The role of nerves in asthma: insights from the study of cough

A Thesis Submitted to the University of Manchester for the Degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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List of Abbreviations

95% CI	95% confidence intervals
ACCP	American College of Chest Physicians
ACE	Angiotensin converting enzyme
ACT	Asthma Control Test
ACQ	Asthma Control Questionnaire
AMP	Adenosine 5'-monophoshate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
APC	Antigen presenting cells
ASIC	Acid-sensing ion channel
ASM	Airway smooth muscle
ATP	Adenosine-5-triphosphate
ATS	American Thoracic Society
B1/2	Bradykinin receptor 1/2
BDNF	Brain derived neurotrophic factor
BHR	Bronchial hyper-responsiveness
BK	Bradykinin
BMI	Body mass index
BTS	British Thoracic Society
C2	Concentration of tussive agent inducing at least 2 coughs
C5	Concentration of tussive agent inducing at least 5 coughs
CA	Classical asthma
Ca	Calcium
CC	Chronic cough
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase 2
CQLQ	Cough-specific quality of life questionnaire
CS	Central sensitisation
C/hr	Coughs per hour
CT	Computed tomography
CVA	Cough variant asthma
CysLT	Cysteinyl leukotrienes
DAG	Diacylglycerol
DNIC	Diffuse noxious inhibitory controls
DRG	Dorsal root ganglion
EAR	Early asthmatic response

EB	Eosinophilic bronchitis
ECRHS	European Community Respiratory Health Survey
ED50	Dose evoking atleast half the Emax
Emax	Maximum evoked coughs
EP2	Prostaglandin EP2 receptor
ERS	European Respiratory Society
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
fMRI	Functional magnetic resonance imaging
FVC	Forced vital capacity
GABA	Gamma-aminobutyric acid
GC	Guanylate cyclase
GEE	Generalised estimating equations
GINA	Global Initiative for Asthma
GMP	Guanosine 3′,5′-monophosphate
GPCR	G-protein coupled receptors
GORD	Gastro-oesophageal reflux disease
HDM	House dust mites
HV	Healthy volunteers
ICS	Inhaled corticosteroids
IP3	Inositol phosphate 3
IQR	Inter-quartile range
LABA	Long acting beta 2 agonist
LAMA	Long acting muscarinic antagonist
LAR	Late asthmatic response
LCQ	Leicester cough questionnaire
LGIC	Ligand gated ion channels
LPR	Laryngo-pharyngeal reflux
LTRA	Leukotriene receptor antagonists
mDC	Myeloid dendritic cells
mRNA	messenger ribonucleic acid
NANC	Non-adrenergic non-cholinergic
NGF	Nerve growth factor
NK1	Neurokinin receptor 1
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NONMEM	Non-linear mixed effects modelling
NOS	Nitric oxide synthase
NT-3	Neurotrophin 3

nTS	nucleus of tractus solitarius
P2X/Y	Purinoceptor 2
PAF	Platelet-activating factor
PAG	Periaqueductal grey
PC20	Provocative concentration causing a 20% fall in FEV1 from baseline
PD	Pharmacodynamic
pDC	Plasmacytoid dendritic cells
PEFR	Peak expiratory flow rate
PGE2	Prostaglandin E2
PGD2	Prostaglandin D2
PK	Pharmacokinetic
PKA	Protein kinase A
PLC	Phospholipase C
PND	Post-nasal drip
RAR	Rapidly adapting receptors
RVM	Rostral ventral medulla
SAP	Symptom association probability
SAR	Slowly adapting receptors
SD	Standard deviation
SP	Substance P
SPT	Skin prick testing
TrkA/B	Tropomyosin-related kinase A/B
TRPA1	Transient receptor potential cation channel member A1
TRPM8	Transient receptor potential cation channel member M8
TRPV1	Transient receptor potential cation channel member 1
TSLP	Thymic stromal lymphopoetin
UTC	Urge to cough
VGIC	Voltage gated ion channels
VIP	Vasointestinal peptide

Abstract

The role of nerves in asthma: insights from the study of cough
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Introduction: Cough in asthma predicts disease severity, poor prognosis, and is a common troublesome symptom, yet remarkably, little is understood about the underlying neuronal mechanisms. Current dogma suggests asthma symptoms arise as a consequence of bronchial hyper-responsiveness and airway inflammation. Yet despite effective treatments targeting these pathologies, many patients have substantial residual disease and symptoms. Symptoms such as wheeze, cough, chest tightness and breathlessness occur as a consequence of neuronal input from the airways and chest wall. However, there is a paucity of data investigating whether sensory nerve function is heightened in patients with asthma. Many symptoms are challenging to study as they can only be reported subjectively. In contrast, cough is more readily accessible to objective quantification and hence has the most potential for providing insights into the role of nerves in asthma.

Methods: We performed capsaicin cough challenges to experimentally evoke the cough reflex via activation of the transient receptor potential vanilloid type-1 (TRPV1) receptor found characteristically on airway c-fibres. Evoked and spontaneous coughs were objectively quantified and verified using a semi-automated cough recorder (VitaloJAK). We first performed an observational cross-sectional study comparing full dose capsaicin cough responses in patients with mild to moderate stable asthma with healthy volunteers. We performed non-linear pharmacodynamic modelling (NONMEM) to compare differences in the maximum evoked coughs (Emax) and the dose of capsaicin that evoked at least half the responses (ED50). This was followed by two separate randomised, single-blind, placebo controlled cross-over studies investigating the effects of methacholine induced bronchoconstriction and inhaled allergen challenge on capsaicin ED50 evoked coughs. Generalised estimating equations (GEE) were used to model the effects of bronchoconstriction on cough responses.

Results:

i. Compared with healthy volunteers (HV), patients with asthma (A) displayed higher Emax and lower ED50 on full dose capsaicin evoked cough challenge. Emax was influenced by disease group (HV or A), gender, atopic status and objective cough rates, whilst ED50 was influenced by disease group, gender, asthma control (ACQ), and total serum IgE.

ii. Methacholine induced bronchoconstriction was associated with an increase in capsaicin evoked cough response, but capsaicin evoked coughs had no influence on bronchial hyper-responsiveness to methacholine.

iii. Inhaled allergen challenge induced an acute early asthmatic response (EAR) and a late asthmatic response (LAR). There was an increase in capsaicin evoked coughs in the EAR and 24 hours after the start of inhaled allergen challenge.

Conclusions: Patients with asthma exhibited heightened cough responses to capsaicin at baseline, which increased further after inducing bronchoconstriction and allergen exposure. The exaggerated cough reflex observed could be due to peripheral sensitisation and/or central sensitisation. The exact mechanism of how bronchoconstriction and airway inflammation influences the cough reflex to capsaicin requires further evaluation.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

All the work contained in this thesis is my own, unless otherwise specified.

Particular note should be made of:

i. Mrs Kimberley Holt recruited and performed capsaicin cough challenges in 47 healthy volunteers. This data was used in the publication of the first study comparing capsaicin evoked coughs and objective cough rates between healthy volunteers and patients with asthma.

ii. Objective 24 hour recordings were verified and quantified by trained members of the Cough Research Team at the University of Manchester.

iii. Pharmacodynamic modelling (NONMEM) was performed by Dr Nikolaos Tsamandouras and Dr Kayode Ogungbenro at the Manchester Pharmacy School, University of Manchester.

Copyright Statement

Dedication

To my whole family, particularly my parents Fackeerbhai and Nasima Satia, my wife Aasiya and children Hafsah, Sumayyah and Zaynab for their unconditional support, patience and resilience throughout my PhD.

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This PhD would not have been possible without the mentorship and supervision of Professor Jacky Smith, Professor Paul O'Byrne and Dr Stephen Fowler. They have provided me with endless support, an opportunity to develop and overlooked by mistakes and shortcoming.

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I have benefitted immensely from the facilities available at the NIHR Clinical Research Facilities at South Manchester and Central Manchester and I am eternally grateful to Dr Angela Kelsall, Helen Pidd and Paul Brown.

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Finally, the results of my research depended on the goodwill and generosity of every single participant who attended. They inhaled chilli pepper extracts, methacholine, allergen and walked around wearing a cough monitor for 24 hours numerous times – all in the name of science and furthering our understanding of cough in asthma.

The Author

I graduated in medicine from the University of Cambridge in 2006, with an intercalated degree in Neurophysiology. Between 2006 -2010, I completed Foundation and Core Medical Training and became a Member of the Royal College of Physicians (2010). I started my specialist training in Respiratory and General Internal Medicine in August 2010 in the North West Deanery. In January 2013 I took time out of my clinical programme to take up a post as Clinical Research Fellow at the NIHR Clinical Research Facility in South Manchester. During my PhD I have presented at local, national and international conferences and been the recipient of a few awards and grants:

1. European Academy of Allergy and Clinical Immunology Travel Award (November 2016)
2. British Medical Association James Trust Award (July 2015, £55,000)
3. American Thoracic Society International Trainee Scholarship Award (May 2015)
4. North West Lung Centre Asthma Research Grant (£15,000)
5. Best National SPR Research Presentation at GSK Sparrows Symposium (December 2014)
6. Best SPR Research Abstract; North West Thoracic Society, October 2014

I have been back in my clinical programme as a respiratory and general internal medicine specialist trainee since August 2016.

Selected Publications

1. **Capsaicin cough responses in Asthma; Evidence for neuronal dysfunction.**
   Satia I, Tsamandouras N, K Holt, H Badri, M Woodhead, Ogungbenro K, Felton TP O'Byrne, SJ Fowler, Smith JA**.** *Journal of Allergy and Clinical Immunology June 2016*

2. **Objective Cough Frequency, Airway Inflammation And Disease Control In Asthma.** Paul A Marsden, **Imran Satia**, Baharudin Ibrahim, Ashley Woodcock, Lucy Yates, Iona Donnely, Lisa Jolly, Neil C. Thomson, Stephen J Fowler, Jaclyn A Smith. *Chest. 2016 Jun; 149(6):1460-6.*

3. **Towards Understanding and Managing Chronic Cough.**
   Satia I, Al-Sheklly B, Badri H, Woodcock AA, Smith JA. (Review) *Horizons in Medicine, Clinical Medicine, RCP London. Clinical Medicine 2016 Vol 16, No 6: s92–s97*

4. **Investigating the interactions between bronchoconstriction and cough in asthma. Satia I,** Badri H, Woodhead M, O'Byrne P, Fowler SJ, Smith JA. *Thorax 2017 Feb 24.*

Spoken Presentations and Abstracts

1. **A Randomised, double-blind, placebo controlled crossover study to assess the efficacy of a single dose of 100 mg of vrp700 by inhalation in reducing the frequency and severity of cough in adult patients with idiopathic pulmonary fibrosis.** I Satia, H Badri, R Dockry, N Chaudhuri, G Brown, K Abbott-Banner, JA Smith. British Thoracic Society, 3rd December 2015.

2. **Investigating Neuronal Responses By Assessing Capsaicin Evoked Cough Responses In An Allergen Challenge Model Of Asthma (INCA): Satia I,** Badri H, Woodhead M, O'Byrne PM, Fowler SJ, Smith JA. International Severe Asthma Forum. European Academic of Allergy and Clinical Immunology. 17th November 2016

3. **The Interaction between bronchoconstriction and cough in Asthma: Satia I,** Badri H, Woodhead M, O'Byrne PM, Fowler SJ, Smith JA. ERS Abstract 357

4. **Investigating Neuronal responses to Capsaicin after inhaling Allergen; Resurrecting the legacy of Blackley and Altounyan: Satia I,** Al-Sheklly B, Gibbard C, Sen S, Stephan S, Kelsall A, Howard L, Pidd H, Brown P, Woodhead M, Fowler, SJ, O'Byrne PM, Smith JA. UK CRF 12th Annual Conference, May 2016.

5. **South and Central CRF Collaboration in Manchester- A platform for future success: Satia I,** Badri H, Holt K, Al-Sheklly B, Mawn S, Gibbard C, Sen S, Stephan S, Mayo S, Kelsall A, Howard L, Pidd H, Brown P, Woodhead M, Fowler, SJ, O'Byrne PM, Smith JA. UK CRF 12th Annual Conference, May 2016.

6. **A Randomised, Double-Blind (Sponsor-Unblind), Placebo Controlled, Cross-Over Study To Investigate The Efficacy, Effect On Cough Reflex Sensitivity, Safety, Tolerability And Pharmacokinetics Of Inhaled GSK2339345 In Patients With Chronic Idiopathic Cough Using An Aqueous Droplet Inhaler.** H Badri, **I Satia,** R Dockry, L McGarvey, J Marks-Konczalik, RD Murdoch, A Cheesbrough, F Warren, S Siederer, JA Smith. *Thorax 2015;70 Suppl 3 A196.*

7. **Neuronal dysfunction in asthma; novel mechanistic insights from the study of the cough reflex. (Satia I,** K Holt, H Badri, M Woodhead, P O'Byrne, SJ Fowler, JA Smith) *Am J Respir Crit Care Med 191;2015:A4103*

8. **The use of low dose morphine for the management of chronic cough in a tertiary cough clinic.** Badri H, **Satia I,** Woodcock AA, Smith JA. *Am J of Respir Crit Care Med 191;2015:A4117*

9. **The usefulness of heartburn as a marker of the success of acid suppression therapy in chronic cough. (**H Badri, **I Satia**, AA Woodcock, JA Smith) *Am J Respir Crit Care Med 191;2015:A4124*

10. **The effect of naltrexone, an opioid receptor antagonist, on capsaicin evoked cough, in male healthy subjects (NORAH).** (**I Satia,** K Holt, E Hilton, AA Woodcock, JA Smith). *Thorax 2014;69: Suppl 2 A81-A82*

11. **The Role of GABAB receptor mechanisms in the human cough reflex** (H Badri, C Gibbard, D Valdramidou, **I Satia,** B Canning, LA Houghton, A Holt, G Wilkinson, JA Smith) *Thorax 2014;69:Suppl 2 A82*

Thesis Format

This thesis has been written in an alternative format starting with an introduction and general methods followed by three papers in manuscript form. The first paper (Chapter 3) has been accepted for publication in the Journal of Allergy and Clinical Immunology, the second paper (Chapter 4) is currently under review in Thorax, and the third study (Chapter 5) is written in a manuscript format awaiting further recruitment at McMaster University, Canada. The thesis concludes with a final summative discussion chapter.

I have incorporated a list of references along with tables and figures after each individual paper as they would appear in the published manuscript. Please note that chapter 3 and 5 both contain online supplements with additional information.

1 Introduction

1.1 Cough in Asthma

1.1.1 Background

Asthma is a chronic inflammatory disease of the airways associated with bronchial hyper-responsiveness (BHR) and variable airflow obstruction and the presence of symptoms such as wheeze, cough, chest tightness and shortness of breath. An estimated 300 million worldwide suffer from asthma with 10% of adults and up to 30% of children affected in the western world [1, 2]. Symptoms of asthma can vary and hence some patients classically describe all four symptoms, but there are some patients with asthma who may have one predominant symptom or have one symptom only, for example, cough variant asthma first described by McFadden [3] and Corrao et al [4]. Cough variant asthma (CVA) has features of BHR and typically respond well to bronchodilators. Importantly, some patients may be persistently coughing and demonstrate eosinophilic airway inflammation without BHR and are thought of as having eosinophilic bronchitis (EB) [5, 6].

My studies are focused on understanding the mechanism of cough in classical asthma and not specifically cough variant asthma or eosinophilic bronchitis, or those with chronic cough who may have concomitant asthma. Of all the symptoms of asthma, cough is a symptom which can be objectively quantified; hence this provides a unique tool to understand how the pathophysiology underlying asthma may drive symptoms.

1.1.2 Prevalence

In patients with a diagnosis of asthma with the classical symptoms of wheeze, cough, chest tightness and shortness of breath, the exact prevalence of cough has large variations in different countries. The European Community Respiratory Health Survey (ECRHS) was a multi-centre, multinational, self-reported questionnaire study which asked participants: *"Have you been woken by an attack of coughing at any time in the last 12 months?"* The median percent prevalence of 'waking with cough' across 48 centres was 27.9% (IQR 25.6-29.5), but there were considerable geographical variations. The authors commented that this could be due to language, cultural and differences in response rates [7]. In a follow up study, a random sample from the ECRHS were invited to attend a more detailed interview led-questionnaire and included 8748 males and 9531 females (mean age 34, range 20-48 years). This study specifically asked about productive, non-productive and nocturnal cough and found percent prevalence of 10.2, 10.2 and 31.2% respectively. However, this variation was significantly related to the presence of asthma, tobacco smoking, and obesity. Furthermore, female sex was associated with a higher prevalence of nocturnal and non-productive cough [8]. Similarly, in Canada, a large population-

based postal survey of asthma suggested nocturnal cough was more commonly reported than wheezing, especially in women (37.6% vs. 27.6% in males) [9].

There are many limitations in these population based surveys because they depend on accurate patient recall over 12 months, self-reported asthma diagnosis, different health care systems, and are confounded by the presence of variables such as smoking. Furthermore, although assessment of the level of asthma control has been performed using validated questionnaires such as the Asthma Control Questionnaire (ACQ) and Asthma Control Test (ACT), these questionnaires do not directly assess symptoms of cough, and hence large scale epidemiological data specifically on cough is lacking.

1.1.3 Impact

Evidence exists to suggest that increased coughing is associated with worsening asthma severity, poor prognosis and quality of life. Cough with sputum production has been shown to be associated with worse asthma disease severity and higher exacerbations rates [10]. In a questionnaire study in West Sweden, long-standing cough, morning cough, and productive cough were all increased in subjects with asthma compared to healthy controls and associated with worse asthma control [11]. In an observational outpatient study of patients with moderate and severe asthma, 61% complained of cough on most days, despite treatment in a tertiary referral centre [12]. Cough also gave the highest predicted weighting for asthma severity in a 9-year prospective cohort study using the Global Initiative for Asthma (GINA) categorisation of asthma severity [13].

Cough may also be more troublesome in chronic stable asthma than wheeze, shortness of breath or sleep disturbance [14]. Analysis of cough-related quality of life questionnaires [(Leicester Cough Questionnaire (LCQ), and the Cough Quality of Life Questionnaire (CQLQ)] demonstrated no significant differences in scores between patients with chronic cough, asthma, bronchiectasis and COPD suggesting that cough in asthma might be just as disabling to patients in comparison to the other three disease groups [15].

In a 15 year longitudinal study of patients with self-reported asthma in Denmark, those with chronic mucus hypersecretion had a significantly greater decline in FEV1 than those without [16]. Chronic mucus hypersecretion was defined as expectorating phlegm during at least three months per year for at least two consecutive years, and hence implies the presence of cough. Similar findings have been showed in previous studies and could be regarded as a marker of poor control and worsening severity [17], but this could also be related to smoking [18].

More recently, there have been studies that have objectively quantified cough rates over 24 hours using a portable cough monitor (see methods section 2.4.2 for further details). For the first

time this has allowed objective quantification of coughs and comparison with other disease groups, and attempts made to understand the relationships with important measures of asthma. Firstly, these studies demonstrate that most patients with asthma do not cough to the same extent as seen in patients with chronic cough (Table 1.1). Secondly, there exists variability in cough rates which may be depend on asthma control and severity [19, 20]. Thirdly, most of the studies listed in Table 1.1 have recruited patients with asthma from secondary care, who tended to be less well controlled with more severe disease. There is no study to have performed a direct comparison between healthy volunteers and patients with stable mild to moderate asthma.

Objective cough monitoring has also demonstrated that although subjective questionnaires are useful for assessing impact on patient's lives, only a moderate relationship exists between objective cough monitoring and LCQ, and cough rates did not correlate with measures of asthma such as %FEV1 predicted, fractional exhaled nitric oxide (FeNO), or with the dose of inhaled steroid [20]. A further follow up study investigating the relationship between objective cough rates, airway inflammation and airflow obstruction revealed that increasing cough rates and lower FEV1 independently predicted ACQ and GINA control [19].

Table 1.1 Comparison of objective cough rates in patients with asthma, chronic cough and healthy volunteers

Disease Group	N	Gender (M:F)	Age	24 hr cough rate (c/hr)	Daytime cough rate (c/hr)	Night-time cough rate (c/hr)	Additional Information	Monitor
Asthma [19]	86	37:49	57.3** (±11.9)	2.5* (0.2-27.6)	3.7 * (0.2-41.3)	0.5* (0-29.6)	GINA: Well-controlled (20%), Partly Controlled (44%), Un-controlled (36%) FEV1 % predicted 86.4% (±22.1)	VitaloJAK
Asthma [20]	56	22:34	41.5** (±13.6)	2.6* (0-14)	3.9* (0-18)	0.3* (0-9)	FEV1 % predicted 93.9% (30-127)	VitaloJAK
Asthma [21]	9	4:5	52 (22-77)	4.5	-	-	FEV1 % predicted 85% (±12)	Leicester Cough Monitor
Unexplained Chronic Cough [21]	34	7:27	60 (46–80)	19.9	-	-	FEV1 % predicted 91% (±10)	Leicester Cough Monitor
Chronic Cough [22]	62	23:39	54.9** (±12.2)	11.36* (1.06-46)	15.59* (2-74.8)	2.94* (0-26.7)	FEV1 % predicted 101.8% (±16.2). Median duration of cough 5.5yrs (1–30)	VitaloJAK
Chronic Cough [23]	100	35:65	55.8** (±11.0)	Females			Median duration of cough 4.0 yrs (2–10)	VitaloJAK
				16.6* (13.1-21.0)	23.7* (12.4–37.5)	4.5* (2.1–12.9)		
				Males				
				9.4* (6.4 to 13.9)	15.6* (11.1–24.3)	0.8* (0.1–4.5)		

Data quoted as median (IQR), * geometric mean (95% confidence interval); **mean (±S.D)

1.1.4 Triggers and co-morbidities

Although asthma is thought of as a common cause of cough, particular in patients presenting with isolated chronic cough, there are some co-existing conditions which could have the potential to also trigger coughing. Examples of such co-morbidities include bronchiectasis, allergic rhinitis, and gastro-oesophageal reflux disease (GORD). However, these are less well studied and the mechanism causing cough is poorly understood.

Mucus hyper-secretion and sputum production are not typically associated with mild to moderate asthma, however, its presence could suggest the presence of bronchiectasis or cigarette smoking [10, 24]. In such instances, coughing to clear the airways and preventing mucus impaction of the lower airways would be an important defensive mechanism. Hence treatment is often directed at airway clearance techniques, smoking cessation, mucolytics, and long term low dose prophylactic anti-biotics.

The prevalence of asthma in patients with rhinitis varied from 10%-40% in the Allergic Rhinitis and its Impact on Asthma (ARIA) update review [25]. There is some evidence to suggest the nasal mucosa and lower airways are connected in a theory of ‘one unified airway’; nasal allergen challenge can induce eosinophils in bronchial and nasal biopsies, whilst segmental bronchial allergen challenge in the lower airways can induce mast cell degranulation in both the bronchi and nasal mucosa [26, 27]. Furthermore, subjects with allergic rhinitis but not asthma show increased BHR to methacholine and adenosine 5’-monophoshate (AMP) during the pollen season, similar to that seen in asthmatics [28]. Persistent nasal disease predicts the presence of asthma [29] and severe uncontrolled asthma commonly have severe nasal disease [30]. Treatment with nasal corticosteroids, oral anti-histamines, leukotriene receptor antagonists (LTRA), reduced asthma morbidity and improved asthma control in some studies but not all [25]. However, there exists little data specific to coughing in asthma being related to allergic rhinitis, and even in patients with chronic cough, in our clinical experience treatment for allergic rhinitis very seldom cures coughing and randomised clinical trials are lacking.

A limited number of studies have performed capsaicin challenge in patients with allergic rhinitis. Patients with allergic rhinitis coughed more to inhaled capsaicin after intranasal histamine challenge [31] and those who are pollen sensitive are also more sensitive to capsaicin during and out of season [32].

There is still considerable debate about the relationship and potential mechanisms between GORD and cough, and it is beyond this review to fully explain all the postulated mechanisms. In brief, controversy exists in attributing causation or association between reflux and cough and the lack of a clear mechanism to explain causality. This is partly complicated by definitions of GORD, and the reliability of the methods/techniques used to quantify reflux and cough

episodes. The influence of reflux on respiratory disease and of respiratory disease on reflux has been extensively reviewed by Houghton and colleagues [33]. Specific to asthma, there have been reports of increased level of pepsin and/or bile in broncho-alveolar lavage [34] and exhaled breath condensate [35] suggesting possible micro-aspiration. However, sampling techniques can often be contaminated, and the kits available to measure pepsin/bile are often not validated and maybe measuring non-specific pepsin, which can be found expressed in alveolar type 2 pneumocytes [36]. The number of episodes of laryngopharyngeal reflux (LPR) as measured by impedance-pH monitoring in patients with asthma was only two to three in a 24 hour episode [37, 38], but this monitoring can be highly dependent on correct placement of the sensor as many episodes may be related to swallowing.

Evidence of neuronal sensitisation comes from a study which objectively recorded ambulatory coughs over 24 hours whilst having an impedance-pH sensor simultaneously in patients with chronic cough [39]. The important findings were; “48% had a positive symptom association probability (SAP) of coughs preceded by (mainly distal) reflux, 56% had positive SAP for reflux preceded by cough, and 32% both”. Importantly, when cough did occur, the pH of the acid was irrelevant and those with a positive SAP actually had physiological levels of events and exposure to acid. No such study has been performed in patients with asthma.

These studies raise the possibility that reflux episodes may trigger an oesophageo-bronchial cough reflex as they share a common vagal afferent which synapses in the brainstem, hence, raising the possibility of neuronal ‘cross-talk’ (see section 1.3). Consistence with this theory is the observation that in patients with asthma, infusion of hydrochloric increases BHR to histamine and methacholine [40, 41]. Interestingly, in another study, methacholine induced bronchoconstriction in asthma was associated with transient reduction in the pressure of the lower oesophageal sphincter resulting in an increase in acid reflux episodes [42].

1.1.5 Treatments

Despite cough being a major symptom associated with asthma severity and poor patient prognosis, current medication is not designed to directly treat cough. Steps 1 and 2 in the management of asthma recommend a short acting beta 2 agonists (SABA) as required and to consider introducing a low dose inhaled corticosteroids (ICS). If symptoms persist, step 3 recommends a trial of combined ICS and long acting beta 2 agonist (ICS/LABA). Should this not provide adequate control, then higher doses of ICS can be initiated at step 4 with or without a LABA. Additional medication that can be offered to control symptoms would be LTRA and low dose theophylline at steps 2,3, and 4 before reaching step 5, where referral for treatment with monoclonal antibodies (anti-IgE or anti-IL 5) or a regular maintenance dose of low dose oral steroid be made. The most recent GINA update also recommends the use of long-acting anti-muscarinic inhalers (LAMA) as an add-on treatment for persistently symptomatic patients at step 4 and 5 [2].

Unfortunately, there are few high quality data available which have assessed the effects of these medications on objective cough frequency and there is no licensed medication for the management of cough related to asthma. The American College of Chest Physicians has published a clinical practice guideline on chronic cough due to asthma [43]. This recommends cough due to asthma be initially treated with bronchodilators and ICS, however, they caution the use of aerosol inhalers that may exacerbate cough [44]. In cases where there is partial improvement with ICS, the guideline recommends a trial of oral steroids [45]. However, this leaves some diagnostic uncertainty, as the true underlying diagnosis could be asthma, eosinophilic bronchitis, or very rarely an interstitial lung disease. As such, an assessment on airway inflammation, bronchial hyper-responsiveness and chest imaging becomes necessary in order to decipher a more clear diagnosis.

In subjects with a clear diagnosis of cough variant asthma (CVA), compared to placebo, the leukotriene receptor antagonist (LTRA) zafirlukast has shown subjective improvements in mean cough scores (0-10) from 7.75±0.56 to 3.25±0.84 (p=0.0006) [46]. The mechanism of this anti-tussive effect is unknown and whether this can be used a monotherapy in asthma is still unclear and not recommended. Table 1.2 shows a list of medications that have been investigated in an attempt to reduce subjective or objective measures of cough in patients with chronic cough or cough variant asthma. However, these studies have been graded as 'low' or 'fair' evidence as improvements in cough were mainly reliant on subjective cough scoring systems or on tussive challenges, with small numbers, confounding aetiologies or possible interactions with concomitant medications. Overall, none of the studied medication been shown to be better than inhaled bronchodilator or ICS, with or without an LTRA.

The most recent ACCP and ERS consensus statement on management of chronic cough has acknowledged that cough is an airway neuronal reflex, and have coined the term "Cough Hypersensitivity Syndrome" [47, 48]. This term is used mainly in a clinical context where patients with unexplained, or refractory to treatment (of an identifiable condition), continue to have a dry, irritating, non-productive cough. Subjects describe sensitivity to change in temperature, strong smells, talking, laughing or singing. In this context, after all potential common causes have been investigated or optimally treated, the medication to trial include low dose morphine sulphate [49], gabapentin [50], pregabalin with or without speech and language therapy [51].

Table 1.2: Studies of anti-tussives with cough variant asthma

Reproduced directly from the ACCP guideline. PRDBPC = prospective, randomised, double-blind, placebo-controlled study; ICS = inhaled corticosteroid. † Values are given as mean ± SD, ‡ Values in parentheses are the mean.

Study/Year	Age	Study Design	Patients, No.	Treatment	Response Rate, %	Other Details	Quality of Evidence
Corrao et al 1979 [4]	16–40	Prospective, descriptive	6	Terbutaline (inhaled)	100	Duration of cough 1-48 months.	Low
Irwin et al 1997 [52]	55 ± 16†	PRDBPC crossover	15	Metaproterenol (inhaled)	60	In 40%, cough due to other aetiologies	Fair
Corrao et al 1979 (4)	16–40	Prospective, descriptive	6	Theophylline	100	Duration of cough 1-48 months.	Low
Crimi et al 1995 [53]	20–76 (44)‡	PRDBPC	62	Theophylline	83	Response rate for all asthma symptoms	Fair
Crimi et al 1995 [53]	18–55 (37)‡	PRDBPC	43	Nedocromil sodium	78	Response rate for all asthma symptoms	Fair
North American Tilade Study Group 1990 [54]	12–70 (35.2)‡	PRDBPC	121	Nedocromil sodium	Improvement in treated patients. (p = 0.02)	Patients also on theophylline and oral β-agonists	Fair
Dicpinigaitis et al 2002 [46]	27–62	PRDBPC crossover	8	Zafirlukast	88	Suppression of cough reflex sensitivity 100%	Fair
Irwin et al 1997 [52]	55 ± 16†	PRDBPC crossover	15	Beclomethasone diproprionate (inhaled)	60	In 40%, cough due to other aetiologies	Fair

Cheriyan et al 1994 [55]		Retrospective, descriptive	10	Prednisone 7–14 d, followed by beclomethasone diproprionate	100	80% required long-term ICSs for cough suppression	Low
Di Franco et al 2001 [56]	36 ± 16†	PRDBPC	36	Beclomethasone diproprionate (and albuterol)	Improvement in treated patients ($p < 0.01$)	Compared to placebo and albuterol	Fair
Doan et al 1992 [45]	4–71	Prospective, descriptive	10	Prednisone (20–60 mg/d)	100	Subsequent therapy with ICSs	Low
Shioya et al 1998 [57]	25–63 (47.1)‡	Prospective, unblinded, uncontrolled	22	Azelastine hydrochloride	Improvement in treated patients ($p < 0.001$)		Low
Shioya et al 2002 [58]	22–69 (44.7)‡	PRDBPC	20	Suplatast tosilate	Improvement in treated patients ($p < 0.01$)		Fair

Reproduced directly from the ACCP guideline. PRDBPC = prospective, randomized, double-blind, placebo-controlled study; ICS = inhaled corticosteroid. † Values are given as mean ± SD, ‡ Values in parentheses are the mean.

1.2 Neurophysiology of airway nerves

Airway nerves are crucial to the development of chest symptoms such as cough, shortness of breath, chest tightness and pain. These are all sensory phenomena that are dependent on nerve activation and transmission to the central nervous system resulting in a sensory perception, a motor function, or both. Indeed, this may explain why in general, these symptoms are blocked or inhibited by vagal anaesthesia or transection. This has been reviewed extensively previously [59, 60]. Our current understanding of the role of nerves in the airways can be broadly divided into sensory afferents and motor efferents and is mainly derived from pre-clinical studies in rodents.

1.2.1 Sensory Afferent Nerves

Airway afferent nerves have been classified by conduction velocity, location, myelination, chemical or mechanical sensitivity, reflexes associated with their stimulation and sites of termination. Although individually these variables are not specific for different nerve types, used in combination, they reveal four major types of nerves fibres summarised below in

Table 1.3. The low pressure mechanosensors have been discussed together and summarised below.

Table 1.3: Characteristics of airway vagal afferent fibre types involved in cough reflex.

	Aδ Fibres "Cough Receptors"	**C-Fibres**		**Slowly adapting receptors**	**Rapidly adapting Receptors**
Somatic origin	Nodose ganglion	**Jugular ganglion**	**Nodose ganglion**	Nodose ganglion	Nodose ganglion
Mechanical Sensitivity	High (touch only)	Low		High (stretch and touch)	High (stretch and touch)
Chemical Sensitivity	Citric acid, low chloride	Capsaicin, BK, PGE2, citric acid		None	None
Conduction Velocity	~5m/s	~1m/s		~18 m/s	14-13m/s
Location of termini	Extra-, few Intra-pulmonary	Extra-, few Intra-pulmonary	Intra, few extra pulmonary	Extra- and Intra-pulmonary, ASM	Extra- and Intra-pulmonary

Abbreviation: ASM; airway smooth muscle, BK; bradykinin, PGE2; prostaglandin E2. Information adapted from [61-63].

i) **C fibres**: networks of un-myelinated nerves with a conduction velocity of 1 m/sec can be found throughout the airways, are sensitive to chemical stimuli including inflammatory mediators (e.g. bradykinin, prostaglandins), environmental stimuli (e.g. pollutants, temperature) and are characteristically activated by capsaicin via the transient receptor potential vanilloid type 1 (TRPV1) receptor. Activation initiates cough and transient bronchoconstriction in guinea pigs due to tachykinin release but not in humans. C-fibres originating from the jugular ganglion are thought to project to mainly extra-pulmonary airways with few intra-pulmonary projections, whilst C-fibres originating from the nodose ganglion mainly project to the peripheral airways and lung parenchyma. The origin of this pattern of nerve distribution is related to the embryological origins of the cell bodies; the nodose originating from the placode whilst the jugular from the neural crest [64]. This may be important in understanding the opposing effects on cough and respiration upon activation of these sub-types of c-fibres, i.e. the location of nerve terminals, whether they receive blood supply from the bronchial or pulmonary circulation, and where their respective cell bodies lie have important phenotypic characteristics. It is currently thought that activation of jugular c-fibres evoke coughs whilst nodose c-fibres do not, and may in fact be inhibitory to cough [61, 65].

Further sub-typing of C-fibres can be made by expression of neuropeptides such as substance P, CGRP or neurokinin A and their responsiveness to adenosine, serotonin 5HT, and ATP receptor antagonists [64, 66-68].

ii) **Aδ fibres:** the proximal airways are also innnervated by sub-epithelial myelinated nerves with a conduction velocity of 5 m/sec and which are also known as ‘cough receptors’, as they evoke cough. They respond to punctate mechanical stimuli, low osmolarity and acidity but are insensitive to capsaicin and inflammatory mediators [69]. As they are myelinated and originate from the nodose ganglion they share similar characteristics to rapidly adapting receptors (RARs) but their conduction velocities are slower (4-6m/s as opposed to 14-23 m/s) and they are sensitive to light punctate touch rather than stretch.

The literature is sometimes confusing when it describes a group of Aδ nociceptor fibres, which originate in the jugular ganglion with terminals in both the intra and extra pulmonary airways and express TRPV1 ion channels [61]. These are not to be confused with the “polymodal” cough receptors described above, nor are they to be confused with C-fibres based on their TRPV1 expression. These fibres are distinct from the cough receptor because they have a low response to mechanical stimulation and distinct from C-fibres because they do not express substance P or tachykinins [70-72].

iii) **Low Pressure Mechanosensors - (SARs) and (RARs):** Low pressure mechanosensors also exist which, depending on their speed of adaptation to sustained lung inflation, are classified into slowly adapting receptors (SARs) or rapidly adapting receptors (RARs). These are located mainly in the intra-pulmonary airways and lung parenchyma [73-75] but a third type of extra-pulmonary low pressure mechanosensor has been described in guinea pig trachea and larynx [69]. The conduction velocity of transmission from SARS and RARs is 15-18 m/sec suggesting Aβ range [76, 77]. Both SARs and RARs are important in detecting lung volume, airway calibre, bronchoconstriction and airway oedema. SARs are thought to be important in maintaining breathing at tidal volume hence crucial in the Hering-Breuer inflation reflex [75]. However RARs are activated by dynamic lung inflation, pulmonary oedema, lung collapse, negative airway luminal pressures but quiescent during static lung inflations [63, 69, 76]. Much of this work has been done in guinea pigs and rats, and hence translation to humans is still questionable. Furthermore, it is unclear whether or not these low pressure mechanosensors have a role to play in cough. Smooth muscle contraction using methacholine, histamine or leukotrienes stimulate RARs but are not thought to cause significant spontaneous coughing [78, 79].

In addition to sensory vagal afferents, there is evidence of a minor contribution from sensory sympathetic nerves which transmit via the dorsal root ganglion (DRG) in the spinal cord. These nerves have been difficult to study experimentally in humans because isolation of these nerves would require transection of the spinal cord. However, in animal models of vagal section, inhaled capsaicin caused muscle relaxation which was inhibited by propranolol, sympathetic denervation of the trachealis muscle, dorsal rhizotomy (T1-T4) and substance P antagonist[17]. This suggests the presence of a possible bronchodilatatory sympathetic adrenergic reflex being mediated by substance P via the DRG.

1.2.2 Initiating the cough reflex

Upon activation of vagal sensory afferents, be they C-fibres or Aδ fibres, the action potentials generated from these nerves travel to their respective cell bodies from where they originate, located either in the nodose (mainly Aδ fibres) or jugular ganglion (mainly C fibres) before arriving at the nucleus tractus solitarius (NTS) and paratrigeminal nucleus in the medulla. Here they synapse with second order neurons to activate complex neural networks projecting to cortical and sub-cortical areas. This produces a sensation of urge to cough and, if the stimulus is large enough, a co-ordinated sequence of impulses via the spinal motor neurons transmitted to the abdominal muscles, intercostal muscles, diaphragm, glottis and vocal cords to cause cough. See Figure 1.1.

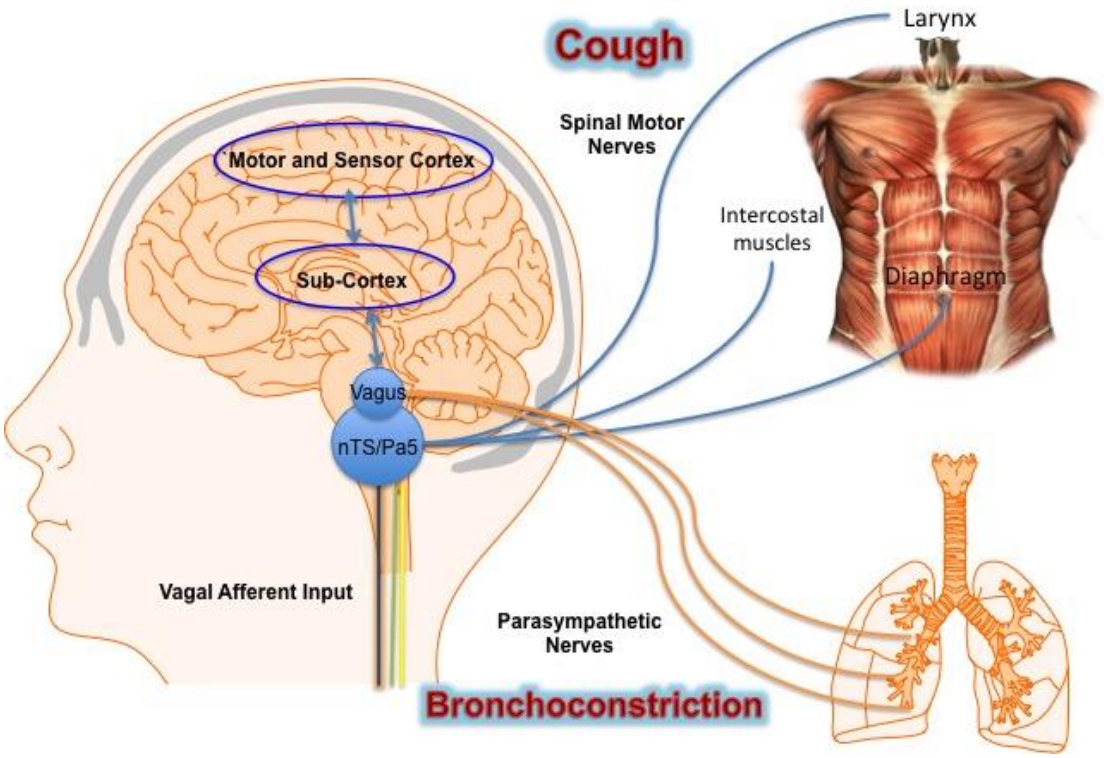

Figure 1.1: A summary diagram of putative nerves and central networks involved in the initiation of cough and bronchoconstriction.

Abbreviations: nTS, nucleus tractus solitarius; Pa5, paratrigeminal nucleus. See text above for explanation. Adopted and reproduced from [61, 80].

1.2.3 Motor Efferent Nerves

The efferent nerves supplying the airways are part of the autonomic nervous system, divided into the adrenergic (sympathetic), cholinergic (parasympathetic) and non-adrenergic non-cholinergic (NANC) nerves. The properties, interactions and functions of these nerves differ and largely depend on the neurotransmitters secreted from post-ganglionic vesicles and the receptors they bind to on the airway smooth muscle, vascular endothelium or mucus glands. However, there is general agreement that the parasympathetic cholinergic system with acetylcholine as its neurotransmitter causes bronchoconstriction, whilst the NANC system with nitric oxide (NO) and vasointestinal peptide (VIP) oppose this effect [61].

The parasympathetic nervous system plays a key role not only in airway smooth muscle tone, but also in regulating mucus gland secretions. This involves release of acetylcholine from the post-ganglionic synapse binding on to M3 muscurinic receptors on smooth cells to cause contraction. In addition, there are M2 receptors on the post-ganglionic synapse which function as an auto-inhibitory receptor to inhibit further acetylcholine release, and hence are broncho-

protective. Of note, these cholinergic nerves are tonically active at rest, but their responses can be modulated by activation of vagal sensory afferents after a mechanical or chemical stimulus in the airway [81]. See Figure 1.2. Hence, it is possible that airflow obstruction can occur secondary to increased basal cholinergic tone independent of vagal sensory activity or that the sensory afferents have a heightened activity, which augments cholinergic activity. In addition, there is some evidence to suggest that inflammation caused by allergen, ozone or virus causes inhibition of the auto-inhibitory M2 receptor, hence augmenting the cholinergic response [82-84].

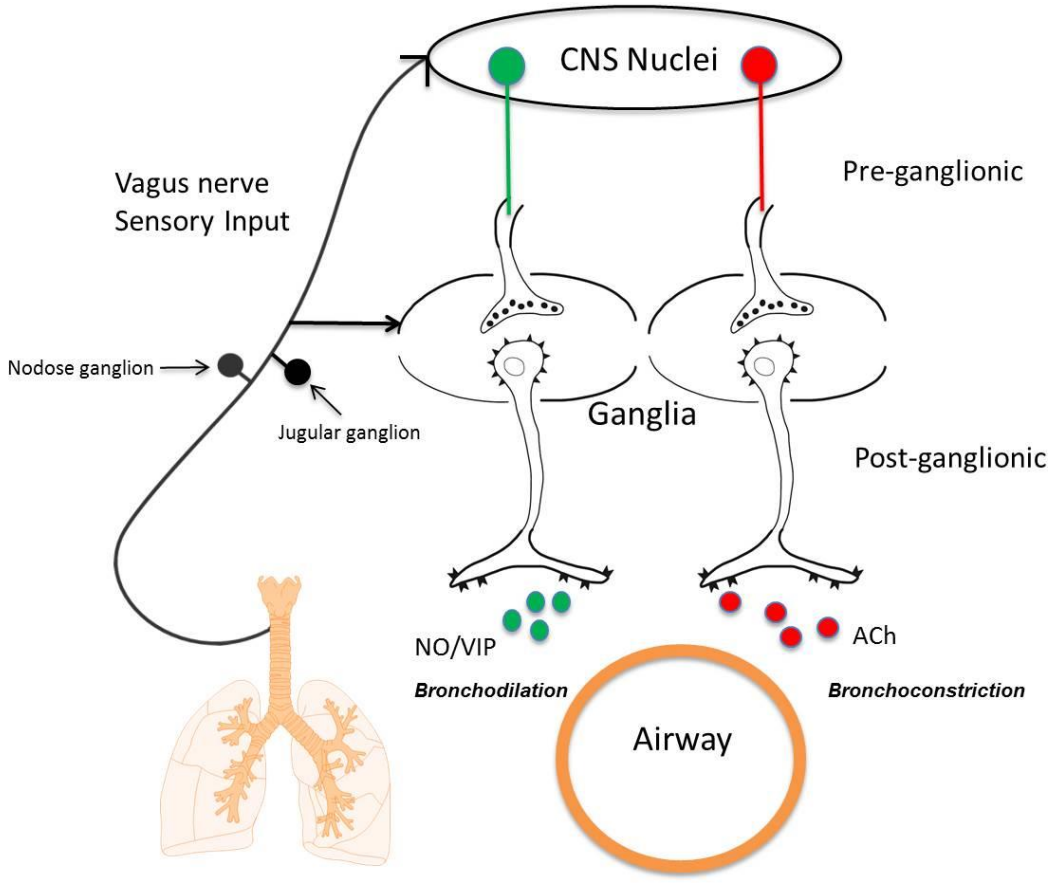

Figure 1.2: Two distinct vagal parasympathetic nerves regulate airway calibre.

Adapted and reproduced from [81]. Abbreviations: Ach; acetylcholine, NO; nitric oxide, VIP; vasointestinal peptide.

Opposing this cholinergic activity are the NANC nerves which mediate bronchodilation via the GC-cGMP pathway to reduce intracellular calcium [85]. Nitric oxide (NO) and vaso-intestinal peptide (VIP) are the major neurotransmitters of NANC nerves. Apart from the action of NANC nerves on the airway smooth muscle, they evoke vascular dilation and mucus secretion. These NANC nerves are physiologically and anatomically distinct to the parasympathetic cholinergic nerves [86]. This is important as it clarifies the concept that bronchoconstriction can occur as a result of an increase in cholinergic activity or a decrease in nitrergic activity, with the opposite

(bronchodilation) also possible. Furthermore, airway sensory afferents can potentially regulate these two sets of nerves both at the level of the brainstem, and/or airway ganglia.

Sympathetic nerves release noradrenaline from their nerve endings but adrenaline is also found circulating in peripheral blood. The relative influence of these sympathetic fibres on smooth muscle fibres has been called into question because in both humans and in many animal species, these nerves are absent in the airways and do not affect muscle function [87]. The sympathetic effects seem to be via indirect inhibitory actions of noradrenaline on pre-ganglionic and post-ganglionic synapses of the cholinergic nerves but also via the direct action of adrenaline on beta-2 receptors found on airway smooth muscle [88].

1.3 Neuronal Sensitisation of the cough reflex

There are two broad mechanisms by which the cough reflex can become sensitised and/or exaggerated; peripheral and central sensitisation. These mechanisms are still poorly understood in cough and have been more extensively studied in the pain literature [89]. Peripheral sensitisation has broadly been defined as any mechanism related to an exaggerated activation of the peripheral nerves most often observed in the context of injury and inflammation. This could be due to receptor modification and/or an increase in the expression of receptors on nerve endings lowering the threshold for the activation of a nerve. Central sensitisation reflects an increase in the synaptic gain of the complex neuronal pathways of the central nervous system resulting in amplified responses to innocuous stimuli. Central sensitisation rarely occurs in isolation and can be driven by excessive peripheral afferent activation, and once established, heightened sensitivity can be maintained even after the resolution of the initial injury.

1.3.1 Peripheral Sensitisation

Understanding the generation and propagation of an action potential on the nerve terminals of vagal fibres is central to understanding peripheral sensitisation. Ligand gated ion channels (LGIC) initiating the cough reflex include TRP channels, of which the most extensively studied in the cough literature is transient receptor vanilloid type 1 (TRPV1). This is a non-selective cation permeable transducer channel, which upon activation (by capsaicin, H+, heat) can cause a depolarisation of the nerve with the inward flux of mainly calcium ions. If sufficient depolarisation is achieved and the membrane threshold for an action potential is achieved then an action potential is generated at the nerve terminal. Thereafter, voltage gated ion channels (VGIC), which are predominantly sodium and some calcium channels are able to propagate the action potentials to the central nervous system. The movement of ion channels across the nerve membrane is the key defining feature of how an action potential is generated and propagated.

Inflammatory mediators such as prostaglandin E2 (PGE2) and bradykinin (BK) and their respective G-protein coupled receptors (GPCRs) EP3 and B2 are important in sensitising the peripheral nerve endings of vagal C-fibres which initiate coughing [90]. Much of this work is based on pre-clinical animal models and has been extensively reviewed previously [91].

Bradykinin and prostanoids can sensitise/activate TRPV1 indirectly via intracellular pathways involving protein kinase A (PKA), phospholipase C (PLC), diacylglycerol (DAG) and inositol phosphate 3 (IP3) [92, 93]. This causes release of calcium from the endoplasmic reticulum, phosphorylation of the TRP channel which results in a conformational change in the structure of TRP channel. The outcome of these changes is that a lower stimulus for opening TRP channels is needed and hence the threshold for the generation of an action potential is reached more readily. The consequence of this is that previously sub-threshold stimuli can now reach the threshold for action potential generation. See Figure 1.3.

In an *ex-vivo* animal model of trachea, larynx, bronchia and vagus nerve, exposure to antigen caused a 4 fold decrease in the mechanical threshold needed to generate an action potential [70]. Likewise, in an *in-vivo* experiment using the trachea/bronchus in a guinea pig, exposure to prostaglandin resulted in a lower mechanical stimulation threshold of airway jugular derived Aδ fibres [94].

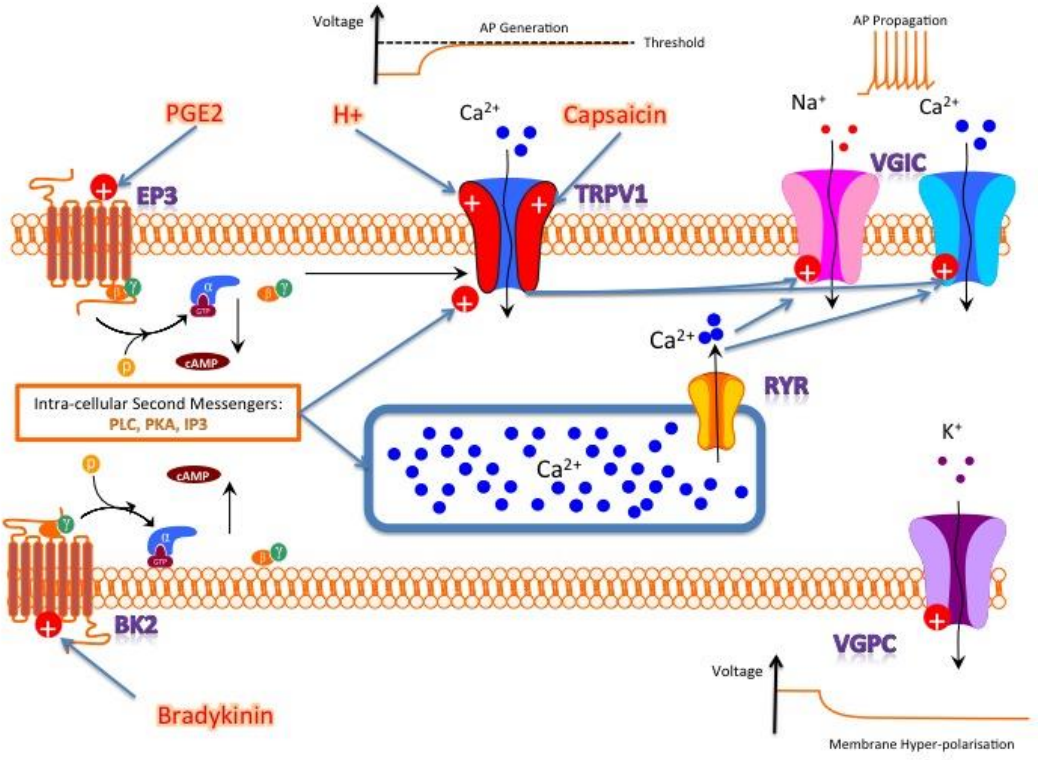

Figure 1.3: Diagram showing the interactions on nerve endings between different receptors in the generation and propogation of action potentials.

Abbreviations: TRPV1; transient receptor potential vanilloid type 1, VGIC; volgate gated ion channel, EP3; epoprostenol type 3, BK2; bradykinin receptor type 2, VGPC; voltage gated potassium channel, RYR; ryanodine receptor, Na+; sodium, Ca^{2+}; calcium, H^{+}; hydrogen, PGE2; prostaglandin.

Sensory nerves can also increase the expression of TRP receptors on their nerve terminals to increase the flow of sodium channels across the membrane. This would make it easier to reach the threshold for generating an action potential as more channel pores could open up to allow the influx of calcium ions. There is some evidence of increased density of TRPV1 axons in the trachea of ovalbumin sensitised guinea pigs [95]. Some investigators have also found increased TRPV1 mRNA expression on human bronchial fibroblast after stimulation with the inflammatory mediators TNF-alpha, LPS and IL-1a [96].

Human studies to demonstrate an increase in TRPV1 expression on a nerve fibre have been limited by difficulties in showing an accurate quantifiable change in TRPV1 expression using immunohistochemistry staining on mucosal bronchial biopsies. Chung and colleagues took

endobronchial biopsies and showed an increase in TRPV1 expression on nerve fibres taken from a group of patients with chronic cough compared with healthy volunteers and correlated this with an increased capsaicin sensitivity [97]. Controversy exists because this has not been reproduced by other groups and it is possible that the TRPV1 antibody had low specificity. More recently, McGarvey et al have reported an increase in functional TRPV1 receptors in the epithelium of severe asthmatics, although from cultured bronchial epithelial cells in only four patients [98].

1.3.2 Phenotypic switching

Demonstration of novel expression of TRPV1 on a fibre type which was previously insensitive to capsaicin is more in keeping with a phenotypic switch and is considered a type of peripheral sensitisation. This can be made possible by the cell bodies of the nerve fibres switching gene expression to produce novel ion receptors on nerve afferents. In the context of cough and inflammation, de novo expression of TRPV1 has been demonstrated in rats after allergen challenge [99] and guinea pigs [100]. The implication of this phenotypic switch is that previously capsaicin unresponsive nerves can become responsive and hence lead to coughing.

Neurotrophins are a family of proteins that regulate the survival, growth and differentiation of neurons in the peripheral and central nervous system [101]. They are secreted from a range of cells including epithelia, mast cells, and also macrophages and T lymphocytes [102, 103]. They have been extensively studied in the somatic nervous system, and in particular, to understand their role in the modulation of pain [104]. Examples of such neurotrophins are nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) binding to tropomyosin-related kinase A (TrKA) and kinase B (TrKB) receptors, respectively.

A role for neutrophins in airway inflammatory disorders has been recently suggested as they have been found to be increased in several clinical studies where elevated neurotrophin levels have been detected in serum and airway samples in allergic asthma [105-107] and after a viral exacerbation [108]. NGF was also found to be higher in severe allergic asthmatics, with a high degree of bronchial hyper-responsiveness and high total IgE [109]. Recently, higher BDNF protein and mRNA was found to be associated with asthma severity and type 2 inflammatory processes [110]. A study of the functional polymorphism of Val66Met in the BDNF gene suggested the Met/Met variant genotype to be associated with asthma, whilst the Val/Met was protective against asthma, particularly in female children [111].

It has been postulated that neurotrophins have an important role to play in the development of symptoms of cough, as pre-clinical animal studies have shown they can influence the expression of receptors on the terminals of different nerve fibres, particularly of TRPV1 [112] and P2X3 receptors [113].

Pre-clinical animal studies have suggested nerves can also change their patterns of response, when the gene expression of ion channels/receptors is changed by the presence of neurotrophins [100, 114]. Lieu et al first showed TRPV1 and TrKA receptors were mainly expressed in the jugular neurons. However, the nodose ganglion, which is predominantly capsaicin insensitive, was TRPV1 deplete and preferentially expressed TrKB receptors. But after an allergen challenge, they demonstrated an increase in TRPV1 mRNA in the nodose ganglion. In a separate experiment, instilling BDNF in the trachea of sensitised guinea pigs mimicked allergen challenge. Furthermore, calcium studies showed these de novo TRPV1 channels to be functional. Evidence of phenotypic switching has also been demonstrated after parainfluenza virus [115]. Thus, Aδ fibres which were previously capsaicin insensitive, were phenotypically switched to becoming capsaicin sensitive and so this may another mechanism explaining heightened cough responses. Similarly, neurotrophin-3 (NT-3) mimicked repeated allergen challenge in a guinea pig, but this time, the NANC neurons changed to a cholinergic phenotype [116]. This is an additional mechanism of how inflammation may affect airway muscle tone.

Theoretically, this could also explain the increase in coughing seen during an allergic or viral exacerbation of asthma. However, at present, no clinical data exists in humans to prove a direct link with a functional change in nerve fibres.

1.3.3 Central Sensitisation

Central sensitisation was first described in the somatic nervous system as a mechanism to explain heightened pain [89]. The broad concept is that that the relationship between the activating stimulus and response is disturbed due to an alteration in the central nervous system rendering a non-painful or innocuous stimulus to cause sensations of pain. Both of these are features of central sensitisation and are termed allodynia and hyperalgesia respectively, and for some people these changes can become chronic.

There is a similar clinical manifestation in patients with chronic cough, where a non-harmful stimulus such as a change in temperature, strong smells from aerosols or cleaning products elicits a strong urge to cough and chronic cough [117]. However the role of central sensitisation in classical asthma has not been studied. A brief description is provided here of the mechanism of central sensitisation of pain and possible similarities from selected studies in chronic cough.

The mechanisms underlying central sensitisation were first described in the spinal dorsal horn of somatic sensory nerves. It is currently thought that the main receptor involved in the mechanism of central sensitisation in pain is the N-Methyl-D-Aspartate (NMDA) receptor [118]. This is an ion channel activated by glutamate and glycine which upon activation allow sodium and calcium ions to pass through causing depolarisation. Sufficient depolarisation dislodges magnesium from its pore, thus allowing for a voltage dependent flow of further sodium and

calcium ions. The passing of intracellular calcium is crucial for trafficking and phosphorylation of NMDA receptors leading to upregulation of receptors on the post synaptic membrane [118]. Intra-cellular calcium also enhances the transcription of novel pro-inflammatory proteins such as nitric oxide synthase (NOS) and cyclooxygenase (Cox-2) [119]. These enzymes lead to the production of nitric oxide and prostanoids leading to more long term sensitisation secondary to disinhibition of pre-synaptic inhibitory inputs controlling the excitability of the spinal neuron. It is important to note that there are other synaptic mediators acting non-NMDA receptors on the post-synaptic membrane which play an important role in the development of the post-synaptic depolarisation and central transmission. These include substance P on neurokinin 1 receptors (NK1), neurotrophins on Trk receptors, and glutamate on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors [89].

In addition to these complex mechanisms occurring at the first order synapse, there is the potential for the higher cortex to control spinal cord excitability via descending inhibitory pathways [120]. These are considered to be top-down pathways originating in the limbic system and hypothalamus and projecting to the midbrain periaqueductal grey (PAG), through to the rostral ventral medulla (RVM). The lower brainstem projects neurons to the dorsal horn of the spinal cord in order to inhibit nociceptive sensory transmission. Opioids are thought to function via activation of this inhibitory system to reduce pain. Another form of inhibiting pain transmission is via diffuse noxious inhibitory control (DNIC) and is considered an endogenous inhibitory mechanism [121]. This is when a painful stimulus is applied to a one part of the body in order to inhibit pain perceived in another location.

Similar mechanism might be involved in chronic cough but this has not been studied as extensively as in pain. Animal studies have implicated the role of NMDA receptors in the NTS and antagonising the NMDA receptor with memantine reduced cumulative coughs evoked by citric acid and bradykinin [122]. Translation to patients with chronic cough showed a median reduction in cough frequency of 17% but this was statistically non-significant and higher doses of memantine were not tolerated; 10 of the 14 subjects could not tolerate more than the starting dose of 10mg [123]. Side effects included dizziness (71%), tiredness (43%) and drowsiness (36%).

Studies performed in anaesthetised guinea pigs have demonstrated that although administration of capsaicin and bradykinin did not evoke coughs, the electrical threshold for initiating cough was reduced, which was prevented by capsazepine, a TRPV1 antagonist. Furthermore, these effects were mimicked by microinjection of capsaicin and substance P in the NTS and also prevented by neurokinin receptor antagonist. The authors suggest a synergy between capsaicin sensitive vagal c-fibres and mechanically sensitive neurons mediates centrally via substance P acting on NK1 receptors [124]. Furthermore, guinea pigs exposed to tobacco smoke for 6 weeks during early life coughed more to citric acid, and this was attenuated with a NK1 antagonist [125]. Recently, an NK1 receptor antagonist aprepitant, which is currently

available for the treatment of nausea in cancer patients was trialled and found to reduce cough scores after just 3 days of treatment [126].

Patients with chronic cough may also present with concomitant gastro-oesophageal reflux disease (GORD). In a study where patients wore a 24 cough monitor along with a pH/impendence probe to objectively investigate the temporal relationship between cough and reflux episodes found that in nearly 50% of patients, there was a positive relationship between cough being preceded reflux [39]. Importantly, reflux events were mainly in the distal oesophagus. This could potentially be explained by neuronal synergy at the level of the NTS between vagal afferents from the lung and oesophagus. This would also explain why instilling acid in the oesophagus increased cough frequency [127] and increased cough reflex sensitivity [128] but only in patients with cough and GORD.

Evidence for failure of descending inhibitory controls and heightened central activations comes from a functional magnetic resonance imaging study (fMRI) performed by Ando et al [129]. In an elegant study design, healthy volunteers and patients with chronic cough first inhaled capsaicin to determine individual capsaicin thresholds for a sensation of urge to cough (UTC), 2 coughs (C2) and maximum suppressible dose during 24 seconds of repeated inhalations (Smax). As expected, their first finding was that chronic cough patients had lower thresholds in all three domains, and there was a linear relationship between UTC and 5 doses of capsaicin above and below C2. Whilst in the fMRI scanner, all participants were stimulated with a ‘high’ dose (Smax) and a ‘low’ dose (two doubling doses below Smax) of capsaicin. They demonstrated a much lower capsaicin concentration was required to match the UTC sensation in chronic cough patients than in healthy volunteers, and when a matched stimulus was inhaled, UTC ratings were higher in the cough patients. This is in keeping with central sensitisation. However, of interest, cough patients showed enhanced activation in the midbrain most likely to incorporate the nucleus cuneiformis, PAG and dorsal raphe, whilst controls showed enhanced activation in the dorsomedial prefrontal cortex and anterior mid-cingulate cortices. The latter areas have been implicated in cough suppression previously [130]. Taken together, these data suggest that patients with chronic cough may have central sensitisation and a lack of inhibitory control mechanisms.

1.4 The role of sensory nerves in asthma

As discussed above, efferent nerves play a major contribution in airway smooth muscle tone and the production of cough. Given asthma is a disease primarily attributed to bronchial hyper-responsiveness and variable airflow obstruction, investigating how inflammation and/or bronchoconstriction, if at all, affects neuronal function is the fundamental question of my study. Many consider bronchoconstriction to be mediated by release of local inflammatory mediators such as histamine, and rarely is the possibility of a reflex bronchoconstriction considered. Understanding such a possibility requires an understanding of the relationship between the sensory afferents, motor efferents and the development of symptoms.

An analysis of neuronal dysfunction needs to take into account a number of possible processes:

i) Sensory nerve endings releases neuro-inflammatory mediators through an axonal reflex

Evidence for the presence of an axonal reflex in humans is lacking [131]. Evidence from animal models suggested that activation of sensory afferents caused release of neuropeptides such as substance P, neurokinin A, and calcitonin gene related peptide (CGRP) from nerve terminals. These mediators could then potentially mediate their effects on blood vessels, mucus glands, blood vessels and smooth muscle and also sensitise the sensory nerve themselves. A number of studies were done in humans on the basis that evidence showed this reflex existed in rats and guinea pigs. However clinical trials to date have shown little evidence that this axonal reflex exists in humans and antagonising or inhibiting the release of neuropeptides have not shown any significant improvement in asthma symptoms or lung physiology [132, 133].

Secondly, cough challenges in humans to stimulate vagal c-fibres by inhaled capsaicin, does not cause significant sustained bronchoconstriction [134]. However, capsaicin challenges have been performed in subjects who are clinically stable and not during an exacerbation. How these receptors and nerves function in the presence of airway inflammation is currently unclear. Hence, it is also unclear whether symptoms during an exacerbation are driven by sensitisation of afferent nerves secondary to inflammation, which then also activate the efferent nerves in a reflex manner to cause bronchoconstriction, or whether inflammation directly affects smooth muscle contractility.

ii) Sensory afferents influencing motor efferent activity

Studies in animal models by James and Mei in cats [135] and independently by Kesler and Canning in guinea pigs suggest that sensory lung afferents drives baseline smooth muscle cholinergic tone [136]. The former attribute this to C-fibers whilst the latter to RARs. However, these studies in cats and guinea pigs cannot be assumed to translate to humans.

The role of inflammatory mediators has been studied extensively in animals because they can potentially affect the sensory nerves endings as well as cholinergic nerves. Evidence for the latter comes from a range of studies which show that, along with their direct effect on smooth muscle, histamine, serotonin, PGD2, thromboxane and even methacholine and acetylcholine can partially cause bronchoconstriction by activating a parasympathetic cholinergic reflex involving intrapulmonary RARs [73, 137-140]. On the contrary, many inflammatory mediators have only a weak direct action on the smooth muscle, but can evoke reflex bronchoconstriction. These include bradykinin, PGE2, prostacyclin, platelet-activating factor (PAF) and adenosine [141]. The majority of these are thought to directly activate intrapulmonary c-fibres and RARs but the latter two via indirect mechanisms - adenosine can cause degranulation of mast cells to release leukotrienes, histamine and serotonin, whilst PAF induces the synthesis of the lipoxygenase product 15-HTE from airway epithelial cells which is a TRPV1 agonist [142-144].

Recent evidence from a guinea pig model of allergic asthma has also demonstrated the importance of TRPA1 in initiating a reflex cholinergic response, which can be inhibited by a specific TRPA1 antagonist, an anti-cholinergic (tiotropium) and a non-selective cation channel blocker (ruthenium red) [145]. In a review, Canning concludes that anti-cholinergics reduce BHR to a wide range of smooth muscle agonists [81]. This could be an additional mechanism by which tiotropium has been recently found to be beneficial as an add at step 4 treatment of asthma [146].

iii) Sensory afferents have an increased expression of receptors causing cough or bronchoconstriction

Discussed in section 1.3.1

iv) Inflammatory mediators cause the phenotypic switching of nerve fibres

Phenotypic switching has been discussed in section 1.3.2.

v) Neuronal convergence

In addition to intrapulmonary afferents, extra-pulmonary vagal and non-vagal afferents may be important in asthma where there is a significant nasal and upper airway inflammation or in patients with reflux disease [147-152]. Evidence of these fibres initiating reflex bronchoconstriction maybe important in overlapping groups of diseases and different asthma phenotypes, for example, instilling intranasal histamine enhances capsaicin sensitivity [153, 154]. Although circumstantial, there is evidence for neuronal cross-talk or convergence at the level of the brainstem, particularly, as the vagal trunk synapses in the NTS. Mazzone and Canning showed that denervating the airway by cutting the vagus nerve caudal to the recurrent laryngeal nerves reversed the cholinergic tone but also prevented any reflex effects in the

trachea evoked by a laryngeal capsaicin challenge [124, 141]. The authors concluded that *“laryngeal C-fibres act synergistically with continuously active intrapulmonary mechanoreceptors to initiate reflex bronchospasm”.* A similar synergism was shown to exist in the sensory afferents regulating the cough reflex and highlighted the importance of convergence of sensory neurons in the caudal NTS and the importance of substance P and neurokinins centrally [124, 141].

1.5 Studies of cough reflex sensitivity in asthma

Inhaled capsaicin is the most widely used tussive agent in studies assessing the cough reflex, but other agents such as citric acid, tartaric acid, prostaglandin and bradykinin have been used. However, there are few studies which have directly compared cough reflex sensitivity in healthy volunteers with patients with classical asthma. Doherty et al demonstrated that the concentration required to cause at least 5 coughs, C5 was much lower than healthy volunteers (median C5 = 62mM vs. C5<500mM) [155]. However, the patients with asthma were all on inhaled steroids, 21% on anti-cholinergics, and 8% on theophylline suggesting they selected a more severe group. Indeed, the mean % predicted FEV1 of this this group was 71% and only 43% were non-smokers. The presence of these demographics and medication history would make it difficult to rule out an interaction with capsaicin evoked cough responses. Fujimura et al also compared C5 to tartaric acid in healthy volunteers (n=38) and patients with asthma (n=11) and found no significant differences [79]. More recently, there has been a direct comparison of capsaicin, citric acid and prostaglandin challenges in five different disease groups; healthy controls, healthy smokers, chronic obstructive pulmonary disease (COPD), asthma and chronic cough [156]. This study demonstrated that there were no significant differences in the C5 to capsaicin and prostaglandin between healthy volunteers and patients with asthma, but there was a significant difference in the C5 to citric acid. This suggests that citric acid might be activating different receptors on nerve endings compared to capsaicin.

There have been some studies which has assessed capsaicin evoked cough responses in patients presenting with chronic cough with an underlying diagnosis of asthma. Nieto et al recruited 101 patients with chronic cough, of whom 54 had asthma, and showed C5 responses to be lower in asthma than in healthy controls (33.3 µmol in asthma vs 151 µmol in healthy) [157]. However, Prudon et al suggested that capsaicin C5 was lower, i.e. more sensitive, only in patients with cough variant asthma (CVA) and not classical asthma (CA) when compared with healthy subjects [158]. This finding was similar to that seen in a clinical trial investigating the effects of zafirlikast on cough in asthma, where patients with CVA demonstrated a lower C5 to capsaicin (0.19 µmol vs. 1.13 µmol) and responded better to zafirlukast that patient with CA [46]. However these findings have not been found to be universal as another study found no difference in cough reflex sensitivity [159].

Until recently, the vast majority of investigators studying cough have used C2 and C5 as endpoints to study cough reflex sensitivity, however, data now exist to suggest that these might not be the optimum endpoints to study the cough reflex. Hilton et al performed an ascending doubling dose capsaicin evoked cough challenge and documented the maximum number of evoked coughs at any concentration of capsaicin as Emax, and the excitatory dose that caused at least half this response ED50 [160]. Using non-linear mixed effects modelling, they demonstrated that Emax better discriminates between patients with chronic cough and healthy volunteers and also correlated with 24 hour spontaneous cough frequency. In their study, there was no significant different in Emax/ED50 between twenty healthy subjects and 18 patients with

asthma, because differences in Emax failed to reached the *a priori* significance which was set relatively high ($p<0.001$). It is possible that with larger numbers of a better defined asthma population, there may be differences in Emax and ED50. A full explanation of the capsaicin full dose response challenge can be found in the methodology chapter.

1.7 Bronchoconstriction and cough

The interaction between airway smooth muscle function and thereby airflow obstruction and cough in unclear. Healthy volunteers and the majority of patients with asthma do not cough significantly after inhaling methacholine. A previous study showed that only one out of 7 (14%) asthmatics coughed [161], whilst in a more recent study, 17% patients with classical asthma and 35% of cough variant asthma (CVA) coughed at least once during a methacholine challenge [162].These studies measured spontaneous coughs but there have been no controlled studies in patients with mild asthma to assess the effects of acute bronchoconstriction on capsaicin evoked coughs responses. Hence investigating the interaction between bronchoconstriction and cough is a key study of my thesis.

The effect of altering airway calibre on capsaicin evoked cough responses (capsaicin concentration causing 2 coughs, C2) has been studied in healthy volunteers using salbutamol, methacholine and 0.9% saline. However, only small changes in airway calibre was demonstrated (FEV1 changed by 6.2 +/- 2.6%, -8.8 +/- 3.2% and -0.18 +/- 1.38%, respectively) and this resulted in no changes in C2 [163]. A similar study in healthy volunteers also found no difference in capsaicin C5 before, and 10 mins after methacholine induced bronchoconstriction [164]. Reassuringly, inhalational cough challenges with capsaicin have not been shown to cause any significant bronchoconstriction [20, 160].

Cough has been assessed during inhalational challenges with mannitol, hypertonic saline, and histamine in subjects with asthma. The first two cause smooth muscle contraction indirectly, whilst the latter does via its direct action on smooth muscle. After inhaling mannitol, asthmatics coughed and bronchoconstricted, however giving one dose of nedocromil (a mast cell stabiliser) reduced bronchoconstriction by 55% but had no effect on coughing after adjusting for the inhaled mannitol dose [165]. A further study compared cough and bronchoconstriction in a group of asthmatics and healthy volunteers by asking subjects to inhale hypertonic (5%) saline, isotonic histamine and hypertonic histamine on different days [166]. They counted the cumulative number of coughs and showed both asthmatics coughed more to hypertonic saline in the absence of bronchoconstriction. However, during the isotonic histamine challenge, asthmatics coughed as much as healthy volunteers when adjusted for the degree of bronchoconstriction. Finally, after inhaling hypertonic histamine, asthmatics coughed more frequently than healthy volunteers when bronchoconstriction had not yet even developed. This suggests that bronchoconstriction and cough were independent, unrelated and occurring via different mechanisms.

Assessing cough sensitivity using the concentration of hypertonic saline causing 15 cumulative coughs showed that pre-treatment with salbutamol to prevent bronchoconstriction showed significant differences in cough sensitivity between asthmatics, chronic coughers and healthy subjects [167]. The implication was that asthmatic patients were hypersensitive (coughed more at lower concentrations) even without bronchoconstriction.

These experiments have provided some insight to suggest that bronchoconstriction and cough are independent and may occur because of different airway mechanisms. However, which receptors, or which nerves are involved, are unknown. It is likely that challenge agents of different osmolarities affect different TRP channels. Recently, TRPV4 has been implicated in functioning as an osmosensor in the lung of guinea pigs [168]. Furthermore, the studies described using mannitol, hypertonic histamine and saline have used unconventional challenge methods, different endpoints to assess cough and are indirect challenge agents via degranulation of mast cells. Not surprisingly, there were significant side effects including dyspnoea, throat irritation, and skin flushing [169].

The potential mechanism of bronchoconstriction causing cough is unclear and there are conflicting results from animal studies. Some have suggested that cough receptors are insensitive to bronchoconstriction [170] and mediated by RARs [166, 171]. Patients with cough variant asthma often improve after short acting beta-2 agonists, so it is tempting to attribute coughing to bronchoconstriction. However, new evidence suggests salbutamol could hyper-polarise the nerve membrane potential secondary to opening of potassium channels and hence reduce the sensitivity of the cough reflex [172].

More recently, the role of ATP in bronchoconstriction-induced activation of guinea pig vagal intra-pulmonary C-fibres has been studied [173]. This has suggested that bronchoconstriction causes release of ATP which activates nodose C-fibres, but not jugular. This can potentially lead to a parasympathetic reflex bronchoconstriction. Furthermore, this also suggests that methacholine and histamine, which are currently thought of as causing smooth muscle contraction directly, may also cause indirect reflex effects via activation of these nodose c-fibre afferents.

1.9 Airway inflammation and cough

The role of inflammation in asthma has been extensively studied over the last 50 years, but there are some important observations which question whether direct causality can be attributed to asthma pathophysiology. Atopic patients who are not asthmatic develop extensive airway eosinophilia to inhaled allergen challenge, but do not develop symptoms of asthma [174, 175], and likewise patients with hyper-eosinophilic syndrome have no increased risk of asthma [176]. When compared to normal healthy volunteers, a large proportion of patients with asthma have similar levels of airway eosinophilia suggesting a discordance between airway inflammation and asthma symptoms [177, 178]. This concept of discordant symptoms and inflammation has been replicated in cluster analysis of patients with severe asthma, i.e. excessive symptoms but little eosinophilic inflammation or high levels of inflammation but few symptoms [179-181]. Treatment strategies aimed at reducing airway inflammation, using treatment algorithms based on sputum eosinophilia or exhaled nitric oxide, either reduced the risk of exacerbations or allowed a reduction in inhaled steroid dose, but there was no effect of asthma symptoms [182, 183]. Studies targeting IL-5, IL-12 dramatically reduced airway eosinophilia after allergen challenge, but little effect on BHR [184-187] or asthma control [188, 189]. However, the more recent studies of mepolizumab (anti IL-5) did show a modest reduction in ACQ-5 score of -0.52 (95% CI-0.87 to -0.017, p=0.004) in a group of patients with severe asthma [190].

There are few data exploring the direct role of airway inflammation and cough in asthma. Marsden et al assessed the relationship between objective cough frequency, airway inflammation and disease control in asthma [19]. They demonstrated that levels of asthma disease control classified according to GINA categories are unrelated to measures of inflammatory cells or mediators. Minoguchi et al showed no change in capsaicin C5 after exposing mild atopic asthmatics to inhaled house dust mite allergen challenge in a randomised placebo controlled parallel group study [191]. This lack of change in capsaicin was despite a significant increase in airway eosinophilia and histamine BHR. Dicpinigaitis et al showed no change in cough reflex sensitivity, C2 or C5, in asthmatics after 14 days treatment with zafirlukast (144). These findings raise doubts about the relationship between airway inflammation and the mechanism of cough.

Circumstantial evidence to suggest airway inflammation and cough are related comes from a study showing improvement in citric acid cough reflex sensitivity, as measured by the concentration causing 2 and 4 coughs, C2 and C4 after one month treatment of inhaled salbutamol and beclomethasone [56].

Controversy exists regarding the relationship between airway eosinophilia and cough. Thirteen percent of patients with chronic cough can be accounted for by non-asthmatic eosinophilic bronchitis [6] and can show a short term clinical improvement with inhaled and occasional oral steroids [192]. Treatment with inhaled budesonide for 4 weeks has also shown to reduce eosinophil count and decrease capsaicin sensitivity [193]. On the contrary, long term

observational data suggests that 66% of patients with chronic cough secondary to eosinophilic bronchitis continue with persistent symptoms despite treatment [194]. Likewise, as mentioned earlier, anti–IL5 antibody treatment reduced exacerbations and eosinophils, but had no effects on cough severity [188]. This could imply that eosinophilic inflammation is an “epiphenomenon” rather than a direct cause of cough.

Apart from an interest in airway eosinophils, Marsden and colleagues did not find any significant correlations between airway neutrophils or inflammatory cytokines with objective cough frequency [19]. Human studies directly assessing the effects of other inflammatory cells or mediators on coughing are limited. Niimi and colleagues demonstrated and increase in submucosal neutrophils and eosinophils in asthmatic patients with chronic cough, but only mast cells in non-asthmatics [195]. In contrast, Brightling et al compared inflammatory mediators in patients with eosinophilic bronchitis (EB), asthma and healthy subjects and found that despite similar levels of eosinophils, patients with EB demonstrated significantly higher amounts of PGD2 and histamine [196, 197]. The gene expression of IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) has also been found to be higher in subjects with asthma and chronic cough responsive to inhaled corticosteroids (ICS) but not in those who were not responsive to ICS [198]. The hypothesis that mast cells mediator release might be important in the production of cough is also supported by a study of patients investigating the effects of montelukast in patients with cough variant asthma; Kawai et al found that only the subset of patients with a high proportion of CD63 positive cells in tryptase positive mast cells benefitted from treatment [199]. Furthermore, when washed BAL samples from patients with CVA, non-asthmatic cough (NAC) and non-atopic controls are stimulated with CGRP, neurokinin A and substance P, only CGRP induced significantly more histamine release from patients with CVA and NAC [200]. However, no studies have yet investigated the correlation between objective cough frequency and capsaicin cough reflex sensitivity with mast cells in patients with mild/moderate asthma.

1.10 General Hypotheses and Aims

Given cough is the archetypal airway neuronal reflex, I hypothesise that airway nerves are dysfunctional in asthma. To test this hypothesis I aim to complete 3 studies:

1. Capsaicin cough responses in patients with stable asthma (COAST Study)
2. The interaction between bronchoconstriction and capsaicin evoked cough responses (BEACH Study)
3. Investigating neuronal responses by assessing capsaicin evoked cough responses in an allergen challenge model of asthma (INCA study)

The chapters that follow include a general methodology and an overview of the statistical modelling techniques, three chapters in paper format for each of the studies, and ending with a summative discussion.

1.11 Objectives

For each study, the specific objectives are described below and further detailed in subsequent chapters.

a) Study 1: Capsaicin cough responses in stable asthma (COAST)

1. Assess capsaicin evoked cough responses in a large group of mild to moderate stable asthmatics and compare with healthy volunteers
2. Assess how capsaicin evoked cough responses differ between different asthma phenotypes.
3. Explore the relationships between objective measures of cough, airflow obstruction, bronchial hyper-responsiveness (BHR) and airway inflammation.
4. Explore the relationship between capsaicin evoked cough responses and asthma control.

b) Study 2: The interaction between bronchoconstriction and cough in asthma (BEACH)

1. To assess capsaicin evoked coughing using the ED_{50} dose immediately after a methacholine challenge which causes a 20% drop in FEV_1 compared with normal saline (0.9%), in participants with mild/moderate asthma.
2. To assess the % drop in FEV_1 after administering methacholine (PC_{20}) immediately after inhaling the ED_{50} dose of capsaicin compared with normal saline (0.9%), in participants with mild/moderate asthma.

3. To compare capsaicin evoked coughs using the ED_{50} dose immediately after, 30 mins and 60 mins after a methacholine challenge which causes a 20% drop in FEV_1 compared with normal saline (0.9%).

c) *Study 3:* Investigating neuronal responses by assessing capsaicin evoked cough responses in an allergen challenge model of asthma

To assess in mild atopic subjects with asthma:

Primary:

1. Capsaicin evoked coughing using the ED_{50} dose 30 mins and 24 hours after an allergen challenge compared with a diluent (saline, 0.9%) challenge.

Secondary:

1. Spontaneous coughs 24 hours after an allergen challenge compared with a diluent challenge.
2. Airway inflammatory cells before after an allergen challenge compared with a diluent challenge.
3. Bronchial hyper-responsiveness to methacholine before after an allergen compared with a diluent challenge.

2 General Methodology

2.1 Assessment of Asthma

2.1.1 Asthma Control Questionnaire

Clinicians and patients aim for asthma symptoms to be well controlled, however, how that is objectively measured and monitored in clinical asthma trials and studies has varied. In this context a number of questionnaires and diaries were developed. The Asthma Control Questionnaire (ACQ) was developed by Juniper which has since been used as a standard tool in numerous asthma studies and trials [201]. This consists of five symptoms based questions and are scored from 0 (no impairment) to 6 (maximum impairment), one question based on short acting B2 use and the seventh question based on the degree of pre-bronchodilator FEV1. Each of the seven questions is scored out of 6 and a mean is calculated ranging from 0-6. It was developed and validated in Hamilton, Canada in 50 patients (18 male, mean age 37±13 (S.D.) with mild to moderate asthma. Lists of questions were gathered from responses taken from 91 experts who served on asthma guidelines committees in 18 countries. They were asked to grade the importance of 10 symptom questions ranging from extremely important, very important, moderately important, not very important and useless. The five highest scoring items were selected for the ACQ along with beta 2 agonist use and pre-bronchodilator % predicted FEV1. The validation of the ACQ was then done in 50 adults in the clinic at enrolment, then at weeks 1, 5, and 9 along with measuring other questionnaires such as the asthma quality of life questionnaire (AQLQ) and clinician's judgment of change in a patient's asthma control . This questionnaire has been validated to be reliable and responsive to change in asthma control [201]. Furthermore, when compared against a goal standard composite of the GINA and National Institute of Health (NIH) guidelines, an average cut-off point of 0.75 (negative predictive value=0.85) for 'well-controlled' and 1.5 (negative predictive value=0.88) for 'not well-controlled' was calculated [202]. A change in ACQ of 0.5 is considered the minimally clinically important difference. It is important to note here that shortness of breath and wheeze are assessed in the questionnaire but there is no specific question about cough symptoms, nor has the ACQ ever been validated in patients with chronic cough. The participants of my study were mild to moderate and hence suitable for use in assessing asthma control.

Permission has been sought from Professor Elizabeth Juniper for the ACQ to be used in this study.

2.1.2 GINA Criteria

The Global Initiative for Asthma (GINA) criteria has been proposed by experts also been used extensively in asthma studies but has not been validated. In my studies we have used the GINA criteria as an inclusion criteria tool to identify well controlled and partial controlled asthma (GINA, 2015). It is similar to the ACQ in that in asks the participants three symptoms based

questions, need for reliever/rescue treatment, and FEV1 but also includes exacerbation history. However, the responses are not numerically graded but binary and hence categorises patients into well-controlled, partly controlled and not well-controlled depending on the number of positive responses. Hence, compared to the numerical ACQ, GINA tends to be less powerful when performing statistical correlations. For this reason, I opted to use the GINA as part of the inclusion criteria but ACQ for the statistical calculations. Another difference between the ACQ and GINA criteria is that the former is based on symptoms over the last one week whilst the latter is designed for retrospective symptoms analysis over the previous four weeks and hence is dependent on the memory recall of the patient. Table 2.1 below explains the criteria for the three classifications.

Characteristics	**Controlled (all of the following)**	**Partly Controlled (1-2 features in any week)**	**Uncontrolled**
Daytime symptoms	None	More than twice/week	Three of more features or partly controlled asthma present in any week
Limitations of activities	None	Any	
Nocturnal symptoms/awakening	None	Any	
Need for reliever/rescue treatment	None	More than twice/week	
Lung Function (PEFR or FEV_1)	Normal	<80% predicted or personal best (if known)	
Exacerbations	None	One or more/year	One in any week

Table 2.1; Characteristics and symptoms that are used to categorise patients into controlled, partly controlled and well-controlled. Adapted from GINA guidelines.

It should be noted that in contrast to the ACQ, the GINA criteria

2.1.3 Allergy Assessment

For the purpose of this study, I relied on the clinical history and a single positive skin prick test to diagnose atopy. I assessed the following aero-allergens that are commonly found in the UK along with a positive and negative control (Diagenics, UK):

1. House dust mite
2. Mixed Moulds I: *Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Curvularia lunata, Fusarium moniliforme, Helminthosporium halodes*

3. Mixed Moulds II: *Aspergillus fumigatus, Mucor mucedo, Penicillium notatum, Pullularia pullulans, Rhizopus Nigricans, Serpula lacrymans*
4. Grass Mix: yorkshire fog/velvet grass, cocksfoot, rye grass, timothy, meadow grass/kentucky blue grass, tall fescue/meadow fescue
5. Tree Mix (Mid Blossoming): birch, beech, oak, plane
6. Cat
7. Dog
8. Histamine (positive control)
9. Saline (negative control)

A positive test was where the weal was more than 3mm with no weal with saline 15 minutes after breaking the skin epithelial barrier.
In addition, I have also measured serum total IgE levels as higher levels are associated with higher probability of asthma diagnosis [203], presence of wheeze, poorer lung function [204] and risk of hospitalisation after a virus induced exacerbation [205].

2.2 Assessment of Airway inflammation

2.2.1 Fractional exhaled Nitric Oxide (FeNO)

The major advantage of this test is it being an easily measured, quick and non-invasive biomarker of airway inflammation, emitted from the bronchial epithelial cells after activation of the inducible form of NO synthase (iNOS). Although there is no direct evidence to suggest that eosinophils directly activate iNOS there are correlation studies with serum, sputum and bronchial alveolar fluid lavage eosinophils to suggest a moderate correlation [206].

Clinically, higher FeNO levels have been noted in asthmatics compared to healthy controls [207] and decrease after initiating oral [208] or inhaled steroid treatment [209]. Importantly, FeNO levels above 50ppb suggest steroid responsiveness whilst levels below 25 would suggest otherwise [210, 211]. This led to studies asthma using treatment algorithms based on FeNO levels either used as an adjunct or as an alternative to standard clinical management, but the results were disappointing [212-215]; at best, only a reduction in the inhaled steroid dose was achieved.

The ATS/ERS have published guidelines on the measurement [216] and interpretation of FeNO [217]. I have used the Niox MINO monitor (Aerocrine, Sweden) during my study. Participants are asked to inhale through a mouthpiece attached to the monitor and exhale for 10 seconds at a steady flow rate of 50 ±5 millilitres/seconds. A visual aid on the monitor is used to help with maintaining a steady exhalation flow rate. Results are recorded in part per billion (ppb). Previously, the mean of 3 reproducible reading were used, however studies have suggested that 1 recording is sufficient [218, 219].

2.2.2 Sputum Induction

Inhaling hypertonic saline to induce sputum from the lower airways has now been established as a safe and suitable technique for over 20 years [220]. Since then, the ERS has published a detailed guideline on the methodology of sputum induction with extra emphasis on monitoring FEV1 for saline induced bronchoconstriction and taking precautions for high risk patients [221]. For low risk patients, using 4.5% hypertonic saline has consensus agreement, however, those who are deemed high risk patient should initiate induction with 0.9% saline for 5 mins, and increase the concentration in a step wise fashion to 3 and 4.5% only if no sputum is produces.

Participants in my study were all stable mild to moderate asthmatics, hence I opted to start induction with 4.5% using a hand held ultrasonic nebuliser (Easy Neb II, Flaem Nuovo, Italy). Sputum induction would be stopped if FEV1 dropped more than 20%. After each 5 mins period, the FEV1 was assessed and the participant is asked blow their nose, rinse their mouth and to voluntarily cough to expectorate sputum. This was repeated a maximum of four times but the process is stopped if sufficient sputum is expectorated or the participant was unwilling to continue.

The study design meant that sputum induction was performed immediately after the methacholine challenge. Hence participants were given 400mcg of salbutamol after the 20% drop in FEV1 caused by methacholine and after fifteen minutes the FEV1 was re-assessed. Sputum induction was only performed if the FEV1 returned to within 90% of the baseline pre-methacholine FEV1, if not, a further 400mcg salbutamol would to be given. If the 90% threshold was not reached then sputum induction was not performed.

Performing sputum induction immediately after methacholine challenge has been found to be safe and reproducible when performed twice in one week [222].Furthermore, repeatedly performing sputum induction at 4, 7 and 24hrs after a methacholine challenge was safe and well tolerated [223].

2.3 Assessment of Airway Function

2.3.1 Spirometry

Spirometry was performed using a handheld digital spirometer (In2itive, Vitalograph, UK) which allows measurement of FEV1 and FVC and stores data on the digital device and is synchronised to the Vitalograph Spirotrac software. Digital spirometers were calibrated on every study day using a 3-litre syringe to within ± 3% of the 3 L (Vitalograph, UK).

The ATS/ERS guidelines were used to perform reproducible and valid spirometry [224]. Of note, participants were asked not to take salbutamol before study visit 1 and to abstain from coffee and exercise 12 hours prior to testing. Measurements were performed using a nose clip.

In visit 1, when spirometry was assessed for the first time, participants were asked to exhale forcefully until all air had been exhaled and a plateau was demonstrated. This was repeated three times to assess FEV1 and FVC. The best reproducible values, both actual and predicted, were recorded. From then on, only the FEV1 was measured for the remainder of the study and hence participants were asked to exhale forcefully for only 1-2 seconds.

2.3.2 Reversibility Assessment

This assesses the volume of air and thereby percentage improvement after inhaling a short acting bronchodilator. In my study I asked participants to sequentially inhale 4 puffs of 100 mcg of salbutamol, using a volumatic spacer device, with intervals of 10-20 seconds between each inhalation. The pre-bronchodilator FEV1 was treated as the baseline and spirometry was repeated three times 15 mins after inhaling 400mcg of salbutamol. The cut-off value for significant reversibility was 12% improvement or 200ml (if FEV1 was below 1.0L).

2.3.3 Bronchial Hyper-Responsiveness (Methacholine)

Methacholine challenge is a well-established method of assessing bronchial hyper-responsiveness (BHR). The ATS have produced detailed guidelines on how to perform the challenge and the two different methodologies used [225]. In my study I have opted to use the 2-minute tidal breathing rather than the 5 breath dosimeter methodology because of its higher sensitivity for asthma [226]. Secondly, my third study involves performing allergen challenge which required inputting the provocative concentration causing a 20% drop in the FEV1 (PC_{20}) values rather than PD_{20} in the Cockcroft formula.

Before the methacholine was performed participants were asked to withdraw their usual inhalers if they were taking regular medication. Table 2.2 below indicates the withdrawal time needed.

Medications	**Time for withdrawal before Methacholine challenge**
Short-acting bronchodilators such as salbutamol	6-8h
Medium acting bronchodilators such as ipratropium	24h
Hydroxazine, cetirizine	72h
Leukotriene Inhibitors	24h
Inhaled corticosteroids	48h

Table 2.2: Time needed for withdrawal of specific medication prior to methacholine challenge

A Devilbiss 646 nebuliser pot was calibrated using an air source to an output of 0.13ml/min ±10%. Contra-indications to BHR testing include: severe airflow obstruction (FEV1 <50% predicted or <1.0l); myocardial infarction or stroke within the previous 3 months, uncontrolled hypertension; known aortic aneurysm. Relative contra-indications include pregnancy and current use of cholinesterase inhibitor medication.

Baseline FEV1 measurements (best of 3) were taken. Participants first inhale 0.9% saline for 2 minutes with a nose clip, followed by one FEV1 measurement at 30 seconds and 90 seconds. The best FEV1 is taken from the two and used to calculate the target FEV1, i.e. a 20% drop post-saline. This was done as a safety step to ensure that participants did not bronchoconstrict more than 10% of baseline, but also to negate the effect of the 0.9% saline in the subsequent methacholine vials. Participants were then given increasing concentrations of methacholine, quadruple in strength, starting from 0.0625mg/ml, 0.25, 1, 4, 16 for 2 minutes. After each concentration the FEV1 was measured at 30 seconds and 90 seconds. The FEV1 was considered to be the same if within 5% of the previous measurement. If the FEV1 at 90 seconds was lower than that at 30 seconds by more than 5%, the measurement was repeated at 3 minutes post inhalation and at 2-minute intervals thereafter until the FEV1 started to rise. The FEV1 was measured once at each interval, unless the measurement was not technically satisfactory, in which case it was measured immediately. The highest FEV1 was used to calculate the % change. If the FEV1 fell by 20% or more below the highest post-saline value, or to below one litre, the next higher concentration of methacholine was not given. Four puffs of 100mcg of salbutamol was administered immediately after via a volumatic spacer device and FEV1 re-checked 15 mins later to ensure the FEV1 returned to within 90% of the pre-methacholine FEV1. If not, a further 400mcg of salbutamol was administered and FEV1 checked after 15 mins.

The PC20 is then calculated using the following formula:

$$PC_{20} = \text{antilog}_{10}\ \frac{[\log_{10} C_1 + (\log_{10} C_2 - \log_{10} C_1)(20 - R_1)]}{(R2\text{-}R1)}$$

Where:

C_1 is the second-to-last **concentration** administered (<20% fall in FEV_1);
C_2 is the final **concentration** administered (inducing a ≥20% fall in FEV_1);
R_1 is the **% fall in FEV_1** observed following C_1; and
R_2 is the **% fall in FEV_1** observed following C_2

For the purpose of my study, a cut-off PC20 of 8mg/ml was used to confirm asthma.

2.4 Assessment of Cough

2.4.1 Leicester Cough Questionnaire (LCQ)

The LCQ is a cough specific quality of life tool which has 19 questions relating to the impact cough is having on their physical, psychological and social wellbeing [227]. The questionnaire was developed in three phases; first, 15 structured interview were completed with chronic cough patients which generated 44 items; second, 104 patients completed the 44 item questionnaire and rated the importance of each item, which were reduced down to 19 items and divided into three domains by experts in the field; third, repeatability after 2 weeks and responsiveness testing after 2 months of treatment. Each question is scored from 1 (maximum impact) to 7 (minimal impact) and an average score calculated for each domain (physical, psychological and social) ranging from 1-7 and hence a total score between 3 -21. The higher score the less the impact. The LCQ was developed and validated in patients with chronic cough but not in patients with asthma. The Leicester Severe Asthma Service has recently published data showing a modest correlation between LCQ and ACQ-6 in a group of patients with severe asthma (r=-0.605) [228]. Many cough studies in asthma have used this questionnaire as it the most widely used and accepted questionnaire to assess the impact of cough on patients. The minimal clinically important difference has been calculated to be 1.3 [229] and has been shown to have moderate negative correlation with objective cough rates (r=-0.53 to -0.62) [230] . It has shown to be reproducible and responsive to change in cough within a patient [227] and has therefore also been used an outcome measure in a clinical trial involving gapapentin [50].

Permission has been sought from Dr Birring for the LCQ to be used in my study.

2.4.2 24 hour cough monitoring

There are a number of subjective and objective measures of cough currently available. In clinical practice, subjective measures such as questionnaires and visual analogue scales can be used to assess a patient's perception of their symptoms. However these are limited and unreliable because they can be affected by mood, expectations and memory recall. As such they do not correlate well with objective measures [22]. The gold standard for objective cough monitoring would be manual cough counting however this is time consuming, labour intensive [223] and not practical to be used in large-scale clinical trials or for academic studies.

A number of automated devices have been developed but with limited success. The major difficulties such technologies face is to correctly identify non-cough sounds such as speech, throat clearing, sneezing and laughing whilst also taking into account differences in cough sounds within and between subjects. Furthermore, subjects with different respiratory diseases may have different cough sounds. As such the sensitivity and specificities between different ambulatory cough monitors have varied and are tabulated below. I have used the VitaloJAK (Vitalograph Ltd, UK) cough monitor for my study (Figure 2.1).

Figure 2.1 VitaloJAK cough monitor showing the device, air microphone and adhesive chest sensor

The VitaloJAK cough monitor has an air microphone which is attached to the patient, along with an adhesive chest sensor which enables chest vibrations during a cough to be picked up and correlated with cough sounds. The air microphone records all sounds (8 kHz, 16 bit format) over a complete 24 hours and is stored on a 4GB data card. Once the recording period ends, data are downloaded onto a secure university server and also copied onto a compact disc for storage. Instead of manually listening to a complete 24 hour period, a validated custom written software is used to compress the 24 hour file into a shorter file (usually less than 90 mins) which is then manually verified by trained cough counters using an audio editing software package (Adobe Audition v3.0). The results are expressed as cough per hour (c/hr). This process has been validated, reproducible [22] with high within and between observer agreement [230]. Furthermore, 10% of every 24 hour recording is double checked by experienced research associates (Holt K, Dockry R) to quality control those reported by the cough counters.

A comparison between other devices which detect cough sounds are tabulated below.

Table 2.3: Comparison of the sensitivity and specificity of different cough monitors

Device	Sensitivity	Specificity	Comments/References
Vivometrics lifeshirt ™	78.2	99.6	8 patients with COPD compared with video surveillance [231]
Hull Automated Cough Counter	80	96	33 patients with chronic cough [232]
Leicester Cough Monitor	85.7	100	28 cough patients, 9 healthy [233]
Leicester Cough Monitor	91	99	19 patients with chronic cough [234]
VitaloJAK™	98	100	[235]

2.4.3 Inhaled Capsaicin Cough Challenge

The use of inhalational agents to assess cough reflex sensitivity has been well established since it was first used in humans in 1984 [236] and has been reviewed in the European Respiratory Society guidelines on the Assessment of Cough [237]. Previous to this, a number of attempts had been made in the 1950's using ammonia vapour, sulphur dioxide, nebulised citric acid and intravenous paraldehyde to investigate cough. However, these were quickly abandoned due to toxicity, laryngeal irritation, choking sensations and bronchospasm [238, 239]. In contrast, Collier and Fuller demonstrated in 1984 that in contrast to citric acid, inhaled capsaicin did not result in bronchoconstriction and much less tachyphylaxis [240, 241]. The safety of inhaled capsaicin has since been reviewed in 122 published studies conducted in 4,833 subjects [134]. This review included 2,671 adult healthy volunteers, 788 adult patients with chronic cough and 538 adult asthmatics. There were no serious adverse events reported (>90% of all site investigators were contacted to verify this). Nine of the 4,833 subjects experienced transient symptom-free airway constriction measured by lung function tests after inhaling capsaicin. The most commonly reported side-effect was transient throat irritation.

The advantage of capsaicin is that is a potent tussive agent which specifically activates TRPV1 found predominantly, but not exclusively on vagal c-fibres. Therefore, this property of capsaicin allows assessment of the airway neuronal reflex in humans mediated by TRPV1. Other challenge agents, such as citric acid could have been used, however, how it causes cough and which nerve receptors and nerve endings it activates is currently unclear.

2.4.4 C2/C5 vs. E_{max}/ED_{50} as endpoints

As mentioned in section 1.8, the most common method to assess cough involved inhaling increasing concentrations of a single dose of capsaicin and the concentration causing 2 coughs (C2) and/or 5 coughs (C5) used as endpoints. These are the most commonly used endpoints as they were shown to be reproducible over the short (14 days) and long term (>6 months) [242]. However, this was done in healthy volunteers only and do not discriminate well between health and disease. More recently, Hilton et al have evolved this methodology by asking participants to inhale four doses of capsaicin at every doubling concentration (121). In contrast to C2/5, where the cough challenge is stopped when 2 or 5 coughs is reached, this method continues with the challenge to the maximum tolerated dose. The total number of coughs that occur in the immediate 15 seconds after the dose inhalation is counted by the investigator, verified by a separate cough counter and the total number of coughs (after 4 inhalations) at each concentration is added together. This results in two separate outcome measures; the maximum evoked coughs (Emax) and the excitatory dose that results in half that response – ED50 (Figure 2.2).

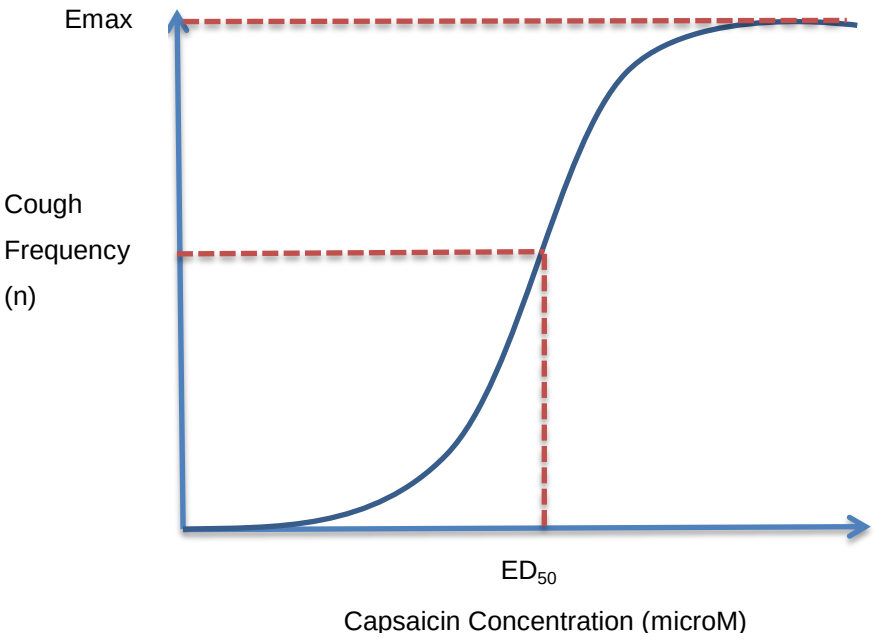

Figure 2.2: Capsaicin dose response curves showing Emax and ED50

These two novel endpoints better discriminate between healthy participants and chronic coughers. My first study investigated whether these differences can be replicated in a well phenotyped group of asthmatics. Furthermore, in contrast to C2/5, Emax highly correlates with objective 24 hour cough frequency.

2.4.5 *Mechanistic insights from the full dose response cough challenge*

The dose response curve gives an insight into to the possible mechanisms involved in the cough reflex. The cough responses evoked may vary such that there is a left shift or an upwards shift of the curve, resulting in a hyper-sensitivity and hyper-responsiveness respectively (Figure 2.3).

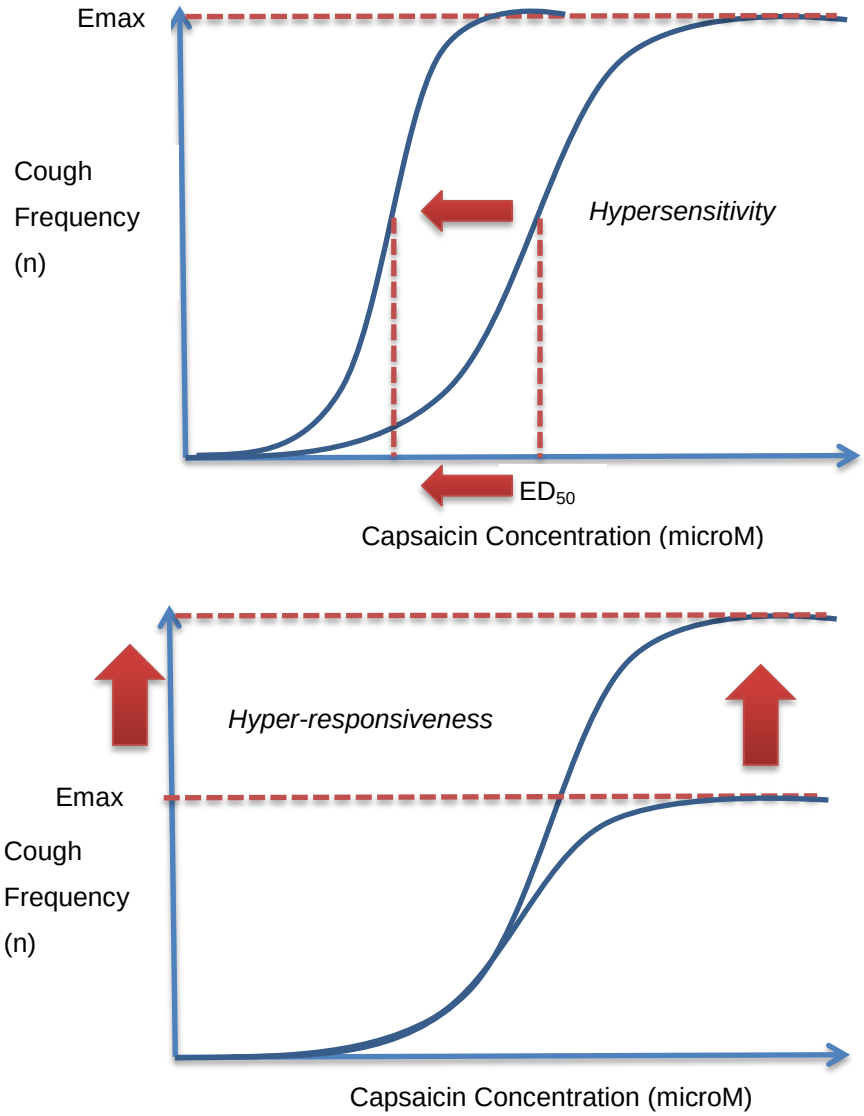

Figure 2.3: Schematic representation of a dose response curve illustrating the difference between hyper-sensitivity and hyper-responsiveness

A left shift in the dose response curve would suggest that the nerves evoking cough would be activated at a lower concentration thereby reducing the threshold for activation. Despite this hyper-sensitivity there is a maximum ceiling beyond which nerves cannot be over stimulated. In neuro-physiological terms this is because a fixed population of nerves, due to refractory period

in between individual action potentials, has a maximum number of action potentials it can generate and transmit.

An upwards shift in the dose response curve would suggest that the nerves evoking cough are responding more vigorously and are exceeding the Emax. This could mean that previously dormant nerves which were not responding to capsaicin are now active, i.e. recruitment of additional nerve fibres. Another possibility is the loss of the descending inhibitory control pathways or central sensitisation resulting in higher Emax values. Evidence for these explanations are yet lacking in human studies. However, pre-clinical animal studies have suggested that there is a phenotypic switching of nerve fibre responses during allergic inflammation mediated by neurotrophins, such that additional nerve fibres are recruited. This is discussed in detail in the previous chapter. This type of dose response curve is used commonly in clinical pharmacology and requires non-linear mixed effects modelling.

Based on these reasons I decided to adopt the full dose response methodology developed in Manchester.

2.4.6 How to perform capsaicin full dose response challenge

Full details of how to perform the capsaicin full dose challenge is located within the online supplement of Chapter 3. The method is summarised here below.

Step1: Calibrate the nebuliser pot and check dosimeter settings

To ensure accurate dosing is achieved at each inhalation, a Devilbiss 646 nebuliser pot fitted with an inspiratory flow limiter (Figure 2.4) is calibrated to emit between 10-12 microlitres per actuation, at a pressure of 30 psi via a dosimeter (KoKo) (see Figure 2.5) and maximum flow of 0.5 l/s.

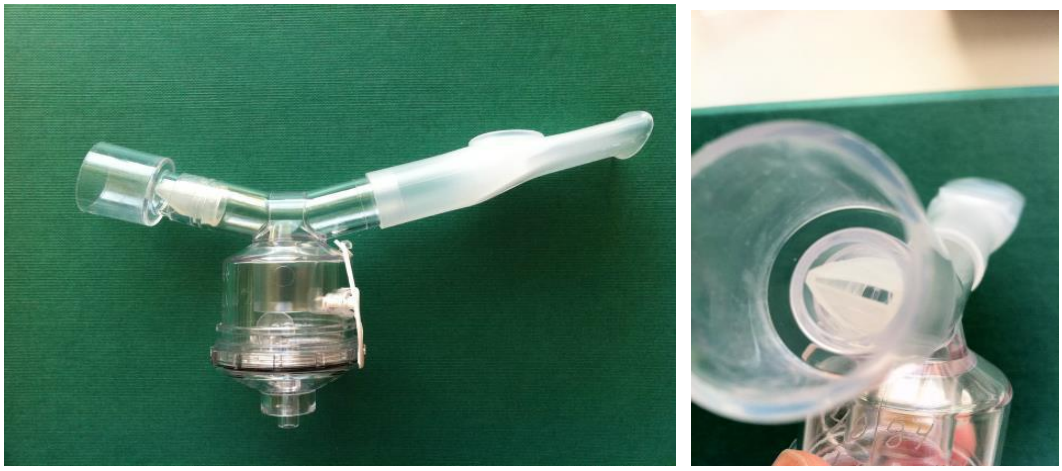

Figure 2.4; Devilbiss 646 nebuliser pot showing the inspiratory flow regulator

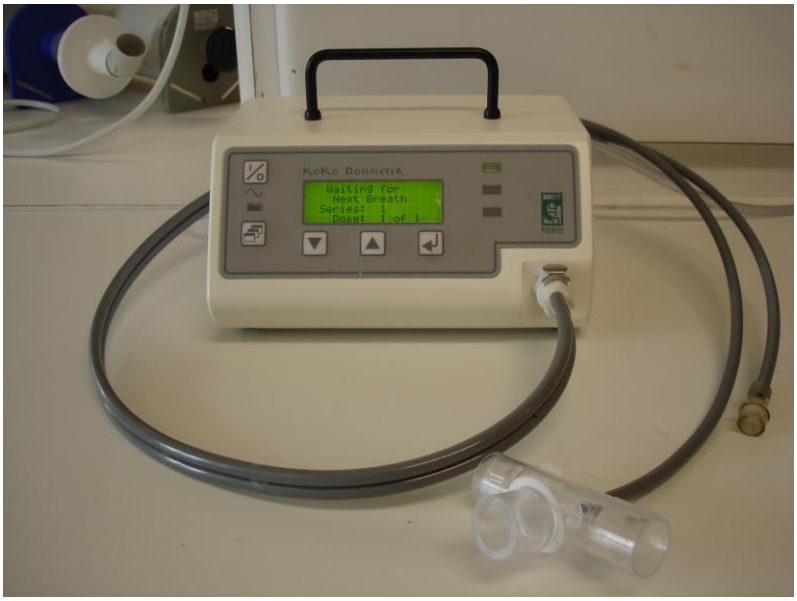

Figure 2.5; Koko dosimeter used for all cough challenges

The settings on the dosimeter are checked before each challenge to ensure each inhalation is set at 30 second intervals and that the duration of the dose emitted matches each individual pre-calibrated nebuliser pot. This usually ranged from 0.9 to 1.2 seconds, but care is taken to ensure that the same nebuliser pot is used in the same study for the same patient each time. This is in order to reduce any variability in dose emitted at each actuation.

Step 2: Prepare capsaicin doses

The starting dose of capsaicin will be 0.49 microM, doubling to 1000 micoM. Serial dilutions will be performed using the 4ml of stock solution of 1000 microM (Stockport Pharmaceuticals, UK), and diluted using 2ml of 0.9% saline. In addition to capsaicin, there are 3 placebo pots of 0.9% saline in pot 1, 6 and 15.

Figure 2.6 below shows the dilution process including the placebo saline doses.

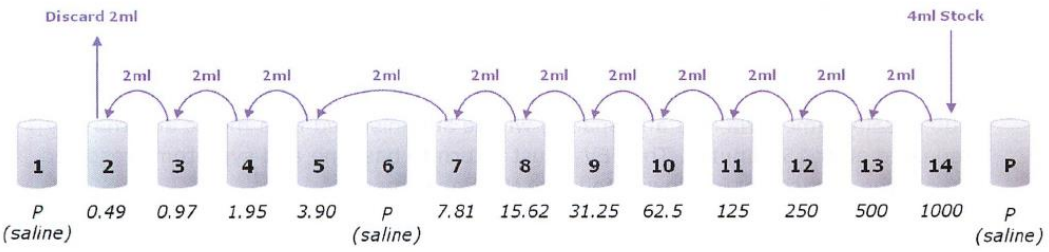

Figure 2.6; Capsaicin serial dilution methodology

Step 3: Check lung function and attach cough monitor

Each subject has their lung function measured prior to starting the cough challenge and has the cough monitor (VitaloJak) attached for the duration of the challenge. The purpose of recording the whole challenge is to be able to verify the number of evoked coughs later in order to reduce errors in counting. Lung function is also measured at the end of the challenge and if there is a drop in FEV1 of more than 10% or patients became symptomatic then salbutamol is administered via a spacer.

Step 4: Start the challenge

Each participant begins the challenge at pot 1, which is a placebo pot containing 0.9% saline. At each dose, four inhalations 30 seconds apart is administered, and the number of coughs evoked in the first 15 seconds after inhalation documented. Doubling doses of capsaicin are sequentially administered and the coughs evoked documented and later verified. Very rarely are patients able to reach the highest 1000 microM concentration and it is more usual to end the challenge at the maximum tolerated dose of capsaicin. Wherever in the dose sequence the participant decides to stop, pot 15 (saline 0.9%) is always administered.

The sum of the four inhalations is documented as the total for each dose. The maximum number of coughs evoked at any dose is recorded as the E_{max} and the excitatory dose causing half the response ED_{50}.

2.4.7 Use of single ED50 for cough challenge

During the latter 2 studies (Chapter 4 and 5) four inhalations of one single dose of the ED50 capsaicin dose was administered after methacholine and allergen challenge. The main advantage of performing a single ED50 challenge is the ability to very quickly assess in 2 mins whether cough responses increase or decrease as airway calibre changes. In contrast, the full dose response challenge takes approximately 40 mins to complete and provides individualised Emax and ED50. However, the objectives of the second and third studies were to investigate the effects of acute bronchoconstriction and allergen exposure on capsaicin evoked cough responses, but not changes in Emax or ED50. Performing a challenge over 40 mins would also be impractical to perform because FEV1 improves quickly after finishing the methacholine challenge and during an allergen challenge. Furthermore, because capsaicin challenge had previously not been attempted immediately after a 20% drop in FEV1 or during allergen challenge, hence in the interest of patient safety, I felt it would be sensible to limit the amount of capsaicin being inhaled.

2.5 Inhaled allergen challenge

2.5.1 Brief history of allergen challenge

It is acknowledged that asthma and allergy are related, and this is most evident in the grass pollen season when many sensitised patients with asthma often develop an increase in symptoms. This observation of seasonality was first published by Floyer in 1698, and Bostock in 1819 provided the first clinical description of seasonal allergic rhinitis and asthma. The term hay fever was first coined around a decade later because these symptoms seemed to occur in the haying season. In 1873, Charles Blackley inhaled grass pollen himself to reproduce these symptoms and demonstrated a causal link [243]. He also described an early symptom response and a much later response, but because the standardisation of measuring lung vital capacity did not occur till the 1940s [244], he was unable to quantitatively measure a changes in lung function. Herxheimer performed allergen challenge to describe the late response in more detail, and by the late 1960's the biphasic reaction of early and late response was documented to occur in approximately 50% of subjects with asthma [245-247]. Hargreaves et al demonstrated increases in non-specific bronchial hyper-responsiveness (BHR) after allergen challenge [248], which was later reported by Cockcroft et al to be linked to the late asthmatic response (LAR) [249]. There was then a further focus on understanding how inhaled allergen changed the inflammatory mediators in the airways by detecting changes in bronchoalveolar lavage [250], induced sputum [251] and breath [252]. Over 3 decades later, performing inhaled allergen challenge has now become an established model to study asthma pathophysiology and to test the efficacy of potential new asthma drugs.

2.5.2 Understanding the early and late asthmatic responses

Inhaled allergen testing is an indirect bronchial provocation test causing an initial early asthmatic response (EAR), and in up to 70% of asthmatics, a much prolonged deeper late asthmatic response (LAR). The EAR defined as a drop of FEV1 ≥20% occurs within 10-15 mins after inhaling allergen but the FEV1 can continue to fall up to 30 mins after. The magnitude of the EAR is closely related to the baseline BHR, the dose of allergen inhaled and serum IgE levels [253]. The FEV1 slowly returns to baseline within 1-3 hours. The LAR is defined as a drop in FEV1 ≥15% between 3-7 hours, but can continue for 6-12 hours after [254]. The LAR occurs in between 50-70% of atopic asthmatics [255] and more commonly associated with house dust mites (HDM) than grass pollen [256]. The magnitude of the LAR has been linked to the magnitude of the EAR which suggests a common IgE mediated pathway [254], however, an isolated LAR has been shown after inhalation of peptide independent of IgE mediated pathways [257].

Both the early and late responses are expressed as the maximum % drop in FEV1 from baseline, but for the LAR, the area under the curve ($AUC_{3\text{-}8h}$) of the % decrease in FEV1 between 3-8 hours has also been used.

Allergen inhalation is an indirect bronchial challenge. Direct bronchial challenges such as methacholine or histamine act on specific airway smooth muscle receptors to cause transient bronchoconstriction. The mechanism of this biphasic early and late response is associated with an increased in pro-inflammatory mediators and BHR, which may last days to weeks [255, 258].

2.5.3 Mechanisms of allergen induced bronchoconstriction

In sensitised asthmatics, pre-formed IgE antibodies bound to mast cells and basophils, which upon inhaling allergen, form cross-links with two or more IgE antibodies causing degranulation. This causes release of preformed histamine, proteases such as tryptase and newly formed eicosanoids particularly cysteinyl leukotrienes (CysLT C4, D4 and mainly E4) and prostaglandins (PGD2). Cysteinyl leukotrienes and histamine are considered to be potent bronchoconstrictors, but they also contribute to increasing vascular permeability and mucus secretion. Treatment with anti-histamines and leukotriene receptor antagonists (LTRAs) abolish the EAR [259, 260]. Similarly, the LAR can also be attributed to release of CysLT and histamine as LTRAs and 5-lipoxygenase activating protein antagonist partially attenuated the LAR, which can be further reduced by the addition of an anti-histamine [261-263]. These antagonists are also associated with a reduction in airway eosinophilia and the prevention of increased BHR after an allergen challenge [262, 264, 265].

Controversy exists about the source of CysLT and histamine in the LAR. Basophils can produce both CysLT and histamine but eosinophils which are increased in the LAR only produce CysLT. Blocking IL-5 reduced the influx of eosinophils in the airway after inhaling allergen but had no effect on the LAR [186]. However, blocking the receptors of cytokines which stimulate eosinophil production such ash IL-3,IL- 5 and granulocyte-macrophage colony stimulating factor together with the chemokine receptor CCR3 has been shown to attenuate airway eosinophils and the LAR [266]. Therefore, this suggests that purely blocking one receptor may not be enough in preventing the LAR, but requires inhibition of the maturation, production and trafficking of eosinophils along with blocking the effects of mast cells and basophils which also release CysLT and histamine.

2.5.4 Mechanisms of allergen induced airway inflammation

The exact nature of the inflammatory pathways and mediators involved after inhaling remains an area of intense research. However, it is clear is that inhaled allergen stimulates the development of Th-2 cells (type 2) via complex pathways involving the airway epithelium, innate and adaptive immune cells, and the bone marrow.

Evidence is emerging suggesting that inhaling allergen causes the release of epithelial derived cytokines IL-25, IL-33, and thymic stromal lymphopoetin (TSLP) which acts a master switch in ‘alarming’ the airways of incoming allergen, and blocking TSLP causes an attenuation in both the EAR and LAR [267]. The cross-linking of IgE described above causes release of pro-

inflammatory cytokines IL-4, 5, 9, and 13 [268, 269]. These cytokines are crucial for the production of IgE (IL-4), eosinophilopoiesis (IL-5), mast cell development (IL-9), goblet cell hyperplasia and increased BHR (IL-13) [270-272]. These type-2 cytokines can also be released from type 2 innate lymphoid cells and Th-2 cells, however activating the latter requires professional antigen presenting cells (APC), particularly the myeloid dendritic cells (mDC). These mDCs are able to phagocytose allergen peptide and present to both naïve T cells, which become Th-2 cells secreting IL-4, 5, 9, and 13. This leads to plasma cells becoming B cells producing IgE and up regulation of the IL-5 receptor on immature eosinophils progenitors in the bone marrow [273]. Given that allergen inhalation also causes increases in basophils and neutrophils it is likely that their progenitors are also stimulated in the bone marrow [108, 274]. Furthermore, mDCs are also trafficked from the bone marrow and blood into the airways ready to act as APC and also via the lymphatic system to regional lymph nodes to be presented to previously primed Th-2 cells [275]. This causes further production of eosinophils, basophils and neutrophils from the bone marrow to be recruited into the airways [276].

This inflammatory response can be controlled by T regulatory cells (T-reg) and plasmacytoid DCs (pDCs) which seek to attenuate the effects of the LAR. Indeed, patients who develop a LAR show evidence of impaired T-reg cells [277]. Likewise, in animal models, pDCs have been shown to suppress the generation of Th-2 cells and stop an unnecessary reaction to allergens [278, 279].

Mechanisms independent of IgE also need to be considered as asthmatics who are dual responders, demonstrating an EAR and LAR, have been shown to have a similar magnitude of bronchoconstriction to single responders (EAR only). Secondly, exercise causes mast cell degranulation independent of IgE, and this does not results in eosinophilic airway inflammation [280, 281].

In summary, in the context of allergen challenge the inflammatory profile of the EAR is mainly a type 1 hypersensitivity reaction involving IgE, mast cells and basophils. Whilst the late response is secondary to dendritic cells orchestrating the presentation of allergen to T cells, which release inflammatory cytokines that stimulates a range of progenitor cell lines in the bone marrow. This leads to generation of further DCs, eosinophils, basophils and neutrophils which are trafficked into airways where they degranulate upon cross-linking with IgE molecules to release CysLT and histamine. This elaborate response can take up to 3 hours and last between 8-12 hours after inhaling allergen.

2.5.5 Lung challenge methods

My study involved performing total lung inhaled challenges via the mouth. Validated methods for performing total lung inhaled challenges exist; incremental, bolus (high dose), repeated low-dose and ‘real life’ allergen exposure rooms [282-285]. I was trained to perform the incremental

doubling dose tidal breathing method by Professor Paul O'Byrne's team in McMaster University, Canada. In comparison to the other methods, the main advantages are: firstly, starting at 3 doubling doses below the predicted dose to cause an EAR reduces the chances of large unexpected drops in FEV1, secondly, the tidal breathing negates any effects of deep breathing on FEV1, thirdly, deep breathing in the bolus high dose method might lead to more inaccurate dosing due to variations in respiratory manoeuvres. The 2 disadvantages are that incremental doses prolongs the allergen challenge by at least 30 mins, and inhaling relatively high doses of allergen might not mimic natural allergen exposure. For this latter reason, repeated low dose exposure of an allergen over 5 days has been used. The low dose method does not result in a late asthmatic response, but does cause airway eosinophilia and increased BHR.

2.5.6 Performing inhaled allergen challenge

Selecting mild atopic patients with asthma who are not on inhaled steroids, LTRA or anti-histamines is of paramount importance as these medications are known to effects the LAR. Similarly, regular use of SABA has been shown to affect the LAR, with frequent use associated with a more pronounced LAR and airway eosinophilia [286, 287]. The reason for this is likely due the bronchodilatatory effects masking airway inflammation.

i) Safety precautions

Allergen challenge resulting in loss of asthma control is minimised by carefully predicting the allergen doses and ensuring asthma stability before the challenge. There is a risk that inhaled allergen can induce severe acute bronchoconstriction or generalised anaphylaxis. Hence, staff with ample experience in dealing with a potential life-threatening situation and resuscitation equipment always needs to be available. Despite this risk, the challenge is well tolerated because of the careful method for selecting patients and the dose of inhaled allergen.

After the measurements are completed on the day of the challenge all patients are given inhaled salbutamol to ensure that FEV1 has returned to approximately 90% of baseline. Patients are sent home with bronchodilator, prednisolone tablets, contact numbers for emergency on call study doctors and given clear advice explaining the possibility of symptoms recurring later in the evening or overnight. Should this occur to take additional inhaled salbutamol. Patients are also told not to perform any exercise after the allergen challenge, and if possible, not to be left alone at home.

The time interval between successive allergen challenges in a study needs be considered. The main determinant of the EAR and LAR is the degree of BHR, and this can be affected for up to 2 weeks after an allergen challenge [288] . Furthermore, patients may also have come into contact with another allergen such as from a cat, dog or from grass pollen. Hence, before any allergen challenge, the methacholine PC20 needs to return within 1 doubling concentration of

baseline PC20 and it is recommended that FEV1 is within 10% of baseline. If the PC20 has not returned to near baseline values then the visit postponed for at least another 7 days.

The safety of inhaled allergen challenge has been evaluated for 965 allergen challenges carried out in 310 subjects at McMaster University between 2003-8. There were 5 adverse events related to allergen challenges. Three subjects experienced persistent symptoms after allergen challenge at screening, and one subject while on treatment. These 4 subjects were administered inhaled steroids and withdrawn from the study. One subject experienced exacerbation after allergen challenge and was treated with oral steroids. All subjects fully recovered.

ii) Equipment

There are some important considerations before setting up an allergen study in relation to the equipment to use. My purpose was to re-create the exact methodology as has been performed in McMaster University over the last 30 years.

The prediction formula to calculate the starting dose of allergen is dependent on the 2-minute tidal breathing methacholine challenge using a jet nebuliser calibrated to 0.13ml/min. The 3 types of nebulisers that have been used for the methacholine challenge are:

1. DeVilbiss 646 (Somerset, Pa)
2. English Wright (Roxon, Quebec, Canada)
3. Bennett Twin (Puritan Bennett Corporation)

The English wright nebuliser is also the most commonly used for allergen challenge. For the purpose of this study, methacholine was performed using the Devilbiss 646 and English Wright nebuliser for performing allergen. Dedicated nebuliser pots should be used for methacholine and allergen to ensure no mixing of solutions. One of the logistical problems that other investigators may find is that Roxon has terminated making the English Wright nebulisers and hence I had to loan two English Wright nebulisers from McMaster University for the duration of my allergen study. These pots were used solely for performing allergen challenge and not for methacholine challenge.

There are also a number of companies making allergen extract at different concentrations and at substantially different costs. I was able to source the same supplier as McMaster University to ensure the house dust mite extract was of identical quality (Omega Laboratories, Montreal, Canada). The allergen is stored in a glass vial between 4-8 °C. When diluting allergen, care must be taken not to use plastic vials as the allergen will stick and degrade the plastic making it unsuitable for use.

iii) Allergen Selection

The choice of allergen is dependent on the allergen which caused the largest wheal by skin prick testing (SPT). For the purpose of my allergen study, I was recruiting from a cohort of 97 mild to moderate asthmatics who all previously had SPT, and over 90% of those who were atopic were sensitive to house dust mite (HDM). It has been noted that allergen extract can cause a stronger LAR compared to purified allergen and this might be due to activation of non-IgE mediated mechanisms and protease activated receptors [289]. Hence, because my purpose was to study mechanisms of disease I decided to use HDM allergen extract only to reduce the variability of responses.

iv) Calculating the starting allergen concentration at the screening visit

The formula for predicted concentration allergen challenge that will cause a 20% drop in FEV1 (EAR) is called the Cockcroft formula:

$$\text{Log}_{10}\,(PC_{20}\text{allergen}) = 0.68 \times \log_{10}\,(PC_{20}\ \text{methacholine} \times SS)$$

where PC20 methacholine refers to the calculated PC20 the day before the allergen challenge as measured by the tidal breathing method, and SS refers to the lowest concentration of allergen which caused an average 2mm x 2mm skin wheal at screening. This gives an estimate which is accurate to within 2 (approx. 80%) or 3 (92-94%) doubling concentrations, hence it is recommended and safer to start the first concentration at least 3 doubling concentrations lower. For example, if the formula predicts the allergen PC20 to be 1:32, then the first concentration to administer the patient would be 1:256.The patients would begin the 2 minute tidal breathing of allergen, wait for 10 mins thereafter to measure FEV1, and if there is <10% drop in FEV1, then the next doubling concentration 1:128 is again administered for 2 mins and FEV1 checked 10 mins after inhalation. If there is a drop in FEV1 of between 10-20% of baseline, then FEV1 is checked after a further 10mins. If at that time point, the FEV1 begins to improve, then 1:64 is administered, but if the FEV1 drops >20%, then no further allergen needs to be administered. If there is an insufficient drop in FEV1 even after 3 doses, then doubling incremental concentrations are administered until the 20% threshold is reached, or until the highest concentration of 1:8 is reached.

For subsequent allergen challenges during an individual study, only 3 doses of allergen are administered. For example, if the formula predicted 1:32, but the actual concentration which caused an EAR at the screening challenge was 1:16, then the starting dose would be 1: 64, then 1:32 followed by 1:16. If the EAR occurred at lower than predicted concentration such as 1:128, then likewise, start at 1:512.

v) Monitoring during visit

After the last inhalation of allergen the FEV1 is measured at 20, 30, 45, 60, 90 and 120 minutes, and then at hourly intervals at 3h, 4h, 5h, 6h until 7 hours. The challenge may be terminated by administration of bronchodilator and/or steroids at the investigator's discretion if it is deemed unsafe to continue. The first 2 hours of the challenge are the most intense as they require regular FEV1. After the first 2 hours, one technically acceptable FEV1 is performed every hour, and during this time, patients often prefer to move into a more comfortable seating area, however care must be taken to ensure the patient is never left unattended.

2.5.7 Application for studying nerves

Allergen challenge is a safe, validated and reproducible model for studying allergic airway inflammation. Likewise, capsaicin evoked cough challenge is a model for studying airway nerve function. By combining these two models, which test different airway pathologies we tested the effects of bronchoconstriction and airway inflammation on airway nerves. Firstly, 30 mins after allergen inhalation, during the EAR, we tested the effects of bronchoconstriction secondary to CysLT and histamine release on capsaicin ED50 evoked cough responses. This is in contrast to my second study which investigated the effects of methacholine induced bronchoconstriction, a direct muscarinic agonist. Secondly, in patients who had a LAR, we also performed capsaicin ED50 cough challenge at 24 hours. It has been explained above that there will be an influx of predominantly eosinophils and basophils but also some neutrophils 3 hours after allergen inhalation. Importantly, 24 hours after allergen, the FEV1 begins to improve to baseline values. In addition to measuring capsaicin evoked cough responses at 30 mins (EAR) and 24 hours (after LAR) we measured spontaneous cough rates from the start of inhalation, and were able to quantify cough rates per hour.

There are some important considerations to this methodology. Firstly, inhaling allergen may cause release of other inflammatory mediators which we did not seek to measure. These include neurotrophins such as BDNF, NGF and NT-3. As discussed in chapter 1, evidence exists in animals and humans that allergen challenge induces the release of neurotrophins which can phenotypically switch A-δ fibres to becoming capsaicin sensitive by expressing novel TRPV1 [100, 105, 290, 291]. Other mediators such as substance P, ATP or its breakdown product adenosine could also be confounding variables in sensitising or directly activating the cough reflex [168, 173, 292, 293]. Although these mediators could be important, reliably and sensitively quantifying these mediators has not been well established and hence at this stage, we chose to focus on inflammatory cells which we can reliably quantify.

Secondly, we chose not to perform capsaicin ED50 challenge at the 7 hour time point because we were unsure whether inhaling capsaicin would affect the nature of the ensuing inflammatory cascade. Based on the results of my second study we knew that inhaling capsaicin after a transient bronchoconstriction does not affect subsequent FEV1, so we were re-assured that inhaling capsaicin at 30 mins would be safe. However, the prolonged nature of allergen challenge and the on-going inflammatory cascade made us reluctant to send patients home at the end of 7 hours having just inhaled capsaicin. We also felt more comfortable performing capsaicin ED50 challenge 24 hours later because one study has safely performed capsaicin challenge by measuring C5 before and 24 hours after allergen challenge [191]. Our reason for performing ED50 challenges during and after allergen challenge has been explained in the section above.

2.6 Statistical Modelling

Data collected during my studies included quantitative, categorical and repeat measures in individual subjects over time. Parametric and non-parametric single point data between subjects was analysed using independent t-test or the Mann-Whitney U test. Categorical data was compared using chi-squared or fisher's exact test.

For the purpose of my studies, *non-linear* mixed effects modelling was performed for analysing population responses to compare capsaicin evoked full dose response challenge between 97 asthmatics and 47 healthy volunteers (Study 1). For studies 2 and 3, a form of generalised *linear* model, Generalised Estimating Equations (GEE), was used because cough responses to ED50 capsaicin was measured in a smaller number of patients at different time points.

There exists other methods for repeat data analysis such as ANOVA/MANOVA but these are limited in there use because they:

i) assume categorical predictors
ii) are unable to incorporate time-dependent co-variates
iii) unable to provide parameter estimates and
iv) missing data must be imputed.

In the context of cough challenge data there are categorical data and continuous data, some missing data, and time-dependent co-variates. Importantly, the variability within the whole population of data needs to be understood in terms of important characteristics such as disease state, gender, atopic status, age, BMI etc. Both GEE and non-linear mixed effects modelling can provide this type of analysis, giving insights to the possible mechanisms of capsaicin cough responses.

GEE was performed by myself under the supervision of Professor Jacky Smith.

NONMEM is an advanced statistical modelling package and is the gold standard for performing population pharmacodynamic modelling. This was performed by Dr Nikolaos Tsamandouras and Dr Kayode Ogungbenro at the Manchester Pharmacy School, University of Manchester.

I have given a brief overview of the two modelling techniques below, but further specific details of the models are found within the individual studies.

2.6.1 Generalised Estimating Equations (GEE)

GEE is an extension of a generalised linear model and is suitable to estimate population average parameters. The key feature of this type of model is that it treats the variability in the data as nuisance and models the mean responses. It does allow continuous and categorical variables to be modelled in order to give parameter estimates and assess whether such co-variates significantly influence cough responses. The mathematical method of comparing differences is based on a linear equation of y=ax +b. As such, differences between different co-variates are analysed for whether the gradient between two lines are different, whether the intercept on the y-axis is different, and whether the mean responses are different. A basic example of this is demonstrated below.

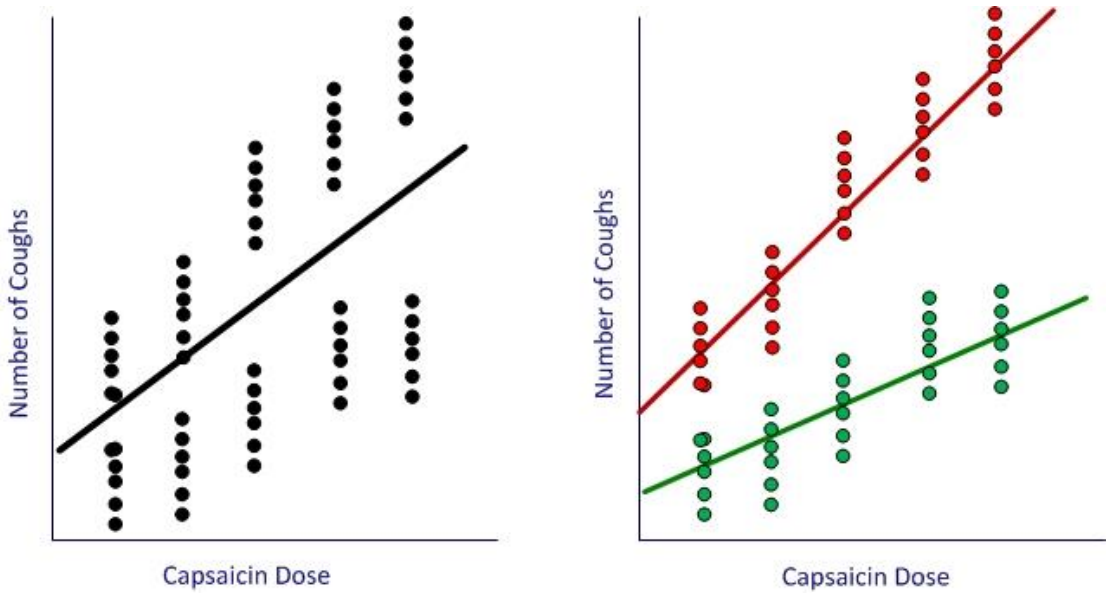

Figure 2.7: Schematic showing the principle of generalised estimating equation (GEE)

The example above demonstrates the broad principle of GEE. On the left, are all the population responses demonstrating that as capsaicin dose responses increase, the number of coughs also increase. The GEE model is able to produce a line of best fit as the population average. If however, we add the co-variate of disease state where green represent healthy volunteers, and red represent subjects with asthma, the GEE model produces two separate population averages across each dose to demonstrate that there is a significant group effect, i.e. the average cough responses are higher, the gradient is steeper, and the intercept is higher in the asthmatics. The GEE model can also analyse within every subject whether there is an interaction between sequential capsaicin doses, and is also able to compare between subject differences at every dose. This prevents the need for laborious t-tests between every single dose and every single patient. This type of analysis can be expanded to add additional co-variates such as gender, atopic status etc., however, doing so may make the model unstable as there will be fewer subjects in each category.

One limitation of the GEE model is that it is linear, and cough responses, like many biological processes, are non-linear, i.e. dose escalation results in a sigmoid shaped relationship with cough response until a maximum cough plateau is reached (Emax). Therefore, I chose to adopt a non-linear mixed effects model for assessing the dose response curves generated in study 1. In study 2 and 3, dose response curves were not being compared, but rather the influence of FEV1 and time on ED50 capsaicin cough responses which can be performed in a GEE model.

2.6.2 Non-linear Mixed Effects Modelling

Population pharmacokinetics/pharmacodynamics (PK/PD) is a well-established tool to compare the variability in plasma drug concentrations between individuals when standard doses are administered (PK) and the physiological response of such drugs (PD). The variability within a population can be investigated for by patient fixed factors such as age, gender, weight, renal function, disease state or an intervention or random factors which are unknown subject factors. This approach has been very useful in determining drug doses in different patient groups in different clinical scenarios when drug doses may need to be altered. The use of such techniques can also be replicated in other scenarios where there are repeated measures in groups of patients, where the variability of responses can be attempted to be modelled using known patient factors. The first time this was performed in the context of cough was PD modelling of capsaicin evoked cough responses in patients with chronic cough, asthma and healthy volunteers [160]. This demonstrated the feasibility of performing a population non-linear modelling technique on cough responses and concluded that Emax and ED50 were superior to C2 and C5 in differentiating health from disease.

In my study, I adopted this same technique to analyse the influence of a range of variables which influenced Emax and ED50 in subjects with asthma and healthy volunteers. The variables which influenced the model were: disease state (asthma or healthy), gender, atopic status, serum IgE, ACQ score and 24 hour rates. Full details of the modelling can be found in the online supplement of the manuscript.

3 Capsaicin Cough Responses in Asthma: Evidence for Airway Neuronal Dysfunction

Capsaicin Cough Responses in Asthma: Evidence for Airway Neuronal Dysfunction

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3.1 Abstract:

Background: Cough in asthma is a common and troublesome symptom. It is generally assumed coughing occurs as a consequence of bronchial hyper-responsiveness and inflammation, but the possibility that airway nerves are dysfunctional has not been fully explored.

Objectives: We sought to investigate capsaicin evoked cough responses in a group of well-characterised mild to moderate asthma patients compared with healthy volunteers, and assess the influences of gender, atopy, lung physiology, inflammation and asthma control on these responses.

Methods: Capsaicin inhalational challenge was performed and cough responses analysed using non-linear mixed effects modelling to estimate maximal cough responses (E_{max}) and the dose evoking half this response (ED_{50}).

Results: Ninety-seven stable asthmatics (median age 23yrs (IQR 21-27), 60% female) and 47 healthy volunteers (38yrs (29-47), 64% female) were recruited. Asthmatics had a higher E_{max} and lower ED_{50} than healthy volunteers. E_{max} was 27% higher in females (p=0.006), 46% higher in non-atopic asthma (p=0.003) compared with healthy volunteers. Also, atopic asthmatics had a 21% lower E_{max} than non-atopic asthmatics (p=0.04). ED_{50} was 65% lower in females (p=0.0001) and 71% lower in all asthmatics (p=0.0008). ED_{50} was also influenced by asthma control and serum IgE, whilst E_{max} was related to 24hr cough frequency. Age, BMI, FEV1, PC_{20}, FeNO, blood eosinophils and inhaled steroid treatment did not influence cough parameters.

Conclusion: Stable asthmatics exhibited exaggerated capsaicin cough responses consistent with neuronal dysfunction. Non-atopic asthmatics had the highest cough responses, suggesting this mechanism might be most important in type 2-low asthma phenotypes.

Key Messages

- Using a novel challenge methodology and pharmacodynamic modelling we have demonstrated that mild/moderate subjects with asthma have a heightened cough response to inhaled capsaicin, most evident in female non-atopic subjects.
- Unlike standard cough challenge endpoints (C2 and C5), the capsaicin Emax and ED50 were influenced by gender, spontaneous cough frequency, asthma control and measures of atopy (IgE and Skin prick testing).

Capsule Summary

This study shows that performing non-linear modelling of cough responses to full dose capsaicin challenges discriminates healthy volunteers from subjects with mild/moderate asthma, and reveals novel relationships between cough responses, atopy and asthma control.

3.2 Introduction

Asthma affects an estimated 300 million people worldwide, and is characterised by symptoms of cough, wheeze, chest tightness and shortness of breath. Current dogma suggests asthma symptoms arise as a consequence of airway narrowing, bronchial hyper-responsiveness (BHR) and airway inflammation. Yet despite effective treatments targeting each of these components of asthma, many patients have substantial residual symptoms. Even in a clinical trial setting, with optimal inhaled treatment, up to 50% of asthma patients are not well controlled (1). Whilst for some patients adherence might be an issue (2) it is also likely that undiscovered mechanisms explain the heterogeneity in asthma clinical phenotypes and treatment responses.

Symptoms are often challenging to study as they can only be reported subjectively; cough however, is readily amenable to objective quantification (3). Cough in asthma is not only a common (4) and troublesome symptom (5), but also predicts disease severity (6) and poor prognosis (7), suggesting it reflects important pathophysiological processes, yet remarkably little is understood about the underlying mechanisms. The general assumption is that airway afferent nerves activating the cough reflex are stimulated by inflammatory mediators, mucous and bronchospasm, and the possibility that these neuronal pathways are dysfunctional is rarely considered.

Vagal afferent fibres innervate the airways and are responsible for mediating symptoms and airway reflexes (8,9). Coughing is readily evoked by activation of C fibres; these networks of un-myelinated chemically sensitive afferents are characteristically sensitive to capsaicin (chilli pepper extract) through activation of the transient receptor potential vanilloid type 1 (TRPV1) channel. Aδ fibres are sparsely distributed thinly myelinated fibres in the proximal airways and also evoke cough. They protect the airways by responding to mechanical stimuli (e.g. foreign objects), and changes in osmolarity and acidity. Importantly, they are typically insensitive to capsaicin and inflammatory mediators and do not usually express TRPV1.

Experimentally evoked cough responses to inhaled irritants are an established tool for studying the cough reflex, and thus airway nerve function. Capsaicin is the most widely used agent and the concentration causing ≥5 coughs (C5) considered a measure of cough reflex sensitivity (3). However, previous studies in asthma have produced conflicting results, with some studies suggesting sensitisation of the cough reflex (reduced C5), whilst others found no difference from healthy controls (10-13). We have recently investigated capsaicin evoked cough responses using repeat inhalations of capsaicin and concentrations beyond the C5. Non-linear mixed effects modelling of this data found maximal cough responses (E_{max}) best discriminated patients with chronic cough from healthy controls/mild asthma subjects; the difference between healthy and asthma subjects did not quite reach the *a priori* statistical significance (14). Therefore, we have studied capsaicin evoked cough responses in a larger group of well-characterised mild to moderate asthma patients and healthy volunteers. We also investigated the influences of gender, atopic status, lung physiology, inflammation and asthma control on capsaicin cough

responses. Some of the results of these studies have been previously reported in the form an abstract (15,16).

3.3 Methods

3.3.1 Subjects:

Subjects with a physician diagnosis of asthma were recruited, but not selected for symptoms of cough. Treatment with salbutamol as required, and/or inhaled corticosteroid (ICS) ≤500mcg of fluticasone propionate equivalent daily, with or without a long acting bronchodilator (LABA) was permitted. Subjects uncontrolled according to Global Initiative for Asthma (GINA) classification or not on stable medication over the previous four weeks were excluded. Healthy controls, approximately matched for age were also recruited. We excluded current smokers, those with a recent chest infection or exacerbation, and use of any medication which may alter the cough responses (e.g. opiates, gabapentin, anti-cholinergics, and theophylline). The study protocols for healthy controls and subjects with asthma were approved by the local research ethics committee (13/COA/002 and 13/CLU/001) and all subjects provided written informed consent.

3.3.2 Study Protocol and Procedures:

For full details see the online repository. Subjects with asthma attended on three occasions. On visit 1, subjects underwent history and examination, completed the Asthma Control Questionnaire (ACQ), Leicester Cough Questionnaire (LCQ), exhaled breath nitric oxide (FeNO) (NIOX, Aerocrine), spirometry, bronchodilator reversibility and an ambulatory cough monitor (VitaloJAK™; Vitalograph, Buckinghamshire, UK) was fitted for the next 24 hours. At Visit 2, at least 48 hours later, subjects underwent full blood count, serum IgE, skin prick testing, and the provocative concentration of methacholine challenge causing a 20% drop in FEV_1 (PC_{20}). Subjects completed a peak flow diary twice a day for 7 days after visit 2.

Visit 3 took place at least one week later and a capsaicin cough challenge was performed as previously described (14), using a dosimeter (Koko Dosimeter; Ferraris Ltd, Hertford, UK) and a nebuliser pot (Model 646; Devilbiss Healthcare LLC) with an inspiratory flow limiter. Briefly, four inhalations were administered, thirty seconds apart, of doubling doses of capsaicin (0.48-1000µmol/L). After each inhalation, the number of coughs in the first 15 seconds was counted and later verified using a cough monitor (VitaloJAK™). The challenge was completed when the patient reached the final dose or the maximal tolerated dose. Spirometry was performed before and after each challenge.

Healthy volunteers attended on two occasions. On visit 1 consent, screening, spirometry, and the ambulatory cough monitor was attached. On visit 2, the capsaicin challenge was performed.

3.3.3 Statistical Analysis:

Cough responses to capsaicin were analysed using nonlinear mixed effects modelling software (NONMEM® 7.3, ICON Development Solutions) and the Laplace estimation method (17,18). Additional investigations of the NONMEM output, statistical and graphical analyses were performed in Matlab R2014a (The MathWorks, Inc., Natick, MA). We applied a modelling approach developed previously (14); the number of coughs was assumed to follow a Poisson distribution, adjusted for tachyphylaxis evoked by repeat inhalations of the same capsaicin dose. The capsaicin cough response curve was assumed to follow a sigmoid shape where the maximum response was denoted E_{max} and the dose evoking half this response ED_{50}.The effect of continuous and categorical covariates were investigated including: age, gender, body mass index, disease state (healthy or asthmatic), atopy (atopic or non-atopic), predicted FEV1, cough frequency, serum IgE, blood eosinophil count, FeNO, methacholine PC_{20}, ACQ, and LCQ questionnaires. The pharmacodynamic model was used to simulate typical dose response curves for significant covariates. Finally, we also calculated the traditional C2 and C5 endpoints from our challenges to explore the differences between healthy controls and subjects with asthma, and the effects of the same continuous and categorical covariates. See the online repository for full details of the non-linear model and C2/C5 analyses.

3.4 Results

3.4.1 Subjects

Ninety-seven subjects with asthma and 47 healthy volunteers were recruited and completed all visits; see online repository Figure E1 for exclusions, withdrawals and missing data. Asthma patients and healthy volunteers were well matched for gender, body mass index (BMI) and smoking history, but asthmatic subjects were significantly younger and had slightly reduced lung volumes compared with healthy volunteers (Table 1). Asthmatic subjects had low cough frequencies but these were statistically higher than healthy volunteers.

Asthma subjects were well or partly controlled (Table 2). Almost 50% were steroid naïve and one third on a low dose of inhaled steroid. The majority were atopic based on ≥1 positive skin prick test to a common aero-allergen, and exhibited bronchial hyper-responsiveness to methacholine.

3.4.2 Application of pharmacodynamic model

The model parameters are described in Table 3, and Figure E2 shows a very good fit of the model to the observed raw capsaicin evoked cough data. The model fit was also accurate at the individual level (see Figure E3). The subject characteristics that significantly affected E_{max} and ED_{50} are also summarised in Table 3.

3.4.3 Asthma, gender, and atopic status significantly affect capsaicin cough responses

Asthmatic subjects (both atopic and non-atopic) had a higher E_{max} and lower ED_{50} compared with healthy volunteers (Tables 3, 4 and Figure 1a). Specifically, asthmatics had a 71% lower ED50 (p=0.0008) than healthy volunteers, with no difference in ED50 between atopic and non-atopic asthma subjects. Non-atopic asthmatics had a 46% higher Emax (p=0.003) compared with healthy volunteers but atopic asthmatics had a 21% lower Emax (p=0.04) than non-atopic asthmatics (Tables 3, 4 and Figure 1c). In addition, female gender increased Emax by 27% (p=0.006) and decreased ED50 by 65% (p=0.0001) (Tables 3, 4 and Figure 1b).

The interaction between these characteristics was simulated to create typical dose-response curves shown in Figure 2. Healthy male subjects had the lowest cough responses, whereas female non-atopic asthmatics had the highest cough responses to capsaicin.

3.4.4 Capsaicin cough responses are associated with cough frequency and asthma control

Higher 24 hour cough frequency (coughs/hr) was associated with increased E_{max} (p=0.006) (Figure 3a, Table 3). Specifically, for every unit that cough frequency increased, E_{max} increases by approximately 5%. Also, higher ACQ scores were associated with lower ED_{50} (p=0.02) (Figure 3b, Table 3). The median ACQ score in the studied asthmatic population was 0.71; if, for example, this score increased by one unit (1.71) ED_{50} decreased by 48%. Finally, higher IgE levels were associated with an increase in ED_{50} (p=0.01) (Figure 3c and Table 3). For every unit that IgE increased, ED_{50} increased approximately by 0.15%. The effect of these continuous covariates on the simulated dose response curves is illustrated in Figure 4.

Other co-variates such as age, BMI, % FEV1 predicted, the PC_{20}, FeNO, serum eosinophils and LCQ had no significant influence on model parameters (E_{max}, ED_{50}, γ). There was a non-significant trend (p=0.09) that asthmatics on steroids had a lower maximal number of coughs (E_{max}) compared with asthmatics not on steroids, but no differences were observed for ED_{50} or γ. In addition, the magnitude of steroid dose (in subjects on steroids) did not impact on the model parameters.

3.4.5 Termination of the cough challenge

At higher doses of capsaicin, increasing numbers of patients elected to terminate the challenge (Figures E2 and E4a). The most important determinant of whether an individual was likely to terminate the challenge at a given dose level was the total cumulative number of coughs, up to the maximum tolerated dose (see Figure E4b). When subjects reached an approximate threshold of 40 to 60 cumulative coughs, they tended to terminate no matter whether this threshold was reached at a low or high capsaicin dose (see Figure E4b).

3.4.6 Safety of full dose capsaicin challenge

Transient bronchoconstriction after inhaling capsaicin has been reported in patients with asthma (19). In this study there was no significant bronchoconstriction after inhaling high dose capsaicin; the median change in % FEV1 post capsaicin challenge was -1.7% (IQR 0.8 to - 4.3). However, one subject did drop their FEV1 by 54% and coughed a total of 38 times at a low concentration of capsaicin (15.6µmol/L). The subject received 4 inhalation of salbutamol (100mcg) after which the FEV1 improved to baseline.

3.4.7 Exploratory analysis of C2 and C5 endpoints

Subjects with asthma demonstrated a significantly lower C2 and C5 than healthy controls (p=0.002 and p=0.013 respective, see Table E1). However, there was substantial variability between individuals and overlap between the two groups for C2 and C5; 42% of healthy volunteers and 30% of subjects with asthma did not have a measureable C5 (see Figure E5). Furthermore, multiple linear regression models failed to show significant relationships between covariates which were identified as important in the non-linear model, only log ACQ was related to log C5 (see online repository for full details).

3.5 Discussion

This study is the first to show evidence of heightened capsaicin cough responses and thus neuronal dysfunction in stable, mild to moderate asthma. These changes in capsaicin responses can only be fully appreciated by extending cough challenge beyond the standard C5 endpoint and with the implementation of population pharmacodynamic modelling to provide individual estimates of ED_{50} and E_{max}. Using this methodology, we showed that compared with healthy volunteers, asthma patients started to cough at lower capsaicin doses (lower ED_{50}) and had greater maximal cough responses (higher E_{max}), both indicative of increased excitability of the neuronal pathways controlling cough. Notably, both gender and atopic status significantly influenced cough responses, with non-atopic female asthma patients exhibiting the greatest degree of neuronal dysfunction. Importantly, measures of inflammation such as FeNO, bronchial hyper-responsiveness (BHR, PC_{20}) or lung function did not influence E_{max} or ED_{50}, suggesting this neuronal dysfunction was independent of airway inflammation and bronchial hyper-responsiveness.

It is difficult to directly compare these results with other studies which have used the standard C5 endpoint, because the patient demographics are very different to those in our study. Doherty and colleagues compared C5 in a group of asthmatics and healthy volunteers and demonstrated an increased sensitivity to capsaicin (10). However, subjects in that study were older with more severe asthma (mean FEV1 % predicted (71%), all on inhaled steroids, 21% on an inhaled anti-cholinergic and 8% on theophylline), and only 43% were non-smokers. Fujimura and colleagues evaluated just 18 asthmatics with worse lung function (mean %FEV1 predicted

67%) yet found no difference in C5 from healthy controls (13). It was striking that we demonstrated highly statistically significant differences in capsaicin responses in a cohort of younger patients who were all non-smokers, with good lung function and almost half were steroid naïve. Our exploratory analysis extrapolating C2 and C5 from our challenge protocol, shows these endpoints are statistically different in asthma compared with healthy volunteers. However, unlike Emax and ED50, C2 and C5 did not relate to any of the clinical features of asthma apart from control; not even with cough frequency which might be expected. This suggests C2 and C5 are not only less powerful than Emax and ED50 but also do not represent the underlying mechanisms important in different asthma phenotypes.

Gender differences in evoked cough have not previously been specifically described in asthma but have been repeatedly shown in both healthy volunteers and chronic cough patients, with females demonstrating heightened responses compared with males (14,20). However, the observation that non-atopic asthma subjects have exaggerated responses (increased E_{max}) compared with atopic asthma is a novel finding that was unexpected and requires further exploration. Consistent with this, lower IgE was associated with reduced threshold for capsaicin evoked coughs (reduced ED_{50}). The combined effects of gender and atopy suggest that the highest cough responses and thus, the greatest degree of neuronal dysfunction, was exhibited by female, non-atopic asthmatics. By comparison, atopic male asthmatics displayed the lowest levels of dysfunction in subjects with asthma and healthy male subjects the lowest responses overall (Figure 2). Our findings could help explain the results of a cluster analysis of asthmatics, highlighting two discordant groups where symptoms did not match the degree of airway inflammation (21). Interestingly, excessive symptoms were observed in the predominantly female cluster with fewer atopics, whilst low symptoms were observed in the cluster who were predominantly male and atopic. Therefore we speculate that neuronal dysfunction could explain the discordance between such clinical phenotypes of asthma.

We also found capsaicin cough responses were relevant to the clinical manifestations of asthma. Poorer asthma control, as measured by ACQ scores, was associated with lower cough thresholds (ED_{50}), and higher 24hr objective cough frequencies associated with higher maximal cough responses (E_{max}). It is interesting to speculate that these differences in capsaicin cough responses may represent different mechanisms of neuronal dysfunction, either in the peripheral or central nervous system. Moreover these changes in cough reflex responses may also provide a surrogate for changes in other populations of airway nerves responsible for mediating symptoms that are less easily quantified such as chest tightness and breathlessness.

Nerve fibres have a maximum frequency of action potential firing, determined by the rate of membrane repolarisation (refractory period). However, the threshold for action potential generation can be lowered by changes in the membrane resting potential or ion channels at the nerve terminal e.g. increased expression, membrane insertion, and conformational changes (22). Such changes can be induced by a range of inflammatory mediators including cytokines, chemokines and growth factors. Aδ fibres can also become responsive to capsaicin following

airway exposure to allergen and cigarette smoke, with novel gene expression of TRPV1, known as phenotypic switching (23). This can potentially affect both the ED_{50} and E_{max}; the membrane depolarisation threshold can be reached more easily causing a left shift (lower ED_{50}) but in addition, the usual E_{max} ceiling can be exceeded by the recruitment of a newly capsaicin responsive nerve fibre sub-type. Whilst changes to the afferent fibres innervating the airways are the most plausible explanation for the exaggerated cough responses we have observed in asthma, modification of action potentials at the first synapse in the brainstem and in the cortical and sub-cortical pathways could also occur. Processes analogous to central sensitisation (24,25), and/or loss of descending neural inhibitory control mechanisms, as described in chronic pain states, have the potential to produce similar effects (26). However, these possible mechanisms are largely unexplored in asthma.

As asthma is generally considered to be a chronic inflammatory disease, with the role of airway innervation or even the contribution of neuro-immune interactions rarely investigated. Cytokines, chemokines, growth factors and lipids released by immune cells have all been shown to induce profound changes in the activity and sensitivity of peripheral nerve terminals in the somatosensory system and have the potential to explain the changes in neuronal function that we have observed (27, 28). In particular, growth factors e.g. nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) have the potential to induce long term qualitative changes to a range of stimuli to which nerves respond, and this has been demonstrated in an animal model of allergic asthma (23). However, the most heightened neuronal dysfunction was observed in non-atopic asthma and perhaps therefore subjects with 'low Th-2' disease. Therefore perhaps neuronal dysfunction has the potential to provide insights into mechanisms underlying this phenotype and suggest new treatment targets.

There are some limitations to this study. Firstly, the study population was young, mainly atopic, and predominantly steroid naïve. It is currently unclear how generalisable these findings are to other age groups with more severe disease. Secondly, apart from measuring serum eosinophils, IgE and FeNO, we did not make direct measures of airway inflammation and hence were not able to investigate whether these influenced capsaicin responses. Finally, we chose capsaicin as it is the most widely used cough challenge agent, and it is with this methodology that we developed the previous pharmacodynamic model (14). However, it remains to be seen whether other challenge agents, such as citric acid, provide different results and hence novel insights into cough mechanisms.

In conclusion, these data are consistent with the concept that neuronal dysfunction is a feature of asthma, even in mild stable disease. Assessing capsaicin evoked cough responses may therefore provide an additional tool in phenotyping asthma and identifying those in whom this mechanism may be most prominent. Although our data suggests neuronal dysfunction seems to be independent of indirect measures of airway inflammation, studies are required to directly assess the effects of airway inflammation on capsaicin evoked coughs. If neuronal dysfunction is truly independent of airway inflammation it is unlikely to be addressed by current therapies.

Hence, novel neuro-modulatory treatments may be a useful adjunct in treating asthma, and perhaps most effective in non-atopic patients.

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3.6 Tables

Table 1: Comparison of subjects with asthma and healthy volunteers.
Data quoted as median and interquartile range (IQR) and compared using the Mann Whitney U Test.

		Asthmatics	**Healthy Volunteers (HV)**	**P-value**
Participants (N)		97	47	
Age (Years)		23.0 (21.0-27.0)	38.0 (29.0-47.0)	<0.001
Gender (M:F)		39:58	17:30	0.64
BMI (kg/m^2)		24.1 (21.8-27.0)	25.0 (22.2-28.6)	0.25
Smoking History (Pack Years)		0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.34
FEV_1 (%Predicted)		95 (87.0-103.0)	103.0 (97.0-115.0)	<0.001
FVC (%Predicted)		102 (95-110)	106.0 (99.0-118.0)	0.02
Cough Frequency (c/h)	24hr	1.1 (0.5-2.4)	0.2 (0.0-0.9)	<0.001
	Day	1.6 (0.7-3.8)	0.2 (0.0-1.3)	<0.001
	Night	0.0 (0.0-0.4)	0.0 (0.0-0.1)	0.25

Table 2: Description of the key characteristics of patients with asthma.

Data quoted as median (IQR)

Characteristic		**All Asthmatics (n=97)**	**Male (n=39)**	**Female (n=58)**
Age		23.0 (21.0-27.0)	23.0 (21.0-25.0)	22.0 (20.0-27.5)
Age of Onset		7.0 (4.0-14.0)	7.0 (4.0-14.0)	7.5 (4.0-14.3)
Exacerbations/year		0(0-0)	0(0-0)	0(0-0)
ACQ Score		0.71 (0.43-1.00)	0.86 (0.50-1.21)	0.64 (0.43-1.00)
Smoker	No (%)	91.8	89.7	93.1
	Ex (%)	8.2	10.3	6.9
	Yes (%)	0	0	0
GINA Category	Well Controlled (%)	50.5	56.4	46.6
	Partly Controlled (%)	49.5	43.6	53.4
Steroid Naïve (%)		48.5	43.6	43.1
On ICS alone (%)		34.0	23.1	41.4
On ICS/LABA combination (%)		17.5	20.5	15.5
Daily ICS Dose (mcg FP equivalent)		200 (100-400)	200 (100-400)	200 (100-400)
Reversible volume (mls)		180 (75-275)	260 (160-420)	135 (48-230)
Proportion with significant reversibility ≥12% (%)		14.4	17.9	12.1
FeNO (ppb)		34 (21-75)	37 (23-91)	30 (21-54)
Methacholine PC_{20} mg/ml		0.94 (0.25-3.26)	1.62 (0.46-3.21)	0.86 (0.24-3.40)
Bronchial Hyper-reactivity (%) ≤8mg/ml		81.4	79.5	82.8
Peak Flow Variability (%)		5.4 (3.4-6.9)	5.7 (4.2-7.7)	5.2 (3.2-6.8)
Atopic* (%)		78.4	87.2	72.4
Serum eosinophils (x10^9/L)		0.21 (0.13-0.35)	0.25 (0.13-0.41)	0.21 (0.13-0.32)
Serum total IgE (ku/L)		200 (58-470)	210 (70.0-460)	175 (41-483)

* At least one positive skin prick test to common aero-allergen. See online repository for full list.

Table 3: Parameter estimates of the final population pharmacodynamic model.

$\theta_{1\text{-}5}$ describe the model parameters for a healthy male with the typical (median) population values for each model incorporated covariate. $\theta_{6\text{-}13}$ describe the influence of each covariate on the model parameters Emax and ED50 (see Eqs.E5, E6 in online repository). For example, the presence of asthma lowers ED50 by 71% (θ_6). $\eta_{1\text{-}3}$ refer to the inter-individual variability with regard to Emax, ED50 and γ (slope).

γ:Hill factor (slope); K: tachyphylaxis parameter; E_0: average cough response at baseline; RSE%: relative standard error %.

Model parameter	NONMEM estimate (RSE%)	Bootstrap estimate (95% CIs) [a]
Structural model		
θ_1: Emax	3.57 (10)	3.59 (2.93, 4.52)
θ_2: ED50	67.6 (33)	68.8 (35.0, 135.9)
θ_3: γ	2.11 (5)	2.13 (1.89, 2.55)
θ_4: E_0	0.063 (23)	0.062 (0.041, 0.091)
θ_5: K	0.142 (10)	0.143 (0.115, 0.173)
Covariate effects [b]		
θ_6: Asthma on ED50	-0.71 (13)	-0.704 (-0.842, -0.462)
θ_7: Female on ED50	-0.647 (15)	-0.654 (-0.807, -0.412)
θ_8: Asthma (non-atopic) on Emax	0.462 (37)	0.448 (0.156, 0.870)
θ_9: Female on Emax	0.269 (39)	0.250 (0.043, 0.494)
θ_{10}: Atopy on Emax	-0.209 (35)	-0.204 (-0.353, -0.033)
θ_{11}: Cough frequency on Emax	0.0482 (29)	0.0482 (0.0137, 0.0807)
θ_{12}: ACQ on ED50	-0.66 (39)	-0.69 (-1.28, -0.17)
θ_{13}: IgE on ED50	0.00145 (49)	0.0014 (0.0002, 0.0029)
Inter-individual variability (%CV)[c]		
η_1: Emax	40.1 (8)	38.0 (29.0, 47.2)
η_2: ED50	281.7 (10)	272.3 (172.9, 446.1)
η_3: γ	36.8 (13)	39.0 (23.8, 55.1)

[a] Estimates obtained from bootstrap with the final population model. The median of the bootstrap sample estimates together with the non-parametric 95% confidence intervals (CIs) are reported for each parameter.

[b] The increase in objective function (-2log-likelihood) after removing each covariate effect from the final model is listed below followed by the corresponding likelihood ratio test p-value in parenthesis. θ_6: 11.25 (p=0.0008); θ_7: 14.43 (p=0.0001); θ_8: 8.55 (p=0.003); θ_9: 7.47 (p=0.006); θ_{10}: 4.24 (p=0.04); θ_{11}: 7.40 (p=0.006); θ_{12}: 5.21 (p=0.02); θ_{13}: 6.58 (p=0.01)

[c] Coefficient of variation (% CV) is calculated as: $\sqrt{(e^{\omega^2}-1)} \cdot 100$

Table 4: Emax and ED50 values for healthy volunteers and asthmatics

Values below represent the E_{max} and ED_{50} values for a typical healthy and asthmatic male/female (i.e. with median values for all model-incorporated covariates).

	Healthy volunteer		**Asthmatic**			
			Atopic		Non-atopic	
	Male	Female	Male	Female	Male	Female
Emax	3.57	4.53	4.13	5.24	5.22	6.62
ED50	67.6	23.86	19.6	6.92	19.6	6.92

3.7 Figures:

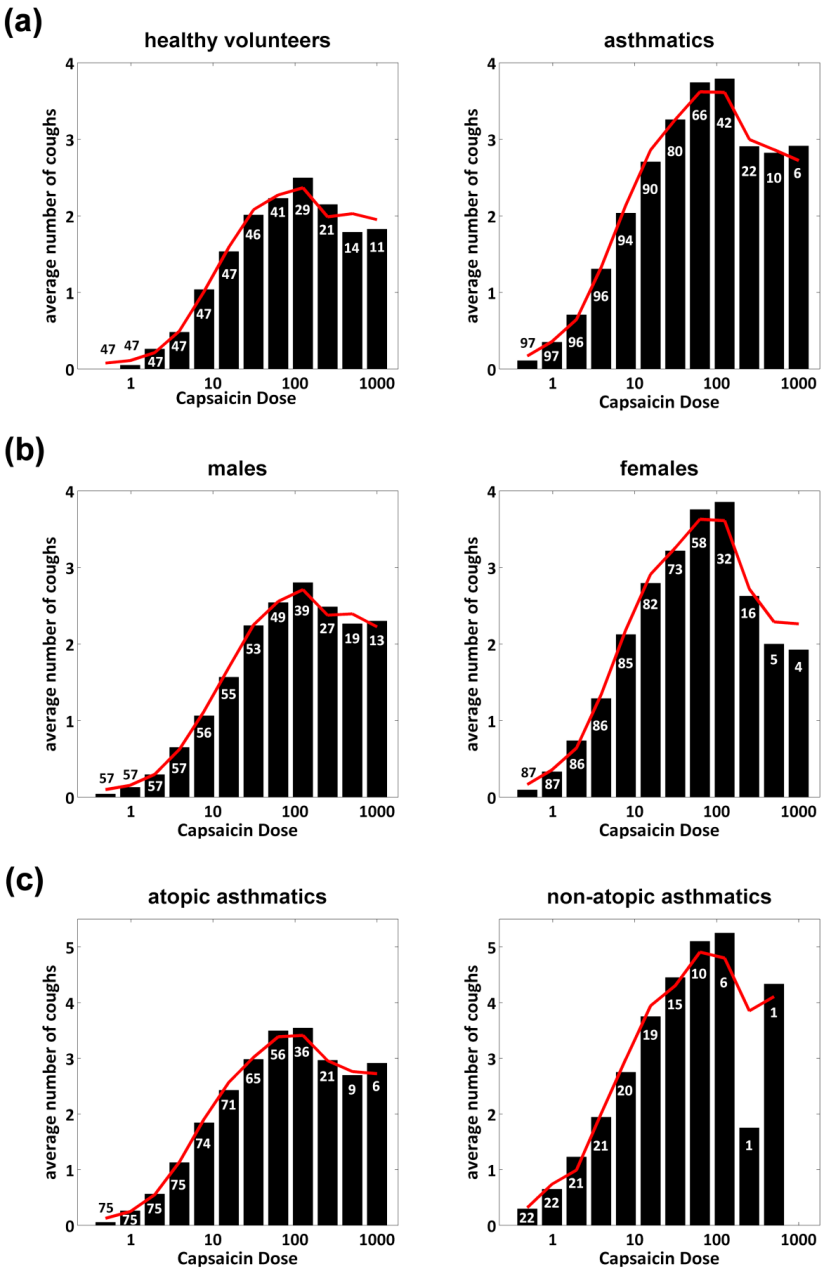

Figure 1: Model fit to the observed dose-response data stratified by significant categorical covariates; (a) healthy volunteers vs asthmatics, (b) males vs females, (c) atopic vs non-atopic asthmatics. The average number of coughs (y-axis) is plotted against the capsaicin dose (x-axis). Bars and red lines represent the observed and model-predicted respectively number of coughs averaged across all individuals in a specific covariate subpopulation and all inhalations at a given capsaicin dose level. The number of individuals in each subpopulation subjected to at least one inhalation at a given dose level are also reported inside (or above) each bar.

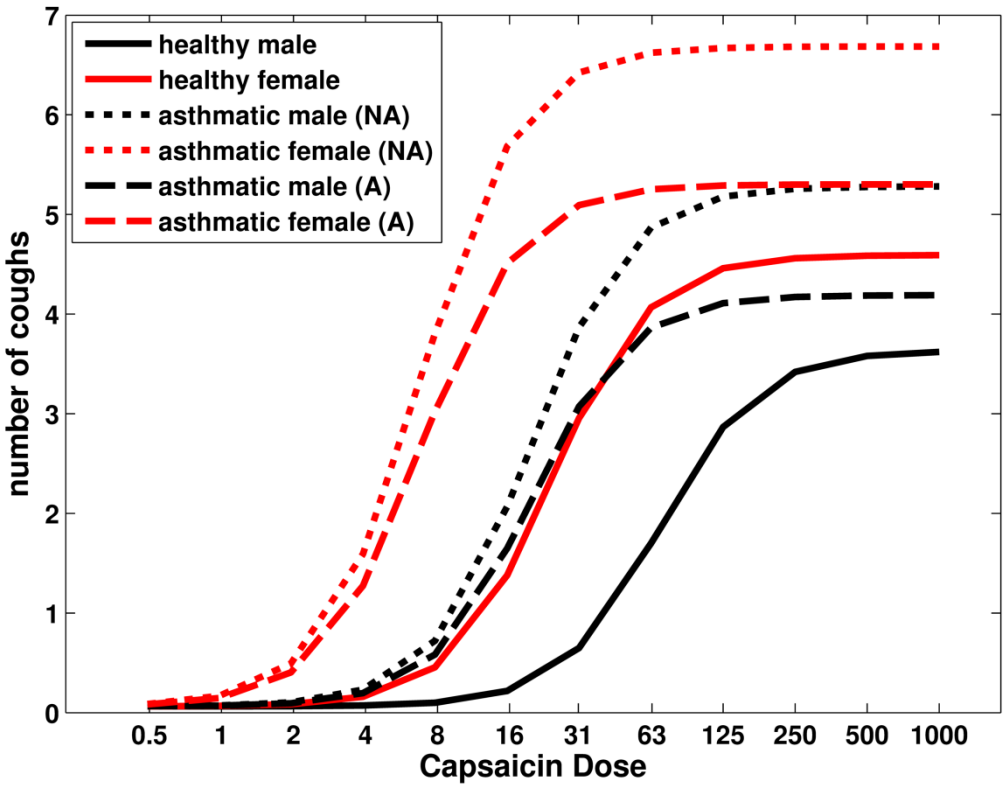

Figure 2: Model-simulated typical dose-response curves for individuals with different population characteristics. The typical (median) population values have been assumed for the model-incorporated continuous covariates; NA: non-atopic, A: atopic

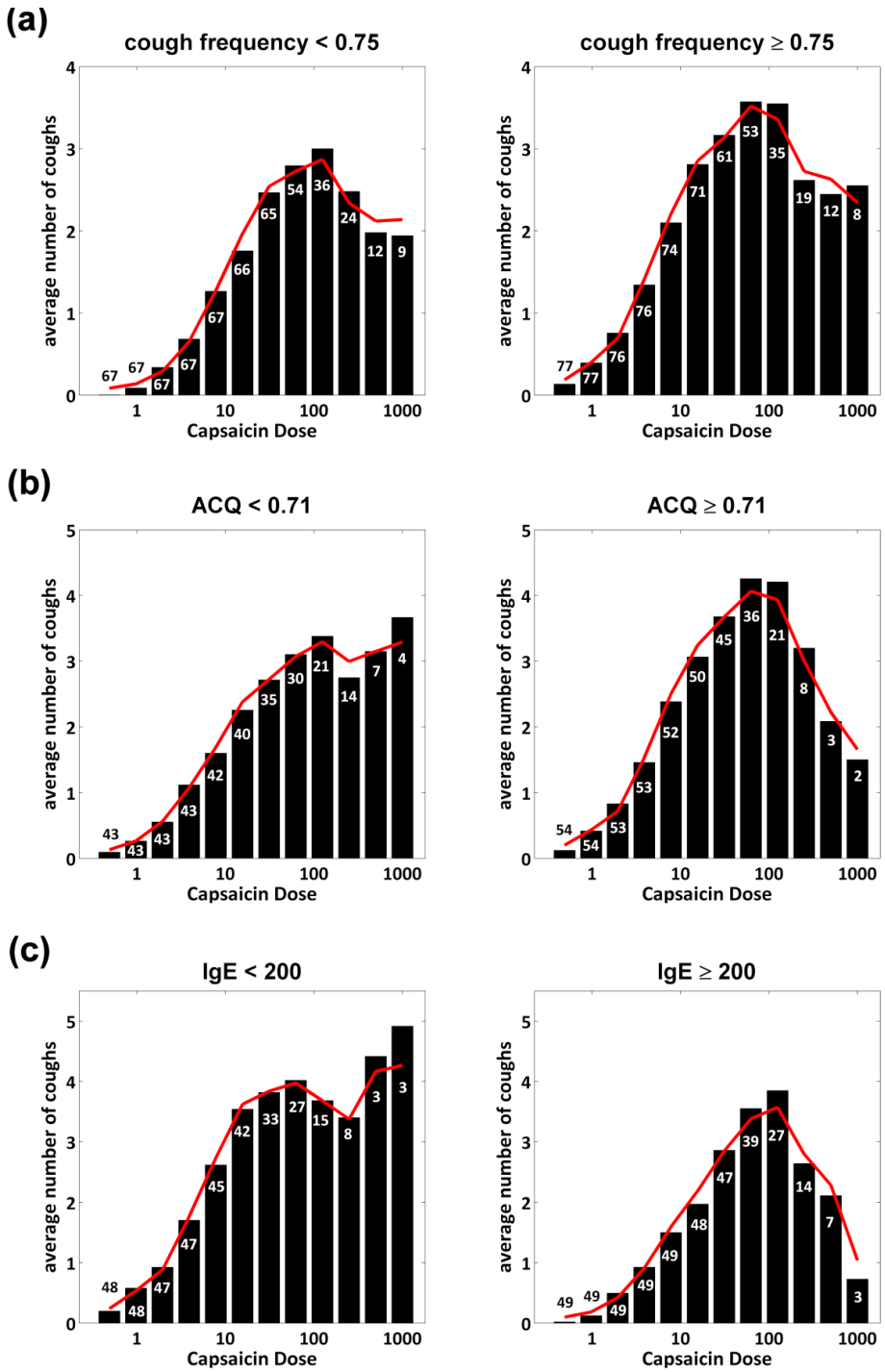

Figure 3: Model fit to the observed dose-response data stratified by significant continuous covariates. Subpopulations are stratified in relation to the median population value of each covariate; (a) low *vs* high cough frequency (in coughs/h), (b) low *vs* high ACQ score, (c) low *vs* high IgE levels (ku/L). The average number of coughs (y-axis) is plotted against the capsaicin dose (x-axis). Bars and red lines represent the observed and model-predicted respectively number of coughs averaged across all individuals in a specific covariate subpopulation and all inhalations at a given capsaicin dose level. The number of individuals in each subpopulation subjected to at least one inhalation at a given dose level are also reported inside (or above) each bar.

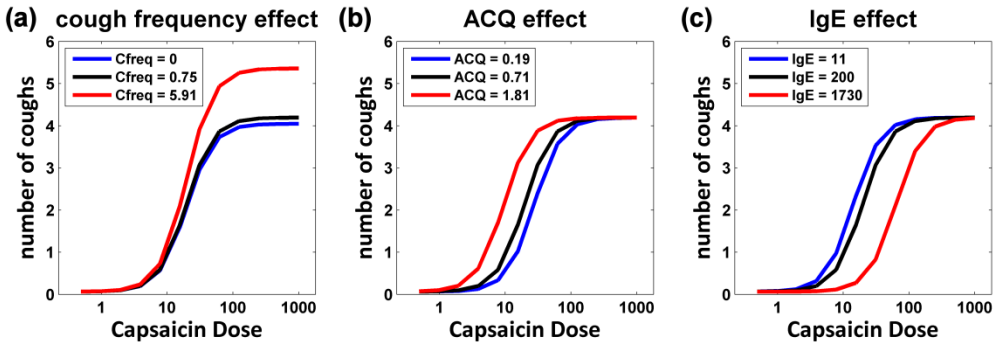

Figure 4: Effect of the model-incorporated continuous co-variates on the simulated dose-response curves; (a) cough frequency (Cfreq, coughs/h) (b) ACQ score, and (c) serum IgE levels (ku/L). Model simulated dose-response curves show the influence of continuous covariates at 3 incremental values (5th, 50th and 95th percentile in the analysed dataset) using an atopic asthmatic male as an example reference. The typical (median) population values have been assumed each time for the remaining continuous co-variates.

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3.9 Online Supplement

3.9.1 Methods:

Procedural Details

Fraction of Exhaled Nitric Oxide: Exhaled nitric oxide (eNO) was measured at a rate of 50ml/sec (NIOX, Aerocrine) prior to spirometry.

Spirometry: Spirometry was performed (In2itive, Vitalograph) according to standard American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines. Volume (ml) and percentage reversibility was assessed after administering 400mcg of salbutamol using a volumatic spacer device.

Questionnaires: Subjects completed the full Asthma Control Questionnaire (ACQ) and Leicester Cough Questionnaire (LCQ) described previously. Subjects were also classified on the basis of GINA categories; well controlled, partly controlled, not well controlled. The latter group were excluded from the study.

Cough Monitoring: Objective 24 hour cough monitoring was performed using the VitaloJAK cough recorder (Vitalojak; Vitalograph Ltd, Buckinghamshire, UK). Twenty-four hour ambulatory cough sound recordings were performed using a custom-built validated recording device and microphone. Briefly, this consists of a digital data logger recording sounds at a sample rate of 8 kHz, 16-bit resolution and in wav format, which is a commonly used uncompressed sound file format. Recordings were transferred to a personal computer; silences and background noise removed by validated, custom-written software (1); and cough sounds counted using an audio editing package (Audition version 3; Adobe Systems Inc., San Jose, CA) and the number of coughs expressed as coughs per hour (c/h).

Blood Sampling: Two samples of blood tests were taken from subjects with asthma for full blood count (to assess serum eosinophils) and total IgE.

Skin Prick Testing: Atopy was defined by the presence of at least one positive skin prick test (≥3mm) to commonly inhaled aeroallergens. The following were tested:

1. House Dust Mite
2. Mixed Moulds I: *Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Curvularia lunata, Fusarium moniliforme, Helminthosporium halodes*
3. Mixed Moulds II: *Aspergillus fumigatus, Mucor mucedo, Penicillium notatum, Pullularia pullulans, Rhizopus Nigricans, Serpula lacrymans*

4. Grass Mix: yorkshire fog/velvet grass, cocksfoot, rye grass, timothy, meadow grass/kentucky blue grass, tall fescue/meadow fescue

5. Tree Mix (Mid Blossoming): birch, beech, oak, plane

6. Cat

7. Dog

8. Histamine (positive control)

9. Saline (negative control)

Bronchial Hyper-responsiveness Testing: Methacholine challenge was performed to assess bronchial hyper-responsiveness using the 2-minute tidal breathing methodology according to standard ATS guidelines. Subjects were withdrawn off any inhaled medications as per ATS guidelines (2). The concentration causing a 20% drop from baseline FEV_1 in response methacholine (PC_{20}) was documented.

Capsaicin Cough Challenge: Full dose capsaicin evoked cough challenge was performed using the methodology described previously (3) using a nebuliser pot (Model 646; Devilbiss Healthcare LLC) and dosimeter (Koko Dosimeter; Ferraris Ltd, Hertford, United Kingdom). Two millilitres (ml) of Capsaicin solution at 1000 µmol/L (Stockport Pharmaceuticals, Stockport, UK) was serially diluted with 2 ml saline (0.9%) to create solutions of concentrations 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.95, 1.95, 0.98 and 0.48 µmol/L. Spirometry was performed and a cough monitor (VitaloJAK) attached to aid in the manual verification of capsaicin evoked coughs later on. To ensure accurate dosing was achieved, calibrated devilbiss 646 nebuliser pots were fitted with an inspiratory flow limiter and connected to a dosimeter (KoKo) at a pressure of 30 psi. This emitted between 10-12 µL per actuation. The full dose capsaicin evoked cough challenge involved administering four inhalations, thirty seconds apart, of doubling doses of capsaicin, starting from 0.48 to 1000 µmol/L. After each inhalation, the number of coughs in the first 15 seconds were recorded and later verified. The highest total number of coughs evoked at any dose of capsaicin is denoted E_{max} and the dose evoking half this response ED_{50}. To explore how these novel endpoints compare with traditional cough challenge endpoints, we also calculated the concentration of capsaicin evoking at least 2 and 5 coughs, i.e. the C2 and C5. Our challenge methodology is slightly different from the traditional challenge as four rather than one inhalation are performed at each concentration. Therefore, to calculate the C2 and C5 we simply used the number coughs evoked by the first of these four inhalations. If subjects did not cough 2 or 5 times during the whole challenge then for the purposes of analysis a value of 2000µmol/L was assigned. Spirometry was performed after the challenge and if there was more than a 10% drop in FEV1 compared to baseline or subjects complained of any chest symptoms then 4 puffs of salbutamol (100mcg) via a spacer was administered. The challenge ended when the patient reached the final concentration of capsaicin or reached the maximal tolerated dose.

3.9.2 Non-linear mixed effects modelling in detail

Population pharmacodynamic model

Model structure

Population pharmacodynamic modelling was performed using nonlinear mixed effects modelling software NONMEM version 7.3 (ICON Development Solutions) and the Laplace estimation method (4, 5). Goodness-of-fit plots, statistical analysis and simulations were performed in Matlab R2014a (The MathWorks, Inc.). In total 6606 observations (verified cough measurements) from 144 individuals were analysed for the development of the population dose-response model. The response variable (number of coughs) is discrete (count data) and was assumed to follow the Poisson distribution (6) (Eq.E1).

$$P(Y_i = n) = \frac{e^{-\lambda_i} \cdot {\lambda_i}^n}{n!} \qquad (\boldsymbol{Eq.E1})$$

, where $P(Y_i = n)$ is the probability that individual i is having n $(= 0,1,2, \dots)$ number of coughs per interval of time and λ_i is the individual mean count response. The individual mean count response (λ_i) is expressed as a function of capsaicin dose according to Eq.E2.

$$\lambda_i = E_0 + \frac{Emax_i \cdot D^{\gamma_i}}{{ED50_i}^{\gamma_i} + D^{\gamma_i}} \qquad (\boldsymbol{Eq.E2})$$

, where E_0 represents the mean cough count at baseline (placebo), $Emax_i$ is the maximum number of coughs in individual i, $ED50_i$ is the capsaicin dose that induces half of the maximum effect in individual i, D is the administered capsaicin dose and γ_i is the Hill factor that controls the steepness of the dose-response sigmoidal curve in individual i.

Inter-individual variability random effect terms were assigned on model parameters ($Emax$, $ED50$, γ) using an exponential relationship (Eq.E3).

$$Emax_i = \theta_{Emax} \cdot e^{\eta_{i_{Emax}}} \qquad (\boldsymbol{Eq.E3})$$

, where θ_{Emax} is the typical $Emax$ parameter value in the population and $\eta_{i_{Emax}}$ is the $Emax$ inter-individual variability random effect parameter which is assumed to be normally distributed with mean zero and variance, ω^2_{Emax}.

Tachyphylaxis effect

In a previous study (3) it was identified that the incorporation of a tachyphylaxis parameter substantially improved the description of the observed cough response data after serial capsaicin inhalations. Therefore in the current work, we carefully examined the data with regard to the occurrence of a tachyphylaxis pattern between consecutive inhalations of the same capsaicin dose. In the case that such a pattern was apparent we subsequently incorporated a tachyphylaxis parameter (K) in the model (similarly to (3)) according to Eq.E4 and investigated the extent that model fit was improved.

$$Emax_i(j+1) = Emax_i(j) \cdot e^{-K} \qquad (\boldsymbol{Eq.E4})$$

, where j =1,2,3; $Emax_i(j)$ and $Emax_i(j+1)$ correspond to the individual $Emax$ referring to the j^{th} and $j+1^{th}$ inhalation respectively of the same capsaicin dose; and K is a positive real

number. The equation above implies that tachyphylaxis (reduced response) occurs between consecutive inhalations of the same capsaicin dose.

Covariate model building

After the development of the base population pharmacodynamic model, a covariate analysis was performed in an effort to explain some of the observed inter-individual variability in cough responses. Both continuous and categorical covariates were investigated in the covariate model building procedure including: age, gender, body mass index, disease state (health or asthma), atopy (atopic or non-atopic), predicted FEV_1, cough frequency (Cfreq) from the monitoring of spontaneous cough, IgE levels, eosinophil levels, FeNO levels and ACQ, LCQ questionnaire scores. Empirical Bayes estimates of the inter-individual variability random effects (η_i) from the base model (without any covariates) were used for an initial screening of the covariates, given a low η -shrinkage (7). Subsequently covariate model building was performed with a stepwise forward inclusion – backwards deletion procedure where covariate selection is guided at each step by likelihood ratio tests between nested models (8, 9). A bootstrapping procedure (n=1,000) was performed with PsN 3.7.6 (Perl-speaks-NONMEM) (10) for the final model in order to evaluate the robustness of the parameter estimates and provide non-parametric 95% confidence intervals. A covariate effect was retained in the final model only if all the following conditions applied: i) the direction of the covariate effect was physiologically / mechanistically plausible based on our understanding of the underlying system and prior knowledge; ii) removal of the covariate caused the model to be inferior at the 0.05 statistical level (assessed by likelihood ratio test); iii) the bootstrap-obtained non-parametric 95% confidence interval for the covariate effect did not include zero.

Investigation of termination of the cough challenge

Due to the nature of the ascending-dose capsaicin challenge it is expected that a substantial number of participants will elect to terminate the challenge before reaching the maximum capsaicin dose. Possible reasons for terminating the challenge include discomfort due to excessive coughing or sensations of heat/stinging/burning in the throat at higher concentrations of capsaicin. Termination of the cough challenge results in missing data at higher capsaicin concentrations which may have implications for the modelling strategy / methodology. Therefore, an exploratory statistical and graphical analysis of the raw cough response data was performed to further understand the reasons for termination of the cough challenge.

Linear regression modelling of C2 and C5

In order to explore the utility of C2 and C5, we compared log base 10 transformed values in health and disease using an independent t-test. To see how these endpoints performed compared with Emax and ED50, we carried out linear regression modelling to test whether the features of asthma found to influence Emax and ED50 similarly influenced logC2 and logC5.

3.9.3 Results

Detailed Results of Modelling

Population pharmacodynamic model

The parameter estimation process and the covariance step for the final population pharmacodynamic model (including covariates) converged successfully under the Laplace estimation method and a requested precision of more than three significant digits in the parameter estimates. The parameter estimates of the final population model are reported in Table 3 (main manuscript), together with the bootstrap results and the 95% non-parametric confidence intervals around these estimates. All model parameters (including both fixed and random effects) were estimated with adequate precision (see Table 3 in main manuscript). The average cough response at baseline, referred as E_0, was estimated to be only 0.06 indicating that cough response is very rare when the capsaicin dose is zero.

Tachyphylaxis effect

A tachyphylaxis pattern was apparent after examination of the raw cough response data, as the magnitude of response (number of coughs) was decreased with consecutive inhalations of the same capsaicin dose. Similar to the tachyphylaxis pattern previously reported (3), this was apparent in all the capsaicin dose levels apart from the two low doses (0.48, 0.98μM) where cough responses were minimal and the highest dose (1000μM) where the sample size was small as many subjects had terminated the challenge before this dose. Incorporation of a tachyphylaxis parameter (K), substantially improved the model fit and model diagnostics and was retained in the final model. More specifically incorporation of this parameter in the model decreased the objective function value (-2log-likelihood) by 181 units. The tachyphylaxis parameter (K) was estimated to be 0.142 (see Table 3 in main manuscript), which practically means that the $Emax$ decreases approximately by 13% (calculated as $1 - e^{-K} = 0.13$) for any capsaicin inhalation preceded by another inhalation at the same dose level, in agreement with our previous work where using another population the decrease in $Emax$ due to tachyphylaxis was estimated to be around 15% (3). This replication provides additional confidence that the model adequately captures the true quantitative effect of the underlying tachyphylaxis physiological mechanism on the observed cough response.

Inter-individual variability random effect terms were assigned on the following structural model parameters: $Emax$, $ED50$ and γ. The estimates of these variability terms (see Table 3 in main manuscript) clearly indicated that the magnitude of population variability in $ED50$ was vast (CV (coefficient of variation) of 282%) and in particular, higher than the population variability in $Emax$ (CV of 40%). The magnitude of η -shrinkage in the final population model was 19%, 3% and 32% for the inter-individual variability terms of $Emax$, $ED50$ and γ respectively.
The observed raw dose-response data together with the model fit are illustrated in Figure E2, where it is apparent that the developed model provided a very good fit to the observed data.

The pattern of decreased average number of coughs observed in the last few high doses of capsaicin did not represent a true dose-response relationship, but was due to the subset of individuals that reached these high dose levels having substantially lower $Emax$ and higher $ED50$ values i.e. they had overall reduced cough responses to capsaicin.

The model fit to the observed dose-response data at the individual level is illustrated in Figure E3 for 16 representative subjects. It is clearly demonstrated that observed dose-response relationship is completely different between individuals. For example, some subjects had a substantial number of coughs relatively early in the ascending dose challenge (e.g. ID=6), whilst others had only a limited number of coughs even in the highest dose levels (e.g. ID=31, ID=300). However, it is apparent from Figure E3, that the developed mixed-effects population model is flexible enough to very accurately capture all these patterns of different dose-response relationships across different individuals.

Identification of covariate effects

The final population model included the influences of both categorical and continuous covariates: disease state (health or asthma), gender, atopy (atopic or non-atopic), cough frequency (Cfreq), ACQ questionnaire score and IgE levels. The inclusion of these covariates resulted in a substantial improvement of the model as they decreased the objective function (-2log-likelihood) by approximately 68 units compared to the objective function of the base model (model with no covariates). All the covariates retained in the final model offered additional and at least partly unique information, as removal of each of the covariates causes the model to be statistically inferior. The level of statistical evidence regarding each covariate (increase in objective function after removing each covariate from the final model and the corresponding likelihood ratio test p-value) is presented in the legend of Table 3 (main manuscript). In addition the bootstrap-obtained non-parametric 95% confidence intervals regarding all the covariate effects in the final model are reported in Table 3 (main manuscript) where it is apparent that they do not include zero. The equations that described the typical values of the model parameters including covariate effects are listed below:

$$Emax = \theta_1 \cdot (1 + GROU \cdot \theta_8) \cdot (1 + GEN \cdot \theta_9) \cdot (1 + GROU \cdot ATO \cdot \theta_{10}) \cdot e^{\theta_{11} \cdot (Cfreq - 0.75)} \quad (\boldsymbol{Eq.5})$$

$$ED50 = \theta_2 \cdot (1 + GROU \cdot \theta_6) \cdot (1 + GEN \cdot \theta_7) \cdot \left(1 + GROU \cdot \theta_{13} \cdot (IgE - 200)\right) \cdot e^{GROU \cdot \theta_{12} \cdot (ACQ - 0.71)} \quad (\boldsymbol{Eq.6})$$

, where $GROU$ is a dummy variable that takes the value 1 for asthmatics and 0 for healthy volunteers; GEN is a dummy variable that takes the value 1 for females and 0 for males; ATO is a dummy variable that takes the value 1 for atopic asthmatics and 0 for non-atopic asthmatics; $Cfreq$ is the spontaneous cough frequency (coughs/h) over 24hrs; IgE is the IgE levels measurement (ku/L); and ACQ is the ACQ questionnaire score.

For all continuous covariates in the model ($Cfreq$, IgE and ACQ), covariate effects were centred around the median population values in the analysed dataset (0.75, 200 and 0.71 respectively) to increase model stability and allow parameter interpretation with respect to a typical/reference individual. It should be noted that $Cfreq$ values were missing for 7 out of the 144 studied individuals, so these values were imputed with the population median. This imputation did not have an impact on the results, as when these individuals were excluded from the analysis all the parameter estimates (including all covariate effects) were comparable.

Figures 1 and 3 (main manuscript) illustrate the model fit to the observed dose-response data stratified by the significant categorical and continuous respectively model covariates. It is apparent from these figures that substantially different dose-response patterns were observed across the different covariate subpopulations (e.g. healthy volunteers vs asthma, males vs females etc.). However the developed covariate-incorporated population model very accurately described the observed dose-response relationships within each of these subpopulations.

Model-simulated typical dose-response curves for individuals with different population characteristics are presented in Figure 2 (main manuscript), to illustrate the effect of the model-incorporated categorical covariates on the dose-response relationship. Similarly, the effect of the model-incorporated continuous covariates on the simulated dose-response curves is illustrated in Figure 4 (main manuscript).

All the significant covariate effects (see Table 3 and Figures 1-4) are described in the main manuscript. It should be noted that although it was possible in the current work to explain part of the observed variability in cough response through the incorporation of several covariates, the extent of unexplained inter-individual variability remains substantial.

Investigation of termination of the cough challenge

An exploratory analysis of the raw cough response data with regard to termination of the challenge is illustrated in Figure E4. The number of individuals that performed at least one inhalation at a given dose level decreased as the capsaicin dose increased (Figure E4a). The most important determinant of termination of the challenge at a given dose level, was the total number of coughs in the entire challenge up to that specific dose. Figure E4b shows that individuals who terminated the challenge at a given dose level had substantially more coughs in the challenge up to this dose compared with individuals who continued to the next capsaicin dose level; bootstrap 95% confidence intervals (using bootci in MATLAB) indicated a statistically significant difference in the number of coughs for the majority of the capsaicin dose levels.

Figure E4b suggests that when individuals reach a threshold of approximately 40 to 60 coughs, they tend to terminate the challenge, irrespective of the dose of capsaicin at which this occurs.

Although the missing data mechanism (termination of the challenge) was not independent of the response values, it depends on them only through the observed components of the response (number of coughs up the point of drop-out). Therefore, valid estimation-based inferences could be obtained with the maximum likelihood mixed effects modelling approach, without the need to simultaneously develop a model for the missing data (11). The development of a drop-out model for the capsaicin cough challenge, although not necessary for the analysis of this data set represents a significant task. This is currently in progress as it will inform the design of future clinical studies and will allow the performance of clinical trial simulation.

3.9.4 Exploratory analysis of C2 and C5 endpoints

Comparison of healthy volunteers and asthmatics

As illustrated by Table E1, asthmatics demonstrated a significantly lower C2 and C5, i.e. were more sensitive to capsaicin, than healthy volunteers. However, as shown in Figure E5, there was substantial variability in these endpoints and overlap between health and disease. Moreover, many individuals in both groups did not achieve a measureable C5, particularly for the C5 endpoint (42% of healthy volunteers and 30% of asthmatics).

Predictors of C2 responses

Analysing healthy volunteers and asthma data combined in the simplest model, both gender ($p<0.001$) and disease group ($p<0.001$) significantly predicted log C2, explaining 17.5% of the variance; females were more sensitive to capsaicin than males and asthmatics more sensitive than healthy volunteers. However, when the predictors of capsaicin responses found to be significant in our non-linear modelling approach (disease group, gender, atopic status, log cough frequency, log total IgE, log ACQ) were introduced in the linear regression model, none significantly predicted logC2.

Predictors of C5 responses

Again in the simplest model, both gender ($p=0.002$) and disease group ($p=0.002$) significantly predicted log C5, explaining 11.2% of the variance. When the predictors of capsaicin responses found to be significant in our non-linear modelling approach were introduced in the linear regression model, only log ACQ significantly predicted logC5 (Beta=-0.8, $p=0.012$), i.e. worse asthma control was associated with a lower C5.

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3.11 Supplementary Tables

Table E1: Comparison of C2 and C5 in patients with asthma and healthy volunteers

	Group	Capsaicin µmol/L Geometric Mean, (95% CI)	p-value
C2	Healthy Volunteers (n=47)	22.6 (12.1-42.1)	0.002
	Asthmatics (n=97)	7.3 (5.4-9.8)	
C5	Healthy Volunteers (n=47)	209.5 (108.5-404.5)	0.013
	Asthmatics (n=97)	78.2 (48.8-125.1)	

3.12 Supplementary Figures

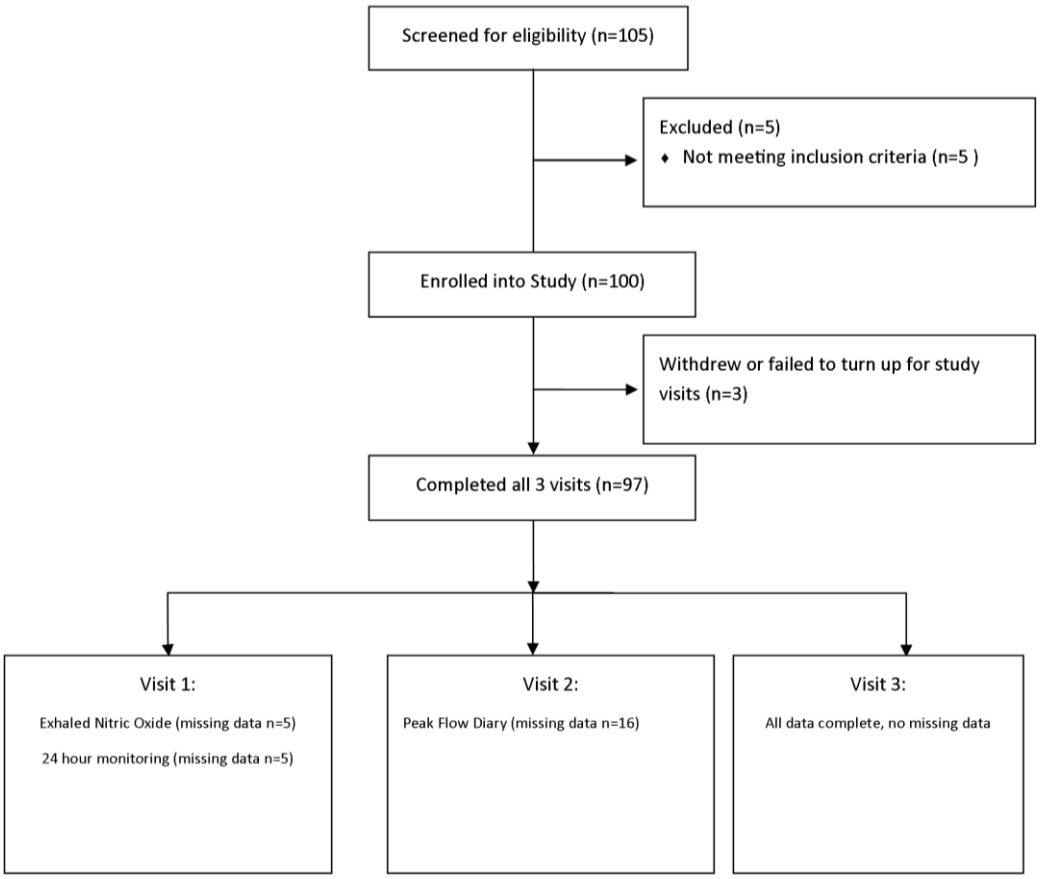

Figure E1: Patient flow diagram illustrating number of patients screened, withdrawals and missing data.

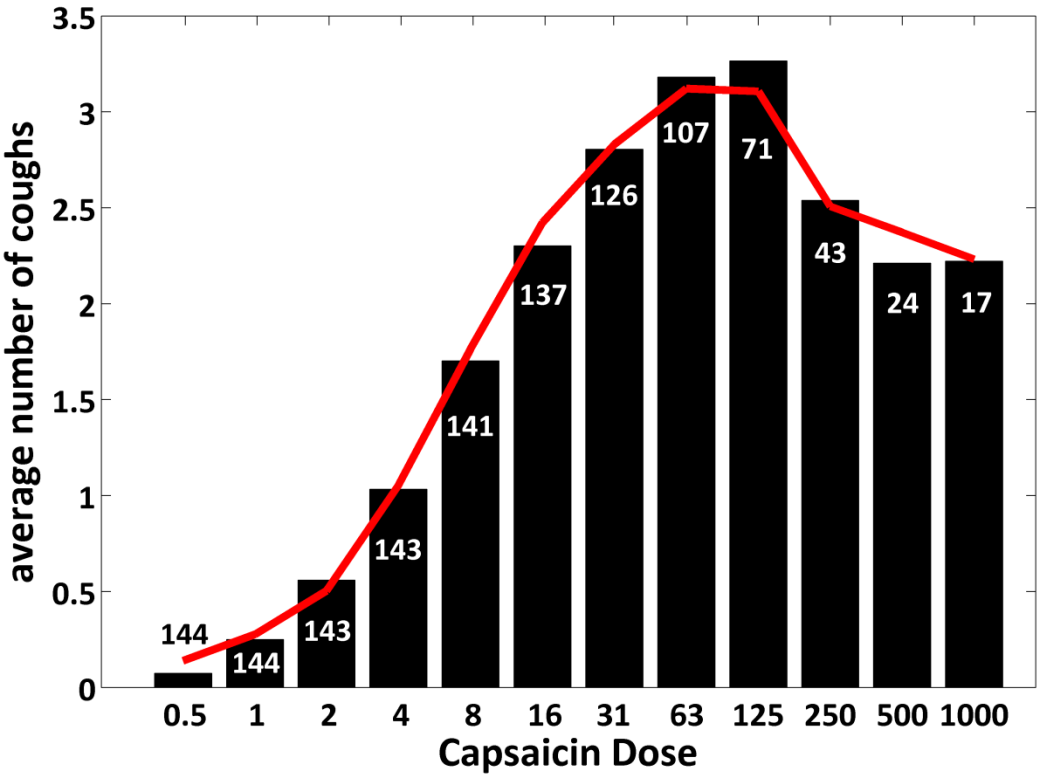

Figure E2: Model fit to the observed dose-response data. The average number of coughs (y-axis) is plotted against the capsaicin dose (x-axis). Bars and red line represent the observed and model-predicted respectively number of coughs averaged across all individuals and inhalations at a given capsaicin dose level. The number of individuals subjected to at least one inhalation at a given dose level are also reported inside (or above) each bar.

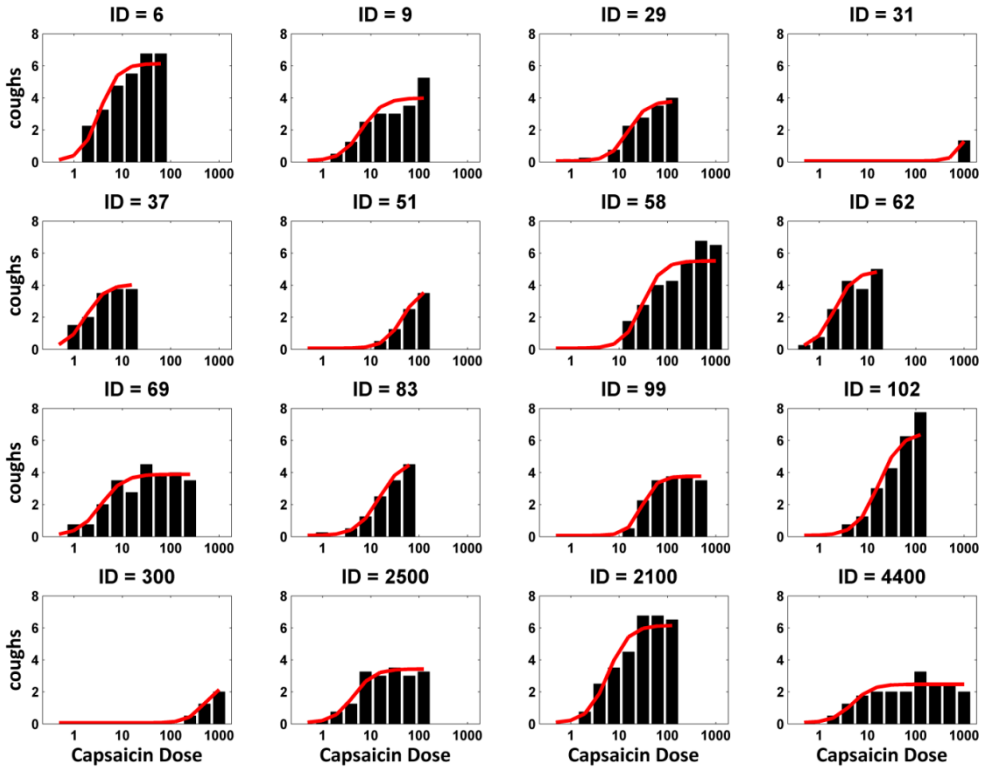

Figure E3: Model fit to the observed dose-response data at the individual level. Observed data (bars) and individual model predictions (red lines) of 16 representative of the population subjects are presented. The number of coughs (y-axis) averaged across all inhalations at a given capsaicin dose level for a given individual is plotted against the capsaicin dose (x-axis).

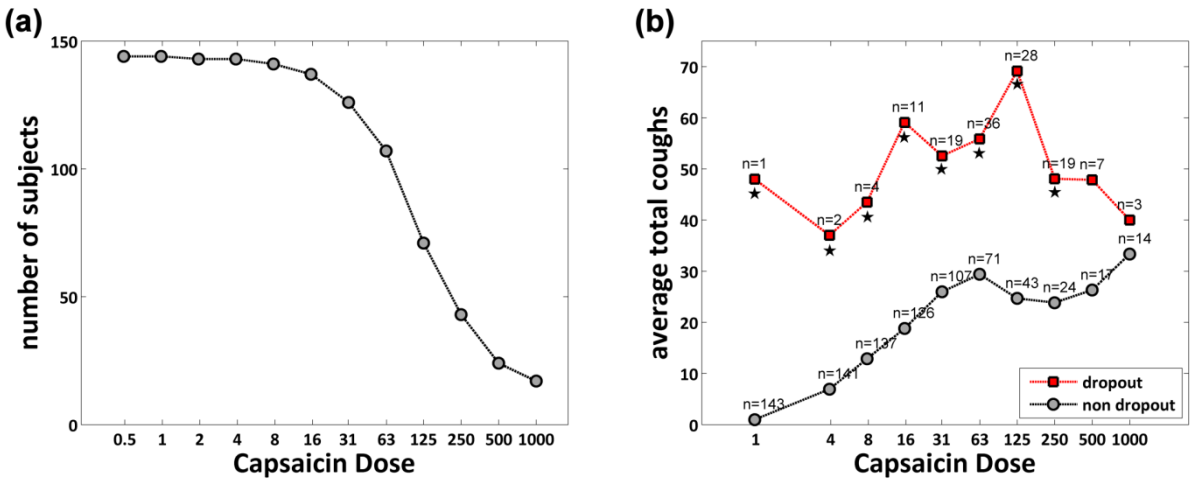

Figure E4: Investigation of the challenge termination pattern in the raw cough response data. (a): The number of individuals (y-axis) performing at least one inhalation at a given dose level is plotted against the capsaicin dose (x-axis); (b): average number of total coughs in the challenge (y-axis) up to a given capsaicin dose (x-axis) for individuals that do or do not terminate the challenge after this specific dose. For example the 4 individuals that dropped-out after inhaling the 7.81 µmol/L dose (3rd marker from the left) had on average 43 coughs up to this point of the challenge; the 137 individuals that did not drop-out and continued to the higher dose level had on average only 13 coughs up to this point of the challenge. Markers highlighted with a star indicate a statistically significant difference in total coughs between the drop-out and the non-drop-out groups at the given dose level.

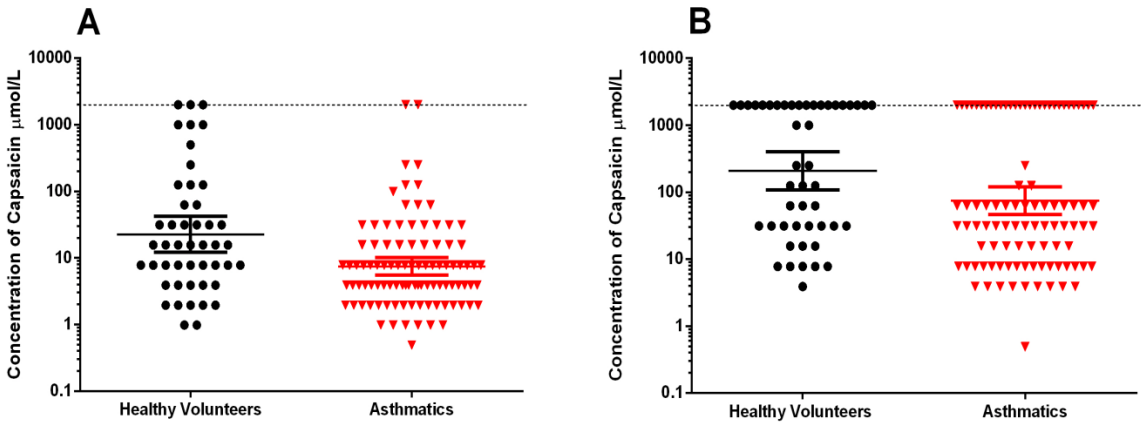

Figure E5: Comparison of traditional capsaicin challenge endpoints C2 (A) and C5 (B). Note logarithmic scale (base 10) of y axis and error bars show geometric mean and 95% confidence intervals. Dashed reference lines at 2000 µmol/L capsaicin represent value assigned to those subjects who did not achieve C2 or C5

4 The Interaction between Bronchoconstriction and Cough in Asthma

The Interaction between Bronchoconstriction and Cough in Asthma

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Clinical Trial Registration: ISRCTN14900082

Total Word Count (exc abstract): 2286

4.1 Abstract:

Background: Cough is an airway neuronal reflex and a common troublesome symptom in asthma, but it is unclear if there is an interaction between bronchoconstriction and cough reflex responses.

Objective: We sought to investigate the interaction between acute bronchoconstriction and cough in mild atopic asthma.

Methods: Methacholine was used to induce bronchoconstriction and capsaicin to evoke cough. Full dose capsaicin cough challenge and methacholine challenge were performed on all subjects on separate occasions. Subjects then underwent three interventions in a randomised, single-blinded, placebo controlled manner to assess: i) the effect of induced bronchoconstriction on evoked coughs, ii) the effect of evoked coughs on induced bronchoconstriction, iii) the change in evoked coughing during spontaneous resolution of induced bronchoconstriction. Generalised estimating equations were used to model the interaction between bronchoconstriction and capsaicin evoked coughs.

Results: Fourteen subjects were enrolled [median (IQR) age 23 (21.0-28.5) yrs, 64% female]. Compared to placebo, methacholine induced bronchoconstriction was associated with a 34.2% increase in capsaicin evoked coughs (geometric mean 8.4 coughs (95% CI 6.6-10.7) vs. 13.9 coughs (10.9-17.8) $p<0.001$). There was no difference in methacholine induced bronchoconstriction following capsaicin evoked coughing, compared with placebo (mean 13.7% (95% CI 8.7-18.7) vs. 12.3% (8.9-15.7), $p=0.49$). Spontaneous resolution of methacholine induced bronchoconstriction over 60 mins was associated with a significant reduction in capsaicin evoked coughing ($p<0.001$).

Conclusion: Bronchoconstriction heightens cough responses to capsaicin in subjects with asthma.

Clinical Trials Registration: ISRCTN14900082

Key question?

What is the interaction between bronchoconstriction and cough in patients with mild asthma?

What is the bottom Line?

Acute bronchoconstriction was associated with increased capsaicin evoked coughing in patients with mild stable asthma suggesting neuronal sensitisation. However, capsaicin evoked coughing had no impact on methacholine induced bronchoconstriction.

Why read on?

This mechanistic study provides evidence that induced bronchoconstriction significantly increases coughing in response to inhaled capsaicin implying a sensitisation of airway nerves.

4.2 Introduction

The pathophysiological hallmarks of asthma are the presence of airway inflammation, bronchial hyper-responsiveness (BHR) and variable airflow obstruction, which manifest in patients with symptoms of wheeze, cough, shortness of breath and chest tightness. Current dogma assumes that the pathophysiology directly causes symptoms; however, the mechanisms linking the pathophysiology and the development of symptoms are poorly understood.

Cough is considered to be the archetypal airway neuronal reflex, whilst wheeze mainly occurs as a result of airway-obstruction occurring as a direct response to local mediator release e.g. histamine, or by reflex activation of parasympathetic fibres releasing acetylcholine. The interaction and relationships between airway smooth muscle and nerve function is unclear, but our previous work demonstrated that cough responses to capsaicin were not correlated with BHR to methacholine and forced expiratory volume in 1 second FEV1 (1, 2). This is consistent with previous uncontrolled cross-sectional studies which found only 14-17% of subjects with asthma spontaneously coughed after methacholine induced bronchoconstriction (3, 4), and another study where healthy volunteers coughed more than subjects with asthma up to 30 mins after a methacholine challenge (5). However, none of these studies directly assessed the acute effects of bronchoconstriction on cough reflex sensitivity, nor did they objectively record cough frequency.

The effects of bronchoconstriction on cough responses evoked by capsaicin inhalation have been studied in healthy volunteers using methacholine and 0.9% saline. However, only minor bronchoconstriction was achieved (mean fall in FEV1 of 8.8%) and this resulted in no changes in the capsaicin concentration provoking two coughs (C2) (6). As such, there is a paucity of human data which has investigated the effects of smooth muscle contraction on the cough reflex in individuals with asthma.

In this study, we aimed to directly investigate the interaction between acute bronchoconstriction and cough in subjects with mild atopic asthma. We used specific agonists to achieve bronchoconstriction (methacholine via muscarinic receptors) and cough (capsaicin via transient receptor potential vanilloid type-1 (TRPV1) receptors). We hypothesised that neuronal function and smooth muscle function are independent in asthma. Hence, we predicted that capsaicin evoked coughing would not be influenced by changes in airway calibre and vice-versa.

4.3 Methods:

4.3.1 Participants:

Participants with mild atopic asthma were recruited, but not selected for symptoms of cough. All subjects had evidence of bronchial hyper-responsiveness to methacholine (PC20<8mg/ml) and at least one positive skin prick test to an inhaled aeroallergen. Treatment with salbutamol as required, and/or inhaled corticosteroid (ICS) ≤250mcg of fluticasone propionate equivalent daily were permitted. Subjects uncontrolled according to Global Initiative for Asthma (GINA) classification or not on stable medication ≥4 weeks were excluded. We also excluded current smokers, those with a recent exacerbation, and use of medication which may alter cough responses (e.g. opiates, gabapentin, anti-cholinergics, and theophylline). The protocol was approved by the local research ethics committee (15/NW/0052) and all subjects provided written informed consent.

4.3.2 Study Protocol and Procedures:

Subjects were initially invited to attend on six occasions, and then on a further two occasions following a protocol amendment (Figure 1).

On visit 1, subjects underwent history and examination, spirometry, and a capsaicin cough challenge, as previously described (7). Briefly, four inhalations were administered, thirty seconds apart, of doubling concentrations of capsaicin (12 concentrations, 0.48-1000µmol/L); after each inhalation, the numbers of coughs in the first 15 seconds were counted. The challenge was completed when the patient reached the final dose or the maximal tolerated dose. The maximum evoked coughs were denoted E_{max} and the dose evoking half this response ED_{50}. Individual ED_{50} doses of capsaicin were used to evoke cough in subsequent visits.
At Visit 2, ≥48 hours later, subjects underwent a methacholine challenge (2 minute tidal breathing) (8) to evaluate PC_{20}; the concentration preceding this was used to initiate bronchoconstriction in future visits. Subjects with no evidence of BHR (PC20>8mg/ml) were excluded.

Participants then entered a three-period, single-blinded, randomised, crossover study with ≥48 hours between visits (Figure 1). At visits 3/4 (period 1), the effect of bronchoconstriction on capsaicin cough responses was assessed. Subjects were randomised to inhale methacholine to achieve a fall in %FEV1 between 15-25% (starting concentration determined at visit 2, next doubling concentration inhaled if required), or placebo (saline) for two minutes followed immediately by ED_{50} capsaicin (4 inhalations, 30s apart). At visits 5/6 (period 2), the effect of cough reflex activation on bronchial hyper-responsiveness was assessed. Subjects were randomised to either ED_{50} capsaicin or placebo (saline), followed by a 2-minute inhalation of the methacholine concentration selected at visit 2.

At visits 7/8 (period 3), we assessed the effect of spontaneous recovery of bronchoconstriction on evoked cough responses. Visits were identical to visits 3/4, except after inhaling methacholine or placebo, subjects received ED_{50} capsaicin immediately and again at 30 and 60mins thereafter.

Spirometry was performed before and after all challenges and participants were discharged when FEV1 had returned to ≥90% of baseline. A cough monitor (VitaloJAK™; Vitalograph, Buckinghamshire, UK) was worn throughout visits to record coughing.

4.3.3 Statistical Analysis:

Generalised estimating equations (GEE) were used to model the effect of bronchoconstriction on capsaicin evoked coughs (periods 1 and 3), and the effect of capsaicin evoked cough on FEV1 (period 2); reported as estimated means and 95% confidence intervals (SPSS Version 22.0, IBM Corp., NY). Exploratory analyses also compared the effects of methacholine and saline inhalation on spontaneous coughing during visits. Cough data was log transformed for analysis to normalise distribution.

4.4 Results:

4.4.1 Subjects

Two subjects did not meet BHR criteria (PC20>16mg/ml), but fourteen subjects completed the first six visits. Ten of those subjects (four males) returned to complete the additional two visits (visits 7 and 8). Table 1 shows the demographics and key baseline data of the subjects enrolled.

4.4.2 Induced bronchoconstriction increased capsaicin evoked coughing

The mean % fall in FEV1 following methacholine was 19.1% (95% CI; 17.3 to 21.0) and after saline was 1.3% (1.0 to 3.5). Bronchoconstriction was associated with an increase in capsaicin evoked coughs (geometric mean 8.4 coughs (95%CI 6.6-10.7) vs. 13.9 coughs (10.9-17.8), 34.2% increase, $p<0.001$) (Figure 2). The analysis was adjusted for baseline ED50 coughs and there was no significant effect of gender and sequence.

4.4.3 No change in bronchial hyper-responsiveness following capsaicin evoked coughing

Four inhalations of one dose of capsaicin at ED50 evoked a mean cough count of 8.9 (95% CI; 6.8-10.9) whilst saline evoked 0 coughs (0.0-0.0). There was no difference in the mean fall in %FEV1 with methacholine after capsaicin or saline (12.3% (95% CI 8.9-15.7) vs. 13.7% (8.7-18.7) of baseline respectively, $p=0.49$) (Figure 3).

4.4.4 Capsaicin evoked coughs during spontaneous resolution of bronchoconstriction

Compared with placebo inhalation, there was a significant drop in %FEV1 from baseline with methacholine, which spontaneously resolved over 60 mins ($p<0.001$, Figure 3). Spontaneous resolution of FEV1 was associated with a significant reduction in coughs after inhaling the ED_{50} dose of capsaicin (beta=-0.24, $p<0.001$, Figure 4). Therefore, each 10% improvement in FEV1 equated to a reduction of 2.4 coughs evoked by capsaicin.

4.4.5 Exploratory analysis of spontaneous coughing

A post-hoc exploratory analysis of the cough monitor data showed that in the time periods when subjects were not inhaling capsaicin, asthmatics coughed more on the visits when methacholine was inhaled compared with placebo (mean coughs 1.59 (95% CI 1.2-2.0) vs. 0.73 (0.3-1.1) per visit, $p<0.001$). Each visit was divided into five time periods to compare differences in spontaneous coughs; pre-challenge spirometry, after methacholine/placebo challenge, post-challenge spirometry, post-salbutamol (SABA) recovery and after the final spirometry. Compared with placebo inhalation, significant differences in spontaneous coughs were seen immediately after methacholine inhalation, and after performing spirometry subsequently (both $p=0.001$, Figure 5).

We compared also spontaneous coughing in visits 7 and 8 by dividing the 60-minute recovery period after methacholine/placebo inhalation and the capsaicin challenges into two time periods; 0-30 and 30-60 mins. There was a significant increase in spontaneous coughs 0-30 mins after methacholine challenge compared with placebo ($p=0.05$, Figure 6).

4.4.6 Safety of inhaled capsaicin

Inhaling ED50 dose capsaicin did not cause any further bronchoconstriction after methacholine induced bronchoconstriction. Instead, FEV1 tended to increase (mean fall in %FEV1 from baseline before capsaicin was 19.1% (S.D. 3.6), and after, 15.9% (5.5).

4.5 Discussion

To our knowledge this is the first study that has investigated the interaction between acute bronchoconstriction and cough in asthma. We have demonstrated that methacholine induced bronchoconstriction causes a significant increase in capsaicin evoked cough responses, which gradually resolves as airway calibre returns to baseline. In contrast capsaicin evoked coughing has no influence on bronchial hyper-responsiveness. Our data therefore challenges the idea that cough reflex responses and bronchoconstriction are completely independent in asthma. An exploratory analysis of spontaneous cough frequency also demonstrated that bronchoconstriction was associated with increased coughing.

Our data would suggest that subjects with asthma might behave differently to healthy volunteers, as two controlled studies in healthy participants found bronchoconstriction induced by methacholine did not alter capsaicin sensitivity measured by C2 (6) or C5 (9). However, there are fundamental differences between our study and those performed in healthy controls. In the first study, cough responses were not affected by either bronchoconstriction with methacholine or bronchodilation with salbutamol, however the induced changes in FEV1 were all small, less than 10% of baseline. In addition, both studies relied on the conventional C2 and C5 endpoints, which we have recently shown may not be optimal (7) especially in subjects with asthma (1). The relationship between bronchoconstriction and cough has also previously been explored to some extent by inhalational of hypertonic saline, hypertonic histamine and mannitol, which evoke both cough and bronchoconstriction but with different endpoints and non-conventional challenge protocols (10-12). During hypertonic challenges asthmatics cumulatively coughed more, but these hyperosmolar challenge agents may have acted via multiple ion channels on nerves and smooth muscle and hence it is difficult to draw conclusions about the interactions between airway nerves and smooth muscle from those studies.

There are a number of potential explanations for the effects of bronchoconstriction on cough responses found in this study. Firstly the mechanical effect of bronchoconstriction on the epithelium, smooth muscle and other structural cells may result in the release of mediators capable of sensitising airway nerves and hence increasing cough responses to capsaicin. For example, preclinical evidence supports the release of ATP following airway constriction with subsequent airway nerve activation, blocked by a specific antagonist (P2X2/3 receptors) (13). Furthermore TGF-β, a mediator typically associated with mechanical stress, has also been shown to sensitise nerve terminals via ATP release (14, 15). Secondly, mechano-sensitive afferent nerves in the airways are tonically active during tidal breathing and provide feedback to the central nervous system about changes in lung pressures/volumes. These same fibres are activated by bronchoconstriction, and converge with those evoking cough in the brainstem, providing the opportunity for cough responses to be modified by bronchoconstriction (16, 17). Finally, bronchoconstriction alters the deposition of inhaled particles within the airways [294]. During bronchoconstriction capsaicin may have been deposited in areas more densely innervated with cough fibres. Such effects could similarly apply to real world irritant exposures,

however as capsaicin has a very short-lived effect, the increase in spontaneous cough frequency during bronchoconstriction would argue against this explanation.

We have previously found in patients with mild/moderate stable asthma that capsaicin evoked cough responses were heightened compared with healthy controls, but this was not related to the degree of airflow obstruction (1). However, in this current study we have demonstrated that dynamically changing FEV1 in an individual subject with asthma does heighten cough sensitivity, which then recovers as FEV1 returns to baseline. Hence, patients with stable asthma have a greater tendency to cough than healthy volunteers, but a dynamic drop in FEV1 increases the propensity to cough further. Therefore, we speculate that variations in airflow obstruction, which are a fundamental feature of asthma, may be accompanied by a similar variability in airway neuronal sensitivity, superimposed on elevated baseline neuronal excitability previously demonstrated in stable disease. Bronchodilator treatments might be expected to counteract the sensitising effects of bronchoconstriction, but not abolish the baseline neuronal excitability. Indeed, cough in stable asthma patients suggest that despite adequate treatment, capsaicin cough responses and spontaneous cough frequency are increased compared with healthy controls (1). It also remains to be determined whether sensory nerves and neuronal pathways mediating other symptoms such as shortness of breath and chest tightness are similarly sensitised. Therefore, this study may provide much broader mechanistic insights describing the link between intermittent episodes of bronchoconstriction typical of asthma and heightened symptoms.

An important unique feature of this study was the use of an individualised ED50 capsaicin dose serially in order to compare differences in coughing. Although single doses of tussive agents are commonly used in animal studies, this is the first study to use this particular method in humans to assess cough. The main advantages of performing a single ED50 challenge is the ability to very quickly and safely assess in two minutes whether cough responses increase or decrease as airway calibre changes with minimal exposure to capsaicin. The choice of individualised ED50 rather than an arbitrary single dose ensures changes in cough responses are readily detected; this stimulus level is on the steepest portion of the dose response curve. Hence, this can be a useful tool particularly in mechanistic or interventional studies where there are time restraints due to rapid changes in physiology.

There are a number of limitations in this study. Firstly, we have shown changes in coughs induced by capsaicin, which is a very specific TRPV1 agonist. It is possible that other tussive challenge agents may show different results and further insights into changes in nerve function. Secondly, we recruited young mild atopic subjects with early onset asthma, and it is unclear whether similar results would be achieved in other asthma phenotypes, and moderate/severe disease. Thirdly, we deliberately did not measure any markers of airway inflammation in sputum or exhaled breath because performing these tests may have interfered with inhaling methacholine and capsaicin immediately after each other.

In conclusion, this study provides important mechanistic insights about how airway pathophysiology in asthma relates to the development of symptoms through neuronal activation. These data suggest that bronchoconstriction increases the activation of capsaicin-responsive airway nerves but the precise mechanisms and mediators involved require further evaluation.

ACKNOWLEDGMENTS

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4.7 Tables:

Table 1: Demographics and baseline data

Characteristic		**Asthma Participants (n=14)**
Age, years		23.0 (21.0-28.5)
Gender, % Female		64.3
BMI, kg/m^2*		23.8 (21.7-25.1)
Smoker, %	Never	100.0
Treatment, %	Salbutamol PRN only	50.0
	ICS	50.0
ICS Dose, µg FP**		200 (50.0-200.0)
Asthma age of onset, Years		7.5 (5.0-14.3)
FEV_1, L		3.2 (2.8-3.8)
FEV_1, % predicted		95.9 (85.9-106.4)
E_{max} (coughs, n)		17 (13.8-20.0)
ED_{50} (capsaicin, µM)		15.6 (6.8-15.60)
Methacholine PC_{20} (mg/ml)		0.95 (0.4-3.1)

Data quoted as median (Interquartile range), * Body Mass Index **fluticasone propionate equivalent dose in those treated with ICS. There were no significant differences in the 10 patients who returned for visits 7 and 8.

4.8 Figures:

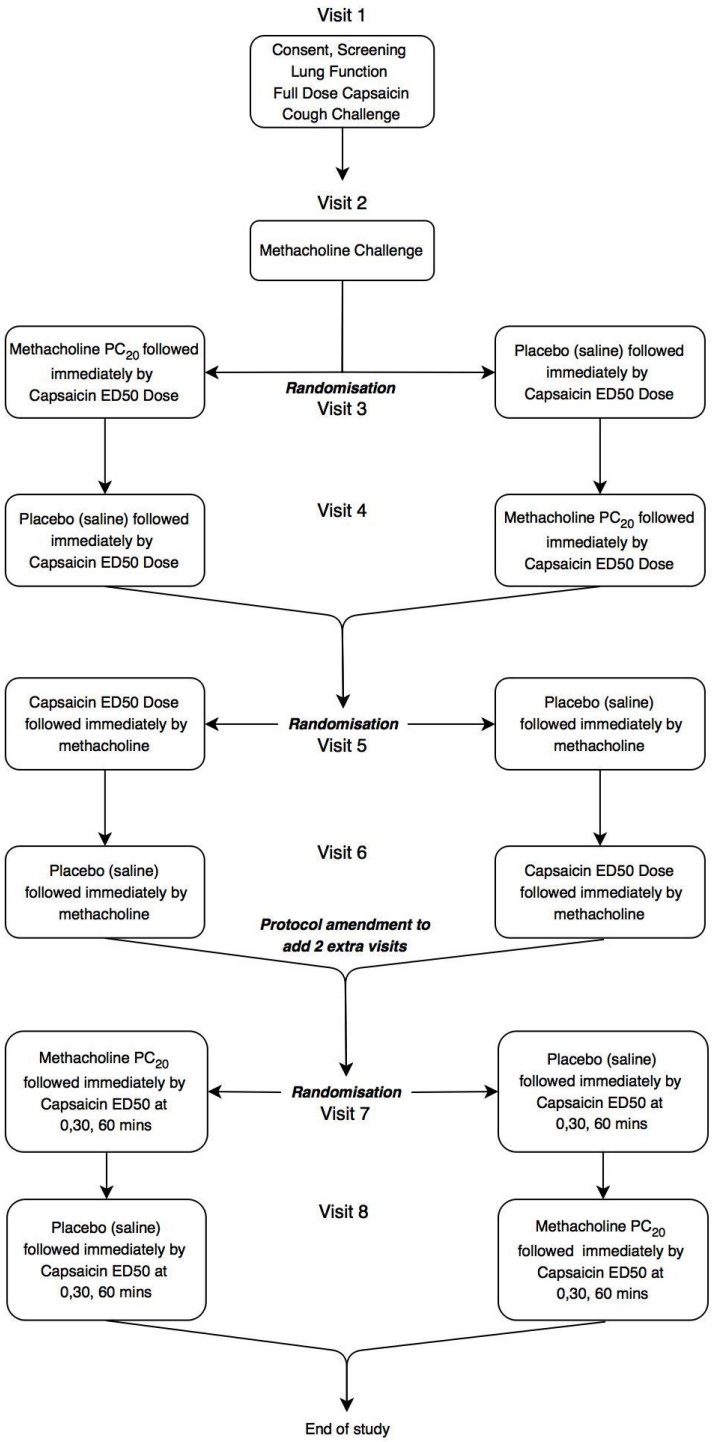

Figure 1: Flow chart summarising the study design

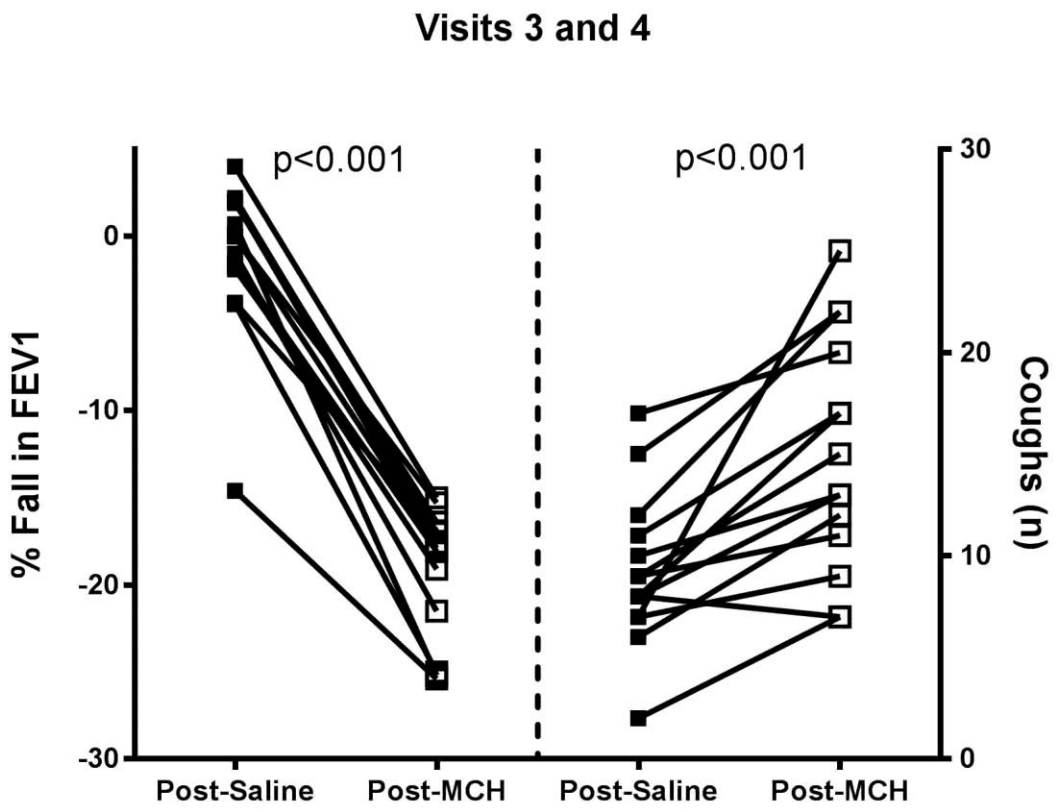

Figure 2: **The effects of bronchoconstriction on capsaicin evoked coughs A:** Fall in %FEV1 from baseline (left y-axis) after methacholine and saline challenge with corresponding capsaicin evoked coughs (right y-axis)**.**

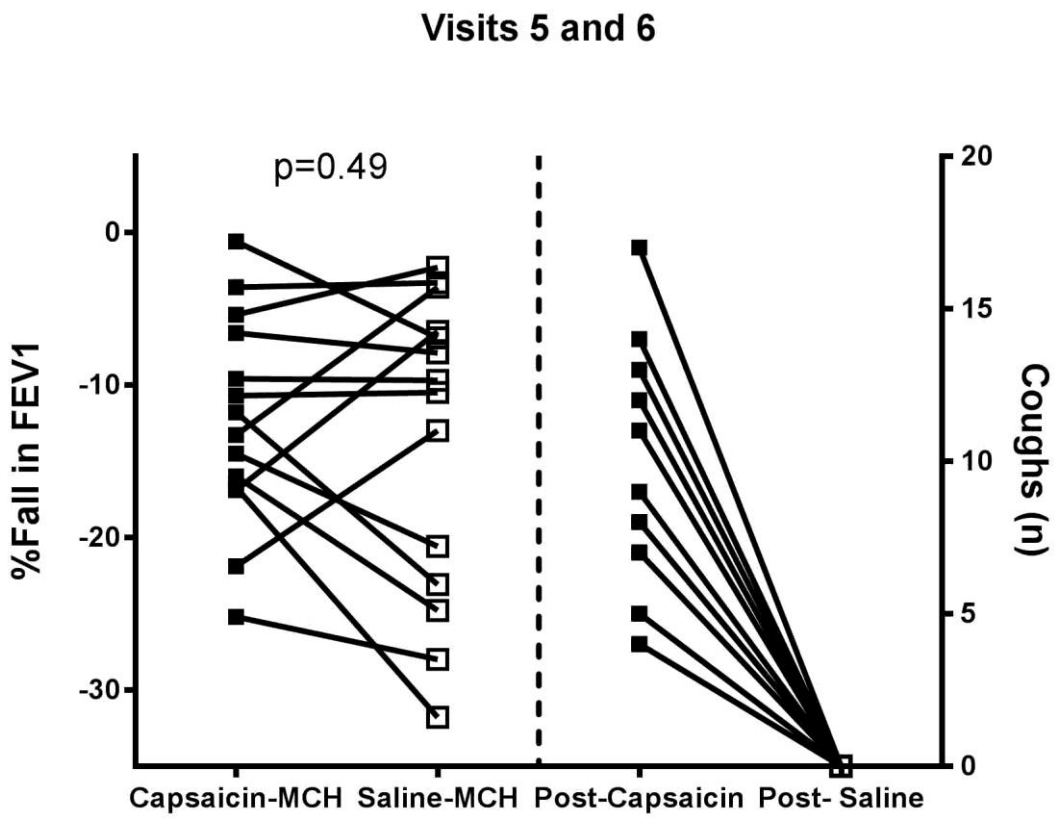

Figure 3: The effects of capsaicin evoked coughing on methacholine bronchial hyper-responsiveness. Fall in %FEV1 from baseline (left y-axis) after methacholine and saline challenge with corresponding capsaicin evoked coughs (right y-axis)**.**

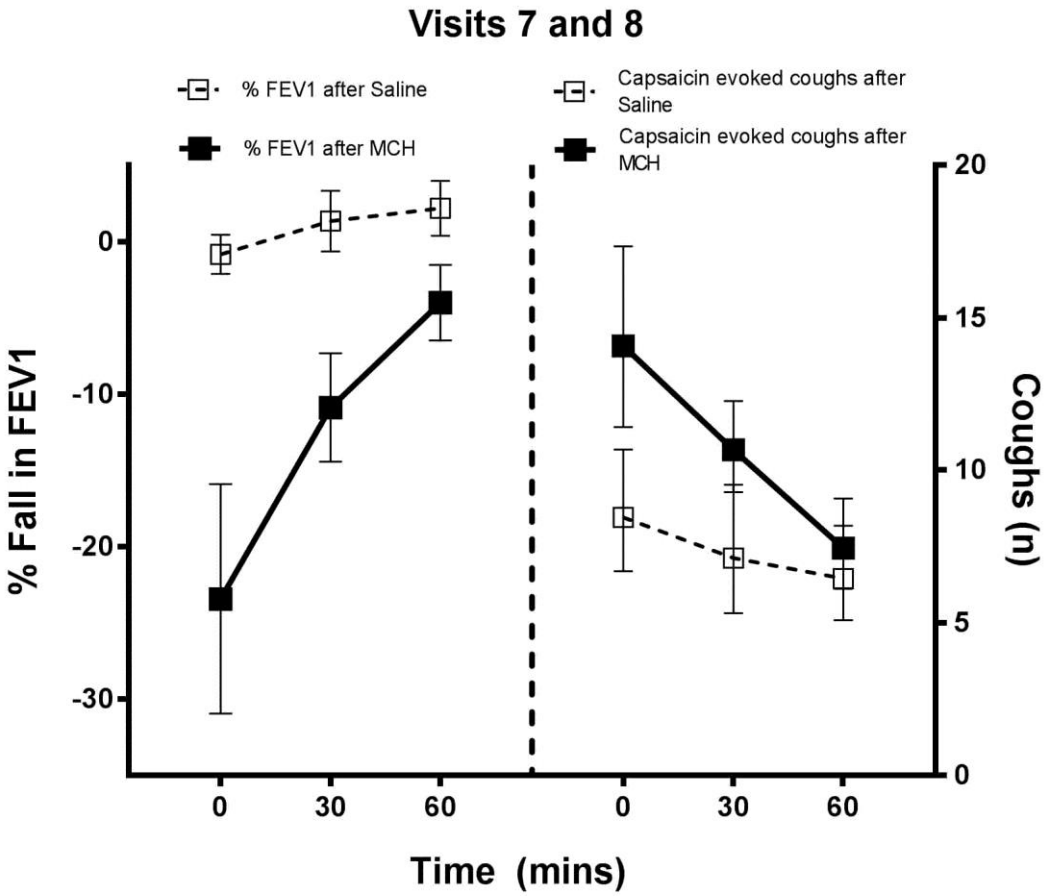

Figure 4: The effects of spontaneous recovery of FEV1 on capsaicin evoked coughs. Spontaneous recovery of FEV1 after methacholine inhalation shown as arithmetic mean and 95% CI (left y-axis) with corresponding reduction in capsaicin evoked coughs shown as geometric mean and 95% confidence intervals (right axis).

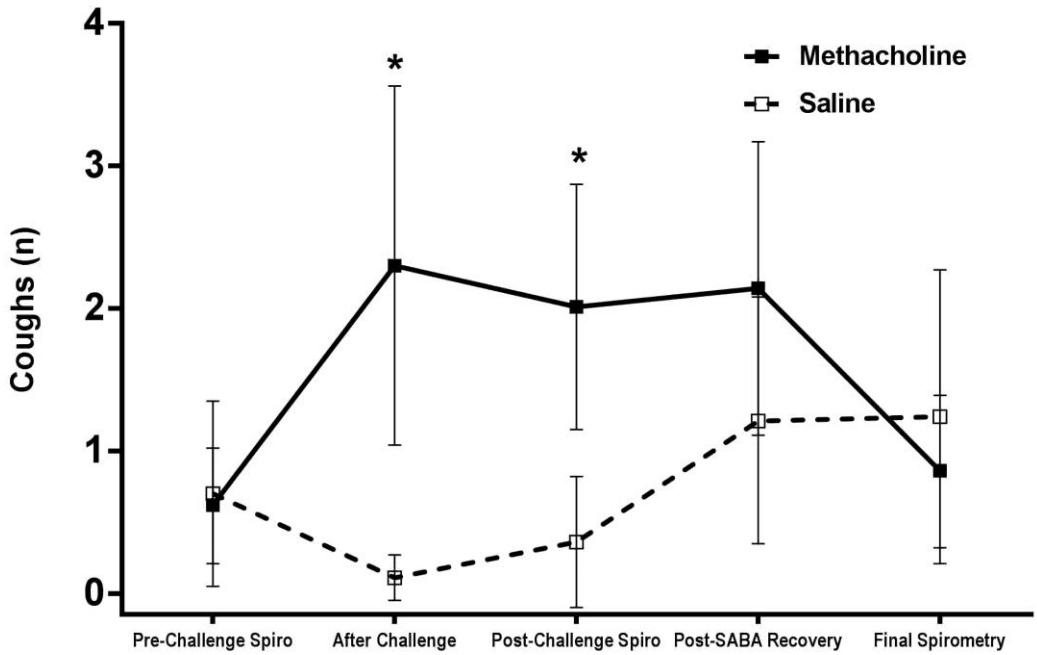

Figure 5: Spontaneous coughs during five chronological time periods for each visit after inhaling methacholine and placebo. Coughs shown are arithmetic mean and 95% CI. * indicates p=0.001

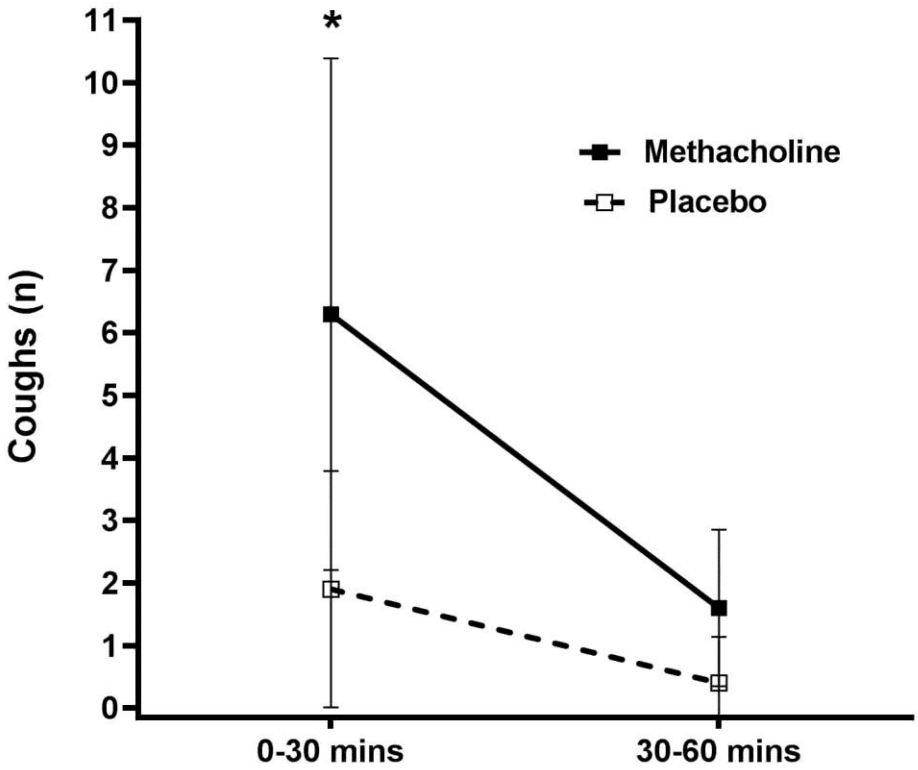

Figure 6: Spontaneous coughs during 0-30 mins and 30-60 mins after inhaling methacholine and placebo. Mean and 95% CI shown.* indicates p=0.05.

5 Investigating neuronal responses by assessing capsaicin evoked cough responses in an allergen challenge model of asthma (INCA)

5.1 Abstract

Introduction

Cough is a common and troublesome symptom in asthma but little is known about the neuronal pathways that trigger cough. The mechanisms by which airway inflammation, bronchial hyper-responsiveness and variable airflow obstruction cause cough are unclear. We have previously shown that methacholine induced bronchoconstriction heightens cough responses to inhaled capsaicin. The aim of the current study was to investigate the effects of allergen exposure on cough reflex sensitivity.

Method

We performed a 8 visit randomised, single-blind, placebo controlled, two-way cross-over study comparing cough responses to inhaled capsaicin in allergic asthma patients 30 mins and 24 hours after exposure to allergen compared with diluent (saline) control.

Mild atopic asthmatics who were dual responders to inhaled allergen were administered allergen/diluent (saline) challenge. Early asthmatic responses (EAR) (fall in FEV1>20% at 30mins) and late asthmatic responses (LAR) (fall in FEV1>15% at 3-7 hours) were documented. Full dose capsaicin challenge was performed at screening to determine the excitatory dose evoking half the maximum cough response (ED50). The ED50 concentration of capsaicin was inhaled four times, 30 seconds apart, at 30 mins and 24hrs after inhaled allergen/diluent challenge. Spontaneous coughing was also measured throughout, along with methacholine challenge and induced sputum before and after allergen/diluent challenge.

Results

We report interim analysis of the first 5 completed patients recruited to this study (2 female, mean age 23.4yrs (S.D ±4.6), BMI 23.9 kg/m^2 (±4.3), FEV1% predicted 97.4% (±8.0), methacholine PC20 1.55 mg/ml (±1.72)). Median baseline ED50 concentration was 15.6µM of capsaicin (IQR 5.9-47.0). All patients were steroid naïve, sensitive to house dust mites and demonstrated a dual response to inhaled allergen. During the screening allergen challenge, mean % fall in FEV1 from baseline in the EAR was 34.9% (S.D ±6.4) and LAR 29.2% (±20.6). After randomisation to allergen/diluent, there was an increase in capsaicin evoked coughs after allergen exposure compared to diluent at both 30 mins and 24 hours (mean coughs after allergen vs. diluent at 30mins 15.0 (±S.D. 4.9) vs. 9.6 (±3.5), at 24hrs 12.2 (±8.0) vs. 6.0 (±2.6).

Conclusion

We have safely performed capsaicin challenge after allergen exposure. Our data suggests cough responses may be heightened for up to 24 hours after allergen exposure.

5.2 Introduction

Asthma is a complex heterogeneous disease whose pathophysiology is currently thought to consist of two predominant dimensions; airway inflammation and bronchial hyper-responsiveness (BHR). However, the relationship between the fundamental pathophysiology and the development of symptoms such as cough, wheeze, shortness of breath, and chest tightness is unclear. Cohorts of patients with severe asthma have demonstrated discordance between airway eosinophilia and symptoms [179-181], and although recent novel biologics have demonstrated improvements in airway eosinophilia, lung function and risks of exacerbations, there has been a paucity of data showing improvements in day to day symptoms and quality of life [186, 187]. Cough is a common [9, 11], troublesome [14] symptom in asthma which is triggered by airway nerves sending impulses to the central nervous system, and unlike other symptoms can be quantified objectively and evoked experimentally. The relationship between airway inflammation, BHR and cough require further investigation in order to ascertain if neuronal function is an important dimension in the pathophysiology of cough in asthma.

Our previous study provided evidence for neuronal dysfunction in mild to moderate stable asthma as demonstrated by an exaggerated response to inhaled capsaicin compared to healthy volunteers [295]. Cough responses were independent of BHR to methacholine, baseline lung function or fractional concentration of exhaled nitric oxide (FeNO), however, this was in a stable group of patients with asthma with good forced expiratory volumes in 1 second (FEV1). Our follow up study systematically investigated the interaction between directly induced bronchoconstriction and capsaicin evoked cough and showed that bronchoconstriction induced by inhaling methacholine produced an increase in cough responses to inhaled capsaicin, which gradually diminished as airway calibre improved [296]. This suggested that smooth muscle contraction increases neuronal function as measured by the cough reflex. An important missing component in these studies was directly assessing the influence of airway inflammation on airway nerves that trigger the cough reflex. Previous observational cross-sectional studies have demonstrated no relationships between airway inflammation, asthma control and spontaneous coughs [19]. However, the effect of directly increasing airway inflammation in individual patients on spontaneous or experimentally evoked coughs using capsaicin was not assessed.

We therefore performed a randomised, single-blind, placebo controlled, two-way cross-over study comparing cough responses to inhaled capsaicin in patients with mild steroid naïve atopic asthma during and 24 hours after exposure to allergen compared with diluent (saline) control. The primary endpoint was number of evoked coughs after inhaling the dose of capsaicin evoking at least half the maximum cough response after a full dose capsaicin challenge (ED50). We also explored differences in spontaneous hourly cough rates for 24 hours after the allergen and diluent challenges.

5.3 Methods

5.3.1 Participants

Participants with mild steroid naïve atopic asthma who demonstrated an early and late bronchoconstriction response to an inhaled allergen were recruited. All participants had evidence of bronchial hyper-responsiveness (BHR) to methacholine (PC20<16mg/ml), were controlled according to Global Initiative for Asthma (GINA) classification, and were on stable medication ≥4 weeks. We excluded current smokers, those with a recent exacerbation or uncontrolled symptoms, and use of medication which may have altered cough responses (e.g. opiates, gabapentin, anti-cholinergics, and theophylline). The protocol was approved by the local research ethics committee (15/NW/0787) and all subjects provided written informed consent.

5.3.2 Study Design and Procedures

This was a 8 visit, randomised, single-blind, placebo controlled, two-way cross-over study comparing cough responses to inhaled capsaicin in allergic asthma patients 30 mins and 24 hours after exposure to allergen compared with diluent (saline) control (Table 1). *Full details of all study procedures are found in the online supplement.*

Participants were invited for two initial screening visits. On day 1, history and examination was performed followed by spirometry, full dose capsaicin cough challenge as described previously [295], skin prick testing and dose titration [282, 297], and the provocative concentration of methacholine causing a 20% drop in FEV1 (PC_{20}) using the 2 minute tidal breathing method [225]. The dose of capsaicin that evoked at least half the maximum coughs (ED50) during the full dose capsaicin challenge was used as a single dose challenge in visits 4, 5, 7 and 8 (See Table 1). On day 2, participants returned for an incremental inhaled allergen challenge to calculate the dose of allergen needed to induce an early asthmatic response (EAR, fall in FEV1>20% at 30mins,) and to monitor the fall in the late asthmatic response (LAR, fall in FEV1>15% between 3-7 hours) [254]. Only patients who demonstrated the EAR and LAR were included in the subsequent visits.

After at least two weeks, patients were randomised to two study periods of 3 consecutive days at least 2-4 weeks apart. At visit 3, methacholine challenge was performed followed by sputum induction. At visit 4, the day after, participants were fitted with an ambulatory cough monitor (VitaloJAK™; Vitalograph, Buckinghamshire, UK) for the next 24 hours and asked to inhale either allergen/diluent in a randomised order with visit 7. Thirty minutes after the start of the challenge, participants inhaled four inhalations of capsaicin ED50 thirty seconds apart and the number of evoked coughs measured. Patients were monitored for 7 hours after the allergen/diluent challenge with regular measurements of FEV1. Salbutamol was administered at the end of 7 hours and patients told to take additional inhalations at home as and when required according to symptoms. At visit 5, 24 hours after the start of the challenge, patients performed

baseline spirometry followed by repeat capsaicin ED50 challenge. This was followed by a methacholine challenge and sputum induction. At least 2 weeks later, participants returned to repeat the above triad at visits 6, 7, and 8.

5.3.3 Statistical Analysis

Interim analysis on data were analysed using SPSS Version 20.0 (IBM Corp., NY). Individual data are shown for baseline demographics and changes within patients during the study. This study generated data for numbers of capsaicin evoked coughs, changes in spontaneous 24 hour cough rates (c/h), %FEV1, and PC_{20}. Summary data are presented either as median and interquartile range (IQR) or as mean and standard deviation (S.D). The effect of bronchoconstriction on capsaicin evoked coughs, and spontaneous coughs after allergen/diluent challenge was analysed using general estimating equations (GEE). These models were used to estimate the mean and 95% confidence intervals. As there were only five completed patients, we have not performed any statistical testing, however a total of 12 patients will be recruited to complete the study.

5.4 Results

5.4.1 Subjects

We recruited seven patients with mild steroid naïve asthma and completed five who are described in Table 2. One patient developed an upper respiratory tract infection and was excluded from the study, and one failed to develop a LAR. All participants had good baseline lung function (mean %FEV1 predicted 97.4 % (S.D ±8.0), demonstrated evidence of BHR to methacholine (median 0.7mg/ml (0.17-3.35), and were sensitive to house dust mite allergen extract (HDM). During the screening allergen challenge all participants demonstrated an EAR and LAR to inhaled allergen; mean % fall in FEV1 in the EAR was 34.9% (S.D ±6.4) and LAR 29.2% (±20.6) relative to baseline. All patients coughed during the baseline full dose capsaicin cough challenge; median E_{max} was 15.0 coughs (13.5-17.5) and ED_{50} 15.6µM of capsaicin (5.9-47.0).

Recruitment for a further seven patients is planned at McMaster University but has been delayed due to approval of capsaicin challenge by Health Canada.

5.4.2 Lung function after allergen and diluent challenge after randomisation

There were differences in the fall in %FEV1 from baseline following inhaled allergen compared to diluent challenge in both the EAR (mean fall in %FEV1 after allergen 34.6% (±7.1) vs. diluent 2.7% (±2.0)) and LAR (mean fall in %FEV1 after allergen 26.8% (±18.4) vs. 2.0% (±0.6) (Figure 1). There was an improvement in the FEV1 24 hours after allergen and diluent challenge (mean % fall in FEV1 after allergen 12.7% (±13.5) vs. diluent -4.0% (±8.1)), i.e. there was a 4% improvement in %FEV1 24 hours after diluent challenge. However, three patients still had reduced lung function 24 hours after allergen challenge and did not recover to near baseline FEV1; 34.4, 14.4 and 11.6% below baseline. The patient with the FEV1 still 34% below baseline did not undergo a methacholine challenge.

5.4.3 Capsaicin cough responses after allergen and diluent challenge

There was an increase in capsaicin ED50 evoked coughs 30 mins after an allergen challenge compared with diluent (mean 15.0 (95% C.I 11.1-18.9) vs. 9.6 coughs (6.9-12.3), Figure 2). There was a reduction in capsaicin ED50 coughs 24 hours after allergen/diluent challenge, but participants still coughed more after allergen (mean 6.0 (95% C.I 3.9-8.1) vs. 12.2 coughs (5.9-18.5), Figure 3).

5.4.4 Spontaneous 24 hour cough rates (c/h) after allergen and diluent challenge

There was an increase in spontaneous coughs over 24 hours after allergen compared with diluent challenge (mean 4.7 c/h (95% C.I. 1.28-8.17) vs. 1.1c/h (0.45-1.72). Spontaneous coughs were highest in the first 2 hours after allergen challenge which decreased over time (Figure 4 and Table E1).

5.4.5 Changes in methacholine PC20

There was a fall in methacholine PC20 after inhaled allergen challenge compared with diluent (mean PC_{20} pre-allergen 1.00mg/ml (± S.D.1.14), post-allergen 0.31mg/ml (±0.33), pre-diluent 1.77mg/ml (±1.38), post-diluent 2.00 mg/ml (±3.03) (Figure 5).

5.5 Discussion

To our knowledge, this is the first study to investigate both experimentally evoked cough responses and objectively quantified spontaneous coughs both during and after an allergen challenge. Our data provide evidence to suggest that inhaled allergen is associated with an increase in capsaicin evoked cough responses at 30 mins and 24 hours later. Allergen inhalation was also associated with an increase in overall spontaneous coughs, and as expected, an increase in bronchial hyper-responsiveness (BHR). These results suggest that inhaled allergen challenge, an indirect bronchial challenge, may sensitise sensory nerve afferents responsible for causing cough.

The results of our study are in contrast to the results demonstrated by Minogushi et al who, despite an increase in sputum eosinophils and BHR, showed no change in the dose of capsaicin causing atleast five coughs (C5) after allergen challenge [191]. However, that study was in a parallel group design with the capsaicin C5 endpoint only 24 hours after allergen, which we have recently demonstrated may not be the optimum endpoint [160, 295]. Also, three out of the nine patients did not develop a LAR. There have been no other studies to have directly investigated the effects of inducing airway inflammation and BHR on cough and hence our understanding of asthma pathophysiology and cough has been based on observational studies in humans, which previously showed no relationship [295]. Evidence that airway inflammation and cough are related comes from indirect circumstantial evidence demonstrating an increase in the concentration of citric acid causing 2 and 4 coughs one month after treatment with an inhaled steroid and salbutamol [56].

To fully untangle the possible mechanisms involved, careful consideration must be given to the relationships between airflow obstruction, airway inflammation, and BHR after inhaled allergen challenge. In the early asthmatic response (EAR) the mean fall in %FEV1 after inhaled allergen was 34.6% compared with only 2.7% after diluent and capsaicin evoked coughs were higher (mean 9.6 to 15.0 coughs). Compare this to our previous study which showed a 19% fall in %FEV1 after methacholine resulted in a similar increase in coughs (geometric mean 8.4 increasing to 13.9) (10). Despite the smaller sample size in the allergen challenge study, we have now shown that bronchoconstriction after a direct and indirect challenge increases cough responses implying sensitisation of the cough reflex, with almost exact beta (gradient) values of 0.24 and 0.25, suggesting a consistent relationship between cough and airway calibre.

Unlike the shorter acting methacholine challenge, allergen challenge resulted in a late asthmatic response (LAR) in all our patients, with one patient dropping by nearly 60% of baseline. The morning after allergen challenge, FEV1 did improve by 22% overall, but the mean was still 12.7% below baseline and this was associated in a fall in mean coughs after allergen (15.0 decreasing to 12.2). Compared with the EAR, where the difference in FEV1 after allergen/diluent was nearly 32% and this resulted in a difference of 5.4 coughs, 24 hours later, the difference in FEV1 was halved to 16%, but there was still a difference of 5.6 coughs (see

Figure 6). Therefore, we could possibly speculate that the higher than expected coughs at 24 hours could be due to the effects of airway inflammation on airway nerves.

Interestingly, after the diluent challenge, there was a 6% increase in FEV1 after 24 hours and a corresponding mean reduction of 3.6 coughs. This fall in coughs could either due to natural variation in ED50 evoked coughs, or the consequence of repeated deep breathing manoeuvres from measuring FEV1 during the challenge. Indeed, there is some evidence to suggest that deep inspiration is a strong stimulus for activating slowly adapting receptors (SARS) which can inhibit parasympathetic drive at the level of the brain stem [124, 141].

There are a number of possible neuronal mechanisms by which the exaggerated cough responses after inhaled allergen can be explained. Acute bronchoconstriction in the EAR is thought to be elicited by IgE mediated release of histamine, tryptase, cyteinyl leukotrienes (CysLT) and prostaglandins. The LAR is less well understood but current evidence suggests that allergen inhalation activate dendritic cells in the airway which orchestrate a complex type 2 inflammatory response resulting in the stimulation, maturation, trafficking, and degranulation of eosinophils, basophils and neutrophils to release CysLT and histamine[298]. These mediators are thought to directly cause smooth muscle contraction, hence, capsaicin sensitive airway nerves could be sensitised directly due to the mechanical effects of bronchoconstriction or indirectly via the release of inflammatory mediators in the airways. Evidence for either mechanism from human studies is currently lacking, however, studies in guinea pigs have implicated a central role for ATP activating purinergic receptors on nerves [173], mechanical stress stimulating ATP release via TGF-B [299], and histamine indirectly stimulating nerves via ATP release from smooth muscle contraction [173]. Prostaglandin D2 and E2 (PGD2/E2) has been shown to cause depolarisation in an isolated guinea pigs and human vagus nerve preparation [300, 301].

Phenotypic switching is an alternative mechanism for our results seen which has been shown to occur in sensitised guinea pigs who express novel TRPV1 receptors on Aδ fibres after allergen challenge making them capsaicin sensitive [100]. The switching of nerve fibres to express de novo functional TRPV1 ion channels was mimicked by instilling either brain-derived neurotrophic factor (BDNF) or glial-derived neurotrophic factor (GDNF) to the trachea. It is plausible that the increase in coughs seen at 24 hours after allergen was due to the addition of a new set of Aδ fibres now becoming capsaicin responsive.

Animal studies have also suggested an alternative ion channel from the TRP family; transient ankyrin-1 (TRPA1). In a series of experiments in rats and mice, Raemdonck et al showed the LAR can be attenuated by anaesthesia, ruthenium red (a non-selective TRP blocker), TRPA1 antagonist and an anti-cholinergic, but not TRPV1 [145]. This suggests that the LAR is driven by TRPA1 found on sensory nerve endings, which synapse in the brainstem ultimately causing bronchoconstriction via the parasympathetic efferent nerves. However, it is still unclear how

allergen induced inflammation activates the TRPA1 channel and this is yet to be translated to humans.

There are several limitations to this study worth noting. Firstly, this is an interim analysis of only 5 completed patients, and completion of at least 12 patients will provide the opportunity to perform statistical testing. Secondly, we were unable to analyse the sputum differential cell count in order to quantify changes in inflammatory mediators after the allergen challenge. Thirdly, our patients were mild steroid naïve atopics with early onset asthma. It is unclear how applicable these results are to a much broader asthma population. Fourthly, we have used capsaicin to evoke coughs, which is a specific TRPV1 agonist. It is unclear if other tussive challenges such as citric acid, or hypo/hyperosmolar challenge agents would produce similar results. Fifthly, it difficult to completely separate the effects of bronchoconstriction and airway inflammation *in vivo* in humans, hence the relative contribution of each pathophysiology to neuronal sensitivity is still unclear.

Conclusion

We have safely performed capsaicin cough challenge with allergen challenge in this proof of concept mechanistic study in patients with mild atopic asthma. The results of this study suggest that airway inflammation and airflow obstruction both contribute to exaggerated cough responses to inhaled capsaicin, implying neuronal sensitisation.

Acknowledgments

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Author's contributions: Concept and design; IS, MW, POB, SJF, JAS. Data generation; IS. Statistical analysis and modelling; IS and JAS. All authors reviewed the manuscript and approved the final draft.

Role of the Sponsor: No input in design, analysis or in manuscript writing.

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5.7 Tables:

Table 1: Summary of Study Visits

Visit	Procedures	Approximate Time	Comments
1	Consent, screening, history and examination, spirometry, full dose capsaicin challenge, skin prick testing, methacholine challenge	3 hours	***Screening Tests***
2	Screening Allergen Challenge	7 hours	
Within 1-2 weeks			
3	Methacholine challenge, sputum induction	1 hour 30 mins	***Allergen/diluent randomised***
4	Attach Cough Monitor *Allergen/Diluent Challenge* Capsaicin ED50 Dose at 30 mins	7 hours	
5	Capsaicin ED50 Dose Methacholine challenge, sputum induction Remove Cough Monitor	1 hour 30 mins	
After 2-4 weeks			
6	Methacholine challenge, sputum induction	1 hour 30 mins	***Allergen/diluent randomised***
7	Attach Cough Monitor *Allergen/Diluent Challenge* Capsaicin ED50 Dose at 30 mins	7 hours	
8	Capsaicin ED50 Dose Methacholine Challenge, Sputum induction Remove Cough Monitor	1 hour 30 mins	

Table 2: Baseline demographics and screening data of individual participants.

Patient ID	**1**	**2**	**3**	**4**	**5**
Age	19	19	25	24	30
Gender	M	F	M	F	M
BMI (kg/m^2)	23.1	21.3	25.9	19.1	29.9
FEV1 (L) (% predicted)	3.51 (91)	3.70 (97)	3.84 (110)	2.60 (90)	3.77 (99)
FVC (L) (%predicted)	4.76 (105)	4.54 (103)	4.40 (108)	3.03 (89)	4.51 (100)
Emax (coughs, n)	18	17	14	13	15
ED50 (µM Capsaicin)	7.8	15.6	62.5	3.9	31.3
Methacholine PC20 (mg/ml)	0.095	2.7	0.7	4	0.25
Allergen	HDM	HDM	HDM	HDM	HDM
SPT Endpoint	1:16384	1:2048	1:8192	1:1024	1:256
EAR Screen (% fall in FEV1 from baseline)	31.8	28.1	31.1	41.5	42.1
LAR Screen (% fall in FEV1 from baseline)	65.2	16.4	27.8	19.8	16.9

Abbreviations: BMI; Body Mass Index, Emax; maximum evoked coughs during full dose capsaicin challenge, ED50; the dose of capsaicin evoking atleast half the Emax, PC20; provocative concentration of methacholine causing a 20% fall in FEV1, HDM; house dust mites, SPT Endpoint; skin prick titration, the dilution factor of allergen causing a 2mm x 2mm weal on the skin, EAR; early asthmatic response, LAR; late asthmatic response.

5.8 Figures

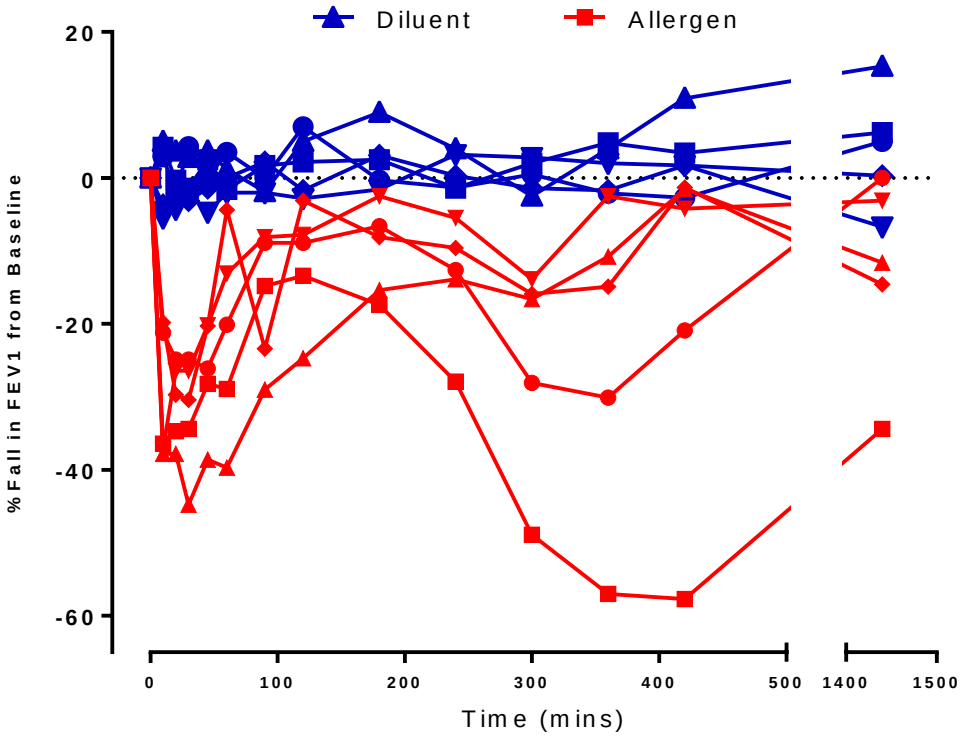

Figure 1: Changes in FEV1 after inhaled allergen and diluent challenge after randomisation

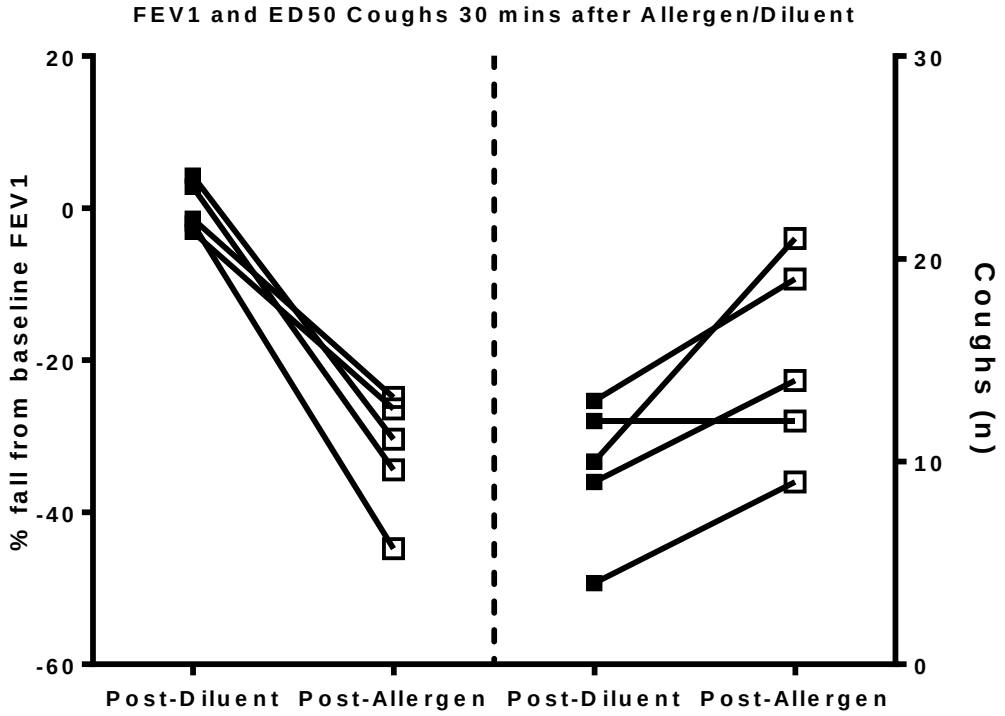

Figure 2: Comparison of the % fall in FEV1 from baseline and capsaicin ED50 evoked coughs 30 mins after allergen/diluent challenge

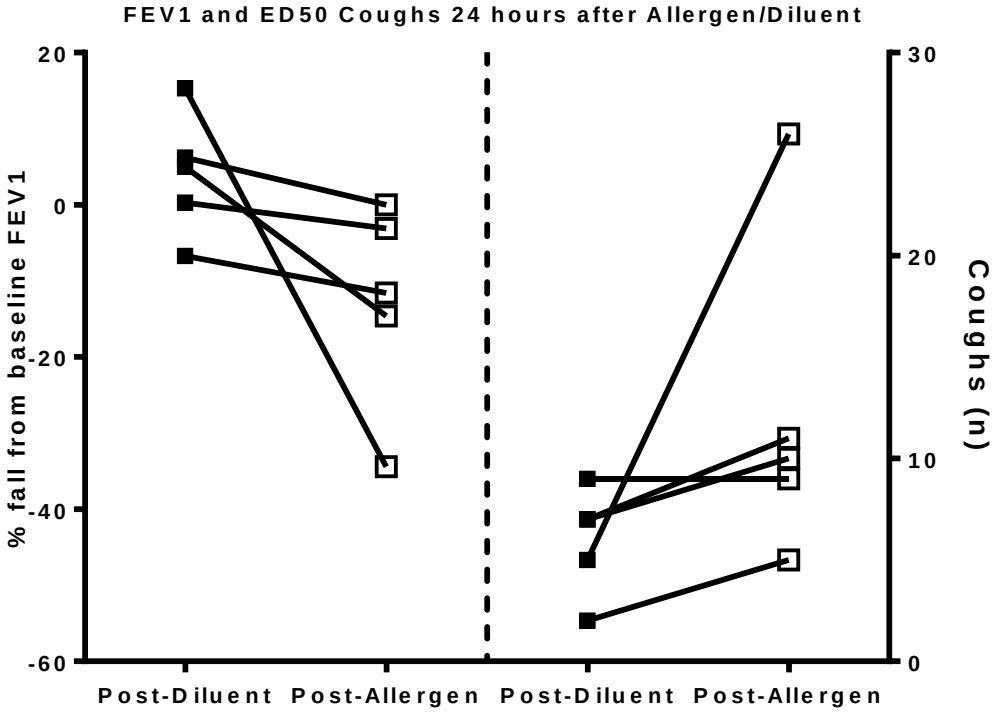

Figure 3: Comparison of the % fall in FEV1 from baseline and ED50 capsaicin evoked coughs 24 hours after allergen/diluent challenge

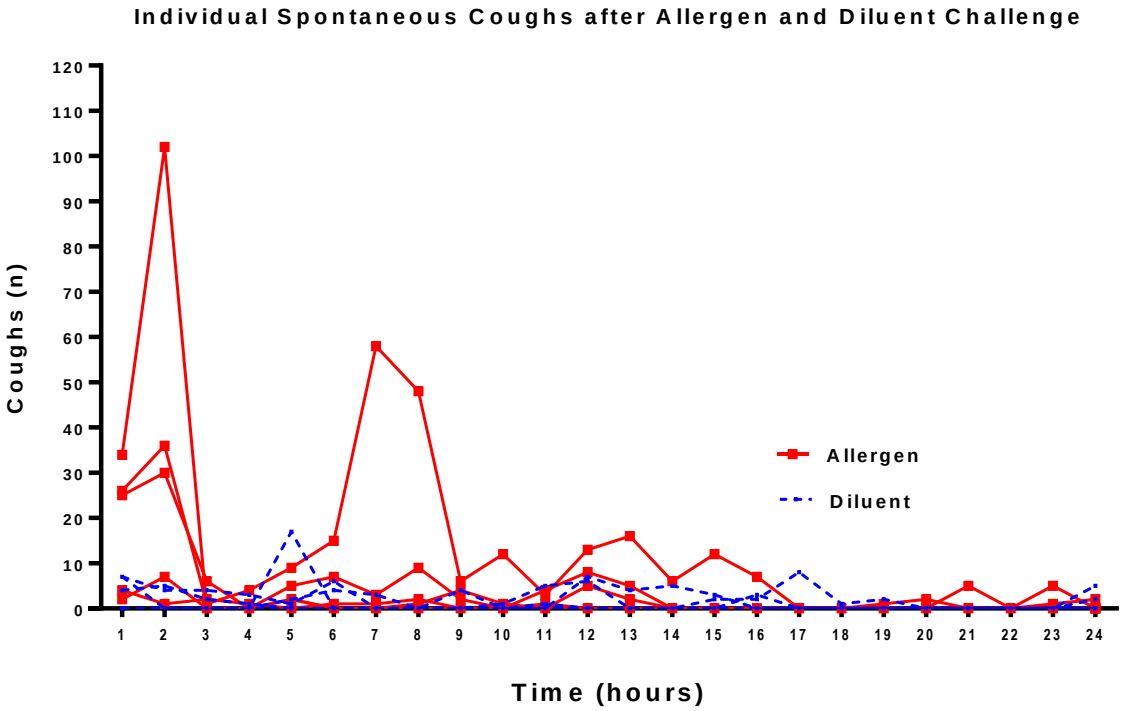

Figure 4: Individual spontaneous coughs for 24 hours after allergen and diluent challenge

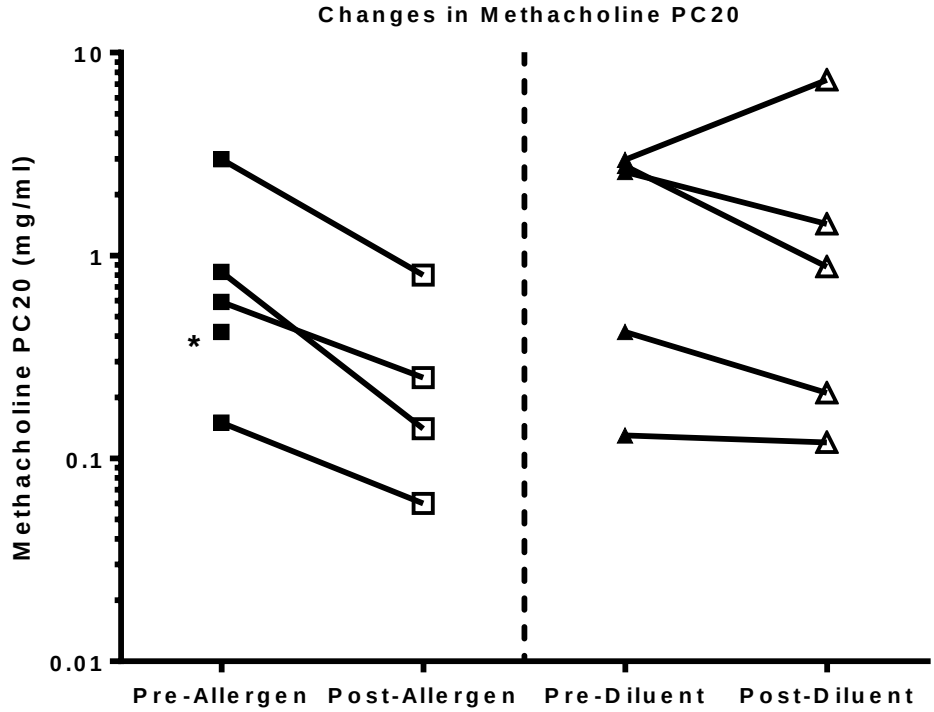

Figure 5: Changes in methacholine PC20 before and after allergen and diluent challenge. y-axis shown as $\log_{10}$ scale. * indicates one patient whose FEV1 was more than 20% below baseline and no methacholine challenge was performed.

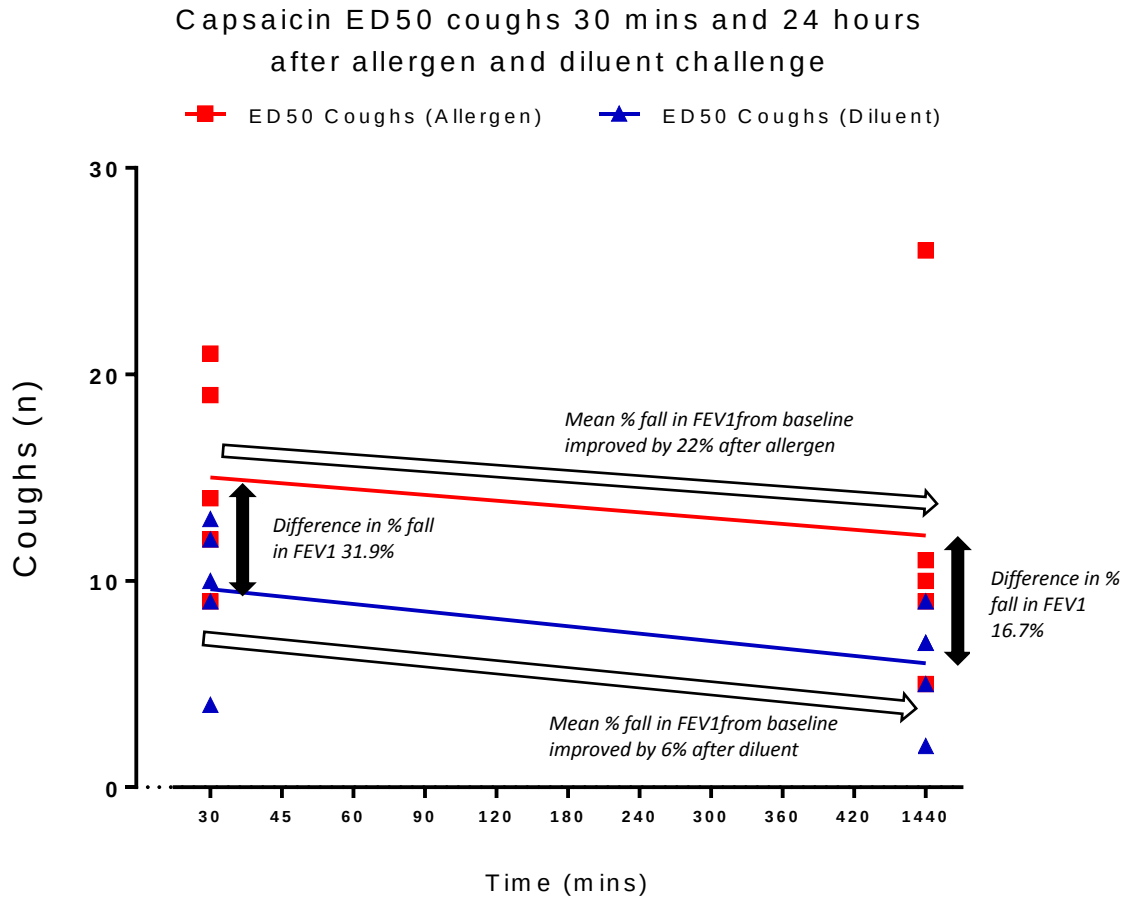

Figure 6: Comparison of capsaicin ED50 coughs 30 mins and 24 hours after allergen/diluent challenge. Line denotes mean. Annotated to show differences in % fall in FEV1 at 30 mins and 24 hours between and longitudinally after allergen/diluent.

5.9 Online Supplement

5.9.1 Study Procedures in Detail:

All procedures listed were performed according to local standard operating procedures (SOPs):

A. Allergen Challenge

The concentration of allergen causing 20% fall in FEV1 is predicted using the methacholine PC20, and the titration of allergen determined from the skin prick testing. The starting dose of allergen concentration was determined using the Cockcroft formula and the allergen challenge was performed as previously described (1). Briefly, subjects were asked to inhale a low concentration of an allergen for two mins through a nebuliser. Lung function was measured 10 mins after the end of the 2 minutes of inhalation. If FEV1 fell by less than 10% from the highest baseline FEV1, the next concentration was given. If the fall was between 10-20%, the FEV1 was measured again after a further 10 minutes. If it was the same or had started to rise, the next concentration or half concentration was given. The inhalations were stopped when the FEV1 had fallen by 20% or more, or at the discretion of the investigator. After the last inhalation, the FEV1 was measured at 20, 30, 45, 60, 90 and 120 mins and then at hourly intervals until 7 hours after the last allergen inhalation. The early asthma response (EAR) was defined by >20% fall in FEV1 between 0 and 3h after allergen, while the late asthma response (LAR) was defined by >15% fall in FEV1 between 3h and 7h after allergen inhalation.

B. Full Dose-Response Capsaicin Challenge Testing

A full dose-response capsaicin challenge was performed at visit 1 (2). Patients inhaled increasing doubling concentrations (0.49-1000µM) of capsaicin through a nebuliser adapted to control dosage and inspiratory flow rate. Each dose of capsaicin was inhaled in 4 single breaths separated by 30 seconds. Following each inhalation, the total number of coughs evoked (defined as explosive cough sounds) at each concentration within the first 15 seconds were recorded. The total maximum number of coughs evoked by any dose (Emax) is documented along with the dose of capsaicin that induced at least 50% of the maximal cough response (ED50). The ED50 concentration was used throughout visits 3-8. The lapel microphone of the VitaloJAK cough recorder was attached to the patient throughout the duration of the challenge and an independent observer verified the number of coughs induced at each dose of capsaicin.

C. Single Dose Capsaicin Challenge

The dose of capsaicin that evoked at least 50% of the maximum coughs (ED50) during the full challenge at visit 1 was used as a single dose challenge in visits 4, 5, 7 and 8. Four inhalations were administered 30 seconds apart and the number of coughs evoked in the first 15 seconds were counted and verified manually.

D. Cough Monitoring:

Participants were asked to wear a cough monitor (VitaloJAKTM, Vitalograph, UK) for 24 hours from the start of the allergen/diluent challenge (Visit 4 and 7) but also for a short duration whilst four inhalations of capsaicin were being performed (Visits 5 and 8). This involved attaching an air microphone to the lapel of the clothing which recorded all the evoked coughs. The verification process was done after the end of the visits. Twenty four hour files were compressed into filed of shorter duration using custom built software and individual coughs tagged and counted using an audio editing package (Audition version 3; Adobe Systems Inc., San Jose, CA). The number of coughs were expressed as coughs per hour (c/h).

E. Methacholine Challenge Test (MCT)

Nebulised methacholine was administered in doubling concentrations from 0.0625mg/mL up to 16mg/ml via the 2 minute tidal breathing method (3). At each concentration, FEV1 was measured 30s and 90s after the end of each inhalation. The % fall in FEV1 from baseline was calculated after each dose using the best FEV1 and the test was terminated once a 20% fall in FEV1 was documented or the maximum concentration of 16 mg/mL was reached. Salbutamol was withheld for at least 4 hours.

F. Sputum Induction:

Prior to performing the sputum induction, spirometry was repeated to ensure FEV1 had returned to within 10% of the baseline prior to the MCT. This occasionally required 400mcg of salbutamol in addition to the post-MCT salbutamol. Nebulised hypertonic saline was administered at 5 min intervals provided the FEV1 did not fall by more than 20% from pre-sputum baseline. If there was a drop in FEV1 between 10-20% then a lower saline concentration was administered.

H. Skin Prick Testing (SPT)

A panel of allergen extracts using a single head metal lancet on the patient's forearm. A negative control and a positive histamine control was also assessed. A positive result was demonstrate by a weal of ≥3mm. The allergen with the largest wheal was used to titrate down to much lower concentrations, and the concentration of allergen which caused a skin wheal at least 2 x 2 mm in size was noted. This was used to calculate the starting dose of the allergen challenge described previously (1).

5.9.2 Supplementary Tables

Table E1: Comparison of spontaneous coughs over 24 hours after allergen and diluent challenge. Mean and 95% confidence intervals estimated using generalised estimating equations (GEE).

	Allergen			Diluent		
Time	Mean	95% CI		Mean	95% CI	
1	18.20	29.43	6.97	3.60	6.35	0.85
2	35.20	66.69	3.71	1.80	3.75	-0.15
3	1.80	3.75	-0.15	1.20	2.60	-0.20
4	1.00	2.36	-0.36	0.80	1.82	-0.22
5	3.60	6.35	0.85	4.00	9.73	-1.73
6	4.60	9.70	-0.50	2.00	4.22	-0.22
7	12.40	32.41	-7.61	0.60	1.65	-0.45
8	12.00	28.02	-4.02	0.00	0.00	0.00
9	2.40	4.44	0.36	0.80	2.20	-0.60
10	2.60	6.73	-1.53	0.20	0.55	-0.15
11	1.60	3.02	0.18	1.20	2.90	-0.50
12	5.20	9.54	0.86	2.60	5.40	-0.20
13	4.60	9.85	-0.65	0.80	2.20	-0.60
14	1.20	3.30	-0.90	1.00	2.75	-0.75
15	2.40	6.61	-1.81	1.00	2.11	-0.11
16	1.40	3.85	-1.05	1.00	2.11	-0.11
17	0.00	0.00	0.00	1.60	4.40	-1.20
18	0.00	0.00	0.00	0.20	0.55	-0.15
19	0.20	0.55	-0.15	0.40	1.10	-0.30
20	0.40	1.10	-0.30	0.00	0.00	0.00
21	1.00	2.75	-0.75	0.00	0.00	0.00
22	0.00	0.00	0.00	0.00	0.00	0.00
23	1.20	2.90	-0.50	0.00	0.00	0.00
24	0.40	1.10	-0.30	1.40	3.12	-0.32

5.9.3 References

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6 Final Discussion

The fundamental aim of medicine is to improve the health of our patients, which includes both the morbidity and mortality associated with disease. However, the greatest challenge in all disciplines of medicine is to connect symptoms of illness with a mechanism causing the disease and providing a treatment. A breakdown in any one side of this triangle leaves a hole in our ability to confidently treat patients. This triangle of medicine is no different in asthma, except, there are many phenotypes of asthma, the mechanisms causing symptoms poorly understood, and treatment options limited; instead of connecting the sides of one triangle, it is rather like connecting the sides of a polygonal triangle in three dimensions.

The broad aim of this thesis was to try to explore just one facet of this polygonal structure; cough in asthma. The motivation for studying cough was because, unlike shortness of breath, chest tightness or wheeze, cough can be objectively quantified using a cough monitor and experimentally evoked using tussive challenge agents. A number of observational studies have shown that cough in asthma is common, troublesome and predicts poor prognosis. However, current asthma treatment with corticosteroids and bronchodilators are directed towards reducing airway inflammation, bronchial hyper-responsiveness (BHR), and variable airflow obstruction, but the evidence that these medications reduce objective coughs is currently lacking. An important reason for the lack of development of novel anti-tussives is that the mechanisms linking airway inflammation, BHR, and cough are unclear. Cough is widely thought to be neuronally mediated, but the interaction between airway nerves, inflammation and airflow obstruction is poorly understood.

The three studies that are presented in this thesis have provided some mechanistic insights in to the role of nerves in asthma. The objective of this final discussion is to summarise the main findings, explore the limitations, and discuss areas for future research.

6.1 Summary of main findings of thesis

i) Study 1: Capsaicin cough responses in asthma; evidence for neuronal dysfunction

The primary objective of the first study was to compare capsaicin cough responses in patients with mild/moderate stable asthma with healthy volunteers. This was an observational study where we performed a full dose response capsaicin cough challenge and performed non-linear pharmacodynamic (PD) modelling and demonstrated patients with asthma have a higher Emax, and lower ED50. We also demonstrated that Emax is influenced by gender, presence of asthma, atopic status and cough frequency with the highest Emax in non-atopic females with asthma. We also showed that ED50 is influenced by gender, presence of asthma, asthma control (ACQ), and IgE. Importantly, neither Emax nor ED50 were influenced by methacholine PC20, lung function, FeNO or serum eosinophils. The main conclusion from this study was that even during stability, patients with asthma demonstrated an exaggerated cough response to capsaicin; a left shift, increased gradient, and higher plateau. This is not unlike the increase in smooth muscle BHR that is demonstrated with methacholine or histamine, except that capsaicin activates the cough reflex, and hence demonstrates nerve function.

The influence of gender on capsaicin evoked cough responses in both the asthmatic and healthy volunteers suggests this is an innate fixed feature. An explanation for this difference is currently speculative. We analysed absolute values of lung function in our study and found no differences, suggesting this difference was unrelated to lung volumes. One study has demonstrated higher levels of TRPV1 mRNA expression from the peritoneum of women with chronic pelvic pain secondary to endometriosis, incubation of sensory neurons with 17B-estradiol or an agonist to this oestrogen receptor increased TRPV1 mRNA expression [302]. However, two-thirds of patients with chronic cough are female and presenting in their 50's and 60's, when oestrogen levels will be low. It is unclear what effect the role of a lifetime of oestrogen has on the cough reflex sensitivity, and whether the lack of oestrogen subsequently induces any neuroplastic changes in the peripheral or central nervous system. As yet, clear differences in capsaicin evoked coughs or objective cough frequencies during different phases of the menstrual cycle have not yet been demonstrated.

An important feature of this study was the safety and feasibility of performing a full dose capsaicin challenge in asthma patients and modelling the data using advanced pharmacodynamic (PD) modelling. This was only the second time such modelling techniques had been used for analysing capsaicin cough responses, and Emax and ED50 parameters from the model performed better than the traditional C2 and C5 cough challenge endpoints. In a multi-variate linear regression model, none of the co-variates that we identified in the PD non-linear model were found to be significant (disease group, gender, atopic status, cough frequency, or serum IgE). The implication is that comparing a whole set of dose response data in a non-linear model with cough as the output is a more useful model than comparing one

value, i.e. C2 or C5, in a linear regression model. This makes neurophysiological sense when testing the cough reflex; we are more interested in the output (coughs) of the reflex rather than the stimulus (capsaicin dose).

The use of Emax/ED50 is not common in the vast majority of studies in chronic cough, and consensus statements have relied on using C2 or C5 endpoints and subsequently coined the term 'cough hypersensitivity syndrome' for those patients with a low C2 and C5. However, the implication of this study in patients with asthma [296] and that of Hilton and colleagues in chronic cough [117], is that there is a hyper-responsiveness (upward shift in Emax) and a hyper-sensitivity (left shift in ED50). But adapting this novel nomenclature in the field of cough will be challenging because it relies on changing the cough challenge methodology.

ii) Study 2: The interaction between bronchoconstriction and cough in asthma

The primary objective of the second study was to understand how airway calibre affects capsaicin cough responses and thereby neuronal function. Unlike the first study, in which we obtained observational data from a cohort of patients with mild/moderate stable asthma, we performed a randomised, single blind, placebo controlled, cross-over study. The motivation for performing this was that our first study suggested that capsaicin responses were independent of airflow obstruction; however, this was in a stable group of patients with good lung function. In order to test the hypothesis that airway nerves and airway calibre are truly independent, we altered the stable baseline lung function by inducing bronchoconstriction using methacholine.

The key finding was that bronchoconstriction increased capsaicin cough responses, and gradual spontaneous recovery over 60 minutes was associated with an improvement in cough responses. However, capsaicin evoked coughing did not alter BHR to methacholine nor did it cause a worsening of FEV1. These findings are important for two main reasons. Firstly, contrary to our first study, it suggested that neuronal activity is dependent on airflow obstruction but this was only demonstrated when changing the baseline lung function in individual patients. Secondly, it was safe to inhale an ED50 dose of capsaicin when FEV1 was 20% below baseline. The latter finding gave us some confidence in performing inhaled capsaicin during an allergen challenge when we anticipated lung function would fall to similar levels.

An important methodological difference was the use of an individualised single dose of capsaicin ED50 rather than performing the whole dose response. This dramatically reduces the time needed to assess the cough reflex, reduces the exposure to capsaicin, and is a useful tool at analysing differences within patients at different time points and intervention periods.

iii) Study 3: Investigating neuronal responses by assessing capsaicin cough responses in an allergen challenge model of asthma

The primary objective of the allergen challenge study was to investigate how allergic airway inflammation affects airway nerves. This was a randomised, single-blind, placebo controlled, cross-over study in mild steroid naïve patients with asthma who had previously demonstrated an early and late asthmatic response. The early asthmatic response (EAR) was associated with an increase in capsaicin evoked coughs. This validated our previous finding that bronchoconstriction does indeed cause an exaggerated cough response.

At 24 hours, cough responses were still heightened despite an improvement in FEV1, but with just five completed patients, we would cautiously speculate that this is related to airway inflammation. The alternative hypothesis would be that airway inflammation has no influence on airway nerves, and this would also be an important finding as it would imply that experimentally evoked coughs and airway inflammation are independent.

iv) 24 hour objective coughs and capsaicin evoked coughs

Analysing objective coughs was a secondary objective in all my three studies, but we demonstrated they are associated with Emax, increased with bronchoconstriction during the methacholine and allergen challenge studies. This suggests that capsaicin evoked coughs does provide a useful clinical tool which mirrors real life day-to-day spontaneous symptoms.

6.3 Limitations

In order to fully contextualise the implications of these findings, it is important to highlight and consider some important limitations of these studies. These can broadly be divided and focused into areas related to patients, methods, data and interpretation of results.

i. Patient factors

In all three studies, patients with asthma were selected who had stable mild/moderate disease, who were young, predominantly atopic and developed asthma in early childhood. We excluded patients with uncontrolled symptoms or those using high dose inhaled steroids (fluticasone propionate >500mcg) as we were unsure about the safety of performing full dose capsaicin challenge in patients with more severe asthma. We deliberately selected mild patients as we intended to perform allergen challenge during the planning stage of my thesis, and given this is only performed in steroid naïve subjects, we aimed to create a cohort of such patients who could be recruited for our subsequent allergen challenge study. Secondly, our clinical research facilities are based on large university campuses where conditions for the recruitment of students and staff were optimal. We used university mailing lists, student's union newsletters and social media to advertise.

The advantage of studying this group of mild/moderate patients with asthma was that most of these patients had asthma as their sole medical condition and were not taking many other medications, therefore limiting confounding factors. The limitation is that one could argue how applicable our findings are to the much broader heterogeneous asthma population, such as those with severe asthma and late onset adult asthma. I would propose that the aim of my studies was to investigate a proof of concept in mechanisms of disease, and given the lack of previous studies of capsaicin in severe asthma, it was safer to start in a milder stable group.

ii. Methodological limitations

In the first observational study we recruited 47 healthy volunteers as the control group. These participants did not have any blood tests, skin prick tests to test for any allergic conditions. Given our findings about the influence of atopy, and IgE on capsaicin dose response curves, it would have been interesting to have such data available. However, these results were not expected at the start of the study, and hence could not have been planned for in advance.

It must be noted that the inclusion criteria for allergen challenge studies requires mild steroid naïve patients with asthma who are well controlled and demonstrate a dual response to inhaled allergen. It can be argued that this is a very small percentage of the whole asthma population and hence the results are not widely applicable. However, it has been shown over the last three decades that this is a safe model to study the mechanisms of asthma pathophysiology and efficacy of novel anti-inflammatory drugs. Novel drugs that successfully inhibition of the late

asthmatic response have subsequently shown to be beneficial in a much broader asthma population as a whole demonstrating the usefulness of the allergen challenge model [298].

It could be argued that as inhaled allergen challenge induces bronchoconstriction and airway inflammation, attempts could have been in advance to negate the bronchoconstrictor response. This would have made the effects of inflammation on capsaicin cough responses more apparent. A range of potential bronchodilators could have been used such as salbutamol or a long or short acting anti-cholinergic. However, as discussed in the introduction, there is evidence that salbutamol and anti-cholinergic medication could also influence airway nerves via actions on the potassium channels or TRPA1 channel respectively. This will be an objective of my future research studies and requires further investigations.

It has been emphasised throughout the thesis that we investigated the role of TRPV1 in asthma, however other receptors on nerve endings may still be important. The advantage of using capsaicin is that it directly and specifically activates TRPV1. Other challenge agents such as citric acid are commonly used, however it is unclear which receptors are activated. Developing specific challenge agents which are safe are challenging. For example, TRPA1 can be stimulated by aldehydes but these are extremely pungent to inhale, can cause occupational asthma, and are carcinogenic (acrolein in cigarette smoke). Hence, safer specific TRPA1 challenge agents are difficult to find, but may provide important mechanistic insights in the future. Transient receptor potential vanilloid type-4 (TRPV4) is both a mechanoreceptor and responds to changes in osmolality so potentially responds to hypertonic saline or mannitol, but these do carry a greater risk of bronchoconstriction. Furthermore, the finding of TRPV4 channels on airway smooth muscle does not make it an ideal candidate to study airway nerves is isolation.

An important lesson to be learnt from my three studies is that it is difficult to exclude significant associations between co-variates from just observational studies. In the first study, there was no significant association between cough responses to capsaicin and airflow obstruction and indirect measures of inflammation (serum eosinophils and FeNO), however, in the second and third, which were interventional, changing the physiology of individual patients and comparing changes within patients, we did demonstrate associations.

iii. Data Collection

The patients with asthma could have been better phenotyped with a direct measure of airway inflammation. Apart from documenting a medical history, we did not collect any other questionnaires or analyse more direct measure of airway inflammation such as induced sputum. The majority of patients who inhaled hypertonic saline solution were unable to expectorate a substantial quantity of sputum to be analysed. Secondly, there was a lack of availability of staff and equipment during a substantial time period of the study at our clinical research facilities. As

this was not the primary objective of our first study, we decided to continue without induced sputum results. In the second study, we deliberately chose not to perform induced sputum as this would have interfered with the methacholine and capsaicin challenge. In the third study, sputum has been collected, however, they are yet to be analysed. We are planning to analyse all the sputum data once the remaining patients have been recruited and completed. The fact that only five patients have completed the allergen challenge study makes the results of this study still speculative, despite the changes we have described so far. Delays in recruitment were due to an unanticipated delay in the approval of inhaled capsaicin by Health Canada.

The lack of sputum and supernatant thus far, has meant that we were unable to investigate possible important effects of inflammatory cells such as eosinophils, neutrophils, or neurotrophins such as BDNF or NGF on capsaicin cough responses. Therefore, although we have demonstrated a change in function of the cough reflex with airflow obstruction and inflammation, we have been unable to determine a link between how or where this occurs in the reflex arc. It is possible that the increased sensitisation could be in the peripheral afferent nerves or the central nervous system. Hence, exploring neuro-immune interactions would be an important next step.

iv. Interpretation of results

One possible anatomical interpretation for the results found in the methacholine and allergen challenge studies is the effect of bronchoconstriction on the deposition of capsaicin in the bronchial tree. It is possible that changes in airway calibre can worsen ventilation heterogeneity within the lungs [303, 304] leading to capsaicin being deposited in the more proximal central airways, where bronchial c-fibres are thought to be located. However, given that bronchoconstriction was also associated with an increase in spontaneous cough frequency, this possible explanation is less likely.

As we have utilised a TRPV1 agonist to demonstrate heightened cough responses, it would suggest that inhibiting TRPV1 would be the obvious candidate for suppressing cough. However, a clinical trial of a TRPV1 antagonists in patients with chronic cough showed no improvements in 24 hour objective coughs, despite showing an improvement in cough reflex sensitivity to capsaicin [305]. This one study suggests that experimentally evoked capsaicin challenge testing and objective spontaneous coughs may involve different mechanisms in patients with chronic cough. In my study there was an association between Emax and 24 hour objective cough frequency, but it is still unknown whether a reduction in Emax to capsaicin evoked coughs would also translate to a reduction in spontaneous coughs in patients with asthma. Furthermore, although TRPV1 antagonists failed to show improvements in patients with chronic cough, this does not exclude the possibility it might still be useful in patients with asthma during periods of variable airflow obstruction, acute bronchoconstriction or exposure to allergen.

More recently, antagonism of P2X3 and neurokinin 1(NK-1) receptors have shown more promise as they have demonstrated a reduction in objective cough frequency in patients with chronic idiopathic cough [306], and cough in lung cancer [126] , but these has not been not yet been studied in patients with asthma. The nature of coughing in patients with chronic cough is vastly different; they cough to innocuous triggers such as perfumes, aerosols, changes in temperature, talking, laughing and cough tens to sometimes hundreds of times per hour. These clinical features of cough hypersensitivity are not typical in patients with mild/moderate stable asthma and hence novel anti-tussives used in chronic cough may not be effective.

The difference in full dose capsaicin evoked cough responses and single dose capsaicin ED50 needs to be clarified. In the first observational study, we compared full dose capsaicin dose response curves using advanced non-linear PD modelling, whilst in the latter 2 studies, we opted to use just four inhalations of one ED50 dose of capsaicin. Our reasons for using an individualised single dose challenge have been justified in the general methods section and individual studies, however, interpreting ED50 evoked coughs needs to be clarified. Firstly, the ED50 coughs have been modelled using generalised estimating equations (GEE), which is a linear model. Secondly, the increase in coughs after a single dose of ED50 seen after methacholine and allergen challenge does not inform us about how the full dose response capsaicin may have changed; it is possible there is a left shift (lower ED50, hypersensitivity), an upwards shifts (higher Emax, hyper-responsiveness) or both (See Figure 6.1). This makes it difficult to fully interpret the increase in ED50 coughs with a neuronal mechanism; peripheral and/or central sensitisation and/or phenotypic switching.

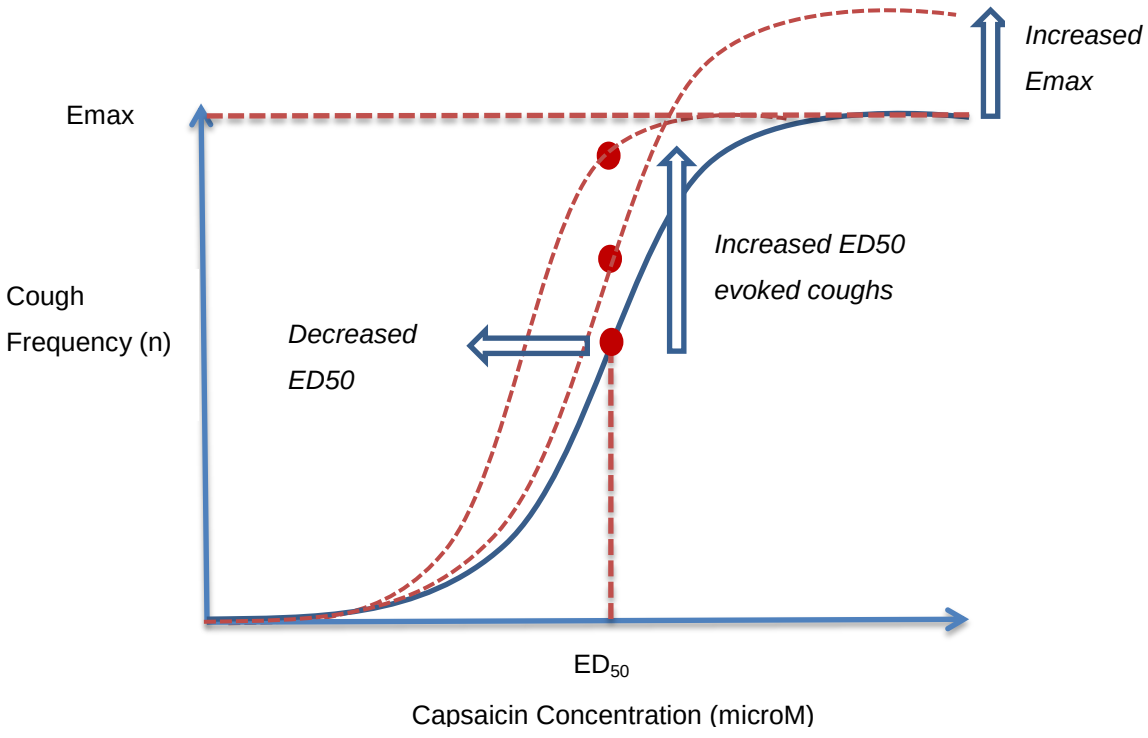

Figure 6.1: Schematic showing the possible effects of increased capsaicin ED50 coughs on the full dose capsaicin challenge. An increase in capsaicin ED50 coughs could be due to a left shift, an upwards shift or both.

6.4 Directions for future work

There are a number of different lines of research that could be undertaken to take this work forward both academically and clinically. However, the central theme of my future work is to focus broadly on the mechanisms of neuro-immune interactions in asthma; how do sensory nerves interact with airway inflammatory cells to cause excessive coughing? The key questions which I feel need further experimental investigations are:

1. Is there a spatial interaction between airway nerves and inflammatory cells?
2. Do airway sensory nerves directly or indirectly interact with airway inflammatory cells? And if so, which neurotransmitters are involved?
3. Is there any evidence of central sensitisation in patients with asthma?

The anatomical work is focused on investigating whether or not nerves and airway inflammatory cells are in close enough proximity to interact. To untangle the cellular basis of how bronchoconstriction and airway inflammation might interact with nerves to evoke coughing, a number of different of airway and tussive challenge models are available. Although my work has focused on methacholine and allergen challenge, alternative challenges are currently in use and these may provide important mechanistic insights. For example, viruses are the commonest cause of exacerbations of asthma, but there is very little data in humans about the role of cough and nerves during an acute exacerbation. Safe models of rhinovirus infections in patients with mild asthma are now being used to study mechanisms of virus induced exacerbations and hence it would be feasible to also study cough and nerves in that setting [307]. A range of possible receptor targets could be antagonised to investigate the mechanism of the cough reflex after different experimental challenges and this can now readily be supplemented by objective cough monitoring.

The possible interactions between airway structural cells such as the epithelium, smooth muscle, and fibroblasts and airway nerves also needs to be considered, as a recent clinical study with bronchial thermoplasty has demonstrated a reduction in airway nerves [308]. Finally, changes in evoked and spontaneous coughs could also be due to changes in central sensitivity in the brainstem, therefore, how bronchial provocation and cough challenges affect the complex neuronal pathways of the central nervous system requires further investigation. One study has recently demonstrated the feasibility combining functional MRI and tussive challenges in patients with chronic cough to understand the possible mechanism of central pathways [129].

From a clinical perspective, it would be useful to validate the Emax and ED50 capsaicin cough challenge in different asthma phenotypes, assess if ED50 coughs is a useful tool to identify patients with an exaggerated cough response, and test the efficacy of anti-tussives in those patients coming back for clinical review. This can then also be correlated with objective cough rates, and questionnaires such as the LCQ and ACQ. Analysing such data longitudinally would

help determine the variability and repeatability in a real life cohort of patients and this would help in the development of studies with sufficient power to detect clinically meaningful differences.

The role of airway nerves and cough could also be investigated in other respiratory diseases such as chronic obstructive pulmonary disease (COPD), lung cancer and interstitial lung diseases where cough is a troublesome symptom. A recent study has demonstrated different neuro-phenotypes in airway diseases by investigating cough responses to a range of inhaled irritants in humans and guinea pigs [156].

Finally, given airway nerves transmit information to the central nervous system, the role of nerves in other symptoms such as shortness of breath, chest tightness, and wheeze also needs further investigation.

6.5 Conclusion

In this thesis, I have made an attempt at understanding the mechanism by which airway nerves evoke cough in patients with asthma. These experiments have shown that there is evidence of neuronal dysfunction in patients with asthma even during stability, but these can be altered by bronchoconstriction and allergen exposure. Importantly, we have demonstrated the safety of performing full dose capsaicin cough challenge in patients with asthma during stability and capsaicin ED50 challenges after methacholine and allergen challenge. Given the heterogeneity of asthma phenotypes, it would be important to understand whether different neuro-phenotypes exist in different asthma phenotypes. Given the importance of airway inflammation and bronchial hyper-responsiveness in asthma, more research needs to be done to unravel the interactions between immune cells, airway structural cells and nerves.

7 References

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8 Appendix:

8.1 Published Papers:

1. Capsaicin-evoked cough responses in asthmatic patients: Evidence for airway neuronal dysfunction

2. Objective Cough Frequency, Airway Inflammation, and Disease Control in Asthma

3. Toward understanding and managing chronic cough

Capsaicin-evoked cough responses in asthmatic patients: Evidence for airway neuronal dysfunction

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Background: Cough in asthmatic patients is a common and troublesome symptom. It is generally assumed coughing occurs as a consequence of bronchial hyperresponsiveness and inflammation, but the possibility that airway nerves are dysfunctional has not been fully explored.

Objectives: We sought to investigate capsaicin-evoked cough responses in a group of patients with well-characterized mild-to-moderate asthma compared with healthy volunteers and assess the influences of sex, atopy, lung physiology, inflammation, and asthma control on these responses.

Methods: Capsaicin inhalational challenge was performed, and cough responses were analyzed by using nonlinear mixed-effects modeling to estimate the maximum cough response evoked by any concentration of capsaicin (E_{max}) and the capsaicin dose inducing half-maximal response (ED_{50}).

Results: Ninety-seven patients with stable asthma (median age, 23 years [interquartile range, 21-27 years]; 60% female) and 47 healthy volunteers (median age, 38 years [interquartile range, 29-47 years]; 64% female) were recruited. Asthmatic patients had higher E_{max} and lower ED_{50} values than healthy volunteers. E_{max} values were 27% higher in female subjects ($P = .006$) and 46% higher in patients with nonatopic asthma ($P = .003$) compared with healthy volunteers. Also, patients with atopic asthma had a 21% lower E_{max} value than nonatopic asthmatic patients ($P = .04$). The ED_{50} value was 65% lower in female patients ($P = .0001$) and 71% lower in all asthmatic patients ($P = .0008$). ED_{50} values were also influenced by asthma control and serum IgE levels, whereas E_{max} values were related to 24-hour cough frequency. Age, body mass index, FEV_1, PC_{20}, fraction of exhaled nitric oxide, blood eosinophil counts, and inhaled steroid treatment did not influence cough parameters.

Conclusion: Patients with stable asthma exhibited exaggerated capsaicin-evoked cough responses consistent with neuronal dysfunction. Nonatopic asthmatic patients had the highest cough responses, suggesting this mechanism might be most important in type 2–low asthma phenotypes. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Atopy, transient receptor potential vanilloid type 1, vagus, pharmacodynamic modeling

Asthma affects an estimated 300 million persons worldwide and is characterized by symptoms of cough, wheeze, chest tightness, and shortness of breath. Current dogma suggests asthma symptoms arise as a consequence of airway narrowing, bronchial hyperresponsiveness, and airway inflammation. Yet despite effective treatments targeting each of these components of asthma, many patients have substantial residual symptoms. Even in a clinical trial setting with optimal inhaled treatment, up to 50% of asthmatic patients have symptoms that are not well controlled.[1] Although for some patients adherence might be an issue,[2] it is also likely that undiscovered mechanisms explain the heterogeneity in asthma clinical phenotypes and treatment responses.

Symptoms are often challenging to study because they can only be reported subjectively; however, cough is readily amenable to objective quantification.[3] Cough in asthmatic patients is not only a common[4] and troublesome[5] symptom but also predicts disease severity[6] and poor prognosis,[7] suggesting it reflects important pathophysiologic processes, yet remarkably little is understood about the underlying mechanisms. The general assumption is that airway afferent nerves activating the cough reflex are stimulated by inflammatory mediators, mucus, and bronchospasm, and the possibility that these neuronal pathways are dysfunctional is rarely considered.

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Abbreviations used
ACQ: Asthma Control Questionnaire
ATS: American Thoracic Society
C2: Concentration of capsaicin inducing at least 2 coughs
C5: Concentration of capsaicin inducing at least 5 coughs
ED_{20}: Capsaicin dose inducing half-maximal response
E_{max}: Maximum cough response evoked by any concentration of capsaicin
FENO: Fraction of exhaled nitric oxide
LCQ: Leicester Cough Questionnaire
TRPV1: Transient receptor potential vanilloid type 1

Vagal afferent fibers innervate the airways and are responsible for mediating symptoms and airway reflexes.[8,9] Coughing is readily evoked by activation of C fibers; these networks of unmyelinated and chemically sensitive afferents are characteristically sensitive to capsaicin (chili pepper extract) through activation of the transient receptor potential vanilloid type 1 (TRPV1) channel. Aδ fibers are sparsely distributed, thinly myelinated fibers in the proximal airways that also evoke cough. They protect the airways by responding to mechanical stimuli (eg, foreign objects) and changes in osmolarity and acidity. Importantly, they are typically insensitive to capsaicin and inflammatory mediators and do not usually express TRPV1.

Experimentally evoked cough responses to inhaled irritants are an established tool for studying the cough reflex and thus airway nerve function. Capsaicin is the most widely used agent, and the concentration of capsaicin causing 5 or more coughs (C5) is considered a measure of cough reflex sensitivity.[5] However, previous studies in asthmatic patients have produced conflicting results, with some studies suggesting sensitization of the cough reflex (reduced C5) and others finding no difference from healthy control subjects.[10-13]

We have recently investigated capsaicin-evoked cough responses using repeat inhalations of capsaicin and concentrations beyond C5. Nonlinear mixed-effects modeling of these data found maximum cough response evoked by any concentration of capsaicin (E_{max}) best discriminated patients with chronic cough from healthy control subjects/patients with mild asthma; the difference between healthy and asthmatic subjects did not quite reach *a priori* statistical significance.[14] Therefore we have studied capsaicin-evoked cough responses in a larger group of patients with well-characterized mild-to-moderate asthma and healthy volunteers. We also investigated the influence of sex, atopic status, lung physiology, inflammation, and asthma control on capsaicin-evoked cough responses. Some of the results of these studies have been previously reported in the form an abstract.[15,16]

METHODS

Subjects

Subjects with a physician's diagnosis of asthma were recruited but not selected for symptoms of cough. Treatment with salbutamol as required and/or inhaled corticosteroids at 500 μg or less of fluticasone propionate equivalent daily with or without a long-acting bronchodilator was permitted. Subjects with uncontrolled symptoms according to Global Initiative for Asthma classification or not receiving stable medication over the previous 4 weeks were excluded. Healthy control subjects approximately matched for age were also recruited. We excluded current smokers, those with a recent chest infection or exacerbation, and those using any medication that might alter the cough responses (eg, opiates, gabapentin, anticholinergics, and theophylline).

TABLE I. Comparison of asthmatic patients and healthy volunteers

	Asthmatic patients	Healthy volunteers	*P* value
Participants (no.)	97	47	
Age (y)	23.0 (21.0-27.0)	38.0 (29.0-47.0)	<.001
Sex (male/female)	39/58	17/30	.64
BMI (kg/m^2)	24.1 (21.8-27.0)	25.0 (22.2-28.6)	.25
Smoking history (pack years)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	.34
FEV_1 (% predicted)	95 (87.0-103.0)	103.0 (97.0-115.0)	<.001
FVC (% predicted)	102 (95-110)	106.0 (99.0-118.0)	.02
Cough frequency (coughs/h)			
24 h	1.1 (0.5-2.4)	0.2 (0.0-0.9)	<.001
Day	1.6 (0.7-3.8)	0.2 (0.0-1.3)	<.001
Night	0.0 (0.0-0.4)	0.0 (0.0-0.1)	.25

Data are presented as medians and interquartile ranges and compared by using the Mann-Whitney *U* test.
BMI, Body mass index; *FVC*, forced vital capacity.

The study protocols for healthy control subjects and asthmatic patients were approved by the local research ethics committee (13/COA/002 and 13/CLU/001), and all subjects provided written informed consent.

Study protocol and procedures

For full details, see the Methods section in this article's Online Repository at www.jacionline.org. Asthmatic patients attended on 3 occasions. On visit 1, subjects underwent history and examination and completed the Asthma Control Questionnaire (ACQ), Leicester Cough Questionnaire (LCQ), fraction of exhaled nitric oxide measurement (FENO; NIOX, Aerocrine, Solna, Sweden), spirometry, and bronchodilator reversibility measurement, and an ambulatory cough monitor (VitaloJAK; Vitalograph, Buckinghamshire, United Kingdom) was fitted for the next 24 hours. At visit 2, at least 48 hours later, subjects underwent full blood count, serum IgE measurement, skin prick testing, and PC_{20} measurement. Subjects completed a peak flow diary twice a day for 7 days after visit 2.

Visit 3 took place at least 1 week later, and a capsaicin cough challenge was performed, as previously described,[14] by using a dosimeter (KoKo Dosimeter; Ferraris, Hertford, United Kingdom) and a nebulizer pot (Model 646; Devilbiss Healthcare, Somerset, Pa) with an inspiratory flow limiter. Briefly, 4 inhalations were administered, 30 seconds apart, of doubling doses of capsaicin (0.48-1000 μmol/L). After each inhalation, the number of coughs in the first 15 seconds was counted and later verified by using a cough monitor (VitaloJAK). The challenge was completed when the patient reached the final dose or the maximal tolerated dose. Spirometry was performed before and after each challenge.

Healthy volunteers attended on 2 occasions. On visit 1, consent was obtained, screening and spirometry were performed, and the ambulatory cough monitor was attached. On visit 2, the capsaicin challenge was performed.

Statistical analysis

Cough responses to capsaicin were analyzed by using nonlinear mixed-effects modeling software (NONMEM 7.3; ICON Development Solutions, Ellicott City, Md) and the Laplace estimation method.[17,18] Additional investigations of the NONMEM output and statistical and graphic analyses were performed in Matlab R2014a (MathWorks, Natick, Mass). We applied a modeling approach developed previously[14]; the number of coughs was assumed to follow a Poisson distribution adjusted for tachyphylaxis evoked by repeat inhalations of the same capsaicin dose. The capsaicin-evoked cough response curve was assumed to follow a sigmoid shape in which the maximum response was denoted as E_{max} and the capsaicin dose inducing half-maximal response (ED_{50}). The effect of continuous and categorical covariates were investigated, including age, sex, body mass index, disease state (healthy or

TABLE II. Description of the key characteristics of asthmatic patients

Characteristic	All asthmatic patients (n = 97)	Male subjects (n = 39)	Female subjects (n = 58)
Age (y)	23.0 (21.0-27.0)	23.0 (21.0-25.0)	22.0 (20.0-27.5)
Age of onset (y)	7.0 (4.0-14.0)	7.0 (4.0-14.0)	7.5 (4.0-14.3)
Exacerbations/y	0 (0-0)	0 (0-0)	0 (0-0)
ACQ score	0.71 (0.43-1.00)	0.86 (0.50-1.21)	0.64 (0.43-1.00)
Smoker			
No (%)	91.8	89.7	93.1
Ex-smoker (%)	8.2	10.3	6.9
Yes (%)	0	0	0
GINA category			
Well controlled (%)	50.5	56.4	46.6
Partly controlled (%)	49.5	43.6	53.4
Steroid naive (%)	48.5	43.6	43.1
Receiving ICS alone (%)	34.0	23.1	41.4
Receiving ICS/LABA combination (%)	17.5	20.5	15.5
Daily ICS dose (μg of FP equivalent)	200 (100-400)	200 (100-400)	200 (100-400)
Reversible volume (mL)	180 (75-275)	260 (160-420)	135 (48-230)
Proportion with significant reversibility ≥12% (%)	14.4	17.9	12.1
FENO (ppb)	34 (21-75)	37 (23-91)	30 (21-54)
Methacholine PC_{20} (mg/mL)	0.94 (0.25-3.26)	1.62 (0.46-3.21)	0.86 (0.24-3.40)
Bronchial hyperreactivity ≤8 mg/mL (%)	81.4	79.5	82.8
Peak flow variability (%)	5.4 (3.4-6.9)	5.7 (4.2-7.7)	5.2 (3.2-6.8)
Atopic* (%)	78.4	87.2	72.4
Serum eosinophils ($\times 10^9$/L)	0.21 (0.13-0.35)	0.25 (0.13-0.41)	0.21 (0.13-0.32)
Serum total IgE (kU/L)	200 (58-470)	210 (70.0-460)	175 (41-483)

Data are presented as medians (interquartile ranges).
FP, Fluticasone propionate; *GINA*, Global Initiative for Asthma; *ICS*, inhaled corticosteroid; *LABA*, long-acting bronchodilator.
*At least 1 positive skin prick test response to common aeroallergen. See this article's Online Repository at www.jacionline.org for the full list.

asthmatic), atopy (atopic or nonatopic), predicted FEV_1, cough frequency, serum IgE measurement, blood eosinophil count, FENO value, methacholine PC_{20}, and ACQ and LCQ score. The pharmacodynamic model was used to simulate typical dose-response curves for significant covariates. Finally, we also calculated the traditional concentration of capsaicin inducing at least 2 coughs (C2) and C5 end points from our challenges to explore the differences between healthy control subjects and asthmatic patients and the effects of the same continuous and categorical covariates. See the Methods section in this article's Online Repository for full details of the nonlinear model and C2/C5 analyses.

RESULTS

Subjects

Ninety-seven asthmatic patients and 47 healthy volunteers were recruited and completed all visits; see Fig E1 in this article's Online Repository at www.jacionline.org for exclusions, withdrawals, and missing data. Asthmatic patients and healthy volunteers were well matched for sex, body mass index, and smoking history, but asthmatic patients were significantly younger and had slightly reduced lung volumes compared with healthy volunteers (Table I). Asthmatic patients had low cough frequencies, but these were statistically higher than those in healthy volunteers.

Asthmatic patients' symptoms were well or partly controlled (Table II). Almost 50% were steroid naive, and one third were receiving a low dose of inhaled steroid. The majority were atopic based on 1 or more positive skin prick test responses to a common aeroallergen and exhibited bronchial hyperresponsiveness to methacholine.

Application of the pharmacodynamic model

The model parameters are described in Table III, and Fig E2 in this article's Online Repository at www.jacionline.org shows a very good fit of the model to the observed raw capsaicin-evoked cough data. The model fit was also accurate at the individual level (see Fig E3 in this article's Online Repository at www.jacionline.org). The characteristics that significantly affected E_{max} and ED_{50} values are also summarized in Table III.

Asthma, sex, and atopic status significantly affect capsaicin-evoked cough responses

Asthmatic patients (both atopic and nonatopic) had higher E_{max} and lower ED_{50} values compared with healthy volunteers (Tables III and IV and Fig 1, *A*). Specifically, asthmatic patients had a 71% lower ED_{50} value ($P = .0008$) than healthy volunteers, with no difference in ED_{50} values between atopic and nonatopic asthmatic patients. Nonatopic asthmatic patients had a 46% higher E_{max} value ($P = .003$) compared with healthy volunteers, but atopic asthmatic patients had a 21% lower E_{max} value ($P = .04$) than nonatopic asthmatic patients (Tables III and IV and Fig 1, *C*). In addition, female sex increased E_{max} values by 27% ($P = .006$) and decreased ED_{50} values by 65% ($P = .0001$; Tables III and IV and Fig 1, *B*).

The interaction between these characteristics was simulated to create the typical dose-response curves shown in Fig 2. Healthy male subjects had the lowest cough responses, whereas female nonatopic asthmatic patients had the highest cough responses to capsaicin.

Capsaicin-evoked cough responses are associated with cough frequency and asthma control

Higher 24-hour cough frequency (coughs per hour) was associated with increased E_{max} values ($P = .006$; Fig 3, *A*, and Table III). Specifically, for every unit that cough frequency

TABLE III. Parameter estimates of the final population pharmacodynamic model

Model parameter	NONMEM estimate (RSE%)	Bootstrap estimate (95% CIs)*
Structural model		
θ_1: E_{max}	3.57 (10)	3.59 (2.93 to 4.52)
θ_2: ED_{50}	67.6 (33)	68.8 (35.0 to 135.9)
θ_3: γ	2.11 (5)	2.13 (1.89 to 2.55)
θ_4: E_0	0.063 (23)	0.062 (0.041 to 0.091)
θ_5: K	0.142 (10)	0.143 (0.115 to 0.173)
Covariate effects†		
θ_6: Asthma on ED_{50}	−0.71 (13)	−0.704 (−0.842 to −0.462)
θ_7: Female sex on ED_{50}	−0.647 (15)	−0.654 (−0.807 to −0.412)
θ_8: Asthma (nonatopic) on E_{max}	0.462 (37)	0.448 (0.156 to 0.870)
θ_9: Female sex on E_{max}	0.269 (39)	0.250 (0.043 to 0.494)
θ_{10}: Atopy on E_{max}	−0.209 (35)	−0.204 (−0.353 to −0.033)
θ_{11}: Cough frequency on E_{max}	0.0482 (29)	0.0482 (0.0137 to 0.0807)
θ_{12}: ACQ score on ED_{50}	−0.66 (39)	−0.69 (−1.28 to −0.17)
θ_{13}: IgE level on ED_{50}	0.00145 (49)	0.0014 (0.0002 to 0.0029)
Interindividual variability (% CV)‡		
η_1: E_{max}	40.1 (8)	38.0 (29.0 to 47.2)
η_2: ED_{50}	281.7 (10)	272.3 (172.9 to 446.1)
η_3: γ	36.8 (13)	39.0 (23.8 to 55.1)

$\theta_{1\text{-}5}$ define model parameters for a healthy male subject with the typical (median) population values for each model-incorporated covariate. $\theta_{6\text{-}13}$ define the influence of each covariate on the model parameters E_{max} and ED_{50} (see equations E5 and E6 in this article's Online Repository at www.jacionline.org). For example, the presence of asthma decreases ED_{50} by 71% (θ_6). $\eta_{1\text{-}3}$ refer to the interindividual variability with regard to E_{max}, ED_{50}, and γ (slope).

CV, Coefficient of variation; *E_0*, average cough response at baseline; γ, Hill factor (slope); *K*, tachyphylaxis parameter; *RSE*, relative standard error.

*Estimates were obtained from bootstrapping with the final population model. The median of the bootstrap sample estimates together with nonparametric 95% CIs are reported for each parameter.

†The increase in objective function (−2 log-likelihood) after removing each covariate effect from the final model is listed below, followed by the corresponding likelihood ratio test *P* value in parentheses: θ_6 = 11.25 (*P* = .0008); θ_7 = 14.43 (*P* = .0001); θ_8 = 8.55 (*P* = .003); θ_9 = 7.47 (*P* = .006); θ_{10} = 4.24 (*P* = .04); θ_{11} = 7.40 (*P* = .006); θ_{12} = 5.21 (*P* = .02); θ_{13} = 6.58 (*P* = .01).

‡Coefficient of variation is calculated as follows: $\sqrt{(e^{\omega^2}-1)}\cdot 100$.

TABLE IV. E_{max} and ED_{50} values for healthy volunteers and asthmatic patients

	Healthy volunteer		Asthmatic patient			
			Atopic		Nonatopic	
	Male	Female	Male	Female	Male	Female
E_{max}	3.57	4.53	4.13	5.24	5.22	6.62
ED_{50}	67.6	23.86	19.6	6.92	19.6	6.92

Values represent E_{max} and ED_{50} values for a typical healthy and asthmatic male/female (ie, with median values for all model-incorporated covariates).

increased, E_{max} values increase by approximately 5%. Also, higher ACQ scores were associated with lower ED_{50} values (*P* = .02; Fig 3, *B*, and Table III). The median ACQ score in the studied asthmatic population was 0.71; if, for example, this score increased by 1 unit (1.71), the ED_{50} value decreased by 48%. Finally, higher IgE levels were associated with an increase in ED_{50} values (*P* = .01; Fig 3, *C*, and Table III). For every unit that IgE increased, ED_{50} increased approximately by 0.15%. The effect of these continuous covariates on the simulated dose-response curves is shown in Fig 4.

Other covariates, such as age, body mass index, FEV_1 percent predicted, PC_{20} and FENO values, serum eosinophil counts, and LCQ scores had no significant influence on model parameters (E_{max}, ED_{50}, and γ). There was a nonsignificant trend (*P* = .09) that asthmatic patients receiving steroids had a lower maximal number of coughs (E_{max}) compared with asthmatic patients not receiving steroids, but no differences were observed for ED_{50} or γ values. In addition, the magnitude of the steroid dose (in subjects receiving steroids) did not affect the model parameters.

Termination of the cough challenge

At higher doses of capsaicin, increasing numbers of patients elected to terminate the challenge (se Figs E2 and E4, *A*, in this article's Online Repository at www.jacionline.org). The most important determinant of whether a subject was likely to terminate the challenge at a given dose level was the total cumulative number of coughs up to the maximum tolerated dose (see Fig E4, *B*). When subjects reached an approximate threshold of 40 to 60 cumulative coughs, they tended to terminate no matter whether this threshold was reached at a low or high capsaicin dose (see Fig E4, *B*).

Safety of full-dose capsaicin challenge

Transient bronchoconstriction after inhaling capsaicin has been reported in patients with asthma.[19] In this study there was no significant bronchoconstriction after inhaling high-dose capsaicin; the median change in percentage FEV_1 after capsaicin challenge was −1.7% (interquartile range, 0.8% to −4.3%). However, 1 subject did decrease their FEV_1 by 54% and coughed a total of 38 times at a low concentration of capsaicin (15.6 μmol/L). The subject received 4 inhalations of salbutamol (100 μg), after which FEV_1 improved to baseline.

Exploratory analysis of C2 and C5 end points

Asthmatic patients demonstrated significantly lower C2 and C5 than healthy control subjects (*P* = .002 and *P* = .013, respectively; see Table E1 in this article's Online Repository at www.jacionline.org). However, there was substantial variability

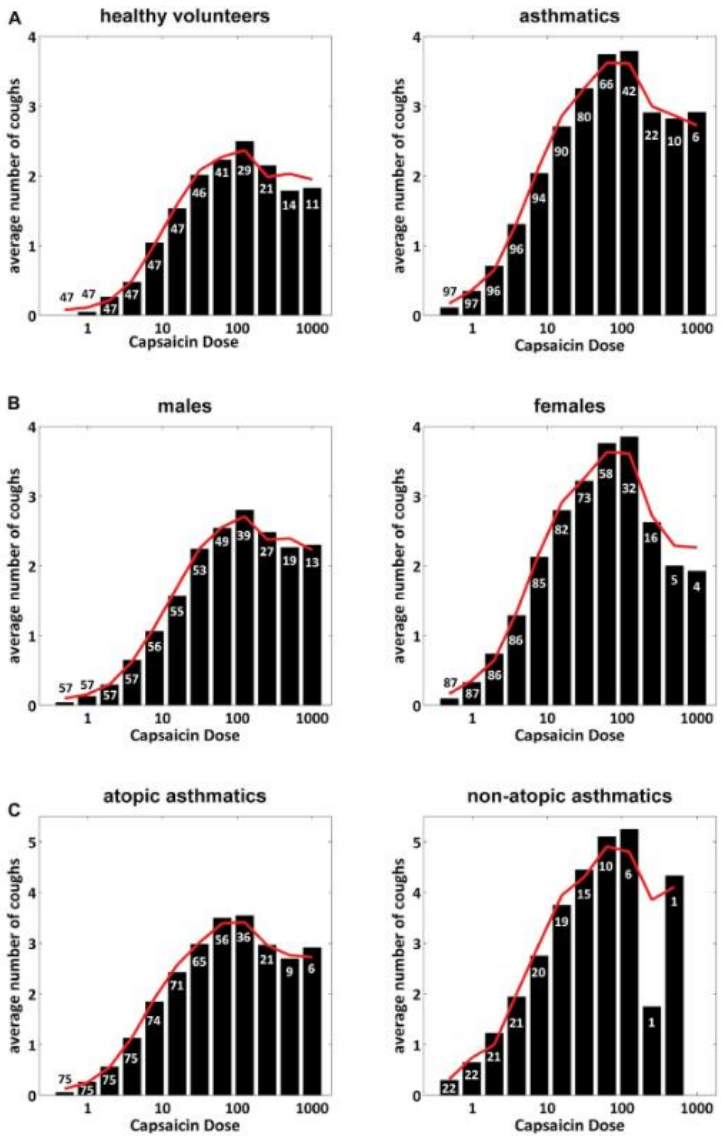

FIG 1. Model fit to the observed dose-response data stratified by significant categorical covariates: **A,** healthy volunteers versus asthmatic patients; **B,** male versus female subject; **C,** atopic vs nonatopic asthmatic patients. Average number of coughs *(y-axis)* is plotted against capsaicin dose *(x-axis). Bars* and *red lines* represent the observed and model-predicted number of coughs, respectively, averaged across all subjects in a specific covariate subpopulation and all inhalations at a given capsaicin dose level. The number of subjects in each subpopulation subjected to at least 1 inhalation at a given dose level are also reported inside (or above) each bar.

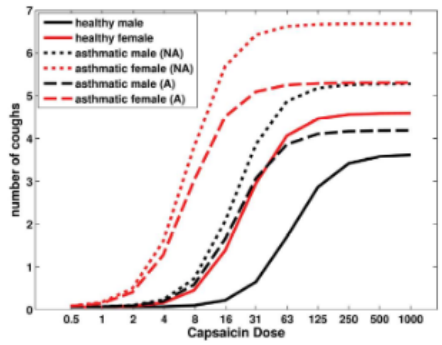

FIG 2. Model-simulated typical dose-response curves for subjects with different population characteristics. The typical (median) population values have been assumed for the model-incorporated continuous covariates. *A*, Atopic; *NA*, nonatopic.

between subjects and overlap between the 2 groups for C2 and C5; 42% of healthy volunteers and 30% of asthmatic patients did not have a measureable C5 (see Fig E5 in this article's Online Repository at www.jacionline.org). Furthermore, multiple linear regression models did not show significant relationships between covariates identified as important in the nonlinear model; only log ACQ scores were related to log C5 (see the Results section in this article's Online Repository at www.jacionline.org for full details).

DISCUSSION

This study is the first to show evidence of heightened capsaicin-evoked cough responses and thus neuronal dysfunction in patients with stable mild-to-moderate asthma. These changes in capsaicin responses can only be fully appreciated by extending cough challenge beyond the standard C5 end point and with implementation of population pharmacodynamic modeling to provide individual estimates of ED_{50} and E_{max} values. Using this methodology, we showed that asthmatic patients, when compared with healthy volunteers, started to cough at lower capsaicin doses (lower ED_{50} values) and had greater maximal cough responses (higher E_{max} values), both of which are indicative of increased excitability of the neuronal pathways controlling cough. Notably, both sex and atopic status significantly influenced cough responses, with nonatopic female asthmatic patients exhibiting the greatest degree of neuronal dysfunction. Importantly, measures of inflammation, such as FENO, bronchial hyperresponsiveness (PC_{20}), or lung function, did not influence E_{max} or ED_{50} values, suggesting this neuronal dysfunction was independent of airway inflammation and bronchial hyperresponsiveness.

It is difficult to directly compare these results with other studies that have used the standard C5 end point because the patients' demographics are very different from those in our study. Doherty et al[10] compared C5 values in a group of asthmatic patients and healthy volunteers and demonstrated an increased sensitivity to capsaicin. However, subjects in that study were older and had more severe asthma (mean FEV_1 percent predicted [71%], all receiving inhaled steroids, 21% receiving an inhaled anticholinergic agent, and 8% receiving theophylline), and only 43% were nonsmokers. Fujimura et al[13] evaluated just 18 asthmatic patients with worse lung function (mean FEV_1 percent predicted, 67%) yet found no difference in C5 from healthy control subjects. It was striking that we demonstrated highly statistically significant differences in capsaicin-evoked responses in a cohort of younger patients who were all nonsmokers with good lung function, and almost half were steroid naive. Our exploratory analysis extrapolating C2 and C5 from our challenge protocol shows these end points are statistically different in asthmatic patients compared with healthy volunteers. However, unlike E_{max} and ED_{50} values, C2 and C5 did not relate to any of the clinical features of asthma apart from control, not even with cough frequency, which might be expected. This suggests C2 and C5 are not only less powerful than E_{max} and ED_{50} but also do not represent the underlying mechanisms important in different asthma phenotypes.

Sex differences in evoked cough have not previously been specifically described in asthmatic patients but have been repeatedly shown in both healthy volunteers and patients with chronic cough, with female patients demonstrating heightened responses compared with male patints.[14,20] However, the observation that patients with nonatopic asthma have exaggerated responses (increased E_{max} values) compared with those with atopic asthma is a novel finding that was unexpected and requires further exploration. Consistent with this, lower IgE levels were associated with reduced threshold for capsaicin-evoked coughs (reduced ED_{50} values). The combined effects of sex and atopy suggest that the highest cough responses and thus the greatest degree of neuronal dysfunction were exhibited by female nonatopic asthmatic patients. By comparison, atopic male asthmatic patients displayed the lowest levels of dysfunction among asthmatic patients, and healthy male subjects had the lowest responses overall (Fig 2). Our findings could help explain the results of a cluster analysis of asthmatic patients, highlighting 2 discordant groups in which symptoms did not match the degree of airway inflammation.[21] Interestingly, excessive symptoms were observed in the predominantly female cluster with fewer atopic subjects, whereas low symptoms were observed in the cluster who were predominantly male and atopic. Therefore we speculate that neuronal dysfunction could explain the discordance between such clinical phenotypes of asthma.

We also found capsaicin-evoked cough responses were relevant to the clinical manifestations of asthma. Poorer asthma control, as measured by ACQ scores, was associated with lower cough thresholds (ED_{50}), and higher 24-hour objective cough frequencies were associated with higher maximal cough responses (E_{max}). It is interesting to speculate that these differences in capsaicin-evoked cough responses might represent different mechanisms of neuronal dysfunction in either the peripheral or central nervous system. Moreover, these changes in cough reflex responses might also provide a surrogate for changes in other populations of airway nerves responsible for mediating symptoms that are less easily quantified, such as chest tightness and breathlessness.

Nerve fibers have a maximum frequency of action potential firing, as determined based on the rate of membrane repolarization (refractory period). However, the threshold for action potential generation can be decreased by changes in the membrane resting potential or ion channels at the nerve terminal (eg, increased expression, membrane insertion, and conformational changes).[22] Such changes can be induced by a range of inflammatory mediators, including cytokines, chemokines, and growth factors. Aδ fibers can also become responsive to capsaicin

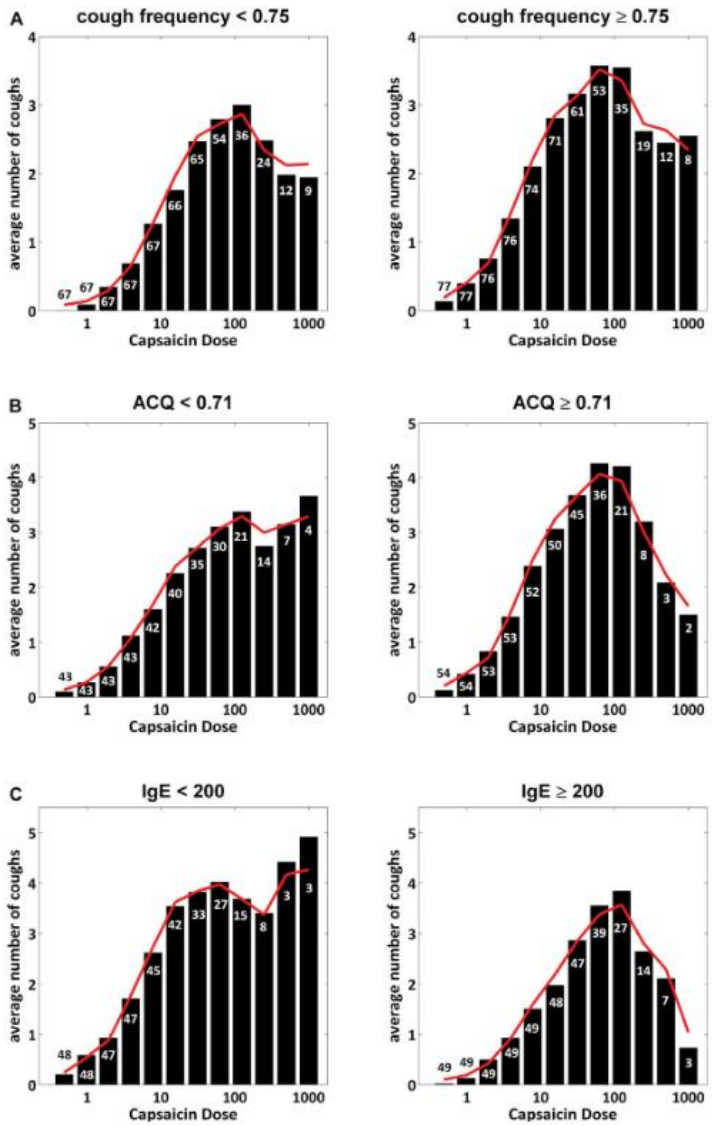

FIG 3. Model fit to the observed dose-response data stratified by significant continuous covariates. Subpopulations are stratified in relation to the median population value of each covariate: **A,** low versus high cough frequency (in coughs per hour); **B,** low versus high ACQ score; **C,** low versus high IgE levels (in kIlounits per liter). Average number of coughs *(y-axis)* is plotted against capsaicin dose *(x-axis)*. *Bars* and *red lines* represent observed and model-predicted number of coughs, respectively, averaged across all subjects in a specific covariate subpopulation and all inhalations at a given capsaicin dose level. The number of subjects in each subpopulation subjected to at least 1 inhalation at a given dose level are also reported inside (or above) each bar.

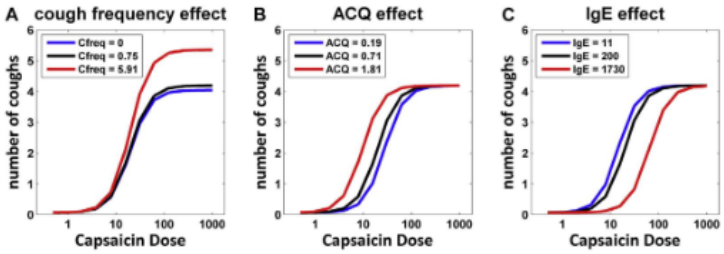

FIG 4. Effect of model-incorporated continuous covariates on simulated dose-response curves: **A,** cough frequency (in coughs per hour); **B,** ACQ score; **C,** serum IgE levels (in kIlounits per liter). Model-simulated dose-response curves show the influence of continuous covariates at 3 incremental values (5th, 50th, and 95th percentiles in the analyzed data set) by using an atopic asthmatic male subject as an example reference. The typical (median) population values have been assumed each time for the remaining continuous covariates.

after airway exposure to allergen and cigarette smoke, with novel gene expression of TRPV1, which is known as phenotypic switching.[23] This can potentially affect both ED_{50} and E_{max} values; the membrane depolarization threshold can be reached more easily, causing a leftward shift (lower ED_{50} value), but in addition, the usual E_{max} ceiling can be exceeded by the recruitment of a newly capsaicin-responsive nerve fiber subtype. Although changes to the afferent fibers innervating the airways are the most plausible explanation for the exaggerated cough responses we have observed in asthmatic patients, modification of action potentials at the first synapse in the brainstem and in the cortical and subcortical pathways could also occur. Processes analogous to central sensitization[24,25] and/or loss of descending neural inhibitory control mechanisms, as described in chronic pain states, have the potential to produce similar effects.[26] However, these possible mechanisms are largely unexplored in asthmatic patients.

Asthma is generally considered a chronic inflammatory disease, with the role of airway innervation or even the contribution of neuroimmune interactions rarely investigated. Cytokines, chemokines, growth factors, and lipids released by immune cells have all been shown to induce profound changes in the activity and sensitivity of peripheral nerve terminals in the somatosensory system and have the potential to explain the changes in neuronal function that we have observed.[27,28] In particular, growth factors, such as nerve growth factor and brain-derived neurotrophic factor, have the potential to induce long-term qualitative changes to a range of stimuli to which nerves respond, and this has been demonstrated in an animal model of allergic asthma.[23] However, the most heightened neuronal dysfunction was observed in patients with nonatopic asthma and perhaps low T_H2 disease. Therefore neuronal dysfunction has the potential to provide insights into mechanisms underlying this phenotype and suggest new treatment targets.

There are some limitations to this study. First, the study population was young, mainly atopic, and predominantly steroid naive. It is currently unclear how generalizable these findings are to other age groups with more severe disease. Second, apart from measuring serum eosinophil counts, IgE levels, and FENO values, we did not make direct measures of airway inflammation and hence were not able to investigate whether these influenced capsaicin-evoked responses. Finally, we chose capsaicin because it is the most widely used cough challenge agent, and it is with this methodology that we developed the previous pharmacodynamic model.[14] However, it remains to be seen whether other challenge agents, such as citric acid, provide different results and hence novel insights into cough mechanisms.

In conclusion, these data are consistent with the concept that neuronal dysfunction is a feature of asthma, even in those with mild stable disease. Therefore assessing capsaicin-evoked cough responses might provide an additional tool in phenotyping asthma and identifying those in whom this mechanism might be most prominent. Although our data suggest neuronal dysfunction seems to be independent of indirect measures of airway inflammation, studies are required to directly assess the effects of airway inflammation on capsaicin-evoked coughs. If neuronal dysfunction is truly independent of airway inflammation, it is unlikely to be addressed by current therapies. Hence novel neuromodulatory treatments could be a useful adjunct in treating asthma and perhaps most effective in nonatopic patients.

We thank all the subjects who participated in the study and also the National Institute for Health Research (NIHR) South Manchester Clinical Research Facility (CRF) and the NIHR/Wellcome Trust Central Manchester CRF. We also thank Dr Elizabeth Juniper and Dr Surinder Birring who gave permission to use the ACQ and LCQ, respectively, and Dr Piet van der Graaf and Dr Paul Baverel, who first developed the modeling approach for capsaicin-evoked cough responses.

Key messages

- **Using a novel challenge methodology and pharmacodynamic modeling, we have demonstrated that patients with mild-to-moderate asthma have a heightened cough response to inhaled capsaicin that is most evident in female nonatopic subjects.**
- **Unlike standard cough challenge end points (C2 and C5), capsaicin E_{max} and ED_{50} values were influenced by sex, spontaneous cough frequency, asthma control, and measures of atopy (IgE measurement and skin prick testing).**

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METHODS

Procedural details

FENO. Exhaled nitric oxide was measured at a rate of 50 mL/s (NIOX, Aerocrine) before spirometry.

Spirometry. Spirometry was performed (In2itive, Vitalograph) according to standard American Thoracic Society (ATS) and European Respiratory Society guidelines. Volume (in milliliters) and percentage reversibility were assessed after administering 400 μg of salbutamol with a Volumatic spacer device.

Questionnaires. Subjects completed the full ACQ and LCQ, as described previously. Subjects were also classified on the basis of Global Initiative for Asthma categories as having well-controlled, partly controlled, not well-controlled asthma. The latter group were excluded from the study.

Cough monitoring. Objective 24-hour cough monitoring was performed with the VitaloJAK cough recorder (VitaloJAK, Vitalograph). Twenty-four-hour ambulatory cough sound recordings were performed with a custom-built validated recording device and microphone. Briefly, this consists of a digital data logger recording sounds at a sample rate of 8 kHz, with 16-bit resolution and in wav format, which is a commonly used uncompressed sound file format. Recordings were transferred to a personal computer; silences and background noise were removed by using validated, custom-written software[E1]; and cough sounds were counted by using an audio editing package (Audition version 3; Adobe Systems, San Jose, Calif). The number of coughs was expressed as coughs per hour.

Blood sampling. Two samples of blood tests were taken from asthmatic patients for full blood counts (to assess serum eosinophils) and total IgE measurements.

Skin prick testing. Atopy was defined by the presence of at least 1 positive skin prick test response (≥3 mm) to commonly inhaled aeroallergens. The following were tested:

1. house dust mite;
2. mixed molds I: *Alternaria tenuis, Botrytis cinerea, Cladosporium herbarum, Curvularia lunata, Fusarium moniliforme,* and *Helminthosporium halodes*;
3. mixed molds II: *Aspergillus fumigatus, Mucor mucedo, Penicillium notatum, Pullularia pullulans, Rhizopus nigricans,* and *Serpula lacrymans*;
4. grass mix: Yorkshire fog/velvet grass, cocksfoot, rye grass, timothy, meadow grass/Kentucky blue grass, and tall fescue/meadow fescue;
5. tree mix (mid blossoming): birch, beech, oak, and plane;
6. cat;
7. dog;
8. histamine (positive control); and
9. saline (negative control).

Bronchial hyperresponsiveness testing. Methacholine challenge was performed to assess bronchial hyperresponsiveness by using the 2-minute tidal breathing methodology, according to standard ATS guidelines. Any inhaled medications were withdrawn per ATS guidelines.[E2] The concentration causing a 20% decrease from baseline FEV_1 in response to methacholine (PC_{20}) was documented.

Capsaicin cough challenge. A full-dose capsaicin-evoked cough challenge was performed by using the methodology described previously[E3] with a nebulizer pot (Model 646, Devilbiss Healthcare) and dosimeter (KoKo Dosimeter; Ferraris, Hertford, United Kingdom). Two milliliters of capsaicin solution at 1000 μmol/L (Stockport Pharmaceuticals, Stockport, United Kingdom) was serially diluted with 2 mL of saline (0.9%) to create solutions of concentrations of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.95, 1.95, 0.98, and 0.48 μmol/L. Spirometry was performed, and a cough monitor (VitaloJAK) was attached to aid in manual verification of capsaicin-evoked coughs. Calibrated Devilbiss 646 nebulizer pots were fitted with an inspiratory flow limiter and connected to a dosimeter (KoKo) at a pressure of 30 psi to ensure accurate dosing was achieved, which emitted between 10 to 12 μL per actuation. The full-dose capsaicin-evoked cough challenge involved administering 4 inhalations 30 seconds apart of doubling doses of capsaicin, starting from 0.48 to 1000 μmol/L. After each inhalation, the number of coughs in the first 15 seconds was recorded and later verified. The highest total number of coughs evoked at any dose of capsaicin is denoted as E_{max}, and the dose evoking half this response is denoted as ED_{50}. To explore how these novel end points compare with traditional cough challenge end points, we also calculated the concentration of capsaicin evoking at least 2 and 5 coughs (ie, C2 and C5). Our challenge methodology is slightly different from the traditional challenge because 4 inhalations rather than 1 inhalation are performed at each concentration. Therefore to calculate C2 and C5, we simply used the number of coughs evoked by the first of these 4 inhalations. If subjects did not cough 2 or 5 times during the whole challenge, then for the purposes of this analysis, a value of 2000 μmol/L was assigned. Spirometry was performed after challenge, and if there was a greater than 10% decrease in FEV_1 compared with baseline or subjects complained of any chest symptoms, then 4 puffs of salbutamol (100 μg) was administered through a spacer. The challenge ended when the patient reached the final concentration of capsaicin or the maximal tolerated dose.

Nonlinear mixed-effects modeling in detail

Population pharmacodynamic model: Model structure. Population pharmacodynamic modeling was performed with the nonlinear mixed-effects modeling software NONMEM (version 7.3, ICON Development Solutions) and the Laplace estimation method.[E4,E5] Goodness-of-fit plots, statistical analyses, and simulations were performed in Matlab R2014a (MathWorks). In total, 6606 observations (verified cough measurements) from 144 subjects were analyzed for development of the population dose-response model. The response variable (number of coughs) is discrete (count data) and was assumed to follow the Poisson distribution (Equation E1)[E6]:

$$P(Y_i = n) = \frac{e^{-\lambda_i} \cdot \lambda_i^n}{n!}, \tag{E1}$$

where $P(Y_i = n)$ is the probability that individual i is having n (=0,1,2,…) number of coughs per interval of time, and λ_i is the individual mean count response. The individual mean count response (λ_i) is expressed as a function of capsaicin dose according to Equation E2:

$$\lambda_i = E_0 + \frac{Emax_i \cdot D^{\gamma_i}}{ED50_i^{\gamma_i} + D^{\gamma_i}}, \tag{E2}$$

where E_0 represents the mean cough count at baseline (placebo), $Emax_i$ is the maximum number of coughs in individual i, $ED50_i$ is the capsaicin dose that induces half of the maximum effect in individual i, D is the administered capsaicin dose, and γ_i is the Hill factor that controls the steepness of the dose-response sigmoidal curve in individual i.

Interindividual variability random effect terms were assigned on model parameters (E_{max}, ED_{50}, and γ) by using an exponential relationship (Equation E3):

$$Emax_i = \theta_{Emax} \cdot e^{\eta_{iEmax}}, \tag{E3}$$

where θ_{Emax} is the typical E_{max} parameter value in the population, and η_{iEmax} is the E_{max} interindividual variability random effect parameter, which is assumed to be normally distributed with mean zero and variance as follows: ω^2_{Emax}.

Tachyphylaxis effect. In a previous study[E3] it was identified that incorporation of a tachyphylaxis parameter substantially improved the description of the observed cough response data after serial capsaicin inhalations. Therefore in the current work we carefully examined data with regard to the occurrence of a tachyphylaxis pattern between consecutive inhalations of the same capsaicin dose. If such a pattern was apparent, we subsequently incorporated a tachyphylaxis parameter (K) in the model (similar to Hilton et al[E3]) according to Equation E4 and investigated the extent that model fit was improved:

$$Emax_i(j+1) = Emax_i(j) \cdot e^{-K}, \tag{E4}$$

where $j = 1,2,3$; $Emax_i(j)$ and $Emax_i(j+1)$ correspond to the individual E_{max} value referring to the jth and $j+1th$ inhalation, respectively, of the same

capsaicin dose, and K is a positive real number. The above equation implies that tachyphylaxis (reduced response) occurs between consecutive inhalations of the same capsaicin dose.

Covariate model building. After development of the base population pharmacodynamic model, a covariate analysis was performed in an effort to explain some of the observed interindividual variability in cough responses. Both continuous and categorical covariates were investigated in the covariate model–building procedure, including age, sex, body mass index, disease state (health or asthma), atopy (atopic or nonatopic), predicted FEV_1, cough frequency from the monitoring of spontaneous cough, IgE levels, eosinophil levels, FENO levels, and ACQ and LCQ scores. Empiric Bayes estimates of the interindividual variability random effects (η_i) from the base model (without any covariates) were used for an initial screening of the covariates, given a low η shrinkage.[E27] Subsequently, covariate model building was performed with a stepwise forward inclusion–backwards deletion procedure in which covariate selection is guided at each step by likelihood ratio test between nested models.[E28,E29] A bootstrapping procedure (n = 1000) was performed with PsN 3.7.6 (Perl-speaks-NONMEM)[E30] for the final model to evaluate the robustness of the parameter estimates and provide nonparametric 95% CIs. A covariate effect was retained in the final model only if all of the following conditions applied: (1) the direction of the covariate effect was physiologically/mechanistically plausible based on our understanding of the underlying system and prior knowledge; (2) removal of the covariate caused the model to be inferior at the .05 statistical level (assessed by using a likelihood ratio test); and (3) the bootstrap-obtained nonparametric 95% CI for the covariate effect did not include zero.

Investigation of termination of the cough challenge. Because of the nature of the ascending-dose capsaicin challenge, it is expected that a substantial number of participants will elect to terminate the challenge before reaching the maximum capsaicin dose. Possible reasons for terminating the challenge include discomfort because of excessive coughing or sensations of heat/stinging/burning in the throat at higher concentrations of capsaicin. Termination of the cough challenge results in missing data at higher capsaicin concentrations, which might have implications for the modeling strategy/methodology. Therefore an exploratory statistical and graphical analysis of the raw cough response data was performed to further understand the reasons for termination of the cough challenge.

Linear regression modeling of C2 and C5

To explore the utility of C2 and C5, we compared log base 10–transformed values in health and disease using an independent t test. To see how these end points performed compared with E_{max} and ED_{50}, we carried out linear regression modeling to test whether the features of asthma found to influence E_{max} and ED_{50} similarly influenced log C2 and log C5.

RESULTS

Detailed results of modeling

Population pharmacodynamic model. The parameter estimation process and covariance step for the final population pharmacodynamic model (including covariates) converged successfully under the Laplace estimation method and a requested precision of more than 3 significant digits in the parameter estimates. The parameter estimates of the final population model are reported in Table III, together with the bootstrap results and 95% nonparametric CIs around these estimates. All model parameters (including both fixed and random effects) were estimated with adequate precision (see Table III). The average cough response at baseline, referred as E_0, was estimated to be only 0.06, indicating that cough response is very rare when the capsaicin dose is zero.

Tachyphylaxis effect. A tachyphylaxis pattern was apparent after examination of the raw cough response data because the magnitude of response (number of coughs) was decreased with consecutive inhalations of the same capsaicin dose. Similar to the tachyphylaxis pattern previously reported,[E3] this was apparent in all the capsaicin dose levels apart from the 2 low doses (0.48 and 0.98 μmol/L) at which cough responses were minimal, and the highest dose (1000 μmol/L), at which the sample size was small, because many subjects had terminated the challenge before this dose. Incorporation of a tachyphylaxis parameter (K) substantially improved the model fit and model diagnostics and was retained in the final model. More specifically, incorporation of this parameter in the model decreased the objective function value (−2 log-likelihood) by 181 units. The tachyphylaxis parameter (K) was estimated to be 0.142 (see Table III), which practically means that the E_{max} value decreases approximately by 13% (calculated as $1-e^{-K} = 0.13$) for any capsaicin inhalation preceded by another inhalation at the same dose level. This is in agreement with our previous work, in which the decrease in E_{max} value because of tachyphylaxis was estimated to be around 15% in another population.[E3] This replication provides additional confidence that the model adequately captures the true quantitative effect of the underlying tachyphylaxis physiologic mechanism on the observed cough response.

Interindividual variability random effect terms were assigned on the following structural model parameters: E_{max}, ED_{50}, and γ. The estimates of these variability terms (see Table III) clearly indicated that the magnitude of population variability in ED_{50} values was vast (coefficient of variation, 282%) and in particular higher than the population variability in E_{max} values (coefficient of variation, 40%). The magnitude of η shrinkage in the final population model was 19%, 3%, and 32% for the interindividual variability terms of E_{max}, ED_{50}, and γ, respectively.

The observed raw dose-response data together with the model fit are illustrated in Fig E2, in which it is apparent that the developed model provided a very good fit to the observed data. The pattern of decreased average number of coughs observed in the last few high doses of capsaicin did not represent a true dose-response relationship but was due to the subset of subjects who reached these high dose levels having substantially lower E_{max} and higher ED_{50} values (ie, they had overall reduced cough responses to capsaicin).

The model fit to the observed dose-response data at the individual level is illustrated in Fig E3 for 16 representative subjects. It is clearly demonstrated that the observed dose-response relationship is completely different between subjects. For example, some subjects had a substantial number of coughs relatively early in the ascending-dose challenge (eg, ID = 6), whereas others had only a limited number of coughs, even at the highest dose levels (eg, ID = 31 and ID = 300). However, it is apparent from Fig E3 that the developed mixed-effects population model is flexible enough to very accurately capture all these patterns of different dose-response relationships across different subjects.

Identification of covariate effects. The final population model included the influences of both categorical and continuous covariates: disease state (health or asthma), sex, atopy (atopic or nonatopic), cough frequency, ACQ questionnaire score, and IgE levels. Inclusion of these covariates resulted in a substantial improvement of the model because they decreased the objective function (−2 log-likelihood) by approximately 68 units compared with the objective function of the base model (model with no covariates). All the covariates retained in the final model offered additional and at least partly unique information because removal of each of the covariates causes the model to be statistically inferior. The level of statistical evidence regarding

each covariate (increase in objective function after removing each covariate from the final model and the corresponding likelihood ratio test P value) is presented in the legend of Table III. In addition, the bootstrap-obtained nonparametric 95% CIs regarding all the covariate effects in the final model are reported in Table III, where it is apparent that they do not include zero. The equations that described the typical values of the model parameters, including covariate effects, are listed below:

$$Emax = \theta_1 \cdot (1+GROU \cdot \theta_8) \cdot (1+GEN \cdot \theta_9) \cdot (1+GROU \cdot ATO \cdot \theta_{10}) \cdot e^{\theta_{11} \cdot (Cfreq-0.75)}$$

$$ED50 = \theta_2 \cdot (1+GROU \cdot \theta_6) \cdot (1+GEN \cdot \theta_7) \cdot (1+GROU \cdot \theta_{13} \cdot (IgE-200)) \cdot e^{GROU \cdot \theta_{12} \cdot (ACQ-0.71)},$$

where $GROU$ is a dummy variable that takes a value of 1 for asthmatic patients and 0 for healthy volunteers; GEN is a dummy variable that takes a value of 1 for female and 0 for male subjects; ATO is a dummy variable that takes the value 1 for atopic asthmatic and 0 for nonatopic asthmatic patients; $Cfreq$ is the spontaneous cough frequency (coughs per hour) over 24 hours; IgE is the IgE level measurement (in kilounits per liter); and ACQ is the ACQ questionnaire score.

For all continuous covariates in the model ($Cfreq$, IgE, and ACQ), covariate effects were centered around the median population values in the analyzed data set (0.75, 200, and 0.71, respectively) to increase model stability and allow parameter interpretation with respect to a typical/reference subject. It should be noted that $Cfreq$ values were missing for 7 of the 144 studied subjects, and therefore these values were imputed with the population median. This imputation did not have an effect on the results because when these subjects were excluded from the analysis, all the parameter estimates (including all covariate effects) were comparable.

Figs 1 and 3 show the model fit to the observed dose-response data stratified by the significant categorical and continuous model covariates, respectively. It is apparent from these figures that substantially different dose-response patterns were observed across the different covariate subpopulations (eg, healthy volunteers vs asthmatic patients and male vs female subjects). However, the developed covariate-incorporated population model very accurately described the observed dose-response relationships within each of these subpopulations.

Model-simulated typical dose-response curves for subjects with different population characteristics are presented in Fig 2 to illustrate the effect of the model-incorporated categorical covariates on the dose-response relationship. Similarly, the effect of the model-incorporated continuous covariates on the simulated dose-response curves is shown in Fig 4.

All the significant covariate effects (see Table III and Figs 1-4) are described in the main article. It should be noted that although it was possible in the current work to explain part of the observed variability in cough response through incorporation of several covariates, the extent of unexplained interindividual variability remains substantial.

Investigation of termination of the cough challenge. An exploratory analysis of the raw cough response data with regard to termination of the challenge is shown in Fig E4. The number of subjects who performed at least 1 inhalation at a given dose level decreased as the capsaicin dose increased (Fig E4, *A*). The most important determinant of termination of the challenge at a given dose level was the total number of coughs in the entire challenge up to that specific dose. Fig E4, *B*, shows that subjects who terminated the challenge at a given dose level had substantially more coughs in the challenge up to this dose compared with subjects who continued to the next capsaicin dose level; bootstrap 95% CIs (using bootci in MATLAB) indicated a statistically significant difference in the number of coughs for the majority of the capsaicin dose levels. Fig E4, *B*, suggests that when subjects reach a threshold of approximately 40 to 60 coughs, they tend to terminate the challenge, irrespective of the dose of capsaicin at which this occurs.

Although the missing data mechanism (termination of the challenge) was not independent of the response values, it depends on them only through the observed components of the response (number of coughs up the point of dropout). Therefore valid estimation-based inferences could be obtained with the maximum likelihood mixed-effects modeling approach, without the need to simultaneously develop a model for the missing data.[E11] The development of a dropout model for the capsaicin cough challenge, although not necessary for analysis of this data set, represents a significant task. This is currently in progress because it will inform the design of future clinical studies and allow the performance of clinical trial simulation.

Exploratory analysis of C2 and C5 end points

Comparison of healthy volunteers and asthmatic patients. As shown in Table E1, asthmatic patients demonstrated significantly lower C2 and C5 (ie, were more sensitive to capsaicin) than healthy volunteers. However, as shown in Fig E5, there was substantial variability in these end points and overlap between health and disease. Moreover, many subjects in both groups did not achieve measureable C5, particularly for the C5 end point (42% of healthy volunteers and 30% of asthmatic patients).

Predictors of C2 responses. Analyzing healthy volunteers and asthma data combined in the simplest model, both sex ($P < .001$) and disease group ($P < .001$) significantly predicted log C2, explaining 17.5% of the variance; female subjects were more sensitive to capsaicin than male subjects, and asthmatic patients were more sensitive than healthy volunteers. However, when the predictors of capsaicin responses found to be significant in our nonlinear modeling approach (disease group, sex, atopic status, log cough frequency, log total IgE level, and log ACQ score) were introduced in the linear regression model, none significantly predicted log C2.

Predictors of C5 responses. Again, in the simplest model both sex ($P = .002$) and disease group ($P = .002$) significantly predicted log C5, explaining 11.2% of the variance. When the predictors of capsaicin responses found to be significant in our nonlinear modeling approach were introduced in the linear regression model, only log ACQ score significantly predicted log C5 ($\beta = -0.8$, $P = .012$; ie, worse asthma control was associated with a lower C5 value).

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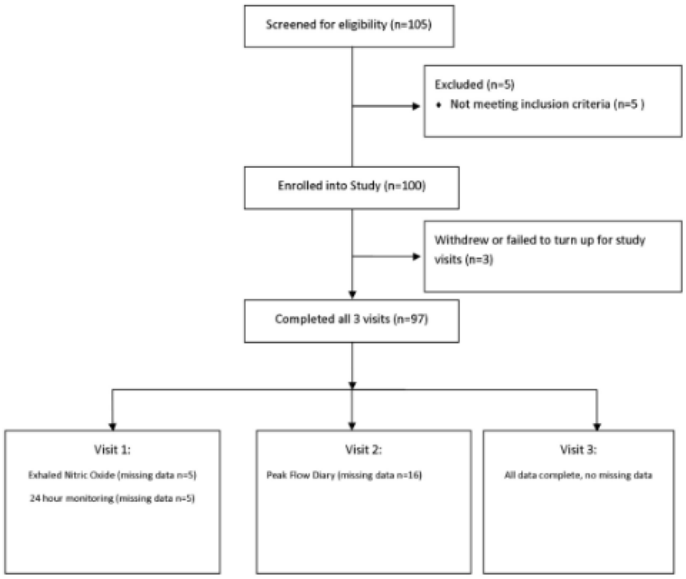

FIG E1. Patient flow diagram illustrating the number of patients screened, withdrawals, and missing data.

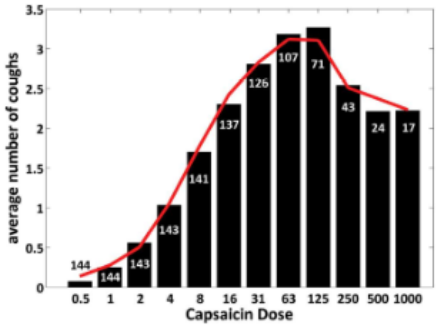

FIG E2. Model fit to the observed dose-response data. The average number of coughs *(y-axis)* is plotted against the capsaicin dose *(x-axis)*. *Bars* and the *red line* represent the observed and model-predicted number of coughs, respectively, averaged across all subjects and inhalations at a given capsaicin dose level. The number of subjects subjected to at least 1 inhalation at a given dose level is also reported inside (or above) each bar.

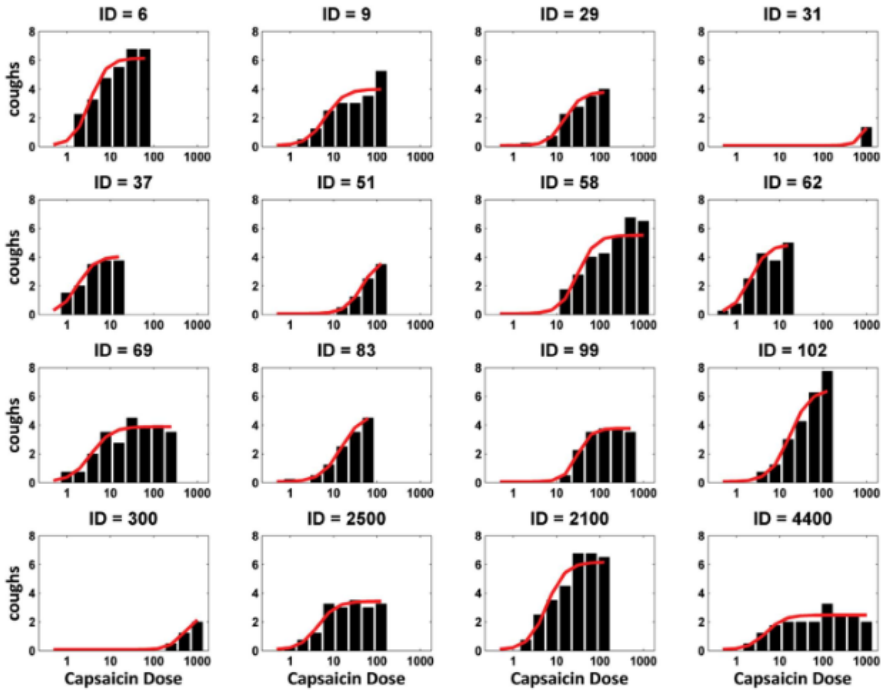

FIG E3. Model fit to the observed dose-response data at the individual level. Observed data *(bars)* and individual model predictions *(red lines)* of 16 representatives of the population subjects are presented. The number of coughs *(y-axis)* averaged across all inhalations at a given capsaicin dose level for a given subject is plotted against the capsaicin dose *(x-axis)*.

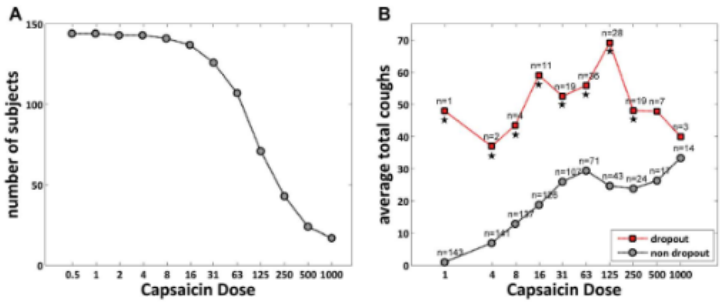

FIG E4. Investigation of the challenge termination pattern in the raw cough response data. **A,** Number of subjects *(y-axis)* performing at least 1 inhalation at a given dose level is plotted against the capsaicin dose *(x-axis)*. **B,** Average number of total coughs in the challenge *(y-axis)* up to a given capsaicin dose *(x-axis)* for subjects who do or do not terminate the challenge after this specific dose. For example, the 4 subjects who dropped out after inhaling the 7.81 μmol/L dose (third marker from the left) had, on average, of 43 coughs up to this point of the challenge; the 137 subjects that did not drop out and continued to the higher dose level had, on average, only 13 coughs up to this point of the challenge. Markers highlighted with an *asterisk* indicate a statistically significant difference in total coughs between the dropout and nondropout groups at a given dose level.

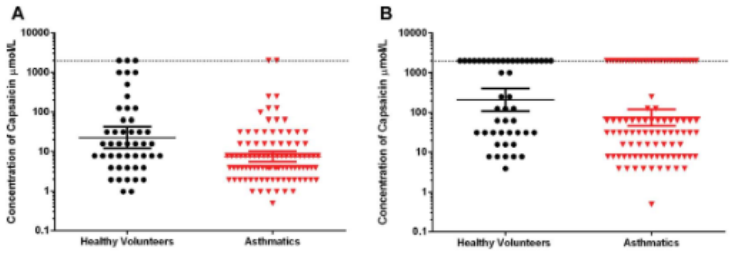

FIG E5. Comparison of the traditional capsaicin challenge end points C2 **(A)** and C5 **(B)**. Note the logarithmic scale (base 10) of the *y-axis*. *Error bars* show geometric means and 95% CIs. *Dashed reference lines* at 2000 µmol/L capsaicin represent values assigned to those subjects who did not achieve C2 or C5.

TABLE E1. Traditional capsaicin endpoints in asthma and healthy volunteers

	Group	Capsaicin (μmol/L), geometric mean (95% CI)	*P* value
C2	Healthy volunteers (n = 47)	22.6 (12.1-42.1)	.002
	Asthmatic patients (n = 97)	7.3 (5.4-9.8)	
C5	Healthy volunteers (n = 47)	209.5 (108.5-404.5)	.013
	Asthmatic patients (n = 97)	78.2 (48.8-125.1)	

CHEST

Objective Cough Frequency, Airway Inflammation, and Disease Control in Asthma

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BACKGROUND: Cough is recognized as an important troublesome symptom in the diagnosis and monitoring of asthma. Asthma control is thought to be determined by the degree of airway inflammation and hyperresponsiveness but how these factors relate to cough frequency is unclear. The goal of this study was to investigate the relationships between objective cough frequency, disease control, airflow obstruction, and airway inflammation in asthma.

METHODS: Participants with asthma underwent 24-h ambulatory cough monitoring and assessment of exhaled nitric oxide, spirometry, methacholine challenge, and sputum induction (cell counts and inflammatory mediator levels). Asthma control was assessed by using the Global Initiative for Asthma (GINA) classification and the Asthma Control Questionnaire (ACQ). The number of cough sounds was manually counted and expressed as coughs per hour (c/h).

RESULTS: Eighty-nine subjects with asthma (mean ± SD age, 57 ± 12 years; 57% female) were recruited. According to GINA criteria, 18 (20.2%) patients were classified as controlled, 39 (43.8%) partly controlled, and 32 (36%) uncontrolled; the median ACQ score was 1 (range, 0.0-4.4). The 6-item ACQ correlated with 24-h cough frequency ($r = 0.40$; $P < .001$), and patients with uncontrolled asthma (per GINA criteria) had higher median 24-h cough frequency (4.2 c/h; range, 0.3-27.6) compared with partially controlled asthma (1.8 c/h; range, 0.2-25.3; $P = .01$) and controlled asthma (1.7 c/h; range, 0.3-6.7; $P = .002$). Measures of airway inflammation were not significantly different between GINA categories and were not correlated with ACQ. In multivariate analyses, increasing cough frequency and worsening FEV_1 independently predicted measures of asthma control.

CONCLUSIONS: Ambulatory cough frequency monitoring provides an objective assessment of asthma symptoms that correlates with standard measures of asthma control but not airflow obstruction or airway inflammation. Moreover, cough frequency and airflow obstruction represent independent dimensions of asthma control. CHEST 2016; 149(6):1460-1466

KEY WORDS: airway inflammation; asthma; cough

ABBREVIATIONS: ACQ = Asthma Control Questionnaire; BHR = bronchial hyperresponsiveness; eNO = exhaled nitric oxide; GINA = Global Initiative for Asthma

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Part of this article has been presented in abstract form (Marsden PA, Ibrahim B, Woodcock AA, Fowler SJ, Smith JA.

Asthma is a chronic inflammatory disease of the airways, estimated to affect 300 million people worldwide.[1] The aim of asthma treatment is to achieve and maintain control of the clinical manifestations of the disease; this approach includes managing current symptoms but also reducing the risk of future exacerbations. However, despite an increase in our understanding of the mechanisms underlying asthma and the availability of effective treatments, optimal asthma control is often not achieved.[2] Several tools are currently in use for describing asthma control; a categorical classification based on expert opinion has been suggested by the Global Initiative for Asthma (GINA), and clinical trials have commonly used the Asthma Control Questionnaire (ACQ),[3] which gives a numerical score and is responsive to treatment.[4-6] These tools rely on patient recall of symptom frequency, severity, and medication use over several weeks. Such reporting is inevitably influenced by a range of external factors unrelated to asthma, such as vigilance, mood, and social interactions, as well as memory. A more objective measure of asthma symptoms may therefore be a useful tool for assessing control and treatment responses.

Cough is an important symptom in asthma because it predicts disease severity,[7,8] poor prognosis,[9] and is a common,[10] troublesome symptom.[11] Unlike wheezing, breathlessness, and chest tightness, coughing is readily objectively quantified by using ambulatory monitoring systems.[12] We have previously shown that objective cough counts are elevated in patients with asthma compared with healthy control subjects but are poorly represented by patient reports of cough.[13] However, it is unknown whether the objective measurement of cough frequency is a useful marker of asthma control. Furthermore, it is unknown whether cough frequency is related to elements of asthma pathophysiology such as airflow obstruction and inflammation. Therefore, the aim of the current study was to examine, in a group of patients with asthma not selected for cough, the relationships between objective cough frequency, asthma control (using the GINA classification and the ACQ), and measures of airway obstruction and inflammation.

Subjects and Methods

Subjects

We studied subjects with asthma recruited from a longitudinal cohort of adult patients with asthma that had been established in 1997.[14,15] Inclusion criteria were: physician diagnosis of asthma, age ≥ 16 years, symptoms of asthma in the preceding 12 months, and minimum treatment with a short-acting bronchodilator. Ethical approval was obtained from the regional Research Ethics Committee (06/Q1403/110), and subjects provided written informed consent.

Study Procedures

All subjects attended the North West Lung Research Centre, University Hospital of South Manchester, on two occasions, at least 1 week apart and within a 2-week period. Prior to both visits, subjects withheld medication as follows: short-acting bronchodilators for 6 h; long-acting bronchodilators, theophyllines, and leukotriene receptor antagonists for 12 h; and antihistamines and inhaled steroids for 48 h.

At visit 1, subjects performed exhaled nitric oxide (eNO) testing (NIOX, Aerocrine Inc) followed by spirometry (Jaeger, Viasys Healthcare) and reversibility of FEV_1 to 400 μg of salbutamol determined according to American Thoracic Society criteria.[16] Reversible airflow obstruction was defined by an increase in FEV_1 of ≥ 12%. Sputum was induced by using 3%, 4%, and 5% saline sequentially via an ultrasonic nebulizer (Sonix 2000, Clement Clarke). Samples were processed as previously described.[17] A total of 400 nonsquamous cells were counted by a fully trained observer and were expressed both as a percentage and as a number of cells per gram of selected sputum. Competitive or sandwich enzyme-linked immunosorbent assays were performed to measure inflammatory mediators; leukotriene B_4, prostaglandin E_2, 8-isoprostane, leukotriene C_4, IL-8, myeloperoxidase, and eosinophilic cationic protein. Subjects completed the 7-item Asthma Control Questionnaire (ACQ-7).[3] The subjects were categorized into levels of asthma control according to GINA guidelines by using information given in questionnaires, clinical history, and pulmonary function. Finally, subjects underwent ambulatory 24-h cough monitoring by using the VitaloJAK cough recorder (Vitalograph Ltd) as previously described.[18,19] Recordings were analyzed by using an audio editing package (Audition 3.0, Adobe Systems Inc). The number of cough sounds was manually counted and expressed as coughs per hour (c/h).

At visit 2, bronchial hyperresponsiveness (BHR) was assessed by using the 5-breath dosimeter method (KoKo, Ferraris Respiratory, Inc) according to published guidelines, and it was defined by the dose causing a 20% drop from baseline FEV_1 in response to methacholine.[20] Skin prick testing was performed, and atopy was defined by the presence of at least one positive skin prick test result to common inhaled allergens (eg, house dust mite, cat, dog, grass, mixed molds).

Statistical Analysis

Data were analyzed by using SPSS version 20.0 (IBM SPSS Statistics, IBM Corporation). Overall 24-h and daytime cough rates were log transformed prior to analysis and geometric mean (95% CI)

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summary data given. Spearman rank correlations were used throughout. Nonparametric data were compared by using the Mann-Whitney *U* test. Multinomial logistic regression and general linear models were used to investigate possible predictors of GINA control category and ACQ scores, respectively. Only parameters correlated with control in the univariate analysis ($P < .1$) were included in the models, and nonstatistically significant variables were sequentially removed from each model to determine which model explained the greatest proportion of variance in asthma control. Because the ACQ-7 includes a score for FEV_1 % predicted (which would inevitably be correlated with the FEV_1 % predicted), we opted to also use the 6-item Asthma Control Questionnaire (ACQ-6) score for the general linear models; this score only includes scores for symptoms and reliever medication use.

Results

Subjects

We studied 89 subjects with asthma; their baseline characteristics are summarized in Table 1. Of the 88 subjects who underwent reversibility testing (one subject with β_2-agonist intolerance was not tested), 23 (26.1%) had bronchodilator reversibility; 37 of 73 subjects (50.7%) tested had a positive methacholine challenge. Testing was not conducted in 16 subjects (three subjects did not attend; one subject refused testing; two subjects had used a β_2-agonist; and 10 subjects were below the FEV_1 safety cutoff for the test). A total of 45 subjects had bronchodilator reversibility and/or positive methacholine challenge, and 32 (44% of those tested) had neither. Subjects were evenly distributed between British Thoracic Society steps 1 through 4 (e-Table 1).

Asthma Control

According to GINA criteria, 18 (20.2%) patients were classified as controlled, 39 (43.8%) were partly controlled, and 32 (36%) uncontrolled. GINA category was not influenced by sex, but patients with uncontrolled asthma were older than those with partly controlled and controlled (62.4 ± 9.0 years vs 54.8 ± 12.1 years and 54 ± 13.7 years, respectively; $P = .019$) asthma. ACQ-7 data were available for 88 (98.9%) subjects (median score, 1; range, 0.0-4.4). There was also a correlation between ACQ score and age ($r = 0.22$; $P = .04$). Uncontrolled patients were treated with higher doses of inhaled corticosteroids ($P < .001$), and there was a positive correlation between ACQ score and inhaled corticosteroid dose ($r = 0.39$; $P < .001$). Smoking status did not influence measures of asthma control (ACQ score or GINA control).

TABLE 1] Subject Characteristics

Characteristic	Subjects With Asthma (N = 89)
Age, y	57.3 ± 11.9
Sex, % female	57.3
BMI, kg/m^2	28.6 ± 5.3
Smoker, %	
Never	52.8
Ex	39.3
Current	7.9
Pack-year history	0.0 (0.0-67.5)
Asthma duration, y	29.0 (10.0-68.0)
Atopy	83.5%
FEV_1 % predicted	86.4 ± 22.1
ICS dose, μg BDP	800 (0-4,000)

Data presented as mean ± SD or median (range) unless otherwise indicated. BDP = beclomethasone dipropionate; ICS = inhaled corticosteroid.

Objective Cough Rates

Ambulatory cough monitoring was performed in 86 subjects. Nighttime data were missing in four patients due to battery failure. Cough rate varied widely between subjects (geometric mean, 2.5 c/h [95% CI, 0.2-27.6]) and was higher by day than by night (median for day, 3.7 c/h [range, 0.2-41.3]; median for night, 0.5 c/h [range, 0-29.6]; $P < .001$]. There were no sex differences in cough frequency (log 24-h cough rate), nor was this variable influenced by age, BMI, FEV_1 % predicted, log eNO, or dose–response ratio to methacholine. There was a tendency for the small number of current smokers to have a higher cough frequency than ex-smokers or nonsmokers ($P = .09$). Thirteen subjects were taking angiotensin converting-enzyme (ACE) inhibitors, but cough frequency was not elevated in these patients (ACE inhibitor geometric mean, 2.8 c/h [95% CI, 1.5-5.2]; no ACE inhibitor geometric mean, 2.4 c/h [95% CI, 1.8-3.2]; $P = .64$).

Airway Inflammation

The geometric mean of eNO was 23.2 parts per billion (95% CI, 19.9-27.1). There was a negative correlation between eNO and inhaled corticosteroid dose ($r = -0.29$; $P = .007$).

Induced sputum data were available for 55 subjects (61.8%). The interobserver agreement for counts of 400 nonsquamous cells were as follows: eosinophil mean difference, 3.3 cells (equivalent to 0.8% of total count; 95% CI, −9.5 to 16.0); neutrophil mean difference,

7.6 cells (equivalent to 1.9% of total count; 95% CI, −41.5 to 56.6). Geometric mean of percent sputum eosinophils was 2.1% (95% CI, 1.5 to 2.9), with a median 0.11 × 10^{-6} cells/g of sputum (interquartile range, 0.01-0.11 × 10^{-6}). Thirty subjects (54.5%) had increased sputum eosinophils (ie, ≥ 2%). Median percentage of sputum neutrophils was 68% (interquartile range, 57.3-77.3) with 2.25 × 10^{-6} cells/g of sputum (interquartile range, 0.76-2.96 × 10^{-6}).

Levels of airway inflammatory mediators were measured in subjects with satisfactory cell counts (e-Table 2). There were correlations between sputum eosinophilic cationic protein levels and eosinophil counts (percentage, $r = 0.43$; $P = .002$) as well as between sputum IL-8 and neutrophils (percentage, $r = 0.44$; $P = .001$). There were no significant correlations between objective cough frequency and sputum eosinophils or neutrophils (e-Table 3). For sputum inflammatory mediators, only 8-isoprostane showed a correlation with objective cough frequency (e-Table 4).

Predictors of Asthma Control

GINA Categories: Cough frequency and FEV_1 significantly differed between GINA categories ($P = .003$ and $P < .001$, respectively) (Figs 1A, 1B). Subjects in the uncontrolled group had higher cough rates than both partially controlled ($P = .01$) and controlled ($P = .002$) subjects. This outcome was also the case for daytime cough counts ($P = .004$ and $P = .002$) and nocturnal cough counts ($P = .004$ and $P = .04$). Subjects with uncontrolled asthma had greater airflow obstruction than both partly controlled ($P = .002$) and controlled ($P < .001$) asthma. Although sputum eosinophils increased with worsening asthma control, the differences between groups did not reach statistical significance (Fig 1C). There were no statistically significant differences in sputum neutrophils (percentage or cells × 10^6/g sputum) (Fig 1D), levels of airway inflammatory mediators (e-Table 5), methacholine challenge, or log eNO between GINA control categories.

In a multinomial logistic regression model with GINA control category as the outcome variable, higher log 24-h cough frequency ($P < .001$) and lower % FEV_1 ($P < .001$) were independently predictive of GINA category. The numbers in each group were too small for odds ratios to be calculated.

ACQ Scores: There were positive correlations between ACQ-6 and 24-h cough frequency ($r = 0.40$; $P < .001$) (Fig 2), daytime cough rate ($r = 0.40$; $P < 0.001$), and overnight cough rate ($r = 0.30$; $P = .006$). There was a correlation between ACQ-6 and log eNO ($r = 0.24$; $P = .03$) and borderline correlation with FEV_1 % predicted ($r = -0.19$; $P = .07$). There were no statistically significant correlations between ACQ-6 and percent sputum eosinophils/neutrophils (percent or cells × 10^6/g sputum) (Table 2), inflammatory mediators, or methacholine challenge (data not shown).

In the general linear model, log ACQ-6 was only significantly predicted by log 24-h cough rate ($P = .008$), which accounted for 17% of the variance. When the model was repeated with ACQ-7 (including FEV_1 % predicted) as the outcome variable, log total cough rate ($P = .004$) and FEV_1 % predicted ($P = .001$) independently predicted asthma control, accounting for 37% of the variance.

Discussion

Until recently, few studies have sought to objectively quantify cough in patients with asthma. Our previous research found that objective measures of cough were poorly represented by subjective patient reporting of cough severity and were also not related to standard measures of asthma such as FEV_1, methacholine responsiveness, or eNO.[13] To the best of our knowledge, the current study is the first to examine the relationships between objective cough frequency, asthma control, airflow obstruction, and airway inflammation. We have shown that increasing cough rates are associated with worse asthma control, as assessed by using both the GINA criteria and the ACQ score. In addition, we found that objective cough frequency and airflow obstruction independently predicted disease control, whereas measures of airway inflammation did not.

BHR and airway inflammation are acknowledged as important components of asthma pathophysiology, but how they relate to asthma control is less clear. Previously published data suggest BHR and inflammation do not discriminate well between different levels of asthma control.[21,22] Furthermore, treatment algorithms that incorporate measures of sputum eosinophils[23,24] or BHR[25] to titrate treatment improved exacerbation rates and reduced airway remodeling, respectively, but failed to show improvements in asthma control. Similarly disappointing were the outcomes based on eNO algorithms when used as an adjunct or as an alternative to standard clinical management[26-28]; at best, only a reduction in the inhaled steroid dose was achieved. Together, these observations suggest that there may be

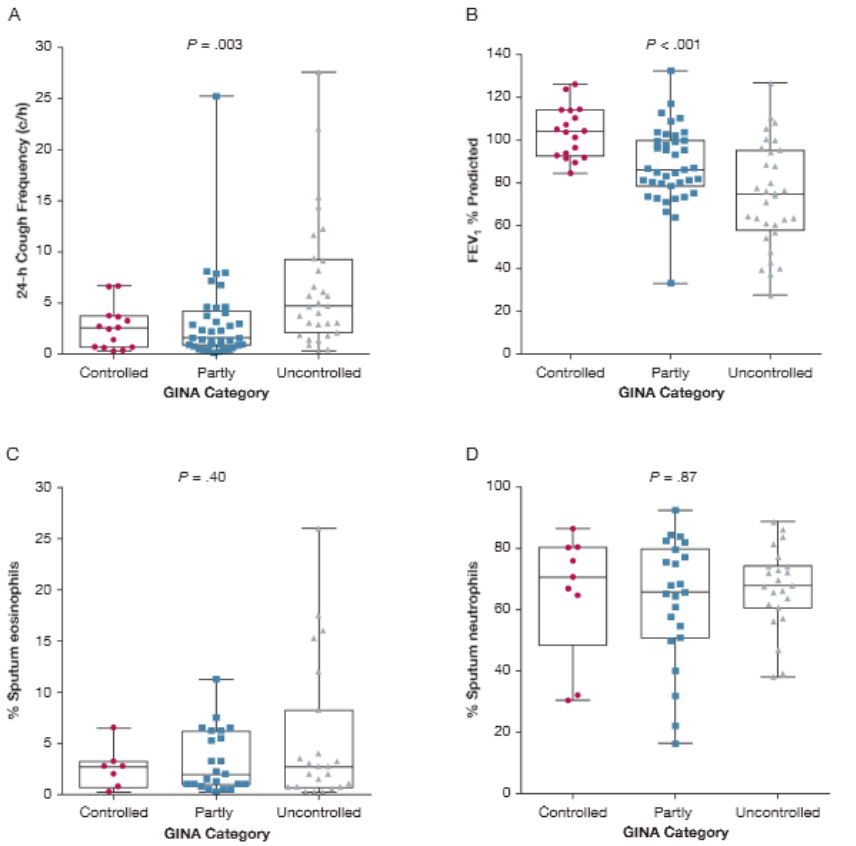

Figure 1 – A-D, Comparison of patients with controlled, partially controlled, and uncontrolled asthma (based on GINA classification). The number of cough sounds was manually counted and expressed as coughs per hour (c/h). A, Objective cough frequency over 24 h; B, FEV_1, % predicted; C, Percent sputum eosinophils; D, Percent sputum neutrophils. GINA = Global Initiative for Asthma.

other important determinants of day-to-day asthma control.

This study is consistent with a cluster analysis of subjects with asthma[29] which showed that some groups of patients exhibited discordance between airway inflammation and symptoms (ie, high levels of symptoms despite low levels of eosinophilic inflammation [discordant symptoms] or high levels of eosinophilic inflammation but low expression of symptoms [discordant inflammation]). Our data relating symptoms to inflammatory cells and mediators similarly failed to show any overall relationship, even when the symptom of cough was quantified objectively and for mediators with known tussive effects (eg, prostaglandin E_2). These data would therefore suggest that inflammation does not directly trigger asthma symptoms and that other mechanisms may play an important role. Airway nerves are crucial to the development of symptoms such as cough, shortness of breath, chest tightness, and pain. These symptoms are dependent on nerve activation and transmission

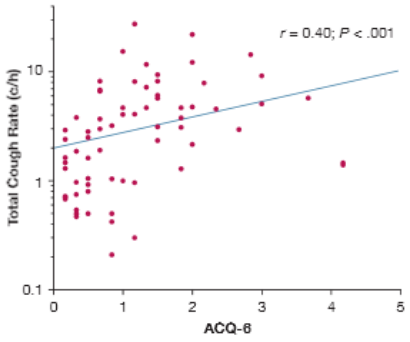

Figure 2 – *Correlation between ACQ-6 score and total cough rate. The number of cough sounds was manually counted and expressed as coughs per hour (c/h). ACQ = Asthma Control Questionnaire.*

to the central nervous system, predominantly via vagal afferents; hence, these symptoms are blocked or inhibited by vagal anesthesia or transection.[30] We speculate that neuronal dysfunction in asthma could be the missing link in explaining the discordance between airway inflammation and symptoms.

Our goal was to study a "real-life" asthma population, and the inclusion criteria for the study reflected this goal; patients had a physician diagnosis of asthma but were not selected for cough, reversibility, or BHR as a prerequisite. However, subjects were recruited from a previously established much larger Assessment of Manchester Asthmatics Longitudinally (ASMAL) cohort established in 1996, in which > 88% had evidence of BHR at baseline.[31] More than 80% of the study patients were established on inhaled corticosteroid treatment, and the sensitivity of methacholine challenge in the diagnosis of asthma is reduced by undergoing this treatment.[32] Furthermore, compared with the tidal breathing method, the 5-breath dosimeter method may underestimate BHR because of the protective bronchodilator effect of deep breathing.[33] Finally,

TABLE 2] Correlation Between ACQ-6, Cough Rates, and Sputum Cell Counts

Parameter	ACQ-6
Cough rate, coughs/hour	
Day	$r = 0.40$ $P < .001$
Night	$r = 0.30$ $P = .006$
Total	$r = 0.40$ $P < .001$
Sputum cell counts	
Eosinophil	
%	$r = 0.12$ $P = .37$
Cells × 10^6/g sputum	$r = 0.12$ $P = .37$
Neutrophil	
%	$r = -0.10$ $P = .45$
Cells × 10^6/g sputum	$r = -0.10$ $P = .45$

Spearman rank correlations were used throughout. ACQ-6 = 6-item Asthma Control Questionnaire.

although this study found an association between objective cough frequency and asthma control, further interventional studies would be required to confirm that objective cough frequency is a useful and sensitive marker of improved control.

Conclusions

Data from this study suggest that objective measurements of cough provide unique information about asthma control that is not fully captured by ACQ or GINA questionnaires. Importantly, cough frequency reflected asthma control independent of airflow obstruction and inflammation. The inclusion of measures of cough frequency in future asthma studies offers an objective measure of day-to-day control that may be better for assessing the impact of novel asthma therapies.

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Towards understanding and managing chronic cough

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ABSTRACT

Chronic cough is a common and troublesome condition affecting approximately 12% of the general population. It is associated with poor quality of life with psychological, social and physical consequences. Patients typically complain of a dry irritating cough, driven by a strong urge to cough associated with a sensation or irritation located in the throat. Treatment of potential 'causes', ie asthma, gastro-oesophageal reflux disease and rhino-sinusitis, may produce a complete or partial response, but the response of some patients to opiates and alpha-2-delta ligand antagonists (gabapentin and pregabalin) supports the concept that this is primarily a neurological disorder, characterised by hyper-responsiveness of the nerves. Novel and highly effective neuronal treatments are in development and offer hope of better symptom control with fewer side effects within a few years. This review focuses on understanding the mechanism of chronic cough, current management approaches and research that may lead to novel therapies.

KEYWORDS: Capsaicin, chronic cough, cough hypersensitivity syndrome, vagus nerve, P2X3

Introduction

Cough is the single most common reason for seeking medical attention and the lack of suitable anti-tussives makes this a major unmet clinical need. Many patients experience acute coughing lasting up to 3 weeks after a viral respiratory tract infection. However, a substantial proportion of the population (12–16%) suffer from chronic cough (lasting more than 8 weeks) with detrimental effects on quality of life.[1–3] The stereotypical patient is a woman in her 50s with a non-productive cough for many years. Chronic cough is twice as common in women as in men.[4] Chronic cough often occurs in distressing bouts, which the patient cannot control, and is usually preceded by an irresistible urge to cough associated with an irritating sensation in the throat. Cough can be precipitated by changes in temperature, strong smells from perfumes or aerosols, dust and passive smoking.[5,6] Chronic cough has major psychological, social and physical consequences. Patients are embarrassed socially and in the work place. They are physically exhausted by the frequent prolonged coughing bouts, and women can have distressing stress incontinence. The treatment options have been limited. In the USA, over $7 billion is spent each year on over-the-counter cough and cold medications, most of which contain dextromethorphan, which has very limited if any clinical effectiveness.[7] Understanding the mechanisms underpinning coughing in order to develop novel treatments is a significant challenge to the medical profession and pharmaceutical industry.

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Neurophysiology of cough

Cough is normally a defensive reflex that protects the airways. It is a response to an inhaled foreign body or excessive mucus, and to noxious and harmful environmental irritants. Activated sensory airway nerves transmit information via the vagus nerve to first synapse in the brainstem, which rapidly initiates the motor cough response.

The cough reflex is thought to involve two main subtypes of sensory vagal afferent nerves.[8] The first subtype is c-fibres; these form networks of unmyelinated nerves throughout the airways and are characteristically sensitive to capsaicin (chilli pepper extract) through activation of the transient receptor potential vanilloid type 1 (TRPV1) receptor and other irritant chemicals. They can also respond to other stimuli such as heat, acidity and inflammatory mediators. The second type, myelinated sub-epithelial Aδ fibres, are found in the proximal airways and respond to mechanical stimuli, osmolarity and acidity but do not typically express TRPV1, and are normally insensitive to capsaicin and inflammatory mediators. The morphology of these airway nerves has recently been delineated in human airway tissue and shows remarkable similarity to that seen in animal models (Fig 1).[9]

Stimulating these airway nerves generates action potentials that synapse in the nucleus tractus solitarius (NTS) and paratrigeminal nucleus of the brainstem.[10] These afferent nerves then activate complex neural networks, projecting to cortical and sub-cortical areas responsible for sensations of airway irritation and the urge to cough and ultimately, if the stimulus is sufficient, results in coughing via activation of spinal motor nerves to the diaphragm, intercostal muscles and larynx (Fig 2). Importantly, coughing can also

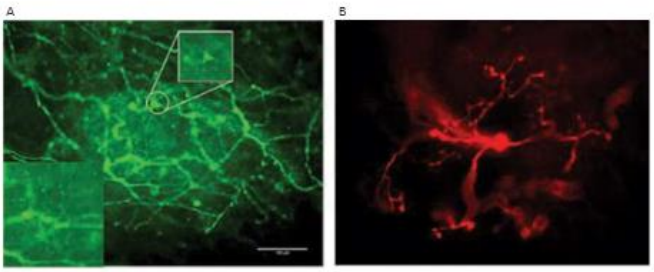

Fig 1. Structure of human airway nerves. A – thin unmyelinated c-fibres located near the epithelial membrane; B – sub-epithelial myelinated Aδ fibres. Reprinted with permission of the American Thoracic Society.[9] Copyright © 2016 American Thoracic Society.

be initiated voluntarily without any peripheral stimulus or precipitating sensations, and in some cases voluntarily supressed.[11] Thus, the potential drivers of excessive cough could originate either in the peripheral nerves or central nervous system, including the brainstem. There have been case report associations noted between chronic cough and conditions affecting the autonomic nervous system such as Holmes-Adie syndrome and autosomal dominant hereditary sensory neuropathy.[12–15]

Hypersensitive or hyper-responsive cough?

A recent consensus statement has suggested the term 'cough hypersensitivity syndrome' be used to describe patients with chronic cough.[16] However, evidence from experimentally evoked cough suggests that the neuronal pathways exhibit hyper-responsiveness rather than hypersensitivity.[17]
In pharmacokinetic terms, the dose response curve is predominantly shifted upwards with much less left-shift, ie the dose required to evoke a cough is marginally reduced, but the maximal number of coughs to a given stimulus is substantially increased (Fig 3). This is reflected in what patients describe; anecdotally, patients complain of an inability to stop coughing and quality of life is most severely impacted by the length and severity of coughing bouts.

Aetiology of chronic cough

Chronic cough can occur in many common respiratory conditions such as asthma, bronchitis in smokers and chronic obstructive pulmonary disease (COPD), where the cough is typically related to the pathophysiology of the disease, eg excessive airway mucus and inhalation of irritants in cigarette smoke for COPD.[18] Other causes include eosinophilic

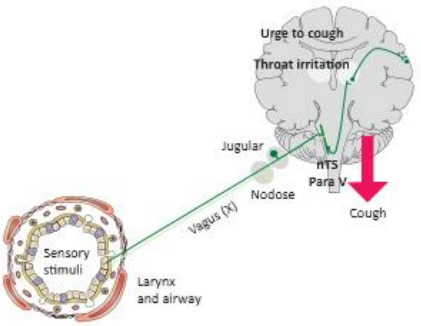

Fig 2. Schematic diagram representing the cough reflex. Vagal afferents transmit stimuli from the airways to the nucleus tractus solitarius (nTS) and paratrigeminal nucleus (Para V) in the brainstem. Neuronal signals are then transmitted to the somatosensory cortex via the thalamus causing throat irritation and urge to cough. These sensations, if great enough, lead to cough via activation of spinal motor neurons.

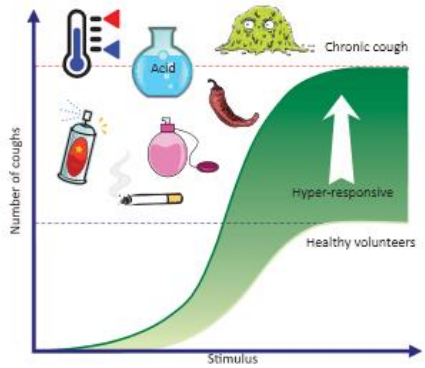

Fig 3. Schematic diagram representing cough hyper-responsiveness to airway stimuli displayed by chronic cough patients. Compared with healthy volunteers, patients with chronic cough predominantly exhibit heightened cough responses to airway stimuli, such as inhaled capsaicin and citric acid, in cough challenge test and report coughing triggered by changes in temperature, perfumes, aerosols, smoke and to small volumes of mucus.

bronchitis, interstitial lung diseases, bronchiectasis or use of angiotensin-converting enzyme inhibitors. Extra-pulmonary diseases, such as gastro-oesophageal reflux disease (GORD) and post-nasal drip secondary to rhinosinusitis, can also be identified as potential triggers.

Observational studies detailing the aetiology of chronic cough in patients presenting to specialist cough clinics show large variations in the prevalence of asthma (6–36%), GORD (0–41%) and rhinitis (8–56%).[19] Furthermore, up to a quarter of patients may have multiple aetiologies combined.[20]

In the past three decades, this diagnostic triad of asthma, GORD or rhinosinusitis have been asserted to be the 'cause' of chronic cough. However, the vast majority of patients with these common conditions do not complain of coughing or have features suggestive of cough hypersensitivity. Treatment of these conditions in patients with chronic cough may improve cough but rarely stops it completely. After numerous investigations, some patients have no evidence of any of the above causes, and hence the use of the term idiopathic chronic cough.[21]

Given our recent understanding of the neurophysiology of cough, it seems likely that neuronal dysfunction is the primary cause of chronic cough. Indeed, evidence for such has been demonstrated by heightened cough responses to inhaled capsaicin in patients with chronic cough[17,22] and asthma.[23] In the context of cough hyper-responsiveness, cough can be triggered by endogenous factors (asthma, GORD, post-nasal drip, even speaking and laughing) or exogenous factors (eg cold air, passive smoking, deodorants etc).

Management approach to chronic cough

There are many international guidelines based on consensus opinion and observational data that describe the approaches taken in managing patients presenting with chronic cough.[19,24–26] The applicability of these guidelines will vary based on the availability of specialised investigations and clinical experience at primary, secondary and tertiary levels. Despite the complexity of these diagnostic algorithms, the basic approach can be summarised in four main steps,[27] which can be performed with appropriate referral made to secondary or tertiary care if needed.

1. Exclude and treat obvious causes

A detailed history and examination should aim to identify obvious causes and exclude sinister pathologies, such as malignancy. The duration and nature of symptoms is useful, as a cough lasting less than 3 weeks is usually secondary to an upper respiratory tract viral infection, while a cough lasting 3–8 weeks can often be associated with a prolonged post-infectious period, and is occasionally secondary to *Mycoplasma* or *Bordetella pertussis*. The majority of acute coughs are self-limiting and unfortunately very few clinical studies exist to support the use of over the counter (OTC) anti-tussives, which generally contain dextromethorphan.[7] Smoking, even in otherwise healthy subjects, has now been shown to be associated with an increased frequency of objective 24-hour coughs.[18] Approximately 15% of patients on an angiotensin-converting enzyme inhibitor may develop a chronic cough and hence changing to the alternative angiotensin receptor blocker would be appropriate,[28] but the improvement may take several months to take effect. For patients with chronic cough, an initial evaluation should also include a chest radiograph and spirometry. It is important to differentiate a dry, minimally productive cough and a cough with a high daily volume of mucus. The latter would suggest bronchiectasis and would need further investigations to confirm.

2. Investigate and treat common triggers of chronic cough

If the chest radiograph, spirometry and clinical examination are normal, then investigations for asthma, GORD and rhinosinusitis should be considered. Although most patients with asthma have a history of wheeze and shortness of breath as the predominant feature, some patients have cough as the key feature and have been previously coined 'cough-variant asthma'.[29,30]

Objective evidence of asthma requires evidence of variable airflow obstruction – such as peak flow variability of more than 20% or reversibility to salbutamol of more than 12%. However, both of these investigations have a very low negative predictive value and, therefore, further investigation of bronchial hyper-responsiveness (BHR) may be required using either methacholine or histamine inhalational challenge. These tests are not always available in all secondary care settings so a treatment trial of inhaled corticosteroids for 6 weeks is often a more pragmatic approach. In our experience, a trial of inhaler therapy can be limited by the patient's technique and compliance given inhaled treatments often trigger coughing; an alternative is to prescribe 14 days of oral steroids, prednisolone 30 mg daily is our preference. However, a dramatic improvement in cough still leaves some diagnostic uncertainty; a response to inhaled therapy suggests either asthma or eosinophilic bronchitis (EB) and a response to oral steroids could also indicates allergic nasal disease or interstitial lung diseases.

Elevated eosinophils (>3%) in the airways in the absence of BHR suggests EB, which has been reported in up to 13% of patients attending cough clinics.[31] This can be evaluated in samples of induced sputum or from broncho-alveolar lavage (BAL) performed at bronchoscopy, but performing different cell counts on these airway samples is not routinely available in many hospitals. An easier alternative, if available, is exhaled nitric oxide in breath as a surrogate marker to assess airway eosinophils, but its utility in aiding diagnosis in patients with chronic cough has not yet been systematically evaluated. Overall, the key recommendation is that inhaled steroids be trialled in patients who have evidence of BHR or airway eosinophilia in sputum, BAL or exhaled nitric oxide.

Objectively investigating GORD is a challenge and previous guidelines advised empirical acid suppression treatment. A 2011 Cochrane review concluded 'in adults, there is insufficient evidence to conclude definitely that GERD treatment with PPI is universally beneficial for cough associated with GORD'.[32] However, those that demonstrate objective evidence of increased oesophageal acid exposure on pH monitoring or complained of heartburn were most likely to benefit from proton pump inhibitor (PPI) therapy in a retrospective review of the PPI studies.[33,34] In our practice,

we use a trial of twice daily PPI 30 minutes before food, and ranitidine 300 mg at bedtime for 8 weeks.

The mechanisms linking cough and reflux are still unclear. No clear evidence exists to support the concept of micro-aspiration of refluxate into the airways nor that refluxate reaches the proximal oesophagus or enters the larynx/pharynx.[35,36] Furthermore, most chronic cough patients have similar numbers of reflux events to healthy controls; however, in 50% there is a temporal relationship between reflux and cough,[37] suggesting that in some patients stimulation of oesophageal afferents may be capable of triggering coughing, again consistent with sensitisation of neuronal pathways. No evidence exists for the use of pro-kinetics such as domperidone, which has recently been associated with ventricular arrhythmias and sudden cardiac death. Likewise, metoclopramide should be avoided because of its neurological side effects and the lack of clear indication for use in chronic cough.

Patients with chronic cough also often report sensations of post-nasal drip so guidelines recommend nasal corticosteroids and anti-histamines for those thought to have underlying allergic rhinitis and antibiotics/decongestants for sinusitis.[24] Many patients with chronic cough are often assessed by ear, nose and throat surgeons and, upon inspection, the larynx are found to be red and inflamed; this is often attributed to laryngo-pharyngeal reflux (LPR) secondary to gastric refluxate reaching the larynx. However as discussed earlier, evidence of gastric reflux reaching the larynx is lacking and patients who are often coughing hundreds of times may show signs of trauma to the larynx.

3. Exclude and treat rarer triggers of chronic cough

For those without a clear diagnosis or chronic cough that is refractory to treatment of associated conditions, it is important to exclude rarer conditions by performing a high-resolution computerised tomography scan of the chest. This can reveal interstitial lung diseases not easily visible on chest radiograph, eg pulmonary fibrosis, sarcoidosis and bronchiectasis. When other investigations are normal, bronchoscopy is performed in all our patients to assess vocal cord movements, identify tracheobronchomalacia or tracheopathia osteochondroplastica and take BAL and biopsies to evaluate eosinophils. Thick purulent secretions are occasionally found in patients with a productive cough; these secretions are cleared and cultured for bacteria, fungus and acid-fast bacilli. Bronchoscopy can also reveal naso-pharyngeal pathologies such rhinitis, polyps and large tonsils, which the patient may benefit from having removed.[38]

4. Neuro-modulatory treatments

Unfortunately, there are no licensed drugs for the treatment of chronic cough, so in most specialist clinics management relies on using interventions with evidence of efficacy in clinical trials. The most commonly prescribed medication in our clinic is slow release morphine sulphate (MST) starting at 5 mg twice daily and occasionally increasing to 10 mg twice daily.[39] If MST is ineffective then a discussion of the potential risks and benefits of trialling gabapentin or pregabalin is crucial.[40,41] These drugs bind the $\alpha 2\delta$ subunit of the voltage dependent calcium channel and are commonly used for neuropathic pain. The risks need to be carefully considered as unsteadiness, drowsiness, severe depression, hallucinations and, occasionally, suicidal ideation have been reported. Like MST, the benefits in cough have been reported by subjective improvements in the cough-specific quality of life questionnaire or a 100 mm visual analogue score. Low dose gapabentin has also been shown to be effective in cough syncope.[42] Our practice with gabapentin has been to slowly up-titrate doses to 300 mg three times a day and likewise with pregabalin, to a maximum of 150 mg twice daily. This allows for an individualised dose for each patient and side effects are identified at lower doses. Amitriptyline 10 mg at night has been assessed in a randomised controlled trial (RCT) without a placebo control,[43] but our experiences with it have been disappointing.

Some patients decide against trying any of the above medication and opt for speech and language therapy (SALT). Given many patients report laryngeal symptoms and dysphonia, this can be an appealing option. One RCT and several observational studies have reported improvements in reported cough voice and quality of life.[44] This intervention involves a multi-modality approach of education, reducing laryngeal irritation with relaxation exercises, cough suppression techniques and counselling. The major advantage is the lack of adverse events, but the disadvantage is that it is often dependent on the commitment of patients to continue their exercise routines outside the therapy sessions and on the expertise of the therapist in assessing and treating cough patients. Those that benefit often have SALT as an adjunct to medication and this was assessed in a 2016 RCT of SALT with placebo or pregabalin.[41] The study showed that combining pregabalin with SALT improved cough-specific quality of life and visual analogue score, but disappointingly showed no additional improvement in actual objective cough frequency.

Future directions

There have been a number of studies using pharmacological therapies targeting specific receptors on nerve terminals either on the peripheral nerves or brainstem. Unfortunately, the vast majority have been negative, although one positive study targeting the P2X3 receptor on peripheral nerves reported an unprecedented 75% reduction in 24-hour objective cough frequency after just 2 weeks of treatment.[45] This has prompted investigators in the field to further explore the role of ATP on nerves and cough, and it is hoped that results from a large multicentre study will pave the way for a future anti-tussive.

Conclusion

Despite the impression that chronic cough is a difficult condition to investigate and manage, a stepwise approach to investigate and identify associated treatable diseases can be successful. In the absence of treatment responses, neuro-modulating pharmacological and physical therapies can provide relief, but this needs to be managed in specialist clinics. It is likely that over the coming years we will have novel anti-tussives targeting P2X3 and other neuronal receptors with better efficacy and tolerability than current treatment options. ■

Conflicts of Interest

AAW and JAS are named inventors on a patent, owned by University Hospital of South Manchester (UHSM), describing a method for generating output data licensed to Vitalograph Ltd; however, no financial benefits have been received

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