‘An Investigation into the Mechanism of Inhalational Cough Challenge’

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Abstract

Chronic cough is a common problem. Historically treatments have focussed on treating underlying physiological causes. More recently an overarching theory of cough hypersensitivity syndrome has developed. In-vitro models of cough have not successfully translated into human studies. Testing the cough reflex in humans via inhalational cough challenge has been utilised since the 1950s. The mechanisms of cough challenge are poorly understood. This thesis sets out to investigate these mechanisms further in three different experiments. By altering pH in a citric acid challenge and measuring cough response, I show that cough hypersensitivity is not due purely to a shift in the dose response curve to pH, but also an alteration in the pattern of response to a given stimulus. Designing a cough challenge with a novel agent (ATP) revealed that the cough response to ATP is clearly delineated from that of AMP. The response to ATP in chronic cough is heightened, but not to such a degree as to implicate the acute response to inhalation of ATP in the pathophysiology of cough hypersensitivity syndrome. Comparing four cough challenges – the commonly used citric acid and capsaicin; the slightly less utilised distilled water fog challenge; and the new ATP challenge – proved that all challenges show less intra-patient reproducibility in chronic cough patients. Inhaled ATP cough challenge responses correlated with citric acid and capsaicin challenge suggesting overlap in mode of action. All experiments explore the cough challenge further in a group who have had little previous cough challenge investigation: the patient with chronic cough. They reveal that patients with cough hypersensitivity syndrome have not only a heightened but an unpredictable cough reflex, and that this is not due solely to upregulation of the cough receptors at peripheral nerve endings. Inhalational cough challenge plays an important role in further elucidating the mechanisms of chronic cough.

List of Contents

1 List of Tables and Illustrations

Tables

Illustrations

2 Acknowledgements

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3 Dedication

For Bunty and John, George and Carys (Nanna and Grandpa, Bampy and Gu).

4 Author's 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 (are) 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 confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

5 List of Publications Arising from this Thesis

5.1 Presentations and Abstracts

ATP Cough Challenge in Healthy Volunteers; H.E. Fowles, A.H. Morice; Presented at Fifth American Cough Conference June 2015 – winner of John Widdicombe prize for best abstract. Published in 'Abstracts from the Fifth American Cough Conference' February 2016 *Lung 194(1) pp1-7* F. McCool

Inhalational Cough Challenges; H Fowles, A Morice; Won SpR Presentation prize for best Abstract Welsh Thoracic Society Autumn meeting 2015

S89 Hypersensitivity to Adenosine Triphosphate in Chronic Cough Patients; Dec 2015; *Thorax*; Fowles HE, Morice A.

P243 Assessing the Effect of pH on Citric Acid Cough Challenges in Chronic Cough Patients and Healthy Volunteers; Dec 2015; *Thorax 70(Suppl 3):A199.1-A199*; Z Rai, H Fowles, J Howard, C Wright, A Morice.

ATP: A novel Cough Challenge Agent; Poster Presentation at Allam Lecture (CCMR at HYMS); 2016

ATP Cough Challenge; 3 Minute Thesis presentation at Hull York Medical school Post-Graduate Conference 2016

S31 Reproducibility of four challenge modalities for chronic cough; Dec 2016; *Thorax 71(Suppl 3):A19-A20;* L Douglas, H Fowles, K Arnell, S Thackray-Nocera, A Morice.

5.2 Publications

Tussive challenge with ATP and AMP: does it reveal cough hypersensitivity?; Feb 2017; *European Respiratory Journal 8;49(2)* Fowles HE, Rowland T, Wright C, Morice A.

ATP and cough reflex hypersensitivity: a confusion of goals?; Jul 2017; *European Respiratory Journal;* Fowles HE, Rowland T, Wright C, Morice A.

The effect of pH on citric acid cough challenge: A randomised control trial in chronic cough and healthy volunteers. March 2018; Respir Physiol Neurobiol; Rai ZL, Fowles HE, Wright C, Howard J, Morice AH. [Epub ahead of print]

6 Introduction

6.1 Cough – clinical aspects

The Oxford English Dictionary gives the following definition of the verb 'to cough':

> *To expel the air from the lungs with a more or less violent effort and characteristic noise, produced by the abrupt forcible opening of the previously closed glottis; usually in order to remove something that obstructs or irritates the air-passages.* (Dictionary)

The reflex of cough is a protective mechanism in humans and other mammals, aimed at protecting the airway from foreign matter and clearing the mucus produced by the airways.

It is normal to cough on average about 20 times a day (Yousaf et al, 2013). When coughing becomes more frequent than this, awareness of cough is increased and patients may seek treatment or advice from their healthcare provider. Approximately four and a half million patients attend their GPs complaining of the symptom of cough annually in the UK (Morice et al, 2001). Over £100 million is spent annually in the UK on over the counter cough remedies.

Acute cough is very common, is defined as lasting less than three weeks, and commonly results from viral upper respiratory tract infection which is usually self-limiting (Morice et al, 2006).

A chronic cough is usually defined as a cough that persists for more than eight weeks. This is, however, an arbitrary definition which whilst agreed in both the American and European guidelines does vary somewhat in cough research literature. Most cases of chronic cough referred to secondary care in the UK have persisted for much longer than eight weeks (Irwin et al, 2006; Morice et al, 2004; Morice et al, 2006).

The prevalence of chronic cough is difficult to estimate, and suggested figures vary wildly from 3-40% (Cullinan, 1992; Ford et al, 2006; Fuller & Jackson, 1990; Janson et al, 2001; Wynder et al, 1965). These variations probably reflect the variation in the specific question asked in different prevalence studies. A recent comprehensive literature review of 90 studies found the overall global prevalence of chronic cough to be 9.6%. The most common time definition used was greater than three months, rather than the guideline stated time of more than eight weeks. There were regional differences in chronic cough with prevalence being higher in Oceania, Europe and America than in Asia and Africa.

The authors speculate that this may be due to environmental factors or comorbidities, such as obesity, but recognise that the majority of the studies considered were carried out in Europe (Song et al, 2015).

Chronic cough seems to be twice as common in women compared to men, and more prevalent in post-menopausal women (Janson et al, 2001). Cough frequency is also higher in female healthy volunteers and respiratory patients (Kelsall et al, 2009; Yousaf et al, 2013). Women have been shown to have increased sensitivity of their cough reflex (Kastelik et al, 2002), and functional MRI studies suggest that their 'sensory cough centre' is more pronounced (Morice et al, 2014a).

Tobacco smokers have a higher prevalence of chronic cough than non-smokers, and this effect is dose-related (Ford et al, 2006; Janson et al, 2001). Smokers, however, seem to be less likely to present to healthcare providers complaining of cough, which may be due to a cultural acceptance that smoking causes cough (Everett et al, 2007). Interestingly, nicotine delivered either by cigarette or by e-cigarette has been shown to suppress the cough reflex and smokers have a lower cough sensitivity than normal subjects. Smoking cessation removes this inhibition and explains the frequent observation of patients complaining of increased cough in the month or two after quitting (Dicpinigaitis et al, 2006).

Obesity has been shown to be a risk factor for cough (Ford et al, 2006), and the rising prevalence of obesity in the United States and Europe may account for the increased prevalence in these areas. With rising levels of obesity in the United Kingdom, chronic cough is predicted to become an even greater cause of morbidity.

Other risk factors for chronic cough include an underlying diagnosis of asthma, living in an area with higher particulate matter and pollution, symptoms of gastro-oesophageal reflux, and irritable bowel syndrome (Morice et al, 2006).

Whilst it could be argued that a chronic cough is unlikely to be associated with an increased mortality and therefore is a nuisance rather than a disease, patients suffering from this condition are likely to disagree. Chronic cough has been found in a number of quality of life surveys to often be debilitating and significantly impact patient's work and social life (Chamberlain et al, 2015; Everett et al, 2007). Some patients may have to cease employment due to their cough, particularly those who rely on talking. Many patients will report that they avoid public gatherings such as concerts and the theatre for fear of having

a coughing attack. The fact that coughing is culturally associated with a risk of infection also tends to limit patient's social activities (from personal discussion with patients).

With targeted treatment, many patients see improvement in their cough. However, in a small group of patients, no treatment appears to be successful and the cough may be lifelong (McGarvey et al, 1998).

Physical complications of persistent coughing include incontinence (with urinary incontinence in the female cohort being very common), and uterine prolapse is also seen in chronic cough patients. Severe coughing bouts often lead to musculoskeletal chest wall pain or even rib fractures, vomiting and cough syncope. Syncopal episodes precipitated by coughing are likely to be due to the elevated intrathoracic pressures caused by a coughing bout. Typical patients who report episodes of cough syncope are males in their middle ages who are overweight or large-framed with obstructive airways disease. Elimination of cough will resolve the syncopal episodes (Dicpinigaitis et al, 2014).

6.1.1 Causes of chronic cough

A variety of respiratory diseases can present with the symptom of cough. These include asthma, COPD, bronchiectasis and idiopathic pulmonary fibrosis.

Patients who are referred to specialist cough clinics with persistent cough are typically middle aged female non-smokers who have no apparent underlying respiratory disease. In these cases, traditionally the causes of cough considered have been GORD, cough variant asthma and post nasal drip. However, more recently Cough Hypersensitivity Syndrome has been agreed as the over-arching diagnosis by key opinion leaders in respiratory medicine (Morice et al, 2014b).

6.1.1.1 Reflux

Gastro-oesophageal reflux disease is a widely accepted cause of chronic cough (Irwin et al, 1993; Irwin & Richter, 2000; Irwin et al, 1989). It accounts for between 5-41% of chronic cough (Kastelik et al, 2005; Morice et al, 2006; Palombini et al, 1999). This wide variation in reported incidence is probably due to the lack of recognition of its existence as a cause of cough in non-specialist clinics, highlighting again the importance of thinking 'outside the lung' when managing chronic cough.

More recently it has become clear that GORD is an inaccurate descriptor for the reflux that causes cough, and that many patients do not have features such as heartburn and indigestion, typical of acid related liquid reflux. This was first recognised in the ENT

world where the term laryngopharyngeal reflux was coined (Koufman et al, 1996). Because of the loss of voice it was also called silent reflux, hardly an appropriate term if it is causing a noisy cough. The term airway reflux has been coined to describe this phenomenon. Controlling the acidity of the refluxate does not appear to consistently treat the cough (Kilduff et al, 2014).

Patients often provide a classical history with coughing occurring at peak times of reflux and lower oesophageal sphincter relaxation (after meals, on rising from bed, on bending over) (Morice et al, 2011).

There are two proposed mechanisms whereby this 'non-acid' reflux precipitates coughing. Firstly, that micro-aspiration of oesophageal contents into the larynx and lungs occurs, leading to irritation of vagus nerve endings in these areas (Patterson et al, 2009). Secondly, that reflux into the oesophagus itself stimulates a vagal reflex which leads to cough (Ing, 1997; McGarvey & Ing, 2004; Woodcock et al, 2017). It is probable that these are both true. A further mechanism has been postulated in that, because of the hypersensitivity, spasm or dysmotility of the oesophagus may lead to the urge to cough via an aberrant or 'referred' sensation (Kastelik et al, 2003). Thus, reflux may not be a pre-requisite for production of the symptom by the oesophageal sensory nerves.

6.1.1.2 Eosinophilic cough syndromes

A number of patients with chronic cough appear to have a cough that is responsive to steroid treatment. This often shows some features of overlap with asthma in the form of nocturnal cough, airway hyper-responsiveness on methacholine challenge testing, and positive markers of eosinophilic airways inflammation (blood eosinophilia, sputum eosinophilia, or raised exhaled nitric oxide). However there seems to be marked variation in the airways hyper-responsiveness in such patients, which has led to a variety of diagnostic labels being applied including cough variant asthma, atopic cough and eosinophilic bronchitis (Chung & Pavord, 2008; Corrao et al, 1979). More recently it has been posited that these are all variations on a single clinical syndrome, which can be expressed in lay terms as 'asthmatic cough'. Thus, classic asthma includes variable bronchoconstriction, bronchial hyper-responsiveness and sputum eosinophilia; cough variant asthma does not exhibit bronchoconstriction; and eosinophilic bronchitis is only characterised by sputum eosinophilia. All respond to steroid treatment, although perhaps less well than in classic asthma. The 'asthma-like' cough syndromes account for about 20% of referrals to cough clinics (Irwin & Madison, 2000).

Recent evidence provides an explanation for the diverse nature of these asthmatic cough syndromes. Unlike the classic asthma of childhood, which is mediated through allergic adaptive immunity, the main trigger in the older coughing patient is through the innate immune system. Epithelial damage caused by reflux, infection and pollution causes the release of interleukin 33 which then activates the innate lymphocyte type 2. This cell releases IL5 and IL13 calling in the eosinophil. Thus there is no need to invoke allergy in this 'allergic' response (Woodcock et al, 2017).

6.1.1.3 Post nasal drip

Whilst widely described as a cause of cough, post nasal drip has been the subject of some debate. The prevalence of post nasal drip also appears to vary, with much higher incidence in the United States, suggesting a possible cultural aspect (Irwin et al, 1981). This also means that it features much more heavily in the United States guidelines than the European ones. The US guidelines now refer to the existence of nasal stuffiness, sinusitis, or the sensation of secretions draining into the posterior pharynx from the nose or sinuses in association with cough as the Upper Airways Cough Syndrome (UACS) (Irwin et al, 2006). In my opinion, whilst rhinitis is associated with chronic cough, there are many patients who have rhinitis, post nasal drip or sinus disease without cough and the association remains dubious. Reflux of gaseous non-acid refluxate throughout the airways, including the nose seems a much more likely aetiology (Morice, 2004; Morice et al, 2004).

6.1.1.4 ACE-inhibitor induced cough

The association between ACE-inhibitors and cough is well established (Morice et al, 1987). ACE-inhibitors lead to increased cough sensitivity. In some patients, this sensitivity is sufficient to reveal previously sub-clinical irritation, to produce a clinically noticeable persistent cough. Stopping the ACE-inhibitor usually leads to resolution of the cough, although it may require many months for the cough sensitivity to reset.

6.1.1.5 Auricular nerve stimulation

Irritation of the auricular branch of the vagal nerve by a substance in the external acoustic meatus can stimulate cough. Removal of the irritant (cerumen, foreign body or a hair) should have effect within a few days. In some patients, sensitivity of the auricular branch can be associated with other vagal nerve dysfunction (Ryan et al, 2014).

6.1.1.6 Other clinical conditions associated with isolated chronic cough

Patients with a congenital trans-oesophageal fistula (TOF)/ oesophageal atresia with subsequent repair are often left with a dysfunctional oesophagus. These patients often

present with a typical cough or bronchiectasis due to recurrent aspiration – the 'TOF-cough' (Love & Morice, 2012).

Associations between chronic cough and various neurological conditions have been described. These include motor, sensory and autonomic neuropathies such as Holmes-Adie Syndrome, and Hereditary Sensory Neuropathy Type 1. These associations support the role of an abnormality in the autonomic nervous system as a cause of chronic cough (Karur et al, 2012).

6.1.1.7 Linking these Phenotypes together

An overarching theory for the stimulation of cough in all of these patients has been labelled 'Cough Hypersensitivity Syndrome'. Some of the above conditions appear to lead to damage within the airways, which in turn is postulated to the production of pro-inflammatory components which then sensitise the airways and lead to plasticity of the afferent nerves of cough, separately to the nerves that lead to bronchoconstriction (Fujimura et al, 1992b). Some of the conditions associated with cough are neurological conditions in themselves. It could be debated whether oesophageal dysmotility falls into this category. Other conditions (such as COPD) which involve cough have been seen to have different neurophenotypes (Belvisi et al, 2016). The theory has therefore developed that all of these conditions lead to a change in the nerves of the cough reflex and the term Cough Hyersensitivity was coined.

6.1.1.8 Hypersensitivity cough

The concept of cough hypersensitivity is key to the understanding of both acute and chronic cough. Objective testing in a wide range of cough syndromes has demonstrated increased sensitivity when the cough receptors (outlined in more detail below) are challenged by inhalation of protussive agents. Patients with excessive cough can be provoked by minimal stimulation which in the normal subject would not lead to the urge to cough. It is clear that this hypersensitivity does not arise purely from the upregulation of cough receptors, since specific drugs blocking these receptors have no important effect in clinical cough (Khalid et al, 2014). Recently mediators such as ATP have been suggested to 'irritate' the afferent nerves, leading to a syndrome of cough hypersensitivity akin to that of neuropathic pain (Morice et al, 2014b). Another hypothesis is that a normal stimuli (such as liquid reaching the pharynx) in a chronic cough patient can produce an exaggerated response which could potentially be due to up-regulation or over production of the cough receptors in this individual. A more central nervous hypothesis for the

mechanism of cough hypersensitivity would suggest that in some patients their cough response is permanently 'switched on' and appears to respond to no stimuli at all. Yet a fourth hypothesis has been suggested in that it is possible that it is the inhibitory neural pathways that are abnormal and patients with chronic cough are unable to exert the level of voluntary control over their cough reflex as seen in Healthy volunteers. (Chung, 2014; Undem et al, 2015)

The concept of cough hypersensitivity syndrome helps to explain why some patients with other respiratory conditions present with a cough which is resistant to therapies for that condition. The cough thus represents a separate disease of cough hypersensitivity which is associated rather than directly caused by, for example, asthma.

6.2 The cough reflex

Like any other reflex in the body the cough reflex is made up of an afferent arc (the vagus nerve) and an efferent arc – the nerves supplying the inspiratory and expiratory respiratory muscles.

The nerves that appear to be implicated in the afferent limb of the cough reflex are myelinated a-delta fibres (sometimes also referred to as Rapidly Activated Receptors or RARs), and non-myelinated C-fibres of the vagal nerve (Karlsson, 1996). The involvement of these nerves in cough is better established in animals than in humans, although recent studies suggest that similar entities do exist in humans (West et al, 2015).

The receptors involved in signalling at these afferent nerve endings are of interest in targets for therapies for chronic cough hypersensitivity syndrome, as well as potential diagnostic tests.

This is also an area of potential interest as patients often describe their cough as being stimulated by exposure to extremes of temperature, perfumes and other scented products (Johansson et al, 2002).

Whilst coughing is often unavoidable when certain stimuli are introduced, the cough reflex in humans is under a considerable degree of voluntary control. Simply instructing patients with an acute cough not to cough can reduce their coughing levels (Hutchings et al, 1993). This cortical influence leads to a high placebo effect in antitussive trials, making

study of antitussives difficult. In addition, it means that some therapies which show good effect in animal studies show very little effect in human trials (Khalid et al, 2014).

It would be incorrect, however to suggest that chronic cough is 'all in the mind'. Psychogenic cough (a form of Tourette's syndrome) is very rare in adults.

6.3 Receptors involved in cough

6.3.1 TRPV1

The vanilloid receptor 1 (VR1), now more commonly known as TRPV1, was first cloned in 1997 (Caterina et al, 1997). Like other TRP channels, it is a non-selective cation channel which responds to external stimuli and can open to allow an influx of ions, causing depolarisation of the cell it is located on (Bevan et al, 2014). It is activated by capsaicin (found in chilli peppers) and in fact, this is how it was discovered. Capsaicin had been used in studies of pain and nociceptor response for some time prior to this (Caterina et al, 1997).

The TRPV1 receptors were the first described as a potential 'cough receptor'. Similarly to their response to pain, they produce a tussive response to inhalation of heat and irritant substances such as capsaicin and acids (Caterina et al, 1997). They also appear to be implicated in cough response to endogenous agents such as prostacyclins and bradykinins (Grace et al, 2012).

Inhalation of capsaicin provokes a reliable cough response which has been utilised to produce a cough challenge (Dicpinigaitis, 2003; Fuller, 1991; Morice, 1996).

6.3.2 TRPA1

Another member of the cation channel TRP family, TRPA1 has been implicated in the cough reflex. Experimentation in vitro and in vivo in humans and guinea pigs has shown that the TRPA1 agonists stimulate vagal nerves (Birrell et al, 2009). The TRPA1 agonist cinnamaldehyde provokes a cough response in healthy volunteers and appears to do so independently of other cough challenge stimuli (Birrell et al, 2009).

A number of other substances stimulate TRPA1 receptors in vitro, although these have not yet been studied in vivo. These include a number of substances that patients commonly describe as provoking their cough, such as smoke, perfumes and other strong smells (Birrell et al, 2009).

Like TRPV1, TRPA1 appears to be stimulated by endogenous as well as exogenous stimuli (Grace et al, 2012). In experimentation utilising anaesthetised guinea pigs, TRPA1 is also stimulated by citric acid (Mukhopadhyay et al, 2014).

6.3.3 Acid Sensing Ion Channels (ASICs)

Another commonly used cough challenge substance is citric acid. Whilst TRPV1 seems to respond to citric acid, there also seems to be a cough response to citric acid even when TRPV1 is blocked, suggesting the presence of other acid sensing channels (Kollarik et al, 2007). However, other TRP channels also appear to be stimulated by inhalation of citric acid, and blocking ASICs in guinea pigs does not seem to reduce the tussive effect of citric acid (Canning et al, 2006).

6.3.4 Other TRP receptors

TRPM8 receptors respond to cold temperatures and menthol, and have been implicated in the cough response given the antitussive effects of menthol (Yu et al, 2015). All of the above mentioned TRP receptors are also sensitive to change in temperature. The archetypal TRPV1 is a 'hot' receptor, explaining capsaicin's oral sensation when in chilli peppers. This also explains why patients frequently complain of their cough being precipitated by a change in atmosphere.

There are many other receptors which appear to be implicated in the cough response including voltage-gated sodium channels, acid sensing receptors and other TRP classes such as TRPV4 (Bonvini et al, 2016).

6.3.5 Purinergic receptors and ATP

Recently the demonstration that blockade of ATP-preferring purinergic receptors led to a marked reduction in cough frequency in chronic cough (Abdulqawi et al, 2015) suggested that ATP may be a key mediator of cough hypersensitivity, and thus ATP challenge may differentiate between a normal cough reflex and cough hypersensitivity.

The theory of ATP as an extracellular signalling molecule was first proposed in the 1970s (Burnstock, 1972) but was not widely accepted until the first purinergic receptors were cloned in the 1990s. Receptors responsive to ATP have been found within the lungs and have been implicated in the pathogenesis of a number of respiratory diseases including Asthma and COPD.

P2X3 receptors are therefore a viable candidate for cough receptors.

6.4 Inhalational challenges

Many different inhaled substances have been used with an aim to stimulate cough in humans. The most commonly used are citric acid, capsaicin and fog challenge.

A consistent, reproducible cough challenge is useful for assessing the antitussive properties of therapies but the various cough challenges developed have also provided information about epidemiology, aetiology and pathology of chronic cough. This includes supporting the involvement of the aforementioned receptors TRPV1 and TRPA1 in the cough reflex.

6.4.1 Citric acid challenge

The citric acid tussive challenge has been used to assess the cough reflex in humans for over 50 years. It was first described by Bickerman and Barach in 1954 (Bickerman & Barach, 1954). Since then the technique has been used in a number of different settings.

Quite how inhaling nebulised citric acid leads to the initiation of the cough reflex is not fully understood. It does seem to cause some agonistic effect at both TRP receptors and non-capsaicin responsive receptors (Canning et al, 2006). However, the lack of correlation between the capsaicin and citric acid challenges (Wong et al, 1999) would suggest that citric acid is working via a different mechanism to capsaicin (Canning et al, 2006).

It is thought that some of the tussive effect is due to its weak acidity and the protons it carries, and this is borne out by work comparing it with other acids which seem to have similar tussive effects. The suggestion is that the pH of Citric acid is an important factor rather than its molecular structure as citrate (Wong et al, 1999).

6.4.2 Capsaicin challenge

It is often claimed by advocates of the capsaicin cough challenge, that whilst the citric acid challenge has been in use the longest, it is the capsaicin challenge that is the most commonly used cough challenge. This is borne out by a simple PubMed search performed on 19th May 2016 by this author (limited to English language, human studies and clinical trials) of the terms ‘capsaicin’ and ‘cough’ which produced 108 results. When the same tactic was employed with citric acid as a search term in place of capsaicin there were 55 hits; fog or distilled water produced 22 papers. From this one can gather that certainly the capsaicin challenge seems to have produced the most published outcomes, although it is

possible that unpublished cough challenges of other substances are taking place, and this method of gathering data did not include other acidic challenges such as tartaric acid.

Capsaicin as a cough challenge was first employed in 1984, when a one-minute continuous nebulisation with tidal breathing of capsaicin was found to provoke a dose dependent cough response in all 15 volunteers in the initial study.(Collier & Fuller, 1984)

Unlike citric acid, which is readily soluble in normal saline at tussive concentrations, capsaicin requires the addition of other solvents such as DMSO (Tween 80) (Collier & Fuller, 1984) and ethanol (Midgren et al, 1992). This substance can then be diluted with normal saline. However, it is noted that in the initial studies of capsaicin, the DMSO solvent alone provoked a cough response in 2 out of 15 healthy volunteers.

As with other cough challenges there is some debate about which is the best method of nebulisation (single breath dosimeter versus tidal breathing for a fixed period of time). This debate is further contributed to by the fact that slightly higher doses of capsaicin are required with the single breath method. These higher doses mean that about 1 in 5 patients experience a 'burning' sensation in the throat with this method. It is suggested that this is less of a problem with the tidal-breathing method (Nejla et al, 2000).

As well as being less tolerable for the patient, the effect of the higher doses required for the dosimeter single breath method can mean that patients don't complete their inhalation through the length of the dosimeter administration. This in turn leads to the incorrect dose being administered and may lead to a falsely high end point (Dicpinigaitis, 2003).

6.4.3 Fog challenge

An observation that fog in London in the 1960s seemed to lead to increased episodes of bronchoconstriction in patients with respiratory disease led to the further investigation of nebulised fog as an inhaled challenge.

Following the initial publication of two papers using ultrasonically nebulised distilled water (commonly referred to as 'Fog') to cause bronchoconstriction (Abernethy, 1968; Cheney & Butler, 1968) it continued to be used as a method to induce airways hyper-reactivity.

Fog was also noted to cause cough in both bronchial hyper-reactive patients and healthy volunteers but initially tended to be less widely used to measure cough. Cough is however not dependent on the bronchoconstriction as it has been demonstrated that inhibiting the

bronchoconstriction does not inhibit the cough (Fuller & Collier, 1984; Sheppard et al, 1983).

Fog challenges have been utilised as a method of measuring the antitussive effect of a variety of pharmacological agents, including diuretics (Foresi et al, 1996; Lowry et al, 1988a; Stone et al, 1993a; Stone et al, 1993b; Tanaka et al, 1996).

Much of the experimentation involving a fog cough challenge has focussed on investigating the fact that inhalation of normal saline doesn't reliably produce cough, suggesting that there is something about the hypo-osmolarity of the distilled water that stimulates cough.

Investigators appear to be looking to answer the questions: Is it the water causing the cells to swell and provoking a mechanical response? Or is lack of chloride ions provoking a shift in chloride out of cells leading to a shift in other ions and generation of an action potential?

Varying the osmolarity of the solution does not appear to change the tussive response. Cough response seems to be similar unless the concentration of chloride ions is altered (Godden et al, 1986). This observation is, however, based on a very small number of participants, all of whom were healthy volunteers.

Other investigators have found that cough is stimulated by inhalation of solutions with low chloride concentration, extremes of pH and very high osmolarity (Lowry et al, 1988b).

It is generally accepted that the absence of permeant anions in an ultrasonically nebulised, distilled water (Fog) challenge causes cough (Eschenbacher et al, 1984).

5-20% of healthy subjects do not cough with fog. This does not seem to be the same group that don't cough with other cough challenges, but is purely an observational statement from the authors (Lowry et al, 1987).

6.4.4 ATP challenge

ATP challenges have not previously been carried out specifically looking to objectively measure cough; however, a number of challenge experiments have been performed considering bronchoconstriction with ATP and AMP (as an adenosine substitute given its better solubility).

Inhalation challenges using ATP and AMP in COPD patients, smokers and healthy volunteers found that ATP appeared to cause increased breathlessness and cough compared to AMP. The Borg score difference was only significant in the COPD patients – there was no comment on any significant difference between groups with cough (Basoglu et al, 2015).

Inhalation challenges using ATP and AMP in asthmatics versus healthy volunteers found that there was more of a distinction between the two substances in cough symptoms and throat irritation in the healthy volunteers than there was in the asthmatics. The cough symptoms were recorded before and 30 minutes after the challenge (Basoglu et al, 2005). ATP also caused greater bronchoconstriction in the asthmatics.

ATP caused bronchoconstriction in both healthy and asthmatic volunteers, but more so in the asthmatics (Pellegrino et al, 1996).

6.5 Designing an inhalational cough challenge

There are a number of factors to be considered when designing an inhalational cough challenge. Previous studies, particularly those involving citric acid and capsaicin challenges, have highlighted or clarified a number of these considerations.

Capsaicin and citric acid both have accepted ERS standards for cough challenge administration.

6.5.1 Diurnal variation

One study claims that the concentration of citric acid that leads to cough is higher in the morning than in the afternoon (Pounsford & Saunders, **1985**). This has not been replicated since but may need to be considered when designing a novel cough challenge.

6.5.2 Substrate factors

Substances for cough challenges need to be able to be nebulised as liquid, and ideally diluted if a dose response is to be assessed. The choice of diluent may affect the cough response particularly in chronic cough patients. The stability in solution of the substances in question also needs to be considered.

6.5.3 Patient factors

Some experimenters discuss the need to control the variation in the flow rate at which a subject inhales the nebulised challenge substance on the cough response. However, a study considering the effect of different inhalation flow rates found that significant differences in cough response were only found at very wide variations in inspiratory flow rates (Barros et al, 1990; Barros et al, 1991). Therefore, whilst some consideration needs to be given to this, very strict control appears to be unnecessary.

Some studies also utilise cohorts of volunteers and patients who have been 'trained' in how to undertake a cough challenge (Belcher & Rees, 1986; Bickerman et al, 1956; Dilworth et al, 1990). It may be worth considering that participants seem to demonstrate a learning effect. However, this is often the case in any test which requires an element of patient cooperation, the immediate one springing to mind in a respiratory setting being the 'Six-Minute Walk' test.

Some patients appear not to cough at any concentration of the commonly used challenge substances. In experiments particularly measuring the antitussive effects of medications, this issue has been bypassed by pre-screening volunteers and excluding those without a cough response to the challenge being used. It has become generally accepted, that in order to measure tussive effect, participants who have a baseline cough response need to be pre-selected (Empey et al, 1979; Foresi et al, 1996).

Results of cough challenges may be further confounded by the fact that cough does appear to have some degree of voluntary control (Hegland et al, 2011; Hutchings et al, 1993; Young et al, 2009). There is some evidence that giving the cough challenges in a random order or interspersing the substance with saline 'placebo' challenges provides additional blinding of the patient (Wright et al, 2010). However, as often the patient is aware of other sensations such as taste and laryngeal irritation other than cough with each inhalation, the actual true 'blinding' effect is debatable.

Tachyphylaxis is also an issue with repeated challenges. In the tidal breathing method of capsaicin inhalation, cough started during or after the first or second inhalation of the threshold dose of capsaicin, then diminished over the first 30 seconds (Midgren et al, 1992). The cough reflex appears to adapt after first exposure to challenge substances such as capsaicin, distilled water and citric acid. Acute, long-term and cross tachyphylaxis (between different challenges) have been described (Morice et al, 1992). This needs to be

taken into consideration when designing an experiment that involves repeated challenge testing.

6.5.4 Safety and tolerance

Cough inhalational challenge has been demonstrated to be reasonably tolerated and safe. Minimal bronchoconstriction was demonstrated in early studies with capsaicin (Collier & Fuller, 1984) mainly in one patient who had a URTI developing (Midgren et al, 1992). At higher concentrations of capsaicin (50microM) some patients could not complete full breath due to coughing, burning taste, pharyngeal irritation and hypersalivation (Midgren et al, 1992). There have been no serious adverse events in over 20 years of usage of capsaicin (Dicpinigaitis & Alva, 2005).

6.5.5 Nebuliser factors

The two types of nebuliser that have been used for cough challenges are ultrasonic and jet nebulisers. An ultrasonic nebuliser is required for a 'Fog' challenge whilst jet nebulisers can produce an accurate dose of inhalant using a dosimeter (Morice, 1996).

Some experiments have used repeated inhalations of challenge substances (Fujimura et al, 1994; Fujimura et al, 1992a; Fujimura et al, 1996; Fujimura et al, **1992b;** Fujimura et al, 1990; 1992c). But it is generally accepted that the single breath method (where only one inhalation is taken at each concentration of a challenge substance) is more accurate (Morice et al, 2007a; Wright et al, 2010). Whilst some studies suggest that tidal breathing is as reproducible as single breath, (Nejla et al, 2000) it is difficult to accurately measure dose of substance delivered during tidal breathing. This is due both to the decreased tolerance of higher concentrations of challenge substance leading to truncated tidal breathing and variations in different individuals flow rates and tidal volumes which can be controlled better with a single breath (Morice et al, 2007a).

6.5.6 Measuring coughing

The objective measurement of the cough response has been the subject of some debate through the literature, and various complicated devices have been designed to try and measure both an accurate number of coughs and the cough 'intensity' (Bickerman et al, 1956; Cox et al, 1984; Pounsford et al, 1985). There has also been some discussion about whether cough 'latency' i.e. the timing between the inhalation and the cough is important.

One of the considerations in designing a cough challenge for humans is the acceptability and ease of administration of this test. Simple counting of coughs would appear to be easier than applying collars, belts or other detection devices.

6.5.7 End points for Inhalational Cough Challenges

The earliest experiments of cough challenge utilised absolute number of coughs induced by inhaling a substrate as an endpoint (Bickerman & Barach, 1954). This is still used in some experiments where it is difficult to alter the concentration of the substrate (Fog) or where tidal breathing is utilised.

Using a concentration at which patient coughs a certain number of times appears to have been introduced as it is an accepted methodology in other respiratory challenge tests (Methacholine for example), and an equipment overlap was introduced in laboratories which already performed these challenges.

Initial studies showed that using the concentration which elicits the first cough tends to be poorly reproducible therefore either C2 or C5 (the concentration of a substance causing the subject to cough twice or five times respectively) has been adopted in a number of studies. There has been some debate as to which is the more superior value with some believing C5 to be more clinically relevant. (Dicpinigaitis, 2003)

It appears to be difficult with healthy volunteers and mild acids such as citric to elicit 5 coughs at soluble concentrations therefore experiments with this group and Citric acid more commonly utilise C2. (Wright et al, 2010)

Some recent studies comparing investigating the cough response in different populations have utilised EMax (number of coughs at the dose which elicits the highest response). Studies suggest Emax is a measure which is better at discriminating health from disease than C2 or C5. (Hilton et al, 2013)

C5 can seem to be a somewhat arbitrary endpoint. Initial studies however did suggest that following a single inhalation of Capsaicin patients rarely go into a coughing bout. And indeed Emax in Healthy volunteers and cough patients respectively was 4.5 and 8.6 coughs (Hilton et al, 2013) suggesting that setting a C value higher than 5 would be unrealistic to be achieved within the range of most challenges.

The current ERS guidelines on measurement of cough suggest that both C2 and C5 are measured where possible. (Morice et al, 2007a)

6.5.8 Designing a fog challenge

Unlike capsaicin and citric acid challenges, fog challenge as a cough challenge does not have an accepted ERS standard. Whilst many papers have published results of experiments using ultrasonically distilled water, the methodology of these varies greatly. It is therefore difficult to compare results of antitussive effect across centres. Different methods of fog challenge have different advantages and disadvantages.

One minute of inhaled fog exposure at the maximal output of the nebuliser with counting coughs (Godden et al, 1986) seems to be a reasonable method for comparing fog with other substances or to try and determine mechanism of action, but is less helpful for measuring an antitussive response.

Gradually stepping up the output of fog from the nebuliser during a challenge allows the calculation of a cough threshold. But this is again hard to standardise as many factors impact on the output of ultrasonic nebulisers. This is the method that appears to have been used most frequently – although much of the published data is from the same group (Fontana et al, 1999; Lavorini et al, 2001) (Fontana et al, 2002; Fontana GA, 2005). It also requires the use of some more complex equipment to measure nebuliser output accurately – a potentiometer and a DC signal on an oscilloscope – which limits its use in more extensive, multi-centre trials of antitussives.

Varying the ion content has also been utilised as a method of altering the amount of chloride ions inhaled. Using varying combinations of normal saline and distilled water appears to produce a cough response curve. This is expressed as a scale of 0-150mMol of sodium chloride (NaCl) (Lowry et al, 1987). Mixing distilled water with NaCl creates a solution series with gradually reducing concentrations of chloride ions. Based on the findings that a reduction in chloride ions causes the cough response, this appears to be a sound method in terms of basic principles, as well as being reasonably easy to reproduce across different centres and by different administrators.

6.5.9 Cough challenges in chronic cough patients

Whilst there have been a number of studies utilising cough challenges to measure the antitussive effect of medications for chronic cough, there have overall been few studies comparing inhalational cough challenges in cough patients and healthy volunteers. Fewer studies still, have considered whether cough challenge responses are consistent and reproducible in chronic cough patients.

Most studies comparing chronic cough patients with healthy volunteers have utilised the capsaicin challenge. The largest study of 363 participants (Choudry & Fuller, 1992) comparing healthy volunteers to cough patients, appears to have utilised the now somewhat outdated belief that non-productive cough is different to productive cough. Those with productive cough had C5 values similar to healthy volunteers (C5 – 1.81). Whilst those classed as having chronic idiopathic cough had the highest C5 (1.08). It has been confirmed in later studies that patients with chronic cough have a lower capsaicin C5 than healthy volunteers. C5 (median) in chronic cough patients – 6.92. In healthy volunteers – 62.7 (Vertigan et al, 2013).

In a study that did look at repeatability within sub-groups of their respiratory disease and healthy volunteer subjects, the authors state that they found good reproducibility (Prudon et al, **2005**). Another study considers repeatability of the capsaicin cough challenge in chronic cough patients between two visits, and found good correlation between two visits, eight weeks apart, for both C2 and C5. Healthy volunteers were not included as a comparison group in this study (Faruqi et al, 2011). Repeatability was felt to be good over a year, utilising the ERS capsaicin cough challenge in chronic cough patients (Pullerits et al, 2014).

It is difficult to locate any published data which directly compares citric acid challenge in adult healthy volunteers with chronic cough patients, or which considers its reproducibility in chronic cough patients. One study considered children and therefore had quite a different methodology (Riordan et al, 1994). Other studies have compared inhaled citric acid cough response with other measures of cough severity in chronic cough patients (Decalmer et al, 2007), which provides minimal information about whether cough challenges are a good, reproducible method of assessing chronic cough patients.

There appears therefore to be a dearth of information available regarding inhalational cough challenge in chronic cough patients.

6.5.10 Gender and the citric acid cough challenge

As mentioned earlier, women both present with chronic cough more frequently than men, and also have a higher cough frequency. Studies utilising the citric acid cough challenge as well as the capsaicin cough challenge have shown that women appear to have a more sensitive cough response. This is the case in both healthy volunteers and chronic cough patients, although the difference appears to be more pronounced in chronic cough patients (Kastelik et al, 2002; Rostami-Hodjegan et al, 2001). There also appears to be an

association between the increased cough response in women and perception of breathlessness, suggesting that this may be related to central sensory pathways rather than the peripheral response to citric acid being heightened in women (Gui et al, 2012).

6.5.11 Summary

The ideal cough challenge would be consistent, reproducible and targeted at a receptor known to be involved in cough. The substance needs to be soluble at a range of concentrations, safe and stable. The challenge needs to be acceptable to participants and easy to administer. Further research is needed to elucidate a challenge that fully satisfies all of these criteria.

7 Summary of Aims, Objectives and Hypotheses

My overall aim in the experiments described in this thesis was to explore the mechanisms of inhalational cough challenges. One of my objectives was to design cough challenges with novel agents that could be administered easily and were well tolerated by healthy volunteers and patients, as well as to explore some previously used cough challenges in more detail.

Another main objective was to carry out all experiments in both healthy volunteers and chronic cough patients, in order to further explore the cough reflex in patients who suffer with a chronic cough.

In my first experiment I aimed to explore one of the oldest cough challenge agents, citric acid. I hypothesized that the pH of the nebulised solution may be the important factor in precipitating a tussive response in man. I constructed a challenge methodology based around the respective pKa of the three acid moieties in the citric acid molecule. I had previously observed clinically that patients with a chronic cough have an increased sensitivity associated with a greater variability in their pattern of coughing. I sought to characterise this further.

In my second experiment I designed a challenge using Adenosine triphosphate and compared this to Adenosine monophosphate as a control. I tested the hypothesis that ATP when inhaled provokes a reliable cough response in healthy volunteers, and that this response is heightened in chronic cough patients.

My final experiment was designed to compare the repeatability and mode of action of four cough challenges – citric acid, capsaicin, fog and ATP. The hypotheses being challenged were two-fold: that inhaled ATP cough challenge is as reproducible in both chronic cough patients and healthy volunteers as citric acid, capsaicin and fog; that inhaled ATP cough challenge doesn't correlate with fog, citric acid or capsaicin cough challenge due to different mechanism of action

8 Investigating the effect of pH on the citric acid challenge in healthy volunteers and chronic cough patients

8.1 Introduction

8.1.1 Citric acid challenge

The use of citric acid as a tussive challenge in humans was first described by Bickerman and Barach in 1954 (Bickerman & Barach, 1954). The citric acid cough challenge model has become a standard methodology for investigating cough reflex sensitivity.

The challenge is highly reproducible, and has become one of the favoured techniques for studying the pharmacokinetics and pharmacodynamics of antitussive medications. It has been used in this way in a number of published studies which are summarised in **Table 1** below.

A number of studies have used the citric acid challenge to investigate differences in cough sensitivity between sub-groups of normal population. These relate in particular to gender and smoking status. Other studies have considered the varying responses to the citric acid challenge in disease states such as COPD and asthma, and considered whether it would be useful in the detection of patients at risk of aspiration.

Only one study has directly considered the effect of age on the cough response to citric acid and this found no difference in the cough response between the younger and older cohorts (Ebihara et al, 2011).

8.1.2 Gender and the citric acid cough challenge

As mentioned earlier, women both present with chronic cough more frequently than men, and also have a higher cough frequency. Studies utilising the citric acid cough challenge as well as the capsaicin cough challenge have shown that women appear to have a more sensitive cough response. This is the case in both healthy volunteers and chronic cough patients although the difference appears to be more pronounced in chronic cough patients (Rostami-Hodjegan et al, 2001). There also appears to be an association between the increased cough response in women and perception of breathlessness, suggesting that this may be related to central sensory pathways rather than the peripheral response to citric acid being heightened in women (Gui et al, 2012).

Table 1: Assessment of antitussives using citric acid cough challenge.

Authors	Antitussive Substance
(Bickerman et al, 1957)	
(Empey et al, 1979)	Codeine Dextromethorphan Noscapine
(Franzone et al, 1981)	2-(7'theophyllinemethyl)-1,3-dioxolane
(Rees & Clark, 1983)	Glaucine Codeine
(Belcher & Rees, 1986)	Pholcodine and salbutamol
(Chakrabarti et al, 1987)	Hexapneumine Clistine
(Karttunen et al, 1987)	Dextromethorphan Dextromethorphan-salbutamol combination
(Bossi et al, 1988)	Levodropropizine
(Karttunen, 1988)	Vadocaine hydrochloride Codeine
(Packman EW et al, 1991)	Diphenhydramine
(Fumagalli et al, 1992)	Levodroproprizine Droproprizine
(Morice et al, 1994)	Menthol (inhaled)
(Grattan et al, 1995)	Dextromethorphan (oral and inhaled)
(Abdul Manap et al, 1999)	Dextromethorphan (in relation to CYP2D6 activity)
(Moghadamnia et al, 2003)	Dextromethorphan and dextrorphan
(Usmani et al, 2005)	Theobromine
(Smith et al, 2006b)	Codeine (in COPD patients)
(Morice et al, 2007b)	Morphine Sulphate
(Xu et al, 2007)	Verticinone-cholic acid salt (based on Shedan Chuanbei powder – traditional Chinese medicine)
(Kenia et al, 2008)	Menthol (inhaled)
(Ramsay et al, 2008)	Dextromethorphan (in smokers)
(Sutovska et al, 2009)	Polysaccharides isolated form Malian medicinal plants
(Mincheva et al, 2014)	Montelukast

8.1.3 Smoking status and the citric acid cough challenge

Research specifically concerning the response to citric acid in smokers has had slightly contradictory results. One study showed a tendency for smokers to cough more than non-smokers although this was not a significant difference. The same study compared occasional smokers with non-smokers and regular smokers, and found that occasional smokers didn't cough. The authors therefore postulated that having a diminished cough reflex gives you the ability to become an occasional smoker, as normally intermittent smoking would precipitate uncomfortable coughing (Pounsford & Saunders, 1986).

A study published a few years later found that in smokers, cough threshold inversely correlated with greater cigarette consumption and depth of inhalation. Suggesting that the more cigarettes smoked and the deeper the deposit of toxic fumes into the lungs, the greater the destruction of the sensory nerve endings and therefore the higher the cough threshold (Taylor et al, 1988). This theory is supported by the fact that in smoker's cough frequency seems to be reduced directly following a cigarette (Mulrennan et al, 2004).

A more recent study also confirmed that cough threshold in smokers was higher than in non-smokers, suggesting that smoking causes decreased cough sensitivity (Kanezaki et al, 2010).

This suggests that the chronic cough in smokers is probably due to a mechanism other than neuronal hypersensitivity.

8.1.4 Citric acid challenge in respiratory disease

There are few published studies that directly compare citric acid cough challenge thresholds in healthy volunteers with those with respiratory illness.

The citric acid cough response is heightened in patients with an upper respiratory tract infection (Empey et al, 1976). It also appears to be heightened in COPD patients (Wong & Morice, 1999) although it is not clear whether the COPD patients included in this study were current smokers. Interestingly, there was no difference in the capsaicin cough challenge outcome in the same group of participants (Wong & Morice, 1999). These patients were not pre-selected as patients complaining of a cough, but similar sensitivities have been shown in later studies of COPD with cough (Smith et al, 2006a).

Asthmatics (again not pre-selected for the symptom of cough) have been shown to have a similar cough response to citric acid as healthy volunteers (Di Franco et al, 2001; Pounsford et al, 1985). Although the results of one of these studies may however be

limited by the utilisation of patients who have 'mild asthma', and haven't required inhaled steroids for four weeks (Di Franco et al, 2001).

8.1.5 Citric acid challenge in chronic cough patients

I have been unable to locate any previous studies that report on a direct comparison of a citric acid cough challenge in healthy volunteers versus chronic cough patients.

Chronic cough patients have been studied with regards to citric acid cough challenge, by comparing males with females (Kastelik et al, 2002) and by comparison with other objective and subjective measures of cough severity (Decalmer et al, 2007).

In 61 patients with idiopathic chronic cough, the median C5 was 250mM citric acid (range 30–4,000mM). There was inverse correlation between $\log_{10}$ daytime cough rates and log C5 (Decalmer et al, 2007).

In the comparison of male and female patients with chronic cough, females coughed at significantly lower citric acid concentrations. The mean and standard deviation results for C2 and C5 are outlined in **Table 2** below.

In the Hull laboratory, with the same equipment as used for my study, the mean C2 for healthy volunteers has previously been found to be 263mM (Wright et al, 2010). C2 was used as the endpoint in this study as insufficient numbers of participants achieved C5 within the concentration range of the study.

Table 2: Mean [SD] of cough response to citric acid.

	Female	**Male**
C2	53.5 [17.3 – 145.4] mM	118.1 [41.4 – 38.1] mM
C5	300 [97.1 - >1000] mM	830.4 [300->1000] mM

Data extracted from (Kastelik et al, 2002)

8.1.6 Safety of citric acid challenge

Citric acid challenges in asthmatics and patients with COPD do not cause significant bronchoconstriction, rendering it safe and well tolerated even in patients with underlying obstructive airways disease (Auffarth et al, 1991).

Citric acid has also been found to remain stable over prolonged periods of time, although it is suggested that individual aliquots are used to make up each challenge to avoid any fungal growth within the solution (Falconer et al, 2014).

8.1.7 Why does citric acid produce a tussive response?

Despite its extensive use, the mechanism whereby citric acid produces cough has never been fully elucidated.

Quite how inhaling nebulised citric acid leads to the initiation of the cough reflex is not fully understood. It is thought to be due to its properties as a weak acid. It does seem to cause some agonistic effect at both TRPV1 receptors and non-capsaicin responsive receptors (Canning et al, 2006).

The lack of correlation with the capsaicin and citric acid challenges (Wong et al, 1999) would, however, suggest that citric acid is working via a different mechanism to capsaicin (Canning et al, 2006).

It has therefore been suggested that unlike capsaicin, citric acid does not act solely via the TRPV1 receptor. Other candidate receptors for producing a tussive response by citric acid include the acid sensitive channel receptor (ASCR) and other members of the TRP family, such as the TRPA1 receptor.

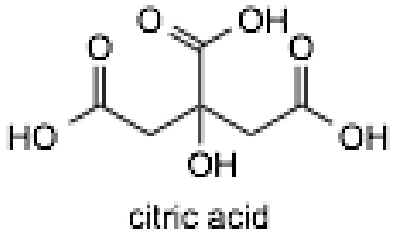
It is thought that some of citric acid's effect is due to the protons it can release, and this is borne out by work comparing it with other acids which seem to have similar tussive effects, suggesting that the pH of citric acid is an important factor rather than its molecular structure as citrate (Wong et al, 1999).

Previous studies have shown that citric acid, along with other complex acids, demonstrates a variable response within the population. Indeed, a proportion of both normal volunteers and chronic cough patients fail to cough even at high inhaled concentrations. Within individuals tussive response between the different complex acids is consistent (Wong et al, 1999).

8.1.8 Rationale behind choice of pH values used

Citric acid has three carboxylic acid groups (**Figure 1**), at each of which it can lose a proton. Dependent on the environment, as a weak acid, it loses or holds onto its protons.

Figure 1: The chemical structure of citric acid.



citric acid

Depending on the pH of the solution four different types of citric acid may be present depending on how many protons they have lost.

The ratio of each species at a certain pH is interpreted in the **Figure 2** below.

Figure 2: Ratio of species of citric acid in solution at each pH value.

H3A is the fully protonated species and $H2A^-$ has one less hydrogen etc.

At pH 3, 5 and 6.5 there is a 50/50 split between two of the species. This is its pKa value. Citric acid has three pKa values (3, 5 and 6.5) (Goldberg et al, 2002).

8.2 Hypothesis

I hypothesized that the pH of the nebulised solution may be the important factor in precipitating a tussive response in man. I therefore constructed a challenge methodology based around the respective pKa of the three acid moieties in the citric acid molecule. I have previously observed that patients with a chronic cough have an increased sensitivity

associated with a greater variability in their pattern of coughing. I sought to characterise this further, to help the understanding of the phenomenon of cough hypersensitivity.

8.3 Methods

8.3.1 Recruitment

Two study populations were recruited, by myself and by Dr Howard and Dr Rai (Academic F2s) under my supervision. Chronic cough patients were recruited from the Hull Cough Clinic and the Clinical Trials Unit chronic cough database. Chronic cough patients were required to be stable on medication for a month before recruitment, and exhibit a Hull Cough Hypersensitivity Questionnaire (HCHQ) score of 20 or above (see **Appendix 1**).

Healthy volunteers were recruited from the hospital staff and the trials unit healthy volunteer database. Healthy volunteers were required to be free from significant respiratory illness and using no regular medication affecting cough. A HCHQ below the upper limit of normal (13 or less) on entry was required by the healthy volunteers. The two groups were matched for gender by stratification during recruitment.

8.3.2 Inclusion and exclusion criteria

All participants were aged 18-85, current non-smokers who had been stable on medication for at least a month.

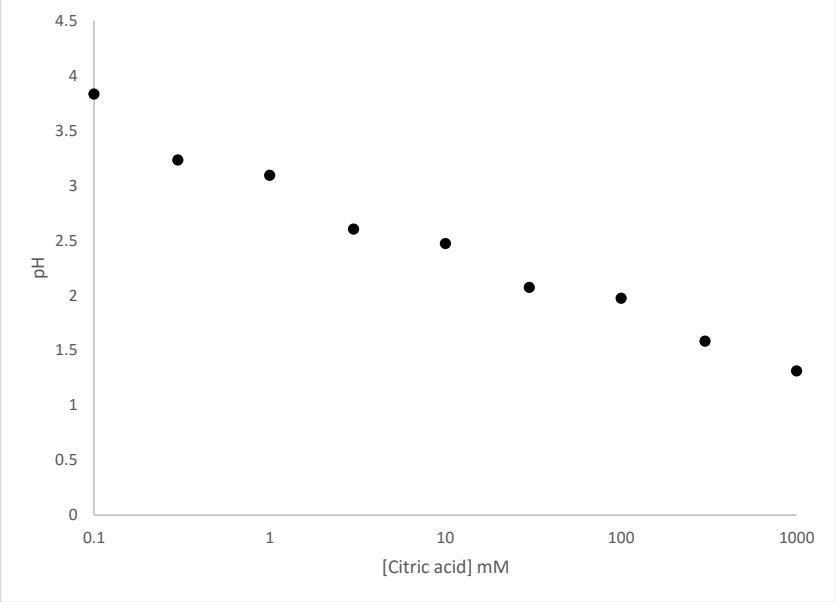
For safety reasons, volunteers were excluded if they had serious co-morbidities, or mental illness, were pregnant or had a pacemaker. Participants also needed to be able to follow instructions closely and were therefore excluded if they did not speak English or had dementia. To ensure the validity of the cough challenges, if the participant had a recent (within the last three weeks) upper respiratory tract infection or asthma exacerbation, they were offered an appointment at least three weeks after this had resolved.

8.3.3 The challenge agents

Following preliminary work, by myself and Dr Howard under my supervision, it was discovered that the pH of the citric acid within the standardised challenge varied throughout the range of concentrations used within the standardised challenge (see **Figure 3** below) and that doing this would introduce too much variation within the pH of the challenge.

As a result, it was decided that to perform five inhalations at one concentration of citric acid would allow for better standardisation of the pH within each challenge. A concentration of 300mM was chosen as this is similar to the mean C2 in healthy volunteers (Wright et al, 2010) and is unlikely to induce too severe a coughing bout in patients. The other aspects of the standardised challenge remained unchanged (equipment, flow limiter etc.).

Figure 3: pH of citric acid at concentrations used in standard cough challenge.



Citric acid solutions of pH 3.13, 5.05 and 5.99 were prepared by diluting 1 molar solution of citric acid (Fisher Chemical, made from citric acid monohydrate) with sodium hydroxide and distilled water (**Table 3**).

Commented [HF1]:

Commented [HF2R1]: This is the company that make it and how I made it – I can't think of a better way to say it

Table 3: Dilution sequence for citric acid.

1M Citric Acid (ml)	**Distilled Water (ml)**	**1M Na OH (ml)**	**Final Citric acid conc^{n} (mM)**	**pH (as measured)**
3	7	0	300	3.13
3	2	5	300	5.05
3	0	7	300	5.99

8.3.4 Delivery of the challenges

The three challenges were administered in the clinical trials unit either by myself or one of two academic foundation doctors under my supervision, on different days, at least 48 hours apart.

The order in which the challenges were administered was randomised using a computer-generated randomisation system. The order of administration was double-blinded, with neither patient nor challenge administrator being aware which challenge was delivered on which day.

The cough challenge methodology was adapted from the standard cough challenge methodology used in the Clinical Trials Unit, and standardised by the ERS.

Spirometry was measured at the first visit of each participant. Salbutamol nebulisers and inhalers were available if bronchoconstriction occurred during the challenge. Adrenaline and a crash trolley were also to hand.

Participants were asked to avoid caffeine and menthol for one hour prior to each cough challenge. Challenges were also delayed if they had used over the counter cough medications within 24 hours of the challenge.

A DeVilbiss spirometer with KoKo DigiDoser was used to nebulise the substrate with a flow limiter and fixed straw and baffle. Following initial tidal breathing and a maximal exhalation, the participant was encouraged to take a steady breath in at which point the nebuliser would deliver the substrate for 1.2 seconds.

All challenges started with a Normal Saline inhalation. Citric Acid was then delivered at a concentration of 300mM. Coughs were counted by the cough challenge administrator in the first 15 seconds after each inhalation (identified by a timer in the software). There was at least one minute between each inhalation. This inhalation was then repeated a further four times.

Cough response to each inhalation was documented, and the number of coughs in response to the five citric acid inhalations was then totalled to give individual patient responses to each pH.

Participants received their second and third challenges at a similar time of day, and as far as possible using the same DigiDoser. If the patient experienced changes in health between the challenges these were noted, and if this was an upper respiratory tract infection, the further challenges were delayed until recovery.

Adverse events were recorded at the next visit, or if the participant actively reported them by contacting the study administrator.

8.3.5 Statistical analysis.

Data was statistically analysed with SPSS (IBM Version 22). Within groups, the comparison of total number of coughs at each pH was done using the Friedman test with Bonferroni correction. Between-group comparison was performed using the Wilcoxon Rank Sum test.

The standard deviation of the number of coughs produced at each of the five inhalations at a given pH value in the chronic cough group was compared with the healthy volunteers, as an analysis of the difference in variation in cough response between the two groups.

Since the effects of these challenge solutions were previously unknown it was impossible to create an accurate power calculation. However, multiple previous studies done in the Hull Clinical Trials Unit in both healthy volunteers and chronic cough patients have demonstrated significant results using 20 subjects.

8.3.6 Approvals

Ethical approval was obtained to complete this study from the English R.E.C. (Study title: An Investigation into the Mechanism of Inhalational Cough Challenge, REC reference: 14/SS/1071, Protocol number: ACADMED240913, IRAS project ID 164364) and the Hull and East Yorkshire Trust R&D department (R1740 Inhalation Cough Challenge). The study is registered on ClinicalTrials.Gov (NCT02039999).

8.4 Results

8.4.1 Demographics

Twenty chronic cough patients and twenty healthy volunteers were prospectively recruited into this study. All subjects were non-smokers. One participant in each group withdrew their consent before completing the cough challenges. Their data was not included in the final analysis.

Participants were gender matched and included 12 females in each group. The median age in the healthy volunteers was 42 with a range of 23-77. The median age in the chronic cough patients was 74 with a range of 50 to 83.

Chronic cough patients were on more medications and had more self-reported co-morbidities than the healthy volunteers. 18 chronic cough patients and 10 of the healthy volunteers were on regular medications which they had been stable on for more than 3 months. One healthy volunteer was on oral steroids, two chronic cough patients were using inhaled steroids. One healthy volunteer and two patients were on ACE-inhibitors. Other common medications included statins (4 in CC, 3 in HV), Proton pump inhibitors (8 in chronic cough, 1 in healthy volunteers). The most common co-morbidities were Gastro-oesophageal reflux disease, Hypertension, Hypercholestrolaemia and Diabetes.

The full demographics for each patient group as well as more detail about their co-morbidities are compared in **Table 4** below.

8.4.2 Response to normal saline

Initial inhalation of normal saline provoked cough in a single subject in the healthy volunteers. In the chronic coughers ten subjects coughed in response to normal saline on at least one challenge day, and two subjects coughed on all three challenge days.

8.4.3 Cough response to different pH

8.4.3.1 Healthy volunteers

Six of the 19 healthy volunteers who completed all three challenges did not cough in response to any of the citric acid pH challenges. **Figure 4** below shows the total number of coughs in response to each pH cough challenge in healthy volunteers. The figure illustrates that the lower the pH, the more coughs tended to be elicited.

At pH 3 the mean total number of coughs was 8.7, however only 63% of volunteers coughed at all (in this group the mean total number of coughs was 13.8). At pH5 the mean total number of coughs was 3.2, only 30% of people coughed at pH5 (mean total number of coughs 9), and at pH6 the overall mean total number of coughs was close to 0 (0.2) as only 10% of people coughed at pH6 (mean total number of coughs 2).

Total cough counts were significantly different between the pH values ($p<0.01$). Post-hoc analysis revealed statistically significant differences in total number of coughs comparing pH 3 to pH 6 ($p<0.01$) and pH3 to pH 5 ($p=0.045$), but not comparing pH5 to pH6 ($p=1.000$).

Cough response in the chronic cough patients was further analysed for order effect and showed no evidence of order effect dependent on which challenge was given first. (p values 0.458, 0.818, and 0.546 for pH3, pH5 and pH6 respectively)

Table 4: Demographics in the study: Investigating the effect of pH on the citric acid challenge in healthy volunteers and chronic cough patients.

	Healthy Volunteers	**Chronic Cough Patients**
Gender (Male: Female)	8:12	8:12
Age (**Median**: Range)	**42**: 23-77	**74**: 50-83
Race (Caucasian: Non-Caucasian)	17:3	20:0
FEV1 % Predicted (**Median**: Range)	**91%**: 55-121	**90%**: 57-128
Hull Cough Hypersensitivity Score (**Median**: Range)	**2**:0-8	**35**:21-50
On Medications (Number)	18	10
Most Common Self Reported Co-morbidities (Number)		
Hypertension	2	6
Hypercholestrolaemia	4	2
Diabetes	1	4
IBS/other bowel	1	1
Iron Deficiency	1	0
GORD	1	6
Polymyalgia	1	0
Asthma	0	2
IHD	0	3

8.4.3.2 Chronic cough patients

One patient did not cough in response to any pH of citric acid. **Figure 5** below shows the total number of coughs at each pH in the chronic cough participants. The graph does not illustrate any discernible pattern to the number of coughs dependent on pH.

At pH 3 the mean total number of coughs was 16.5, 74% of patients coughed (with the mean total number of coughs in this group being 22.4 coughs). At pH5 the mean total number of coughs was 18.1 with 89% of patients coughing at least once (mean coughs 20.2). At pH6 the mean total number of coughs was 7.7 and 57% of patients coughed (mean coughs 12.2).

8.4.3.3 Differences between healthy volunteers and chronic cough patients

A cough response to citric acid was provoked in a greater number of chronic cough patients at all pH values. In those that coughed, the number of coughs also tended to be higher in the chronic cough patients, irrespective of pH.

A comparison of mean total cough responses to each pH between healthy volunteers and chronic cough patients are demonstrated in **Figure 6** below.

The mean cough response at pH 3 was similar (16 for chronic cough patients and 9 coughs for the healthy volunteers), whereas at pH 5 the cough response in the healthy volunteers was lower with a mean of 3 coughs but in chronic cough patients was higher with a mean of 18. At pH 6 mean cough response fell in both groups approaching 0 in healthy volunteers (0.6) whereas chronic cough patients still had substantial cough response, with a mean cough response of 8.

8.4.3.4 Distribution of coughs within patients

Within the five inhalations performed at each pH there appeared to be a much greater variability in response in the chronic cough patients. The variability is illustrated in **Figure 7** below where the individual standard deviations at each challenge concentration are plotted.

Figure 4: Total number of coughs in healthy volunteers.

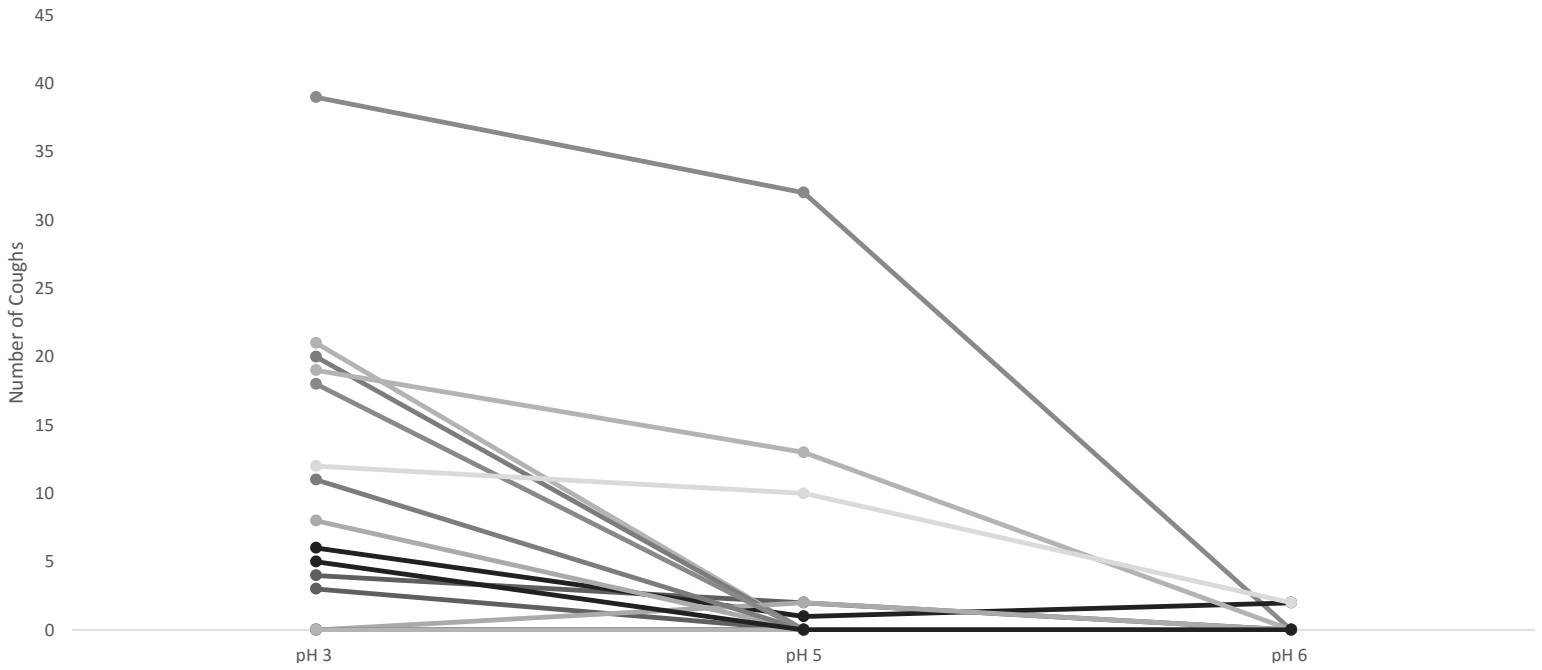


Figure 5: Total number of coughs in chronic cough patients.

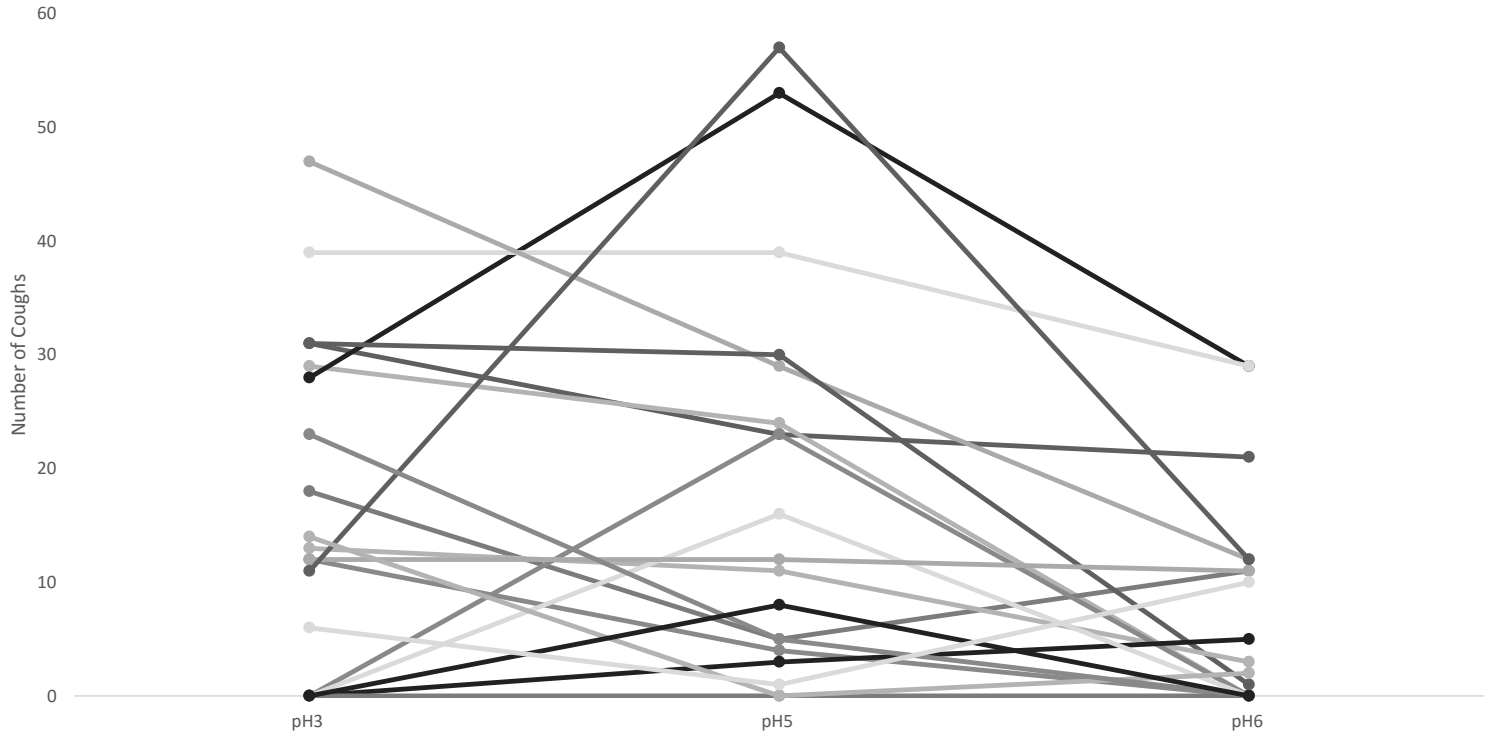


Figure 6: Comparison of means: Healthy volunteers and chronic cough patients at each pH with standard error.

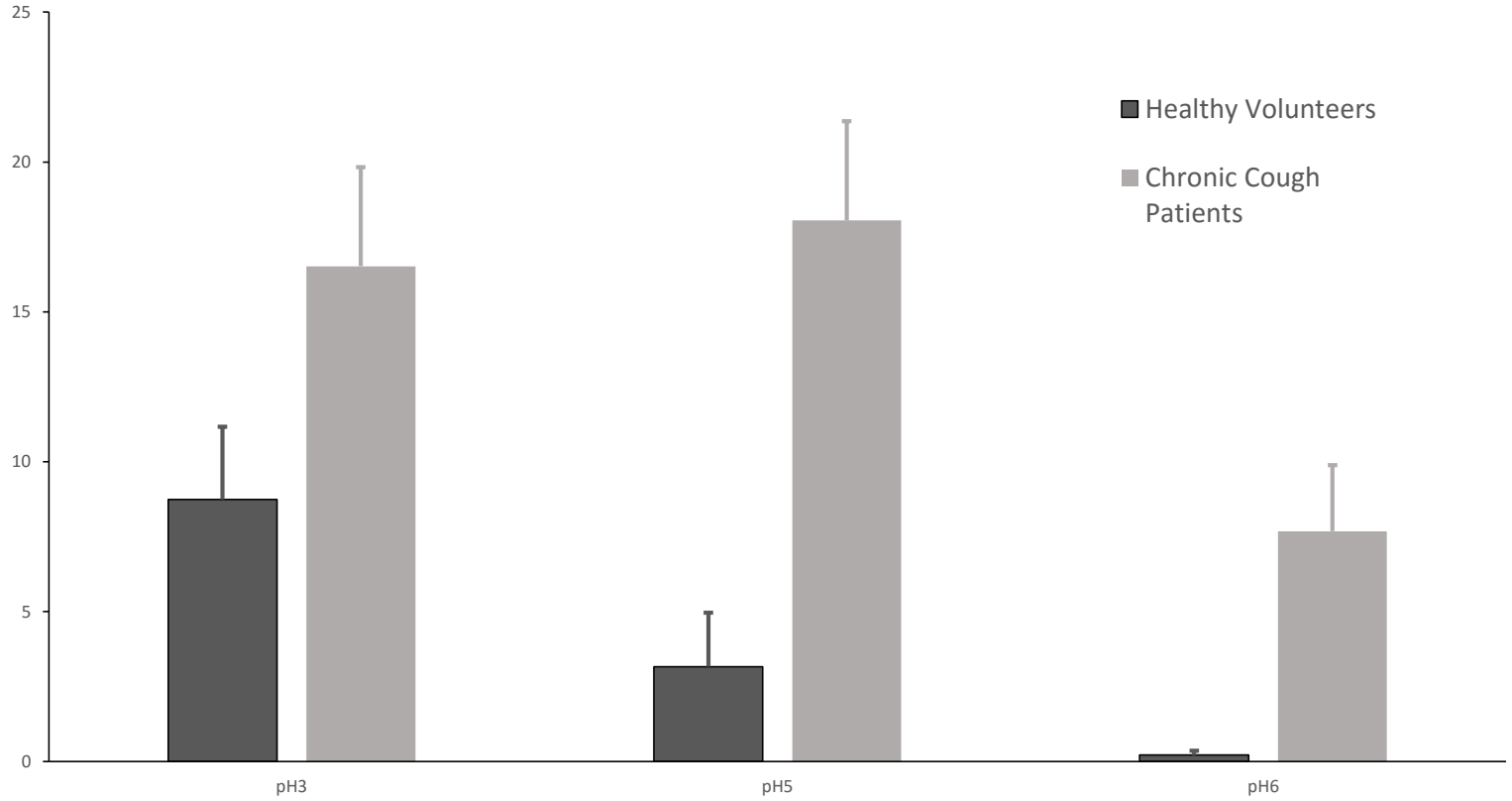
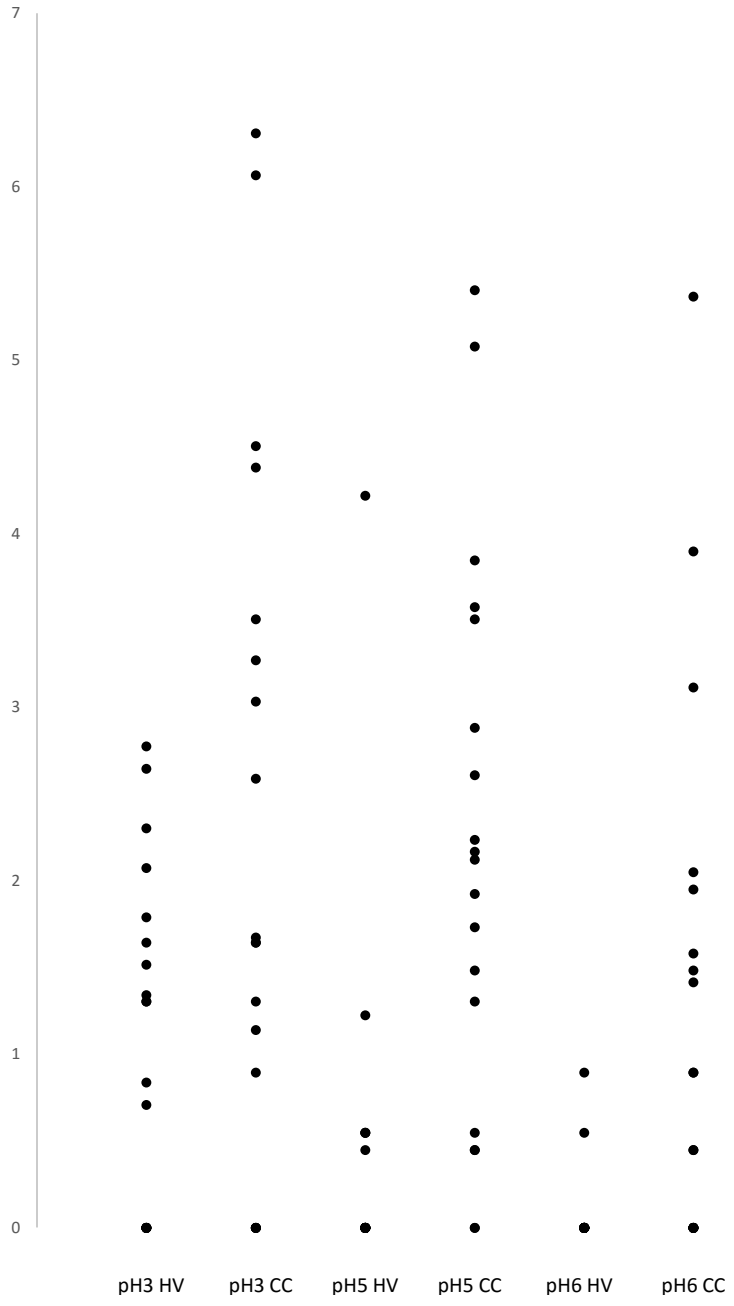


Figure 7: Standard deviations of each participant's individual inhalations of challenge substance.



8.5 Discussion

My objective in this investigation was to characterise the degree of hypersensitivity to citric acid seen in patients with chronic cough. Overall patients coughed more, illustrating cough hypersensitivity syndrome.

It was expected that some healthy volunteers wouldn't cough within the range of the given cough challenge and this was true, but also one chronic cough patient didn't cough. The variability in response to citric acid with considerable overlap between healthy and chronic cough has previously been noted. The isolated chronic cough patient who didn't cough in response to citric acid is slightly unusual, but was in a male patient who often have less cough hypersensitivity (Kastelik et al, 2002). This particular patient may be sensitive to substances other than citric acid. This finding does support the need to pre-challenge patients prior to measuring effect of tussive response.

Chronic cough patients coughed across all pH values of citric acid suggesting a hypersensitivity independent of pH. The chronic cough group overall coughed more across all pH levels when compared to healthy volunteers. In the healthy volunteers, the more hydrogen ions available in the cough challenge the greater the cough response both in terms of the number that cough and the number of coughs. The chronic coughers did not follow this pattern and abolishing the number of hydrogen ions in this group did not significantly alter the tussive response. This is suggestive of cause of hypersensitivity not wholly lying within the response to hydrogen ions.

In studying the effects of citric acid at different pH values, I have illustrated in the healthy volunteers the previously described reproducibility of citric acid challenge in measuring tussive response. However, for the first time I have demonstrated that this relationship does not hold true for patients suffering from chronic cough. Thus, in healthy volunteers the stimulus activating the cough reflex appears to have a more or less linear relationship to the concentration of protons in the nebulised solution. Unfortunately, I have not elucidated which of the several potential receptors have been activated to generate the stimulus for coughing and this would need specific antagonists of the putative acid sensor within the airways.

My results suggest that hypersensitivity in chronic cough patients is not purely due to an upregulation of hydrogen ion activated receptors.

Across the range of the standard citric acid cough challenge pH decreases as concentration becomes stronger, therefore not just increasing concentration of citrate but also number of available hydrogen ions. It is possible that the effect of the standard citric acid challenge is due therefore to its acidity rather than a concentration of citrate.

The lack of relationship to pH in the chronic cough patients was mimicked by their tussive response to normal saline. This was again an erratic phenomenon, some patients coughing vigorously to normal saline with subsequent citric acid challenge evoking no cough. The stimulus for this normal saline 'hypersensitivity' is unclear but jet nebulisation causes a fall in temperature and so a thermal stimulus may be the cough provoking factor. Cough with normal saline does however illustrate both the increased sensitivity to minimal stimulation and the apparent random nature of the excessive response to such stimulation. Patients describe paroxysms of severe cough (excessive response) induced by exposure to ordinarily innocuous agents – 'There is no pattern to it, Doctor'.

The considerable difference in response of patients suffering from chronic cough is illustrated by two measures of variability in the citric acid challenge. Firstly, there is a lack of concentration response over the pH spectrum studied. Secondly at a given pH a patient with chronic cough responds in a highly idiosyncratic and variable manner, sometimes not coughing for the first few inhalations and then coughing excessively to what is an exactly similar stimulus. These altered responses show parallels to chronic pain where the phenomenon of 'wind up'- the perceived increase in intensity over time when a given non-painful stimulus is delivered repeatedly and the concept of allodynia – central sensitisation following painful, often repetitive stimulation. Allodynia can lead to the triggering of a response from stimuli which do not normally provoke pain. These concepts have previously been discussed in the context of cough where the term allotussia has been coined. However, the naming of the phenomenon does not necessarily help one's understanding. Cough is essentially a vagal phenomenon with the afferent pathway relaying in a complex fashion through nodose and jugular ganglia, where the expression of neurotransmitters and receptors may be altered by disease. In addition, the concept of simple afferent neuron to a brainstem 'cough centre' is unlikely to reflect the neurophysiology of plastic neuron to neuron interaction.

My study has several limitations. Whilst I matched the gender between the two study groups I did not match age. The normal volunteers were younger than the chronic cough population. Of these demographics, there is a large body of evidence supporting

heightened cough sensitivity in women, but there is little evidence of an important age-related change in cough sensitivity (Morice et al, 2014a). Indeed if age related neurological conditions such as stroke or dementia are excluded from population surveys of cough reflex sensitivity there appears to be little difference in adults (Ebihara et al, 2011).

In studying citric acid, I have potentially used an agent which is subject to tachyphylaxis. However, there was no evidence of an order effect in the chronic cough subjects and in both arms of the study the order of challenge solution was randomised.

Clinically I have noted a day-to day variability in cough response and severity in cough patients. My study design allowed for quite long periods of time between the cough challenges specifically to avoid any tachyphylaxis but paradoxically this may have allowed for a change in the patients cough hypersensitivity.

8.6 Conclusion

Cough hypersensitivity is not simply a shift in the dose response curve to citric acid but a fundamental alteration in the pattern of response to a given stimulus.

9 Tussive challenge with ATP and AMP: Does it reveal cough hypersensitivity?

9.1 7.1 Introduction

9.1.1 ATP as an extracellular signalling molecule

Adenosine 5' Triphosphate (ATP) is known to be an intracellular energy source and signalling molecule. ATP as an extracellular signal, detected by purinergic receptors, was first postulated by Burnstock in 1972 (Burnstock, 1972) who then went on to define the receptors in 1976 (Burnstock, 1976).

There then followed a recognition of the subclasses of this group of receptors based on whether they had a greater affinity for adenosine or ATP. These were initially designated P1 for the group that had affinity for adenosine, and P2 for those receptors that had affinity for ATP and ADP (Burnstock, 1978). P2 receptors were then further subdivided into P2X and P2Y receptors. This subdivision initially was based on their pharmacology (Burnstock & Kennedy, 1985; Kennedy & Burnstock, 1985).

The role of ATP as an extracellular signal was not widely accepted until the 1990s when the first purinergic receptors were cloned. Once they were cloned the subdivision into P2X and P2Y receptors was confirmed. P2X receptors (**Table 5**) are ligand-gated ion channels (Brake et al, 1994; Valera et al, 1994), whilst P2Y receptors are G-protein coupled receptors (Abbracchio & Burnstock, 1994). The action of ATP at P2 receptors is described as a primitive signalling system. It appears to be involved in both non-neuronal and neuronal mechanisms (Burnstock, 2007).

Table 5: P2X receptor sub-class in the human body.

Subtype	Cell type location
P2X1	Smooth muscle, platelets, cerebellum, dorsal horn
P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia
P2X3	Sensory neurons, NTS, sympathetic neurons
P2X4	CNS, testis, colon
P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord
P2X6	CNS, motor neurones in spinal cord
P2X7	Apoptotic cells

9.1.2 P2 receptors in the lungs

A number of findings support the presence of ATP responsive P2 receptors within mammalian lungs. In the dog, ATP delivered directly into the right atrium is mediated by P2X receptors and activates vagal C-fibre nerve terminals; a similar effect is seen when capsaicin is administered in the same way. There is a difference in the amount of response to the two substances and a different response when a P2 antagonist was used in combination (Pelleg & Hurt, 1996). However, it is possible that there is interplay between TRPV receptors and P2.

9.1.3 ATP in respiratory disease

In vivo and in vitro models of COPD have suggested a role for ATP in the pathogenesis of COPD. Emphasis is placed on the P2X7 receptors on macrophages (Mortaz et al, 2010). P2X7 is upregulated on macrophages in COPD patients. Levels of ATP are also higher in the Broncho Alveolar Lavage (BAL) fluid of these patients (Lommatzsch et al, 2010).

Asthmatic mice and humans show an increase in ATP in BAL fluid after an allergic challenge. This effect is blocked in mice if they are treated with an enzyme which breaks down the ATP (apyrase) or a P2 receptor antagonist (Idzko et al, 2007).

9.1.4 P2X3 receptors

P2X3 receptors are thought to be the main P2X receptor responsible for the effects of ATP in the lung, having been demonstrated by immunohistochemistry on sensory nerve endings in rat lungs (Brouns et al, 2000). P2X3 is also found on C- and A-delta fibres in the dorsal root ganglia, cranial sensory ganglia and peripheral nerve terminals in receptive areas in a number of different tissues. It tends to be found as either a homotrimeric receptor or P2X2/3 (heterotrimeric) (Ford, 2012). Vagal C-fibres may be stimulated by ATP, via heteromeric P2X2/3 receptors (Undem & Nassenstein, 2009). Vagal C-fibres of guinea pigs in ex vivo preparations are blocked from methacholine and histamine response by P2X3/P2x2/3 antagonists (bronchoconstriction is not) (Weigand et al, 2012).

Responses of peripheral neurons to ATP vary within the same ganglia, between different types of ganglia and within species. However the response appears to be consistently due to its effect on P2X2 and P2X3, but there is probably a difference in the proportion of homo/hetero types of receptor expressed (Ford, 2012). Of relevance to cough hypersensitivity, activation of P2X2/3 heterodimers produces a prolonged current, where stimulation of P2X3 receptors produces a rapidly inactivating current (Kwong et al, 2008). A recent study has however suggested that prolonged activation of the P2X3 receptor may be achieved by TRPV4 activation of pannexin causing the continuous stimulation of P2X3, thus leading to prolonged hypersensitivity (Bonvini et al, 2016).

9.1.5 P2 receptors/ATP in cough – animal studies

Animal studies considering the role of ATP and P2X receptors specifically in cough have been limited to guinea pigs. Guinea pigs pre-treated with inhaled ATP coughed more with citric acid afterwards. This was blocked by one P2X antagonist but not another and these results implicated the P2X4 receptor in this species. It is however worth noting that ATP alone didn't stimulate cough in guinea pigs (Kamei et al, 2005).

9.2 Administering ATP – human studies

IV ATP administered to palliative patients caused breathlessness as its most common side effect (Beijer et al, 2007). Inhalation of ATP has previously been noted to cause cough although this was not characterised systematically. Inhalation of ATP caused bronchoconstriction in both healthy and asthmatic volunteers, with a greater response in asthmatics (Basoglu et al, 2005; Pellegrino et al, 1996). Inhalation challenges using ATP

and AMP in COPD patients, smokers and healthy volunteers found that ATP appeared to cause increased breathlessness and cough compared to AMP (Basoglu et al, 2015).

9.3 P2X3 receptors in cough treatments

AF-219 (a P2X3 receptor antagonist) has been trialled in a phase 2 study, and was found to significantly reduce cough in patients with chronic cough. It also caused taste disturbance, leading to early withdrawal by some patients (Abdulqawi et al, 2015)

This recent demonstration that blockade of ATP preferring purinergic receptors leads to a marked reduction in chronic cough, combined with the aforementioned studies of the P2X3 receptors and ATP, suggests that ATP may be a key mediator of cough hypersensitivity and thus an inhalational challenge of ATP may differentiate between a normal cough reflex and cough hypersensitivity.

9.4 Hypothesis

I believe ATP when inhaled should provoke a reliable cough response in healthy volunteers, and that this response is heightened in chronic cough patients.

9.5 Method

9.5.1 Recruitment

Twenty healthy volunteers were recruited from departmental staff and the Clinical Trials Unit database of volunteers. Twenty gender matched patients with hypersensitivity cough syndrome were recruited from the Hull chronic cough clinic and the Clinical Trials Unit database of chronic cough patients.

Healthy volunteers had a Hull Cough Hypersensitivity Questionnaire score of less than 13. Chronic cough patients had a Hull Cough Hypersensitivity Questionnaire score of 20 and above.

9.5.2 Inclusion and exclusion criteria

All participants were aged 18-85, current non-smokers who had been stable on medication for at least a month.

For safety reasons, volunteers were excluded if they had serious co-morbidities, or mental illness, were pregnant or had a pacemaker. Participants also needed to be able to follow instructions closely and were therefore excluded if they did not speak English or had dementia. To ensure the validity of the cough challenges if the participant had a recent (within the last three weeks) upper respiratory tract infection or asthma exacerbation, they were offered an appointment at least three weeks after this had resolved.

9.5.3 The challenge agents

Participants received two cough challenges; one with ATP and one with AMP (Sigma Aldrich). 0.9% saline was chosen as the solvent for dissolving the ATP and AMP, as it is reasonably inert in terms of causing cough, and its slight acidity aids in preserving stability.

Preliminary work with regards to solubility found that adenosine had low solubility in saline in the quantities that would be required for a human cough challenge. Although a more acidic solution could be used to dissolve adenosine, this would have altered the overall properties of the challenge and added another variable factor as one knows that acidity plays a role in stimulation of the cough reflex. Therefore, AMP was chosen as a substitute for adenosine as it rapidly loses its additional phosphate group. This is in common with previous ATP/adenosine inhalational challenges (Basoglu et al, 2015; Basoglu et al, 2005). ATP was readily soluble in normal saline in the quantities required at a maximum concentration of just over 0.3M. As a result, this concentration was chosen as the maximum challenge concentration for both substrates.

Stability of ATP and AMP in solution was confirmed using HPLC analysis in the Hull chemistry department by Juozas Domarkas. Both substrates were found to be stable in solution for at least 72 hours. They were made up in single aliquots and when not used on the same day were stored in the fridge at 4^{o}C.

9.5.4 Delivery of the challenges

The two challenges were administered on different days, at least 48 hours apart.

The order in which the challenges were administered was randomised using a computer-generated randomisation system. The order of administration was double-blinded, with neither patient nor challenge administrator being aware which challenge was delivered on which day, as the challenge substances were prepared by a third party and the two challenge substances looked identical once made up.

The cough challenge methodology was adapted from the standard cough challenge methodology used in the Clinical Trials Unit, and standardised by the ERS (Wright et al, 2010). Spirometry was measured at the first visit of each participant. They were excluded at this stage if they were very severely obstructed. Salbutamol nebulisers and inhalers were available if bronchoconstriction occurred during the challenge. Adrenaline and a crash trolley were also to hand. Participants were asked to avoid caffeine and menthol for one hour prior to each cough challenge. Challenges were also delayed if they had used over the counter cough medications within 24 hours of the challenge.

A single inhalation of each dose of substrate was delivered using a KoKo DigiDoser with flow limiter and fixed straw and baffle, after maximal exhalation. All challenges started with a normal saline inhalation. ATP or AMP was then delivered in increasing concentrations on a half-log scale from 0.1-300mM.

Coughs were counted by the cough challenge administrator in the first 15 seconds after each inhalation (identified by a timer in the software). There was at least one minute between each inhalation. The challenge was completed once the participant coughed at least 5 times following an inhalation or reached the maximal concentration available. Comments about throat sensation during the challenge were not actively sought, but were noted if the participants volunteered them.

Participants received their second challenge at a similar time of day, and as far as possible using the same DigiDoser.

If the patient experienced changes in health between the challenges these were noted, and if this was an upper respiratory tract infection, the second challenge was delayed. Adverse events were recorded at the next visit, or if the participant actively reported them by contacting the study administrator.

9.5.5 Statistics/data analysis

C2 was taken as the first concentration that induced at least 2 coughs. C5 was taken as the first concentration that induced at least 5 coughs. For purposes of statistical analysis during comparison between the 2 groups of participants, if C2 or C5 was not reached – it was set at 1000mM.

Differences in the number of patients reaching C2 and C5 in the ATP and AMP challenges within each group were assessed using McNemar's test for related data. Comparisons of

C2 and C5 between the two groups (healthy volunteers and chronic cough patients) were made using T-test. Statistical analysis was performed using SPSS.

9.5.6 Ethical approval

Ethical approval was obtained to complete this study from the National Research Ethics Committee (Study title: An Investigation into the Mechanism of Inhalational Cough Challenge, REC reference: 14/SS/1071, Protocol number: ACADMED240913, IRAS project ID 164364) and the Hull and East Yorkshire Trust R&D department (R1740 Inhalation Cough Challenge). The study is registered on ClinicalTrials.Gov (NCT02039999).

9.6 Results

9.6.1 Demographics

The two groups of participants were gender matched and there were 14 females and 6 males in each. The healthy volunteer group had a lower age range of 23 years, an upper age range of 74 years with a median of 43 years. The patient group lower age range was 27 years, upper age range was 83 years and the median was 71 years. The healthy volunteer group comprised of 18 Caucasians, 1 Asian and 1 North African. The chronic cough patient group comprised 19 Caucasians, and 1 Asian.

FEV1 as a percentage of predicted was also lower in the chronic cough group with a median of 88% versus 101%

Nine of the healthy volunteers self-reported other comorbidities. All the chronic cough patients had other co-morbidities. Gastrointestinal disturbances (both upper and lower), inflammatory disorders such as arthritis and vasculitis, hypertension and other respiratory conditions were more common in the patient group. Full demographic details for each group are outlined in **Table 6**.

Table 6: Demographics in the study: Tussive challenge with ATP and AMP – does it reveal cough hypersensitivity?

	Healthy Volunteers	**Chronic Cough Patients**
Gender (Male: Female)	6:14	6:14
Age (**Median**: Range)	**43**: 23-74	**71**: 27-83
Race (Caucasian: Non-Caucasian)	18:2	19:1
FEV1 % Predicted (**Median**: Range)	**101%**: 55-121	**88%**: 57-128
Hull Cough Hypersensitivity Score (**Median**: Range)	**1.**5:0-8	**35.5**:21-52
Completed Challenges (AMP: ATP)	19:20	20:19
Number of self-reported co-morbidities (**Median**: Range)	**0**: 0-4	**2**: 1-6

9.6.2 Medications

Similarly, the patient group tended to be taking more medications. One participant in each group was taking ACE-inhibitors. One healthy volunteer was taking a PPI compared to six patients taking a PPI, two taking ranitidine, 2 taking metoclopramide and one patient on both a PPI and ranitidine. One of the healthy volunteers was on the OCP and one was on HRT.

9.6.3 Baseline spirometry

One of the healthy volunteers had mildly obstructed spirometry. Six of the chronic cough patients had obstructed spirometry. One chronic cough patient was unable to fully complete spirometry due to coughing despite multiple attempts.

9.6.4 Hull Cough Hypersensitivity Questionnaire (HCHQ) score

The patient group had HCHQ scores ranging from 21 to 52 out of 70. The median was 35.5 and the mean was 35.65.

The healthy volunteers had HCHQ scores ranging from 0 to 8 out of 70 with a median of 1.5 and a mean of 2.05.

9.6.5 Challenge completion

Twenty healthy volunteers completed the ATP challenge, nineteen completed the AMP challenge as one volunteer was withdrawn after their first challenge for safety reasons (see details under 'adverse events').

Nineteen chronic cough patients completed the ATP challenge, twenty completed the AMP challenge as one patient chose to withdraw from further challenges after their first challenge (see details under 'adverse events').

9.6.6 Healthy volunteers cough challenge

9.6.6.1 Comparing ATP and AMP

2/19 healthy volunteers coughed with AMP (one healthy volunteer was not challenged with AMP due to an adverse event with the previous challenge). One healthy volunteer achieved C2, neither achieved C5. In total throughout the AMP challenges there were only four coughs.

Two healthy volunteers did not cough at all in response to the ATP challenge. The remaining eighteen all achieved C2 with fifteen achieving C5. The difference between the ATP and AMP challenges in the number of healthy volunteers reaching C2 and C5 was statistically significant ($p < 0.001$). The results of the individual healthy volunteer cough challenges are shown in **Figure 8** below.

9.6.6.2 ATP 'threshold'

Only one healthy volunteer reached C5 at a low concentration of ATP (0.3mM). The remainder all reached C5 at the three higher concentrations (30, 100 and 300 mM). Some volunteers achieved C2 and C5 at the same concentration.

9.6.7 Cough hypersensitivity patients

9.6.7.1 ATP vs. AMP

Ten of the chronic cough patients coughed at least once in response to AMP. Of these, eight achieved C2 and two achieved C5. Within individuals cough response was erratic.

Having coughed at least twice patients often then did not cough at any of the other higher concentrations. These results are outlined in **Figure 9**.

All of the patients coughed in response to ATP. They all achieved C2. One patient only did not achieve C5, and they coughed four times at the two highest concentrations. These results are outlined in **Figure 10**.

The two patients who achieved C5 for both challenges, both achieved C5 at a lower concentration of ATP than AMP.

The difference between the ATP cough challenge and the AMP cough challenge in the number of patients reaching C2 and C5 challenges was statistically significant ($p = 0.001$ and <0.001 respectively)

9.6.7.2 ATP 'threshold'

All patients who reached C5 did so by a concentration of 100mM. The C5 in chronic cough patients was in general distributed between 1mM and 100mM.

Figure 8: Number of coughs at each concentration of ATP in 20 individual healthy volunteers. N/S: normal saline; ATP: adenosine triphosphate.

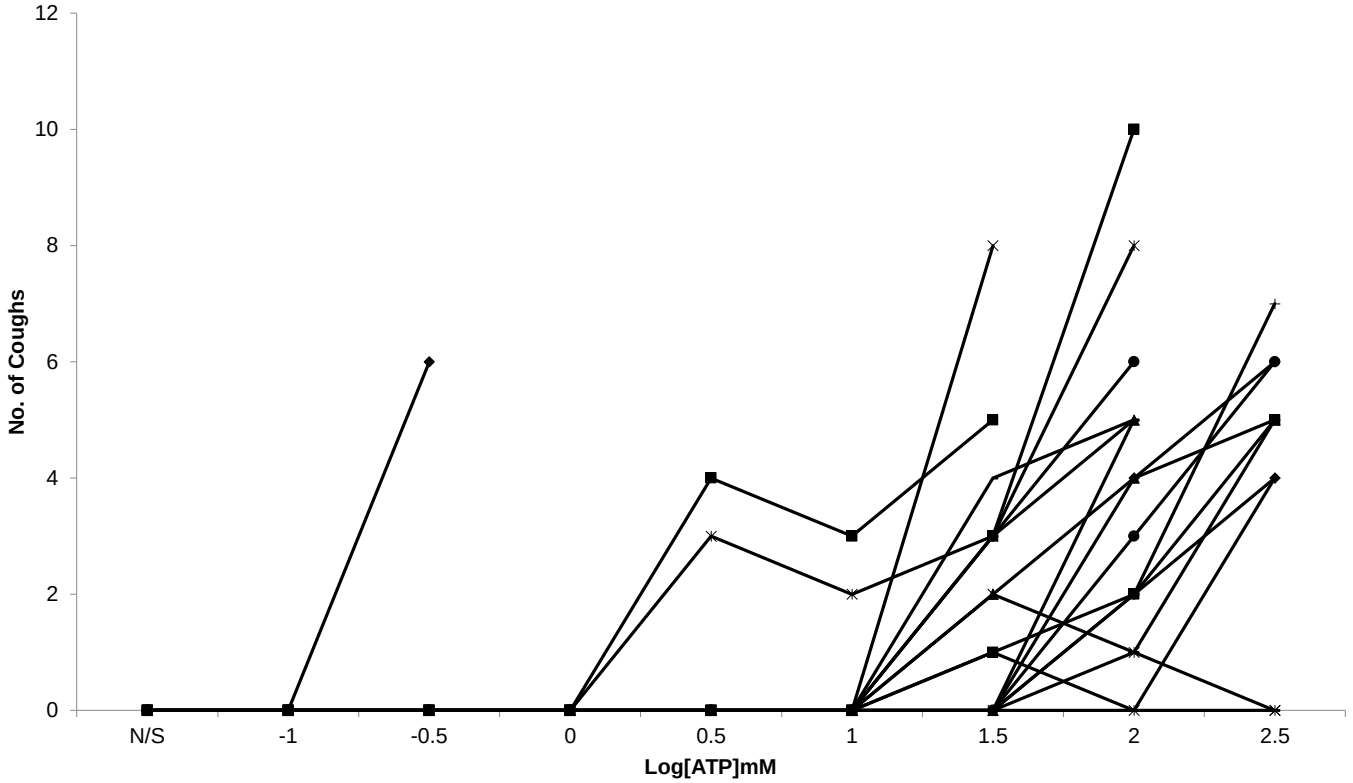


Figure 9: Number of coughs at each concentration of AMP in 20 chronic cough patients. N/S: normal saline; AMP: adenosine monophosphate.

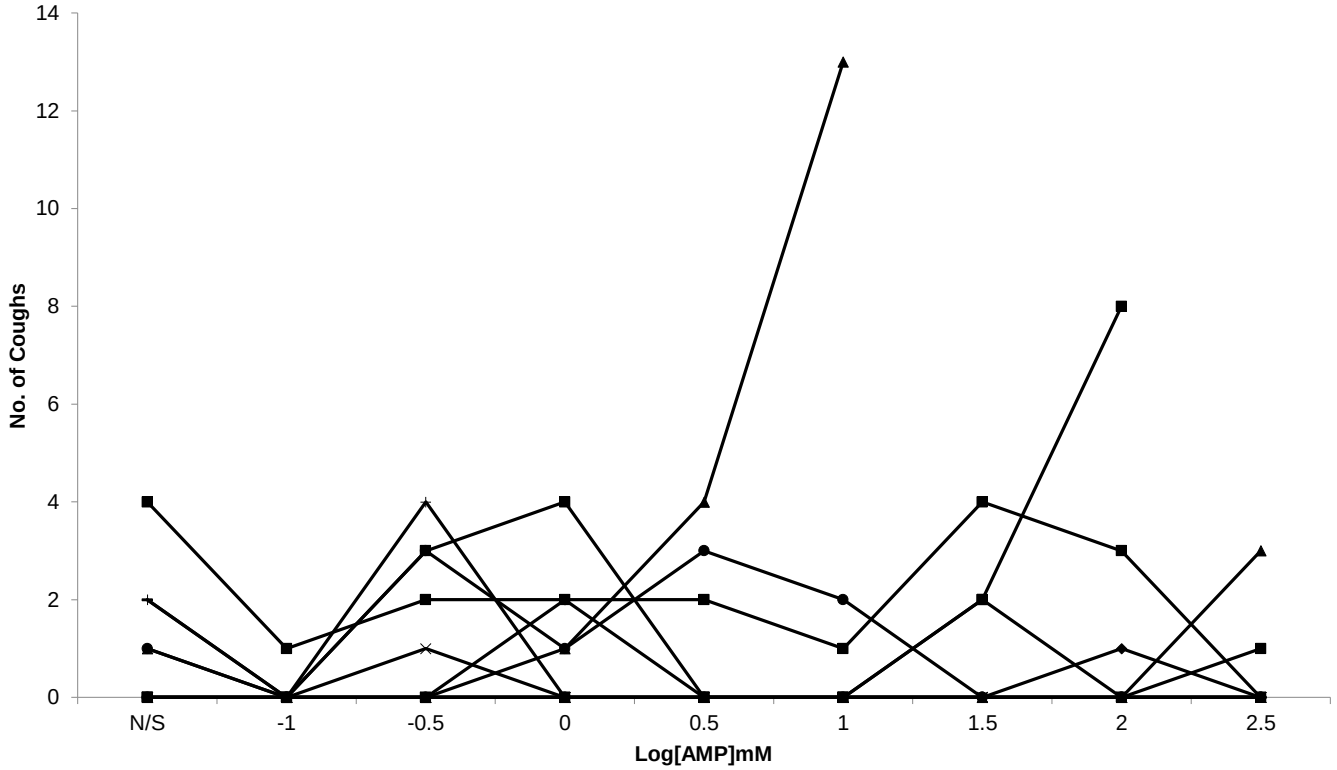
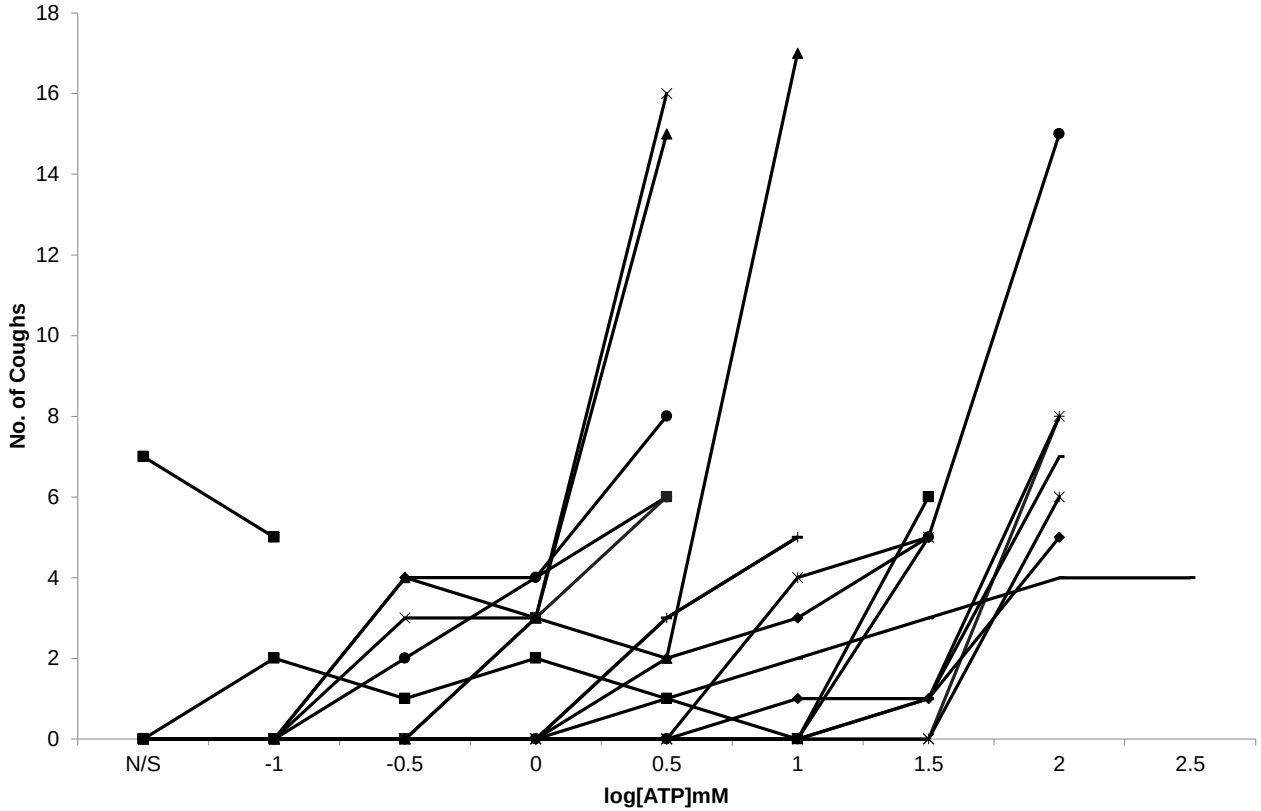


Figure 10: Number of coughs at each concentration of ATP in 19 chronic cough patients. N/S: normal saline; ATP: adenosine triphosphate.



9.6.8 N/saline response

Whereas none of the healthy volunteers coughed in response to the initial inhalation of N/saline, six of the chronic cough patients did. All of these only coughed in response to N/saline prior to one of their challenges. This was not consistently on their first exposure to a cough challenge substance. Three coughed on their first ATP/AMP challenge and three coughed on their second – however some of these would also have participated in other arms of the cough challenge study prior to their ATP/AMP challenges.

Only one patient coughed with the N/saline before their ATP challenge, however they coughed five times in response to N/saline and then achieved C5 at the lowest concentration of ATP.

The remaining five patients who coughed in response to N/saline all did so prior to their AMP challenges. One patient, who coughed four times in response to N/saline, then proceeded to cough in response to all but one of the subsequent inhalations on this challenge, never achieving C5. One patient who coughed with N/saline did not then cough at all in response to AMP. Out of the two patients who achieved C5 with AMP, one coughed in response to N/Saline at the start of the challenge, and one did not. Of the eight patients that achieved C2 with AMP, four coughed in response to N/saline at the start of their challenge and four did not.

9.6.9 Comparing healthy volunteers and cough hypersensitivity patients

The distribution of the C2 and C5 to ATP in healthy volunteers and patients is outlined in **Figures 11** and **12**. Healthy volunteers and patients C2 to ATP was statistically significantly different ($p = 0.047$). This was also the case for C5 ($p < 0.01$).

The average number of coughs at each concentration of ATP for healthy volunteers and chronic cough patients is compared in **Figure 13**.

Figure 11: Box and whisker plots showing distribution of C2 during adenosine triphosphate challenge in healthy volunteers compared to chronic cough patients. C2: the concentration of ATP causing at least 2 coughs.

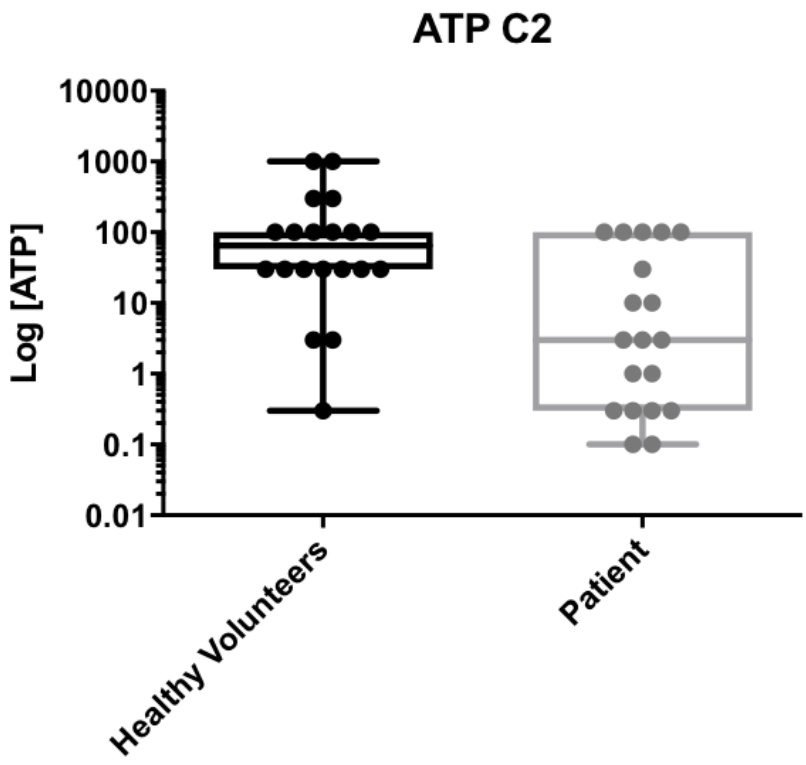


Figure 12: Box and whisker plots showing distribution of C5 during adenosine triphosphate challenge in healthy volunteers compared to chronic cough patients. C5: the concentration of ATP causing at least 5 coughs.

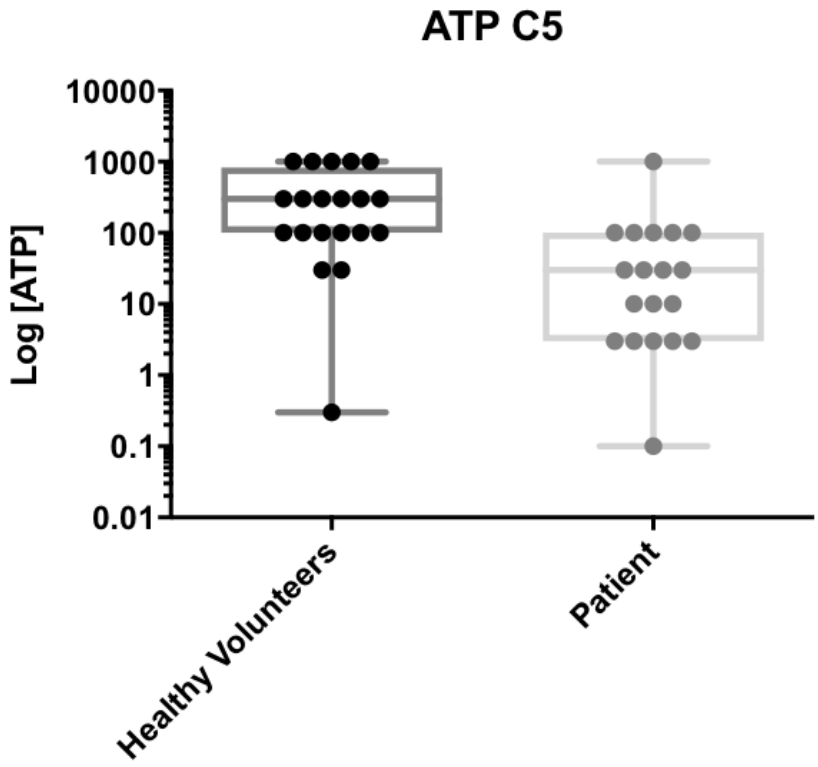
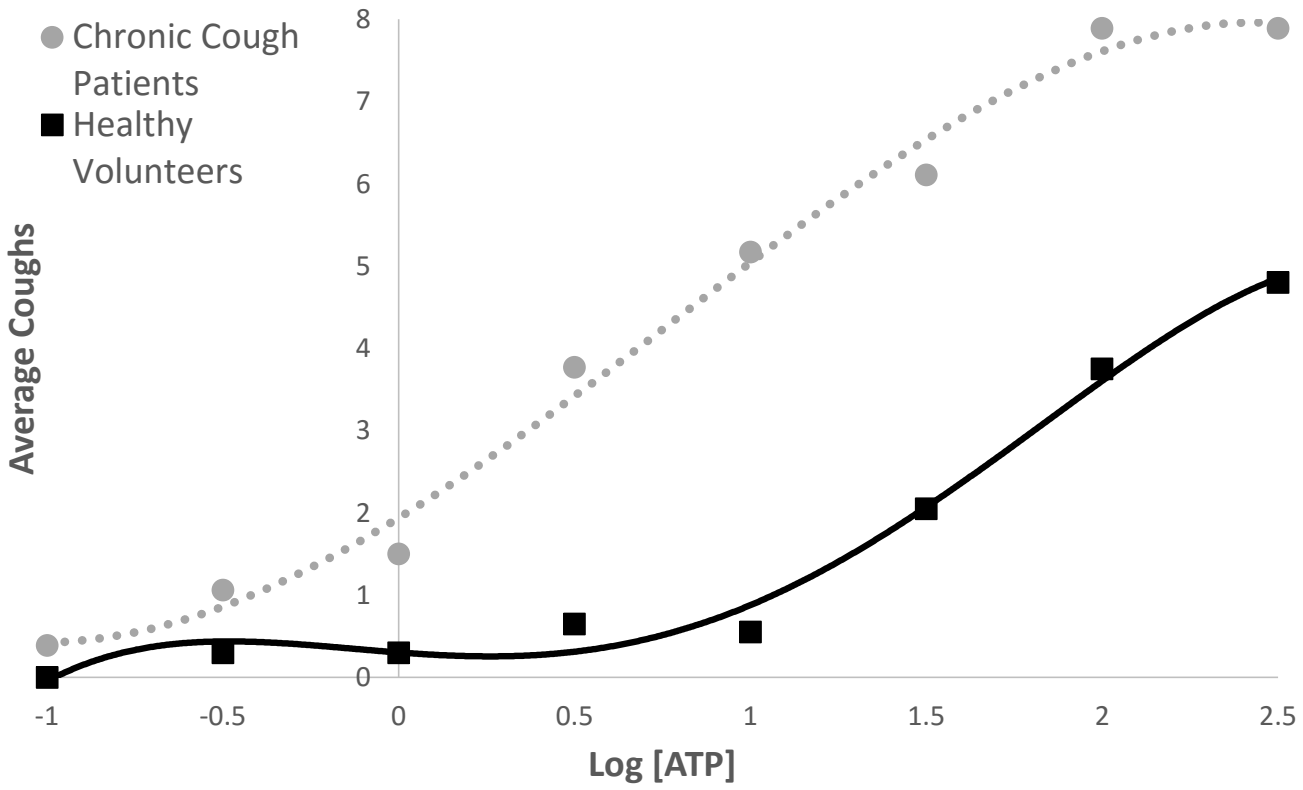


Figure 13: Comparison of mean coughs to each concentration of adenosine triphosphate in healthy volunteers and chronic cough patients.



9.6.10 Adverse events

One healthy volunteer had an episode of urticaria in the 24 hours following inhalation of the ATP challenge and was withdrawn from the study. There was one episode of wheeze following AMP in a hypersensitivity cough patient which resolved following administration of inhaled salbutamol.

One patient withdrew after their first challenge as they had felt that cough was increased in the days after the challenge.

Participants in both groups informally reported that they had throat irritation which lasted for up to several hours after the ATP challenge.

9.7 Discussion

Previous inhalational challenges using ATP and AMP have been carried out comparing healthy volunteers with asthmatics and COPD patients respectively (Basoglu et al, 2015; Basoglu et al, 2005). The main end point of these was considering bronchodilation, however the symptom of cough produced by inhaling both ATP and AMP was commented on. It was not however objectively measured.

I therefore believe that these are the first results objectively measuring and confirming that inhalation of ATP produces a dose dependent cough response in healthy volunteers.

The lack of a significant cough response to inhaled AMP in the healthy participants seems to be in contrast with previous studies. This may be due to methodological differences given the brief exposure of the participants consequent on the use of the single breath inhalation method. Whilst the majority of healthy volunteers coughed with ATP two did not. This is in keeping with the experience of other cough challenges such as citric acid and capsaicin, where a proportion of healthy volunteers do not cough within the range of the challenge. ATP challenge does not appear therefore to be exceptional in its sensitivity or persistence. Thus, while my findings support the importance of purinergic receptors in the normal cough reflex pathway, it is not possible to differentiate between the P2X3 and P2X2/3 as the main modulator of this tussive response to ATP.

When comparing healthy volunteers with chronic cough patients, the patient group coughed significantly more, and at lower concentrations of ATP. However, the degree of hypersensitivity demonstrated by the patient group to ATP does not appear to be any more

than previously seen in other cough inhalational challenges (Kastelik et al, 2002; Wright et al, 2010; Young et al, 2010). This suggests that chronic cough patients do not have an intrinsically heightened sensitivity to ATP and, thus it is not the acute, peripheral response to ATP that underlies the cough hypersensitivity in these patients.

AF-219 (a P2X3 receptor antagonist) (Afferent pharmaceuticals) has been trialled in a phase 2 study and has been found to dramatically reduce 24-hour cough counts after two weeks administration in patients with chronic cough (Abdulqawi et al, 2015). My data would tend to support the hypothesis that while P2X3 receptor activation may be the final common pathway producing hypersensitivity, acute activation of the receptor does not infer this state on afferent nerves and that other mechanisms such as activation of TRPV4 / pannexin are required.

The cough response of the patients to AMP was less clearly delineated from that of ATP. Two patients appeared to have a very dose-dependent response to both substances. Given this, there is a possibility that other purinergic receptors that respond to adenosine rather than ATP (P1 receptors) may also be involved in cough hypersensitivity.

Other patients, however, seemed to cough randomly at various inhalations of AMP. In addition, chronic cough patients were more likely to cough on the first inhalation of normal saline than the healthy volunteers, and this was borne out in some other challenges carried out in tandem with this study. There did not seem to be any relationship between patient's tendency to cough with AMP and their tendency to cough with normal saline.

This supports previous experience in my department of working with cough challenges in chronic cough patients. Their cough reflex seems to be not only hypersensitive but also unpredictable – suggesting an inherently unstable reflex pathway, like a tightly wound spring. It will be set off by lower threshold stimulus than healthy volunteers, but it is very variable what this threshold will be, with both intra- and inter- patient variability. This is consistent with patient reporting in cough clinic, and during the study, that their symptoms (urge to cough, throat sensitivity etc.) seem to vary from day to day.

9.7.1 Differences in demographics between the two groups

It is worth noting that whilst the two groups were gender matched, they were not age matched, with the patient group tending to be older than the healthy volunteers. Chronic cough tends to be more prevalent in older patients (Morice et al, 2014a) however previous studies are not consistent in whether age affects the cough reflex (Ebihara et al, 2011) .

The racial variation in both populations was minimal and reflects the clinical practice in Hull. It would be interesting to carry out the ATP cough challenge in a different country to see if there is any evidence of racial variation. However previous studies with other cough challenges have not found any particular difference dependent on race (Dicpinigaitis et al, 2001).

Co-morbidities were also more prevalent in the patient group. With the majority of these being conditions which are often associated with cough, such as GORD, asthma and PND as well as IBS and lymphoedema. These co-morbidities were self-reported by patients. The increased prevalence of them in the patient group may be a reflection of the older age of these patients. Or may reflect the fact that many of them have been diagnosed with a variety of conditions in the search for a cause of their cough.

ACE-inhibitors are a well-recognised cause of increased cough hypersensitivity (Morice et al, 1987) and interestingly, there was one patient in each of the groups on an ACE-inhibitor. These participants do not appear to be any more sensitive to ATP than others. In this respect, the two groups were well matched, and although the sample is too small to draw any definitive conclusions, it is possible that ACE inhibitors act via a different mechanism to ATP.

Due to the higher prevalence of chronic cough in women there has been some suggestion that female hormones play a role in cough hypersensitivity. A number of the healthy volunteer group were on either the OCP or HRT which may affect their cough reflex.

Cough is documented as a side effect of statins and a number of participants in both groups were on this group of drugs.

Spirometry was generally more obstructed in the patient group, which may have influenced the differing results in the two groups. Whilst none of the participants complained of symptoms of bronchoconstriction with ATP (in contrast to AMP), it is recognised as causing bronchoconstriction and therefore further study may be warranted, analysing whether the brief exposure of a cough challenge to ATP also causes bronchoconstriction.

9.7.2 Limitations in analysis

There has been some debate previously about whether absolute extrapolated values for C2 and C5 are more appropriate than simply using the concentration of challenge substance at which the participant coughs twice or five times. Given the lack of previous

experience with ATP, I didn't know initially whether I was going to reveal a dose-response curve, and therefore I have not extrapolated values. Given the presence of the apparent dose response, it may be possible in future experiments to extrapolate an absolute value.

Whilst there is a precedent for using an upper limit of 1,000mM as the C2 or C5 for those patients who didn't achieve it, this may skew the results analysis that has been completed here.

9.7.3 Safety

Within the population of forty participants, I only had one adverse effect to ATP. This does appear to have been a true hypersensitivity reaction, and occurred in a participant who has had previous hypersensitivity reactions to other substances. As a result of this I would recommend that any future ATP challenges exclude participants who have had previous anaphylactic or severe hypersensitivity reactions.

Spirometry was not measured after inhalation of the cough challenges, although patients were asked to report any symptoms of bronchospasm such as chest tightness or wheeze, and the only patient to report this had an underlying diagnosis of asthma and reported these symptoms after inhalation of AMP which is sometimes used as a challenge substance in the diagnosis of asthma. This patient's symptoms resolved after administration of inhaled salbutamol.

9.7.4 Areas of future interest

This data supports the role of P2X receptors in the pathophysiology of cough hypersensitivity. These are therefore an interesting area for further work, both on a molecular, cellular and clinical level, to investigate further whether there is a difference in these receptors in patients who suffer from hypersensitivity cough syndrome.

Whilst my data has supported the tussive nature of ATP, I have not been able to delineate the mechanism by which ATP is postulated to lead to cough hypersensitivity. It would be of interest to investigate further whether inhalation of ATP leads to sensitisation to other cough challenges.

Further work is already ongoing with clinical trials to further investigate the use of the P2X3 receptor blocker AF-219 in the treatment of chronic cough. Other similar agents may also be of use in the treatment of chronic cough and warrant further investigation. It

is also possible that they may be of use in other vagally related conditions such as irritable bowel syndrome and chronic pain syndrome.

9.8 Conclusion

I believe that this is the first study to compare objective cough response to inhaled ATP and AMP in healthy volunteers and chronic cough patients. The response to ATP in chronic cough appears to be heightened, but not to such a degree to implicate the acute response to inhalation of ATP in the pathophysiology of cough hypersensitivity syndrome.

10 Comparison of four cough challenges – Fog, Capsaicin, Citric Acid and ATP

10.1 Introduction

As previously described, the most common cough challenges employed in clinical trials of antitussives are capsaicin, citric acid and fog. Capsaicin cough challenge has been extensively described as being repeatable (Collier & Fuller, 1984; Dicpinigaitis, 2003; Nejla et al, 2000); citric acid appears to be repeatable (Bickerman & Barach, 1954); there is less information available about fog, but this has also been suggested to be repeatable (Fontana et al, 2002). However, most previous experiments across these challenges have focussed on healthy volunteers. Limited information is known about their repeatability in chronic cough patients, although they have all been utilised in assessing potential antitussives.

It is therefore difficult to determine which, if any of these challenges would be the most useful in assessing the anti-tussive effect of new agents such as AF 219.

It is also possible that none of these three commonly used challenges would show any effect with a new anti-tussive as their modes of action are not fully understood. Using a specific ATP challenge to measure the effect of a P2X receptor blocker could be a more accurate measure of effect. However, the use of ATP as a cough challenge is novel and its reproducibility is untested.

In order to test new antitussives that come onto the market, and treatments for chronic cough, a reliable, repeatable test is required. It has been assumed that cough challenges may provide this. However, my previous work suggested that given the labile nature of the cough response in hypersensitive cough patients, repeatability of the cough challenge over time cannot be assumed.

Citric acid cough challenge has previously been shown to correlate weakly with capsaicin cough challenge (Wong et al, 1999), suggesting only a partial sharing of mode of action. The correlation between ATP and the other challenges is unknown, although as they are anticipated to act on different receptors, it would be expected that ATP would lack correlation with the other challenges.

This experiment was designed to compare the repeatability and mode of action of four cough challenges – citric acid, capsaicin, fog and ATP.

10.2 Hypotheses

Inhaled ATP cough challenge is as reproducible in both chronic cough patients and healthy volunteers as citric acid, capsaicin and fog.

Inhaled ATP cough challenge doesn't correlate with fog, citric acid or capsaicin cough challenge due to different mechanism of action

10.3 Methods

This was a prospective observational cohort study.

10.3.1 Recruitment

Patients were recruited by Clinical Trials Unit staff and myself from the Hull cough clinic and both patients and healthy volunteers were recruited from the Clinical Trials Unit database of volunteers. The recruitment target was 24 cough patients and 12 healthy volunteers.

All participants had to be between 18-80 years of age, could provide informed consent in English, had a BMI of between 18 and 35, were in good general health and were current non-smokers. Healthy volunteers also had to have normal spirometry.

Chronic cough patients had treatment refractory cough, which was unresponsive to targeted treatment for potential triggers (GORD, asthma or post nasal drip), with no clear underlying cause after investigation, and scored >20/70 on the Hull Cough Hypersensitivity Questionnaire.

10.3.2 Exclusions

Participants were excluded if they had an upper respiratory tract infection or a change in their respiratory status within four weeks of the baseline visit, including an acute asthma exacerbation. Prohibited medications throughout the study period included ACE-inhibitors and opioids.

Screening was failed if participants did not cough to ATP, capsaicin or citric acid, or only achieved C2 at the very top concentrations in two out of the four tests. In addition, any participant who coughed more than twice when inhaling normal saline in any of the challenges was also excluded at this stage.

As these challenges were carried out at the baseline of a drug trial for Afferent Pharmaceuticals, volunteers were also excluded if they did not meet all the criteria to receive an unlicensed product safely.

Participants were asked to refrain from consuming alcohol, caffeine and menthol for eight hours prior to cough challenges.

10.3.3 Baseline measurements

Other measurements recorded at baseline, by Clinical Trial Unit staff, were demographics, medication, spirometry, Hull Cough Hypersensitivity Questionnaire, urge to cough and cough severity visual analogue scores for the chronic cough patients.

Chronic cough patients and healthy volunteers underwent four different cough challenges (ATP, capsaicin, fog, citric acid) at visit one and visit two, separated by at least seven days.

The challenges were administered by Clinical Trial Unit staff who had received training in the study protocol and the standard operating procedures for each challenge (see **Appendices 2, 3, 4** and **5**). They had all performed cough challenges previously, many for other studies.

10.3.4 ATP cough challenge method

ATP cough challenge was performed as per the protocol described previously in chapter seven.

10.3.5 Citric acid cough challenge method

Citric acid cough challenge was performed as per the Hull clinical trials SOP (see **Appendix 3**) which is based on the ERS standardised protocol (Morice et al, 2007a) using the KoKo DigiDoser. For this study serial log doses were utilised.

10.3.6 Capsaicin cough challenge method

Capsaicin cough challenge was performed as per the Hull clinical trials SOP (see **Appendix 5**) which is based on the ERS standardised protocol (Morice et al, 2007a) using the KoKo DigiDoser. For this study serial log doses were utilised.

10.3.7 Nebulisation methodology

All three of the above challenges used the KoKo DigiDoser, single breath method, which has been described more extensively in chapter seven. Unfortunately the previously described fixed straw and baffle device with an incorporated flow rate limiter is no longer available, therefore for all of the challenges using the KoKo DigiDoser, unfixed mouthpieces which incorporated a flow whistle were utilised and patients were trained to control flow rate prior to performing cough challenges.

10.3.8 Fog Challenge methodology

For the Fog challenge, solutions with gradually reducing concentrations of chloride ions were created by combining different combinations of normal saline and distilled water. These were then nebulised Ultrasonically using a DeVilbiss nebuliser. Participants tidally breathed the nebuliser solution for one minute during which time coughs were counted.

Preliminary work had suggested that this method was the easiest way to control concentration of chloride ions produced by the nebuliser in order to measure a cough threshold comparable to the other cough challenges. This is similar to previous methods of fog challenge (Lowry et al, 1988b).

10.3.9 Randomisation of challenges

Participants were randomised to the order in which the four challenges were given. They were also not specifically informed which substance they were inhaling on each challenge, although true blinding was not possible due to the variance in methods required for nebulisation.

The challenges were performed at least an hour apart on visit one, and ten minutes apart on visit two.

C2 and C5 were recorded for each of the four challenges on both occasions.

10.3.10Statistical analysis

For the purposes of statistical analysis, if C2 or C5 wasn't achieved within the concentrations of the soluble solution, this was set at a half log dose above the strongest dose.

C5 was compared between chronic cough patients and healthy volunteers using box and whisker plots, and Mann-Whitney U analysis.

Intra-subject variability was analysed using the Bland-Altman method. Repeatability can be accepted when 95% of the calculated differences between the values of the two visits for each tussive agent, lie within ± 2 standard deviations (SD) of the mean difference (Bland & Altman, 2007). For ease of comparison with previous studies, intraclass co-efficients were also calculated.

Correlation between ATP and the other tussive agents was calculated using Pearson's correlation coefficient

10.3.11Approvals

This data was collected as part of a Study to assess the Effect of AF-219 on Cough Reflex Sensitivity in Both Healthy and Chronic Cough Subjects – Protocol number AF219-014. Sponsored by Afferent Pharmaceuticals. Ethics approval was obtained from the Yorkshire & The Humber – Sheffield Research Ethics Committee. REC reference 15/YH/0400. IRAS ID: 184954. The trial was registered on the UK National clinical trials database. NCT02476890 EUDRACT No: 2015-002034-47

10.4 Results

Table 7: Demographics in the study: Comparison of four cough challenges – Fog, Capsaicin, Citric Acid and ATP.

	Healthy Volunteers (N = 12)	**Chronic Cough Patients (N=24)**
Gender (**F**:M)	**11**:1	**20**:4
Age (**Median**: Range)	**38**:26-52	**63**:48-73
BMI (**Median:** Range)	**24.39**:19.2 - 36.4	**25.4**:18.1 - 33.6
FEV1 % predicted (**Median**: Range)	**100%**:89-117	Not measured

Table 8: Baseline cough severity scores for chronic cough patients.

Score	Median	Range
HCHQ	37.5	20 - 59
VAS Severity Score	71	29 - 91
VAS Urge to cough score	74	22 - 93

10.4.1 Demographics

Twelve healthy volunteers and twenty-four chronic cough patients were included in the data analysis. Both groups had a considerably higher proportion of females than males, with 92% of the healthy volunteers being female, and 83% of the chronic cough patients. The age range was younger in the healthy volunteers group (26 – 52) compared to the chronic cough group (48-73). BMI was similar in both groups with medians of 24.4 and 25.4 respectively. The FEV1 as a percentage of predicted in the healthy group fell within a range of 89 to 117 percent of predicted. Full demographics for each group are outlined in **Table 7** above.

Baseline cough severity scores performed for the chronic cough patients showed a large range. These are outlined in **Table 8** above.

10.4.2 Comparing responses between chronic cough patients and healthy volunteers for each of the four challenges

Table 9 compares the mean and standard deviation of the cough response for chronic cough patients with that of healthy volunteers for each of the four challenges. All means are expressed as Log[C5].

T-tests for differences in the means across all four challenges show significant differences between healthy volunteers and chronic cough patients. The p-values are displayed in **Table 9**.

Table 9: Comparison of the means for the four challenges.

Challenge Agent	**Chronic Cough Patients:** Mean (SD)	**Healthy Volunteers:** Mean (SD)	**p-value**
ATP	0.33 (0.99)	2.12 (0.57)	<0.001
Fog	3.01 (0.16)	3.15 (0.79)	0.001
Capsaicin	0.60 (0.59)	1.71 (0.54)	<0.001
Citric Acid	1.39 (0.91)	2.67 (0.39)	<0.001

Figures 14-17 below summarise the cough responses to each of the four baseline challenges, comparing chronic cough patients with healthy volunteers.

Figure 14: Box and whisker plot comparing cough response to ATP in chronic cough patients and healthy volunteers - expressed as Log[C5] – where [C5] is the concentration of inhaled ATP causing the subject to cough at least 5 times.

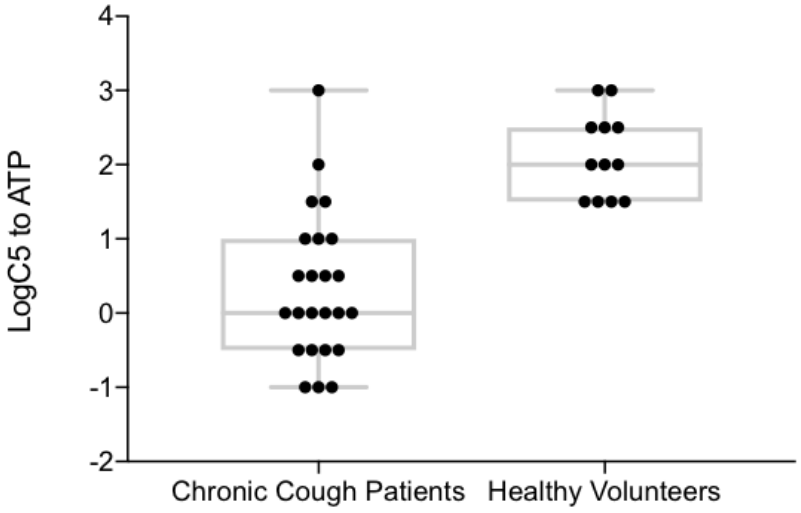


Figure 15: Figure 15 Box and whisker plot comparing cough response to citric acid in chronic cough patients and healthy volunteers - expressed as Log[C5] – where [C5] is the concentration of inhaled citric acid causing the subject to cough at least 5 times.

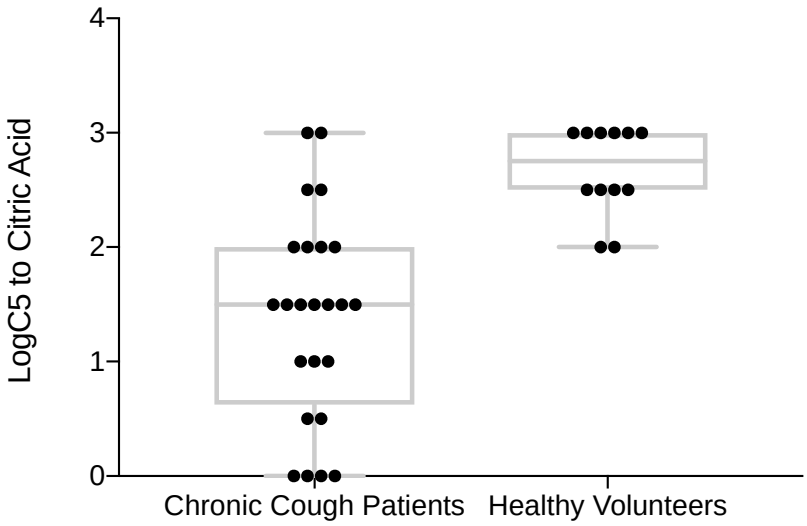


Figure 16: Box and whisker plot comparing cough response to fog in chronic cough patients and healthy volunteers - expressed as Log[C5] – where [C5] is the concentration of inhaled fog causing the subject to cough at least 5 times.

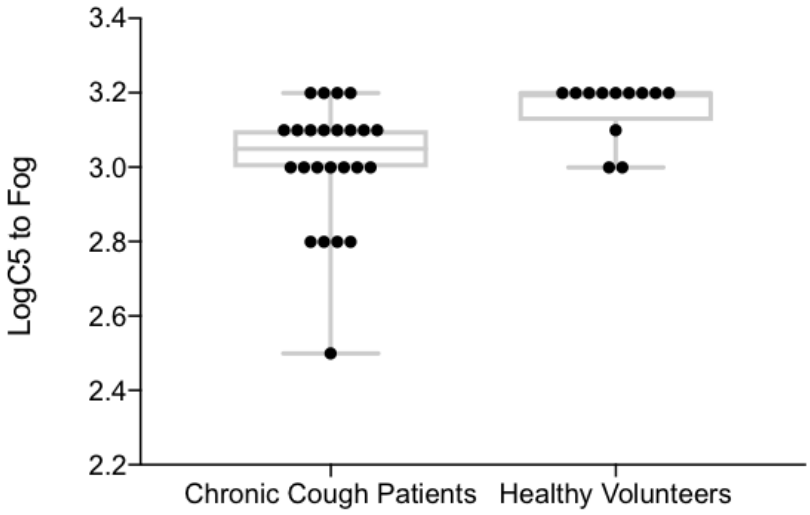
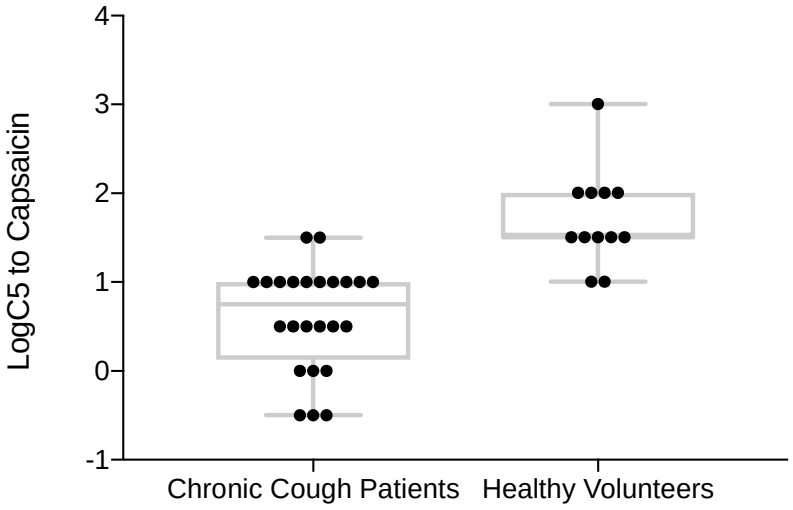


Figure 17: Box and whisker plot comparing cough response to capsaicin in chronic cough patients and healthy volunteers - expressed as Log[C5] – where [C5] is the concentration of inhaled capsaicin causing the subject to cough at least 5 times.



10.4.3 Intra-subject variability

The difference in Log[C5] between first and second cough challenge visit for the study population was compared for each of the four challenges. The Bland-Altman plots are shown in **Figures 18-21**. These plot the mean of the two visits against the absolute difference in the Log[C5] response at first and second visit. Where two or more data points fall at the same point on the graph, these are expressed using larger circles.

Table 10 shows the mean difference, (solid line on **Figures 18-21**) standard deviation of the differences and upper and lower limits (two standard deviations in each direction – the dotted line on **Figures 18-21**) on the Bland-Altman plots. Standard correlation co-efficients are included to allow comparison with previous studies.

Table 10: Bland-Altman values and Intra-class correlation co-efficients

Difference	**ATP**		**Fog**		**Citric Acid**		**Capsaicin**	
	Cough	Healthy	Cough	Healthy	Cough	Healthy	Cough	Healthy
Mean	-0.167	0.250	-0.013	-0.025	-0.167	-0.042	-0.042	0.292
SD	1.100	0.399	0.185	0.075	0.830	0.498	0.706	0.689
Upper limit	2.033	1.048	0.357	0.126	1.493	0.955	1.370	1.671
Lower limit	-2.367	-0.548	-0.382	-0.176	-1.826	-1.038	-1.453	-1.087
Coefficient	0.412	0.772	0.341	0.343	0.528	0.058	0.248	0.320

One chronic cough patient and no healthy volunteers sit outside the limits for ATP cough challenge. Two chronic cough patients and one healthy volunteer sit outside the limits for fog cough challenge. For the citric acid cough challenge, two chronic cough patients and no healthy volunteers lie outside the limits. In these challenges, all those outside the limit have a negative difference. In the capsaicin challenge, one chronic cough patient had a negative difference below the lower limit and one healthy volunteer had a positive difference above the upper limit.

With the exception of the capsaicin cough challenge where the variation of differences in challenge response is similar in chronic cough patients and healthy volunteers, the variance in responses is smaller in healthy volunteers than in chronic cough patients.

Figure 18: Bland-Altman plot of agreements for log[C5] response to ATP inhaled cough challenge.

A: Chronic Cough Patients and **B**: Healthy Volunteers

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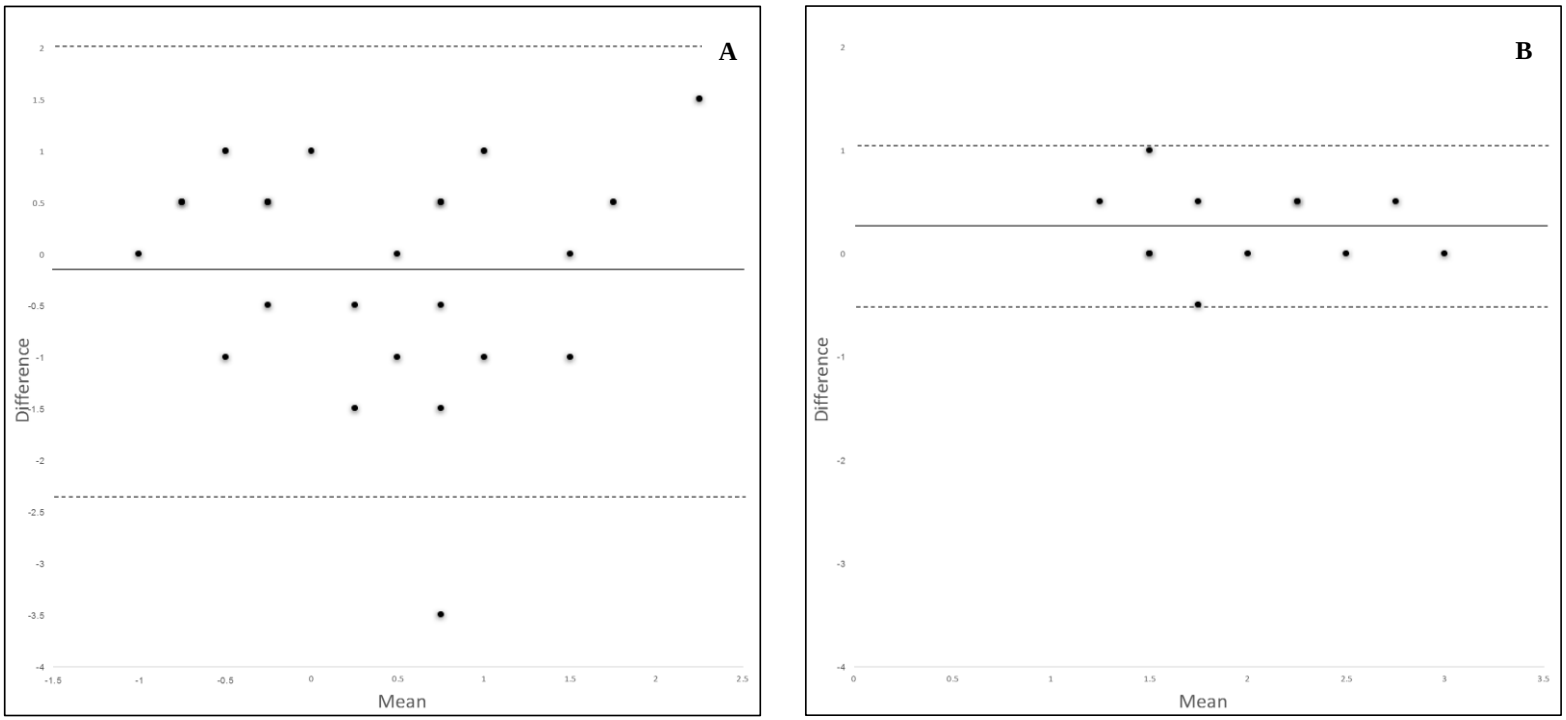
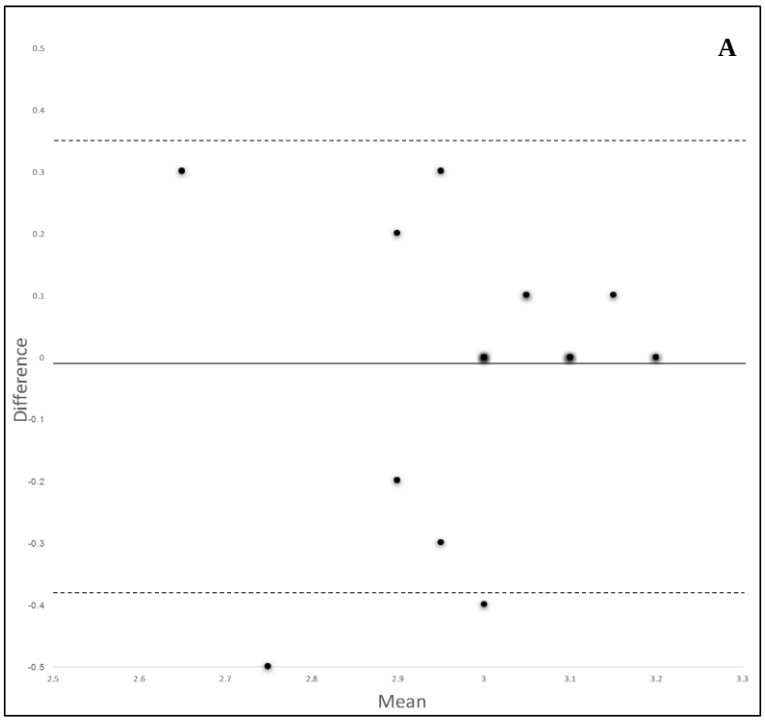


Figure 19: Bland-Altman plot of agreements for log[C5] response to fog inhaled cough challenge.

A: Chronic Cough Patients and **B**: Healthy Volunteers



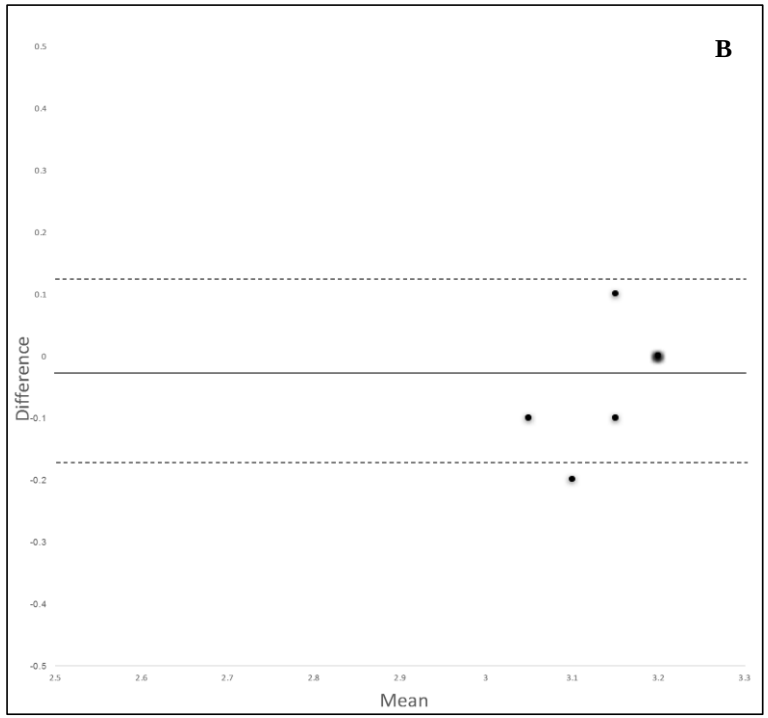


Figure 20: Bland-Altman plot of agreements for log[C5] response to citric acid inhaled cough challenge.

A: Chronic Cough Patients and **B**: Healthy Volunteers

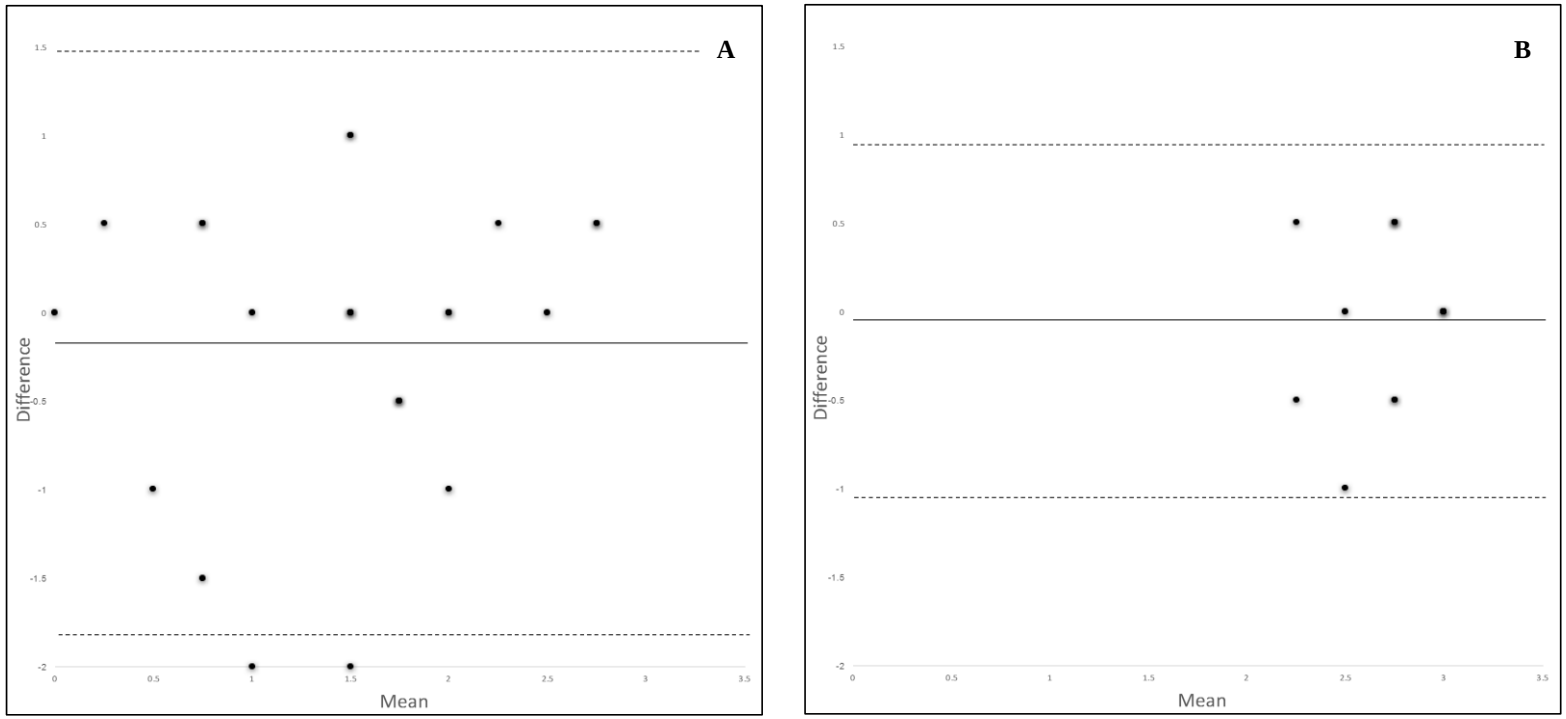


Figure 21: Bland-Altman plot of agreements for log[C5] response to capsaicin inhaled cough challenge.

A: Chronic Cough Patients and **B**: Healthy Volunteers

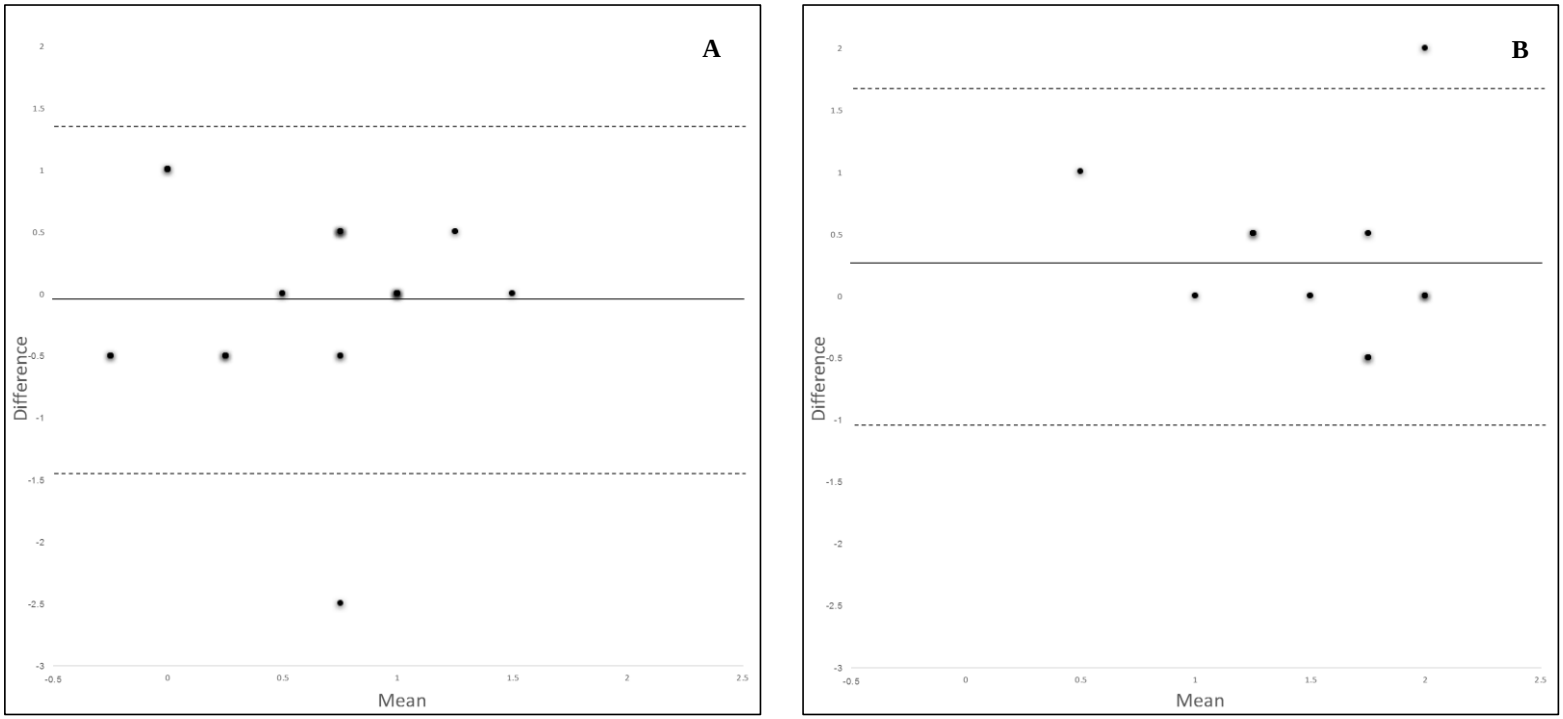


Figure 22: Cough response to ATP challenge compared to cough response to capsaicin challenge.

(A and B – C2, C and D - C5, A and C – Chronic Cough patients, B and D – Healthy Volunteers)

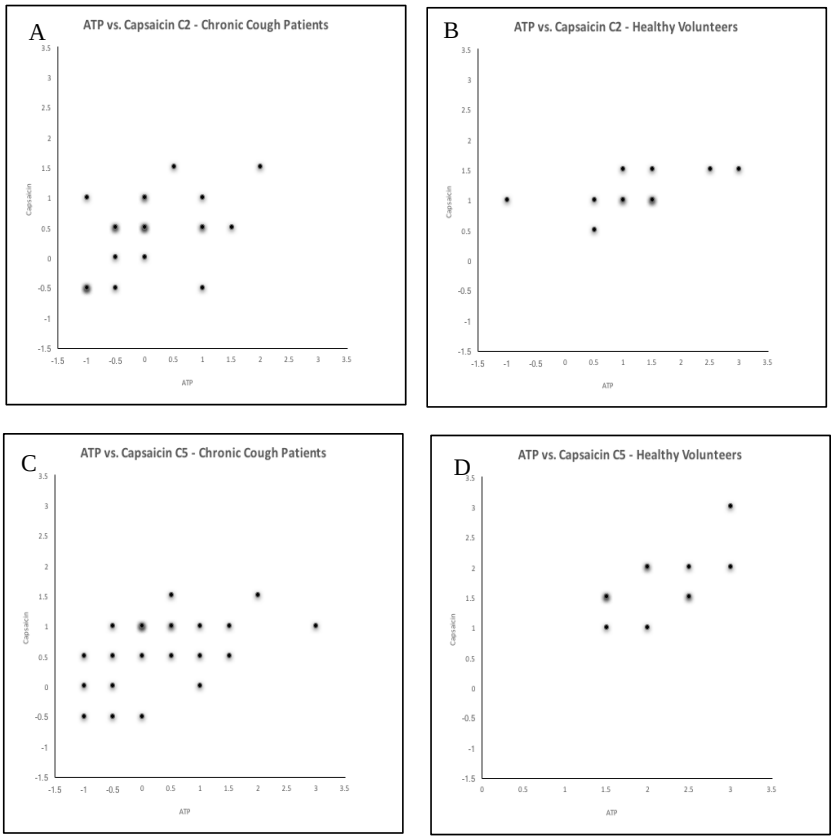


Figure 23: Cough response to ATP challenge compared to cough response to citric acid challenge.

(A and B – C2, C and D - C5, A and C – Chronic Cough patients, B and D – Healthy Volunteers)

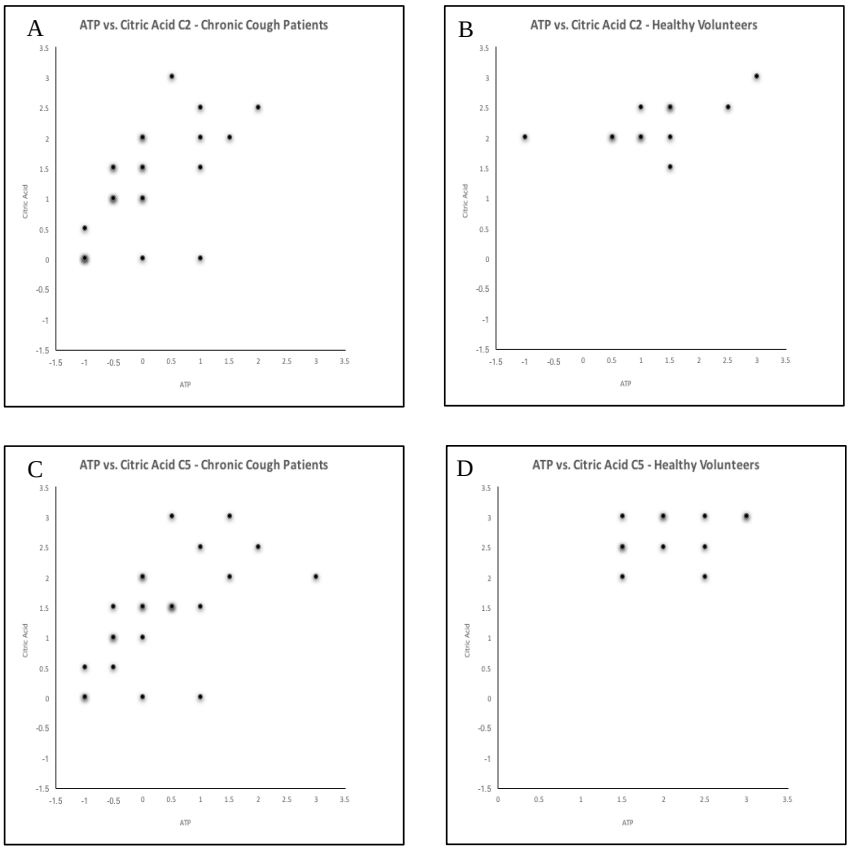
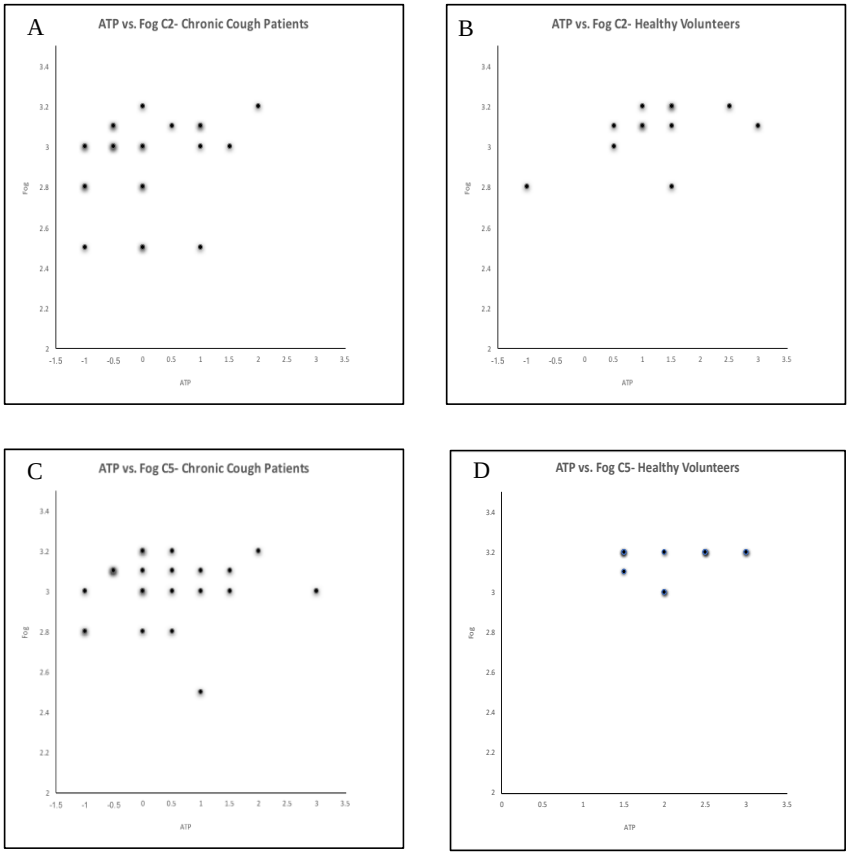


Figure 24: Cough response to ATP challenge compared to cough response to fog challenge.

(A and B – C2, C and D - C5, A and C – Chronic Cough patients, B and D – Healthy Volunteers)



10.4.4 Correlation between challenge response

Figures 22, 23 and **24** above show the correlation between C2 and C5 between the ATP challenges and each of the other three challenges. The correlation co-efficients are outlined in **Table 11** below.

The Log[C2] and Log[C5] values of the ATP cough challenge in both chronic cough patients appears to correlate slightly with both capsaicin and citric acid challenges, but not at all with fog challenge.

Table 11: Correlation co-efficients for data outlined in Figures 22-24.

ATP Cough Challenge Versus			**Correlation Co-efficients**
Capsaicin Cough Challenge (Figure 22)	C2	Chronic Cough	0.511
		Healthy	0.561
	C5	Chronic Cough	0.475
		Healthy	0.645
Citric Acid Cough Challenge (Figure 23)	C2	Chronic Cough	0.640
		Healthy	0.563
	C5	Chronic Cough	0.604
		Healthy	0.308
Fog Cough Challenge (Figure 24)	C2	Chronic Cough	0.227
		Healthy	0.514
	C5	Chronic Cough	0.078
		Healthy	0.227

10.5 Discussion

There have been few previous studies directly comparing inhalational cough challenges in healthy volunteers and chronic cough patients. Data is often extracted from previous studies to provide comparison. This is the first data which has compared healthy volunteers with chronic cough patients across four different cough challenge substrates within the same study, allowing comparison of the cough response of chronic cough patients to healthy volunteers, as well as a comparison of the four different cough challenges.

10.5.1 Comparison of chronic cough patients with healthy volunteers

Whilst all the four cough challenges investigated during this study show a difference in the cough response between healthy volunteers and chronic cough patients, there is overlap between the two groups whichever challenge is considered. In this study, there is very little difference between this overlap for each challenge. This suggests that none of the four challenges are better than each other at discriminating between chronic cough patients and healthy volunteers.

10.5.2 Intra-subject variability

Using the accepted limit that 95% of differences need to lie within 2SD of the mean difference, in this study population where two patients lie outside the limits, or one healthy volunteer does, this criterion is not met. Therefore, in this study the fog challenge and the citric acid challenge do not show good reproducibility. Minimal numbers of cough response differences for ATP and capsaicin in this study population sit outside the accepted limits of reproducibility which suggests good reproducibility of these challenges. However, when considered further there are still big differences between repeated challenges in some subjects across all four challenges. The majority show a tendency towards negative differences (i.e. the second cough challenge response was at a higher value). This is often expected for cough challenge and is why studies are often designed to have a screening test initially. It is possible that therefore this would be less of a problem for subsequent challenges. This has previously been referred to as a 'learning effect' (Morice et al, 1992) or 'startle phenomenon' (Dicpinigaitis, 2003). The startle phenomenon is the term used to describe the occurrence where the participant coughs at a lower dose on their very first exposure to capsaicin than on all their previous challenges. As a result of the startle phenomenon there has also been some debate about whether all first cough challenges should be excluded but this has not been supported during further investigation (Dicpinigaitis, 2003).

Intra-subject variability appears very similar for ATP, fog and citric acid challenges, with little difference in the Bland-Altman plots. Capsaicin appears to be slightly less variable for chronic cough patients, but unlike the others has a similar variability in healthy volunteers.

In this study, the variance in cough response between the two challenge occasions appears to be greater in chronic cough patients than healthy volunteers across all challenges except capsaicin. There are two potential reasons for this. Firstly, as all the cough response C5 concentrations are lower in patients it is possible that the cough challenge tests are more consistent at higher values. This is supported by previous data in repeatability tests for capsaicin cough challenge. Cough counts were similar at higher concentrations but more coughs were seen at lower concentrations on repeat testing (Midgren et al, 1992). However, this does not fully bear out with the capsaicin cough challenge data from this study.

The second possible explanation for the variance in cough response is that it is more marked in chronic cough patients due to a neuronal phenomenon, which would be supported by my previous observations that patients suffering from hypersensitivity cough syndrome have a very unpredictable response to inhalational cough challenge.

It is difficult to directly compare the variability of the cough challenges carried out in this study to previous data, given the usage of the more pharmacologically relevant logarithmic scale rather than doubling doses (which are often used as the solutions are easier to produce via serial dilution). In addition, previous studies of variability have tended to focus solely on healthy volunteers. However, the reproducibility of capsaicin, citric acid and fog appears to be broadly similar to previous studies.

The cough challenge for which there has been most reproducibility information previously is capsaicin. In the initial experiments it appeared to be very reproducible (Collier & Fuller, 1984). This repeatability has subsequently been judged as good in both dosimeter and tidal breathing method (within two doubling concentrations) (Nejla et al, 2000).

The short-term intra-subject reproducibility (less than fourteen days) of the capsaicin challenge is reasonably well established and has been further confirmed up to 100% if C5 is used (Dicpinigaitis, 2003). The longer term reproducibility (over six months) has also been shown to be reasonable at 90% for both C2 and C5 (Dicpinigaitis, 2003). Again, this

reproducibility is judged as good if repeated challenges are within two doubling doses of previous.

There is some contention as to whether C2 or C5 is the most reproducible (Dicpinigaitis, 2003; O'Connell et al, 1996). C2 seems to be more subject to the startle phenomenon (Dicpinigaitis, 2003). C5 requires higher doses of capsaicin to achieve and this in itself can cause problems.

Citric acid reproducibility has been suggested previously to be within one doubling dose. (Schmidt et al, 1997). It appears to be better with the KoKo DigiDoser than with the Mefar (Wright et al, 2010). However, the majority of subjects in these studies appear to have been healthy volunteers. Therefore, its reproducibility in chronic cough patients has not previously been explored, and it appears from my experiments to be less reproducible than previously thought. Direct comparison is difficult given the varying statistical analysis that has been utilised to check variability.

Fog reproducibility has been stated previously to be reproducible (Fontana et al, 1999). And this certainly seems true in my healthy volunteer cohort. It does not appear to be entirely true on the chronic cough patients, in keeping with the other challenges, in which there has been little previous exploration.

The reproducibility of the ATP cough challenge is explored for the first time in this study. It appears to be broadly similar to that of citric acid, although possibly slightly lower than that of capsaicin in the healthy volunteer group.

This data suggests that fog is the least reproducible in chronic cough patients, whilst capsaicin is the least reproducible in healthy volunteers. However, fog is difficult to compare in terms of absolute figures as the scale used is very different to the other challenges.

10.5.3 Correlation of cough challenges

ATP cough challenge responses positively correlate with both citric acid and capsaicin cough challenge. This suggests some overlap between the mechanisms of action in these substances in producing cough. This is a rather unexpected finding as one knows that they all have a different receptor as a target. Although possibly citric acid may target the other two receptors as one still doesn't really understand how it works. There are a number of potential explanations for this phenomenon; either there is some crossover of action of the substrates at different receptor types, or there could be crosstalk between different

receptors (ATP/TRPV4 as an example (Bonvini et al, 2016)), or it is possible that the overlap in action is further up the neural reflex arc.

Fog seems to sit out on its own with regards to its lack of correlation with ATP, suggesting it has a more unique mechanism of action. However, the way in which fog needs to be generated via an ultrasonic nebuliser and the fact that the solutions are generated in a different manner to the other cough challenge solutions means that a very different methodology has to be employed in generating a cough challenge using fog. It is difficult to ignore this when comparing it with the other three challenges and this fact alone may be contributing to the differences in results obtained.

10.5.4 Additions to knowledge about ATP cough challenge

Whilst this data has confirmed that chronic cough patients are more sensitive to ATP than healthy volunteers, it has confirmed that there is an overlap in response between the two groups. I already knew that ATP cough challenge was no more sensitive at identifying chronic cough patients than capsaicin. And I suspected it was similar to citric acid. This data has confirmed these findings, and also shown it has similar overlap between healthy volunteers and chronic cough patients as fog.

Inhaled ATP cough challenge appears to have a similar variability to the other cough challenges commonly in use and may be slightly less variable than fog. Overall though, chronic cough patient's responses are less reproducible in all cough challenges, suggesting careful study design is required if the cough challenge response is to be used to measure anti-tussive effect in these groups.

The correlation of ATP cough response with other cough challenge response was somewhat surprising and suggests some mechanisms of action overlap with citric acid and capsaicin but not with fog.

10.5.5 Limitations of this study

Due to the study design, ethics permission and timing of visits, there were different time lengths between each of the randomised cough challenges at visit one and visit two. The hour-long interval between challenges at visit one is likely to have been long enough to avoid cross-tachyphylaxis; however, the shorter wait at visit two could account for the slight reduction in cough response between visits. However, this reduction could also be due to the recognised startle phenomenon. Cough response at second exposure is often seen to be less than initial exposure and needs to be taken into consideration when

designing a study to test antitussive effect. It has also been previously shown that cross-tachyphylaxis does not vary whichever cough challenge is given first (Morice et al, 1992), therefore randomising the cough challenge order should have negated some of the effect of cross-tachyphylaxis.

A high proportion of this study's participants were female. This may have skewed the data slightly as women have a more hypersensitive cough reflex and cough at lower C5 values (Dicpinigaitis & Rauf, 1998; Kastelik et al, 2002). As there appears to be better reproducibility at higher values of C5, there may be a better reproducibility of the cough challenge response with men. However, more women than men tend to have cough hypersensitivity syndrome (Song et al, 2015) and therefore this data may be more helpful in informing knowledge of this condition. The healthy volunteers were also mainly female, so this should not have affected the results when comparing these two groups unduly.

10.6 Conclusion

Inhaled ATP cough challenge responses show correlation with citric acid and capsaicin challenge responses in both chronic cough patients and healthy volunteers suggesting some overlap in mode of action of these challenges.

ATP has a similar reproducibility to citric acid, capsaicin and fog challenges. However, all of these show less intra-patient reproducibility in chronic cough patients, suggesting that cough challenges are better reserved for experiments considering modes of action and exploring mechanisms of cough hypersensitivity rather than measurement of the effects of antitussives.

11 Discussion

11.1 9.1 Developing the methodology

11.1.1 Solvents and solubility

Throughout all my experiments the range of cough challenge concentrations has been limited by the solubility of the substrates. In some cases it has been difficult to establish an accurate C5 – particularly in healthy volunteers – due to the limited upper concentration available. There has been previous suggestion that the variability of inhaled cough challenge response is negated by using the concept of Emax (Hilton et al, 2013) but this would be limited by the relative insolubility of substrates (particularly ATP) at higher concentrations.

As the most widely used cough challenge substrate available, capsaicin is available to purchase already in solution. However, this solution does utilise DMSO as a solute, and whilst it is generally accepted that this is an inert substance, early challenges did see some coughing with this (Midgren et al, 1992), and this has never been confirmed in chronic cough patients. I chose to avoid using any substance other than normal saline as a solute, although as the results from chapter six illustrate, even inhalation of saline can precipitate cough in some chronic cough patients.

Difficulty in maintaining pH across a range of concentrations meant that whilst initial plans were to utilise a fairly standardised methodology, this cough challenge needed adapting. Repeating the inhalation 5 times at a single concentration in the end revealed some of my most interesting results regarding variability.

11.1.2 Preliminary work with TRP agonists

Whilst designing the cough challenges presented here, I spent quite a lot of time trying to nebulise known TRP agonists such as cinnamaldehyde, citronellol and ginger oil (Birrell et al, 2009). This proved a lot harder than expected as they are all lipid like substances (some are essential oils). Whilst one can diffuse these in oil burners and similar in an uncontrolled manner, measuring a consistent output through a nebuliser proved more challenging. They all only dissolve in ethanol, which preliminary work showed me is in itself a potent tussive agent. Eventually I concluded that challenging chronic cough patients with near 100% ethanol was probably not practical.

In addition, when I did manage to nebulise these substances the (not always entirely pleasant) scent clung to everything in the laboratory, making me the least popular scientist for days afterwards. I discovered that the reason essential oils are stored in glass is somewhat more prosaic than practitioners of aromatherapy and homeopathy would have us believe. They simply melt plastic – some within minutes – others if left in a container for a few days. As the nebulisation equipment – whether single dose or ultrasonic is all plastic – nebulising these substances neat or even slightly diluted was not possible. Whilst glass nebulisers are available – they are now somewhat prehistoric and even the University glassblower was not able to replicate the fine tubes required to construct one. Sterility and ensuring no cross-contamination of inhaled substances also proved problematic even when considering glass equipment.

Whilst my preliminary work with TRP agonists therefore did not produce any useable results in patients it did add to my understanding of the difficulties in producing consistent cough challenges for different receptor agonists.

11.1.3 Nebuliser factors

Previous studies have commented on 'truncation' of inhalation with higher doses of capsaicin. Observationally I have noted that this can also happen with higher doses of ATP. This may affect whether the participant truly manages to inhale the dose of substrate nebulised during a single breath method.

Continuous breathing however, whether using the ultrasonic nebuliser or a more standard air driven nebuliser requires the patient to stop inhalation to cough which is also altering the dose received.

In the pH study and the initial development of the ATP cough challenge, a fixed straw and baffle was used on the Ko-Ko nebuliser chamber. These also had incorporated a flow-limiter valve. These had been individually tested and had a previously measured flow rate which allowed for a more precise understanding of the inhaled dose of challenge substance. Unfortunately, these chambers were some years old at the time I started my research and by the time of the third experiment were no longer useable due to wear and tear. A larger number of chambers were also required than were available in the laboratory. As a result a different device for flow limitation was used: a mouthpiece with a whistle which only made the correct sound when the correct inhalation flow was applied. Subjects were trained on this before they commenced the cough challenges.

One of the challenges of carrying out cough challenges across different studies and sites which can be easily compared is maintaining the same nebuliser equipment. Like all technology, this is under constant development. Companies constantly upgrade to the newest technology rather than producing similar equipment to previous studies. They are also keen to design nebulisers for therapeutic use and bulk sale to patients/ hospitals rather than for research purposes. As a result, much equipment for a research purpose requires the addition of extra equipment to maintain consistency of output – for example utilising spacers and flow limiters. This tends to make the whole process somewhat resemble an episode of Blue Peter. Using a more simple nebuliser without these flow limiting and standardising devices would prove for an easier challenge but whilst flow rate has been shown to be of limited relevance,(Barros et al, 1990; Barros et al, 1991) nebuliser output does impact on dose received and therefore where practical should still be standardised.

11.1.4 Measurement of output

The output measurement throughout these experiments was simple cough counting. A suggestion has been made that utilising 'urge to cough' rather than motor response as an outcome would separate those subjects who are hypersensitive (Birring, 2017). Whilst I agree that this would be an ideal measurement if an objective way of measuring this could be sought, current methods employing a VAS or similar introduce a subjective element to measurement. Previous studies have shown that objective and subjective measures of cough response do not correlate well (Faruqi et al, 2011). Recently presented results suggest that chronic cough patients can't suppress their cough to the same degree as healthy volunteers (Cho et al, 2017). So whilst there may be a significant difference in urge to cough and actual motor response in healthy volunteers, there is unlikely to be in cough patients. This is further supported by a study showed that whilst healthy volunteers tend to experience an urge to cough at a dose lower than they actually produce a motor response, chronic cough patients almost all cough at the same dose they experience an urge to cough. (Hilton et al, 2015) It is useful to stimulate the urge to cough without the actual motor response for radiological studies, for example, where movement would affect imaging, however I feel this would prove difficult in chonic cough patients. This does however raise the interesting question: is cough hypersensitivity actually a failure of inhibitory pathways rather than an upregulation of stimulatory ones?

Whilst most recent studies of cough challenge have utilised C2 or C5 as an outcome measure, Emax has also been postulated as a more reliable outcome measure (Hilton et al., 2013). As well as having some concerns about the tolerability of this to patients in

repeated challenges, as mentioned earlier, I think it would prove difficult to easily define Emax across other challenge solutions such as ATP, particularly in healthy volunteers given the difficulty in producing stronger solutions. However, observationally I feel I possibly came close to it with some of the chronic cough patients. At the dose which led to the outcome of C5, some patients went into a coughing bout which took some time to recover from.

11.1.5 Conclusions regarding cough challenge methodology

I acknowledge that despite ERS standards having been set, there is still much debate as to what methods to employ when designing a cough challenge. My work has shown however that chronic cough patients cannot be assumed as responding to cough challenge in a predictable way, and therefore novel methodologies may need to be sought in order to determine their response to antitussives and further explore their cough response.

Given that different patients seem to respond to different substances in different ways, it would be helpful to know what each patient is hypersensitive to or which receptor is affected in different individuals. Therefore, it would be helpful to have a cough challenge available for each of the current putative 'cough receptors' but as I have shown, this is limited by the methodology.

11.2 Cough challenge in healthy volunteers

Much work previously on cough challenge has centred on healthy volunteers, therefore little of the data presented here is a surprise. The dramatic difference between cough response to ATP versus AMP was, however, the first time this has been objectively measured.

Throughout my investigation healthy volunteers respond predictably, to variations in pH, to the novel ATP cough challenge, and to fog, citric acid and capsaicin.

Overall, cough challenges would appear to be a reliable way of confirming that antitussives reduce cough hypersensitivity in healthy volunteers, so long as the selection of the cough challenge substrate is chosen carefully and participants are pre-screened to ensure they have a tussive response to the molecule in question. In addition, I feel my results would suggest that an initial 'teaching' or screening challenge should be included in any protocol, and that this result should not then be used in analysis of anti-tussive effect.

11.3 Cough challenge in cough hypersensitivity patients

In stark contrast to healthy volunteers, the data I have collected on chronic cough patients is both novel and informative. Lack of consistency and heightened variability as well as sensitivity in chronic cough patients is a theme throughout all three experiments. However, is the increased variability a function of the heightened sensitivity? Are inhaled cough challenge responses less consistent at lower doses?

Whilst the lower concentrations needed to stimulate a cough response in chronic cough patients may play a part in some of the variability. I feel it is an inherent part of the cough hypersensitivity syndrome. Having observed patients clinically – they often tell me that they are having a 'bad day' or a 'good day'. This in part has led me to believe, along with my data, that patients with chronic cough have a highly varied response to the same stimulus. I have termed this the 'Buckaroo theory' of chronic cough. I believe it is much like the child's game where various items gradually loaded onto a donkey will invariably cause it to buck, but the anticipation is gained from the fact that at which point in the loading the bucking will occur is unpredictable. Just like a patient's cough response is unpredictable following loading with various stimuli. This has similarities with the neuronal phenomenon of 'wind-up' which occurs in chronic pain.

11.4 Variability of response to cough challenge

Variation in the response to cough challenge of both healthy volunteers and more so in chronic cough patients means that studies using change in cough challenge response as a measure of efficacy of antitussives require careful design. There is a need to balance tachyphylaxis effect with the newly postulated 'Buckaroo' effect. Should one really be using cough challenges as a measure of antitussive effect? I continue to believe it a useful tool if one ensures careful challenge agent selection and interpret results with caution, alongside other cough measurement mechanisms.

Whilst all the patients selected for these experiments had been established as having a chronic cough which had not resolved following trials of standardised treatment pathways, they were, as patient groups often are, a very heterogeneic group in some respects (in terms of medication and co-morbidities for example) this may have impacted on the inter-patient variability of cough response seen.

It is widely accepted that there are a number of receptors involved in the cough reflex, and whilst ATP is currently thought to stimulate P2X3, it may stimulate other receptors and other receptors such as TRPV4 are thought to be involved in the activation of P2X3. (Bonvini et al, 2017a) Citric acid is known to show cross-reactivity, and Capsaicin may cross react with other TRP receptors. The results of the correlation of the 4 challenge study support this, and this cross reactivity and involvement of different receptors may account for different cough responses in different cough patients, and even account for different responses in patients over time, as the interactions between the receptors could modify in response to a cough challenge.

The other part of the cough reflex process that is likely to modify in response to repeated cough challenges is the central nervous system. A 'learning curve' is seen with a number of challenges in the respiratory world, and may be present with cough challenge. Indeed it is accepted that the startle phenomenon will occur with the majority of challenges, which is why screening challenges are often included.

11.5 pH cough challenge

I was hopeful that carrying out a cough challenge at a variety of pH levels would lead to greater elucidation of the mechanism of action of citric acid. Whilst the results obtained show that in healthy volunteers the more hydrogen ions are present in the solution the greater the cough response, the inconsistency of the response in chronic cough patients actually led to some more interesting conclusions as I have outlined previously.

This data has been presented at Winter BTS 2015 (Rai et al., 2015) and at the time of writing is undergoing revision following review for publication in 'Respiratory Physiology & Neurobiology'.

11.6 ATP cough challenge

I have described a new cough challenge using ATP to stimulate cough. The results outlined in chapter 7 of this thesis have been presented at the American Cough Conference in 2015 (healthy volunteers only) and at Winter BTS conference 2015 (Fowles & Morice, 2015). They have also been published in ERJ (Fowles et al, 2017b; Rai et al, 2015). I have therefore already had the opportunity to receive feedback and discussion points on this. These have led to some debate about the challenge itself, and

the conclusions drawn regarding cough hypersensitivity as a result of the findings (Belvisi & Smith, 2017; Fowles et al, 2017a).

The specific criticisms levelled were: firstly, that the chronic cough patients included in the study were not specifically phenotyped as 'refractory to treatment'; and secondly that the conclusion regarding lack of peripheral stimulation by ATP as a solitary mechanism of hypersensitivity is incorrect.

I disagree with the comment regarding lack of phenotyping on two levels. Firstly, I don't believe patients have to have treatment refractory cough in order to have cough hypersensitivity syndrome. Essentially cough is cough – and hypersensitivity cough syndrome can be present in patients with COPD, asthma and reflux, (for example) but is not present in all patients with these diseases (Morice et al, 2014b). Secondly, the patients were well phenotyped, they had all been treated through the well-established Hull cough clinic. All had tried previous treatments and some were still on them as evidenced by the higher proportion of the patient group on PPIs and inhaled therapies. It is possible that had I specifically chosen patients who had failed on every available treatment option that the curve may have shifted further left however I am not sure that this would have been the case

The lack of entirely peripheral stimulation as a cause of cough hypersensitivity I have discussed elsewhere and I agree that there are alternative potential mechanisms. I do not feel that these have yet been fully elucidated and I look forward to seeing further work carried out exploring this.

The ATP cough challenge I designed has gone on to be used subsequently in a study of the effect of Gefapixant (AF-219/MK-7264) on cough reflex sensitivity, alongside other cough challenge agents. These results have been presented at the ERS conference in 2017 (Morice et al, 2017) and showed that the ATP cough challenge was the only one of the four that was significantly modulated by Gefapixant (AF-219/MK-7264). The ATP challenge has therefore already proved useful confirming that the P2X3 receptor blocker does block cough response to ATP in humans.

It has been suggested during discussion that the biological characteristics lead to the wide range of the sensitivity to ATP (Birring, 2017). As it is present in the human respiratory tract already and has been found in higher levels in those with respiratory disease this may have altered the response seen in a cough challenge. It has also been suggested that

increased ATP in airways of patients with chronic hypersensitivity cough may be the cause of their hypersensitivity. An area for future exploration would be the measurement of intra-airway ATP in bronchial washings from patients with chronic hypersensitivity cough.

The suggestion that the breakdown products may also be active in causation of cough response (Birring, 2017) is somewhat negated by the dramatically negative AMP response in healthy volunteers. It would have been helpful if I had been able to also challenge my subjects with ADP however I was hampered in this attempt as it is less soluble than both AMP and ATP and therefore not readily nebulisable.

Of course, the assumption that the ATP challenge is acting on the P2X3 receptor alone cannot be made from my data and other ATP receptors may well be being activated. However, the subsequent study carried out using a specific P2X3 receptor blocker and the dramatic effect it has on the ATP cough challenge response, would suggest that the majority if not all of the effect of ATP is taking place at neuronal pathways with P2X3 involvement (Morice et al, 2017).

There appears to be general agreement that my work with ATP supports the theory that cough hypersensitivity is not created by upregulation of a single receptor, but that the mechanism is likely to be more subtle and complicated (Birring, 2017).

11.7 Comparison of the different cough challenges

The work on comparison of four challenges was presented whilst in its infant stages at the Winter BTS conference 2016.

I feel that this data hasn't really supported the use of one particular cough challenge as a standard. It suggests that cough challenges should be selected based on the type of study being carried out, whether this be a phenotyping effort, a mechanisms study or a study of an antitussive. The population under consideration also needs to be taken into account as this data supported my previous work in showing that chronic cough patients respond very differently to inhaled cough challenge. It is not likely to be helpful to utilise a non-targeted cough challenge as a measurement of anti-tussive response, particularly in chronic cough patients.

11.8 Further utilisation of cough challenge

Are cough challenges a good mechanism for testing antitussive effect? This is particularly a concern in chronic cough patients given the high degree of day-to-day variability in patient hypersensitivity. However, cough counting also falls down in this respect. It is often measured over 24 hours and cough patients may experience greater variability day to day independent of utilisation of an anti-tussive. In the future in house monitors, portable daily utilised cough challenges or novel mechanisms of monitoring cough may be helpful.

When I started this thesis, I was hopeful that I would discover a new diagnostic test that would help clearly define patients with chronic hypersensitivity cough. If I'm honest I don't think any cough challenge is going to become a widely used part of clinical assessment in chronic cough clinic although they may in the future be useful for patients with a failure of treatment trials. Currently I do not feel that performing a chronic cough challenge would outweigh a careful targeted history in successfully diagnosing cough hypersensitivity syndrome.

Whilst my experiments have not produced a new diagnostic test for chronic cough they have, however added more information to our understanding of the cough reflex, particularly in chronic cough patients. This knowledge would not have been gained by simply counting cough or utilising cough severity scores. Cough challenge therefore remains a useful research tool in further investigating underlying mechanisms of hypersensitivity cough syndrome.

11.9 Additions to understanding of cough hypersensitivity mechanisms

Results of my experiments suggest that cough hypersensitivity does not consistently arise from an upregulation of one single receptor. Cross reactivity of different stimuli is likely; the TRPA agonists also activate other TRP receptors, and it has already been postulated that more than one ATP receptor may be involved in the cough reflex arc. Citric acid and other mild acids appear to act at various receptors.

If increased response to abnormal stimuli is not due to an upregulation of receptors on nerve endings – it could be due to a misinterpretation of that signal further up the reflex arc.

Interpretation of this is complicated by the fact that the cough reflex has a voluntary element. However, healthy volunteers seem to be able to suppress cough even in cough challenge, where cough hypersensitivity patients can't. Supporting that cough hypersensitivity is not all in the head (Cho et al, 2017).

11.9.1 Cough receptors

Correlation of the ATP, capsaicin and citric acid challenges certainly supports some kind of cross-talk between receptors and different substrates. The list of candidates for potential 'cough receptors' seems to be ever increasing, with TRPM3 being a candidate for having some responsibility for the increased response in females (Bonvini et al, 2017b).

However, some patients who respond considerably to one stimulus, do not respond to another. Previously the possibility has been raised that individual patients have changes in different receptors causing different 'genotypes' of cough hypersensitivity (Birring, 2017). It is also likely that even in a single patient a combination of different receptors, such as the recognised association between TRPV4 and ATP (Bonvini et al, 2016), is involved in the hypersensitive pathway.

How does one identify which cough receptor is affected in each patient? Having different cough challenges available targeted specifically at different receptors may help with identifying patients who may respond. For example, should one check whether patients are hypersensitive to ATP before one gives Gefapixant (AF-219/MK-7264)? Particularly given the likely expense of new treatments?

11.9.2 Neural Pathway

Rather than the source of hypersensitivity being located on the nerve endings in 'cough receptors' it has been postulated that the source of hypersensitivity or dysregulation of the cough reflex is higher up the neuronal pathway, en-route to the central nervous system. As I have completed my thesis this theory seems to have gained popularity, and is certainly supported by my finding that stimulation of the peripheral cough response does not appear to be the whole story. As well as peripheral receptors, inhaled ATP could be stimulating upstream receptors at peripheral nerve endings, other than in the airways. One of the difficulties in analysing these downstream nerve bodies is that within the human body they are relatively inaccessible. Hopefully work involving developing these nerve bodies in tooth pulp will allow a non-invasive exploration of upstream ganglionic junctions (McGarvey et al, 2017).

11.9.3 Central Nervous System (CNS)

Another alternative to peripheral receptor dysregulation is central sensitisation either in the brainstem or cortex which could up-regulate cough reflex sensitivity. The striking preponderance of women presenting with chronic cough and the known hypersensitivity of women to inhale tussives challenge has recently been shown to be associated with a greater activation of the somatosensory cortex suggesting the importance of such central mechanisms (Mazzone et al, 2007).

Further use of functional MRI whilst doing cough challenges may be of benefit in further determining the effect of cough challenge on the central cortex, as well as the role of the central cortex in cough hypersensitivity syndrome. This would certainly lead to some highly complicated protocols and may only be possible in very small groups. Further results of fMRI on patients doing a cough challenge are likely to prove interesting.

11.10 An overarching theory for cough hypersensitivity?

From the results I have presented here, from clinical experience and listening to patient stories, I think that the root of chronic hypersensitivity cough syndrome is likely to lie not in a single one of these areas of the cough reflex pathway but rather in different areas or even a combination of different areas in different phenotypes of patients. While analogies have been drawn to chronic pain syndrome, the analogous condition I prefer to consider is one that has much overlap and similarity with both chronic pain and chronic cough – irritable bowel syndrome.

Clinical experience has shown us that some patients with irritable bowel syndrome respond well to interventions that act locally in the gut – adaptation of diet, others require a more neurally acting agent such as peppermint, and some respond well to centrally acting agents such as antidepressants.

A similar situation whereby some chronic cough patients respond to treatment directly to the airways (whether it be reducing airway reflux or inhaled corticosteroids); others have receptor dysregulation, and will thereby respond to receptor antagonism; some patients have a hereto undiscovered dysregulation in their neuronal pathway that may be responsive to some form of yet to be discovered medication, and others have a central sensitisation which is modulated by morphine or gabapentin.

Current clinical management of chronic cough often involves extreme patience on the part of both patient and clinician whilst different trials of treatment are carried out. Future work in researching treatment of chronic cough should also focus on defining and identifying these different phenotypes further in order to select the right treatment for the right patient first time.

11.11 Final Thoughts

Since I started this research project I feel that there has been an ever-increasing interest in understanding the mechanisms of cough hypersensitivity. This is partly driven by its increasing presentation to outpatient clinics, particularly general respiratory clinics, where my personal experience is it accounts for upwards of a third of referrals not pre-selected out into specialist clinics.

It is also partly driven by societal pressures whilst the somewhat similar conditions of chronic pain and symptoms of IBS are often very private battles for a patient, the explosive and intrusive nature of a cough can have an effect on all around a patient. Patients often report ‘My wife told me I had to get it sorted’ or ‘Everyone keeps asking if I’ve got an infection’.

It has also been driven by public health campaigns aimed at lung cancer (‘Cough for 6 weeks’ campaign).

Its apparent refractory nature seems to add to the mystique in managing chronic cough. Although I feel this is in a smaller percentage if a carefully structured approach to diagnosis and management is adopted (McGarvey et al, 1998).

This increase in interest was recently emphasised to me on attending the Winter BTS by the interest in the cough SAG which has only recently been established, and by standing room only in the cough presentation session. As well as in my professional experience the apparent desperation of many of my colleagues to refer me their ‘difficult’ cough patients.

One of my aims in completing this research was to produce some information about mechanisms of chronic cough which was directly relevant to patients. Unlike research utilising cell cultures, animal work or ex-vivo nerve preparations, all of my experiments were directly on human subjects. This was something that was important to me not only

because I am primarily a clinician but because previous study has shown that while work on models is helpful, it does not always translate into humans. I feel that this research has shown that it is not only possible to translate previous ideas into human research, but that novel experiments can be carried out on human subjects and provide a wealth of information.

The ATP challenge will I hope continue to be useful in assessing response to Gefapixant (AF-219/MK-7264) which has now undergone Phase 2b Study and is due to enter Phase 3 trials next year (Smith et al, 2017) which is the most promising pharmaceutical agent available in some years for chronic cough.

On a professional and clinical level – I feel my research has allowed me to talk more authoritatively about the mechanisms underlying cough. Reassuring patients that there is ongoing research into diagnosing and treating their condition is in my experience part of the battle in managing patients in clinic. By sharing my research with other professionals, I hope that I have added to some of the understanding of the causes of cough hypersensitivity syndrome.

The results presented in this thesis have dramatically altered the understanding of cough challenges, particularly in chronic cough patients, and the underlying mechanisms of cough hypersensitivity syndrome.

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12 Appendices

1. Hull Cough Hypersensitivity Questionnaire. Version 5, July 2009. Copyright A.H. Morice.

2. Standard Operating Procedure: Cough Challenge with Ultrasonically Nebulised Distilled Water / 0.9%NaCl using the DeVilbiss UltraNeb Nebuliser.

3. Standard Operating Procedure: A Log Dose-Response Cough Challenge with Citric Acid using the KoKo Spirometer & DigiDoser System.

4. Standard Operating Procedure: A Dose-Response Cough Challenge with ATP using the KoKo Spirometer & DigiDoser System.

5. Standard Operating Procedure: A Log Dose-Response Cough Challenge with Capsaicin using the KoKo Spirometer & DigiDoser system.

6. Full List of Inclusion and Exclusion Criteria for Afferent Pharmaceuticals Study AF219-014.

12.1 Appendix 1

HULL COUGH HYPERSENSITIVITY QUESTIONNAIRE

Name: _____

D.O.B: _____ UN: _____

DATE OF TEST: _____

Please circle the most appropriate response for each question

Within the last MONTH, how did the following problems affect you? **0 = no problem and 5 = severe/frequent problem**						
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

12.2 Appendix 2

THE CLINICAL TRIALS UNIT

Centre for Cardiovascular and Metabolic Research

Standard Operating Procedure

Title: Cough Challenge with Ultrasonically Nebulised Distilled Water / 0.9%NaCl using the DeVilbiss UltraNeb Nebulizer

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Author	**Caroline Wright**
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Target audience	**Clinical Trials Unit staff**

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This page details the version history for this SOP and the main changes corresponding to the versions.

VERSION LOG		
Version number and Date	**Author**	**Details of significant changes**
Version 1, 03.07.2015	Caroline Wright	Original authorised by respiratory Medicine
Version 2, 23.10.2015	Caroline Wright	Changed from Variable Nebuliser Output, to Fixed Nebuliser Output with variable concentrations of Distilled Water/0.9%NaCl Solution
Version3, 15.12.2015	Caroline Wright	Altered the table for preparation so that dosing goes from most concentrated to the least concentrated Table selecting the dilution below a C" has been altered as was completely in accurate Removed examples at the end of previous SOP as irrelevant
Version 4 21.12.2015	Caroline Wright	Altered details re: recording of cough challenge. Have been more explicit re the counting of coughs over 30 secs period

Principle

To measure the sensitivity of the cough reflex within:

- Healthy volunteers
- Chronic cough patients
- Patients with other respiratory disorders

The method of administration of the distilled water is via ultrasonic DeVilbiss UltraNeb nebuliser. This standard operating procedure is intended for all appropriately qualified staff and physicians within the Department of Respiratory Medicine.

INDEX

1. Things to consider before performing ultrasonically nebulised distilled water cough challenge

i Personnel

Before performing the cough challenge on a patient, you must fulfil the following criteria:

1. Be capable of managing the equipment including set-up, proper function, maintenance and cleaning.
2. Be proficient at spirometry.
3. Know the exclusions (APPENDIX 2) to cough challenge testing.
4. Be familiar with safety and emergency procedures.
5. Know when to stop further testing.
6. Be proficient in the administration of inhaled bronchodilators and evaluation of the response to them.

ii Safety

Inhaled distilled water / 0.9% NaCl aerosol is not anticipated to, but may potentially cause bronchoconstriction. Thus, you should consider the safety of the patient.

Precautions for patient safety

A physician or other person appropriately trained to treat acute bronchospasm including appropriate use of resuscitation equipment should be close enough to respond to an emergency quickly.

You should make sure that medications to treat severe bronchospasm are present within the testing area. These include epinephrine and atropine for subcutaneous injection and salbutamol and ipratropium in metered dose inhalers or pre-mixed solutions for inhalation, oxygen must also be available. A small volume nebuliser should be readily available for the administration of bronchodilators. A stethoscope, sphygmomanometer, and pulse oximeter should also be available.

2. Equipment and Materials

2.i Solutions

Distilled water
0.9% NaCl
β adrenergic agonist nebule- 2.5 mg (Ventolin)

2.ii Storage

Distilled water should be stored at room temperature, below 25ºC in a tightly sealed container.

0.9% NaCl should be stored at room temperature, below 25ºC in a tightly sealed container.

β adrenergic agonist nebule (2.5mg) stored at room temperature.

2.iii Equipment

A DeVilbiss UltraNeb ultrasonic nebulizer

Bacterial filter

Tubing with mouthpiece and nose peg

Disposable cup and lid

3. Preparation for testing

3i Patient preparation before testing

a. Explain the test to the patient.
Patients should be told that they might suffer severe bouts of coughing and that they may experience some minor symptoms such as chest tightness or breathlessness.

Care should be taken to ensure that the test description does not bias the result.

b. Ask the patient if they would like to urinate before the test (stress incontinence could be precipitated, especially in older women).

c. Evaluate the patient for exclusions (**APPENDIX 2)** and review medication use, details of medications affecting the cough challenge test are in **APPENDIX 2**.

3ii Distilled water preparation

Measure 15ml of distilled water in a universal pot.

Measure 15ml of 0.9%NaCl in a universal pot.

Label 6 Containers 1-6 and make up concentrations as per the following table:

Pot	Distilled Water	0.9%NaCl	Concentration
1	5ml	0ml	100%V
2	4ml	1ml	80 %v
3	3ml	2ml	60%v
4	2ml	3ml	40%V
5	1ml	4ml	20%v
6	0ml	5ml	0.9% NaCl

4. Performing the test

4i setting the equipment up – Assembling the nebuliser

a. Mount the bacterial filter in place
b. Fill the nebulizer chamber with tap water between lines drawn
c. Place disposable cup and lid into nebulising chamber. Fill it in with the solution to be nebulised.
d. Connect the tubing – one tube will connect bacterial filter with the lid and the other the lid with the mouthpiece.

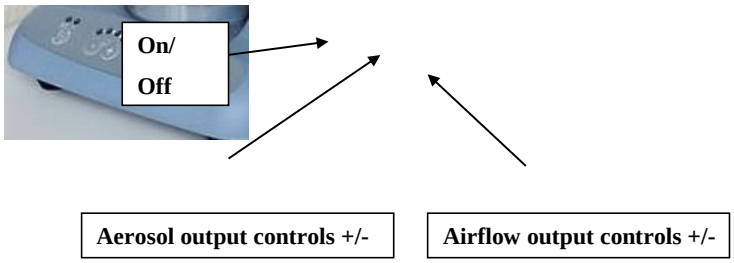
4ii The patient

a. Subjects must be able to understand the procedure
b. Subjects should be seated comfortably throughout the test.

4iii Test sequence – Performing the cough challenge test

Cough reflex sensitivity assessment will consist of two partial stages – determination of threshold output and measurement of coughs.

Operation of DeVilbiss nebuliser for purposes of cough challenge



1. Machine is turned on by pressing the on/off button.
2. Aerosol and airflow output is adjusted by pressing + or – buttons of aerosol or airflow output.
3. After the challenge, the machine is turned off by pressing the on/off button.
4. The machine should be set to the maximum setting. That is, +4 aerosol output and +4 airflow output.

A Determination of cough output.

Threshold output is determined as output, on which test subject coughs 5 **or more times (C5)**. Subject is to **tidally** inhale aerosol of ultrasonically nebulised solution for 30 seconds, followed by a rest period of 1 minute.

Coughs are to be counted during the 30 secs of inhalation only.

If patient coughs 2 or more times during the 30 secs of inhalation of saline, then the challenge will be aborted as patient sensitive to the diluent.

The challenge should be stopped when the subject coughs 5 or more times (C5). You should record the concentration at which patient coughs 2 or more times (C2) and the concentration at which cough 5 or more times (C5). The challenge ends once a C5 is reached.

B Assessment of cough reflex sensitivity

For subsequent visits, the starting concentration should be 1 dilution below the screening C2 concentration. This can be determined from the table below:

Screening C2 Concentration	Starting Concentration for Subsequent Visits
Distilled Water	1:4(80%)
1:4(80%)	2:3(60%)
2:3(60%)	3:2(40%)
3:2(40%)	4:1(20%)
4:1(20%)	0.9% NaCl
0.9% NaCl	0.9% NaCl

The procedure for determining C2 and C5 concentration is then the same as for the screening visit, but starting at the concentration determined from the above table.

APPENDIX 1

Appendix 1: Dismantling the nebuliser

1 Any unused solution from the nebulizer is to be disposed of.
2 All parts are single-use only (exception of nebulizer chamber)
3 Pour out the water from nebulizer chamber and dry it out with a paper towel.

APPENDIX 2

Exclusion Criteria

- *If the subject smokes:* Cough challenge must be performed at least one hour after the last cigarette has been smoked.
- *If the subject has used an inhaler:* Lung function testing should be performed at least one hour after the use of any inhalers.
- *If the subject has used an inhaler that is not a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* Lung function testing is carried out and the data is recorded.
- *If the subject has used an inhaler that is a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* I f the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unable or reluctant to return another time, testing should proceed and the medication used should be recorded.
- *If the subject has taken an oral beta-2-agonist or a theophylline or an oral antimuscarinic within the last eight hours:* If the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded

- *If the subject has taken any over the counter (OTC) cough mixture within the last twelve hours:* If the subject is willing to come back another time for cough challenge testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.
- *If the subject has had a respiratory tract infection in the last three weeks:* Another appointment should be made unless the subject is unwilling to come back, in which case testing should continue. The number of days elapsed since the end of the respiratory infection should be recorded.
- *If the subject has had any food or drink products containing caffeine or menthol within the last hour.* If the subject is unwilling to wait for 1 hour before starting the test, the subject should return another time. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.]

APPENDIX 3

CLINICAL TRIALS UNIT

RESPIRATORY MEDICINE

CARDIOVASCULAR & RESPIRATORY STUDIES

Ultrasonically Nebulised Distilled Water Cough Challenge

Time	Concentration	No. of Coughs	C2 Reached, C5 Reached
30s	**0.9% NaCl**		
60s	*Rest*	-	-
30s	**1:4**		
60s	*Rest*	-	-
30s	**2:3**		
60s	*Rest*	-	-
30s	**3:2**		
60s	*Rest*	-	-
30s	**4:1**		
60s	*Rest*	-	-
30s	**Distilled Water**		

C2 Concentration: _____

C5 Concentration: _____

Date of Test .....

Time .....

Study No:.....

Screening Visit …..Yes/No……..

Visit No. ……..

Name .....

UN .....

DOB .....

Questions

Have you had a recent upper respiratory tract infection?	YES	NO
Are you a current smoker?	YES	NO
	No pack yrs____	
Are you taking any medications?	YES	NO
Do you have a family history of cough?	YES	NO

Medication List

....

12.3 Appendix 3

THE CLINICAL TRIALS UNIT

Centre for Cardiovascular and Metabolic Research

Standard Operating Procedure

Title: A Log Dose-Response Cough Challenge with Citric Acid using the KoKo Spirometer & DigiDoser System

When this document is reviewed as a paper copy, the reader is responsible for checking that it is the most recent version.

The current version is available on:

T:\Cardiovascular and Respiratory Studies\CTU Documentation\Standard Operating Procedures

SOP reference number	**CTU080715**
Author	**Caroline Wright**
Current version and date	**Version 3, 09/03/2016**
Approved by	**Professor Morice**
Approval signature/date	
Approved by CTU Manager signature / date	
Target audience	**Clinical Trials Unit staff**

SOP No: CTU080715

This page details the version history for this SOP and the main changes corresponding to the versions.

VERSION LOG		
Version number and Date	**Author**	**Details of significant changes**
Version 1, 08.05.2015	Caroline Wright	Original authorised by respiratory Medicine
Version 2 15.12.2015	Caroline Wright	Including the saline negative control in dilutions table Reported cleaning of the nebuliser pot between concentrations
Version 3 09/03/2016	Caroline Wright	Change of SOP reference number

Principle

To measure the sensitivity of the cough reflex within:

- Healthy volunteers
- Chronic cough patients
- Patients with other respiratory disorders

The method of administration of the Citric acid is via a nebuliser utilising a KoKo spirometer & DigiDoser. This standard operating procedure is intended for all appropriately qualified staff and physicians within the Department of Respiratory Medicine

INDEX

1. Things to consider before performing the citric acid cough challenge

i Personnel

Before performing the cough challenge on a patient, you must fulfil the following criteria:

1. maintenance and cleaning.
2. Be proficient at spirometry.
3. Know the exclusions (APPENDIX 2) to cough challenge testing.
4. Be familiar with safety and emergency procedures.
5. Know when to stop further testing.
6. Be proficient in the administration of inhaled bronchodilators and evaluation of the response to them.

ii Safety

Inhaled Citric acid may cause bronchoconstriction. Thus, you should consider the safety of the patient.

Precautions for patient safety

A physician or other person appropriately trained to treat acute bronchospasm including appropriate use of resuscitation equipment should be close enough to respond to an emergency quickly.

You should make sure that medications to treat severe bronchospasm are present within the testing area. These include epinephrine and atropine for subcutaneous injection and salbutamol and ipratropium in metered dose inhalers or pre-mixed solutions for inhalation, oxygen must also be available. A small volume nebuliser should be readily available for the administration of bronchodilators. A stethoscope, sphygmomanometer, and pulse oximeter should also be available.

2. Equipment and Materials

2.i Solutions

4M Citric acid stock solution (prepared at Stockport Pharmaceuticals, Pharmacy Department, Stockport)

0.98% (sterile) sodium chloride for irrigation (Baxter, U.K.)

β adrenergic agonist nebule- 2.5 mg (Ventolin)

2.ii Dilution Equipment

x1 5ml micropipette

x8 5ml micropipette tips

x8 sterile universal pots correctly labelled for each concentration of Citric acid/saline

x1 sterile dosimeter pot

gloves

lab coat

2.iii Storage

Citric acid should be stored at room temperature, below 25^0C in a tightly sealed container.

Saline diluent stored at room temperature.

β adrenergic agonist nebule (2.5mg) stored at room temperature.

2 iv Equipment

A KoKo DigiDoser

KoKo filter

A DeVilbiss 646 characterised, flow limited nebuliser with sterile chamber and mouth piece.

Gas cylinder containing compressed air, set at 30psi.pressure.

3. Preparation for testing

3i Patient preparation before testing

a. Explain the test to the patient.

   Patients should be told that they might suffer severe bouts of coughing and that they may experience some minor symptoms such as chest tightness or breathlessness.

   Care should be taken to ensure that the test description does not bias the result.

b. Ask the patient if they would like to urinate before the test (stress incontinence could be precipitated, especially in older women).

c. Evaluate the patient for exclusions (**APPENDIX 2)** and review medication use, details of medications affecting the cough challenge test are in **APPENDIX 2**.

3ii Calibration

Before starting the test make sure the KoKo DigiDoser system is calibrated to the standard procedures outlined in SOP no: CTU080709.

3iii Solution Preparation

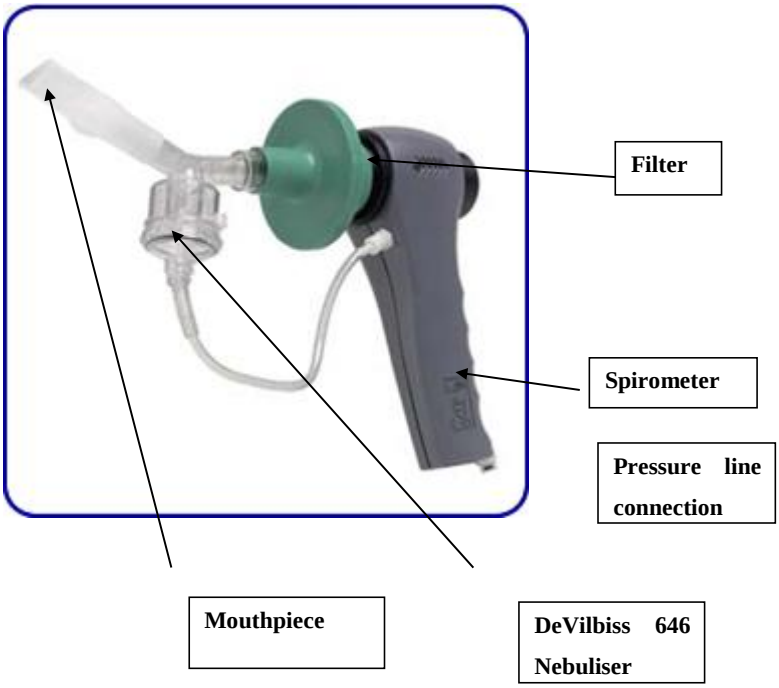
1. Label 9 universal pots with final concentrations as listed in table below
2. Follow the dilution sequence below, ensuring that each concentration of citric acid is in the correctly numbered universal pot and that a new pipette tip is used to make each concentration of citric acid. After addition of sodium chloride, the lid is replaced and the solution is adequately mixed.
3. Make up one universal pot with only 3ml 0.9% saline solution, this solution will be given at the start of the citric acid cough challenge as the diluent test. If subject coughs to this solution the test should be aborted.

Citric Acid		Vol. 0.9% NaCl Sol^{n}	Final [Citric acid] (mM)
Sol^{n} used for dilution	Vol (ml)		
4 M Sol^{n}	7.5	2.5	3M
4M Sol^{n}	2.5	7.5	1 M
3M Sol^{n}	1	9	300
1M Sol^{n}	1	9	100
300mM Sol^{n}	1	9	30
100 mM Sol^{n}	1	9	10
30 mM Sol^{n}	1	9	3
10 mM Sol^{n}	1	9	1
Saline (neg control)	0	9	0

This is enough solution to perform 3 cough challenges

4. Performing the test

4 i setting the equipment up – Assembling the nebuliser



1 Place the KoKo filter on to the spirometer

2 Assemble the nebuliser

Attach the nebuliser adapter, nebuliser and mouthpiece.

Insert the nebuliser into the KoKo Filter cone. The adapter end goes into the filter. The mouthpiece fits on the nebuliser end opposite the air vent.

3 Connect the pressure line outlet on the front of the KoKo DigiDoser handle to the pressure line inlet on the bottom of the supplied nebuliser with the supplied tubing.

4 Adjust the pressurised air source to 30 psi.

5 Connect the pressurised air source to the bottom of the DigiDoser with a long piece of tubing.

4ii Setting up the challenge protocol

a. Enter the challenge protocol by pressing icon
b. Go to set up in the top bar menu
c. Got to protocol set up and create

Enter data as per the table in Appendix 3

4iii The patient

a. Subjects must be able to understand the procedure and perform reliable spirometric manoeuvres.
b. Subjects should be seated comfortably throughout the test.

4iv Test sequence – Entering the patient information and performing the cough challenge test

1. Select the patient icon 
2. Click on the icon and enter patient data in the relevant sections in the window which pops up immediately. Date of birth must be entered as a ***four-digit year*** (eg 1991 ***not*** 91).
3. Adding Extra categories to the "Diagnosis" can be achieved by clicking on the box to the right of the drop-down menu with the dotted lines, for instance you can insert "chronic cough".
4. Add comments relevant to the patient such as whether they are on acid suppression or whether they suffer from heartburn.
5. Add relevant group titles to patient database such as "chronic cough, new patient."
6. Once data inputted close patient details table.
7. From the main menu select "challenge" icon. 
8. This will take you to the screen which requires a baseline Spirometry to be performed prior to challenge.
9. Click on the Start test icon. 
10. Spirometry is performed using only the spirometer and filter. The nebuliser will not be attached at this stage.
11. A grey box appears requesting pneumotach environment data for room temperature and humidity. Click OK.
12. Get patient to perform 4 tidal breaths.
13. The computer screen will prompt the patient to inspire as deeply as possible.
14. The Computer screen will prompt the patient to expire as deeply as possible.
15. A results screen will appear with the spirometry results. Click OK.
16. Click on Go to next stage icon 
17. and select stage 1 from the pop-up screen.
18. Click on Start administration icon. 
19. Place 3ml of the 0.9% saline in a nebuliser pot
20. Attach to spirometer via filter
21. Ask patient to breath normally through the nebuliser
22. Observe 3 relaxed breaths
23. Get patient to then exhale as deeply as possible with the mouthpiece in the mouth. During the expiration press SPACEBAR to start the test.
24. Then the patient will be asked to perform a controlled deep inhalation, during this inhalation the solution will be Nebulised for 1.2 secs. Allow a time interval of 60 seconds between each nebulised citric acid concentration.
25. Details of how to load the nebuliser with each concentration of citric acid are shown in Appendix 1.
26. Count the number of coughs for 15 seconds after each challenge.

27. If the subject coughs 2 or more times in relation to inhaling the saline solution the test will be aborted.
28. Starting from the lowest concentration of citric acid, load 3ml of the solution in to the nebuliser pot and perform cough challenge as per the saline.
29. A positive result is obtained if the patient coughs ***5 or more times*** following nebulisation of a single solution the test is terminated at this point. Continue testing each concentration of citric acid until 5 coughs or more in a single inhalation is reached.
30. The program runs on a timer and alarms 15 secs post nebulisation to allow for the counting of coughs and them alarms again 1 minute post nebulisation to indicate that you are now ready to proceed with next concentration of citric acid.
31. Note clean the inside of the nebuliser with tissue to remove any residual solution before loading the next concentration of citric acid
32. Record results on the sheet Appendix 4.
33. To continue with subsequent concentrations, press icon 

APPENDIX 1

Appendix 1: Dismantling and loading the citric acid in to the nebuliser

1. Pull the short plastic tube out of the bottom of the nebuliser
2. Pull the mouthpiece and nebuliser out of the KoKo filter.
3. Remove the small stopper and dispense 3ml of solution into the nebuliser chamber.
4. After each citric acid concentration has been nebulised, unscrew the bottom chamber from the upper chamber being careful not to spill the contents.
5. Pour any unused fluid into a beaker, clean out the chamber with a dry paper towel and load another dose of citric acid following steps 1-3.

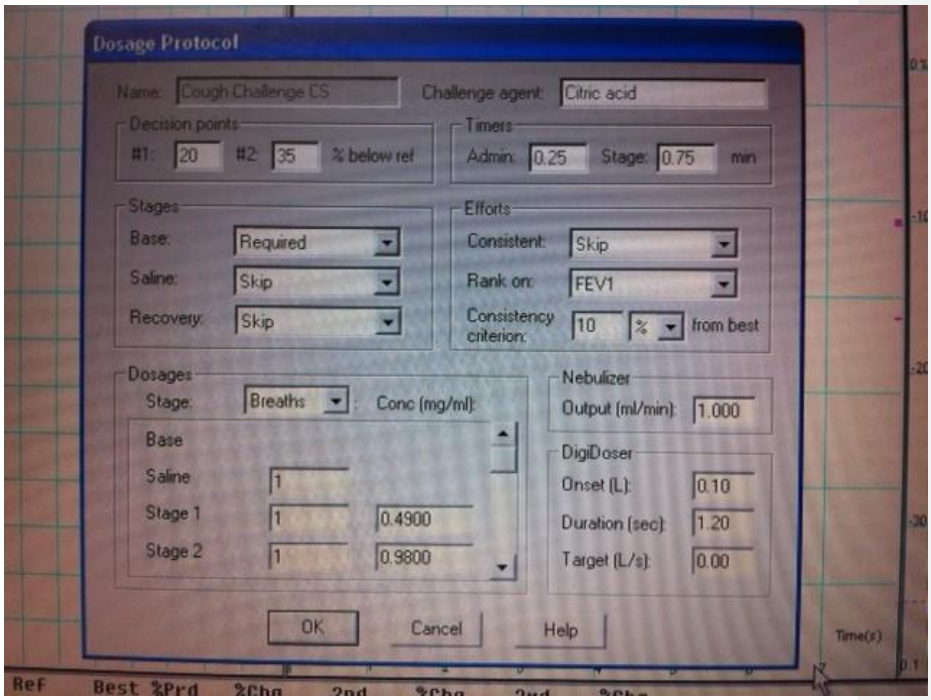
APPENDIX 2

Exclusion Criteria

- *If the subject smokes:* Cough challenge must be performed at least one hour after the last cigarette has been smoked.
- *If the subject has used an inhaler:* Lung function testing should be performed at least one hour after the use of any inhalers.
- *If the subject has used an inhaler that is not a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* Lung function testing is carried out and the data is recorded.
- *If the subject has used an inhaler that is a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* I f the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unable or reluctant to return another time, testing should proceed and the medication used should be recorded.

- *If the subject has taken an oral beta-2-agonist or a theophylline or an oral antimuscarinic within the last eight hours:* If the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded
- *If the subject has taken any over the counter (OTC) cough mixture within the last twelve hours:* If the subject is willing to come back another time for cough challenge testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.
- *If the subject has had a respiratory tract infection in the last three weeks:* Another appointment should be made unless the subject is unwilling to come back, in which case testing should continue. The number of days elapsed since the end of the respiratory infection should be recorded.
- *If the subject has had any food or drink products containing caffeine or menthol within the last hour*. If the subject is unwilling to wait for 1 hour before starting the test, the subject should return another time. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.

APPENDIX 3



APPENDIX 4

CLINICAL TRIALS UNIT

RESPIRATORY MEDICINE

CARDIOVASCULAR & RESPIRATORY STUDIES

DigiDoser Citric Acid Cough Challenge

	Conc Citric acid (mM)	No of Coughs
Saline		
1		
3		
10		
30		
100		
300		
1M		
3M		

(Healthy Volunteer Mean (range) C2 = 263 mM (30.9-1000 mM))

C2 = _____ C5 = _____

Date of Test .....

Time .....

Study No:.....

Name .....

UN .....

DOB .....

Questions

Have you had a recent upper respiratory tract infection?	YES	NO
Are you a current smoker?	YES	NO
	No pack yrs____	
Are you taking any medications?	YES	NO
Do you have a family history of cough?	YES	NO

Medication List

....

12.4 Appendix 4

THE CLINICAL TRIALS UNIT

RESPIRATORY MEDICINE

Standard Operating Procedure

Title: A dose-response cough challenge with ATP using the KoKo Spirometer & DigiDoser system

When this document is reviewed as a paper copy, the reader is responsible for checking that it is the most recent version.

The current version is available on: T:\Cardiovascular and Respiratory Studies\CTU Documentation\Standard Operating Procedures

SOP reference number	**CTU041114**
Author	**Helen Fowles**
Current version and date	**Version 2, 09/12/2015**
Approved by	**Professor Morice**
Approval signature/date	
Approved by CTU Manager/date	
Target audience	**Clinical Trials Unit staff**

SOP No: CTU041114

Version log		
Version number and date	**Author**	**Details of significant changes**
Version 1 04.11.2014	H.Fowles	Original SOP approved by respiratory medicine
Version 2 09.12.15	C.wright	Changed concentrations in the ATP table to mM (pg7) Addition of negative control – saline, in table Addition of spirometry test post challenge as a safety precaution (pt28, pg 10)

Principle

To measure the sensitivity of the cough reflex within:

- Healthy volunteers
- Chronic cough patients
- Patients with other respiratory disorders

The method of administration of the ATP is via a nebuliser utilising a KoKo spirometer & DigiDoser. This standard operating procedure is intended for all appropriately qualified staff and physicians within the Academic department of Respiratory Medicine

INDEX

1. Things to consider before performing the ATP cough challenge

i Personnel

Before performing the cough challenge on a patient, you must fulfil the following criteria:

1. Be capable of managing the equipment including set-up, proper function, maintenance and cleaning.
2. Be proficient at spirometry.
3. Know the exclusions (APPENDIX 2) to cough challenge testing.
4. Be familiar with safety and emergency procedures.
5. Know when to stop further testing.
6. Be proficient in the administration of inhaled bronchodilators and evaluation of the response to them.

ii Safety

Inhaled ATP may cause bronchoconstriction. Thus, you should consider the safety of the patient.

Precautions for patient safety

A physician or other person appropriately trained to treat acute bronchospasm including appropriate use of resuscitation equipment should be close enough to respond to an emergency quickly.

You should make sure that medications to treat severe bronchospasm are present within the testing area. These include epinephrine and atropine for subcutaneous injection and salbutamol metered dose inhaler or pre-mixed solutions for inhalation, oxygen must also be available. A small volume nebuliser should be readily available for the administration of bronchodilators. A stethoscope, sphygmomanometer, and pulse oximeter should also be available.

2. Equipment and Materials

2.i Solutions

ATP - Solid *(SigmaAldrich)* – made up into 0.3M solution with N/Saline (1.65g/1mL)

0.98% (sterile) sodium chloride for irrigation (Baxter, U.K.)

β adrenergic agonist nebule 2.5mg (Ventolin)

2.ii Dilution Equipment

x1 5ml micropipette

x15 5ml micropipette tips

x14 sterile universal pots correctly labelled for each concentration of ATP/saline

x1 sterile modified DeVilbiss pot

gloves

lab coat

2.iii Storage

ATP stored at -20^{o}C in a tightly sealed container

Saline diluent stored at room temperature

β adrenergic agonist nebule (2.5mg) stored at room temperature

2.iv Equipment

A KoKo DigiDoser

KoKo filter

A DeVilbiss 646 nebuliser with sterile chamber and mouth piece

Gas cylinder containing compressed air, set at 30psi.

3. Preparation for testing

3i Patient preparation before testing

a. Explain the test to the patient.
   Patients should be told that they might suffer severe bouts of coughing and that they may experience some minor symptoms such as chest tightness or breathlessness.

   Care should be taken to ensure that the test description does not bias the result.

b. Ask the patient if they would like to urinate before the test (stress incontinence could be precipitated, especially in older women).
c. Evaluate the patient for exclusions (**APPENDIX 2)** and review medication use, details of medications affecting the cough challenge test are in **APPENDIX 2**.

3ii Calibration

Before starting the test make sure the KoKo DigiDoser system is calibrated to the standard procedures outlined in SOP no: CTU 080709

3iii Solution Preparation

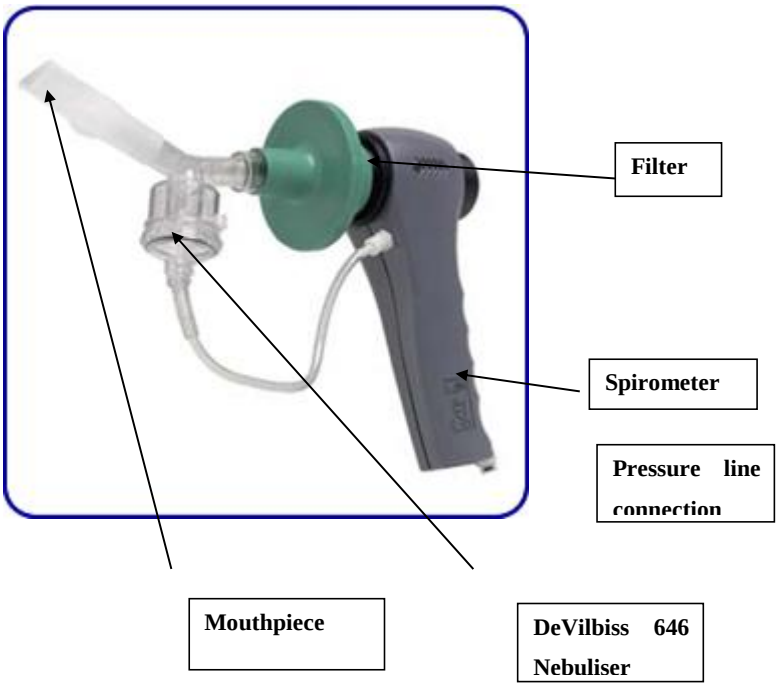
1. Label pots with final solution as in table below
2. Follow the dilution sequences below, ensuring that each concentration of solute is in the correctly labelled universal pot and that a new pipette tip is used to make each concentration. After addition of sodium chloride, the lid is replaced and the solution is adequately mixed.

ATP – won't go into 1M solution – therefore make up 0.3M solution and dilute from there. (0.165g/mL for a 0.3M solution)

ATP			
Sol^{n} used for dilution	Vol (mL)	Vol. 0.9% NaCl Sol^{n}	Final [ATP] (mM)
300 mM	5.0mL	0	**300**
300 mM	1.2mL	2.4mL	**100**
300 mM	0.4mL	3.6mL	**30**
100 mM	0.4mL	3.6mL	**10**
30mM	0.4mL	3.6mL	**3**
10mM	0.4mL	3.6mL	**1**
3mM	0.4mL	3.6mL	**0.3**
1mM	0.4mL	3.6mL	**0.1**
saline		3 ml	**Saline (neg control)**

This will be sufficient solution for one cough challenge

4 i Setting the equipment up – Assembling the nebuliser



Place the KoKo filter on the spirometer

1. Assemble the nebuliser

   Attach the nebuliser adapter, nebuliser and mouthpiece.

   Insert the nebuliser into the KoKo Filter cone. The adapter end goes into the filter. The mouthpiece fits on the nebuliser end opposite the air vent.

2. Connect the pressure line outlet on the front of the KoKo DigiDoser handle to the pressure line inlet on the bottom of the supplied nebuliser with the supplied tubing.

3. Adjust the pressurised air source to 30 psi.

4. Connect the pressurised air source to the bottom of the DigiDoser with a long piece of tubing.

4ii The patient

a. Subjects must be able to understand the procedure and perform reliable spirometric manoeuvres.
b. Subjects should be seated comfortably throughout the test.

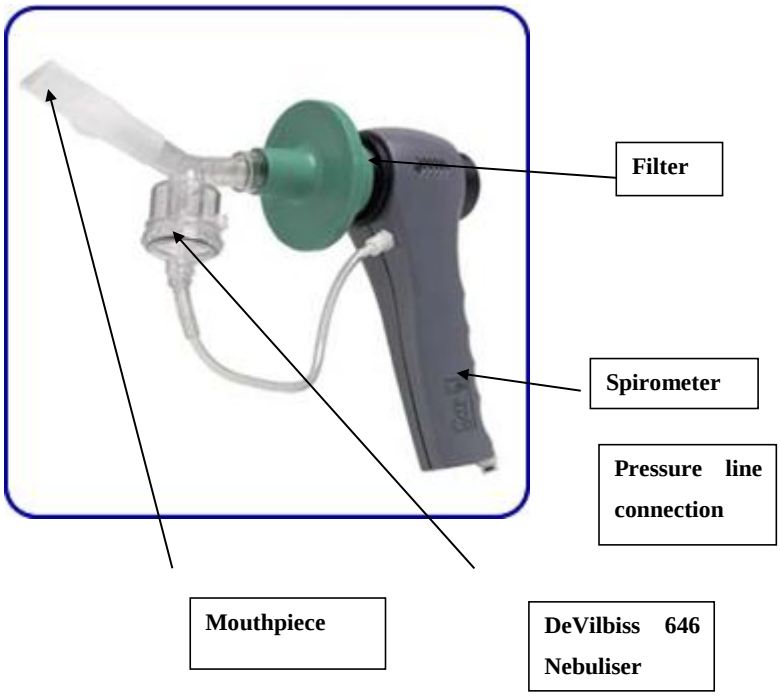
4iii Test sequence – Entering the patient information and performing the cough challenge test

1. Select the patient icon
2. Click on the icon and enter patient data in the relevant sections in the window which pops up immediately. Date of birth must be entered as a ***four-digit year*** (eg 1991 ***not*** 91).
3. Adding Extra categories to the "Diagnosis" can be achieved by clicking on the box to the right of the drop-down menu with the dotted lines, for instance you can insert "chronic cough".
4. Add comments relevant to the patient such as whether they are on acid suppression or whether they suffer from heartburn.
5. Add relevant group titles to patient database such as "chronic cough, new patient"
6. Once data inputted close patient details table
7. From the main menu select "challenge" icon
8. This will take you to the screen which requires spirometry to be performed
9. Click on the Start test icon.
10. Spirometer is performed using only the spirometer and filter. The nebuliser will not be attached at this stage.
11. A grey box appears requesting pneumotach environment data for room temperature and humidity. Click OK.
12. Get patient to perform 4 tidal breaths.
13. The computer screen will prompt the patient to inspire as deeply as possible.
14. The Computer screen will prompt the patient to expire as deeply as possible.
15. A results screen will appear with the spirometry results. Click OK.
16. Click on Go to next stage icon and select Saline test from the pop-up screen.
17. Click on Start administration icon.
18. Place 3ml of saline in a nebuliser pot (this will act as a negative control)
19. Attach to spirometer via filter
20. Ask patient to breath normally through the nebuliser
21. Observe 3 relaxed breaths
22. Get patient to then exhale as deeply as possible with the mouthpiece in the mouth. During the expiration press SPACEBAR to start the test.
23. Then the patient will be asked to perform a controlled deep inhalation, during this in halation the solution will be Nebulised .
24. A bell will sound 15 seconds post nebulisation to indicate the end of cough counting
25. A further bell will ring at 1min post nebulisation to indicate the challenge has completed.
26. Details of how to load the nebuliser with solution are shown in appendix 1.
27. Count the number of coughs for 15 seconds after the challenge.

28. If subject coughs 2 or more times in direct relation to inhaling saline then no further cough challenges should be performed.
29. Starting with the lowest concentration of ATP load the nebuliser pot with 3ml of solution and perform cough challenge as per the saline
30. Continue with each incremental concentration of ATP until 5 coughs or more are reached in one inhalation.
31. A positive result is obtained if the patient coughs ***5 or more times*** following nebulisation of the solution and the test is terminated.
32. At test termination repeat spirometry to measure any change from the pre-test spirometry

Appendix 1: Dismantling and loading the ATP dose in the nebuliser

1. Pull the short plastic tube out of the bottom of the nebuliser
2. Pull the mouthpiece and nebuliser out of the KoKo filter.
3. Remove the small stopper and dispense 3ml of solution into the nebuliser chamber.
4. After each ATP concentration has been nebulised, unscrew the bottom chamber from the upper chamber being careful not to spill the contents.
5. Pour any unused fluid into a beaker, clean out the chamber with a dry paper towel and load another dose of ATP following steps 1-3



APPENDIX 2

Exclusion Criteria

- *If the subject smokes:* Cough challenge must be performed at least one hour after the last cigarette has been smoked
- *If the subject has used an inhaler:* Lung function testing should be performed at least one hour after the use of any inhalers.
- *If the subject has used an inhaler that is not a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* Lung function testing is carried out and the data is recorded.
- *If the subject has used an inhaler that is a beta-2-agonist or an anticholinergic inhaler in the last one to four hours:* If the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unable or reluctant to return another time, testing should proceed and the medication used should be recorded.
- *If the subject has taken an oral beta-2-agonist or a theophylline or an oral antimuscarinic within the last eight hours:* If the subject is willing to come back another time for lung function testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded
- *If the subject has taken any over the counter (OTC) cough mixture within the last twelve hours:* If the subject is willing to come back another time for cough challenge testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.
- *If the subject has had a respiratory tract infection in the last three weeks:* Another appointment should be made unless the subject is unwilling to come back, in which case testing should continue. The number of days elapsed since the end of the respiratory infection should be recorded.
- *If the subject has had any food or drink products containing caffeine or menthol within the last hour*. If the subject is unwilling to wait for 1 hour before starting the test, the subject should return another time. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.

APPENDIX 3

CLINICAL TRIALS UNIT

RESPIRATORY MEDICINE

CARDIOVASCULAR & RESPIRATORY STUDIES

DigiDoser ATP Cough Challenge

	Conc ATP (mM)	No of Coughs
Saline		
0.1		
0.3		
1		
3		
10		
30		
100		
300		

C2 = _____ C5 = _____

Date of Test .....

Time .....

Study No:.....

Name .....

UN .....

DOB .....

Questions

Have you had a recent upper respiratory tract infection?	YES	NO
Are you a current smoker?	YES	NO
	No pack yrs____	
Are you taking any medications?	YES	NO
Do you have a family history of cough?	YES	NO

Medication List

....

12.5 Appendix 5

THE CLINICAL TRIALS UNIT

Centre for Cardiovascular and Metabolic Research

Standard Operating Procedure

Title: A log dose-response cough challenge with Capsaicin using the KoKo Spirometer & DigiDoser system

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T:\Cardiovascular and Respiratory Studies\CTU Documentation\Standard Operating Procedures

SOP reference number	**CTU070715**
Author	**Caroline Wright**
Current version and date	**Version 3, 09/03/16**
Approved by	**Professor Morice**
Approval signature/date	
Approved by CTU Manager signature/date	
Target audience	**Clinical Trials Unit staff**

SOP No: CTU070715

This page details the version history for this SOP and the main changes corresponding to the versions.

VERSION LOG		
Version number and date	**Author**	**Details of significant changes**
Version 1, 07.07.15	C.Wright	Original SOP authorised by Respiratory Medicine
Version 2 15.12.15	c.wright	Updated the table to change the way dilutions performed and to include the saline negative control
Version 3 09.03.16	C.Wright	SOP Number changed in title
Version 4 20.06.17	C.wright	Updated with details of flow limiting mouthpiece

Principle

To measure the sensitivity of the cough reflex within:

- Healthy volunteers
- Chronic cough patients
- Patients with other respiratory disorders

The method of administration of the Capsaicin is via a nebuliser utilising a KoKo spirometer & DigiDoser. This standard operating procedure is intended for all appropriately qualified staff and physicians within the Academic department of Respiratory Medicine

INDEX

1. Things to consider before performing the capsaicin cough challenge

i Personnel

Before performing the cough challenge on a patient, you must fulfil the following criteria:

1. Be capable of managing the equipment including set-up, proper function, maintenance and cleaning.
2. Be proficient at spirometry.
3. Know the exclusions (APPENDIX 2) to cough challenge testing.
4. Be familiar with safety and emergency procedures.
5. Know when to stop further testing.
6. Be proficient in the administration of inhaled bronchodilators and evaluation of the response to them.

ii Safety

Inhaled Capsaicin may cause bronchoconstriction. Thus, you should consider the safety of the patient.

Precautions for patient safety

A physician or other person appropriately trained to treat acute bronchospasm including appropriate use of resuscitation equipment should be close enough to respond to an emergency quickly.

You should make sure that medications to treat severe bronchospasm are present within the testing area. These include epinephrine and atropine for subcutaneous injection and salbutamol metered dose inhaler or pre-mixed solutions for inhalation, oxygen must also be available. A small volume nebuliser should be readily available for the administration of bronchodilators. A stethoscope, sphygmomanometer, and pulse oximeter should also be available.

2. Equipment and Materials

2.i Solutions

Capsaicin (CS): Manufactured by Formosa laboratories Inc., Taoyan, Taiwan, supplied by Stockport Pharmaceuticals, Stockport NHS Foundation Trust. Supplied as 5ml bottled aliquots at 0.03% w/v sterile (= 1mM) stored at 4ºC.

0.98% (sterile) sodium chloride for irrigation (Baxter, U.K.)

β adrenergic agonist nebule 2.5mg (Ventolin)

2.ii Dilution Equipment

x1 5ml micropipette

x10 5ml micropipette tips

x 9 sterile universal pots correctly labelled for each concentration of Capsaicin/saline

x1 sterile modified DeVilbiss pot

gloves

lab coat

2.iii Storage

Capsaicin stored at 4°C in a tightly sealed container

Saline diluent stored at room temperature

β adrenergic agonist nebule (2.5mg) stored at room temperature

2. iv Equipment

A KoKo DigiDoser

KoKo filter

A DeVilbiss 646 nebuliser with sterile chamber and mouth piece

Gas cylinder containing compressed air, set at 30psi.

3. Preparation for testing

3i Patient preparation before testing

a. Explain the test to the patient.

   Patients should be told that they might suffer severe bouts of coughing and that they may experience some minor symptoms such as chest tightness or breathlessness.

   Care should be taken to ensure that the test description does not bias the result.

b. Ask the patient if they would like to urinate before the test (stress incontinence could be precipitated, especially in older women).

c. Evaluate the patient for exclusions (**APPENDIX 2)** and review medication use, details of medications affecting the cough challenge test are in **APPENDIX 2**.

3ii Calibration

Before starting the test make sur the KoKo DigiDoser system is calibrated to the standard procedures outlined in SOP no:CTU080709

3iii Solution Preparation

1. Label 9 universal pots with final concentration

2. Follow the dilution sequence below, ensuring that each concentration of capsaicin is in the correctly numbered universal pot and that a new pipette tip is used to make each concentration of capsaicin. After addition of sodium chloride, the lid is replaced and the solution is adequately mixed.

Capsaicin		Vol. 0.9% NaCl Sol^{n}	Final [Capsaicin] (µM)
Sol^{n} used for dilution	Vol (ml)		
Stock 1mM	3	0	1mM
1mM Sol^{n}	3	7	300
1 mM Sol^{n}	1	9	100
300 µM Sol^{n}	1	9	30
100 µM Sol^{n}	1	9	10
300 µM Sol^{n}	1	9	3
10 µM Sol^{n}	1	9	1
3µM Sol^{n}	1	9	0.3
Saline Neg control		9	0

NOTE: This is enough to perform 3 challenges.

4. Performing the test

4 i Setting the equipment up – Assembling the nebuliser

a. Place the KoKo filter on to the spirometer
b. Assemble the nebuliser:-
c. Attach the nebuliser adapter, nebuliser and mouthpiece.

d. Insert the nebuliser into the KoKo Filter cone. The adapter end goes into the filter. The mouthpiece fits on the nebuliser end opposite the air vent.

e. Connect the pressure line outlet on the front of the KoKo DigiDoser handle to the pressure line inlet on the bottom of the supplied nebuliser with the supplied tubing.
f. Adjust the pressurised air source to 30 psi.

g. Connect the pressurised air source to the bottom of the DigiDoser with a long piece of tubing.

4ii Setting up the challenge protocol

a. Enter the challenge protocol by pressing icon 
b. Go to set up in the top bar menu
c. Go to protocol set up and create
Enter data as per the table in Appendix 3

4iii The patient

a. Subjects must be able to understand the procedure and perform reliable spirometric manoeuvres.
b. Subjects should be seated comfortably throughout the test.

4iv Test sequence – Entering the patient information and performing the cough challenge test

1. Select the patient icon 
2. Click on the icon and enter patient data in the relevant sections in the window which pops up immediately. Date of birth must be entered as a ***four-digit year*** (eg 1991 ***not*** 91).
3. Adding Extra categories to the "Diagnosis" can be achieved by clicking on the box to the right of the drop-down menu with the dotted lines, for instance you can insert "chronic cough".
4. Add comments relevant to the patient such as whether they are on acid suppression or whether they suffer from heartburn.
5. Add relevant group titles to patient database such as "chronic cough, new patient."
6. Once data inputted close patient details table.
7. From the main menu select "challenge" icon. 
8. This will take you to the screen which requires a baseline Spirometry to be performed prior to challenge.
9. Click on the Start test icon. 
10. Spirometry is performed using only the spirometer and filter. The nebuliser will not be attached at this stage.
11. A grey box appears requesting pneumotach environment data for room temperature and humidity. Click OK.
12. Get patient to perform 4 tidal breaths.

13. The computer screen will prompt the patient to inspire as deeply as possible.
14. The Computer screen will prompt the patient to expire as deeply as possible.
15. A results screen will appear with the spirometry results. Click OK.
16. Click on Go to next stage icon 
17. and select stage 1 from the pop-up screen.
18. Click on Start administration icon. 
19. Place 3ml of the 0.9% saline in a nebuliser pot
20. Attach to spirometer via filter
21. Ask patient to breath normally through the nebuliser
22. Observe 3 relaxed breaths
23. Get patient to then exhale as deeply as possible with the mouthpiece in the mouth. During the expiration press SPACEBAR to start the test.
24. Then the patient will be asked to perform a controlled deep inhalation whilst inhaling the flow controlled mouthpieces should make a continuous noise, reflecting the adequate strength of inhalation. During the inhalation the solution will be Nebulised for 1.2 secs. Allow a time interval of 60 seconds between cough challenges.
25. If the patient coughs 2 or more times in direct relation to inhalation of saline, the patient will need to abort the challenge as responsive to saline.
26. Details of how to load the nebuliser with each concentration of capsaicin are shown in Appendix 1.
27. Following the saline, load the lowest concentration of capsaicin perform challenge as per the saline.
28. Count the number of coughs for 15 seconds after each challenge. A positive result is obtained if the patient coughs ***5 or more times*** following nebulisation of a single solution, the test is terminated at this point. Continue testing each concentration of capsaicin until 5 coughs or more in a single inhalation is reached.
29. The program runs on a timer and alarms 15 secs post nebulisation to allow for the counting of coughs and them alarms again 1 minute post nebulisation to indicate that you are now ready to proceed with next concentration of capsaicin. Record results on the sheet Appendix 4.
30. To continue with subsequent concentrations press icon 

APPENDIX 1

Appendix 1: Dismantling and loading the capsaicin in to the nebuliser

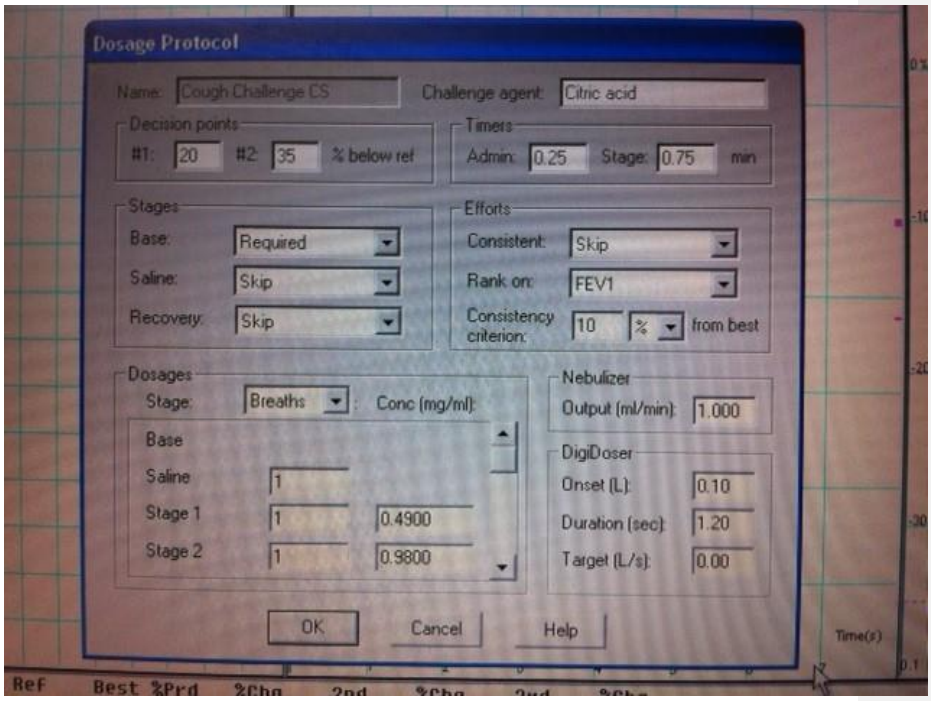
1. Pull the short plastic tube out of the bottom of the nebuliser
2. Pull the mouthpiece and nebuliser out of the KoKo filter.
3. Remove the small stopper and dispense 3ml of solution into the nebuliser chamber.
4. After each capsaicin concentration has been nebulised, unscrew the bottom chamber from the upper chamber being careful not to spill the contents.
5. Pour any unused fluid into a beaker, clean out the chamber with a dry paper towel and load another dose of capsaicin following steps 1-3

APPENDIX 2

Exclusion Criteria

- *If the subject smokes:* Cough challenge must be performed at least one hour after the last cigarette has been smoked.
- *If the subject has used an inhaler: Cough challenge* testing should be performed at least one hour after the use of any inhalers, the inhaler details should be written on the results form and the time of use.
- *If the subject has taken any over the counter (OTC) cough mixture within the last twelve hours:* If the subject is willing to come back another time for cough challenge testing, another appointment should be made. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.
- *If the subject has had a respiratory tract infection in the last three weeks:* Another appointment should be made unless the subject is unwilling to come back, in which case testing should continue. The number of days elapsed since the end of the respiratory infection should be recorded.
- *If the subject has had any food or drink products containing caffeine or menthol within the last hour*. If the subject is unwilling to wait for 1 hour before starting the test, the subject should return another time. If the subject is unwilling to return another time, testing should proceed and the medication used recorded.

APPENDIX 3


Dosage Protocol
Name: Cough Challenge CS
Challenge agent: Citric acid
Decision points
#1: 20
#2: 35
% below ref
Timers
Admin: 0.25
Stage: 0.75
min
Stages
Base: Required
Saline: Skip
Recovery: Skip
Efforts
Consistent: Skip
Rank on: FEV1
Consistency criterion: 10 %
from best
Dosages
Stage: Breaths
Conc (mg/ml):
Base
Saline 1
Stage 1 1 0.4900
Stage 2 1 0.9800
Nebulizer
Output (ml/min): 1.000
DigiDoser
Onset (L): 0.10
Duration (sec): 1.20
Target (L/s): 0.00
OK
Cancel
Help
Time(s)
Ref
Best %Prd
%Chg
2nd
%Chg
3rd
%Chg

APPENDIX 4

CLINICAL TRIALS UNIT

RESPIRATORY MEDICINE

CARDIOVASCULAR & RESPIRATORY STUDIES

DigiDoser Capsaicin Cough Challenge

	Conc Capsaicin (μM)	No of Coughs
Saline		
0.3		
1.0		
3.0		
10		
30		
100		
300		
1000		

C2 = _____ C5 = _____

Date of Test .....

Time .....

Study No:.....

Name .....

UN .....

DOB .....

Questions

Have you had a recent upper respiratory tract infection?	YES	NO
Are you a current smoker?	YES	NO
	No pack yrs____	
Are you taking any medications?	YES	NO
Do you have a family history of cough?	YES	NO

Medication List

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12.6 Appendix 6

Full List of Inclusion and Exclusion Criteria for Afferent Pharmaceuticals Study AF219-014

Inclusion Criteria

Both healthy and chronic cough patients who meet all of the criteria will be included in the study:

1. Be informed of the nature of the study and have provided written informed voluntary consent;
2. Be able to speak, read, and understand English;
3. Be males and females, of any race, between 18 and 8 years of age, inclusive.
4. Have a body mass index (BMI) > 18 and < 35
5. FEV1 > 80% at screening (healthy subjects only)
6. Be in good general health with no clinically relevant abnormalities based on the medical history, physical examination, clnical laboratory evauations (haematology, clnical chemistry, and urinalysis), and 12 lead electrocardiogram;
7. Be non-smokers for at least 5 years;
8. If a female of child bearing potential (i.e. have not undergone a hysterectomy or bilateral oophorectomy) or not post-menopausal (defined as no menses for at least 12 months), agree to use 2 forms of acceptable birth control from Screening through the Follow Up Visit; or if a male they and/or their partner of child-bearing potential agree to use 2 forms of acceptable birth control (defined in Section 5.3) from Screening through the Follow Up Visit;
9. Be able to communicate effectively with the Investigator and other study centre personnel and agree to comply with the study procedures and restrictions.
10. Subjects with a chronic cough must
    a. Have Treatment Refractory cough for at least one year: a cough that is unresponsive to at least 8 weeks of targeted treatment for underlying triggers inclusing reflux disease, asthma and post-nasal drip
    b. Have a cough for which no objective evidence of an underlying trigger can be determined after investigation
    c. Demonstrate significant airway symptoms by a score greater than 20/70 on the Hull Airway Reflux Questionnaire (HARQ)

Exclusion Criteria

Subjects will be excluded if any of the following apply:

1. History of upper respiratory tract infection or recent significant change in pulmonary status within 4 weeks of the Baseline visit (Day 0)
2. Have acute worsening of asthma
3. Do not cough during the ATP or Capsaicin or Citric Acid challenge at screening or only cough twice at the two highest concentrations of the three test solutions
4. Demonstrate more than two coughs to inhalation of the normal saline solution during baseline challenge

5. Treatment with an ACE-inhibitor as the potential cause of a subject's cough, or requiring treatment with an ACE-inhibitor during the study or within 4 weeks pror to Screening
6. History of opiod use within 1 week prior to the baseline visit
7. Requiring concomitant therapy with prohibited medications
8. History or symptoms of renal disease or renal obstructive disease:
    a. History of kidney/bladder stones (nephron/uro-lithiasis) within 5 years of screening
    b. History of conditions or disorders that predispose to nephrolithiasis such as Type I renal tubular acidosis, cystinuria, gout, hyperparathyroidism, inflammatory bowel disease (i.e. ulcerative colitis and Crohn's disease), short bowel syndrome, or bariatric surgery.
    c. Estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m2 at screening
9. History of concurrent malignancy or recurrence of malignancy within 2 years prior to screening (not including subjects with <3 excised basal cell carcinomas)
10. History of a diagnosis of drug an alcohol dependency or abuse within approximately the last 3 years
11. Any condition possibly affecting drug absorption (e.g. gastrectomy, gastroplasty, any type of bariatric surgery, vagotomy or bowel resection)
12. Screening systolic blood pressure (SBP) >160mmHg or a diastolic blood pressure (DBP) >90mmHg
13. Clinically significant abnormal (ECG) at Screening including any of the following:
    a. QT (QTc) interval >450 msec for males and >470 msec for females
    b. Atria fibrillation or Atrial Flutter
    c. Heart rate <40 bpm or >110 bpm
    d. Second degree or third degree AV block
    e. Left bundle branch block (including hemi-block)
    f. Wolf-Parkinson-White Syndrome
14. Personal or family history of congenital long QT syndrome or family history of suddn death
15. Cardiac Pacemaker
16. Significantly abnormal laboratory tests at Screening including:
    a. ALP, ALT, AST, or total bilirubin >150% of the upper limit of normal
    b. Hb <10gm/dL, WBC <2500 mm3, neutrophil count <1500mm3, platelet count <100 x 103/mm3
    c. Positive urine tests for drugs of abuse,
    d. Positive tests at screening for viral hepatitis or HIV
17. History of cutaneous advers drug reaction to sulphonamides or signs and symptoms suggestive of anaphylaxis to sulphonamides
18. Pregnant or breastfeeding
19. Treatment with an investigationsl drug or biologic within 30 days preceding the first dose of study medication or plans to take another investigational drug or biologic within 30 days of study completion
20. Blood donation within 56 days or plasma donation within 7 days prior to dosing;
21. Other severe, acute, or chronic medical or psychiatric condition or laboratory abnormality that may increase the risk associated with the interpretation of trial results and, in the judgement of the Investigator or Sponsor, would make the subject inappropriate for entry into this trial.