J Clin Nutr. 2003 Nov;78(5):1011-7.

184. Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M. Overview of mammalian zinc transporters. Cell Mol Life Sci. 2004;61(49-68).

185. Williams R. Zinc :what is its role in biology? Endeavour 8. 1984;65-70(2).

186. Eide DJ. Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta. 2006;1763(7):711-22.

187. Kabu K, Yamasaki S, Kamimura D, Ito Y, Hasegawa A, Sato E, et al. Zinc is required for Fc epsilon RI-mediated mast cell activation. J Immunol. 2006;177(2):1296-305.

188. Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T, et al. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat Immunol. 2006;7(9):971-7.

189. Ackland ML, Michalczyk A. Zinc deficiency and its inherited disorders -a review. Genes Nutr. 2006;1(1):41-9.

190. Reimold EW, Besch DJ. Detection and elimination of contaminations interfering with the determination of zinc in plasma. Clin Chem. 1978;24(4):675-80.

191. Liska S, Kerkay, J, Pearson, KH. Determination of zinc in whole blood, plasma and serum using Zeeman effect flame atomic absorption spectroscopy. Clin Chim Acta. 1985;151 (3):237-43.

192. Shaw J, Bury, AJ, Barber, A, Mann, L, Taylor, A. A micromethod for the analysis of zinc in plasma or serum by atomic absorption spectrophotometry using graphite furnace. Clin Chim Acta. 1982 118(2-3):229-39.

193. Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. Am J Clin Nutr. 1996;63(5):797S-811.

194. Zalewski PD, Forbes IJ, Seamark RF, Borlinghaus R, Betts WH, Lincoln SF, et al. Flux of intracellular labile zinc during apoptosis (gene-directed cell death) revealed by a specific chemical probe, Zinquin. Chem Biol. 1994;1(3):153-61.

195. Truong-Tran AQ, Grosser D, Ruffin RE, Murgia C, Zalewski P. Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation Biochem Pharmacol. 2003; 66(8):1459-68.

196. Shankar AH, Prasad AS. Zinc and immune function: The biological basis of altered resistance to infection. Am JClin Nutr. 1998; 68 (Suppl):447S–63S.

197. Prasad AS. Zinc in human health: effect of zinc on immune cells. Mol Med. 2008;14(5-6):353-7.

198. Novick SG, Godfrey JC, Pollack RL, Wilder HR. Zinc-induced suppression of inflammation in the respiratory tract, caused by infection with human rhinovirus and other irritants. Medical Hypotheses. 1997 Oct;49(4):347-57.

199. Richter M, Bonneau R, Girard MA, Beaulieu C, Larivee P. Zinc status modulates bronchopulmonary eosinophil infiltration in a murine model of allergic inflammation. Chest. 2003;123(3 Suppl):446S.

200. Wood RJ. Assessment of marginal zinc status in humans. J Nutr. 2000;130(5S Suppl):1350S-4S.

201. el-Kholy MS, Gas Allah MA, el-Shimi S, el-Baz F, el-Tayeb H, Abdel-Hamid MS. Zinc and copper status in children with bronchial asthma and atopic dermatitis. J Egypt Public Health Assoc. 1990;65(5-6):657-68.

202. Ellul-Micallef R, Galdes A, Fenech FF. Serum zinc levels in corticosteroid-treated asthmatic patients. Postgrad Med J. 1976;52(605):148-50.

203. Goldey DH, Mansmann HC, Jr., Rasmussen AI. Zinc status of asthmatic, prednisone-treated asthmatic, and non-asthmatic children. J Am Diet Assoc. 1984;84(2):157-63.

204. Anetor J, Ajose O, Ige O, Oyeleye A, Ojo P. Antioxidant status of adult Nigerian asthmatics: implications for prognosis. Nutr Health 2003;17(3)
221-9.

205. Kocyigit A, Armutcu F, Gurel A, Ermis B. Alterations in plasma essential trace elements selenium, manganese, zinc, copper, and iron concentrations and the possible role of these elements on oxidative status in patients with childhood asthma. Biol Trace Elem Res. 2004;97(1):31-41.

206. Vural H, Uzun K, Uz E, Kocyigit A, Cigli A, Akyol O. Concentrations of copper, zinc and various elements in serum of patients with bronchial asthma. J Trace Elem Med Biol. 2000 Jun;14(2):88-91.

207. Ward R, Woltmann G, Wardlaw AJ, Pavord ID. Between-observer repeatability of sputum differential cell counts. Influence of cell viability and squamous cell contamination. Clin Exp Allergy. 1999;29(2):248-52.

208. Duncan CJ, Lawrie A, Blaylock MG, Douglas JG, Walsh GM. Reduced eosinophil apoptosis in induced sputum correlates with asthma severity. Eur Respir J. 2003;22(3):484-90.

209. Polosa R, Renaud L, Cacciola R, Prosperini G, Crimi N, Djukanovic R. Sputum eosinophilia is more closely associated with airway responsiveness to bradykinin than methacholine in asthma. Eur Respir J. 1998;12(3):551-6.

210. Salome CM, Roberts AM, Brown NJ, Dermand J, Marks GB, Woolcock AJ. Exhaled nitric oxide measurements in a population sample of young adults. Am J Respir Crit Care Med. 1999;159(3):911-6.

211. Mattes J, Storm van's Gravesande K, Reining U, Alving K, Ihorst G, Henschen M, et al. NO in exhaled air is correlated with markers of eosinophilic airway inflammation in corticosteroid-dependent childhood asthma. Eur Respir J. 1999;13(6):1391-5.

212. Berry MA, Shaw DE, Green RH, Brightling CE, Wardlaw AJ, Pavord ID. The use of exhaled nitric oxide concentration to identify eosinophilic airway inflammation: an observational study in adults with asthma. Clin Exp Allergy. 2005;35(9):1175-9.

213. Grant JF, Taylor AW, Ruffin RE, Wilson DH, Phillips PJ, Adams RJ, et al. Cohort Profile: The North West Adelaide Health Study (NWAHS). Int J Epidemiol. 2008.

214. Ware JE, Sherbourne CD. The MOS 36-Item Short-Form Health Survey (SF-36®): I. conceptual framework and item selection. Med Care. 1992;30(6):473-83.

215. Guyatt GH, Townsend M, Berman LB, Keller JL. A comparison of Likert and visual analogue scales for measuring change in function. Journal of Chronic Diseases. 1987;40(12):1129-33.

216. Ruffin RE, Latimer KM, Schembri DA. Longitudinal study of near fatal asthma. Chest. 1991;99(1):77-83.

217. Boulet LP, Cournoyer I, Deschesnes F, Leblanc P, Nouwen A. Perception of airflow obstruction and associated breathlessness in normal and asthmatic subjects: correlation with anxiety and bronchodilator needs. Thorax. 1994;49(10):965-70.

218. American Thoracic Society. Standardization of spirometry.1994 Update. Am Rev Respir Dis. 1995;152:1107-36.

219. Crapo RO, Morris AH, RM. G. Reference spirometric values using techniques and equipment that meets ATS recommendation. Am Rev Respir Dis 1981;123 659-94.

220. Smith CM, Anderson SD. Inhalational challenge using hypertonic saline in asthmatic subjects: a comparison with responses to hyperpnoea, methacholine and water. Eur Respir J. 1990;3(2):144-51.

221. Taylor DR, Bateman ED, Boulet LP, Boushey HA, Busse WW, Casale TB, et al. A new perspective on concepts of asthma severity and control. Eur Respir J. 2008;32(3):545-54.

222. Shaw DE, Berry MA, Thomas M, Green RH, Brightling CE, Wardlaw AJ, et al. The use of exhaled nitric oxide to guide asthma management: a randomized controlled trial. Am J Respir Crit Care Med. 2007;176(3):231-7.

223. Hewitt RS, Modrich CM, Medlicott T, Cowan JO, Taylor DR. Supporting the diagnosis of non-specific respiratory symptoms in primary care: the role of exhaled nitric oxide measurement and spirometry. Prim Care Respir J. 2008;17(2):97-103.

224. Ulrik CS, Postma DS, Backer V. Recognition of asthma in adolescents and young adults: which objective measure is best? J Asthma. 2005;42(7):549-54.

225. Leuppi JD, Anderson SD, Brannan JD, Belousova E, Reddel HK, Rodwell LT. Questionnaire responses that predict airway response to hypertonic saline. Respiration. 2005 Jan-Feb;72(1):52-60.

226. Gibson PG. Tackling asthma phenotypes in community studies. Thorax. 2009;64(5):369-70.

227. Nadif R, Siroux V, Oryszczyn MP, Ravault C, Pison C, Pin I, et al. Heterogeneity of asthma according to blood inflammatory patterns. Thorax. 2009;64(5):374-80.

228. Gibson PG, Fujimura M, Niimi A. Eosinophilic bronchitis: clinical manifestations and implications for treatment. Thorax. 2002 Feb;57(2):178-82.

229. Chhabra SK. Acute bronchodilator response has limited value in differentiating bronchial asthma from COPD. J Asthma. 2005;42(5):367-72.

230. Appleton SL, Adams RJ, Wilson DH, Taylor AW, Ruffin RE. Spirometric criteria for asthma: adding further evidence to the debate. J Allergy Clin Immunol. 2005;116(5):976-82.

231. Strauch E, Neupert T, Ihorst G, Storm van's Gravesande K, Bohnet W, Hoeldke B, et al. Bronchial hyperresponsiveness to 4.5% hypertonic saline indicates a past history of asthma-like symptoms in children. Pediatr Pulmonol. 2001;31(1):44-50.

232. Boulet L. Asymptomatic airway hyperresponsiveness: what does it mean? . Eur Respir J. 2008;32(4):1118-9.

233. Boulet LP. Asymptomatic airway hyperresponsiveness: a curiosity or an opportunity to prevent asthma? Am J Respir Crit Care Med. 2003;167(3):371-8.

234. Hunter CJ, Brightling CE, Woltmann G, Wardlaw AJ, Pavord ID. A Comparison of the Validity of Different Diagnostic Tests in Adults With Asthma. Chest. 2002;121(4):1051-7.

235. Smith AD, Cowan JO, Filsell S, McLachlan C, Monti-Sheehan G, Jackson P, et al. Diagnosing asthma: comparisons between exhaled nitric oxide measurements and conventional tests. Am J Respir Crit Care Med. 2004;169(4):473-8.

236. du Toit JI, Anderson SD, Jenkins CR, Woolcock AJ, Rodwell LT. Airway responsiveness in asthma: bronchial challenge with histamine and 4.5% sodium chloride before and after budesonide. Allergy Asthma Proc. 1997;18(1):7-14.

237. Bosley CM, Fosbury JA, Cochrane GM. The psychological factors associated with poor compliance with treatment in asthma. Eur Respir J. 1995;8(6):899-904.

238. Haida M. Rise in the number of asthma fatalities are poor patient education, lack of compliance, and underlying psychological disturbances. Psychosom Med. 1995;57(5):506.

239. Smith JR, Mildenhall S, Noble M, Mugford M, Shepstone L, Harrison BD. Clinician-assessed poor compliance identifies adults with severe asthma who are at risk of adverse outcomes. J Asthma. 2005;42(6):437-45.

240. Rubinfeld AR, Pain MC. Conscious perception of bronchospasm as a protective phenomenon in asthma. Chest. 1977;72(2):154-8.

241. Barnes PJ. Blunted perception and death from asthma. N Engl J Med. 1994;330(19):1383-4.

242. Magadle R, Berar-Yanay N, Weiner P. The risk of hospitalization and near-fatal and fatal asthma in relation to the perception of dyspnea. Chest. 2002;121(2):329-33.

243. Martinez-Moragon E, Perpina M, Belloch A, de Diego A, Martinez-Frances M. Determinants of dyspnea in patients with different grades of stable asthma. J Asthma. 2003;40(4):375-82.

244. Brand PL, Rijcken B, Schouten JP, Koeter GH, Weiss ST, Postma DS. Perception of airway obstruction in a random population sample. Relationship to airway hyperresponsiveness in the absence of respiratory symptoms. Am Rev Respir Dis. 1992;146(2):396-401.

245. Ellison-Loschmann L, Sunyer J, Plana E, Pearce N, Zock JP, Jarvis D, et al. Socioeconomic status, asthma and chronic bronchitis in a large community-based study. Eur Respir J. 2007;29(5):897-905.

246. Salome CM, Xuan W, Gray EJ, Belooussova E, Peat JK. Perception of airway narrowing in a general population sample. Eur Respir J. 1997;10(5):1052-8.

247. Marks GB, Yates DH, Sist M, Ceyhan B, De Campos M, Scott DM, et al. Respiratory sensation during bronchial challenge testing with methacholine, sodium metabisulphite, and adenosine monophosphate. Thorax. 1996;51(8):793-8.

248. Tetzlaff K, Leplow B, ten Thoren C, Dahme B. Perception of dyspnea during histamine- and methacholine-induced bronchoconstriction. Respiration. 1999;66(5):427-33.

249. Todd DC, Armstrong S, D'Silva L, Allen CJ, Hargreave FE, Parameswaran K. Effect of obesity on airway inflammation: a cross-sectional analysis of body mass index and sputum cell counts. Clin Exp Allergy. 2007;37(7):1049-54.

250. van Veen IH, Ten Brinke A, Sterk PJ, Rabe KF, Bel EH. Airway inflammation in obese and nonobese patients with difficult-to-treat asthma. Allergy. 2008;63(5):570-4.

251. Sutherland TJT, Cowan JO, Young S, Goulding A, Grant AM, Williamson A, et al. The Association between Obesity and Asthma: Interactions between Systemic and Airway Inflammation. Am J Respir Crit Care Med. 2008;178(5):469-75.

252. McLachlan CR, Poulton R, Car G, Cowan J, Filsell S, Greene JM, et al. Adiposity, asthma, and airway inflammation. J Allergy Clin Immunol. 2007;119(3):634-9.

253. Martinez-Moragon E, Perpina M, Belloch A, Serra B, Lloris A, Macian V. Evolution over time in over perceivers of dyspnea in asthma. Arch Bronconeumol. 2006;42(3):120-4.

254. O'Connor PJ, Raglin JS, Morgan WP. Psychometric correlates of perception during arm ergometry in males and females. Int J Sports Med. 1996;17(6):462-6.

255. Tiller J, Pain M, Biddle N. Anxiety disorder and perception of inspiratory resistive loads. Chest. 1987;91(4):547-51.

256. von Leupoldt A, Ambruzsova R, Nordmeyer S, Jeske N, Dahme B. Sensory and affective aspects of dyspnea contribute differentially to the Borg scale's measurement of dyspnea. Respiration. 2006;73(6):762-8.

257. von Leupoldt A, Dahme B. Differentiation between the sensory and affective dimension of dyspnea during resistive load breathing in normal subjects. Chest. 2005;128(5):3345-9.

258. von Leupoldt A, Mertz C, Kegat S, Burmester S, Dahme B. The impact of emotions on the sensory and affective dimension of perceived dyspnea. Psychophysiology. 2006;43(4):382-6.

259. von Leupoldt A, Seemann N, Gugleva T, Dahme B. Attentional distraction reduces the affective but not the sensory dimension of perceived dyspnea. Respir Med. 2007;101(4):839-44.

260. Tufvesson E, Aronsson D, Ankerst J, George SC, Bjermer L. Peripheral nitric oxide is increased in rhinitic patients with asthma compared to bronchial hyperresponsiveness. Respir Med. 2007;101(11):2321-6.

261. Killian KJ. The Objective Measurement of Breathlessness. Chest. 1985;88(2 Supplement):84S-90.

262. Grant S, Aitchison T, Henderson E, Christie J, Zare S, McMurray J, et al. A comparison of the reproducibility and the sensitivity to change of visual analogue scales, Borg scales, and Likert scales in normal subjects during submaximal exercise. Chest. 1999;116(5):1208-17.

263. Wilson RC, Jones PW. Long-term reproducibility of Borg scale estimates of breathlessness during exercise. Clin Sci (Lond). 1991;80(4):309-12.

264. Shore SA. Obesity and asthma: possible mechanisms. J Allergy Clin Immunol. 2008;121(5):1087-93.

265. McClean KM, Kee F, Young IS, Elborn JS. Obesity and the lung: (1) Epidemiology. Thorax. 2008;63(7):649-54.

266. Sin DD, Sutherland ER. Obesity and the lung: (4) Obesity and asthma. Thorax. 2008;63(11):1018-23.

267. Barros R, Moreira A, Fonseca J, Moreira P, Fernandes L, de Oliveira JF, et al. Obesity and airway inflammation in asthma. J Allergy Clin Immunol. 2006;117(6):1501-2.

268. Ford ES. Asthma, body mass index, and C-reactive protein among US adults. J Asthma. 2003;40(7):733-9.

269. von Mutius E, Schwartz J, Neas LM, Dockery D, Weiss ST. Relation of body mass index to asthma and atopy in children: the National Health and Nutrition Examination Study III. Thorax. 2001;56(11):835-8.

270. Santamaria F, Montella S, De Stefano S, Sperli F, Barbarano F, Spadaro R, et al. Asthma, atopy, and airway inflammation in obese children. J Allergy Clin Immunol. 2007;120(4):965-7.

271. Maniscalco M, Zedda A, Faraone S, Cerbone MR, Cristiano S, Giardiello C, et al. Weight loss and asthma control in severely obese asthmatic females. Respiratory Medicine. 2008;102(1):102-8.

272. van den Nieuwenhof L, Schermer T, Heijdra Y, Bottema B, Akkermans R, Folgering H, et al. Are asymptomatic airway hyperresponsiveness and allergy risk factors for asthma? A longitudinal study. Eur Respir J. 2008;32(1):70-6.

273. Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. Thorax. 2002;57(8):667-71.

274. Efthimiadis A, Pizzichini MM, Pizzichini E, Dolovich J, Hargreave FE. Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline. Eur Respir J. 1997;10(6):1336-40.

275. Erin EM, Jenkins GR, Kon OM, Zacharasiewicz AS, Nicholson GC, Neighbour H, et al. Optimized dialysis and protease inhibition of sputum dithiothreitol supernatants. Am J Respir Crit Care Med. 2008;177(2):132-41.

276. Pignatti P, Delmastro M, Perfetti L, Bossi A, Balestrino A, Di Stefano A, et al. Is dithiothreitol affecting cells and soluble mediators during sputum processing? A modified methodology to process sputum. J Allergy Clin Immunol. 2002;110(4):667-8.

277. van Overveld FJ, Demkow U, Gorecka D, Skopinska-Rozewska E, de Backer WA, Zielinski J. Effects of homogenization of induced sputum by dithiothreitol on polymorphonuclear cells. J Physiol Pharmacol. 2005;56 Suppl 4:143-54.

278. Rofe AM, Philcox JC, Haynes DR, Whitehouse MW, Coyle P. Changes in plasma zinc, copper, iron, and hepatic metallothionein in adjuvant-induced arthritis treated with cyclosporin. Biol Trace Elem Res. 1992 Sep;34(3):237-48.

279. Louis R, Shute J, Goldring K, Perks B, Lau LC, Radermecker M, et al. The effect of processing on inflammatory markers in induced sputum. Eur Respir J. 1999;13(3):660-7.

280. Simpson JL, Scott RJ, Boyle MJ, Gibson PG. Differential proteolytic enzyme activity in eosinophilic and neutrophilic asthma. Am J Respir Crit Care Med. 2005;172(5):559-65.

281. Lang C, Murgia C, Leong M, Tan LW, Perozzi G, Knight D, et al. Anti-inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation. Am J Physiol Lung Cell Mol Physiol. 2007;292(2):L577-84.

282. Zalewski PD, Forbes IJ, Giannakis C. Physiological role for zinc in prevention of apoptosis (gene-directed death). Biochem Int. 1991;24(6):1093-101.

283. Holz O, Mucke M, Zarza P, Loppow D, Jorres RA, Magnussen H. Freezing of homogenized sputum samples for intermittent storage. Clin Exp Allergy. 2001;31(8):1328-31.

284. Prince P, Bertrand M, Boulay ME, Bernier MC, Boulet LP. Optimization of the conditions for preservation of induced sputum: influence of freezing on cellular analysis. Chest. 2005;128(2):980-5.

285. Burguera-Pascu M, Rodriguez-Archilla A, Baca P. Substantivity of zinc salts used as rinsing solutions and their effect on the inhibition of Streptococcus mutans. J Trace Elem Med Biol. 2007;21(2):92-101.

286. Greger JL, Sickles VS. Saliva zinc levels: potential indicators of zinc status. Am J Clin Nutr. 1979;32(9):1859-66.

287. Prasad AS. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. J Infect Dis. 2000 Sep;182 Suppl 1:S62-8.

288. Pihl E, Gustafson GT, Josefsson B, Paul KG. Heavy metals in the granules of eosinophilic granylocytes. Scand J Haematol. 1967;4(5):371-9.

289. Danscher G, Obel J, Thorlacius-Ussing O. Electron microscopic demonstration of metals in rat mast cells. A cytochemical study based on an improved sulphide silver method. Histochemistry. 1980;66(3):293-300.

290. Ho LH, Ruffin RE, Murgia C, Li L, Krilis SA, Zalewski PD. Labile zinc and zinc transporter ZnT4 in mast cell granules: role in regulation of caspase activation and NF-kappaB translocation. J Immunol. 2004;172(12):7750-60.

291. Besecker B, Bao S, Bohacova B, Papp A, Sadee W, Knoell DL. The human zinc transporter SLC39A8 (Zip8) is critical in zinc-mediated cytoprotection in lung epithelia. Am J Physiol Lung Cell Mol Physiol. 2008;294(6):1127-36.

292. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. Am J Respir Crit Care Med. 1999;160(5 Pt 1):1532-9.

293. Harlyk C, McCourt J, Bordin G, Rodriguez AR, van der Eeckhout A. Determination of copper, zinc and iron in broncho-alveolar lavages by atomic absorption spectroscopy. J Trace Elem Med Biol. 1997;11(3):137-42.

294. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax. 2002;57(7):643-8.