

The Emerging Role Of The Eosinophil And Its Measurement In
Chronic Cough

Airway Inflammation In Chronic Cough

By

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Submitted in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

The University of Hull and the University of York
Hull York Medical School

October 2017

ABSTRACT

Although the aetiology of chronic cough in guidelines is clearly stated as asthma and related syndromes, gastro-oesophageal reflux disease, and upper airways disease, the inflammatory mechanisms underlying these conditions differ. Recent studies on asthma have increasingly focused on its molecular phenotypes instead of clinical characteristics. Predominantly in this thesis I hypothesize that by dividing cough patients into the clinical characteristics of eosinophilic and neutrophilic groups will enhance our ability to recognise the type of airway inflammation, and consequently will lead us to more targeted treatment approaches.

To investigate this hypothesis I conducted a randomized, single centre, open label, controlled, clinical trial to examine the outcome of anti-inflammatory therapy with either montelukast or prednisolone in 50 patients with chronic cough. Furthermore, I studied the epidemiology of 137 chronic cough patients attending the Hull cough clinic.

Results from the clinical study demonstrated that patients with $\text{FeNO} \leq 20 \text{ppb}$ had twice the number of coughs compared with patients with $\text{FeNO} \geq 30 \text{ppb}$. This was reflected on quality of life as assessed by the LCQ and HARQ. Confirming this finding I found in the epidemiological study, that patients attending the hull cough clinic with $\text{FeNO} \leq 25 \text{ppb}$ scored significantly higher in HARQ compared with $\text{FeNO} \geq 25 \text{ppb}$.

In the clinical trial study I have shown that FeNO was a good marker for eosinophilic inflammation. There was a high degree of correlation with FeNO , blood and sputum eosinophilia thus confirming phenotypic identity. Whether the FeNO can be used to identify the different characteristics between eosinophilic and non-eosinophilic coughs needs further investigation. Cough patients in both low and high FeNO groups have shown a similar response to montelukast despite anticipating little or no effect in those without eosinophilic inflammation. These results suggest that response to montelukast may not be predicted by presence of eosinophilic biomarkers alone but may be act by effecting localised leukotriene mediated inflammation.

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ACKNOWLEDGEMENTS

I would like to thank my supervisors Caroline Wright and Professor Alyn Morice for their continued support and for giving me this opportunity to undertake this research. I would also like to thank Dr Simon Hart and Dr Michael Crooks for their help and guidance.

I would like to thank Professor Peter Dettmar and Andrew Woodcock at Technostics for their remarkable support with evaluation of Peptest study.

I would like to acknowledge that the Utility of FeNO study was founded by Aerocrine Company. I would like to thank the Research and Development Department especially Sarah Moffat for their professional guidance throughout this study.

I would like to thank everyone in the Clinical Trial Unit at Castle Hill Hospital, without whose aid I would not have been able to start this project, let alone finish it. I would also like to thank all volunteers and patients who participated in my research studies.

Finally I would like to thank my husband Mohammad for his endless support and my two beautiful daughters, whose encouragement was priceless throughout this PhD.

AUTHOR DECLARATION

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I also confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

PRESENTATIONS & PUBLICATIONS

PRESENTATIONS

- 1) BTS Winter Meeting December 2017 oral presentation& abstract

The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast (Chapter 3)

- 2) BTS Winter Meeting December 2017 poster presentation & abstract.

Does FeNO predict clinical characteristics in chronic cough? (Chapter 4)

- 3) HYMS Conference 2017 3 minutes thesis Presentation & poster

The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast (Chapter 3)

- 4) Allam Lecturer 2017 poster presentation

The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast (Chapter 3)

- 5) Hull, Belfast and Leicester Conference December 2016 oral presentation for the preliminary results of;

The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast (Chapter 3)

- 6) HYMS Conference 2016 poster presentation

Processing a Sputum Specimen (Chapter 7)

PUBLICATIONS

- 1) Sadeghi, M. H. & Morice, A. H. (2017) The Emerging Role of The Eosinophil and Its Measurement in Chronic Cough. *Open Respir Med J*, 11, 17-30.
- 2) The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast, subject to publication.
- 3) Does FeNO predict clinical characteristics in chronic cough? Subject to publication.

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Cough is one of the commonest symptoms in respiratory disease which force patients to seek medical attention. There is a massive market for cough remedies in the UK and the USA, most of poor evidence base. The etiological mechanisms of cough are poorly understood causing a challenge to management (Morice, 2003). Even after a clear diagnosis, it is still difficult to control and manage cough, and this could decrease the quality of life in patients suffering from cough (French et al., 1998).

For the majority of patients who suffer from acute cough the cause is usually a viral respiratory tract infection. This is initially disruptive but is generally self-limiting and often does not required a great deal of medical involvement (Irwin et al., 1998). Conversely, chronic cough is a continual symptom which is caused by many important chronic respiratory diseases, upper airway diseases and gastrointestinal disease (Morice, 2002).

1.2 ACUTE COUGH

Acute cough is defined as a cough lasting less than three weeks (Morice et al., 2006). Acute cough is generally caused by viral upper respiratory tract infections, most importantly influenza, parainfluenza, rhinovirus, adenovirus, respiratory syncytial and the respiratory corona virus (Irwin et al., 1998). The majority of patients either do not take treatment or use over the counter cough and cold products without seeking medical advice. Some viral infections are more severe and cause distress in patients who then need to consume more cough medicines. Several reviews have shown that such treatments lack clinical evidence (Dicpinigaitis, 2011). Perhaps dosing of cough suppression is not adequate (Manap et al., 1999).

The economic impact of cough on healthcare is enormous. According to the Proprietary Association of Great Britain (2015) only in United Kingdom, the cost of over-the-counter (OTC) treatments for cough is over 400 million pounds per annum. This figure does not include the expenditure on prescribed drugs for cough treatment. Therefore, the total amount of money spent on cough drugs is in excess of 400 million pounds per annum.

1.3 CHRONIC COUGH

Chronic cough is a common and poorly diagnosed condition. It has been typically defined as a cough that persists for more than eight weeks (Morice, 2011). Epidemiological surveys reveal that approximately 10% of the population suffer from a chronic cough (Ford et al., 2006; Everett et al., 2007). Chronic cough is one of the commonest presenting complaints to secondary care. Referrals may be to both chest physicians and departments of Ear, Nose and Throat surgery.

Chronic cough has a significant impact on physical and psychological morbidity. Patients with chronic cough suffer from various physical symptoms including chest pain, headaches, sore throat, voice changes, vomiting, sleep deprivation and syncope (Morice, 2008; Faruqi et al., 2014; Chamberlain et al., 2015). In a questionnaire survey it has shown that chronic cough causes incontinence in 55% of women (Everett et al., 2007). Chronic cough also has a negative impact on patients' relationships and social life and can lead to social isolation as they cannot participate in their usual daily activities. Thus, anxiety and depression is rather common among these patients (Dicpinigaitis et al., 2006; Everett et al., 2007; Chamberlain et al., 2014). These physical and psychological effects of chronic cough lead to a detrimental effect on the quality of life (French et al., 1998). It is important therefore to understand the pathogenesis of chronic cough to treat this symptom.

1.4 THE COUGH REFLEX

Coughing is a sudden expulsion of air from the lungs which is characterized by sound. It is produced by contraction of the respiratory muscles (muscles of chest wall, diaphragm, and abdominal wall) combined with closure of the glottis. As a result of this contraction the intrathoracic pressure increases and opens the glottis. Finally the air is expelled at a fast speed (Polverino et al., 2012) (Morice, 2003). Through such a robust force of air, coughing is an important defence mechanism which helps to prevent aspiration. It clears secretions such as mucus, foreign particles, and infectious organisms from the

airways. However, this protective reflex has been known as a primary symptom of many airway diseases when it becomes excessive and harmful.

1.4.1 COUGH REFLEX MECHANISM

Cough happens as a result of a complex reflex arc stimulation. Anatomically, cough receptors (sensory afferent) are located mainly in the upper airways especially in the lower pharynx, larynx and bronchial tree. As the airways divide cough sensitivity reduces and in the smaller airways cough cannot be produced (Widdicombe, 1998). Cough receptors are thought to comprise of ion channels, one class of which is termed the transient receptor potential (TRP). These ion channels can be stimulated by polymodal stimuli. Initially, cough receptors are stimulated by mechanical and chemical stimulus in the respiratory tract (McLeod et al., 2006). However, it is important to recognise that cough can be stimulated at other sites such as instrumentation of ear and oesophagus (Morice, 2003). All these disparate sites are supplied by the vagus nerve (Figure 1).

AFFERENT PATHWAYS

Cough receptors impulses arise from the afferent nerves in the distribution of the vagus. Afferent nerves can be classified at least to three broad types according to their conductive properties (Figure 2):

1) Rapidly adapting receptors (RARs)

RARs in comparison with other airway afferents have a rapid adaptation (1 - 2 seconds) and are mainly sensitive to mechanical stimulation. RARs are stimulated by changes in airway diameter, length, and interstitial pressures and they are extremely sensitive to lung collapse, pulmonary congestion, reduction in lung compliance and bronchospasm. RARs are activated by the dynamic mechanical forces during the respiratory cycle including lung inflation and deflation, and they are more sensitive to rises in the rate and volume of lung inflation. Stimuli that induce bronchospasm or lung obstruction and consequently increase mucus secretion or cause oedema can activate RARs. RARs can also be activated by substances such as histamine, capsaicin, cigarette smoke, acidic solutions,

alkaline solutions, hypotonic saline and hypertonic saline (Widdicombe, 1998; Polverino et al., 2012).

2) Slowly adapting receptors (SARs)

SARs respond to the lung inflation and their activity increases during inspiration as they are very sensitive to the mechanical forces. SARs are located within the airway smooth muscle layer. SAR central inhibition respiratory effect leads to reduction in phrenic nerve activity with subsequent decrease in airway smooth muscle tone (Widdicombe, 1998; Polverino et al., 2012).

3) C-fibres

Most of the afferent receptors on the airways and lungs are unmyelinated C-fibres. C-fibres are relatively insensitive to mechanical stimulation and lung inflation while they are highly sensitive to chemical stimulus. Therefore, they can be distinguished from RARs and SARs as they are not sensitive to mechanical stimulation and lung inflation. Furthermore, C-fibres are directly activated by chemical stimuli such as bradykinin, capsaicin, SO₂, and citric acid. Whereas, only intrapulmonary RARs are activated indirectly by these chemical stimulus effect on smooth muscle or the airway vasodilation (Chung & Pavord, 2008; Polverino et al., 2012) (Widdicombe, 1998).

Whilst these classifications have been accepted for many years it is still unclear which and in what proportion they play a role in the clinical cough reflex.

CENTRAL PATHWAY (COUGH CENTRE)

Afferent fibres from cough receptors travel via the vagus nerves to a 'cough centre' located in the upper brainstem in the nucleus tractus solitarius. Then the nucleus tractus solitarius connect to respiratory neurons and create an efferent pathway via the vagus nerve, phrenic, and spinal motor nerves (Canning, 2009) . Cough can also be controlled through higher cortical centres, as cough can be started or inhibited voluntarily (Hutchings et al., 1993). This can explain the effect of placebo therapies that inhibit cough

(Eccles, 2008). In addition, during sleep cough is suppressed significantly which could be related to the role of consciousness of this reflex (Hsu et al., 1994). Consequently it can be concluded that both brainstem and cortical centres may have an influence on this complex respiratory reflex (Figure 2).

EFFERENT PATHWAY

Through this pathway the Impulses from the cough centre transfer to the diaphragm, abdominal wall and expiratory muscles to produce the cough. Signals from cough centre travel to the inspiratory and expiratory muscles via phrenic and other spinal motor nerves; and signals are transferred to the larynx through the laryngeal branches of the vagus nerve (Chung & Pavord, 2008).

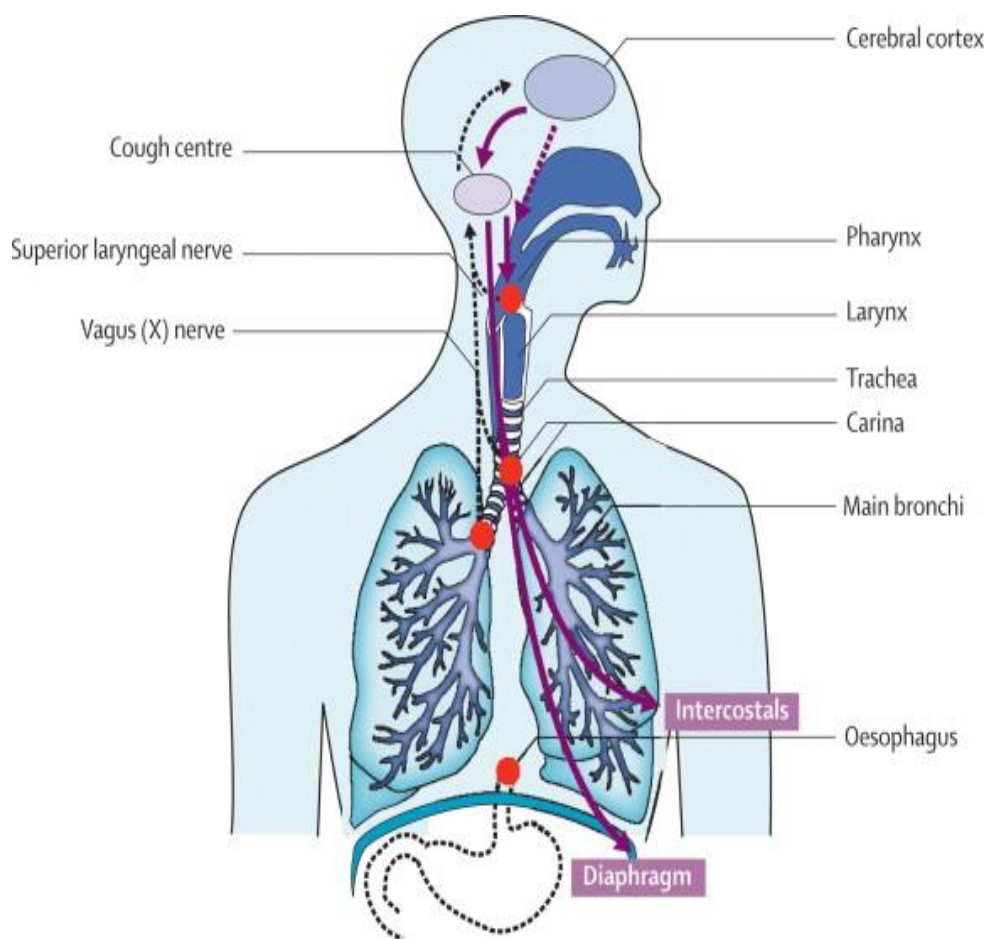


Figure 1 Anatomical representation of neural pathways for cough

Cough receptors (shown in red colour) at the airway bifurcations, in the larynx and at the distal oesophagus, link to cough afferents through the vagus and superior laryngeal nerves to the cough centre and cerebral cortex. Efferent pathways coordinate the muscle response that leads to a cough. Chung & Pavord. Lancet. 2008, 371, 1364 - 1374.

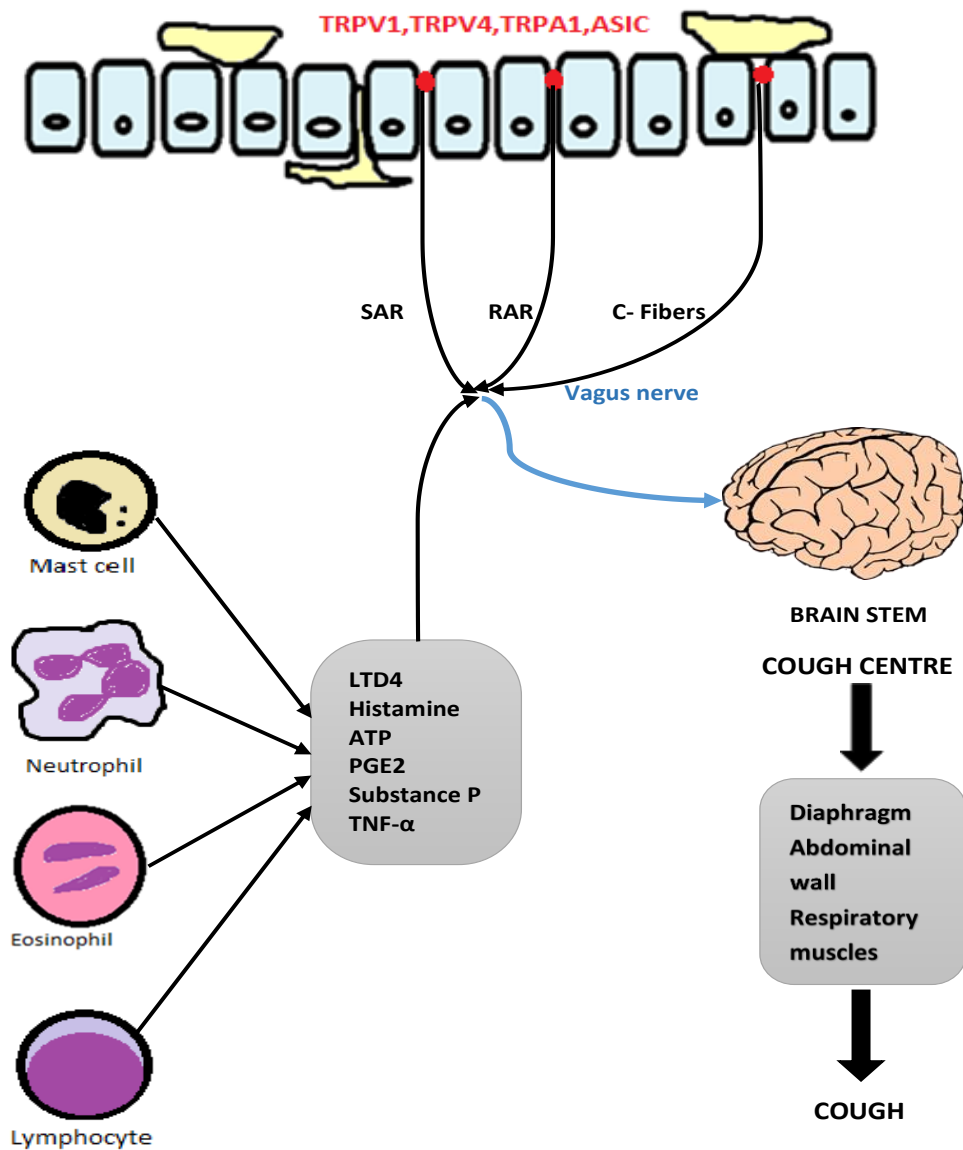


Figure 2 Afferent and efferent neurological pathways of the cough reflex

The cough reflex initiated by the stimulus from the upper respiratory tract. Stimulation of the sensory receptors, such as rapidly adapting receptors (RARs), C-fibres, and slowly adapting fibres (SAR), and cough receptors (TRPV1, TRPV4, TRPA1, ASIC) stems from the vagus nerve to the medulla of the brain and the cough centre. The cough centre then begins to transmit the signals through the efferent pathway to the inspiratory and expiratory muscles that cause cough. Factors that sensitize the cough receptors and lead to hypersensitivity in the cough reflex are demonstrated. LTD4 (leukotriene D4), ATP (adenosine triphosphate), PGE2 (prostaglandin E), TNF (tumour necrosis factor), TRPV (transient receptor potential vanilloid), TRPA1 (Transient Receptor Potential Ankyrin 1), ASIC (Acid-Sensing Ion Channel).

1.5 SYSTEMATIC ASSESSMENT OF CHRONIC COUGH

The accurate assessment of cough frequency is crucially important to define cough severity, evaluate the effectiveness of therapy and it also may be useful to diagnose the disease (Morice et al., 2006). Consequently, having validated measurement tools to effectively assess cough for both clinical and research purposes are necessary.

Currently there are a variety of tools which assess cough. These include a series of quality of life questionnaires such as the Leicester Cough Questionnaire (LCQ), diagnostic questionnaires such as the Hull Airways Reflux Questionnaire (HARQ), visual analog scales, electronic recordings and cough challenge. These tools can be divided into 3 categories; subjective measures, cough counting and cough reflex testing by challenges. Comparison of these tools shows that they represent different facets of cough with modest correlation between them (Faruqi et al., 2011b).

For many years cough frequency was difficult to assess, since by its nature cough is an episodic phenomenon and it is necessary to record cough over a lengthy time period (24 hours) to be able to produce an accurate and reliable result (Yousaf et al., 2013) (Barry et al., 2006). Simple scoring of cough subjectively is not reproducible and could change according to the individual as each person has a different perception of cough. Similarly how this disease affects the quality of life differs (French et al., 1998) (Morice et al., 2007). Moreover, evaluation of cough frequency during the night is another challenge without electronic cough recording (Barry et al., 2006). The absence of well-validated means to assess cough frequency led to difficulties in clinical assessments. The development of objective automated cough counting systems and subjective quality of life questionnaires brought a massive improvement in this field (Birring et al., 2004b; Birring et al., 2006; Kelsall et al., 2009; Boulet et al., 2015b). It has been argued that the frequency of cough may not be the only important parameter that affects patient quality of life, the intensity or physical effort of each cough is also another important parameter that affects patient quality of life (Clare Decalmer et al., 2007). Thus in the CHEST Guideline (Boulet et al., 2015a) cough expert panel recommend validated and reliable health-related quality-of-life

questionnaires be used as the measurement of choice to assess the impact of cough on patient's life. Although multidimensional quality of life questioners are the main tools to evaluate the impact of cough on the patient, it has been challenging to assess their validity in the absence of an objective measure of cough frequency particularly in clinical research settings. In summery it has been suggested a combination of cough frequency measurement and well validated quality of life questioners are should be applied to assess cough appropriately(Clare Decalmer et al., 2007).

At the moment, the automated cough count and tussigenic cough challenge are the two methods that play an important role in research settings (Boulet et al., 2015a). The research studies suggesting that sensitive cough reflex only moderately correlated with higher cough frequency. Although inhalation cough challenge may be a useful method to assess cough reflex mechanisms (Faruqi et al., 2011a), response is highly dependent on the agent used and the drug being tested.

1.5.1 COUGH CHALLENGE

The inhalation cough challenge allows investigation into the sensitivity of the cough reflex and evaluate the antitussive effects of certain treatments.

The cough challenge has been used for more than 50 years as a methodology to investigate the cough reflex (Bickerman & Barach, 1954). Originally, inhalation of citric acid solutions were used to induce cough. Ever since, this methodology has been broadly practiced to investigate the potential antitussive effects of different drugs. There are broadly two different methods to perform cough challenge test, based on use of tussigenic agents (acid or non-acid tussives).

TUSSIGENIC AGENTS

Since cough challenge testing has been recognised, many different tussive agents such as sulphur dioxide, ammonia, and cigarette smoke have been used to induce cough (Gravenstein et al., 1954). However, based on their property and their reproducibility only citric and tartaric acid as acid tussive agents, and capsaicin as a non-acid tussive agent are commonly used to induce cough

in humans (Morice et al., 2007). These antitussives resort in human ‘models’ and were able to increase cough reflex during upper respiratory virus infections and also in patients with chronic cough.

ACID TUSSIGENIC AGENT

Citric acid has been considered to cause cough through the activation of rapidly adapting receptors (RARs). It is believed the acid stimulus activates these receptors which may cause pH gated ion channels such as ASIC to be open and induce cough (Lowry et al., 1988). In another study on humans it has been shown that the tussive effects of acetic acid and phosphoric acid has a similar pathway to citric acid. All three agents cause cough by activating pH sensitive rapidly adapting receptors, this however is different to the capsaicin induced cough pathway (Wong et al., 1999). However *in vitro* in guinea pigs capsaicin and citric acid appear to stimulate C fibres to induce cough (Fox et al., 1993; Lalloo et al., 1995). In guinea pigs the inhalation of citric acid or capsaicin induces both cough and bronchoconstriction (Forsberg et al., 1988). This difference between species is because axon reflex induced bronchoconstriction related to the release of tachykinins does not occur in man (Daoui et al., 1998). Inhalation of citric acid or capsaicin stimulate C-fibres and induced cough only in conscious animals (Canning, 2002).

Acidic solutions and capsaicin have similar effects on pain, bronchoconstriction, cough, and sensory neuropeptide release. It has been reported that capsazepine (a competitive capsaicin antagonist) is able to inhibit capsaicin and citric acid induced cough (Lalloo et al., 1995). It has suggested that both of these agents act through the same pathway, as capsazepine reduces their effects to induce cough (Bevan & Geppetti, 1994).

NON-ACID TUSSIGENIC AGENT

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a pungent agent of hot pepper which mostly stimulates the afferent neurones of the non-myelinated C- fibres by opening a nonselective ion channel of vanilloid receptor (Bevan & Geppetti, 1994). The transient receptor potential vanilloid-1 (TRPV1) channel is a capsaicin receptor which plays a crucial

role to evoke cough in all coughing mammals and humans. This receptor is activated by heat, acid, bradykinin, arachidonic-acid derivatives, and adenosine triphosphate (Caterina et al., 1997). As a result of activation of TRPV1, flow of calcium and sodium reduces which causes depolarization and associated neurotransmitter release (Wood et al., 1988). Administration of TRPV-1 inhibitor in a sensitised guinea pig model reduced the tussive response to the allergen challenge (McLeod et al., 2006). In this study they examined the pharmacology of a TRPV1 receptor antagonist in vivo. They reported that a TRPV1 receptor antagonist blocked the tussigenic effects of capsaicin (300 μ M) and produced a maximum decrease in capsaicin-induced cough. These results shows that TRPV1's role in induced cough. Consequently, regulation of TRPV1 has been suggested to contribute to hypertussivity in inflammatory airway disease (Chung & Pavord, 2008). However recent clinical studies of TRPV1 antagonists in cough have been disappointing (Khalid et al., 2014). Other TRP receptors such as TRPA1 are also implicated in afferent sensation of cough (Birrell et al., 2009).

1.6 AETIOLOGY OF CHRONIC COUGH

In literature it has been reported that there are three common aetiologies of chronic cough: 1) asthma and related syndromes, 2) gastro-oesophageal reflux disease (GORD), and 3) upper airways disease including: rhinitis, sinusitis and post-nasal drip syndrome. However, despite following cough guidelines in detail, in several cases the cause of cough is unclear and has been reported as “idiopathic” chronic cough (Ford et al., 2006; Chung & Pavord, 2008; Magni et al., 2010; Morice, 2011). In a worldwide survey from 11 cough clinics of 10032 patients with persistent cough (cough without any underlying trigger and significant radiological abnormality), it was suggested that these patients suffer from cough hypersensitivity having a single, common, clinical entity (Morice et al., 2014). This survey examined the individual clinic databases and their demographic data for the age and sex and has shown that there is no fundamental difference between the patients' demographics. In another study, HARQ was administered to 185 patients with chronic cough and 70 normal volunteers. According to this study Morice and colleagues (2011b) reported that acid gastro-oesophageal reflux is not greatly

associated with cough hypersensitivity syndrome. Instead, they proposed that gaseous non-acid reflux is highly associated with Cough Hypersensitivity Syndrome. Although, there are some tests available to diagnose acid reflux, detecting gaseous non-acid reflux is a challenge. The prevalence of cough aetiologies are interestingly varied in different reports from different countries. For instance, a higher prevalence of rhinitis followed by asthma has been reported in the United Kingdom. GORD and post-nasal drip are major causes of chronic cough in Western countries (Magni et al., 2010). While, in China and Japan cough variant asthma and upper airways disease are most common and GORD is relatively uncommon (Lai et al., 2013) (Chung & Pavord, 2008). These differences in prevalence may depend on various factors such as; where you live, diet, race, life style but most importantly access to specialists and their particular interest and understanding of the causes of cough (Magni et al., 2010; Lai et al., 2013). However the failure to recognise the different nature of the reflux associated with cough rather than peptic symptoms or GORD underlies most of this confusion.

1.6.1 ASTHMA AND RELATED SYNDROMES

Chronic cough is one of the predominant asthma symptoms. Chronic cough however is not always associated with other clinical symptoms of asthma, such as airway obstruction, wheezing and dyspnea. There are three different asthmatic conditions, which lead to isolated chronic cough: 1) cough variant asthma, 2) atopic cough, and 3) eosinophilic bronchitis.

Cough variant asthma

Patients with cough variant asthma characteristically suffer from non-productive cough in the absence of other asthma symptoms such as wheezing and dyspnea (Corrao et al., 1979). This cough is characterised by bronchial hyper responsiveness and eosinophilia in both sputum and broncho alveolar lavage (BAL), but without bronchoconstriction or airway obstruction. These patients respond to bronchodilator therapy which is the key to the diagnosis of cough variant asthma (Corrao et al., 1979; Fujimura et al., 2003; Magni et al., 2010). Nevertheless, there is a challenge to recognise classic asthma from

cough variant asthma since the difference is not always evident, because a number of patients with cough variant asthma will develop classic asthma characteristics. Therefore, it has been recommended that “cough predominant asthma” is a better term to describe this type of cough which represent a large number of patients (10-66%) with chronic cough (Morice, 2011).

Atopic cough

Atopic cough is characterised by cough hypersensitivity and sputum eosinophilic in the absence of bronchial hyper responsiveness, airway obstruction and eosinophilia in broncho alveolar lavage (BAL) (Fujimura et al., 1992). A history of an atopic constitution can be detected in this group of patients as well (Fujimura et al., 2003; Magni et al., 2010). These patients also respond to anti-asthma therapy such as histamine H1-antagonist and corticosteroid (Fujimura et al., 2003). However, whether these conditions could identify different aspects of clinical features related to airway eosinophilic inflammation is controversial since patients may have eosinophilic inflammation without atopic cough and the majority of older patients have cough without the presence of atopic cough characteristics (McGarvey & Morice, 2003b).

Eosinophilic bronchitis

Eosinophilic bronchitis is a condition in which chronic cough is present with sputum eosinophilia without variable airflow obstruction (bronchoconstriction) or bronchial hyperresponsiveness (Gibson et al., 1989a). Eosinophilic bronchitis chronic cough may be refractory to inhaled anti-asthma therapy but responds to high doses of parenteral steroids (Brightling et al., 2000). This condition has been reported to cause chronic cough among 10–15% of patients who attended respiratory clinics in the UK (Birring et al., 2004a).

1.6.2 GASTRO-OESOPHAGEAL REFLUX DISEASE

Gastro-oesophageal reflux disease (GORD) may cause chronic cough in 2 - 40% of cases in prospective studies. However diagnosis of the more important

extra-oesophageal reflux is problematic as there are no clear diagnostic criteria (Morice et al., 2006).

Classically, the retrograde movement of acid and other gastric contents of the stomach via the oesophagus to the larynx and trachea leads to gastro-oesophageal reflux. In the majority of patients, this happens due to impaired function or brief relaxation of the lower oesophageal sphincter (LOS). GORD causes symptoms such as cough, acid regurgitation, and heart burn which improve by anti-reflux therapy such as the proton pump inhibitors (PPIs) (Chung & Pavord, 2008; Morice, 2011). However, there are some patients that represent non acid reflux which may be an aerosol and gaseous. Morice (2013) believe that *“The reflux which causes respiratory consequences is a gaseous mist which is partially or even wholly non-acid. This mist can travel up the oesophagus without a peristaltic wave since the oesophagus, as is usually seen on thoracic computed tomography, is patent; a so-called common cavity”*. Normally, the LOS opens and allow this gas to pass. However, when this gaseous mist excessively increases in the airway or in some cases if the airway becomes sensitive, it can cause inflammation in the respiratory tract, as well as the nose, ears and sinuses (Morice, 2013). It has been recognised that the reflux reaches beyond the upper oesophagus and into the pharynx, larynx, airways, and middle ear. Consequently this “airway reflux” transfers gastric content (chime, pepsin and bile acids) into the airways, and is extra oesophageal reflux which could be both liquid and aerosol (Bardhan et al., 2012). Associated symptoms with extra oesophageal reflux (EER) are hoarseness, sore throat, voice disorders, throat clearing and cough. However, heartburn and regurgitation which are typical symptoms of GORD are not always present in EER (Hayat et al., 2014). Extra oesophageal reflux (EER) has been referred differently in the literature. It has been known as laryngopharyngeal reflux (LPR), silent reflux or Airway reflux.

The present diagnostic methods of airway reflux are invasive, expensive and have moderate sensitivity. Non acid reflux is untraceable by pH measurement which normally has been used to detect acid reflux and also their symptoms are resistant to the proton pump inhibitors (PPIs) (Morice et al., 2006; Morice, 2011; Herregods et al., 2015). Tests such as Peptest which measure pepsin

(an enzyme which is produced in the stomach) in saliva can be a useful tool to detect airway reflux. Pepsin has been suggested as a promising diagnostic biomarker for reflux (Grabowski et al., 2011; Du et al., 2017). However, this technique still needs further development to increase the sensitivity (Dy et al., 2016; Yadlapati et al., 2016) and for it to be used in routine clinical practice. In the meantime, clinical history is an important tool to identifying patients who suffer from chronic cough associated with airway reflux. In this group of patients cough is evoked by certain foods (chocolate, spicy foods or dry foods), rising from bed, bending and talking, especially on the phone. On holiday or business trips the symptoms get worse. There is no sign of cough through the night when they are asleep because the lower oesophageal sphincter is closed during sleep (Morice, 2003; Morice, 2011). This symptom complex has been codified in a validated questionnaire which is called Hull Airways Reflux Questionnaire (HARQ). This validated questionnaire supports the diagnosis in the majority of patients with chronic cough (Faruqi et al., 2009; Smith et al., 2013).

1.6.3 UPPER AIRWAYS DISEASE

Postnasal drip (PND) is defined as a sense of nasal secretions or dripping into the back of the throat from the nose or sinuses. PND leads to symptoms such as cough, frequently clearing the throat (throat-clearing) and nasal discharge or nasal stuffiness. Belief in the syndrome arose from the observation of first generation of antihistamines caused an improvement in cough in significant number of patients (Irwin et al., 1984). However is this a central effect, since potent second generation of antihistamines which do not pass the blood brain barrier have no effect on chronic cough (Dicpinigaitis & Gayle, 2003)? It is believed that gaseous airway reflux may cause upper airways symptoms by damage to the upper respiratory tract, irritating the larynx and cough receptors which cause both cough and upper airway obstruction (Dettmar et al., 2011; Bardhan et al., 2012).

The reports regarding prevalence of postnasal drip and rhinitis associated with chronic cough in different specialist cough clinics around the world are enormously varied (0% to more than 90%). Research studies from Australia

(Carney et al., 1997) and America (Palombini et al, 1999; Smyrniotis et al, 1995) reported that PND was the most common (40% - 93%) cause of chronic cough in their clinic. While in other studies in Japan (Fujimura et al., 2005), China (Lai et al, 2006), Turkey (Ayik et al, 2003) and United Kingdom (Birring et al, 2004; Kastelik et al, 2005; McGarvey et al, 1998) the proportion of PND in patients presenting with chronic cough was 0-21%. This wide variation is because of the difficulty in defining this condition - if it exists at all (due to the lack of clear diagnostic criteria) and understanding the complex of symptoms related to cough (Morice, 2002; Diczpinigaitis et al., 2006; Morice, 2011).

1.7 *ASTHMA*

Asthma has been known as a pathological disorder since the time of Hypocrates. However, in recent years revolutionary changes to understanding of the nature of asthma and its pathogenesis has led to better diagnoses, control and treatment of the asthmatic patient. By the end of the nineteenth century it had been recognised that asthma is a type of bronchitis (an airway inflammatory disease). In late 1960 onwards eosinophils in blood, sputum and bronchial wall of asthmatic patients were highlighted. However, in most of the twentieth century the focus was on the bronchospasm which was thought to be caused by asthma. As a result of this focus, bronchodilators were developed and methacholine test was recognised and advocated to diagnose bronchial hyperresponsiveness. Consequently asthma was defined as a reversible airway obstruction with hyperresponsiveness while the importance of airway inflammation was not much considered (Busse & Holgate, 2000). In the last two decades, the importance of inflammation in asthma has revived and has been further developed. In early 2000s, researchers working with mouse models of allergic asthma discovered the important role of TH2 immune system on airway inflammation and hyperresponsiveness. This hypothesis changed the perception of asthma, so it was widely believed that asthma was an allergic disease with eosinophilic inflammation which responds well to corticosteroid therapy (Wenzel, 2012). Nevertheless, it had become clear that airway eosinophilia is not limited to patients with allergy (extrinsic asthma); it also can be present in greater

degree in patients with non - allergic asthma (intrinsic asthma). Patients with severe asthma or late onset asthma, chronic infection or smokers might have neutrophilic airway inflammation which is believed to be hallmarks of COPD (chronic obstructive pulmonary disease). More recently it has become clear such simple divisions are inadequate and patients have overlapping features have been described as “asthma-COPD overlap syndrome” (ACOS) (Postma & Rabe, 2015). Thus there are some COPD patients that have eosinophilic inflammation in a pattern that responds to corticosteroids therapy. In contrast there are patients who have severe asthma but they do not have eosinophilic inflammation and they are refractory to corticosteroids therapy. These patients are described as having neutrophilic asthma. In reality there is no clear subdivision into these individual syndromes, but in terms of therapy it is important to consider that absence of eosinophilic inflammation and resistance to corticosteroids therapy (Busse & Holgate, 2000).

1.7.1 *ASTHMA PHENOTYPES*

In last forty years understanding of this heterogeneous syndrome has changed rapidly. Initially, clinical characteristics of asthma were used to identify and group patients. Patients received treatment dependent on their age when their asthma started (early or late onset of asthma), their allergic features, airway obstruction and reversibility or their smoking habits and weight. However, these clinical characteristics were not useful in many cases as they were overlapping and nonspecific enough to identify and treat patients accordingly. For instance, patients were diagnosed as atopy asthma solely in the presence of specific IgE to known allergens without considering biological or clinical responses to the allergy. Although, there are some patients who have atopic asthma without any evidence of the presence of IgE or allergy symptoms. Therefore, molecular phenotypes of asthma (eosinophilic asthma and neutrophilic asthma) based on inflammatory biomarkers have been developed to understand the mechanistic insights and pathology of asthma. Advances in the understanding of the immune system pathways which lead to airway inflammation have been effective in identifying different type of asthma (Ray et al., 2015). Now asthma is known as a chronic inflammatory disease of the airways in which the adaptive immune system and innate immune (i.e. non

atopic) system involved. This inflammation causes airway hyperresponsiveness (AHR) to a range of nonspecific stimuli, variable airway obstruction that is usually reversible, airway remodelling and mucus overproduction. The clinical symptoms of asthma are shortness of breath, wheezing and coughing (Wenzel, 2012; Lambrecht & Hammad, 2015).

1.7.2 COUGH INFLAMMATION PHENOTYPES

Recently molecular phenotypes of asthma based on inflammatory biomarkers have been developed to understand the mechanistic insights and pathology of asthma (Ray et al., 2015). Similarly in cough, it has been evident that cough variant asthma, atopic cough, and eosinophilic bronchitis (EB) present an eosinophilic phenotype in cough patients (Gibson et al., 1989b; Fujimura et al., 1992; Fujimura et al., 2003; Magni et al., 2010; Morice et al., 2011a). Studies from secondary care indicate that about 20% of patients with chronic cough have eosinophilic inflammation present in their airway, whereas the remaining patients tend to have a neutrophilic phenotype (Brightling et al., 1999). This is important because those patients with eosinophilic inflammation respond to anti-inflammatory therapy (Brightling et al., 1999). Treatment of the EB can be highly effective, but classic asthma treatment often does not lead to complete resolution. Because the element of bronchoconstriction is missing in ‘asthmatic cough’ bronchodilators are only partially effective relieving the element of hyperresponsiveness. The inflammation in asthmatic cough may also be more deep seated (Brightling, 2006). Asthmatic cough is a well-recognised phenomenon by physicians and so most patients with a chronic cough receive a trial of anti-asthma medication. However it is not the only phenotype, the majority having neutrophilic inflammation. These patients do not respond and so dose escalation is common. Indeed, many patients are given potentially harmful therapy such as parenteral steroids with little chance of therapeutic response.

1.8 IMPORTANCE OF INNATE IMMUNE SYSTEM IN EOSINOPHILIC INFLAMMATION

Eosinophilic asthma is asthma in which there is prominent eosinophilic airways inflammation as characterised by eosinophils in sputum, bronchoalveolar lavage (BAL) fluid and bronchial biopsies. Eosinophilic inflammation can be seen in patients with allergic or non-allergic asthma (Brusselle et al., 2013; Lambrecht & Hammad, 2015).

Classically, it is believed T helper 2 cells (Th2) by releasing interleukin 4 (IL-4), IL-5, and IL-13 play a vital role to induce eosinophilic allergic asthma. Therefore, new treatment of asthma have been developed which blocks these cytokines (Kabata et al., 2015). However, a recent discovery shows the innate immune system as well as the adaptive immune system play an important role in eosinophilic non-allergic asthma. By recognition of innate lymphoid cells it has been confirmed that ILC group 2 produce type 2 cytokines which might play an important part in pathophysiology of asthma (Fort et al., 2001; Brusselle et al., 2013; Spits et al., 2013; Kabata et al., 2015; Lambrecht & Hammad, 2015).

1.8.1 T HELPER 2 CELLS AND EOSINOPHILIA

The adaptive immune system is crucial to protect the host from helminth pathogens. It can also cause autoimmune disease and allergy conditions such as asthma. Allergic asthma is caused by type 2 response of immune system which is activated by epithelial cell-derived cytokines (TSLP, IL-25 and IL-33). In asthma TSLP, IL-25 and IL-33 are secreted during allergen exposure, for instance to house dust mite, and this activates Th2 cells. As a result, Th2 cells release large amounts of inflammatory cytokines (IL-4, IL-5, and IL-13) (Licona-Limón et al., 2013; Hendriks, 2014) (figure 3).

IL-4 stimulates B cells class switching to IgE, which can bind to high-affinity IgE receptors (FcεR1) on basophils and mast cells. Activated basophils and mast cells release inflammatory mediators such as cytokines, chemokines, histamine, heparin, serotonin and proteases. These mediators cause constriction in smooth muscles, increase vascular permeability and mucus hypersecretion (Paul & Zhu, 2010; Hansbro et al., 2011; Licona-Limón et al.,

2013). IL-5 induces bone marrow to produce eosinophils leading to tissue eosinophilia and airway remodelling (Paul & Zhu, 2010; Hansbro et al., 2011; Lambrecht & Hammad, 2015). IL-13 mediates an increased production of mucus in airway epithelia and smooth muscle cells, and is necessary to promote airway hyperresponsiveness (AHR) (Paul & Zhu, 2010; Hansbro et al., 2011; Licona-Limón et al., 2013).

1.8.2 ILC2 CELLS AND EOSINOPHILIA

An important new development has been discovered through a series of studies on humans and mice, that there is involvement of the innate immune system in airway eosinophilic inflammation. Anti-IL-4 and anti-IL5 therapies in humans led to a favourable result in asthmatic patients with a high level of eosinophils. These patients responded to the therapies regardless of being atopic or not (Lambrecht & Hammad, 2015). In another study, administration of IL-25 to mice induced the production of IL-4, IL-5, IL-13, IgE, IgG1 and IgA. Alternatively, this led to airway eosinophilia in RAG-deficient mice, which do not have B cells and T cells (Fort et al., 2001). Administration of IL-25 and infection of mice with *Nippostrongylus brasiliensis* (Hurst et al., 2002) and helminth (Fallon et al., 2006) in other studies, revealed the same result. These findings indicated that type2 cytokines and eosinophilia can be produced without the adaptive immune system activation. Therefore the recent discovery that innate lymphoid cells (ILC) are another important source of T cell-associated cytokines has important consequences for our understanding of non-atopic asthma.

Innate lymphoid cells (ILCs) react to IL-33 or a combination of IL-2 and IL-25, and they express Sca-1, c-Kit, IL-33R, and IL-7R. However they do not express antigen-specific receptors or lineage markers (CD3, CD4, CD8, TCR, TCR, CD5, CD19, B220, NK1.1, TER119, Gr-1, Mac-1, CD11 and FcεRIα) thus, they cause nonspecific immune responses. Innate lymphoid cells are classified into three groups based on their ability to secrete Th cell-associated cytokines. ILC1 secrete interferon- γ (IFN- γ), ILC2 produce IL-5 and IL13 and finally ILC3 produce IL-17 and IL-22 (Fort et al., 2001; Fallon et al., 2006; Licona-Limón et al., 2013; Walker et al., 2013; Kabata et al., 2015).

ILC2 are activated in the presence of IL-25, IL-33 or thymic stromal lymphopoietin (TSLP). They are dependent to the TH2-defining transcription factor GATA-binding protein 3 (GATA-3) and the transcription factor retinoic acid receptor-related orphan receptor (RORa) for their development. GATA-3 is required to produce ILC lineages in bone marrow for ILC2 differentiation and maintenance (Hoyler et al., 2012) (figure 3).

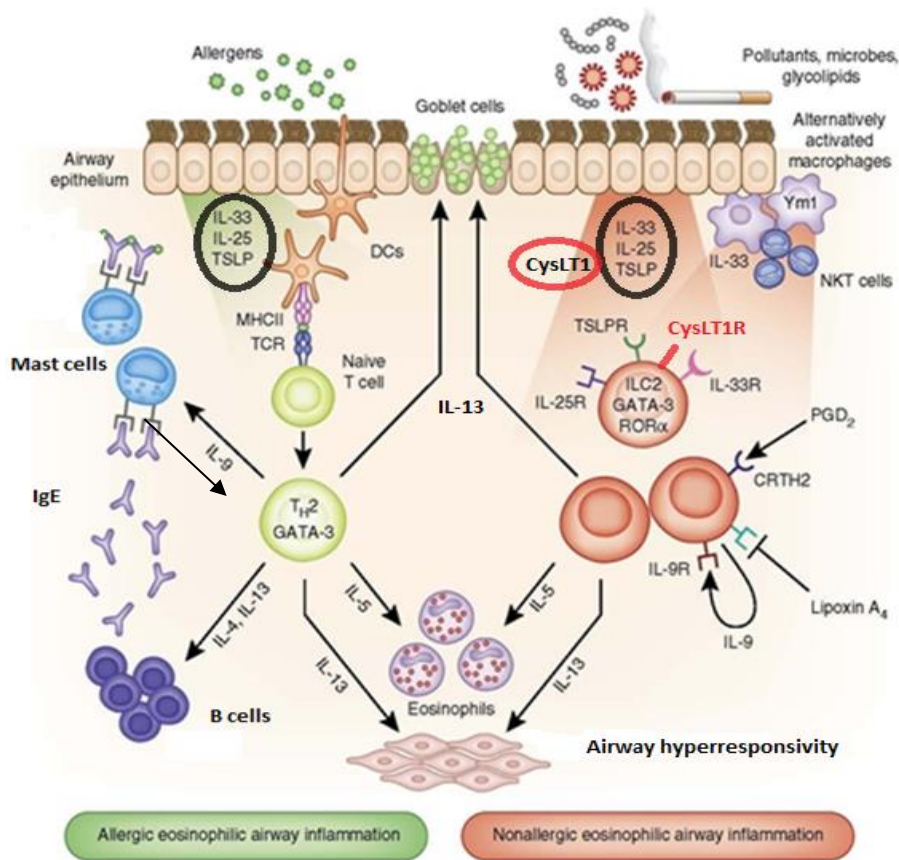


Figure 3 TH2 & ILC2 eosinophilic airway inflammation pathways

Overview of functions of TH2 cells and ILC2 cells that lead to eosinophilic airway inflammation through two different pathways. In allergic eosinophilic airway inflammation, TH2 cells stimulated by dendritic cells in presence of allergens. TH2 cells release IL-4, IL-5 and IL-13, leading to IgE synthesis, eosinophilia, inflammation and bronchial hyperreactivity. In non-allergic eosinophilic airway inflammation, ILC2s are activated in the presence of air pollutants, microbes through an antigen-independent manner via their respective receptors (IL-25R, IL33R, CysLT1R and TSLPR). Activated ILC2s release IL-5 and IL-13, causing eosinophilia, inflammation, mucous hypersecretion and bronchial hyperreactivity. TH2 cells and ILC2 cells have many similar features, both are activated in the presence of IL-33, IL-25 and TSLP and express the transcription factor GATA-3. TH2 cells produce more IL-4 than ILC2s and both produce IL-9. Mast cells also produce IL-9 which stimulates IL-4-driven antibody production by B cells. CRTH2, chemoattractant receptor-homologous molecule expressed on TH2 cells. ALX/FPR2, receptor for lipoxin A₄; FcεRI, high-affinity receptor for IgE; GATA3, GATA-binding protein 3; PGD₂, prostaglandin D₂; RORα, retinoic acid receptor-related orphan receptor α. Modified from Brusselle et al. *Nat Med.* 2013;19 (8):977-9.

1.9 NEUTROPHILIC INFLAMMATION

Neutrophil cells can be seen in the induced sputum of healthy people and asthmatic patients. However, the number of neutrophil cells will increase in the airway secretion of patients with “severe asthma” (Jatakanon et al., 1999). Airway reversibility is also less common in this type of inflammation. Neutrophilic inflammation of airways has been observed in the absence of TH2 cytokines but present in both TH1 and TH17 cytokines (Lambrecht & Hammad, 2015). Airway remodelling is increased in asthmatic patients with neutrophilic inflammation, though these patients take higher doses of inhaled or oral corticosteroids. It is believed that IL-17A may contribute to airways remodelling and steroid-resistance in this type of inflammation (Fahy, 2009; Lambrecht & Hammad, 2015).

1.10 BIOMARKERS OF AIRWAY INFLAMMATION; DIAGNOSTIC APPROACHES

In eosinophilic inflammation, the numbers of eosinophil cells in peripheral blood and in airways secretions are increased. Several studies showed that the number of eosinophil cells have a correlation with the severity of asthma. For example, Bousquet and colleagues (1990) have reported that the number of eosinophils in peripheral blood and in bronchial lavage of asthmatic patients is associated with severity of asthma. Brightling and colleagues (1999) also showed that chronic cough patients with EB had higher numbers of eosinophil in their sputum which reduced significantly after inhaled corticosteroid therapy. This data show that measurement of eosinophils may be a useful biomarker of eosinophil airways inflammation and hence guide therapies which target airway inflammation specifically (Green et al., 2002).

Bronchoalveolar lavage and endobronchial biopsies are the gold standard methods to detect eosinophilic inflammation. However, these methods are invasive and costly and also have many other disadvantages. Therefore, a number of non-invasive sampling methods have been developed which are reliable and complementary to the reference standards. Some of these methods are valid and are in current use in clinical settings for evaluation and therapy monitoring. In this section I will discuss sputum induction, blood

eosinophilic biomarkers and measurement of fractional exhaled nitric oxide (FeNO) methods as diagnostic approaches to the assessment of eosinophilic inflammation (Boot et al., 2007; Korevaar et al., 2015).

1.10.1 SPUTUM INDUCTION

Sputum induction is a validated tool to diagnose respiratory inflammation and monitor anti-inflammatory drug outcomes. Since the end of the 19th century sputum induction has been investigated but researchers used different techniques which led to lack of standardization and the failure to get a sample in many cases. Thus, in 2002 the European Respiratory Society (ERS) guidelines were introduced to apply a universal standardised technique (Boot et al., 2007).

Sputum induction is performed to collect an adequate sample of secretions from lower airways. In this procedure inhalation of hypertonic saline solution by nebulisation helps the subject to produce sputum that can be expectorated (Chanez et al., 2002). It has been demonstrated that an increased number of eosinophils in sputum samples correlates with the presence of tissue eosinophilia as assessed by biopsies (Korevaar et al., 2015). In addition, increase in the total count of inflammatory cells has a direct relationship with severity of disease and duration of exacerbations in asthmatic patients (Louis et al., 2000; Lemièrè et al., 2006). Hence, anti-inflammatory therapies such as corticosteroids reduce the number of eosinophilic cells in sputum which is usually associated with an improvement in disease symptoms and lung function (Boot et al., 2007).

Sputum induction in comparison with bronchial biopsy or BAL is safer, cheaper and easier to administer. Although in bronchial biopsy or BAL samples can be collected from different part of lower airway, analysing the samples from these different techniques indicates similar information regarding inflammatory airway biomarkers. Thus there is a reasonable relationship between them (Boot et al., 2007; Korevaar et al., 2015).

Despite all the advantages of sputum induction there are also some drawbacks. Inhalation of hypertonic saline is an unpleasant experience for subjects and can cause excessive coughing and occasionally vomiting.

Nonetheless, triggering cough is fundamental in this technique and is part of the process. Hypertonic saline also causes bronchoconstriction in asthmatic subjects, therefore pre-treatment with a short acting beta-agonist is recommended to prevent excessive bronchoconstriction. The occurrence of bronchoconstriction after starting the test forces the technician to stop the induction or collect an inadequate sputum sample. Consequently, success rate and repeatability of the results will be affected. Measuring a decline in lung function during to the test (every 5 – 10 minutes) is necessary to detect any symptoms of bronchoconstriction developing. Analysing the sputum sample is a time-consuming and difficult procedure which requires adequate equipment and a highly trained technician. Because of these reasons, processing the sputum sample is only possible in clinical centres with sufficient equipment and staff; therefore the usefulness of this method is limited to the few research centres having the capability to perform it (Chanez et al., 2002; Boot et al., 2007).

1.10.2 BLOOD EOSINOPHIL COUNT

Measurement of eosinophils in induced sputum is a reliable biomarker of airway inflammation as well as a useful predictor of response to treatment with anti-inflammatory therapy (Boot et al., 2007; Korevaar et al., 2015). Nevertheless, this particular test has some limitations (as discussed above) and therefore cannot be used widely in the clinical settings. Alternatively measurement of blood eosinophil count may be a valuable, easy and non-invasive method to identify patients with eosinophilic airway inflammation. Blood eosinophil count measures systemic eosinophilic inflammation that is an indirect but useful tool to assess airways inflammation severity, and consequently predict and direct treatment in respiratory patients (Zhang et al., 2014; Wagener et al., 2015).

Several studies show that there is a positive relationship between sputum eosinophils and blood eosinophils. However, there is only a moderate correlation between these two components (Malinovschi et al., 2013; Zhang et al., 2014; Wagener et al., 2015). In some studies data shows that blood eosinophil count is not always reduced in patients who are treated by inhaled

corticosteroids (Kips & Pauwels, 1998; Malinovschi et al., 2013). In comparison with patients who are on systemic therapies, such as leukotriene receptor antagonists or IL-5 inhibitors these demonstrate greater reduction in the level of the systemic inflammatory markers (Stelmach et al., 2002; Spahn et al., 2006). These findings challenge the old hypothesis that believed that systematic eosinophilic inflammation occurs because of the spillover from the inflamed airways. It is now believed that the systemic inflammation plays an independent role in asthma and other respiratory diseases. Biologically eosinophils are produced in bone marrow consequent to secretion of inflammatory cytokines such as IL-5. Then eosinophils drive in to the blood and are transported to the airway. Thus, IL-5 may increase the number of eosinophilic cells both in blood and sputum. It is possible to suggest that combining the evaluation both local and systemic eosinophilic inflammation in respiratory disease may provide complementary data of greater value (Malinovschi et al., 2013; Katz et al., 2014; Wagener et al., 2015).

1.10.3 MEASUREMENT OF FRACTIONAL EXHALED NITRIC OXIDE (FeNO)

The measurement of exhaled nitric oxide is widely accepted as a non-invasive marker of airway Inflammation and, amongst other uses, has been proposed to monitor the response to anti-inflammatory medications. In 2005 clinical guidelines for the measurements of nitric oxide (NO) from the upper and lower respiratory tract has been published by the American Thoracic Society (ATS) (Boot et al., 2007).

The fraction of exhaled nitric oxide (FeNO) is a marker of local airways inflammation. In humans, nitric oxide mainly comes from the lower airways and synthesis from L-arginine by enzymes called constitutive NO synthase (cNOS) and inducible NO synthase (iNOS). cNOS is activated in endothelial, epithelial cells and neurons, while iNOS can be induced in inflammatory conditions, epithelial and airway smooth muscle cells (Ricciardolo, 2003). Previous studies have shown that a rise in FeNO value can be detected in patients with asthma and a further increase has been seen during asthma exacerbations (Boot et al., 2007). Biologically the level of nitric oxide in

breath is associated with secretion of IL-4 and IL-13 (Malinovschi et al., 2013). Therefore there is a correlation between FeNO concentrations and the IgE levels and the positive skin prick test (Cardinale et al., 2005). Likewise, patients with atopic asthma produce higher levels of FeNO than patients with non-atopic asthma (Gratziou et al., 1999).

In a recent study it has been found that the prevalence of current asthma and wheeze increased 3 times more among patients with high FeNO values than patients with normal FeNO (Malinovschi et al., 2013). Moreover, FeNO level is decreased in response to anti-inflammatory treatments. It had been shown that the level of FeNO decreases with anti-IL-13 treatment (lebrikizumab) (Corren et al., 2011). In response to inhaled corticosteroids FeNO level reduction was dependent on to the dose of treatments (Kharitonov et al., 2002). Administration of leukotriene receptor antagonists also resulted in reduction in FeNO level (Sandrini et al., 2003).

Nevertheless, using the FeNO value as the sole marker of airway inflammation is not recommended (Boot et al., 2007; Malinovschi et al., 2013; Korevaar et al., 2015). It has been reported that the level of FeNO in patients using corticosteroids decreases quickly while airway inflammation and hyperresponsiveness can still be detected by other markers of airway inflammation (Leuppi et al., 2001). Therefore, the reliability of FeNO as a guide to therapy could be questioned and FeNO might be too sensitive to the initiation of corticosteroid therapy.

1.10.4 BLOOD EOSINOPHIL COUNT OR FeNO?

FeNO and blood eosinophilia have been considered as replacement markers for sputum eosinophilia as each of them is able to distinguish eosinophilic inflammation phenotype from neutrophilic. However there is only a modest correlation between FeNO and sputum eosinophilia ($r=0.52$, $p<0.001$), and a moderate to good correlation between blood and sputum eosinophilia ($r=0.59$, $p<0.001$) in asthmatic patients (Wagener et al., 2015). A systematic review and meta-analysis study in asthma investigated the diagnostic accuracy of FENO, blood eosinophil counts and IgE to identify eosinophilic asthma (sputum eosinophils $\geq 3\%$) by using receiver operating characteristics area

under the curve (ROC AUC) (Korevaar et al., 2015). It was reported that the ROC AUC for FeNO in 17 adult studies (3216 patients) was 0.75 (95% CI 0.72–0.78). The ROC AUC for blood eosinophilia in 14 adult studies (2405 patients) was 0.78 (0.74–0.82). Finally, IgE in seven studies (942 patients) had the lowest diagnostic accuracy with ROC AUC of 0.65 (0.61–0.69). In this review summary estimates sensitivity and specificity of FENO value in detecting sputum eosinophils in adults were 0.66 (0.57–0.75) and 0.76 (0.65–0.85) respectively. These values in blood eosinophil counts were 0.71 (0.65–0.76) and 0.77 (0.70–0.83) respectively; and in IgE were 0.64 (0.42–0.81) and 0.71 (0.42–0.89) respectively. Accordingly, blood eosinophilia and FeNO appeared to be the better predictors for eosinophilic inflammation in compared with IgE. Although they consistently reflected a moderate diagnostic accuracy, which will lead to a number of false results (Korevaar et al., 2015; Wagener et al., 2015). However, it is important to consider that studies on asthmatic patients might not reflect results in chronic cough patients. In a systematic review Song and his colleagues (2017) investigated the diagnostic accuracy of FeNO in chronic cough patients with CVA or EB. In this review 15 studies has shown that AUC for FeNO on patients with cough variant asthma was 0.87 (95% CI 0.84-0.90). The diagnostic accuracy of FeNO on chronic cough patients with cough variant asthma or eosinophilic bronchitis was AUC 0.89 (95% CI 0.86-0.92) while this figure was lower [AUC=0.81 (0.77-0.84)] on chronic cough patients with non-asthmatic eosinophilic bronchitis (Song et al., 2017).

The above results indicate that FeNO and blood eosinophilia markers are imperfect tools and there is a possibility that some patients with airway eosinophilia may be falsely considered as normal and would not receive effective treatment. However, moderate accuracy markers still can be useful if they are applied with more consideration, for instance in monitoring therapy.

Consequently, based on the above evidence, it is believed that these biomarkers cannot individually diagnose eosinophilic inflammation accurately. Particularly, it is evident that FeNO and blood eosinophilia are triggered by two different cytokine mechanisms (please see section 1.10.2 & 1.10.3) and there is a weak correlation between these two elements

(Malinovski et al., 2013; Wagener et al., 2015) . As a result, it is suggested that a combination of these markers with other clinical features is more preferable, which is expected to improve diagnostic accuracy (Boot et al., 2007; Malinovski et al., 2013; Korevaar et al., 2015). This however needs to be tested against clinical endpoints, particularly in eosinophilic cough.

1.11 AIRWAY INFLAMMATION; TREATMENT APPROACHES

Treatment of chronic cough caused by airway inflammation is a challenge for the managing physician. Despite substantial clinical investigation on anti-cytokines therapy, treating airway inflammation has, as yet, been only partially successful (Hansbro et al., 2011). Therefore, corticosteroid therapy remains the most widespread anti-inflammatory treatment, despite their side effects. Particularly when the majority of patients with neutrophilic inflammation respond poorly or not at all to high-dose inhaled or oral steroid therapy (Morice et al., 2006).

Montelukast is another treatment option that is generally considered as an add-on therapy in patients with poorly controlled asthma. However, recently it has been suggested that montelukast might have wider range of anti-inflammatory properties than originally thought (Tintinger et al., 2010).

In this section the effect of oral prednisolone and montelukast treatments on patients with airway inflammation will be reviewed.

1.11.1 CORTICOSTEROIDS THERAPY

Corticosteroids have been supported as an anti - inflammatory therapy in airway diseases for five decades. Systemic corticosteroids (SCS) (Administration of corticosteroid through oral, intramuscular or intravenous routes) and inhaled corticosteroids (ICS) (Administration of corticosteroid by dry powder inhaler, metered dose inhaler or nebuliser) are two forms of steroid therapy which have been utilised. Systemic corticosteroids were of course the preferred option before the development of ICS; they were the only option (Rowe et al., 2004). Currently most asthmatic patients are able to manage their symptoms by using ICSs alone or in combination of long acting bronchodilators. Nevertheless, there are still some patients with uncontrolled

asthma for whom administration of oral prednisolone is essential (Bourke, 2015).

ICSs are also widely used in chronic cough (Dicpinigaitis et al., 2014). ICSs are effective on eosinophilic bronchitis (eosinophilic airway inflammation in absent of airway hyperresponsiveness) and they generally improve cough and treat airway eosinophilia (Brightling et al., 1999). ICSs are also useful for patients with cough variant asthma (CVA) and in the long term it has been suggested that it may help to prevent classic asthma in these patients (Cheriyian et al., 1994). Conversely, SCSs are infrequently used to control eosinophilic bronchitis (Brightling, 2006). According to the American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines (Irwin et al., 2006a) *“Patients with cough due to asthma should initially be treated with a standard antiasthmatic regimen of inhaled bronchodilators and inhaled corticosteroids (ICSs). In patients whose cough is refractory to treatment with ICSs, an assessment of airway inflammation should be performed whenever available and feasible. The demonstration of persistent airway eosinophilia during such an assessment will identify those patients who may benefit from more aggressive anti-inflammatory therapy”*.

In adults low dose ICSs are safe and they have limited side effects such as oropharyngeal candidiasis or hoarseness. These side effects can be eliminated by using a spacer device or a dry powder device and rinsing the mouth and the throat with water after inhalation. Conversely, use of high dose ICSs can cause significant systemic adverse effects including suppression of adrenal function and enhanced bone turnover. Accordingly, the safety of repetitive administration of this agent should be considered carefully, since it can lead to severe systematic adverse effects (Bourke, 2015).

Long term usages of steroids can cause adrenal suppression, decreased bone metabolism and thus causing osteoporosis and bone fractures, metabolic disturbances which increase blood glucose, increased eye pressure and causing glaucoma and cataract, decreased skin collagen synthesis, effect on the central nervous system and also, in children suppress their linear growth (Kuna, 1998; Busse & Holgate, 2000). These side effects lead to other diseases such as high blood pressure and diabetes, therefore patients need to use more drugs to control them. Consequently, systemic corticosteroids can

cause further disability for their users (Busse & Holgate, 2000). For that reason, there is an urgent need to replace this agent with another therapy which has less unwanted and damaging effects on patients.

1.11.1.1 CORTICOSTEROIDS MECHANISMS EFFECT ON AIRWAY INFLAMMATION

The number of inflammatory cells in the airways, such as eosinophils, mast cells, T lymphocytes, and dendritic cells are reduced by corticosteroids. These broad spectrum cellular effects of corticosteroids are created by preventing the recruitment of inflammatory cells, decreasing the production of chemotactic mediators and down regulating adhesion molecules (Barnes & Adcock, 2003).

The efficacy of corticosteroids in preventing airway inflammation results from a number of mechanisms based on their long and short term effects on respiratory diseases. Traditionally, it has been suggested that corticosteroids are circulating across the cell membrane and then binding to glucocorticoid receptors (GR). GRs are a type of nuclear receptor normally bound to proteins, known as molecular chaperones (shock protein-90 (hsp90) and FK-binding protein) (Barnes & Adcock, 2003). Chaperone proteins interact with the ligand-free form of GR and cover the parts of the receptor that are needed to translocate from nuclear membrane into the nucleus. Thus these proteins are crucial to protect the glucocorticoid receptors from nuclear localization. Yet, when corticosteroids are binding with GR, this causes changes in the receptor structure which then releases the molecular chaperone proteins exposing GR to nuclear localisation signals and consequently activating them in the cytoplasm. Activated GR–corticosteroid complex move through the nuclear membrane and bind with DNA sequences of corticosteroid-responsive genes called glucocorticoid receptor elements (GRE). Once GR have bound with GRE, it activates transcription of responsive genes and increases gene transcription (*trans-activation*)(Barnes, 2006). Several of these responsive genes that are regulated and prompted by corticosteroids have anti-inflammatory effects, including: lipocortin-1, secretory leukocyte inhibitory protein (SLPI), IL-10 and IL-12. Moreover, GR-GRE complex suppresses pro-inflammatory proteins including: IL-4, IL-5, IL-6, IL-13, and

tumor necrosis factor α . Consequently, corticosteroids effectively inhibit the inflammatory activity of mast cells and T-lymphocytes and as a result, reduce the number of eosinophils in the airway (Liu et al., 2001; Dicpinigaitis et al., 2014). The GR is also able to activate cell signalling pathways by protein phosphorylation independent of transcriptional manner. The GR is phosphorylated by kinases, including MAPKs, CDKs, and GSK-3 β . Phosphorylation reduces the transcriptional activity of the GR within cells. In addition, GR in high concentration can also prompt gene transcription via protein-protein interactions without specific DNA recognition.

1.11.1.2 CLINICAL STUDIES REVIEW OF ORAL PREDNISOLONE

Oral prednisolone is the SCS which is the most commonly used steroid in the treatment of patients with chronic asthma (BTS, 2014).

Regarding cough, there are limited studies that evaluate the effect of oral prednisolone on cough patients. There is a study that evaluates the effect of oral prednisolone on acute cough following respiratory tract infection. In this study, 436 patients with non-asthmatic acute cough participated in a two arm, multi-centre, placebo - controlled, randomised superiority trial. 40 mg daily oral prednisolone was administered for 5 days. The results show that cough duration was reduced at least 20% in the treatment group compared with the control group (Downing et al., 2015). In another study on eight patients with histories of recurrent, post viral, non-asthmatic coughs treatment with oral corticosteroids significantly improved the cough (Strauss, 2013). There are two studies that show oral prednisolone can improve cough which is caused by rhinovirus. In a controlled trial on children with rhinovirus or enterovirus induced recurrent wheezing, oral prednisolone effectiveness was compared with placebo. It has been reported that prednisolone reduced the duration of cough and dyspnea, it also decreased the blood eosinophil count in children. This study did not detect any difference in oxygen saturation or exhaled nitric oxide measurements among the treatment groups (Jartti et al., 2007). Jartti and colleagues (2015), in a double-blind randomised study assessed short term and long term effects of oral prednisolone treatment in young children with the first acute, moderate-to-severe, rhinovirus-induced wheezing

episode. They reported that patients who received the oral prednisolone had shown a significant improvement in severity of symptoms including; cough, rhinitis, and noisy breathing during the two weeks follow-up compared with the patients who were in the placebo group. However no long-term effects between the groups was detected (Jartti et al., 2015). In acute studies perhaps virus release IL33 which leads to secretion of IL-4, IL-5, and IL-13 responses in airway eosinophilic inflammation (Liu et al., 2001; Moriwaki et al., 2011; Baraldo et al., 2012).

However, there are no studies in the literature that assess the effects of oral prednisolone on chronic cough, apart from some small trials which have shown its effects on patients with cough variant asthma (CVA). In a small prospective open label study oral prednisone followed by corticosteroid inhalers were used in a group of patients who suffered from persistent cough from 2 months to 20 years. In this study only patients with non - productive cough with minimal or no wheezing and dyspnea were included when other sources of cough were carefully investigated and excluded. Initially a short course of prednisone as a diagnostic-therapeutic trial were administered and then continued by inhaled corticosteroids to control cough and maintain the effect of oral prednisolone. This diagnostic - therapeutic trial revealed that nine out of 10 patients responded to the therapy (Doan et al., 1992). Similar to this study Cheriyan and colleagues (1994) characterised CVA “*as a persistent nonproductive cough with minimal wheezing or dyspnea*”. They reported similar results on a small study among 10 patients (Cheriyan et al., 1994). However none of these studies characterised the type of inflammation associated with cough.

In addition to the lack of literature and large randomised control trials in this area, there is not any consistent guidelines on dose or duration of corticosteroids therapy for treatment of cough syndromes. Dicipinigitis and colleaguse (2014) believed that “*Current practice has evolved mainly from anecdotal experience, consensus opinion, and the extensive body of evidence showing the efficacy of corticosteroids in controlling symptoms (including cough) in more typical "classic" asthma. However, given the broad range in*

source and quality of evidence, there is no consistent recommendation on dose or duration of corticosteroid therapy for the treatment of cough”.

By considering all the present evidence of prednisolone efficacy on airway inflammation and its severe unwanted effects, we believed that it is necessary to fully evaluate efficacy of prednisolone and montelukast in chronic cough. Brightling (2006) believed that *“The role of other potential therapeutic agents such as antihistamines and antileukotrienes needs to be fully explored”.*

1.11.2 MONTELUKAST

Montelukast is a pharmacological antagonist of type 1 cysteinyl leukotriene receptors (CysLT1Rs). Cysteinyl leukotriene (cysLTs) are the most potent bronchoconstrictors known that have a crucial role in both immediate and late asthmatic responses. Montelukast effectively inhibits the activities of CysLT1Rs and is recognised in international guidelines as a novel therapy in asthma treatment (Gagro et al., 2004). According to the American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines (Irwin et al., 2006a) *“ For patients with asthmatic cough that is refractory to treatment with ICSs and bronchodilators, in whom poor compliance or another contributing condition has been excluded, a leukotriene receptor antagonist may be added to the therapeutic regimen before the escalation of therapy to systemic corticosteroids”.* Nevertheless, there are limited clinical studies in literature that show this anti-inflammatory antagonist could be an effective therapy for chronic cough.

1.11.2.1 LEUKOTRIENES PATHWAY

The Cysteinyl leukotrienes (cysLTs) are a family of inflammatory lipid mediators including leukotriene C4 (LTC4), leukotriene D4 (LTD4) and leukotriene E4 (LTE4). Leukotrienes are produced from nuclear membrane phospholipids in multi-enzymatic cascade. They are synthesised from arachidonic acid through a number of pathways, one of which is the 5-lipoxygenase pathway (Holgate et al., 2003). In this process 5-lipoxygenase oxidated arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (HpETE) and then HpETE converted to LTA4. LTA4 is an unstable leukotriene which

be hydrolysed to LTC₄. Alternatively, LTA₄ can re-form to LTB₄ in neutrophils and other inflammatory cells. LTB₄ is known as a strong neutrophil activator and chemoattractant. LTB₄ also can cause eosinophil chemotaxis. Once LTA₄ is converted to LTC₄, LTC₄ is transported to the extracellular space where is varied between LTD₄ and LTE₄. LTC₄, LTD₄, and LTE₄ all have a cysteine residue and have a very similar effects on the airway smooth muscle. The cysteinyl leukotrienes are abundantly generated in the airway mucosa and submucosa by a selection of cells, mostly mast cells, eosinophils, basophils and macrophages. Cysteinyl leukotriene receptors identify CysLTs and interact with these pro-inflammatory mediators (Samuelsson et al., 1987). As a result of this interaction, CysLTs activate and successively recruit and stimulate inflammatory cells, increase vascular permeability, mucous secretion and bronchial hyperresponsiveness, and promote airway remodelling (Niimi, 2013) (figure 4).

1.11.2.2 LEUKOTRIENE – ILC2 PATHWAY

A crucial question that remains unanswered is that by which mechanism(s) cysteinyl leukotriene receptors antagonists accomplish their antitussive effect on airway inflammation. Previously, it has been proven that CysLT₁R is upregulated by Th₂ cytokines, including IL-4 and IL-13 on human subjects (Thivierge et al., 2001). Furthermore, human Th₂ cells stimulated by CysLTs induced production of IL13 in a CysLT₁R dependant manner. LTE₄ was particularly potent in inducing cytokines production for human Th₂ cells compared with LTD₄ (Xue et al., 2012). Collectively, based on these reports it has been suggested that Th₂ cytokines and CysLTs regulate each other mutually (Doherty et al., 2013). In a recent study in mice it was evident that ILC₂ also can be stimulated by CysLTs. This study demonstrated three important findings. Firstly, it has been reported that lung and bone marrow ILC₂s express CysLT₁R in unchallenged mice and moreover, remains stably expressed in lung ILC₂s after allergen challenges independent of STAT6 and adaptive immune cells. Secondly, stimulation of ILC₂ with LTD₄ (the main ligand for CysLT₁R) after a single exposure to allergens, enhanced cytokine production of IL-4, IL-5 and IL-13 in CysLT₁R dependant manner. Finally, it has been shown that administrating LTD₄ to airways of mice regulate ILC₂

to produce IL-5 and potentially cause airway eosinophilia inflammation independent of adaptive immune cells (Doherty et al., 2013). These results are greatly important in Th2 dependant diseases such as both allergic and non-allergic asthma. Based on documented evidence in the above studies it can be concluded that in presence of allergens Th2 cells produce Th2 cytokines. These cytokines upregulate CysLTs in airway consequently enhance level of CysLTs which is able to active lung ILC2. Activated ILC2s then rapidly produce Th2 cytokines in a CysLT1R dependant manner. In view of that, leukotriene - ILC2 pathway in respiratory diseases (which level of CysLTs increase) might promote airway eosinophilia inflammation and hyper responsiveness by enhancing production of IL4, IL5 and IL13 (Doherty et al., 2013).

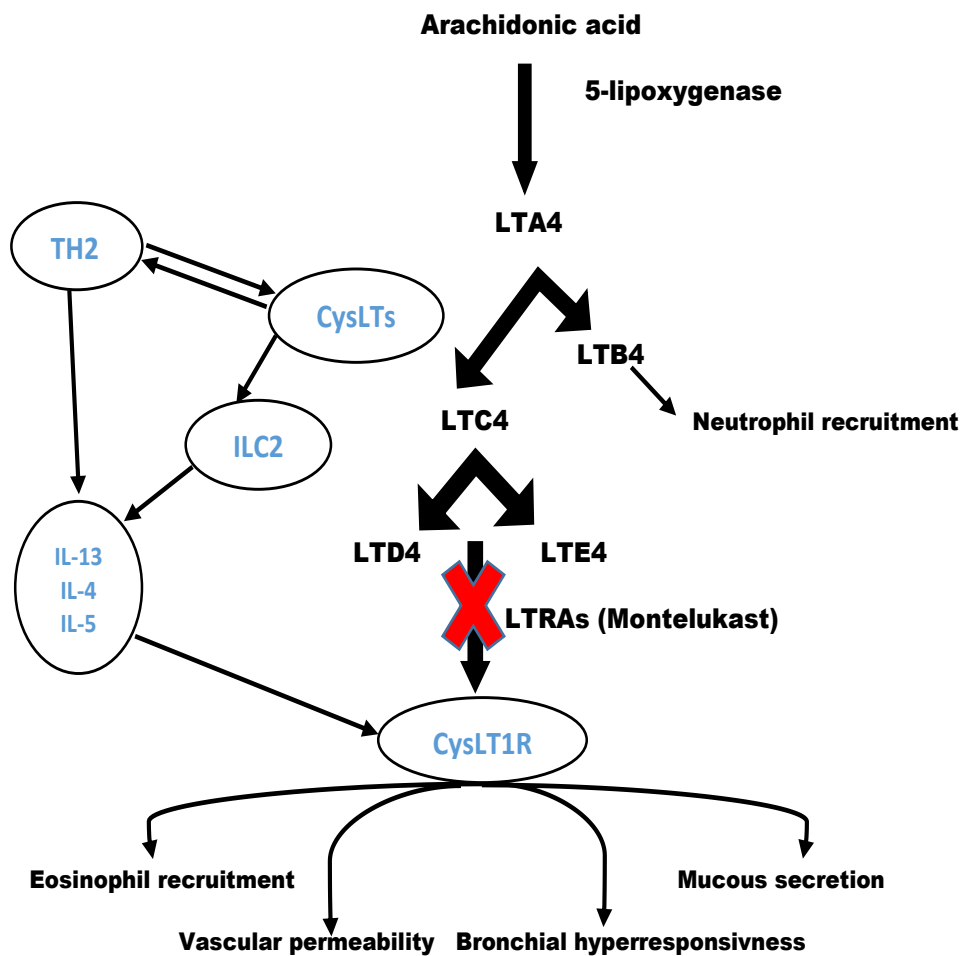


Figure 4 Effects of cysteinyl leukotrienes (CysLTs) on airway eosinophilic inflammation through TH2 and ILC2 pathways.

Arachidonic acid converted LTA4 by 5-lipoxygenase. Then LTA4 converted to LTC4 and then to LTD4 and LTE4 in the extracellular space. These CysLTs can be upregulated by Th2 & ILC2 cytokines and include IL-4 and IL-13 and interacted with Cysteinyl leukotriene receptors (CysLT1R). Cysteinyl leukotriene receptors antagonists block this interaction to accomplish their antitussive effect.

1.11.2.3 CLINICAL STUDIES REVIEW

A review of clinical studies show numerous studies confirming the effect of montelukast in asthmatic patients. Gagro and colleagues (2004) in a study in 14 children with allergic asthma reported that after 6 weeks treatment with montelukast peripheral blood eosinophil count decreased significantly. A significant decrease in the percentage of T lymphocytes and the level of total IgE was observed as well. In a double-blind, randomized, parallel group, placebo-controlled study among 2791 adults with active seasonal allergic rhinitis treatment with montelukast 10 mg (n=813), revealed a significant reduction in peripheral blood eosinophilia in comparison with loratadine 10 mg (n=1275) and placebo (n=703) groups (Ritter et al., 2002). Similar results have been reported when airway tissue inflammatory cells were assessed directly by bronchoscopy. After 6 weeks treatment with montelukast the number of eosinophils cells and mast cell reduced significantly compared with a placebo group (Ramsay et al., 2009).

There are several clinical studies that have reported the effectiveness of montelukast on patients with cough variant asthma (CVA) as defined by cough with bronchial hyper responsiveness and eosinophilic airway inflammation, in absence of bronchoconstriction or airway obstruction. In a study on adults, patients with chronic cough received a diagnosis of CVA and AC (atopic cough). Two weeks therapy with montelukast demonstrated a significant decrease in cough scores which was assessed with a subjective cough symptom scale. However, in subjects with AC, cough scores did not show a significant change, demonstrating montelukast was ineffective in these patients (Kita et al., 2010). In a small, randomized, double blind, placebo controlled trial four weeks montelukast therapy in patients with CVA demonstrated a significant improvement in cough frequency that was evaluated subjectively (Spector & Tan, 2004). In another study in subjects with CVA montelukast significantly decreased the value of FeNO and sputum eosinophil. Moreover, it reduced airway hyperresponsiveness and cough (Shimoda et al., 2006).

Two small studies have been found that evaluate the effects of montelukast in patients with unclassified chronic cough. In a real life observational pilot

study 14 patients with chronic cough (not due to asthma) were given two weeks' treatment with montelukast and assessed both before and after treatment. Cough scores were measured with a validated questionnaire (Leicester Cough Questionnaire) and demonstrated a significant reduction of cough after treatment. Cough reflex sensitivity to capsaicin decreased significantly. While there was a decrease in cough reflex sensitivity for citric acid, it was not significant. Moreover, it has been reported that the eosinophil cationic protein (ECP) value as a marker of eosinophil activation significantly decreased (Mincheva et al., 2014). In an observational study on children (n = 22) with chronic cough, four weeks treatment with montelukast was administered. In 14 children (68%) cough frequency improved within 72 hours of therapy and cough ceased by the third week of treatment. Children who responded to the therapy had a higher level of ECP in their pre-treatment sample compared with children who did not respond to the therapy. Absolute peripheral eosinophil blood counts and IgE levels also were significantly higher in the responders to therapy before treatment. Two of the children who did not respond to montelukast were diagnosed to have GORD (Kopriva et al., 2004). There is no randomized controlled trial in this patient population.

By reviewing current literature to appraise effectiveness of prednisolone and montelukast in chronic cough, it can be concluded that there is a vital need for a real – life observational study to effectively evaluate the potential efficacy of prednisolone and montelukast in patients with chronic cough. To accomplish this aim, it has been decided to run a real life randomised clinical trial to compare the effect of oral prednisolone (20mg) with montelukast (10 mg) on chronic cough patients. This study will comprehensively assess the efficacy of prednisolone and montelukast on severity of cough and inflammatory markers including FeNO and sputum eosinophilia.

1.12 CONCLUSION

In conclusion the role of induced sputum, FeNO and blood eosinophilic to diagnose eosinophilic inflammation and monitor therapies in chronic cough has yet to be fully defined. Currently, all three methods are in use, with anti-inflammatory therapy directed at eosinophilic inflammation prescribed

mainly on clinical judgement rather than evidence. There is an urgent need for the rational application of objective measures of eosinophilic inflammation to avoid excessive and potentially harmful anti-inflammatory treatment in patients with chronic cough.

CHAPTER TWO

METHODOLOGY

2.1 SUBJECTS

Adult patients with chronic cough who attended Castle Hill Hospital Cough Clinic were studied.

Healthy volunteers were recruited from the plastic surgery outpatient clinic in Castle Hill Hospital.

The characteristics of the subjects are described in the individual research studies.

2.2 MEASUREMENT OF FRACTIONAL EXHALED NITRIC OXIDE (FeNO)

FeNO was measured with a NIOX VERO FeNO machine supplied by Aerocrine LTD. The NIOX VERO is a device that employs electrochemical sensors to convert NO gas concentration into electrical signals (Maniscalco et al., 2016). It is a small and portable device that can measure concentration of NO in breath of adults and children.

Fractional exhaled nitric oxide was measured following the NIOX VERO Airway Inflammation Monitor User Manual which was provided by the Aerocrine LTD.

To measure FeNO, patients were seated in front of the equipment at a convenient position. Patients were instructed to breathe out and then close the mouth around the mouthpiece with a tight seal eliminating any air leak. Patients inhaled deeply through the mouthpiece as instructed and guided visually by the machine. The patients then exhaled slowly for 10-second at a constant pressure of 10–20 cmH₂O to sustain a fixed flow rate of 50 ± 5 ml/second (the speed of exhalation is guided by the demo). A calibrated electrochemical sensor analyses the last 3 seconds of the 10-second exhalation to indicate results in parts per billion (ppb) with a measurement range of 5 ppb to 300 ppb.

The NIOX VERO device is pre-calibrated and designed to use without daily calibration (Harman et al., 2015). To ensure validity of data a daily external Quality Control (QC) procedure was performed by a qualified individual (myself) who was presented in the site every morning. If the individual was ill one of the other staff did the test.

In accordance with the ATS and ERS (the published guidelines on standardized techniques as appropriate for measuring exhaled Nitric Oxide (2005)) all FeNO measurements were performed PRIOR to spirometry assessments, (spirometric manoeuvres have been shown to transiently reduce exhaled NO levels). Patients were advised to avoid eating and drinking for at least 2 hours prior to measurement and to avoid any strenuous exercise for one hour. Respiratory tract infections may lead to increased levels of exhaled NO. Patients were asked to report any adverse events at the start of the visits including URTI. The study physician then saw those with an AE and if in their opinion UTRI was present the results were excluded from subsequent analysis.

2.3 PULMONARY FUNCTION (SPIROMETRY)

Equipment

A pneumotach within KoKo DigiDoser Spirometer meeting the specifications and performance criteria recommended in the American Thoracic Society (ATS)/European Respiratory Society (ERS) Standardization of Spirometry (Miller et al., 2005), was used.

The KoKo Spirometer is a device for measuring air flow and when attached and linked to KoKo PFT software can be used to derive flow curves and has the capacity to print FVC tracings.

The pneumotach was calibrated every morning by a calibrated 3-litre volume syringe before any spirometric measurements for the study were performed.

Preparing the test subject

On study days when spirometry was performed, patients refrained from the following:

- Coffee, tea, chocolate, cola and other caffeine-containing beverages and foods and ice cold beverages for 4 hours prior to spirometry
- Alcohol for 4 hours prior to spirometry
- Strenuous activity for 12 hours prior to spirometry
- Exposure to environmental smoke, dust or areas with strong odours

Every effort was made to ensure consistent testing conditions throughout the study. A seated position was provided to reduce risks related to dizziness or syncope. To minimize the effects of diurnal variation on lung function,

spirometry visits were started at approximately the same time of day at each visit.

Performing Spirometry

The subject's age, height and gender were entered into the spirometer. Spirometry, an effort-dependent test, requires careful instruction and cooperation of the subject. The patient was instructed to have a good seal around the mouthpiece in a correct posture. The participants were coached to perform a maximal inspiration, followed by maximum forced expiration for at least 6 seconds. Expiration was rapid with exertion of maximal effort. The results of spirometry met the ATS/ERS criteria for acceptability and repeatability. A minimum of 3 acceptable forced vital capacity (FVC) manoeuvres were performed. If a subject was unable to perform a single acceptable manoeuvre after 8 attempts, testing was discontinued.

Acceptability

An acceptable manoeuvre has the following characteristics:

- No hesitation or false start;
- A rapid start;
- No cough, especially during the first second of the manoeuvre;
- No glottic closure or obstruction by tongue or dentures
- No early termination of exhalation (minimum exhalation time of 6 seconds is recommended, or no volume change for at least 1 second) or the subject cannot continue to exhale further.

Reproducibility

The 2 largest FVC and FEV1 values from 3 acceptable manoeuvres should not vary by more than 0.150 L.

Recording of data

The highest FEV1, FVC, FEV1/FVC, FEF 25-75% and PEFr from any of the acceptable curves was recorded. (The highest FEV1, FVC, FEV1/FVC, FEF 25-75% and PEFr may not necessarily have resulted from the same acceptable curve).

Predicted normal

For subjects greater than 18 years of age, this study utilized the spirometric prediction equation standards for the European Community for Coal and Steel (Quanjer et al., 1993).

2.4 SPUTUM COLLECTION

Collection of sputum sample occurred at visits 1, 3 and 5 for each participating patient. Sputum samples were either collected by spontaneous expectoration or through sputum induction. Previous studies have shown that there is no significant difference in total and differential cell counts between the sputum samples produced with either of these methods, although the cell viability is significantly higher in induced sputum samples in compared with spontaneous sputum samples (Pizzichini et al., 1996; Bhowmik et al., 1998).

2.4.1 COLLECTION OF SPUTUM SPONTANEOUSLY

To collect sputum sample spontaneously patients were instructed to blow their noses and rinse their mouths out with water prior to expectorating, this reduces salivary contamination of the sample. Patients were then asked to take three deep breaths and cough deeply and vigorously when breathing out, and to expectorate into the first labelled pot. The same procedure was repeated and the patients asked to cough up more mucoid sputum into the second labelled pot. The first sample was discarded and second sample was analysed in order to increase viability and quality of collected cells.

2.4.2 SPUTUM INDUCTION

The aim of sputum induction is to obtain satisfactory samples of secretions originating from the airways in a safe and effective manner for subsequent and predetermined analysis.

This methodology uses increasing concentrations of hypertonic saline solution to be inhaled via nebulization, which increases the osmotic pressure and pulls more water from the blood flow surrounding the airway, into the lumen. Diffused water into the lumen causes dilution of mucins in the airways consequently easing sputum expectoration (Seong et al., 2014).

An Ultrasonic Nebuliser (DeVilbiss UltraNeb) with average output of 1 ml/min was used to generate aerosols with a dose of about 5–7mL per

inhalation (Boot et al., 2007). The device was set according to the Standard Operating Procedure of the Clinical Trial Unit No: CTU101099 (Please see appendix 1).

Hypertonic saline was administered with following safety precautions; Medications to treat severe bronchospasm was present within the testing laboratory. These included adrenaline and atropine for subcutaneous or IV injection and salbutamol and ipratropium in metered dose inhalers or pre-mixed solutions for inhalation. Oxygen was also available. A small volume nebulizer was set up ready for the administration of bronchodilators if required. A stethoscope, sphygmomanometer, and pulse oximeter were also accessible.

Airflow was monitored regularly (via FEV1 determination) throughout the test to ensure that the patient was not bronchoconstricting.

There is evidence that hypertonic saline can cause bronchoconstriction in patients (Chanez et al., 2002). To reduce the risk of bronchoconstriction from saline administration, all patients were administered a salbutamol 200ug 20 minutes prior to commencement of saline nebulisation.

Equipment and Materials

- DeVilbiss UltraNeb Ultrasonic Nebuliser
- Micro Medical Micro Plus hand held spirometer
- Volumatic spacer for salbutamol delivery
- Pipettes and tips
- Pari-boy nebuliser
- Sterile hypertonic saline 7% (stored at room temperature)
- Sterile water to dilute hypertonic saline
- Salbutamol MDI (100µg. Stored at room temperature)
- Salbutamol as Salamol Steri-NEB 2.5mg/.2.5ml

Performing the test

Patients were provided with a comfortable chair with back support and arm rests.

Three baseline FEV1 measurements were performed and the highest value was recorded as a baseline value. 200µg Salbutamol was administered to the

patient via a Volumatic spacer 20 minutes prior to saline nebulisation. After 20 minutes resting, the post bronchodilator FEV1 was measured three times and the best value recorded. This value was used to calculate any subsequent fall in FEV1 during the procedure. If there was a 20% drop in FEV1 from baseline the procedure was terminated.

5ml of hypertonic saline (3%) was placed into the cup of the nebuliser and the patient was instructed to tidal breathe through the nebuliser mouthpiece for 1 minute following by a deep breath inhalation. At that point the induction was stopped and 3 FEV1 tests were performed, and the best was recorded. Then the patient was asked to continue breathing through the nebuliser for another 4 minutes after stopping the induction, 3 FEV1 tests were performed and the best was recorded.

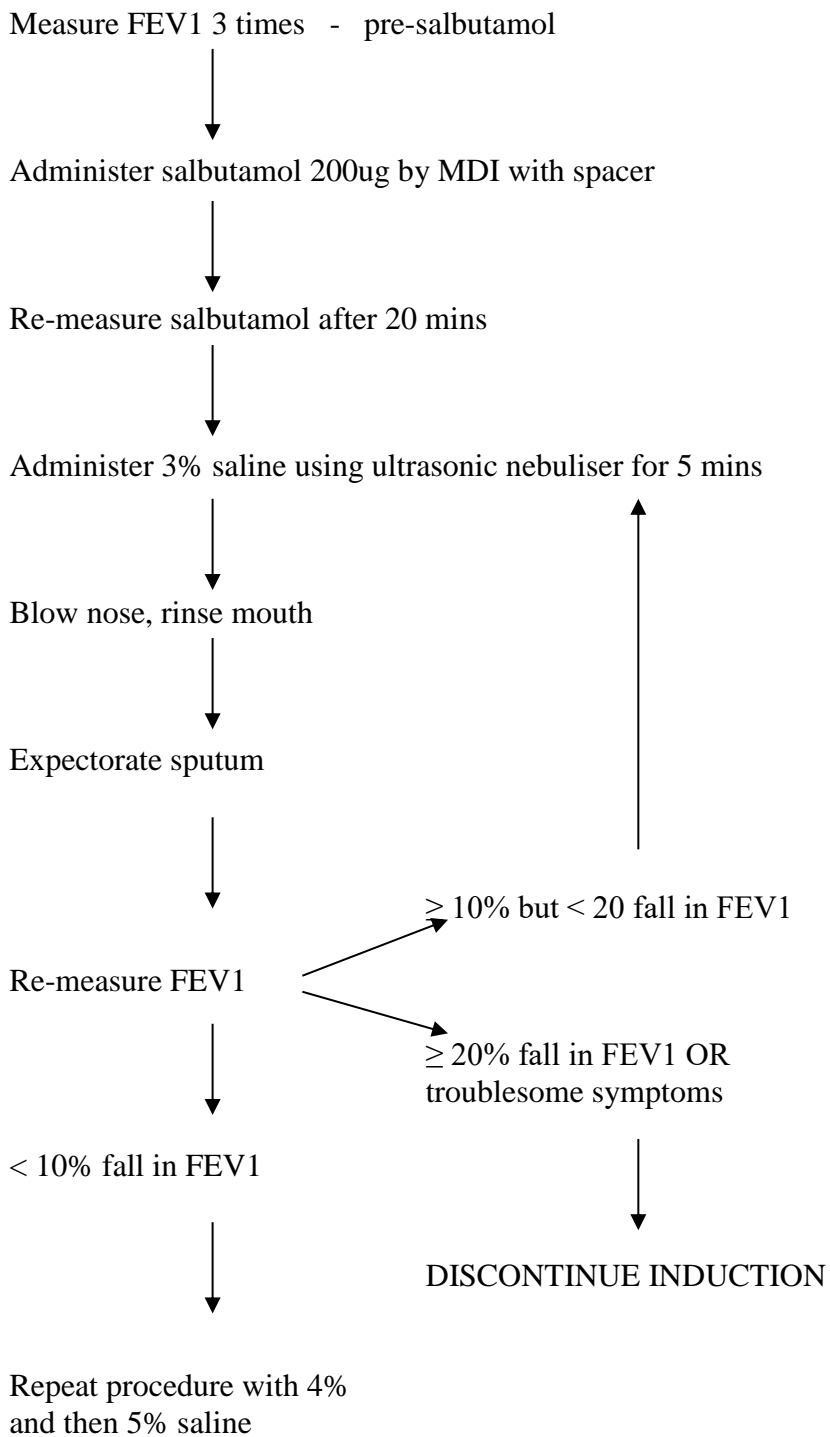
Once nebulisation was completed the patients thoroughly rinsed their mouths with water (not to swallow) and blew their nose. The subject was then asked to cough and collect any sputum into a sputum pot.

If the patient was unable to produce a sputum sample and the patients' FEV1 had not fallen $\geq 10\%$ predicted FEV1 saline dose was escalating from 3-5%. If the patients FEV1 had fallen $\geq 10\%$ but $< 20\%$ of the baseline FEV1 the induction was repeated using 3% saline.

If a sputum sample was not produced by 4% saline the induction was again repeated but using 5% saline instead of 3% saline (if the FEV1 has not fallen by $\geq 10\%$ of the baseline FEV1). After this procedure if the patient could not produce sputum the induction was stopped.

The patient's lung function was measured at completion of the procedure and was required to be within $\leq 5\%$ of the initial baseline before safe to leave the department.

Flowchart for Sputum Induction (non high risk subjects)



2.5 SPUTUM SPECIMEN ANALYSIS

In this study the Standard Operating Procedure of the Clinical Trial Unit SOPCTU100210 (Please see appendix 2) has been used to process the sputum samples, while some minor alterations have been applied.

2.5.1 SPUTUM PLUG SELECTION

Sputum samples were collected and examined within 2 hours of collection to optimise cell viability and staining (Popov et al., 1995; Efthimiadis et al., 2002).

The entire sputum sample was emptied into a clean petri dish, inside a class 2 safety hood. Sputum plugs were separated from saliva by using curved forceps, then transferred onto the petri dish lid. In order to separate the saliva from selected sputum plugs, the entire mass was moved around the lid with small circular motions. This procedure helps to condense the sputum sample and remove saliva, which is important to reduce squamous cell contamination and improve sample quality (Approximately 75 milligram of sputum plugs is preferred to produce adequate samples).

The concentrated sputum was transferred to a pre-weighed polypropylene centrifuge tube. The centrifuge tube was re-weighed to determine the weight of the sputum portion for the process.

2.5.2 SAMPLE PROCESSING AND FILTRATION

Following sample selection, freshly prepared Dithiothreitol (DTT) (in a dilution of one in 10 with distilled water) was added in a volume (in μl) equal to 4 times the sputum weight (in mg). The mixture was then mixed gently by Vortex for 15 seconds and placed on a bench top roller at room temperature for 15 minutes to incubate. This technique homogenises most of the sputum samples but there were some samples in which the mucus was difficult to break down. The time of homogenisation was therefore extended.

Following the homogenisation process further dilution was achieved by adding an equal volume of Dulbecco's phosphate buffered saline (D-PBS) to the sample in order to reach a 9-fold dilution of the sputum plugs and then vortex gently for 15 sec. In order to remove debris and mucus, the clear cell suspension was filtered by using 50 μm nylon gauze.

In the next stage total cell counts (TCC) should be calculated. In current methodology, sputum cells separated from the fluid phase through centrifugation process, however the effect of centrifugation on fluid phase measurement is not clear (Efthimiadis et al., 2002). In Chapter seven I analysis the sputum samples by two methods (with centrifugation & without centrifugation) to calculate total cell counts (TCC). The result of this experiment was fully explained in Chapter 7.

2.5.3 METHOD 1: CELL COUNT WITHOUT CENTRIFUGATION

After filtration the filtered sample was used to determine the total cell count viability and level of squamous cell contamination by using a microscope (x400 magnification), a neubauer haemocytometer and the trypan blue exclusion method (Fahy et al., 1993).

10ml of cell suspension with 10ml of Trypan blue was mixed, this mixture was flooded onto a haemocytometer. The cell count was performed within 5 minutes of mixing. All cells that were located in the central field and the four corner fields of the haemocytometer were counted. To have an accurate result it was aimed to count between 80 and 100 cells. Cells touching the top and left grid lines were counted, cells touching lower and right grid lines were not counted. Cells were classified as viable (live) leukocytes, dead (blue) leukocytes and squamous.

The average live, dead and squamous cell numbers across all 5 counted fields and then the suspended cell concentration per ml ($((\text{total no cells counted} \times 2) \times 10\,000)$) was calculated. The percentage of cell viability ($((\text{number of live cells} \times 100) / \text{total number of leukocyte cells})$) and the percentage of Squamous cells ($((\text{number of Squamous cells} \times 100) / \text{total number of cells})$) was determined. Cell viability less than 40% may affect the accuracy of differential cell counts (DCCs) (Efthimiadis et al., 2002).

Each cytopsin slide should contain 30 000 cells delivered in a 100 μl suspension. To calculate the volume of cell suspension required for each cytopsin, 30 000 was divided by the suspended cell concentration [$30\,000 / ((\text{total no cells counted} \times 2) \times 10\,000)$].

The required volume of D-PBS to be added was calculated to make 100 µl of cell suspension containing 30 000 cells.

After preparing the cytopins they were centrifuged at 450rpm (18.1 x g) for 6 minutes then the slides air dried for at least 15 minutes at room temperature.

2.5.4 METHOD 2: CELL COUNT WITH CENTRIFUGATION

In this method after filtration the filtered sample was used to determine the total cell count viability and level of squamous cell contamination by using a Neubauer haemocytometer and the trypan blue exclusion method. Then cytopin slides were prepared (contain 30 000 delivered in a 100 µl suspension) and centrifuged at 450rpm (18.1 x g) for 6 minutes.

In this method all the calculations were kept the same as SOPCTU100210.

2.5.5 SPUTUM STAINING

Rapi-Diff II stain method was used based on Hull Clinical Trial Unit SOP. Firstly dried slides were fixed in Solution A (methanol) for 15 minutes at that time allowed slides to air dry for 10 minutes. Then immerse slides in Solution B (Eosin) for slides fixed for 1 minute, and slides rinsed in distilled water. They were then dipped in Solution C (Blue) for 1 minute, then rinsed in distilled water and allowed to dry. Glass coverslips were placed over the cells on the slides by using 1 drop of DPX mounting media and allow drying before a differential count on 400 cells was performed.

2.6 HULL AIRWAYS REFLUX QUESTIONNAIRE (HARQ)

Hull Airways Reflux Questionnaire (HARQ) was developed specifically to quantify the symptoms of airway reflux underlying cough hypersensitivity (Morice et al., 2011b). This is a fourteen point questionnaire, which has recently been validated to look at the symptoms often associated with chronic cough (Faruqi et al., 2009) (see appendix 3). The HARQ is a self-administrated instrument which is reproducible and responsive to change (Ternesten-Hasseus et al., 2011).

Participants completed the questionnaire at visits 1, 3 and 5. Each individual question in the HARQ independently tests for the cough hypersensitivity syndrome on a scale of 0-5 (0, no problem; 5, severe/frequent problems), with the total score varying from 0–70 points and the upper limit of normal is 13 out of 70.

2.7 LEICESTER COUGH QUESTIONNAIRE (LCQ)

The LCQ is a quality-of-life questionnaire which subjectively measures cough frequency in patients with chronic cough, easy to administer and well-validated (see appendix 4). The LCQ is highly reproducible and is a useful tool to assess the effectiveness of therapy (Birring et al., 2003).

The LCQ contains 19 items and assess three domains of physical, psychological and social. The questionnaire is self-completed and contains a seven-point Likert response scale, ranging from 1= all of the time to 7= none of the time. A higher score indicates better health status and a change of 2.56 in total LCQ score of the minimum important clinical difference (Birring et al., 2003).

Patients completed LCQ at visit 1 and 3 and asked to indicate how cough had impacted on various aspects of their life during the last two weeks.

LCQ Scoring technique

1. Domains (questions):
Physical: 1,2,3,9,10,11,14,15
Psychological 4,5,6,12,13,16,17
Social: 7,8,18,19
2. Domain Scores: total score from items in domain / number of items in domain (range 1-7)
3. Total Scores: Addition of domain scores (range 3-21)

2.8 24 HOURS COUGH MONITORING

The Hull Automated Cough Counter (HACC) and Leicester Cough Monitor (LCM) software was used to measure the cough frequency over a 24 hour period at the study visits 1, 2 and 4 (The Standard Operating Procedure of the Clinical Trial Unit SOPCTU040610 Please see appendix 5). The automated assessment of cough is valid, reliable and highly reproducible (Barry et al., 2006; Birring et al., 2008) and is significantly correlated with

subjective assessment of cough and cough reflex sensitivity (Faruqi et al., 2011b).

HACC system uses audio recordings from a miniature microphone and the detection algorithm is based on statistical models of the time spectral characteristics of cough sounds. The monitoring should be carry for a prolonged period as cough rates can change from hour to hour.

The HACC is a Marantz PMD620 solid state recorder which digitally records acoustic events onto a 1GB SD card. The recordings are saved as MP3 compression files for subsequent audio analysis. Mp3 files are 'joined' to create single (or multiple) larger files using the Direct MP3 Joiner software which is on the OPTIPLEX 755 Clinical Trial Unit cough computer. Leicester Cough Monitor (LCM) software analysis was used to analyse the number of coughs. This software incorporates a manual input to distinguish between cough and non-cough events. The software requires up to 80 – 100 events. The software algorithms and models that separate cough vs non-cough are automatically adjusted after the first 20 events and then after each 10 events. When the change in the models is below a threshold, this adjustment stops and the process finishes.

2.9 *REFLUX DISEASE QUESTIONNAIRE (RDQ)*

The RDQ assesses the frequency and severity of upper gastrointestinal symptoms such as heartburn, regurgitation and epigastric pain. It is a 12 point questionnaire on a scale of 0-5 (0, no problem; 5, frequent / severe problems), the total score range 0 to 60 (Shaw et al., 2001) (see appendix 6). I used this questionnaire in my second project to evaluate Peptest in healthy subjects and chronic cough patients.

2.10 *STRESS VISUAL ANALOGUE SCALE (VAS)*

The visual analogue scale was used to measure the level of stress in healthy subjects who participated in my second project (the evaluation of Peptest). The subjects rated their perceived stress on a 10-point scale (0=no stress, 10=highest degree of stress). As stress can cause reflux which could affect the Peptest results (see appendix 7).

2.11 SALIVARY PEPSIN ANALYSIS (PEPTEST)

Peptest is a non-invasive, rapid diagnostic test developed to measure pepsin in saliva/sputum of patients as a biomarker for the diagnosis of reflux disease.

Sample collection

Expectorated saliva samples were collected from subjects into sterile plastic tubes containing 0.5 mL of 0.01 M citric acid. The samples were refrigerated at 4°C and were analysed for the presence of pepsin within 2 days of the collection.

Analysis

To identify pepsin in collected samples the Peptest™ an in vitro diagnostic medical device specific for human pepsin A (RD Biomed Ltd, UK) was used. The saliva samples were centrifuged at 4000 rpm for 5 minutes until a clear supernatant was visible. Using an automated pipette, 80 µL was removed from the supernatant layer of the centrifuged sample and then transferred to a screw-top micro tube containing 240 µL of migration buffer solution. This was mixed with a vortex mixer for 10 seconds. A second pipette was used to transfer 80 µL of the mixed sample to the circular well of a Lateral Flow Device (LFD) (Figure 5). Within a few minutes of the sample flowing, a line emerged under the control indicator (C line) on the LFD. If pepsin was present in the saliva sample, a second line emerged under the test indicator (T line) between 5 to 15 minutes after sample was transferred on the LFD. The intensity of the T-line was recorded exactly after 15 minutes of sample application to the LFD. This was then converted to ng/ml pepsin using a conversion spreadsheet.

The LFD device contains two unique monoclonal antibodies (Figure 6) to detect and capture pepsin in the saliva sample; the detection monoclonal antibody is labelled with a blue latex bead and the capture monoclonal antibody is located on the T line. This test is able to detect pepsin concentrations of 16 ng/mL or greater. The limit of quantitation 25 ng/ml.

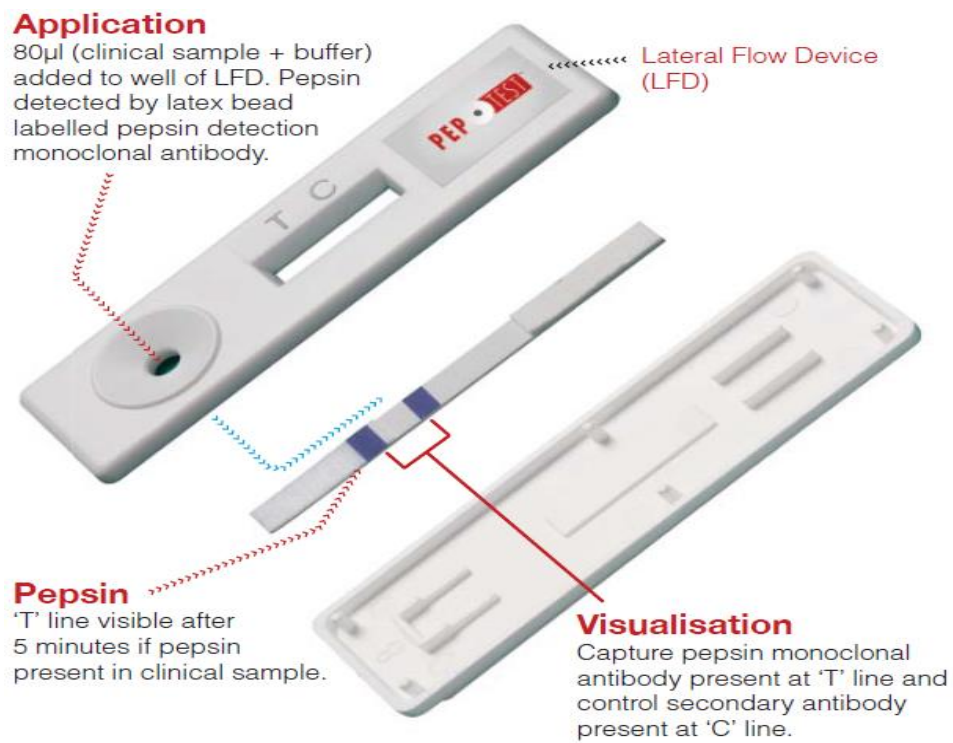


Figure 5 Lateral Flow Device (LFD)

Lateral Flow is a medical device specific for human pepsin to identify pepsin in collected samples in vitro (RD Biomed Ltd, UK).

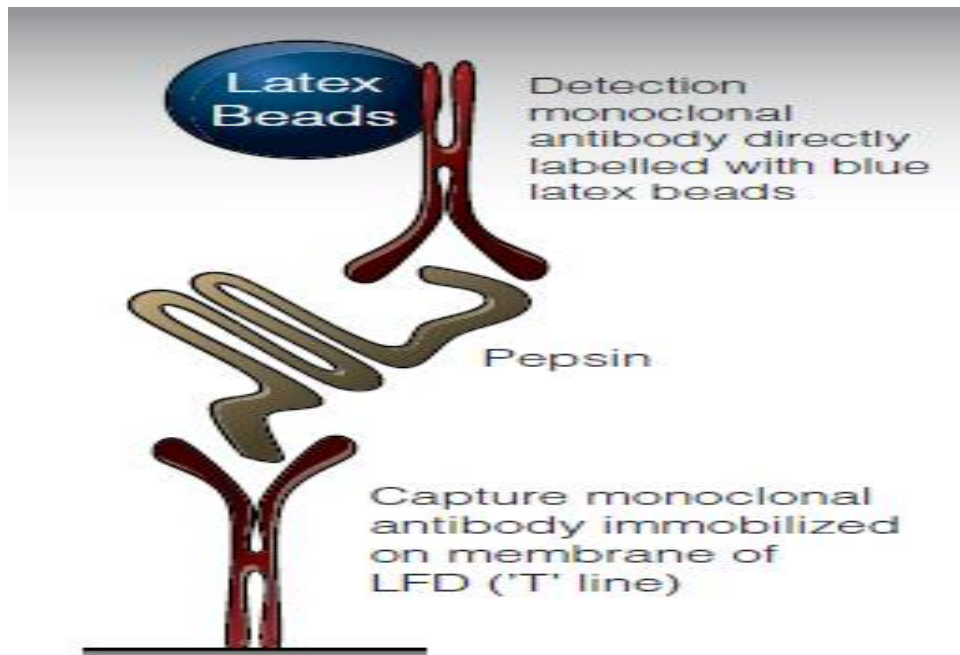


Figure 6 Pepsin detect antibodies

The LFD device contains two unique monoclonal antibodies to detect and capture pepsin in the saliva sample; one is a detection monoclonal antibody and second one is a capture monoclonal antibody; is located on the T line.

2.12 STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package of IBM SPSS Statistics version 23 and Excel 2013. Specific statistical methods are described individually for each study. A p-value of ≤ 0.05 was considered as statistically significant.

CHAPTER THREE

THE UTILITY OF FENO IN THE DIFFERENTIAL DIAGNOSIS OF CHRONIC COUGH: THE RESPONSE TO ANTI-INFLAMMATORY THERAPY WITH PREDNISOLONE AND MONTELUKAST

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3.1 INTRODUCTION

This study sought to establish whether FeNO measurement alone could determine which patients presenting to a clinic were most appropriately treated with anti-inflammatory treatment as first line. It was our hypothesis that FeNO, measured at presentation, will allow the clinician to decide whether patients would be more appropriately treated by anti-inflammatory therapy or whether other options, such as anti-reflux treatment should be administered first line. In the Hull Cough Clinic this strategy is in routine practice. Patients receive FeNO measurement at their initial visit to the clinic and if positive anti-inflammatory therapy is used first line. Those with a FeNO within the normal range are treated by a well established reflux protocol. Based on this practice I performed a randomised controlled study examining the outcome of anti-inflammatory therapy with either the leukotriene antagonist montelukast or oral prednisolone in patients with chronic cough. Patients presenting with a high FeNO measurement were compared with a contemporaneously recruited group with low FeNO.

There are a few studies in chronic cough where the diagnostic value of FeNO measurement has been evaluated (Chatkin et al., 1999; Fujimura et al., 2008; Oh et al., 2008; Maniscalco et al., 2015; Yi et al., 2016). In studies where FeNO value was specifically examined in differentiation of asthmatic cough from non-asthmatic cough (Chatkin et al., 1999; Oh et al., 2008) a cut of point of FeNO>30 ppb was suggested to be more likely to favour asthmatic cough. Others have reported that FeNO value is significantly higher in CVA and EB compared to atopic cough (AC) and upper airway cough syndrome (UACS) (Fujimura et al., 2008; Maniscalco et al., 2015; Yi et al., 2016). However, the value of FeNO to monitor therapeutic response to montelukast in chronic cough has not previously been examined.

A treatment group of chronic cough patients with a low FeNO measurement was chosen to determine whether treatment with montelukast in patients presenting with a low FeNO reduces cough. This allowed us to establish whether the determination of an elevated FeNO predicts the response to drugs or whether the therapy works on cough regardless of the FeNO measurement.

Leukotriene antagonists was proposed as they have been demonstrated to be highly effective in reducing cough reflex sensitivity in patients with asthmatic cough (Dicpinigaitis et al., 2002). Montelukast has been shown to have only a moderate degree of efficacy in classic asthma and it appears to have a unique mode of action in asthmatic cough. Most patients will have been started on inhaled corticosteroid therapy but in asthmatic cough response can be poor presumably because the inflammation is more “deep-seated”.

Prednisolone 20 mg once a day is a treatment recommended in the international cough guidelines to exclude eosinophilic inflammation (Brightling, 2006; Irwin et al., 2006b). Here the effect of the prednisolone trial was used as a positive control to determine the maximum effect of anti-inflammatory therapy on both exhaled NO and symptoms in the selected group of patient.

3.2 METHODS

3.2.1 STUDY DESIGN

This was a randomized, open label, controlled, pilot study to explore the effectiveness of four weeks treatment with montelukast compared with two weeks treatment with prednisolone follow by two weeks treatment with montelukast in patients with chronic cough with an associated elevated FeNO. The low FeNO group received monelukast alone.

The primary objective of this study was to determine the changes in 24 hr cough counts in different treatment periods. 24 hr cough counts measured using the Hull Automated Cough Counter (HACC). Measurements were made at baseline and after two weeks and four weeks treatment. Subjects with a high FENO ($\text{FeNO} \geq 30$ ppb) were randomised to two weeks montelukast 10 mg or prednisolone 20mg followed by montelukast 10 mg for the subsequent two weeks. Patients with low FeNO (defined as ≤ 20 ppb) were given montelukast 10 mg for 4 weeks.

Secondary end points included:

Subjective measures of cough were compared using the Hull Airways Reflux Questionnaire (HARQ) and Leicester Cough Questionnaire (LCQ) at baseline and in second and fourth weeks of therapy.

Changes in the sputum inflammatory cells were evaluated in spontaneously produced or induced sputum to determine whether inflammatory markers present prior to therapy has changed after administration of montelukast or prednisolone to treat chronic cough.

Detailed methodology is contained in chapter two.

This study consisted of 5 visits (see figure 7). In the screening visit informed consent was obtained prior to baseline assessments. Following baseline assessments eligible subjects with high FeNO were randomized in a 1:1 ratio to receive either 4 weeks montelukast 10 mg or 2 weeks Prednisolone 20 mg followed by 2 weeks montelukast 10 mg. Allocation was performed based on a balanced block randomisation scheme, which was prepared using computerised system – sealed envelope. Following baseline assessments subjects eligible for the low FeNO treatment group received montelukast 10 mg for four weeks at the end of the visit.

In the first visit the following assessments and procedures were performed; FeNo measurement, spirometry, sputum collection, full blood count, 24 hour cough count, HARQ and LCQ. After 13 ± 2 and 27 ± 2 days in second and fourth visits the Hull Automated Cough Counter was applied to measure their cough frequency for 24 hours. The next day after these visits in the third and fifth visits the following procedures were repeated: FeNo measurement, spirometry, sputum collection, HARQ and LCQ. At the end of the study all patients were reviewed by a chronic cough registrar or consultant as per the standard care as a clinic patient. All screening procedures and tests establishing eligibility were performed as summarized in the schedule of assessments table.

Subjects' selection

Patients were recruited sequentially from the Hull Cough Clinic. I planned to recruit 40 chronic cough patients with a $\text{FeNO} \geq 30$ ppb. However, patients with high FeNO represented only 10% of the clinic population at the time of

the study and it was decided to stop the study after enrolling 30 patients. As planned 20 subjects with chronic cough and a FeNO \leq 20 ppb were enrolled (see figure 8).

Inclusion Criteria

Subjects must meet all of the following inclusion criteria to be eligible for participation in this study.

- Patients with a history of chronic cough (at least 8 weeks duration)
- Male and female subjects of at least 18 yrs of age
- Subjects able to understand the study and co-operate with the study procedures
- Subjects who consent to their general practitioner (GP) being informed of their study participation
- Patients with a FeNO \geq 30ppb at presentation to the Chronic cough clinic (required for entry on to the high FeNO treatment groups)
- Patients with FeNO \leq 20 ppb at presentation to the chronic cough clinic (required for entry as a low FeNO treatment group)

Exclusion Criteria

- Patients with current diagnosis of asthma
- Female subjects who are pregnant, or lactating, or who are of child bearing potential but are not using contraceptive measures
- Suffering from any concomitant disease (chronic heart, chronic lung such as; COPD, bronchiectasis and cystic fibrosis, chronic renal, chronic liver or neuromuscular disease or immunosuppression; pneumonia and diabetes) which may interfere with study procedures or evaluation.
- A lower respiratory tract infection 4 weeks prior to entry on to study
- Systemic infections
- Live virus immunisation planned within next 3 months
- Subjects with no previous chickenpox who had a recent (\leq 28 days) close personal contact with chickenpox OR herpes zoster (high FeNO treatment groups only)
- Subjects having recent (\leq 28 days) exposure to measles (high FeNO treatment groups only)

- Participation in another study (use of investigational product) within 30 days preceding entry on to study.
- Alcohol or drug abuse
- Inability to follow study procedures
- Regular use of corticosteroids either as inhaled, topical or systemic \geq 4weeks prior to enrolment
- Subjects who were taking bronchodilators should be on it for at least 4 weeks on regular dose and carry on the same dose during the study
- Subjects with known allergy to prednisolone (high FeNO treatment groups only) or montelukast
- Subjects who were taking Angiotensin Converting Enzymes (ACE) inhibitors.
- Current smoker

Prior and concomitant medication

Allowed medication

1. Short acting bronchodilators agonist was permitted provided the subject has been on a stable dose for >12 weeks prior to screening.

Prohibited Medications (for all groups)

Use of the following medications is prohibited during the study:

- Corticosteroids either as inhaled, topical or systemic. (Subjects who are on corticosteroids should be on it for at least 4weeks prior to regular enrolment)
- Long acting **Bronchodilators**. (Subjects who are taking long acting bronchodilators should be on it for at least 4 weeks before the study on regular dose and carry on the same dose during the study, as long acting drugs have longer terminal half-life.)
- **Angiotensin Converting Enzymes (ACE)** inhibitors include; perindopril, captopril, enalapril, lisinopril and ramipril.

Montelukast 10 mg interaction with other medications (for all groups)

- **CYP 3A4 enzyme inducers** such as; phenytoin, phenobarbital and rifampicin
- **CYP 2C8 enzyme inhibitors** such as; paclitaxel, rosiglitazone, and repaglinide

Prednisolone 20 mg interaction with other medications (for high FeNO groups)

- Rifampicin, rifabutin, carbamazepine, phenobarbital, phenytoin, primidone, carbimazole and aminoglutethimide
- Coumarin anticoagulants
- Methotrexate
- Retinoids and tetracyclines
- Antifungals
- Concomitant use of aspirin in patients already having antacid therapy

Monitoring and ethical considerations

The study was monitored in accordance with HEY R &D department's standard operating procedures to ensure compliance with UK Clinical Trial Regulations. All trial related documents was available upon request for monitoring by R&D monitors and for inspection by the MHRA. Monthly monitoring reports was completed by investigators and sent to R&D for regular trial up-dates.

The study was performed subject to Research Ethics Committee favourable opinion (EudraCT No: 2015-001736-38), MHRA clinical trial authorisation (CTA) and HEY Trust R&D approval.

The study also was conducted in compliance with The Medicines for Human Use (Clinicaltrials.gov No: NCT02479074) Regulations 2004 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care 2005.

Data handling and record keeping

Data was collected and retained in accordance with the Data Protection Act 1998. The Data was collected and stored on a local server on a University of Hull computer. The data was entered on to an excel spreadsheet and SPSS format. Spreadsheet was audited. All data was anonymised and only the study related patient numbers were entered on to the spreadsheet or SPSS forms. The excel spreadsheet and SPSS table access were password protected. Only myself and the staff performing quality control (QC) measures had access to the password.

Peer review

Dr Jaymin Morjaria had reviewed this study.

3.2.2 STATISTICAL ANALYSIS

Subjects' ages, FeNO, 24 hours cough count, LCQ & HARQ questionnaires, spirometry measurement, sputum eosinophilic count and blood eosinophilic count (B-Eos) data were expressed as a mean (SD) by using SPSS.

Repeated measures ANCOVA test was used to compare changes in the number of coughs in 24 hr at the baseline, 14 days and 28 days treatment; with either montelukast or prednisolone followed by montelukast in patients with $\text{FeNO} \geq 30$ ppb or montelukast in patients with normal NO measurement of ≤ 20 ppb between three treatment groups.

Comparison of change in subjective measures of cough (HARQ and LCQ questionnaires) between high FeNO treatment groups and low FeNO treatment group at the baseline, after 14 days and 28 days treatment were performed by using repeated measures ANCOVA test.

Inflammatory cell total and differential counts (% neutrophils, eosinophils, macrophages, epithelial cells and lymphocytes) in sputum samples were measured before treatment and after 14 days and 28 days treatment in all groups. Changes in these parameters from baseline will be measured using ANCOVA.

Changes in FVC predicted normal value from baseline were compared between the high FeNO treatment groups and low FeNO treatment group at the baseline, after 14 days and 28 days treatment using ANCOVA.

Finally ANCOVA test was used to compare changes in the FeNO value at the baseline, after 14 days and 28 days treatment between the three treatment groups to assess whether the efficiency of FeNO will help to predict therapeutic response to anti-inflammatory medication in cough. P value <0.05 was considered significant.

In this study for each endpoint (eg 24 hour count), ANCOVA was used for analysis, with repeated measures of count being the dependent variable, and group being the independent variable. Pairwise comparisons was used to establish between which groups the significant difference pertains.

ANCOVA test was used as it was cited on the protocol recommended by Victoria Allgar (Senior Lecturer in Medical Statistics, Statistical Consultancy Service, HYMS and R&D statistician). The data and results were sent to Victoria and she confirmed the results.

3.3 RESULTS

3.3.1 DEMOGRAPHIC DATA

During a 15 month period 50 patients were recruited into the study, 15 patients in each high FeNO group and 20 patients in the low FeNO group. Three patients were withdrawn from the study, one due to an error in the randomization (second arm, high FeNO group) and another two (one from the first arm high FeNO and one from the low FeNO group) due to severe cough associated with montelukast. Thus 47 patients completed the study; 28 in high FeNO and 19 in low FeNO. There were no serious adverse events, however seventeen patients reported minor events during the study of which five complained of symptoms of upper respiratory infection. As upper respiratory infection impacts on FeNO and 24 hours cough count, the data from these patients were not used in the analysis. Thus a total of 41 patients data were included in the analysis (high FeNO first arm=10, high FeNO second arm=14 and low FeNO=17).

Average (\pm SD) age of the subjects studied was 62 ± 9.5 (range, 45-82 years). 32 patients (65%) of the subjects were female while only 17 patients (35%) of the subjects were male. Mean FEV1 and FVC were 105 ± 20 % and 115 ± 21 % of predicted respectively. There was no significant change in lung function during the study. At baseline mean B-Eos in the high FeNO group was $0.34 \pm 0.2 \times 10^9/L$ (range 0.1 to 1) with 50% of the patients having a B-Eos above $0.3 \times 10^9/L$. In the low FeNO group mean B-Eos was $0.16 \pm 0.1 \times 10^9/L$ (range 0.05 to 0.5). In this group a single patient had B-Eos above $0.3 \times 10^9/L$ - $0.56 \times 10^9/L$. There was a marked difference in the gender split with equal numbers of male and female subjects in the high FeNO group whereas the low FeNO group consisted almost entirely of women (See table 2). None of the patients in the low FeNO group were using inhaled corticosteroid neither bronchodilators. Only 4 patients in the high FeNO group were using corticosteroid inhalers.

3.3.2 FeNO

Unsurprisingly at baseline there was a significant difference in mean FeNO value between high FeNO (65 ± 39 ppb) and low FeNO (13 ± 5 ppb) groups. The average FeNO in the montelukast treated high FeNO group was 56 ± 33 ppb at the baseline. After two and four weeks treatment this fell to 41 ± 16 and 39 ± 17 ppb. In the high FeNO prednisolone arm the mean FeNO dropped from 67 ± 30 ppb to 38 ± 18 ppb after two weeks treatment with prednisolone followed by a significant ($p < 0.05$) increase to 50 ± 20 ppb after two weeks further treatment with montelukast (Figure 9, panel 1). Thus at the end of the study there was a significant fall in FeNO of approximately 30% in both high FeNO treatment groups ($p < 0.005$). In the low FeNO group there was no significant change during the study 12 ± 5 ppb at baseline 13 ± 7 ppb at two weeks and 14 ± 7 ppb at four weeks.

3.3.3 24 HOURS COUGH COUNT

Device failure led to loss of data on three occasions. At baseline there was a highly significant difference between high and low FeNO groups total 24 hour cough counts. The predominantly female, low FeNO group had more than twice the number of cough recorded. 24 hour cough count fell after 14

days and 28 days treatment compared to baseline in all 3 groups (F value: 7, $p < 0.005$) (Figure 9, panel 2). In the low FeNO group the mean total 24 hour cough was reduced from 566 ± 388 to 449 ± 315 and 265 ± 267 after two and four weeks of treatment with montelukast representing a 53% overall reduction in recorded cough. In the high FeNO groups a similar trend can be seen. The average number of coughs in 24h reduced 49% from 292 ± 158 to 173 ± 63 and 150 ± 104 with four weeks montelukast. Initial prednisolone therapy reduced cough from 237 ± 223 to 89 ± 132 but then significantly ($p < 0.05$) increased to 114 ± 122 when montelukast was substituted. Thus both high FeNO groups and the low FeNO group cough improved by approximately 50% over the course of the study (Figure 9, panel 2).

3.3.4 HARQ

At baseline there was a significant difference between high (32 ± 11) and low (39 ± 12) FeNO groups. In all treatment groups there was a significant improvement in the HARQ score during the study (F value: 12.5, $p < 0.005$) (Figure 9 panel 4). After two weeks therapy the low FeNO HARQ fell to 37 ± 13 and to 31 ± 14 at end of study. In the high FeNO group montelukast decreased HARQ from 33 ± 5 to 23 ± 8 and 20 ± 11 ; in the prednisolone arm scores almost halved from 27 ± 11 to 14 ± 9 and was 15 ± 10 following montelukast substitution.

3.3.5 LCQ

Again at baseline there was a significant difference in LCQ between high (14 ± 3) and low (12 ± 4) FeNO groups. Similar to the HARQ there was a significant (F value: 6.3, $p < 0.005$) improvement in LCQ scores in all groups during the study. Low FeNO patients on average scored 12 ± 4 at the baseline and 14 ± 3 and 15 ± 3 respectively after two and four weeks treatment. In the montelukast treated high FeNO group the score improved from 14 ± 3 , 15 ± 2 and 16 ± 2 , in the prednisolone arm it also improved from 15 ± 3 to 18 ± 2 and this was maintained for the second treatment period (Figure 9, panel 3).

3.3.6 SPUTUM EOSINOPHILI CELL COUNT

In this study sputum samples were due to be collected from each participant at visit 1, 3 and 5. However, it was challenging to collect sputum samples

especially after treatment in visit 3 and 5. In some cases the produced samples were not cellular enough to process. In this analysis I have included data from each patient who was able to produce a sample at baseline and one from the last or third visit. Overall, there were 25 patients who had two samples before and after treatment (high FeNO first arm=7, high FeNO second arm=8 and Low FeNO=10).

At baseline a substantial difference in sputum eosinophil count between high FeNO (10 ± 21) and low FeNO (0.2 ± 0.2) group was observed. The sputum eosinophil count in the high FeNO group treated by solely with montelukast significantly ($p < 0.05$) fell from $11.7\% \pm 26\%$ to $1.8\% \pm 1.9\%$. In those patients treated initially with prednisolone followed by montelukast counts also fell significantly ($p < 0.05$) from $8.6\% \pm 17\%$ to $2.6\% \pm 5.9\%$. Therefore, anti-inflammatory treatment of which ever regime caused a significant decreased in sputum eosinophils. However it is needed to consider that some of the patients from prednisolone group might have been sampled after 2 weeks of prednisolone, and some might have been sampled after 2 weeks of each of prednisolone and monteleukast. This might cause an error and confound the analysis. In the low FeNO group sputum eosinophilia was below 1% before and after treatment.

3.4 DISCUSSION

There were several anticipated findings from this study. The hypothesis that FeNO could be used as a marker of eosinophilic inflammation in chronic cough was supported by our observation at baseline in the high FeNO group of eosinophilia in both blood and sputum. Only a single patient in the low FeNO group had blood eosinophilia >0.3 .

Montelukast was used as a control treatment in the low FeNO group in the anticipation of having little or no effect in those without eosinophilic inflammation however, I have shown the similar degree of response to therapy in both low and high FeNO groups. This could infer that in our study population there was a large element of the placebo response. However, the markers of eosinophilia, blood and sputum eosinophils fell in the high FeNO group (as they do in allergic asthma) suggesting biological activity.

Perhaps the most surprising aspect of our study is the dramatic response in the low FeNO group to montelukast. In the absence of the placebo it is impossible to rule out a non-pharmacological effect. Montelukast has been repeatedly demonstrated to effect allergic inflammation in asthma (Ritter et al., 2002) and more recently in eosinophilic cough (Kopriva et al., 2004; Mincheva et al., 2014) and it has been assumed that this activity is due to blockade of leukotriene receptors (Gagro et al., 2004). The fact that montelukast appears to be equally effective in the low FeNO group suggest the either the current markers of eosinophilic lung disease are insufficiently sensitive to pick up low levels of leukotriene activation in the low FeNO group or that montelukast has its antitussive activity by an alternative mechanism.

In this study both subjective and objective measures of coughs responded to therapy in a consistent fashion. These metrics do not measure the same domains of cough with there being about a 60% correlation between these measures (Faruqi et al., 2011a). That I saw a similar degree of respond in these diverse metrics suggests that the reduction in the cough was a real observation.

In patients with low FeNO montelukast may have alternative modes of action other than to antagonise eosinophilic inflammation. Anti-inflammatory activity targeting the neuronal hypersensitivity in these patients has been suggested. Mast cells have an ability to interact with airway nerves (Stead et al., 1989; Bienenstock et al., 1991). This localised interaction may be relevant to the pathogenesis of cough in patients without systemic evidence of eosinophilia. Taylor-Clark and his colleagues (2008) demonstrated that application of cysteinyl leukotrienes causes an increase in afferent neuronal excitability as measured by patch clamp and isolated single fibre recordings which was inhibited by CysLT1 antagonists.

Montelukast has previously been shown to reduce subjective cough scores in non-asthmatic chronic cough patients. A small study (Mincheva et al., 2014) also demonstrated that leukotriene receptor antagonists decreased cough reflex sensitivity to capsaicin, supporting the argument for modification of neuronal hypersensitivity. In an airway biopsy study in asthma Ramsay and his colleagues (2009) reported that treatment with montelukast not only

reduced the number of eosinophils but also tissue mast cells were reduced. Therefore, montelukast may inhibit cough by blocking CysLTs produced by mast cells in the immediate vicinity of airway nerves, an observation previously made in cough variant asthma (Kawai et al., 2008).

In conclusion, the results of this study suggest treatment of chronic cough with montelukast is not reliant on the demonstration of eosinophilic inflammation. A larger placebo controlled study is required to confirm the degree of treatment response in the non-eosinophilic population.

Figure 7 Visits schedule diagram

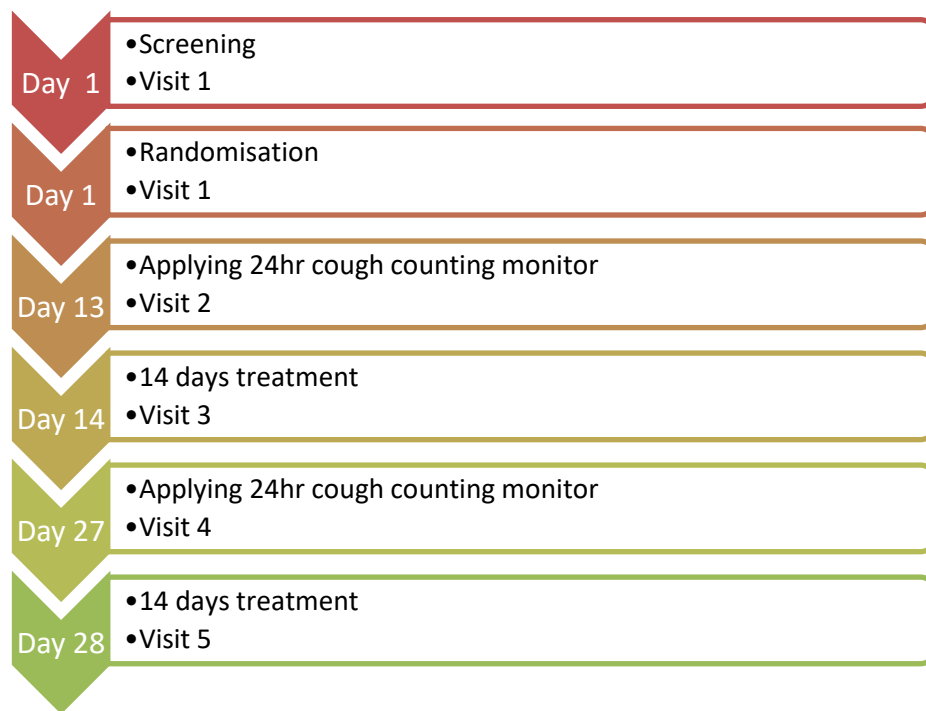


Table 1 Schedule of assessments

Procedures	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
	Day 1	Day 13±2	Day 14±2	Day 27±2	Day 28-2
Informed consent	X				
Medical History	X				
Weight and height	X				
Demographics	X				
Vital signs	X		X		X
Pregnancy test	X				
Spirometry	X		X		X
Sputum sample collection	X		X		X
Full blood count	X				
FeNO	X		X		X
Concomitant medication	X		X		X
24h cough monitoring	X	X		X	
Randomisation (High FeNO Treatment Groups)	X				
Dispense IMP	X		X		
Adverse events			X		X
IMP accountability	X		X		
Hull Airways Reflux Questionnaire	X		X		X
Leicester Cough Questionnaire	X		X		X

Figure 8: Trial profile

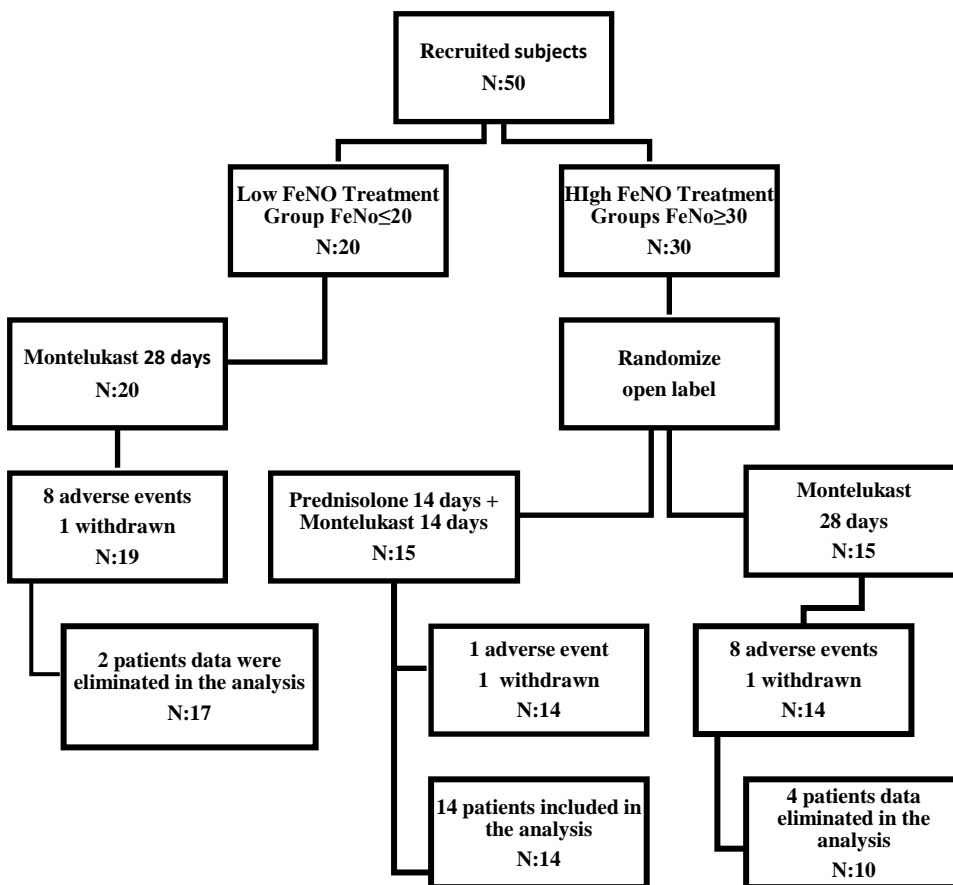


Table 2 Demographic data of age and gender

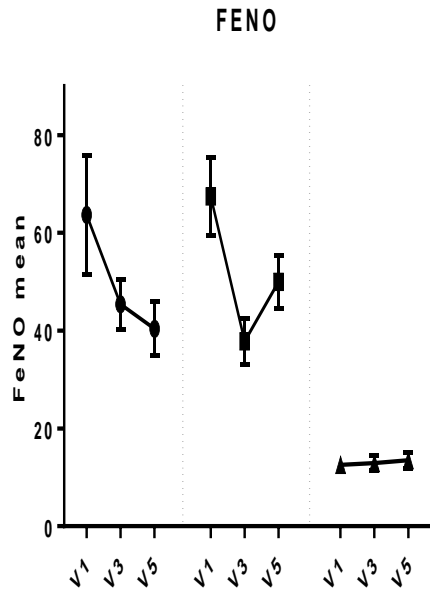
	High FeNO Arm1	High FeNO Arm2	Low FeNO	P Value	Total
Gender (M/F)	8/7	7/7	2/18	0.01	17/32
Age	59	65	62	0.2	62
Number of subjects	15	14	20		59

Figure 9 Measurements of FeNO, 24h cough count, HARQ and LCQ in three treatment Groups in three visits.

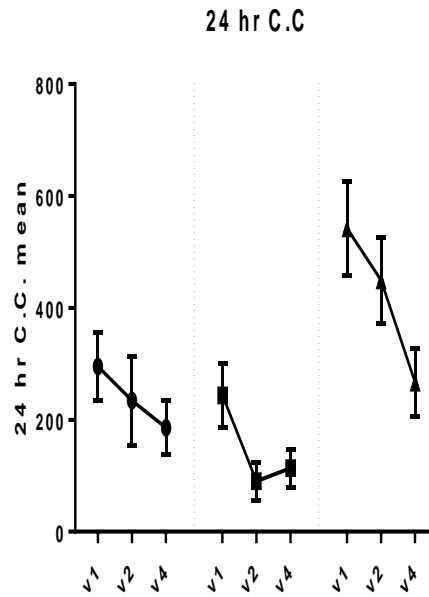
Horizontal bars represent mean and SEM value.

- High FeNO group arm1 (montelukast)
- High FeNO group arm2 (prednisolone + montelukast)
- ▲ Low FeNO group (montelukast)

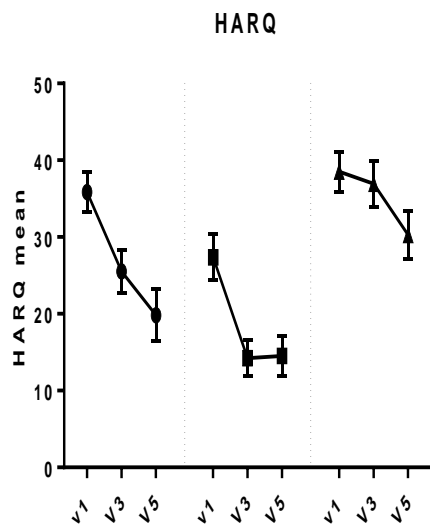
Panel 1



Panel 2



Panel 3



Panel 4

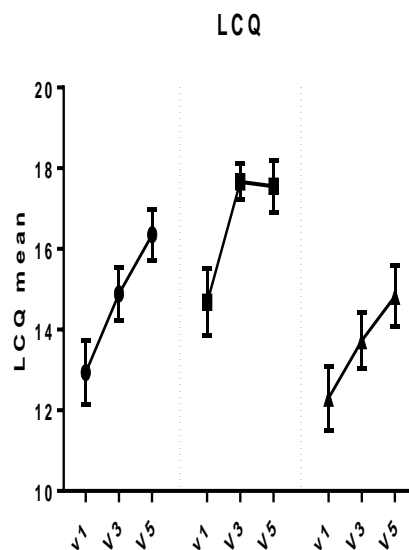


Figure 10 24h cough count changes in the individual patients in three treatment Groups in three visits.

Horizontal bars represent number of the coughs in 24 hours.

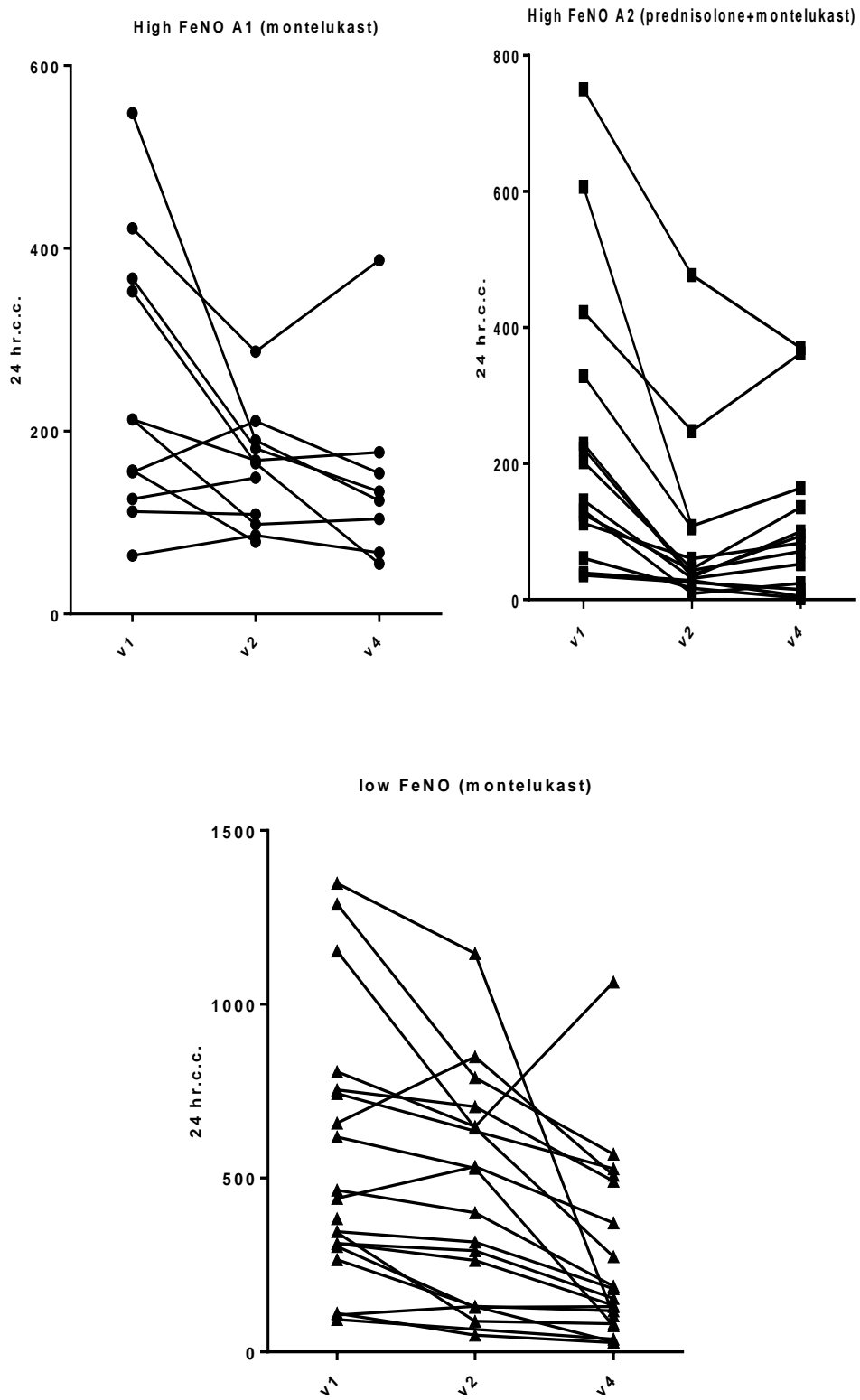


Table 3 Trial summary

Title	The utility of FeNO in the differential diagnosis of chronic cough: The response to anti-inflammatory therapy with prednisolone and montelukast
Short acronym	TUF
Type of trial	CTIMP
Trial design	Randomized, open label, controlled, pilot Study
Medical condition researched	Chronic cough
Trial Treatment	All eligible subjects meeting a FeNo of > 30 ppb will be randomised in a 1:1 ratio to receive either: Montelukast 10 mg daily for 28 days or Prednisolone 20 mg daily for 14 days followed by 14 days treatment with 10 mg Montelukast daily. All eligible patients meeting an FeNo of < 20 PPb will receive Montelukast 10 mg daily only.
Primary Objective	To determine the difference in objective measure of cough as demonstrated by 24 hr cough counts at the baseline, after 2 and 4 weeks treatment between three treatment groups with an associated elevated FeNO.
Secondary Objectives	<ul style="list-style-type: none"> • Compare change in subjective measures on HARQ and LCQ questionnaires of cough between the treatment groups at the baseline and after 2 week and 4 weeks treatment. • Compare change in FVC between treatment groups at the baseline and after 2 weeks and 4 weeks treatment. • Change in Sputum inflammatory markers between treatment groups at the baseline and after 2 weeks and 4 weeks treatment. • Assess whether previous history of blood eosinophils may predict therapeutic response to anti-inflammatory medication in cough. • Assess whether the efficiency of FeNO may help to predict therapeutic response to anti-inflammatory medication in cough.
Target number of participants	60
Duration participant in trial	28 days
Estimated recruitment period	24 months
Estimated total trial duration	30 months
Planned trial sites	Respiratory Medicine, Clinical Trials Unit, Castle Hill Hospital, Cottingham, HU16 5JQ
Main inclusion/exclusion criteria	<p>Inclusion Criteria</p> <ul style="list-style-type: none"> • Patients with a history of chronic cough (at least 8 weeks duration) • Male and female subjects of at least 18 yrs of age • Subjects able to understand the study and co-operate with the study procedures • Subjects who consent to their general practitioner (GP) being informed of their study participation. • Patients with a FeNO of ≥ 30ppb at presentation to the Chronic cough clinic.(required for entry on to the high FeNO treatment groups)

	<ul style="list-style-type: none"> • Patients with FeNO \leq 20 ppb at presentation to the chronic cough clinic (required for entry as low FeNO treatment group) <p>Exclusion Criteria</p> <ul style="list-style-type: none"> • Patients with current diagnosis of asthma. • Female subjects who are pregnant, or lactating, or who are of child bearing potential but are not using contraceptive measures. • Suffering from any concomitant disease (chronic heart, chronic lung such as; COPD, bronchiectasis and cystic fibrosis, chronic renal, chronic liver or neuromuscular disease or immunosuppression; pneumonia and diabetes) which may interfere with study procedures or evaluation. • A lower respiratory tract infection 4 weeks prior to entry on to study • Systemic infections • Live virus immunisation planned within next 3 months • Subjects with no previous chickenpox who had a recent (\leq28 days) close personal contact with chickenpox OR herpes zoster (high FeNO treatment groups only) • Subjects having recent (\leq28 days) exposure to measles (high FeNO treatment groups only) • Participation in another study (use of investigational product) within 30 days preceding entry on to study. • Alcohol or drug abuse • Inability to follow study procedures • Use of corticosteroids either as inhaled, topical or systemic \geq 4weeks prior to enrolment • Subjects with known allergy to prednisolone or montelukast • Subjects who are taking Angiotensin Converting Enzymes (ACE) inhibitors. • Current smoker • Subjects who are taking bronchodilators should be on it for at least 4 weeks on regular dose and carry on the same dose during the study
Investigations performed	Medical history, demographics assessment, prior medications, weight and height and vital signs (pulse, blood pressure, respiratory rate and temperature), spirometry, fractional exhaled nitric oxide (FeNO), sputum induction, full blood count, 24 hours cough monitoring, cough challenge, Leicester cough questionnaire, Hull Airways Reflux Questionnaire (HARQ) and adverse events.
Biological samples to be taken from patients	Urine pregnancy test Sputum induction Full blood count

CHAPTER FOUR

DOES FENO PREDICT CLINICAL CHARACTERISTICS IN CHRONIC COUGH?

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4.1 INTRODUCTION

The diagnosis of chronic cough is controversial with different terms being used to describe similar clinical presentations. Recently a unifying diagnosis of cough hypersensitivity has been proposed with treatment dependent on the type of airway inflammation present. How best to evaluate the inflammatory phenotype in a patient with chronic cough has been studied using fractional exhaled nitric oxide (FeNO) measurement (Chatkin et al., 1999; Fujimura et al., 2008; Oh et al., 2008; Maniscalco et al., 2015; Yi et al., 2016). However the different clinical phenotype of patients with chronic cough based on their inflammatory profiles has not been studied in depth. I therefore divided sequential patients attending a specialist cough clinic into two groups of low FeNO (FeNO \leq 20 ppb) and high FeNO (FeNO \geq 30 ppb) to evaluate the profile of other eosinophilic biomarkers, cough frequency, and demographics to determine if they exhibited phenotypic variability.

4.2 METHODS

4.2.1 Study design

Baseline data from the clinical trial study which was presented in Chapter 3 were explored systematically in this chapter. I aimed to explore the efficacy of FeNO measurement in determining airway inflammatory phenotype in chronic cough patients. Correlation between FeNO, blood and sputum eosinophil cell count was assessed. I then determined the objective and subjective measurements of cough in patients with high FeNO and low FeNO. 24 hr cough counts measured using the Hull Automated Cough Counter (HACC). Hull Airways Reflux Questionnaire (HARQ) and Leicester Cough Questionnaire (LCQ) were applied to measure cough subjectively.

4.2.2 Statistical Analysis

Subjects' ages, FeNO, 24 hours cough count, LCQ & HARQ questionnaires, spirometry measurement, sputum eosinophilic count and blood eosinophil count (B-Eos) data were expressed as a mean \pm (SD), median and range by using SPSS Descriptive statistic test.

ANOVA tests was used to compare differences in the average number of coughs in 24 hr, FeNO value, sputum inflammatory cells, spirometry measurements, B-Eos, HARQ and LCQ score at the baseline between the low FeNO group and high FeNO group. P value <0.05 was considered significant.

I also assessed whether blood eosinophils may predict therapeutic response to anti-inflammatory medication in cough. Pearson's correlation coefficient (r) test was used to evaluate correlation between FeNO, B-Eos and sputum eosinophil count in all the subjects.

This analysis was taken on the advice of the HYMS statistician Victoria Allgar.

4.3 RESULTS

4.3.1 Demographic

During a 15 month period in total 50 patients were recruited into the study, 30 patients in the high FeNO group and 20 patients in the low FeNO group. One patient was withdrawn from the study due to an error in the randomization. In total 49 patients enrolled to the study, 29 in the high FeNO and 20 in the low FeNO group. Mean (\pm SD) age was 62 ± 9.5 (range, 45-82 years) (table 4). Sixty five percentage (n.32) of the subjects were female. There was a marked gender difference between the two cohorts, the low FeNO group having 90% women (18 women and 2 men) whereas the sexes were almost equally represented in the high FeNO group (15 men and 14 women). There was no evidence of airflow obstruction with FEV1 being 96% predicted in the high FeNO and 113% in the low FeNO value (NS).

4.3.2 Airways inflammatory biomarkers (FeNO value, blood and sputum eosinophil count)

Unsurprisingly there was a significant difference in mean FeNO value between high FeNO (65 ± 39 ppb) (median=56 & range=171) and low FeNO (13 ± 5 ppb) (median=12.5 & range=15) groups ($p < 0.005$). Mean B-Eos in the high FeNO group was $0.34 \pm 0.2 \times 10^9/L$, whereas in the low FeNO group it was $0.16 \pm 0.1 \times 10^9/L$ ($p < 0.005$). In the high FeNO group half of the patients (14) had a B-Eos above $0.3 \times 10^9/L$, the rest had a B-Eos between

0.2 and $0.1 \times 10^9/L$ (Table 5). In the low FeNO group all the patients had a B-Eos under $0.3 \times 10^9/L$, only a single patient had a high B-Eos of $0.56 \times 10^9/L$ (Figure 11).

In 30 patients (15 in the high and 15 in the low FeNO group) who had a previous blood test (median=4 months, range= 1month to 26 months) in their clinical record B-Eos results were compared. The mean current B-Eos were highly correlated ($r=0.64$ $p < 0.001$) with the previous B-Eos. Thus the majority of the patients in high FeNO group had a previous history of high blood eosinophil levels. Bland Altman analysis revealed this correlation declined at higher blood eosinophil counts (Figure 12).

Thirty sputum samples successfully processed and counted. The mean eosinophil cell counted in sputum samples in the high FeNO group was $10\% \pm 21\%$ (median=1 & range=71), while in the low FeNO group it was $0.2\% \pm 0.2\%$ (median=0.25 & range=0.5) ($p < 0.05$ equal variances not assumed). Patients with low FeNO all had eosinophil cell count under 0.5%, except one whose eosinophil cell count was 2% which is with the laboratory normal range ($<3\%$). Half of the patients in high FeNO group had an eosinophil cell count under 3%. However, almost all of them had eosinophil cell count above 0.5% except two with 0% (Figure 13). Percentage of macrophages in low FeNO patients (65%) was significantly higher ($P<0.05$) compared with the patients with high FeNO (36%). However, the apparent depression in macrophage count in the high eosinophil group is likely to be a consequence of the excess in the percentage of eosinophils rather than a true fall in macrophage numbers. Other inflammatory cell counts in sputum samples such as neutrophils, epithelial and lymphocytes were similar in both cohorts.

Thirty patients had FeNO, B-Eos and sputum eosinophil count. A strong correlation was observed between FeNO and B-Eos ($r= 0.79$, $p < 0.001$); there was also a strong correlation between FeNO and sputum eosinophil count ($r= 0.65$, $p < 0.001$). The correlation between B-Eos and sputum eosinophil count was more modest ($r= 0.59$, $p<0.001$).

4.3.3 Objective and subjective measurements of cough (24hr cough count, LCQ and HARQ)

Forty eight patients, 20 in the low FeNO group and 28 in the high FeNO group completed 24 hours cough count measurement (device failure led to loss of data on two occasions). There was a highly significant difference ($p < 0.005$) between high and low FeNO groups in the number of recorded coughs in 24 hr. The mean number of coughs in 24 hr in the low FeNO group was 540 ± 376 (median=412 & range=1255), whereas this figure was 270 ± 220 (median=207 & range=865) in the other cohort. A similar significant difference ($p < 0.05$) in the HARQ score between two cohorts observed. The mean HARQ score was 39 ± 12 (median=39.5 & range=46) in the low FeNO group, whereas it was 32 ± 11 (median=31 & range=50) in the high FeNO group (Table 5). The LCQ scores in the low and high FeNO groups on average were 12 ± 4 (median=12.6 & range=12) and 14 ± 3 (median=14.4 & range=11) respectively however this did not achieve statistical significance. Overall, patients with low FeNO suffered greater morbidity in comparison with patients with high FeNO as assessed by 24hr cough count, HARQ and LCQ.

4.4 DISCUSSION

I have evaluated the demographic data, 24 hours cough count, HARQ and LCQ in sequentially recruited patients attending a specialist cough clinic. Patients were stratified into high FeNO and low FeNO groups and the different characteristics of these two cohorts observed.

In contrast to our investigation of unselected patients attending a cough clinic, others have studied the inflammatory profile of patients with a variety of diagnoses such as cough variant asthma, and forms of eosinophilic bronchitis. Whether such conditions are separate disease entities or part of the inflammatory continuum of cough hypersensitivity syndrome is controversial (McGarvey & Morice, 2003a). In none of these studies was cough objectively assessed.

Chatkin and colleagues (1999) determined FeNO values in patients with more than 3 weeks cough and found those with bronchial hyperresponsiveness

and FeNO>30 ppb were more likely to be diagnosed as asthmatic on review. In another study patients with more than 3 weeks cough were classified into three groups of asthmatic cough, non-asthmatic eosinophilic bronchitis (NAEB) and “others” based on spirometric reversibility, methacholine responsiveness and sputum eosinophilia (Oh et al., 2008). They found FeNO values lower than 31 ppb indicated that asthma and NAEB were unlikely. Maniscalco and colleagues (2015) assessed patients with more than 8 weeks cough and classified them in four categories of cough variant asthma (CVA), NAEB, gastro-esophageal reflux disease (GERD) and upper airway cough syndrome (UACS) according to the ACCP guidelines (Irwin et al., 2006a). They reported mean FeNO value were greater than double in CVA and NAEB compared to UACS and GERD. Thus in various groups of cough patients low and high FeNO values have been associated with a different airway inflammatory profile, however the effect on cough frequency has not been examined.

In our study cough frequency in the low FeNO group was double that seen in the high FeNO and this was associated with a greater impact on quality of life as assessed by the LCQ and HARQ. While the airway inflammatory profiles and cough frequency differences between two groups are important there was a miss match between the sexes. Patients in the low FeNO group being predominantly women whereas the high FeNO group have a similar sex distribution. Interestingly a similar disparity was seen in the study by (Oh et al., 2008). Experience of cough clinics around the globe suggest that there is a 2 to 1 preponderance of women attending cough clinics possibly reflecting a greater cough reflex sensitivity (Kastelik et al., 2002; Morice et al., 2014) but the possible relationship between gender and different inflammatory profiles has not previously being described. A recent large database study by Price and colleges (2015) has shown a similar female gender bias of 1.39 in pauci-eosinophilic asthma. Further investigation in a larger number of cough hypersensitive patients will be required to confirm our findings.

Women patients have been shown to have a greater 24 hr cough count than men (Kelsall et al., 2009) and since in our low FeNO cohort women predominated this may explain the almost doubling of mean recorded cough

seen in the low FeNO group. To demonstrate that this difference resides in low FeNO inflammatory profile rather than gender with require study with sexual stratification. However, the observed differences in the low FeNO group appear to be genuine as both the scores of HARQ and LCQ were worse in this cohort. If this were to be confirmed, FeNO might be useful in predicting inflammatory phenotypes cough hypersensitivity. Based on this results a larger study with cluster analysis of phenotypes according to feNO and clinical characteristics should be performed in future.

In this study I found a high degree of correlation between the different measures of airways inflammatory biomarkers. Average FeNO value, blood and sputum eosinophil count were markedly different in the low and high FeNO groups indicating the lack of eosinophil inflammation in low FeNO group patients. To our knowledge this the first study in chronic cough that assess correlation between FeNO and B-Eos and it contrasts with studies in asthma where only a modest ($r=0.51$ $P<0.001$) or weak ($r=0.22$ $P<0.001$) correlation between FeNO value with B-Eos were reported (Strunk et al., 2003; Malinovsky et al., 2013). Thus these biomarkers may have a different profile in chronic cough patients. Our study is consistent with previous observations in cough (Yi et al., 2016) and asthma (Maniscalco et al., 2015; Wagener et al., 2015) which have shown that FeNO has a strong correlation with sputum eosinophil count. The correlation between B-Eos and sputum eosinophil count was modest in our study and similar observations were reported in an asthma study (Wagener et al., 2015).

In conclusion, I showed a meaningful relationship between FeNO, blood eosinophils and sputum eosinophils in chronic cough. Our data indicate that I may use FeNO to phenotype these patients and this may be of therapeutic relevance.

Table 4 Gender & age demographic

			Gender		Age
			Male	Female	
FENO Group	High FeNO	Count	15	14	61.9
		% within FENO Group	51.7%	48.3%	
	Low FeNO	Count	2 _a	18 _b	62.5
		% within FENO Group	10.0%	90.0%	
Total		Count	17	33	62
		% within FENO Group	34%	66%	

Figure 11 Scatter plot of FeNO ppb and B-Eos x 10⁹/L

▲ Low FeNO group

○ High FeNO group

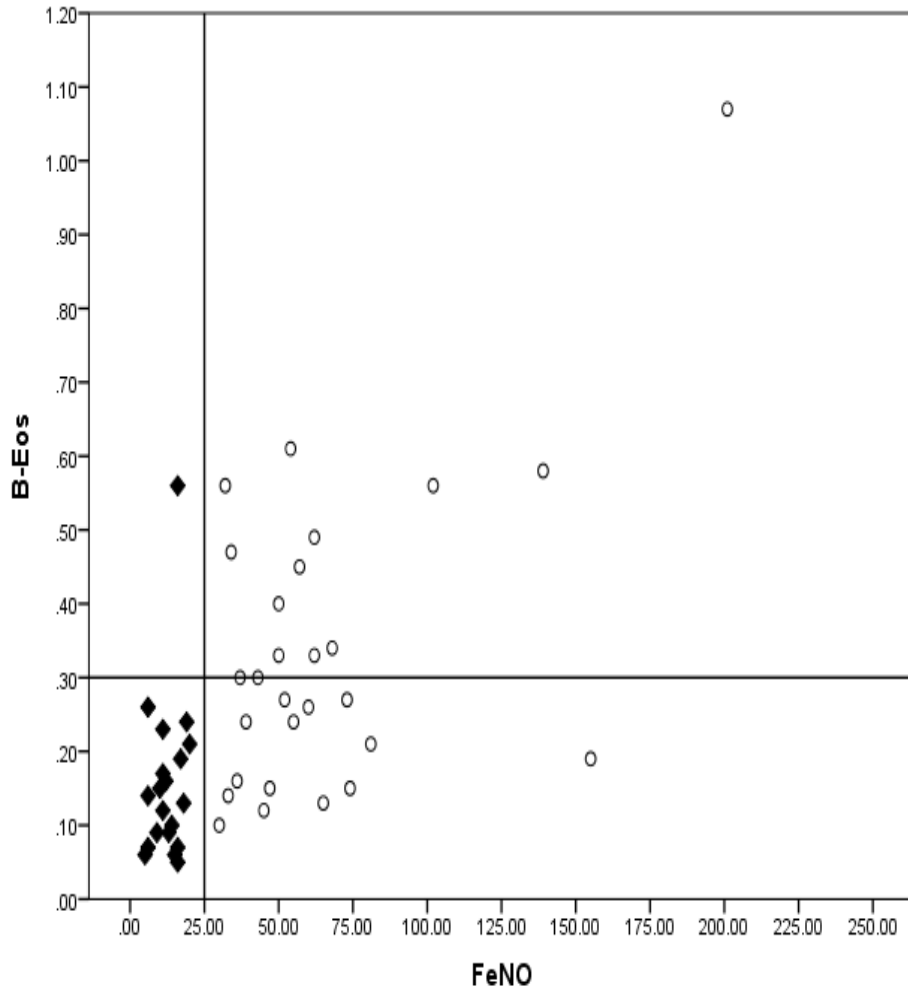


Figure 12 Bland-Altman plot of current B-Eos and previous B-Eos

▲ Low FeNO group

○ High FeNO group

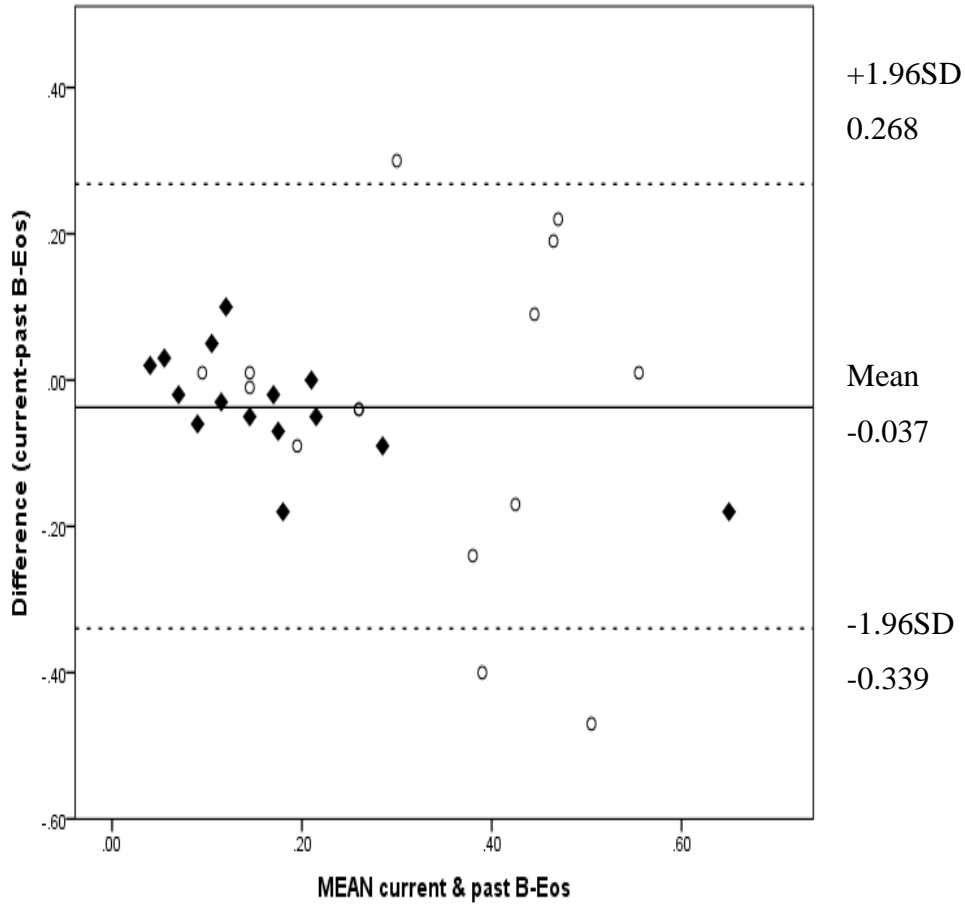


Figure 13 Scatter plot of FeNO ppb and sputum Eos%

▲ Low FeNO group

○ High FeNO group

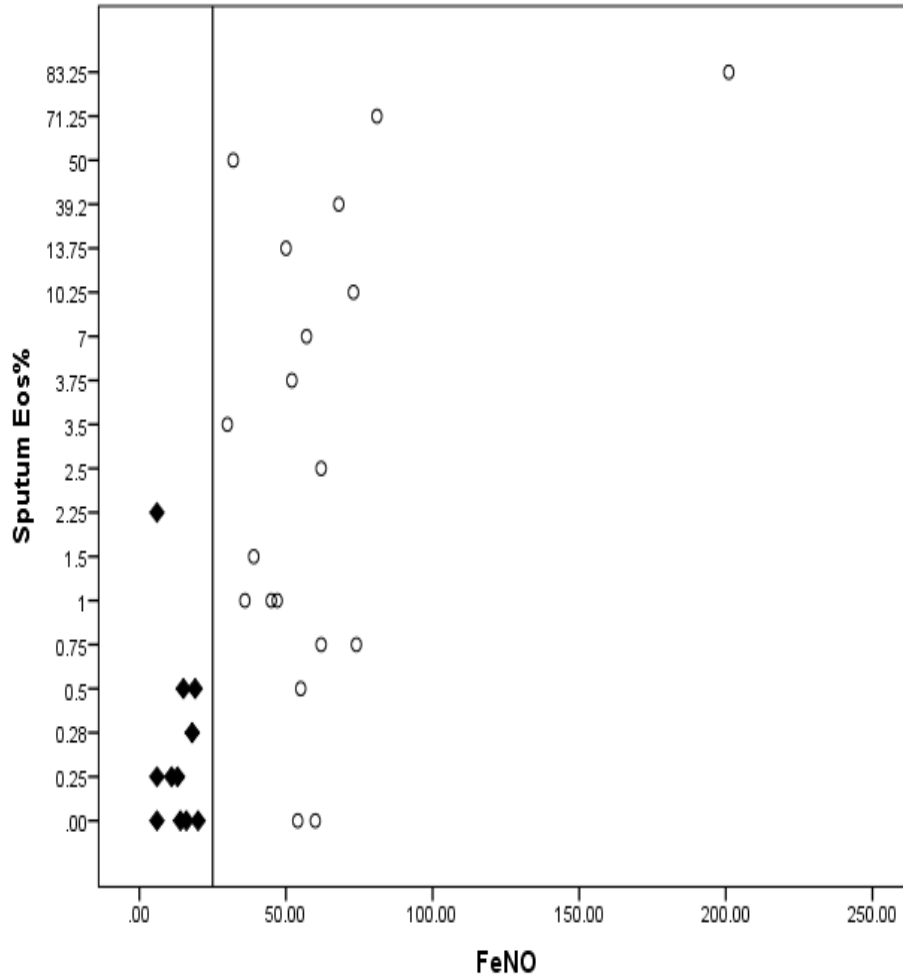


Table 5 Descriptives of FeNO ppb, B-Eos x 10⁹/L, 24hr cough count, LCQ and HARQ in the high FeNO and low FeNO groups

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
FeNo	High FeNO	29	65.48	39	7.2	50.6	80.3	30	201
	Low FeNO	20	12.55	4.6	1	10.4	14.7	5	20
	Total	49	43.87	39.8	5.6	32.4	55.3	5	201
B-Eos	High FeNO	28	.33	.2	.03	.25	.4	.10	1
	Low FeNO	20	.15	.1	.02	.1	.2	.05	.56
	Total	48	.26	.2	.03	.2	.3	.05	1
N.C.C 24h	High FeNO	28	269.6	220	41.6	184	355	36	901
	Low FeNO	20	542.4	376	84	366	718.5	93	1348
	Total	48	383.2	321.8	46.4	289.8	476.7	36	1348
LCQ	High FeNO	29	13.77	3.2	.58	12.57	14.9	7	17.9
	Low FeNO	20	12.30	3.5	.79	10.64	13.9	5.5	17.3
	Total	49	13.17	3.4	.48	12.2	14.1	5.5	17.9
HARQ	High FeNO	29	31.75	11.3	2	27.47	36	11	61
	Low FeNO	20	38.50	11.6	2.6	33	43.9	19	65
	Total	49	34.51	11.8	1.7	31	37.9	11	65

CHAPTER FIVE

PHENOTYPING PATIENTS WITH CHRONIC COUGH

5.1 INTRODUCTION

Chronic cough is a common condition which is difficult to control and manage (Morice, 2003) and often has a massive impact on the quality of life (French et al., 1998). Despite the suggested aetiology of chronic cough being asthma and non-asthmatic eosinophilic bronchitis (NAEB), gastro-oesophageal reflux disease (GORD), and upper airways disease (Pratter, 2006), there are many patients where the cause of cough remains unclear (McGarvey, 2005; Irwin et al., 2006a). In this study I explored the epidemiology of chronic cough patients attending the Hull cough clinic between first of June 2015 and end of November 2015. The patients were categorised based on their FeNO (the fraction of exhaled nitric oxide) measurements.

5.2 METHODS

Study design

Consecutive patients attending the Hull Cough Clinic over a six month period underwent assessment with spirometry, FeNO and Hull Airways Reflux Questionnaire (HARQ) and a cough sensitivity questionnaire as part of their routine clinical care.

Statistical Analysis

Subjects' demographic data (age, gender and geographical location), FeNO, HARQ questionnaire, cough sensitivity questionnaire, lung function, use of bronchodilators, anti-reflux therapy, history of allergy and asthma; and current smoking status was entered into the Excel spreadsheet. These data were expressed as a mean \pm (SD), median and range by using SPSS Descriptive statistic test (version 23).

ANOVA was also used to compare the average FeNO value, spirometry measurements, HARQ and cough sensitivity questionnaire scores between the low FeNO group and high FeNO group. P value <0.05 was considered significant.

Cross-tabulation analysis was used to analyse categorical data such as geographical location, use of bronchodilators, anti-reflux therapy, history of allergy and asthma; and smoking status.

Pearson's correlation coefficient (r) test was used to evaluate correlation between HARQ and cough sensitivity questionnaires.

Logistic regression analysis was used to evaluate the relationship of HARQ score to FeNO and gender as a co-factor.

5.3 RESULTS

138 patients were included in this observational study. Mean \pm SD age of the subjects studied was 61 ± 13 . 85 patients (62%) were female while only 53 patients (38%) were male. To analyse demographic location, the Street Check website (<https://www.streetcheck.co.uk/postcode/>) was used to classify location into rural or urban. Of 138 patients 91 (66%) patients were from urban areas while only 47 (34%) were from rural areas.

At presentation 22% of patients were taking inhaled medication and 34% were using proton pump inhibitors. 27% gave a history of allergy and 32% reported a previous diagnosis of asthma. Only 14% of the population were current smokers.

Mean FEV1% and FVC% predicted was 96 ± 20 and 110 ± 21 respectively. Mean FEV1/FVC ratio predicted was 93 ± 12 , only 3.6% had FEV1/FVC ratio less than 70%.

Average HARQ score was 30 ± 14 (Median=29 & range=68), only 12% of the patients scored below the upper limit of normal of 14. Amongst the 14 symptoms which are listed in this questionnaire two questions, Q7 "Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5) " and Q13 "Coughing more when awake rather than asleep", were scored with the maximum score of 5 by 58% and 45% of the patients respectively (see table 6).

For Q1 "Hoarseness or a problem with your voice", Q10 "Cough with certain foods and Q14 "A strange taste in your mouth" were scored 5 by only 10% of the patients.

On average patients reported “Coughing more when awake rather than asleep” (Q13) and “Clearing your throat” (Q2) with mean score 3 out of 5.

Cough sensitivity questionnaire is a ten point Likert scale with 5 domains (cough frequency, cough intensity, sputum production, wheeziness and breathlessness). Patients on average scored cough frequency and cough intensity 6 ± 2 (Median=6 & range=9) and 5 ± 2 (Median=6 & range=9) out of 9 respectively. Average sputum production scored 4 ± 3 (Median=3 & range=9) whilst wheeziness (Median=2 & range=9) and breathlessness (Median=3 & range=9) was scored 3 ± 3 . When the correlation between individual symptoms and HARQ scores was examined there was the strongest correlation between cough frequency and cough intensity ($p < 0.001$ $r=0.8$). Wheeziness showed a strong correlation with breathlessness ($p < 0.001$ $r=0.65$). Weaker correlation with Sputum production, breathlessness ($p < 0.03$ $r=0.18$) and wheeziness ($p < 0.01$ $r=0.21$) were seen. Sputum production was the least correlated with other symptoms despite having a similar mean score of 3. HARQ was highly correlated with cough frequency and cough intensity ($p < 0.000$ $r=0.5$), but less so with other symptoms (see table 7).

To determine prospectively whether FeNO value can aid the clinician to differentiate phenotypes of cough I divided the patients into two groups of low FeNO (FeNO under 25) and high FeNO (FeNO above 25) (see table 8). Thirty five (25%) patients had high FeNO value while the majority 103 (75%) had a FeNO value in a normal range. The mean lung function was similar in both groups. In the low FeNO group 65 patients (63%) of the subjects were female and 38 patients (37%) of the subjects were male. However, in the high FeNO group there was more balance between the sexes with 20 (57%) of patients being female and 15 (43%) of patients male.

Comparing HARQ score there was a significant difference between the two cohorts with patients in the low FeNO group having a mean HARQ score of $30 (\pm 15)$ (Median=30 & range=66) compared with patients in the high FeNO group $25.5 (\pm 12)$ (Median=26 & range=50) ($p < 0.05$). The same was not shown for the cough frequency and intensity. This latter may be due to a type 2 error with a low number of patients in the high FeNO group. The

relationship of HARQ score to FeNO remained significant when examined using logistic regression analysis with the gender as a co-factor [P=0.05, Exp(B)=1].

In the high FeNO group 31% of patients used inhalers compared with 18% in the low FeNO group (P=0.08). 37% of patients in the high FeNO group gave a history of allergy compared with 23% in the low FeNO group (P=0.08). Percentage with a previous diagnosis of asthma was similar in both groups (32%) (P=0.5). Percentage of patients who used anti reflux medication was 29% in the high FeNO group and 36% in the low FeNO group (P=0.2). In the high FeNO group only 5% of patients were smokers whereas this figure was 12% in the low FeNO group (P=0.2). None of these observed differences were significant between the two cohorts (see table 8).

5.4 DISCUSSION

Age and gender prevalence in chronic cough patients in this study is similar to other reports with the typical patient being a middle aged woman. Similar female gender preponderance of two thirds is reported to other cough studies around the globe. Fujimura and colleagues (2003) in a study in Japan reported that 102/137 of the patients were middle aged women. In a worldwide survey of chronic cough clinics we showed 6591/10032 were female with mean age 55 (Morice et al., 2014). The predominance of women attending cough clinics may be explained by a greater sensitivity of the cough reflex in women (Fujimura et al., 1990; Kelsall et al., 2009). There was a trend for this observation to be particularly prominent in low FeNo population.

Eighty eight percent of patients had a HARQ score above 14 indicating an advanced cough symptoms which likely caused by airway reflux. Cough due to airway reflux can lead to both eosinophilic and neutrophilic inflammation driven by epithelial damage releasing mediators such as IL33 (Sadeghi & Morice, 2017) or IL8 (Sadofsky et al., 2017). The high HARQ score in the low FeNO group indicates greater symptoms burden in this group ($p < 0.05$) even after the correction of the different gender distribution between the two groups and is consistent with the patient reported outcomes cough questions recording intensity and frequency.

In asthma high FeNO is regarded as an indicator of eosinophilic inflammation responding to inhaled corticosteroids. In our cough patients the relative absence of wheeze and normal lung function may have led to a failure to diagnose eosinophil disease either CVA or EB. I observed in the high FeNO group that 69% of the patients were not prescribed any inhaled corticosteroid by referring physicians.

Despite the well-known association of cough with smoking in previous studies (Barbee et al., 1991; Cullinan, 1992) smoking was not a prominent factor in our study. Only 14% of the referred patients to the cough clinic were current smokers. This may be because current smokers do not go to general practitioners regarding their cough as they think smoking is a cause of cough so they ignore their symptoms. In some cases general practitioners may delay referring the current smokers to the secondary care as they might think further investigation is not necessary.

Referring to the results of this study more than two thirds of the patients who visited the Hull Cough Clinic came from urban areas. Previous studies reported that people who have been exposed to air pollution are more likely to develop respiratory symptoms such as cough (Vedal et al., 1998; Montnemery et al., 2001). However, from our data it is impossible to ascertain whether environmental pollution was a risk factor that cause cough and urging people to seek advice in the study.

Examination of the correlation between symptoms and HARQ reveal some interesting findings. The close correlation between cough frequency and cough intensity scores indicates patients view these questions as similar. In contrast sputum production, wheeziness and breathlessness scores showed an intermediate correlation. Since the HARQ is mainly compromised questions regard symptoms associated with airway reflux there was a strong correlation with cough frequency and cough intensity, whilst there was a weak correlation with other symptoms. Therefore, there is a need to develop a questionnaire that delineates these other symptoms.

There are some limitation to refer results of this study to the true chronic cough population because tertiary referral centre may not reflect

characteristics of general clinic. For instance, Q7 “Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)” was scored with the maximum 5 by 58% of the patients while one third of them were on PPI treatment already. In addition, those patients who are responding to the inhalers do not get referred to secondary care thus possibly exaggerating the number of airway reflux we see in the clinic.

Table 6 Descriptive statistics of HARQ scores for each individual question

	Descriptive Statistics						Mean
	0	1	2	3	4	5	
Q1	47	27	15	27	12	10	1.7
Q2	8	12	22	32	36	28	3.1
Q3	64	23	12	11	15	13	1.4
Q4	44	23	21	22	15	13	1.8
Q5	45	16	20	26	15	16	1.9
Q6	45	16	22	25	15	15	1.9
Q7	51	13	6	6	4	58	2.5
Q8	36	17	18	22	21	24	2.3
Q9	56	16	15	14	16	21	1.8
Q10	89	9	9	12	8	11	1.0
Q11	27	15	21	19	23	33	2.6
Q12	34	17	13	19	27	28	2.5
Q13	18	12	10	22	31	45	3.2
Q14	71	20	10	13	13	11	1.3

Table 7 Correlation between cough frequency, cough intensity, wheeziness, breathlessness and HARQ

		Cough Frequen cy	Cough Intensit y	Wheezi ness	Breathles sness	Sputum producti on	HARQ
Cough Frequency	Pearson Correlati on	1	.808**	.295**	.306**	.279**	.558**
Cough Intensity	Pearson Correlati on	.808**	1	.384**	.358**	.333**	.502**
Wheeziness	Pearson Correlati on	.295**	.384**	1	.656**	.182*	.264**
Breathlessn ess	Pearson Correlati on	.306**	.358**	.656**	1	.214*	.341**
Sputum production	Pearson Correlati on	.279**	.333**	.182*	.214*	1	.317**
HARQ	Pearson Correlati on	.558**	.502**	.264**	.341**	.317**	1

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table 8 Different characteristics in chronic cough patients by FeNO value. Symptom scores are expressed as mean out of 9, HARQ score out of 70.

	Low FeNO Group<25	High FeNO group>25	P value
Number of patients	103 (75%)	35 (25%)	
Number of female	65 (63%)	20 (57%)	P=0.3
Using inhalers	19 (18%)	11 (31%)	P=0.08
History of allergy	24 (23%)	13 (37%)	P=0.08
History of asthma	33 (32%)	11 (31%)	P=0.5
Using anti-reflux medication	37 (36%)	10 (29%)	P=0.2
Current smokers	12 (12%)	2 (5%)	P=0.2
HARQ score	Mean=30, Median=30	Mean=25.5 Median=26	P=0.04
Cough Frequency score	Mean=6 Median=6	Mean=5 Median=5	P=0.1
Cough Intensity score	Mean=6 Median=6	Mean=5 Median=5	P=0.1
Wheeziness score	Mean=3 Median=2	Mean=3 Median=3	P=0.8
Breathlessness score	Mean=3.5 Median=3	Mean=3 Median=3	P=0.3
Sputum production score	Mean=4 Median=4	Mean=3 Median=2	P=0.2

CHAPTER SIX

PEPTEST EVALUATION

6.1 INTRODUCTION

6.1.1 CHRONIC COUGH AND GASTRO OESOPHAGEAL REFLUX

Chronic cough is a common problem that is not easy to diagnose and treat accordingly. Gastro oesophageal reflux (GOR) has been long described as one of the common causes for unexplained chronic cough. Recently it has been realised that acidic reflux is not the main irritant in the airways causing the cough hypersensitivity. The present diagnostic methods of both acid and non-acid reflux are invasive, expensive and have moderate sensitivity. Proton pump inhibitors (PPI) treatment is ineffective so therapeutic trials are not to a diagnostic option, there is an urgent need for a non-invasive test which reveals the true incident of airway reflux (Hayat et al., 2015).

6.1.2 PEPSIN IN SALIVA

Pepsin is an enzyme which is produced in the stomach. The gastric chief cells produce and store pepsinogen, the precursor of the active enzyme. Contact with acid activates pepsinogen to pepsin ($\text{pH} < 6$) but it is inactive in high pH environment. It has been observed that the enzyme pepsin was active up to pH 6.5 and inactive but intact between pH 6.8 and 7.8; thus it could be reactivated in low pH environment (Bardhan et al., 2012).

Peptest is a non-invasive, rapid diagnostic test developed to measure pepsin in saliva/sputum of patients as a biomarker for the diagnosis of reflux disease. The PeptestTM is an in vitro diagnostic medical device specific for human pepsin A. This test has been validated in patients presenting with GORD, EER, LPR (Laryngopharyngeal Reflux) and some respiratory diseases (Sifrim, 2015). Peptest has been shown to be a better test for reflux-related aspiration and have greater sensitivity and specificity than the invasive 24-hour pHmetry test (Hayat et al., 2015).

6.2 FIRST EXPERIMENT

This was a baseline study which aimed to measure pepsin in saliva of healthy subjects to explore the normal value of pepsin presence in saliva.

6.2.1 STUDY DESIGN

Healthy subjects were recruited from the plastic surgery outpatient clinic in Castle Hill Hospital. These participants were mainly patient's relatives or patients who had a follow up appointment without any therapeutic procedure requirements. Saliva samples were collected from participants who agreed to take part in the study and met the inclusion and exclusion criteria.

Inclusion Criteria

- Male and female subjects.
- Age range 18 years to 75 years.
- Patient history and family history of no gastrointestinal disease.
- BMI between 18 and 29.
- No prescription gastrointestinal medication.
- No over the counter gastrointestinal medication.
- No health supplements to treat gastrointestinal conditions.
- Reflux Disease Questionnaire (RDQ) must be zero (0).
- Hull Airway Reflux Questionnaire (HARQ) must be 13 or below with the heartburn question zero (0).

Exclusion Criteria

- BMI greater than 29 or below 18.
- RDQ above zero (0)
- HARQ above 13 with heartburn question above zero (0).
- Prescribed gastrointestinal medication such as Lansoprazole and omeprazole in past five years.
- Use of over the counter gastrointestinal medication such as ranitidine and aluminum hydroxide in the past five years.
- Currently taking supplements to treat gastrointestinal symptoms.
- A family member or partner with GERD, EER, LPR or respiratory symptoms or being treated for gastrointestinal or respiratory conditions.

- People who are on nitrates, beta-blockers, calcium channel blockers, bronchodilators for asthma, tricyclic antidepressants, anticholinergics and birth control pills (Progestin).

Prior to providing saliva sample

- No caffeinated or carbonated drinks for 60 minutes before providing saliva sample.
- No alcohol for 18 hours before providing saliva sample.
- No smoking for one hour before providing saliva sample.
- No food for one hour before providing saliva sample.

Subjects needed to complete RDQ (Reflux Disease Questionnaire) (appendix 6) and HARQ (Hull Airways Reflux Questionnaire) (appendix 3) questionnaires to assess if they can be included in the study. When subjects are entered into the study they needed to fill in the Visual Analogue Scale (VAS) to assess level of stress in them, as stress can causes reflux which could affect test results (appendix 7).

Sample collection

Saliva samples were collected into tubes containing 0.5 mL of 0.01 M citric acid and the samples were refrigerated at 4°C and analysed for the presence of pepsin within 2 days of the collection.

Outcomes

The primary outcome was to establish the concentration of pepsin in control subjects' saliva.

The secondary outcome was to assess the relationship between level of the participants' stress and presence of pepsin in their saliva.

6.2.2 ANALYSIS

6.2.2.1 PEPTEST ANALYSIS

To identify pepsin in collected samples the Peptest™ an in vitro diagnostic medical device specific for human pepsin A (RD Biomed Ltd, UK) was used.

The results was interpreted as negative when the detected pepsin in the sample was under 25 ng/mL. Please see chapter two (2.11) for more details.

The diagnostic criteria for positive tests based on a paper by Prof Sifrim (Hayat et al, 2015) is as follow;

Minimal reflux = <25 to 74

Low reflux = 75 to 124

Moderate reflux = 125 to 199

High reflux = 200 to >500

6.2.2.2 STATISTICAL ANALYSIS

Subjects' ages, BMI, HARQ, RDQ frequency and severity questionnaires, and Peptest results were expressed as a mean (SD) and range.

SPSS paired sample T test was used to compare changes in the average pepsin detected in the saliva of the patients at home and in the hospital. Pearson correlation test was used to examine the correlation between Peptest results with HARQ, RDQ frequency and severity and the use of anti-acid reflux medicines by the subjects.

6.2.3 RESULTS

At the beginning 12 saliva samples were collected from healthy volunteers who met the inclusion and exclusion criteria. There was an equal number of males and females among participants with average age of 46 (22-61) and BMI 26. All the subjects scored RDQ zero and HARQ mean 0.3 (0-3) out of 70. They denied having any gastrointestinal symptoms or respiratory disease. The average VAS score (The level of stress) was 0.8 (0-2) out of 10.

The Peptest results were positive in all participants with average 518 ng/mL (Table 8). These results were surprisingly high and apparently suggesting reflux is normal in the setting.

By considering this result it has been decided to stop collecting samples and wait for a new Peptest kits that would apply synthetic antibodies to detect pepsin. Later in the study another 18 samples were collected from healthy volunteers, these samples were analysed by the new Peptest kits. In this group

56% of the volunteers were female and 44% were male. The average age was 44 (26-75) and BMI 24. All the subjects scored RDQ zero and HARQ average 1.3 (0-6) out of 70. They denied having any gastrointestinal symptoms or respiratory disease. The average VAS score (The level of stress) was 1.9 (0-3.5) out of 10.

Once more the Peptest results were positive in all participants with average 141 ng/mL (23 - 212 ng/mL) (Table 9). Only in 1 sample the detected pepsin was <25 ng/mL (minimal reflux), in 5 samples it was 75 to 124 ng/mL (low reflux), in 11 samples it was 125 to 199 ng/mL and only in one sample it was above 200 ng/mL (212). It is clear most of the subjects (61%) indicated a moderate reflux according to the Peptest results.

The samples that were analysed with the old Peptest kits were compared with the samples that were analysed with the new one. The average detection of pepsin with the new Peptest kits dropped by 377 ng/mL from 518 to 141. Nonetheless, all the results were positive while subjects denied having any stress.

Table 9 Old samples from Plastic clinic

S.N	LFD Intensity @15min	Result P/N	Pepsin ng/ml
1	7760	P	1403
2	6571	P	874
3	2681	P	169
4	548	P	21
5	772	P	40
6	6110	P	721
7	6068	P	708
8	2837	P	180
9	5096	P	466
10	745	P	38
11	7016	P	1048
12	5458	P	545
Mean	4305		518

Table 10 New samples from Plastic Clinic

	LFD Intensity @15min	Result P/N	Pepsin ng/ml
1	1777	P	110
2	1692	P	105
3	2299	P	143
4	3015	P	194
5	2227	P	139
6	1518	P	94
7	2027	P	126
8	2751	P	174
9	2149	P	134
10	2837	P	180
11	2966	P	190
12	3227	P	212
13	2470	P	155
14	578	P	23
15	1875	P	116
16	2847	P	181
17	2312	P	144
18	1839	P	114
Mean	2227		140

6.3 SECOND EXPERIMENT

In considering the above results I could not find no obvious reason why all the samples were positive although it has been suggested that the hospital environment where I collected the samples might cause anxiety and stress for the subjects, effectively increasing pepsin production. Therefore, I decided to run another experiment to examine this hypothesis.

6.3.1 STUDY DESIGN

To prove the above hypothesis saliva samples were collected at home from chronic cough patients prior to their attendance to the Hull Cough Clinic. Each week the new patients in the chronic cough clinic received a letter with a Peptest tube, a Peptest leaflet which explained how to collect saliva and a Reflux Disease Questionnaire (RDQ). They were asked to send the sample back to the lab in a provided pre post envelop.

6.3.2 RESULTS

I received 11 samples posted from home by chronic cough patients. 64% of the patients were female and 36% were male. The average age was 65 (54 - 84) and BMI 31. The average RDQ frequency score was 8 (0 – 24) and average RDQ severity score was 8 (0 – 26). The mean HARQ score was 33 (14 - 58) out of 70.

The results showed that the mean pepsin in all samples was 43 ng/mL. Approximately two third of the samples (7 samples) were negative and there was no trace of pepsin in the samples (Table 10).

6.3.3 CONCLUSION

In conclusion it is suggested that the environment of collecting saliva samples may cause changes in the results and the hospital environment may increase the stress level in the subjects and elevate pepsin production. As our samples from hospital and home were from different subjects some healthy and some chronic cough patients I decided to run another study and monitor changes of the pepsin in same subject to get more accurate results.

Before conducting the next experiment I did a quality check to confirm the stability of the pepsin pending assay. I collected 5 samples from 5 different

individuals and transferred to the lab as soon as they produced. The samples were analysed as normal procedure and then they were kept out and analysis was repeated after 3 days. The results were identical.

Table 11 Sample collected at home from cough patients

LFD Intensity @ 15 mins	Result	Pepsin (ng/ml)
	P/N	CALCULATED
2	N	0
118	N	0
283	N	0
44	N	0
14	N	0
279	N	0
48	N	0
3441	P	231
469	P	14
2137	P	133
1604	P	99

6.4 THIRD EXPERIMENT

This study aimed to explore if the environment of collecting saliva samples at home and in a hospital alter the results of the Peptest. I hypothesised that the hospital environment may increase the stress level in the subjects and elevate pepsin production.

6.4.1 STUDY DESIGN

25 chronic cough patients recruited from the Hull Cough Clinic in Castle Hill Hospital. Two saliva samples were collected from cough patients who visited the cough clinic, one in their home environment and one at the hospital. The new cough patients who were due to come to the clinic were sent a letter with a Peptest tube, a Peptest leaflet which explained how to collect saliva and complete a Reflux Disease Questionnaire (RDQ). The patients were asked to send the sample back to the lab by a provided pre post-paid envelop. A second sample was collected when the patients attended the cough clinic. Hull Airways Reflux Questionnaire (HARQ) and demographic data were collected in the clinic.

Sample collection

Saliva samples were collected into tubes containing 0.5 mL of 0.01 M citric acid. The samples that came by post were analysed at the same day that received by post. The samples that collected in the clinic was refrigerated at 4°C and analysed for the presence of pepsin within 2 days of the collection.

Outcomes

The primary outcome was to establish compare the concentration of pepsin in the samples collected at home with samples collected at the hospital from the same patients.

The secondary outcome was to assess the relationship between HARQ and presence of pepsin in their saliva.

6.4.2 RESULTS

Demographics

From the collected samples there were 19 patients that had both samples, one produced at home and the other one in the clinic. The samples were analysed by the same method which was explained in section 3.2. 58% of the patients were female and 42% were male. The average age was 60 (range 30 - 78) and BMI 30 (range 19 – 45). The average RDQ frequency score was 3.9 ± 6.6 (range 0 – 18) and average RDQ severity score was 3.6 ± 5.8 (range 0 – 20). HARQ average was 29 (range 0 – 57) out of 70. Thirty seven % (7 out of 19) of patients were on anti-reflux medicines.

Peptest results

The Peptest results from samples that were collected in the clinic showed that all participants had pepsin in their saliva with average 342 ± 154 ng/mL (range 50 - 711) (Table 13) (Figure 14 & 15). On the other hand the results from samples which were produced from home and sent to the lab showed the average detected pepsin in the saliva was 149 ± 175 ng/mL (range 0 – 463) (Table 14) (Figure 14 & 15). Therefore, there was a significant difference ($P<0.005$) between the results of the Peptest at home and in the clinic (Table 12).

In this study there was no correlation between the Peptest results from home and from the clinic with the HARQ questionnaire. There was no correlation between the Peptest results collected at home and being on or off acid suppression therapy ($r= 0.07$, $P<0.7$). Similar results observed between the Peptest results collected at hospital and being treated by acid suppression medicines ($r= 0.3$, $P<0.1$). There was no correlation between Peptest saliva samples collected at the clinic with RDQ frequency ($r=-0.5$, $P<0.07$) and RDQ severity ($r=-0.3$, $P<0.3$). Similarly, there appeared to be no correlation in samples collected at home with RDQ frequency and severity ($r=0.1$, $P<0.7$ and $r=0.4$, $P<0.2$ respectively).

6.4.3 DISCUSSION

The result of this study shows there was no relationship between the pepsin secretion and acid suppression therapy similar to that which has been recently reported (Fortunato et al., 2017). Thus, it is arguable that silent reflux or gaseous reflux cannot be treated effectively with PPIs (Hayat et al., 2015). There was no correlation between the Peptest results and the HARQ questionnaire, this may be explained as HARQ questionnaire is assessing the symptoms of airway reflux in a period of month while Peptest is testing the patients reflux in a certain time.

As there was no correlation between secretion of pepsin in samples collected in the clinic with RDQ frequency and severity, it can be concluded that the pepsin was extremely elevated in this special circumstance, but it is not a correct indicator to diagnose reflux. In contrast, results of samples collected at home might be a better indicator to use as a diagnostic preference.

Recently, there were two studies (Na et al., 2016; Fortunato et al., 2017) that had shown a wide range of pepsin concentrations within individual saliva samples. Fortunato and colleagues (2017) reported that the wide range of pepsin was detected over 24 hours in some patients with gastro- esophageal reflux disease. Similar results were found in a study on laryngopharyngeal reflux patients (Na et al., 2016). Now this study can report that there was a significant difference ($P<0.005$) between the place of producing the sample from the same individual. Comparing the Peptest results it can be seen that there was a 193 ng/mL (67%) increase in the average pepsin detected in the clinic compared with the average pepsin detected at home. In addition to that the results of 90% of the samples collected in the clinic showed moderate or high reflux while only 37% of the samples collected at home showed high reflux. There are various factors that contribute to escalate secretion of pepsin in saliva, however in this circumstance I suggest that stress can be an important factor which had a dynamic impact on secretion of the pepsin. Previously, in a research study (Nabavizadeh F et al., 2011) it was revealed that physical and psychological stress elevated gastric acid and pepsin secretions in the rat. This might be true in the human as well, so subjects might feel under stress when they are asked to produce a saliva sample in the

clinic, while in a calmer environment like in the home this might not be the case. Therefore, environmental circumstance might affect the result of the Peptest in produced saliva samples.

Table 12 Average detected pepsin in the clinic (PEP.C) and in the home (PEP.H)

	Mean	N	Std. Deviation	Std. Error Mean
Pair 1 PEP.C	341.7895	19	153.83020	35.29107
PEP.H	148.3684	19	175.37047	40.23274

Figure 14 Average detected pepsin in saliva in clinic and home

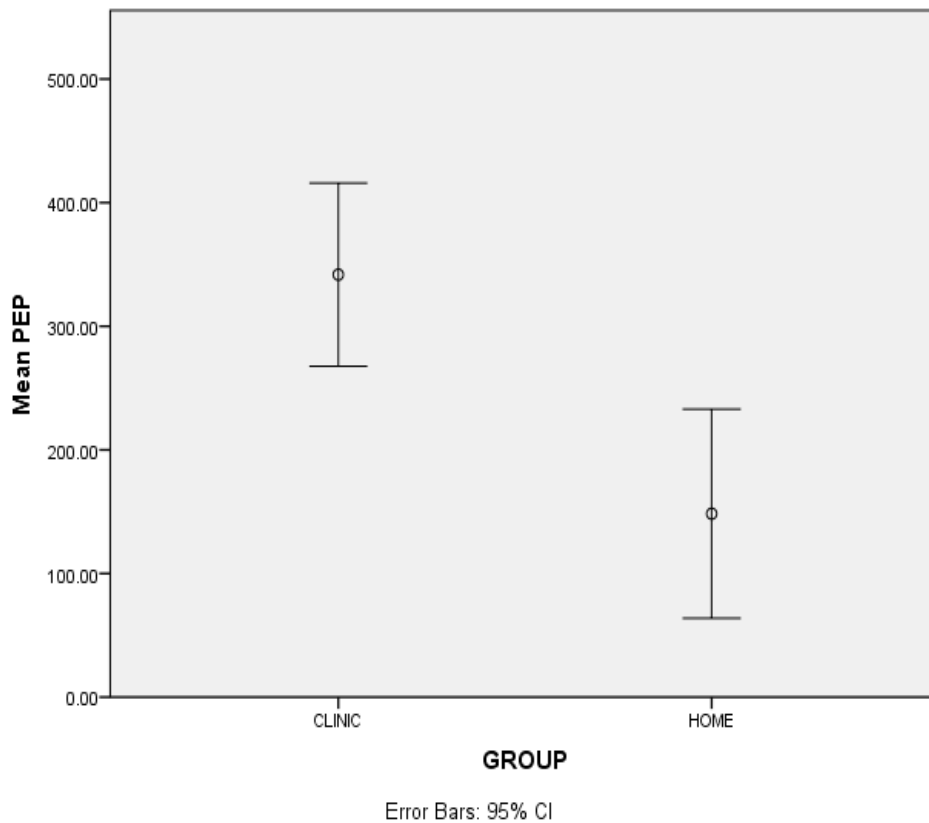


Figure 15 Profile plot of Peptest results in the clinic and at the home for the individual patients

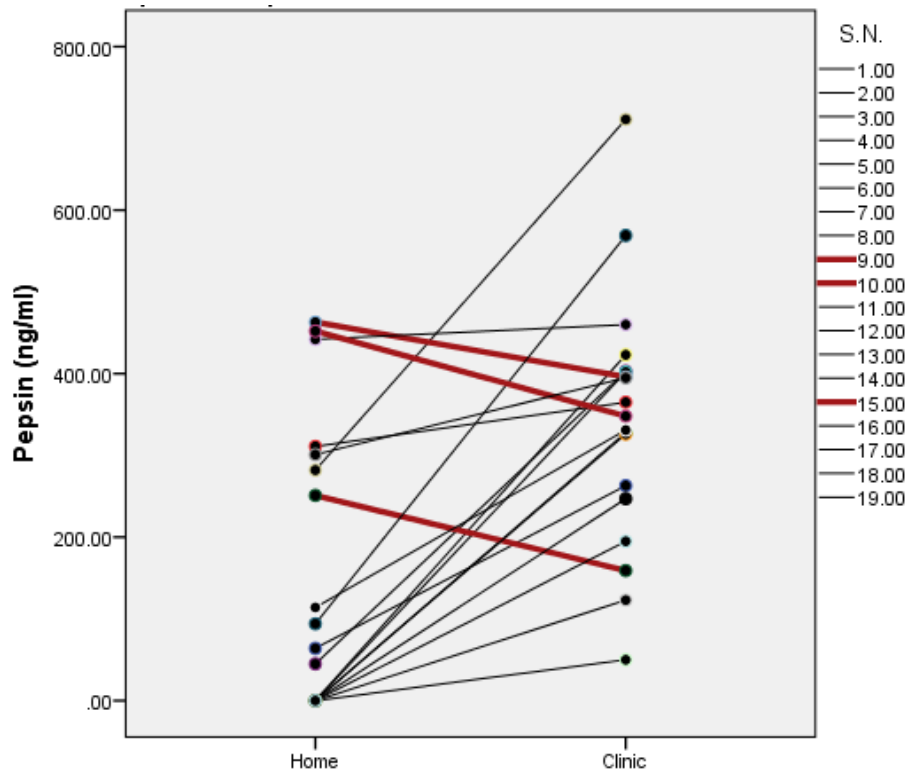


Table 13 Collected samples in the clinic

In Clinic	LFD Intensity @ 15 mins	Result	Pepsin (ng/ml)
Subject N.		P/N	CALCULATED
1	3044	P	263
2	3648	P	328
3	5717	P	711
4	4206	P	403
5	4339	P	423
6	3937	P	365
7	4198	P	402
8	1399	P	123
9	4158	P	396
10	1829	P	159
11	3630	P	326
12	4559	P	460
13	5125	P	569
14	597	P	50
15	3808	P	348
16	4148	P	395
17	2879	P	247
18	2278	P	195
19	3668	P	331

Table 14 Collected samples at home

At Home	LFD Intensity @ 15 mins	Result	Pepsin (ng/ml)
Subject N.		P/N	CALCULATED
1	733	P	64
2	201	N	0
3	3233	P	282
4	841	P	45
5	163	N	0
6	3496	P	311
7	139	N	0
8	189	N	0
9	4577	P	463
10	2916	P	251
11	216	N	0
12	4453	P	442
13	1052	P	94
14	309	P	19
15	4511	P	452
16	3408	P	301
17	53	N	0
18	83	N	0
19	1283	P	114

CHAPTER SEVEN

PROCESSING A SPUTUM SPECIMEN

7.1 INTRODUCTION

There are two methods for processing a sputum sample depending on the processing of the selected sputum plugs or entire sputum sample. In the first method all viscid or denser portions of sputum are selected by using an inverted microscope, and then processed from the expectorated sample (Pin et al., 1992). In the second method the entire sputum sample plus variable amounts of saliva are processed (Fahy et al., 1993). Over the years some modifications have been applied to these methods in order to make them more accurate and easier to perform. However, there is still variability in sputum processing SOPs across the regions.

In this study the Standard Operating Procedure (SOPCTU100210) of the Respiratory Clinical Trials Unit has been followed, however some minor alterations applied.

Current methodology, includes centrifugation as a process to separate sputum cells from the fluid phase, however the effect of centrifugation on fluid phase measurement is not clear (Efthimiadis et al., 2002). Sputum samples can be processed by two possible methods where total cell counts (TCC) are performed either before or after centrifugation. Centrifugation process has previously been shown to reduce TCC. I wish to standardise the sputum process by evaluating the most appropriate method for processing of sputum samples providing accurate differential cell counts (% eosinophils, neutrophils, lymphocytes, macrophages and epithelial). The importance of this method was discussed in chapter 1.8 and it was used in my clinical trial study (chapter 3 & 4).

7.2 METHODOLOGY

This study compared Cell Differential Counts in 14 sputum samples which were processed with centrifugation and without centrifugation. After selecting the sputum plug and filtration process each sample was separated to allow processing half of the sample without centrifugation (method 1) and the other half with centrifugation (method 2) (please see chapter 2 section 4 & 5 for the details of the used methods).

7.3 DATA COLLECTION AND ANALYSIS

I collected 20 sputum samples from cough patients at Clinical Trial Unit. Six samples were discarded because there were not enough cells in the sputum sample to process the slides by both methods to compare them together. Fourteen samples differential cell counts (DCC's) measuring % eosinophils, neutrophils, lymphocytes, macrophages and epithelial were made with and without centrifugation of the sample. For quality control all the coded slides were counted by an experience staff member who was not aware of which method was used for each slide. In addition, three slides from each method (six slides in total) were chosen randomly to be recounted with a second experience staff member.

The results of the DCC of 28 slides from 14 samples are presented in table 14 & 15. Comparison of data between the two methods were analysed using Independent samples T-test to compare mean between groups' values. Data were generated using SPSS (version 23) and Excel 2013 programmes.

7.4 RESULTS

By comparing the data, centrifugation was found to slightly reduce the number of neutrophil cells, with mean % neutrophil DCC reduced from 52.14% (200.8) to 44.72% (205.3) (table 16). There was also a decrease in mean % lymphocytes from 0.88% (2.4) to 0.61% (3.4). Macrophages increased from 34% (2.4) to 36% (3.4) following centrifugation, while mean % eosinophil DCC decreased from 12.8% (51.3) to 12.5% (49.8) and epithelial cells with 0.35% (1.4) remained same. The result has been illustrated in figure 16, clearly indicating that the cell count from both methods are quite identical and none of the changes are significantly different. However, there was an extreme value in sample 14 that eosinophil count with centrifugation and without centrifugation were 340 and 285 respectively. An outlier was excluded, the results were identical and the mean % eosinophil DCC was higher (32=7.9%) without centrifugation in compared with centrifugation (29=7.3%).

Furthermore, by comparing the mean cell concentration of the samples it has been shown that mean cell concentration with centrifugation and the mean

cell concentration without centrifugation were 3635071.429 and 1757857.143 respectively. Therefore, cell concentration after centrifugation is twice the cell concentration before centrifugation. The mean % of viability and the mean % of squamous cells of the samples which were processed with the centrifugation method were %70.7 (2571553) and %4.5 (1063518) respectively. These figures changed to %63.6 (1118311) and %3.55 (639546) when samples were processed without centrifugation.

7.5 DISCUSSION

This study demonstrates that despite centrifugation previously having shown to reduce TCC, this does not appear to alter the proportion of each white blood cell within the sample, ie DCC remains the same.

However, the results shown that centrifugation doubled the concentration of cell within the sample. This makes the cell count performance to calculate the average live, dead and squamous cell numbers more difficult and a lengthier process. In addition to that the centrifugation process itself would make the process longer and more technical to separate the supernatant without disturbance to the cell pellet.

In conclusion by considering all the evidence, our study suggests there is no advantage to performing a centrifugation step in the processing of sputum unless interested in the supernatant for measurement of biomarkers. If this is required there would be no detrimental effect on differential cell counts. Removal of the centrifugation phase will standardise the sputum process within our lab.

Table 15 Fourteen samples Differential cell counts (DCC's) measuring mean % of eosinophils, neutrophils, lymphocytes, macrophages and epithelial without centrifugation

Case n.	Eos n.	Eos %	Neut n.	Neut %	Mac n.	Mac %	Lym ph n.	Lym ph %	Epi n.	Epi %
1	83	20.8	205	51.2	106	26.5	2	0.55	5	1.25
2	1	0.25	379	94.75	20	5	0	0	0	0
3	7	1.8	366	91.5	25	6.25	2	0.5	0	0
4	6	1.5	222	55.5	166	41.5	2	0.5	4	1
5	200	50	99	24.8	97	24.3	4	1	0	0
6	36	9	209	52.2	146	36.5	8	2	1	0.25
7	1	0.25	107	26.75	290	72.5	0	0	2	0.5
8	2	0.5	195	48.8	199	49.8	3	0.75	0	0
9	69	17.25	237	59.25	95	23.8	0	0	0	0
10	7	1.7	339	84.8	39	9.8	10	2.5	6	1.5
11	1	0.25	121	30.3	270	67.5	6	1.5	2	0.5
12	0	0	228	57	171	42.8	3	0.75	0	0
13	0	0	134	44.7	163	54.3	3	1	0	0
14	285	71.25	34	8.4	76	19	5	1.25	0	0
Mean	50	12	205	52	133	34	3.4	0.87	1.4	0.35
SD	87	22	103	25	83.8	21.6	3	0.74	2	0.52

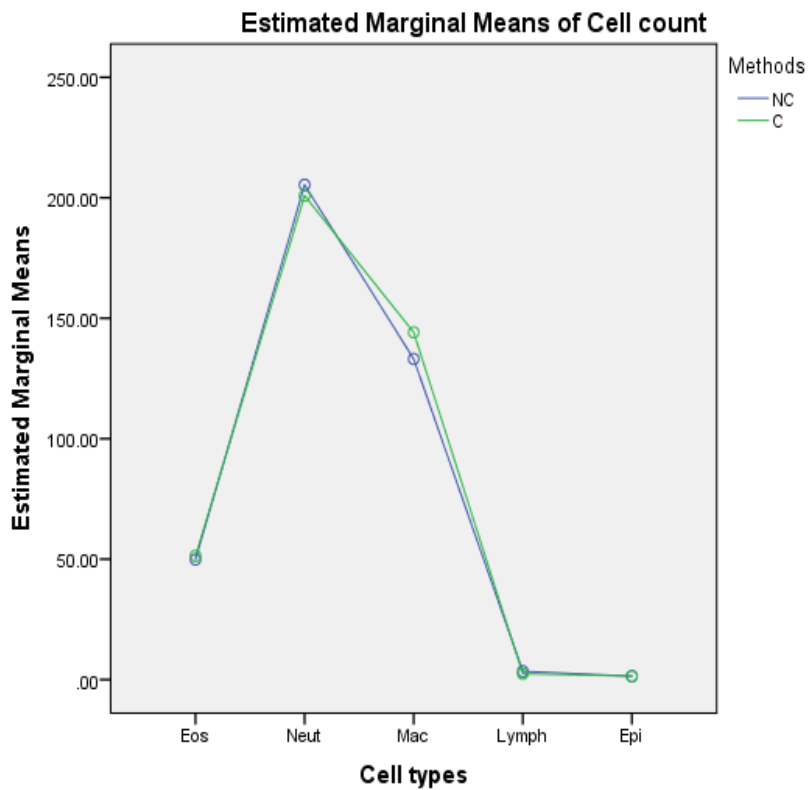
Table 16 Fourteen samples Differential cell counts (DCC's) measuring mean % of eosinophils, neutrophils, lymphocytes, macrophages and epithelial with centrifugation

Case n.	Eos n.	Eos %	Neut n.	Neut %	Mac n.	Mac %	Lym ph n.	Lym ph %	Epi n.	Epi %
1	107	26.8	199	49.8	88	22	3	0.75	3	0.75
2	0	0	365	91.25	36	9	0	0	0	0
3	8	2	355	88.8	35	8.75	2	0.5	0	0
4	15	3.8	245	61.3	136	34	3	0.75	2	0.5
5	144	36	172	43	82	20.5	2	0.5	0	0
6	26	6.5	204	51	157	39.5	6	1.5	7	1.75
7	2	0.5	70	17.5	327	81.75	0	0	1	0.25
8	2	0.5	201	50.2	197	49.3	0	0	0	0
9	68	17	247	61.8	84	21	1	0.25	0	0
10	3	0.75	342	8.5	47	11.8	4	1	4	1
11	0	0	149	37.3	245	61.3	3	0.75	3	0.75
12	3	0.75	163	40.75	231	57.75	4	1	0	0
13	1	0.25	79	19.75	317	79.28	3	0.75	0	0
14	340	85	21	5.2	36	9	3	0.75	0	0
Mean	51	12.8	201	44.7	144	36	2.4	0.6	1.4	0.35
SD	95	24	105	26	103	26	1.7	0.4	2	0.5

Table 17 Comparison of means total cell counts of eosinophils, neutrophils, lymphocytes, macrophages and epithelial with centrifugation © and without centrifugation (NC)

	N	Minimum	Maximum	Mean	Std. Deviation
Eos.NC	14	.00	285.00	49.8571	87.49141
Eos.C	14	.00	340.00	51.3571	94.65533
Neut.NC	14	34.00	379.00	205.3571	103.13815
Neut.C	14	21.00	365.00	200.8571	105.24393
Mac.NC	14	20.00	290.00	133.0714	83.83268
Mac.C	14	35.00	327.00	144.1429	103.56258
Lymph.NC	14	.00	10.00	3.4286	2.97979
Lymph.C	14	.00	6.00	2.4286	1.74154
Epi.NC	14	.00	6.00	1.4286	2.10180
Epi.C	14	.00	7.00	1.4286	2.13809
Valid N (listwise)	14				

Figure 16 Comparison of means total cell counts of eosinophils, neutrophils, lymphocytes, macrophages and epithelial with centrifugation © and without centrifugation (NC)



CHAPTER EIGHT

GENERAL DISCUSSION

8.1 INTRODUCTION

This thesis has concentrated on the diagnostic methods and treatments of chronic cough. The studies have investigated usefulness of current non-invasive diagnostic methods. Objective and subjective measurements of cough were also assessed to discover their differences in eosinophilic and non-eosinophilic inflammation in chronic cough.

As outlined in Chapter One it was argued that although the aetiology of chronic cough in guidelines is clearly stated as asthma and related syndromes, gastro-oesophageal reflux disease (GORD), and upper airways disease, the inflammatory mechanisms underlying these conditions differ (Ford et al., 2006; Chung & Pavord, 2008; Magni et al., 2010; Morice, 2011). Recent studies on asthma have increasingly focused on its molecular phenotypes instead of clinical characteristics (Wenzel, 2012; Lambrecht & Hammad, 2015; Ray et al., 2015). Based on robust evidence in the literature (Gibson et al., 1989b; Fujimura et al., 1992; Fujimura et al., 2003; Magni et al., 2010; Morice et al., 2011a) (Brightling et al., 1999) we propose the hypothesis that dividing cough into two groups; the eosinophilic and neutrophilic will enhance our ability to recognise the type of airway inflammation present which, as a consequence will lead us to more targeted and personalized treatment approaches. To accomplish this target, accurate tools to assess airway inflammation and evaluate the effectiveness of therapy in chronic cough patients is critically essential.

The various diagnostic approaches and available therapies in eosinophilic inflammation are discussed in Chapter One, giving background on this domain used in Chapter Three and Four.

FeNO is an established biomarker of airway eosinophilic inflammation (Boot et al., 2007). A proportion of patients with isolated chronic cough have eosinophilic inflammation present but without bronchoconstriction, a condition termed as cough variant asthma (CVA) or eosinophilic bronchitis (EB). Treatment with anti-inflammatory agents such as prednisolone (Brightling, 2006; Irwin et al., 2006b) and montelukast (Dicpinigaitis et al., 2002) (Mincheva et al., 2014) may be a successful strategy in these patients.

However whether an estimation of FeNO value can help guide therapy in chronic cough is unknown.

In Chapter Three I discussed my randomised controlled study to assess the outcome of anti-inflammatory therapy with either the leukotriene antagonist montelukast or oral prednisolone in patients with chronic cough. Patients presenting with a high FeNO measurement were compared with a contemporaneously recruited group with low FeNO.

The results showed that 24 hours cough count fell in both groups by approximately 50% indicating that montelukast therapy was effective therapy for chronic cough patients regardless of the type of inflammation demonstrated by conventional methods. It is possible that in patients with low FeNO montelukast may have a secondary anti-inflammatory property to targeting nerve hypersensitivity. Mast cells have an ability to interact with airway nerves (unmyelinated C fibres) (Stead et al., 1989; Bienenstock et al., 1991) and drive cytokines and mediators to the airways to stimulate cough nerves. Thus response to montelukast by patients with low FeNO value may not be predicated on systemic eosinophilic inflammation but may be consequent on localised leukotriene mediated inflammation (Taylor-Clark et al., 2008) (Mincheva et al., 2014) (Kawai et al., 2008).

The results of the above study were examined further in Chapter four. I evaluated whether exhaled nitric oxide measurement can assist in the assessment of chronic cough patients based on their airway inflammatory phenotype. The results demonstrated that FeNO was a good marker for eosinophilic inflammation with a high degree of correlation with blood and sputum eosinophilia. This strong relationship between FeNO, blood eosinophils and sputum eosinophils confirms phenotypic identity.

The number of recorded coughs in 24 hr and HARQ scores were significantly ($p < 0.05$) higher in patients with a low FeNO. In the low FeNO group the greater proportion of patients were women compared with the high FeNO group, being 90% and 48% respectively. LCQ scores were not significant between the two groups, however there might be a type 2 error as there are

low numbers and a non-significant trend. Whether the observed gender disparity accounts for the different cough frequency characteristics is unknown.

Chapter Five I examined the epidemiology of chronic cough patients to see whether exhaled nitric oxide measurement (FeNO) can predict different characteristics based on different airway inflammation phenotypes. One hundred and thirty eight consecutive patients who attended to the Hull cough clinic over a 6 months period were studied. In this study I have shown FeNO measurement had only a low yield of diagnostic information. Patients with lower FeNO are more likely to have higher airway reflux symptom (HARQ) scores ($p < 0.05$). There was a greater proportion of women in the low FeNO group. Perhaps the most significant utility was the demonstration that only 31% of patients with high FeNO were on inhaled corticosteroids.

Pepstest is known as a non-invasive diagnostic test, presence of pepsin in saliva being used as a biomarker to diagnose reflux disease in patients presenting with peptic symptoms. In Chapter Six performing three observational studies I examined the presence of pepsin in saliva of different groups of subjects (healthy and cough patients). In the majority of samples collected in a hospital setting, the detected pepsin indicated a moderate to high reflux. However, I witnessed that Pepsin values fell significantly when the samples were collected at home from the same subjects. Therefore, it has been suggested that production of pepsin will be enhanced in a stressful environment.

Sputum eosinophilic cell count is a validated tool to diagnose respiratory inflammation and monitor anti-inflammatory therapy response. However this process is a time-consuming and difficult procedure. In chapter seven Cell Differential Counts (CDF) in sputum samples were processed with centrifugation and without centrifugation. The results shown that CDF was identical in both methods with this difference that processing the samples with centrifugation make the procedure longer and more complex.

8.2 FUTURE RESEARCH

8.2.1 IS MONTELUKAST TREAT NERVE HYPERSENSITIVITY IN CHRONIC COUGH?

In Chapter Three our clinical trial study surprisingly showed a dramatic reduction in cough frequency in the low FeNO group to montelukast. While it was anticipated that montelukast has little or no effect in patients without eosinophilic inflammation. In the absence of the placebo it is impossible to rule out a non-pharmacological effect. However, the strong correlation of FeNO with biomarkers of airway eosinophilia inflammation in blood and sputum suggesting biological activity. Subjective and objective measures of coughs also responded to therapy accordingly.

Montelukast has been repeatedly demonstrated to effect allergic inflammation in asthma (Ritter et al, 2002) and more recently in eosinophilic cough (Kopriva et al, 2004; Mincheva et al, 2014) and it has been assumed that this activity is due to blockade of leukotriene receptors (Gagro et al, 2004). The fact that montelukast appears to be equally effective in the low FeNO group suggests that either the current markers of eosinophilic lung disease are insufficiently sensitive to pick up low levels of leukotriene activation in the low FeNO group or that montelukast has its antitussive activity by an alternative mechanism (see Chapter Three section 3.4). To confirm effectiveness of montelukast in cough patients without presence of eosinophilic biomarkers there is a need for a large placebo controlled study. I also propose to measure of mast cell activation in the sputum samples of patients before and after the therapy.

8.2.2 CAN FENO ASSISTS IN DIAGNOSING DIFFERENT PHENOTYPES OF AIRWAY INFLAMMATION AND DRAW A DIFFERENT CHARACTERISTICS AMONG CHRONIC COUGH PATIENTS?

In Chapter Four baseline data from my clinical trial study has shown a significant correlation between FeNO, blood eosinophils and sputum eosinophils. This result demonstrates the diagnostic value of FeNO measurement in chronic cough patients. I have shown that patients with an

FeNO value of more than 30 ppb are more likely to have eosinophilic inflammation as evidenced by sputum eosinophilia, and observed the lack of eosinophil inflammation in the low FeNO group patients. FeNO value had a strong correlation with blood and sputum eosinophil count ($r= 0.79$ and $r= 0.65$ $p < 0.001$ respectively). This study was the first study in chronic cough that assess correlation between FeNO and B-Eos.

Furthermore, I found that patients with FeNO less than 20 ppb are more likely to have a higher cough frequency and consequently lower quality of life. At the baseline the patients in the low FeNO group had more than twice the number of coughs recorded. Again at baseline there was a significant difference in HARQ & LCQ scores between high and low FeNO groups. Patients with low FeNO suffered more from cough symptoms in comparison with patients with high FeNO according to the 24hr cough count, HARQ and LCQ scores. These patients are more commonly female. Kelsall and his colleagues (2009) in a cough study reported that women had a greater 24 hr cough count than men. Previously it was also reported that women have shown greater sensitivity of the cough reflex compared with men (Fujimura et al., 1990; Kelsall et al., 2009). In the epidemiology study on chronic cough discussed in Chapter Five, I found that HARQ scores in patients with the low FeNO are significantly higher. However, there was not a significant difference in cough frequency and cough intensity scores between the two groups. Based on clinical practice, generally cough frequency is not routinely assessed objectively with a cough monitor for all patients. It is difficult to assess cough objectively and has to rely on the cough questionnaires. Therefore it is important to find out how the number of coughs in a patient effect their quality of life. To confirm these results there is a need to assess cough frequency objectively in larger controlled studies with sexual stratification.

8.2.3 IS PEPTEST A RELIABLE NON-INVASIVE METHOD TO USE AS A DIAGNOSIS TOOL IN CHRONIC COUGH?

Our three experiments have shown that the Peptest is a very sensitive test. The results of this test might be altered by many external factors. In the second

experiment (Chapter 6 section 6.3) I have shown that Peptest results were altered dramatically when the Peptest kit was changed. In the third experiment (Chapter 6 section 6.4) environmental factors showed an impact on the results. Therefore, there is an urgent need for further investigation to eliminate those factors that could affect the test results. Relying on Peptest results without knowing these elements could cause false positive test results.

8.3 CONCLUSION

This thesis has investigated the different types of airway inflammation in chronic cough and assessed usefulness of available diagnostic tools. FeNO is a useful test to recognise patients where TH2 inflammation has not been suspected. There is a strong correlation with other biomarkers of the eosinophilic inflammation. In contrast, Pepsin assay proved to be of no diagnostic value. Objective and subjective measurements of cough were demonstrated to be of value in monitoring therapy response. Future clinical trials can rely on measures of proven accuracy to demonstrate the efficacy of therapeutic effects in chronic cough.

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Standard Operating Procedure

Title: Inducing Sputum with hypertonic saline

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SOP reference number	CTU101099
Author	Rachel Thompson
Current version and date	Version 6, 04/02/2013
Approved by	Professor Morice
Approval signature/date	
Approved by CTU Manager signature / date	
Target audience	Clinical Trials Unit staff

SOP No: CTU101099



PRINCIPLE

The technique of sputum induction has been developed to provide a non-invasive method of sampling airway secretions to assess the cellular content of the lower airways in respiratory diseases.

Assessment of spontaneously produced sputum has been used in clinical practice for several decades to diagnose infection, inflammation and malignancy. Induced sputum analysis has been employed as a specialised research procedure since the late 1980's to help investigate asthma and COPD.

The aim of sputum induction is to obtain satisfactory samples of secretions originating from the airways in a safe and effective manner for subsequent and predetermined analysis.

The method of administration of the hypertonic saline is via an ultrasonic nebuliser. This standard operating procedure is intended for all appropriately qualified staff and physicians within the Academic Department of Medicine, Castle Hill Hospital, Cottingham, UK

1.Safety

1. Staff performing a sputum induction must:

- i.know the contra-indications to sputum induction.
- ii.be aware of, and able to perform, safety and emergency procedures.
- iii.be trained in the administration of inhaled bronchodilators and evaluation of the response to them.

Personnel must not perform a sputum induction if they do not fulfil the above criteria

2. Documented assessment of the infection status. This will have been provided by the referring physician on the request form/letter. For subjects with an infectious respiratory condition, seek guidance from both the referring nurse/physician and the HEY Trust Infectious Control Officer, to assist in determining the level of bio safety/containment that should be used. Any subjects who have a risk of carrying TB must NOT be induced.

3.Medications to treat severe bronchospasm must be present within the testing laboratory. These include epinephrine and atropine for subcutaneous injection/iv and salbutamol and ipratropium in metered dose inhalers or pre-mixed solutions for inhalation, oxygen must also be available. A small volume nebulizer should be readily available for the administration of bronchodilators. A stethoscope, sphygmomanometer, and pulse oximeter should also be available. Staff performing the sputum induction must be trained in how to administer these medications

4. Staff conducting the induction should be aware of the severity of the patient's respiratory condition and any other relevant clinical details (β agonist usage, recent infections/exacerbations) and adopt the appropriate induction protocol. Severe asthmatics (i.e. FEV1<60% predicted should always follow a modified protocol, see below). Sputum induction should NOT proceed on subjects with a FEV1<50% predicted

5. Airflow should be monitored regularly (via FEV1 determination) throughout the test to ensure that the patient is not bronchoconstricting. Patient feedback with regards to dyspnoea should not be relied upon alone.
6. To reduce the risk of bronchoconstriction from saline administration, all patients must be administered a bronchodilator (short-acting β_2 agonist, e.g. salbutamol 200ug) 20 minutes prior to commencement of saline nebulisation.
7. If the patient FEV1 falls by greater than 20% of the best post Salbutamol value the induction is stopped immediately and the patient is given 2.5 mg nebulised Salbutamol.
8. If the FEV1 falls by $>10\%$ $<20\%$ the next concentration of saline is not given and the procedure is continued with the same concentration of saline.
9. If the FEV1 falls to less than 1 litre the induction is stopped immediately and the patient is given 2.5mg of nebulised Salbutamol.
10. If the patient becomes wheezy and develops chest discomfort at any stage check if the FEV1 has fallen by $>10\%$ and then they may be given Salbutamol and the saline inhalation discontinued.
11. All patients must remain in the Unit until their FEV1 has returned to within 5% of baseline.

2. Equipment and Materials

1. Solutions/Medications

Sterile hypertonic saline 7% (stored at room temperature)

Sterile water to dilute hypertonic saline

Salbutamol MDI (100 μ g. Stored at room temperature)

Salbutamol as Salamol Steri-NEB 2.5mg/.2.5ml

2.. Equipment

DeVilbiss UltraNeb Ultrasonic Nebuliser

Micro Medical Micro Plus hand held spirometer

50ml polypropylene sputum pot

Tissues

Water for mouth rinsing

Beaker for mouth rinse waste

Volumatic spacer for salbutamol delivery

Pipettes and tips

Pari-boy nebuliser

3. Patient preparation before testing

1. Ensure that the patient has read and understands the Sputum Information Sheet.
2. Explain to the patient how the test will be performed and how the patient will be monitored. Patients should be told that they may suffer severe bouts of coughing and that they may experience some minor symptoms such as chest tightness or breathlessness.
3. Describe to the patient the most efficient ways to bring up phlegm. Many subjects find it helpful to lean forward and perform 3 phases of sputum expectoration:
 - a.coughing and “huffing”
 - b.clearing the throat: often nosily: “hawking”
 - c.spitting

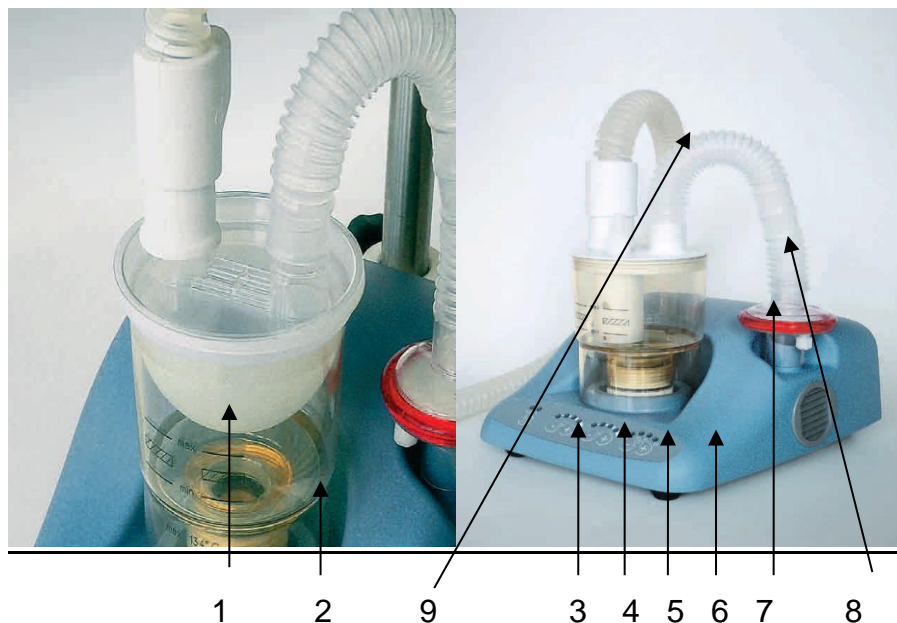
4. Performing the test

1. Patients should be seated comfortably throughout the test, in a chair with back support and arm rests.
2. Three baseline FEV1 measurements are performed and the highest value is recorded as a baseline value and recorded on the Sputum Induction Worksheet.
3. Administer 200µg Salbutamol to the subject via an MDI with a volumatic, 20 minutes prior to saline nebulisation.
4. After 20 minutes resting, the post bronchodilator FEV1 is measured on three occasions and the best value recorded. This value is used to calculate any subsequent fall in FEV1 during the procedure. A 20% drop in this FEV1 is calculated, and used as a safety value for continuation of the procedure. If the FEV1 is <60% predicted post bronchodilator, or the patient is a high risk asthmatic (i.e. severe asthma, exacerbated within the last 4 weeks, is uncontrolled utilising >8puffs of β agonist/day) then the **modified protocol** needs to be followed (see Section 8. below).
5. Determine which protocol is to be used and prepare the saline solutions.
6. To prepare 10mls of saline solutions for nebulisation:

<u>Vol. Stock 7% Saline</u> <u>(mls)</u>	<u>Volume Sterile Water</u> <u>(mls)</u>	<u>Final</u> <u>concentration</u>
7.14	2.86	5%
5.72	4.28	4%
4.28	5.72	3%
1.28	12.72	0.9%

Nebuliser Setup

DeVilbiss UltraNeb Nebuliser setup



1= Medication cup. 10ml of hypertonic saline is placed in here

2=Nebuliser chamber. To be filled between the markings with distilled water.

3=ON/OFF button

4=Aerosol output

5= Air flow rate

6=Timer function

7=Bacterial filter

8=Hosing connection from medication cup to nebuliser

9. Hosing connection from medication cup to patient mouthpiece

1. Pour distilled water into the nebuliser chamber (2) until it reaches about 1/3rd of the way up the medication cup. The distilled water in the chamber is used to transfer vibrations to the medication cup.
2. Put 10ml of the hypertonic saline into the medication cup (1), cover with the lid and place in the nebuliser chamber.

3. Connect the hoses ensuring the hose directly re-connected to the nebuliser (8) has a bacterial filter (7) and the other hose outlet is longer and has a mouthpiece attached (9).
4. Secure the medication cup in position with the clamp.
5. Ensure that the distilled water is changed after use or at least once per day.
6. Connect the device to the power source.
7. To start the nebuliser ensure the power source at the back is switched on (10) and press 0/I button (3) until the green light goes off.
8. Select the aerosol output (4) to the required output (as indicated on the device) by pushing +/- to increase as desired.
9. Select the air flow (5) to the required flow rate (as indicated on the device) by pushing +/- to increase as desired.
NOTE: The output at these settings is ~1.2ml/min.

10. The device is now ready for the patient to start inhaling following one of the 2 protocols below.

7. Standard Protocol for healthy volunteers and stable respiratory

patients with FEV1 >60% predicted

1. Place 5ml of hypertonic saline (3%) into the cup of the nebuliser. The patient is asked to tidal breathe through the nebuliser mouthpiece for 5 minutes after which the induction is then stopped and 3 FEV1 tests are performed, the best being recorded.

2. The patient is then asked to thoroughly rinse their mouth with water (not to swallow) and to blow their nose. The subject is then asked to cough and collect any sputum into the pre-weighed labelled sputum pot (the sputum pot should be correctly labelled with patient's name, DOB and the date and time of collection). ENSURE THAT THE PATIENT IS ALLOWED SOME PRIVACY TO DO THIS.

3. If the subject is unable to produce a sputum sample AND the patients' FEV1 has not fallen $\geq 10\%$ predicted FEV1, steps 1-2 above are then repeated using 5mls of 4% saline instead of 3% saline. If the patients FEV1 has fallen $\geq 10\%$ but $< 20\%$ of the baseline FEV1 then steps 1-2 are repeated using 3% saline.

4. If a sputum sample is not produced following Step 3 then Steps 1-2 are again repeated but using 5% saline instead of 3% saline (if the FEV1 has not fallen by $\geq 10\%$ of the baseline FEV1)

5. The maximum time for nebulised saline inhalation is 15 minutes.

NOTE The induction procedure must be discontinued if the FEV1 falls by $\geq 20\%$ of the post bronchodilator value, significant respiratory symptoms occurs, or if a patient experiences extreme discomfort. Further Salbutamol may be given at any time during or at completion of procedure if lung function has fallen by $\geq 20\%$ post bronchodilator value as discussed in the safety procedures.

6.. Weigh the sputum pot to determine the weight of the sputum.

7. The patient's lung function is measured at completion of the procedure and must be $\leq 5\%$ of the initial baseline before leaving the department.

Or,

8. Modified Protocol for High Risk Patients and Patients with FEV1 < 60% predicted

A high risk patient includes:

- Patients with FEV1 post bronchodilator < 60% predicted
- Patients with uncontrolled asthma (> 8 puffs β agonist/day)
- Patients that have had a recent respiratory exacerbation (< 4 weeks)

IF IN DOUBT, ALWAYS CONSULT WITH THE REFERRING PHYSICIAN/NURSE.

1. Place 5ml of sterile 0.9% saline into the cup of the DeVilbiss UltraNeb nebuliser. The patient is asked to tidal breathe through the nebuliser mouthpiece for 30 seconds. The induction is then stopped and 3 FEV1 tests are performed, the best being recorded.

2. The patient then returns to tidal breathing on the nebuliser for a further 30 seconds and again 3 FEV1 tests are performed as above.

3. Nebulisation continues for a further 3 minutes, a total nebulisation time of 4 minutes stopping after 2 and 4 minutes to perform FEV1 tests as above.

4. Following a total of 4 minutes nebulisation time the subject is asked to thoroughly rinse and gargle with the water, and to blow their nose to reduce saliva contamination. They are then asked to cough and collect any sputum into the sputum pot.

5. Steps 1-4 are repeated but utilising 5ml 3% hypertonic saline. Following this the nebulisation is continued for an additional 4 minutes, to make a total nebulisation time with 3% hypertonic saline 8 minutes.

6. The standard protocol is then followed from Step 6

APPENDIX 2 – PROCESSING A SPUTUM SPECIMEN

Standard Operating Procedure: Processing a Sputum Specimen

1. Introduction

Sputum samples are a useful tool to distinguish eosinophilic from neutrophilic inflammation in patients with a high exhaled nitric oxide measurement. Sputum can be collected from a spontaneously produced sample or induced using standard procedures outlined in SOPCTU101099. Once collected the sputum sample should be refrigerated and processed as soon as possible but within 2 hours of expectoration. Sputum is broken down and cytospin slides are prepared from the resulting cells before being differentially stained and counted on site following procedures in SOPCTU10072014.

IMPORTANT: The Human Tissue Act 2004 states that sputum samples are classified as human material and as such need to be handled and processed under appropriate containment conditions. A Class II microbiological Safety Cabinet must be used to process the sputum sample.

2. Materials

- a) Class 2 Safety hood
- b) Dithiothreitol powder (DTT)
- c) Refrigerator (to store sputum sample, and DTT)
- d) Dulbecco's phosphate buffered saline (D-PBS)
- e) Sterile plasticware (petri-dishes, 15ml centrifuge tubes, 0.5ml eppendorf tubes, pasteur pipettes)
- f) Forceps
- g) Precision balance
- h) Adjustable pipettes and tips
- i) Bench top roller (not essential)
- j) Timer
- k) Plastic funnels and nylon mesh 50µm for filtration
- l) Benchtop centrifuge

- m) Cytocentrifuge, disposable funnels and clasps
- n) Vortexer
- o) Haemocytometer and cover slip
- p) Microscope (x 400 magnifications)
- q) Trypan blue solution
- r) Glass slides

3. Methods

- 3.1 Sputum processing worksheets (see appendix 1) can be found in the red sputum folder located on the shelf in the CTU laboratory. Also in this folder is a sample log to be filled in before each sample is processed.
- 3.2 Turn on the class 2 safety hood. Open the hood door by turning the black handles anticlockwise. Lift the glass screen as far up as it will go and then reclose the door by turning the black handles clockwise. An alarm will sound to remind you to turn the fan on. Press the fan button to switch on followed by the light button to turn the lights on. The hood will begin to initialise airflow and will be safe to use after approximately 1 min.
- 3.3 Inside the class 2 safety hood, empty the entire sputum sample into a clean petri dish. Select sputum plugs, from saliva, using curved forceps and transfer onto the petri dish lid. Gather the sputum plugs into one mass then condense it by moving the entire mass around the lid with small circular motions. *The aim is to spread saliva across the lid but to keep sputum in one mass. The selection procedure and condensation/removal of saliva are important in reducing squamous cell contamination. It is not necessary to select more than approximately 100 milligram of plugs.*
- 3.4 Pre-weigh a clean polypropylene centrifuge tube with screw top. (*Do not use polystyrene tubes as they cause cell adhesion*).
- 3.5 Transfer the concentrated sputum with curved ended forceps to an empty,
- 3.6 Re-weigh the empty sputum pot to determine the weight of the sputum, noting the value on the worksheet.
- 3.7 Subtract the weight of the empty centrifuge tube from the weight of the centrifuge tube plus selected sputum to obtain the weight of sputum portion to be processed. Record this value.
- 3.8 Add 4 times the sputum volume (μl equal to weight in g) of dithiothreitol, freshly diluted 1 in 10 from a stock solution of 1% (i.e. 200mg in 20ml of water stored at 4°C for up to 30 days) using D-PBS.

3.9 Vortex the sample gently for 15 seconds and incubates at room temperature for 15 minutes on a bench top roller. This procedure should homogenise most sputum samples, but there may be some samples in which the mucus is difficult to break down. Consider increasing the temperature during homogenisation and/or extending the time of homogenisation. Avoid foaming of the solution, as this will destroy cells.

3.10 Add an equal volume of D-PBS (i.e. if 2 ml of 0.1% DTT was added to sputum, now add 2 ml D-PBS) to achieve a 9-fold dilution of the sputum plugs and vortex gently for 15 sec.

3.11 Pre-wet a square piece of 50 µm nylon gauze with D-PBS and place in a small plastic funnel. Insert the funnel in to the pre-weighed polypropylene tube and pour the sputum sample through to filter. Discard the gauze and original sample tube but not the funnel (This can be sterilised and re-used)

3.12 Centrifuge the filtered sample at 600 x g for 10 min. at room temperature. Setting 5 labelled 'SPUTUM' on the refrigerated centrifuge.

3.13 Discard the supernatant, carefully to avoid disturbing the cell pellet.

3.14 Add D-PBS to the cell pellet (approximately 200 µl per 100 mg sputum) and gently re-suspend using a pipette. Record the volume of PBS that the pellet is re-suspended in.

3.15 Assess total cell count viability and level of squamous cell contamination using a Neubauer haemocytometer and the trypan blue exclusion method:

3.15.1 Mix 10µl of cell suspension with 10µl of Trypan blue

3.15.2 Secure the glass coverslip over the haemocytometer counting chamber by breathing on the glass chamber before placing the coverslip.

3.15.3 Flood haemocytometer with the cell suspension/trypan blue mix ensuring the counting grid is covered and no air bubbles remain.

3.16 Perform a cell count within 5 minutes. Count all cells in the central field plus the four corner fields of the haemocytometer as indicated in figure 1. Aim to count 100 cells, however if there are 200 cells or more per field, dilute an aliquot of the cell suspension and re-count. Cells touching the top and left lines are counted, cells touching lower and right lines are not counted. Cells are classified as viable (live) leukocytes, dead (blue) leukocytes and squamous (whether viable or not).

3.17 Determine the mean live, dead and squamous cell numbers across all 5 counted fields as directed on the sputum worksheet.

3.18 Calculate the cell concentration per ml ((total no cells counted x 2) x 10 000) as directed by the sputum worksheet.

e.g. mean cell count in 5 large squares is 75, 82, 71, 68 and 85. So sum total = 381 and the mean = $385/5 = 76.2$.

Then cell concentration (cells/ml) = $(76.2 \times 2) \times 10\,000 = 152.4 \times 10\,000 = 1.524 \times 10^6$ cells/ml.

3.19 Calculate the percentage cell viability ((number of live cells x 100) / total number of cells) as directed by the sputum worksheet.

e.g. an average of 21 live cells and 63 dead cells are counted across 5 fields.
So viability (%) = $(21 \times 100) / 84$

3.20 Each of 4 cytopsin slides should contain 30 000 delivered in a 100 μ l suspension. Calculate the volume of cell suspension required to contain 120 000 cells (vol in ml. = $120\,000 / \text{cell suspension}$)

e.g. vol = $120\,000 / 1.524 \times 10^6 = 0.0787401$ ml or 79 μ l.

3.21 Calculate the required volume of D-PBS to be added to make 400 μ l of cell suspension containing 120 000 cells.

e.g. vol. D-PBS = $400 - \text{previous answer in } \mu\text{l} = 400 - 79 = 321 \mu\text{l}$.

3.22 Label 4 glass slides with 1) Patients initial, 2) Casenote number, 3) Date prepared and 4) Referring physician.

3.23 Prepare the apparatus to make 4 cytopsin slides by clamping cytopsin preparation funnels to each of the 4 labelled glass slides using the metal cytopsin clamps.

3.24 Using 100ul of cell suspension per cytopsin, prepare FOUR cytopsin. Centrifuge at 450rpm (18.1 x g) for 6 minutes then air dry the slides for at least 15 minutes at room temperature.

Appendix 1 – Sputum processing worksheet



Respiratory Medicine
Clinical Trials Unit
Academic Medicine
Castle Hill Hospital
Cottingham
HU16 5JG

Sputum Cell Differential

Affix Patient Sticker Here

Date:
Time:
Processed by:
Counted by:

Sample Processing

Time Collected: _____
Time Processed: _____

Empty tube weight (g)	A
Tube + sputum weight (g)	B
Selected sputum weight (g)	C (B-A)

Cell Counts

	Viable Leukocytes	Dead Leukocytes	Squamous Cells	Average leukocyte count	G (=D+E)
Lower left 4x4					
Middle 5x5					
Upper right 4x4					
Lower right 4x4					
Upper left 4x4					
Average	D	E	F		H (=G+F)
				Suspended cell concentration (x 10 ⁶ cells/ml)	I (= [H x 2] x 10 000)

Leukocyte viability (%)	J (= [D x 100] / G)	Squamous cells (%)	K (= F x 100 / H)
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Cytospin Slide Preparation

Volume of cell suspension needed (ml)	L (= 120 000 / I)	Volume of D-PBS to be added (ml)	M (= 0.4 - L)
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Cell Differential Counts

	Eos	Neut	Mac	Lymph	Epi
Absolute count					
% of total					

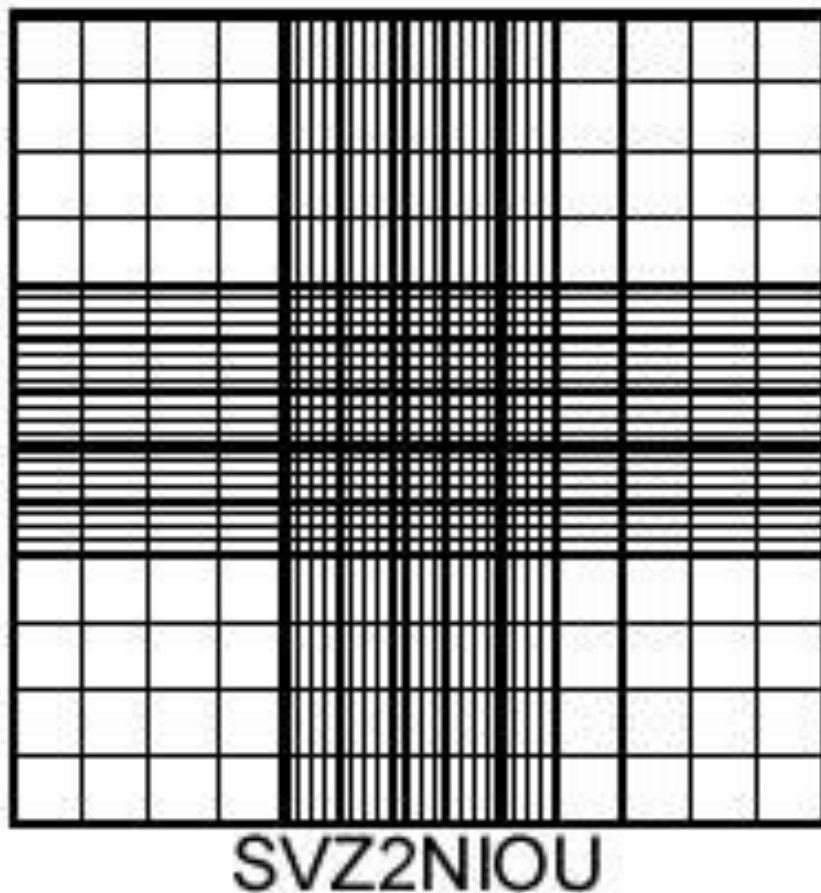


Figure
1 –

Haemocytometer Grid

A haemocytometer grid is divided into 9 large fields. Each field is divided further into 16 smaller squares. The central fields of the first and third rows have vertical lines which further divide the 16 smaller squares. Similarly the central squares of the first and third columns have horizontal lines which further divide the 16 smaller squares. These lines converge within the large central field to divide the 16 smaller squares into a further 16 squares.

When performing cell counts for the purposes of sputum sample processing, cells within the central large field should be counted along with cells in the 4 large corner fields.

Reference

Hayman Y. Processing a Sputum Specimen, 2014, SOP Reference Number CTU100210

**APPENDIX 3 - HULL COUGH HYPERSENSITIVITY
QUESTIONNAIRE**

Name: _____

D.O.B: _____ **UN:** _____

DATE: _____

Please circle the most appropriate response for each question

<p align="center">Within the last MONTH, how did the following problems affect you? 0 = no problem and 5 = severe/frequent problem</p>						
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
The feeling of something dripping down the back of your nose or throat	0	1	2	3	4	5
Retching or vomiting when you cough	0	1	2	3	4	5
Cough on first lying down or bending over	0	1	2	3	4	5
Chest tightness or wheeze when coughing	0	1	2	3	4	5
Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)	0	1	2	3	4	5
A tickle in your throat, or a lump in your throat	0	1	2	3	4	5
Cough with eating (during or soon after meals)	0	1	2	3	4	5
Cough with certain foods	0	1	2	3	4	5
Cough when you get out of bed in the morning	0	1	2	3	4	5
Cough brought on by singing or speaking (for example, on the telephone)	0	1	2	3	4	5
Coughing more when awake rather than asleep	0	1	2	3	4	5
A strange taste in your mouth	0	1	2	3	4	5

TOTAL

SCORE _____ **/70**

APPENDIX 4 - LEICESTER COUGH QUESTIONNAIRE (LCQ)

<p>1 In the last 2 weeks have you had chest or stomach pains as a result of your cough?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>	<p>4 In the last 2 weeks have you felt in control of your cough?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>
<p>2 In the last 2 weeks have you been bothered by sputum (phlegm) production when you cough?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>	<p>5 How often during the last 2 weeks have you felt embarrassed by your coughing?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>
<p>3 In the last 2 weeks have you been tired because of your cough?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <hr/>	<p>6 In the last 2 weeks my cough has made me feel anxious.</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <hr/>

<p>7</p> <p>7</p>	<p>None of the time <input type="text"/></p> <p>In the last 2 weeks my cough has interfered with my job, or other daily tasks.</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p> <p>8</p> <p>In the last 2 weeks I felt that my cough interfered with the overall enjoyment of my life.</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p> <p>9</p> <p>In the last 2 weeks exposure to paints or fumes has made me cough.</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>	<p>7</p> <p>10</p>	<p>None of the time <input type="text"/></p> <p>In the last 2 weeks has your cough disturbed your sleep?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p> <p>11</p> <p>In the last 2 weeks how many times a day have you had coughing bouts?</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p> <p>12</p> <p>In the last 2 weeks my cough has made me feel frustrated.</p> <p>1 All of the time <input type="text"/></p> <p>2 Most of the time <input type="text"/></p> <p>3 A good bit of the time <input type="text"/></p> <p>4 Some of the time <input type="text"/></p> <p>5 A little of the time <input type="text"/></p> <p>6 Hardly any of the time <input type="text"/></p> <p>7 None of the time <input type="text"/></p>
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13 In the last 2 weeks my cough has made me feel fed up.

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time
- 6 Hardly any of the time
- 7 None of the time

14 In the last 2 weeks have you suffered from a hoarse voice as a result of your cough?

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time
- 6 Hardly any of the time
- 7 None of the time

15 In the last 2 weeks have you had a lot of energy?

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time

17 In the last 2 weeks have you been concerned that other people think something is wrong with you because of your cough?

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time
- 6 Hardly any of the time
- 7 None of the time

18 In the last 2 weeks my cough interrupted conversation or telephone calls.

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time
- 6 Hardly any of the time
- 7 None of the time

19 In the last 2 weeks I feel that my cough has annoyed my partner, family or friends.

- 1 All of the time
- 2 Most of the time
- 3 A good bit of the time
- 4 Some of the time
- 5 A little of the time

6 Hardly any of the time

7 None of the time

6 Hardly any of the time

7 None of the time

16 In the last 2 weeks have you worried that your cough may indicate a serious illness?

1 All of the time

2 Most of the time

3 A good bit of the time

4 Some of the time

5 A little of the time

6 Hardly any of the time

7 None of the time

**Leicester
Cough
Questionnaire
(LCQ)**

This questionnaire is designed to assess the impact of cough on various aspects of your life. Read each question carefully and answer by TICKING the response that best applies to you. Please answer ALL questions as honestly as you can.

This questionnaire will remain confidential

APPENDIX 5 – 24 HOURS COUGH COUNTING

Standard Operating Procedure

Title: Digital Cough Recordings using the Marantz PMD620 Solid State Recorder

1. Introduction

This SOP details the procedure to digitally record acoustic events onto a 1GB SD card using a Marantz PMD620 solid state recorder. The recordings are saved as MP3 compression files and may be copied or transferred onto other computers/data storage devices for subsequent audio analysis. The recordings are segmented into 60 minute files for ease of data transfer and analysis, and > 30 hours of recording can be achieved using the recording parameters as described in Section 3.

2. Materials

- a) Marantz PMD620 solid state recorder with attached Audio-Technica cardioid condenser microphone (AT898) with wind cover (Rycote Microphone Windshields Ltd)
- b) 1GB SD card inserted into the recorder
- c) Fully charged Battery Li-Ion Enix battery pack 3.7V 6.8 Ah: Order no. 8809046 Part No. 800040, Farnell and battery charger Enix Li-Ion charger 4.2V 1.3A: Order no 8809054, Farnell)
- d) USB cable
- e) Secure housing with straps.
- f) Marantz Model PMD620 User Guide

3. Methods

3.1 Parameter Settings

- a. Ensure the rechargeable battery is full charged (allow charging time approx 4-6 hours) and the terminal from the battery is securely connected to the terminal from the battery housing within the recording unit.
- b. Ensure a blank 1GB SD card is inserted in to the recorder.
- c. Turn on the recording unit and ensure the recorder has the correct date and time (see page 18 of the User Guide).

- d. Press and hold the **DISPLAY** button for 3 seconds and then press the **VOL+** or **VOL-** button to highlight the **3. PRESET MENU** option.
- e. Press the **ENTER** button and three preset files are presented. Using the **VOL+** or **VOL-** buttons select **PRESET-1** and press **ENTER**.
- f. You will be presented with twenty three (order 1- 23) settings. Scroll up/down to view the settings using the **VOL+** or **VOL-** buttons.
- g. Press **▶▶** or **◀◀** button to tab through the input choices for each setting.
- h. Select the setting selections as detailed in the table below (column d.):

a.Order	b.Setting	c. Choice of setting	d.Select setting
1	Input	Auto/INT MIC/MIC	MIC
2	Rec Format	MP3-H/MP3-M/MP3-L/PCM-16/PCM-24	MP3-M
3	Rec Channel	Stereo/Mono	Mono
4	Input Jack	Stereo/Mono	Mono
5	Sample rate	44.1kHz/48kHz	44.1kHz
6	Manual Track	On/Off	Off
7	Auto Track	Off/1min to 24hour	1 hour
8	Silent Skip	Off/-60dB/-54dB/-38dB/-20dB	Off
9	Level Cont	Manual/ALC	Manual
10	Mic Atten	0dB/-12dB/-24dB	0dB
11	Low Cut	On/Off	On
12	Mic Power	On/Off	On
13	Level LED	-54dB/-38dB/-20dB/-12dB/-6dB	-54dB
14	Skip Back	1sec to 60secs	3secs
15	File Sort	Alphabet or time/date	Time/date
16	Date Form	M/D/Y or D/M/Y	D/M/Y
17	Font Size	Large/Small	Large
18	Auto Power Off	On/Off	Off
19	Battery	Alkaline/Ni-MH	Alkaline
20	Key Lock	All/partial	ALL
21	LED	On/Off	OFF
22	Brightness		1
23	Machine ID		

- i. Press the **DISPLAY/MENU/STORE** button to store the parameter settings in the

Preset-1 file.

3.2 Recording

- a. Press the **REC button** and then set the record level to -10dB using the **REC LEVEL +** or **REC LEVEL –** buttons on the side of the unit.
- b. Slide the **KEY LOCK** switch to lock the keys.
- c. Identify the recording session by speaking into the microphone giving the date, time and subject.
- d. Secure the unit in its housing and place the straps of the unit over the subjects head. Adjust as necessary the unit's straps so that the unit hangs comfortably at the subjects' waist.
- e. Secure the microphone to the subjects' chest clothing approximately 3 – 4 inches below the subjects chin.
- f. Instruct the subject to always have the microphone attached to external clothing and not to cover or mask the microphone.
- g. Fill in a patient log sheet and instruction sheet located in the front of the red cough counting file. This file is kept in the CTU treatment room. Place the instruction sheet in the recorder housing/bag so that the recorder can be identified on its return.
- h. After the recording session, remove the recorder from its secure housing and slide the **KEY LOCK** switch to unlock the keys.
- i. Press the **STOP/CANCEL** button to stop the recording. All audio data is stored on the 1GB SD card. This is the source data and the cards should be securely stored for possible archiving.

3.3 Data Transfer for Analysis

- a. Prior to removing the SD card from the recording unit connect the PMD620 unit to a computer (PC) via the USB cable provided.
- b. Using Windows Explorer on the computer, copy and paste all files from the Marantz PMD620 to a selected directory on the PC or other data storage device.
- c. A one hour recording is approximately 28MB therefore 24 hours recording is <0.7 GB.

4. Additional Information

4.1 Recording level

The MIC ATTENUATION function on the recorder allows the operator to adjust the sensitivity of the microphone (0db, -12dB or -24dB). As such, this function should

be used in conjunction with the RECORD LEVEL function to obtain an optimal recording level. These two parameters should be titrated together depending on the recording environment.

4.2 File size

The AUTO TRACK function allows the operator to determine when a new file begins at specified time intervals (1, 5, 10, 15 or 30 minutes, or every 1, 2, 6, 8, 12 or 24 hours. The time interval will thus determine the file size (see Data Transfer above). Points to consider when determining the file size may include:

- a. Will acoustic analysis be performed on each individual mp3 file or will the files be joined e.g. will 24 hours recording be saved as 24 1 hour files, three 8 hour files or one 24 hour file.
- b. Mp3 files may be 'joined' to create single (or multiple) larger files using the Direct MP3 Joiner software which is on the OPTIPLEX 755 (cough) computer. If files are joined always corroborate that the final file size is the sum of the individual files and that the files are joined in the correct sequence otherwise there may be timing errors.
- c. Leicester Cough Monitor (LCM) software analysis. File size (or 'time size') may affect cough analysis using the LCM. The LCM software incorporates a manual input to distinguish between cough and non-cough events. The software requires up to 80 – 100 events. The software algorithms and models that separate cough vs non-cough are automatically adjusted after the first 20 events and then after each 10 events. When the change in the models is below a threshold, this adjustment stops and the process finishes.

5. Signatories

Reference

Mann V. Digital Cough Recordings using the Marantz PMD620 Solid State Recorder. 2010, SOP Reference Number CTU040610

APPENDIX 6: REFLUX DISEASE QUESTIONNAIRE (RDQ)

Item	Frequency					
	None	Less than one day a week	One day a week	2-3 days a week	4-6 days a week	Daily
Burning behind the breastbone	0	1	2	3	4	5
Pain behind the breastbone	0	1	2	3	4	5
Acid taste in the mouth	0	1	2	3	4	5
Unpleasant movement of material upwards from the stomach	0	1	2	3	4	5
Epigastric burning	0	1	2	3	4	5
Epigastric pain	0	1	2	3	4	5

Item	Severity					
	None	Very mild	Mild	Moderate	Moderately severe	Severe
Burning behind the breastbone	0	1	2	3	4	5
Pain behind the breastbone	0	1	2	3	4	5
Acid taste in the mouth	0	1	2	3	4	5
Unpleasant movement of material upwards from the stomach	0	1	2	3	4	5
Epigastric burning	0	1	2	3	4	5
Epigastric pain	0	1	2	3	4	5

APPENDIX 7: STRESS VISUAL ANALOGUE SCALE (VAS)

Do you feel stressed?



Not at all

Extremely

ABBREVIATIONS AND DEFINITION OF TERMS

° C	degrees Celsius
ACE	Angiotensin Converting Enzymes
ADR	adverse drug reaction
Ah	Ampere-hour
AL	Aluminium
AE	adverse event
ATS/ERS	American Thoracic Society/European Respiratory Society
ppb	parts-per-billion
CRF	Case Report Form(s)
CTU	Clinical Trials Unit
COPD	Chronic Obstructive Pulmonary Disease
dB	Decibel
DPBS	Dulbecco's phosphate buffered saline
DSUR	Development Safety Update Report
DCC	Differential Cell Count
DTT	Dithiothreitol powder
EC	Ethics committee
ECG	Electrocardiogram
EER	Extra oesophageal reflux
FeNO	Fractional exhaled Nitric Oxide
FEV1	forced expiratory volume in one second
FVC	forced vital capacity
g	gram
GCP	Good Clinical Practice (Guidelines)
GERD	Gastro-oesophageal reflux disease
GMP	Good Manufacturing Practices
GP	General Practitioner
HACC	Hull Automated Cough Counts
HARQ	Health Assessment Questionnaire
HEENT	Head ears, eyes, nose , throat
HEY	Hull and East Yorkshire Hospitals
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
ID	Identification
IMP	Investigational Medicinal Product
ITT	Intention to Treat
KHz	kilohertz
LCQ	Leicester Cough Questionnaire
LPR	Laryngopharyngeal Reflux
mg	Milligram
MHRA	Medicines and Healthcare products Regulatory Agency
µl	Microlitre
µm	Micrometer
ml	Millilitre
n	Number
NO	Nitric Oxide
OPA	Oriented Polyamide
OSAC	Oral Steroids for Acute Cough
PE	Physical Examination

PFTs	Pulmonary function tests
PP	Per-Protocol
PVC	polyvinyl Chloride
QC	Quality Control
REB	Research Ethics Board
REC	Research Ethics Committee
RDQ	Reflux Disease Questionnaire
rpm	Revolutions per minute
SAR	Serious adverse reaction
SAS	Safety Analysis Set
SAE	Serious adverse event
sec	second
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
TMF	Trial Master File
TCC	Total Cell Count
v	volts
VOL	Volume