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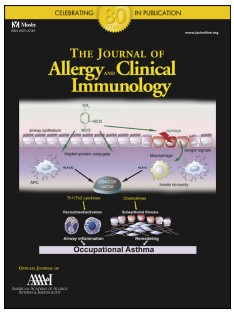

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Capsaicin Cough Responses in Asthma: Evidence for Airway Neuronal Dysfunction

Imran Satia M.D.[1,2], Nikolaos Tsamandouras PhD[3], Kimberley Holt MPhil[1], Huda Badri M.D [1], Mark Woodhead M.D [1,4], Kayode Ogungbenro PhD[4], Timothy W Felton PhD[1,2], Paul M O'Byrne M.D [1], Stephen J Fowler M.D [1], Jaclyn A Smith PhD[1,2]

1. Centre for Respiratory Medicine and Allergy, University of Manchester and Manchester Academic Health Science Centre, Manchester, UK
2. University Hospital of South Manchester, Manchester, UK
3. Centre for Applied Pharmacokinetic Research, Manchester Pharmacy School, University of Manchester, UK
4. Central Manchester NHS Foundation Trust, Manchester, UK

Corresponding Author: Professor Jacky Smith, Centre for Respiratory and Allergy Research, University of Manchester, University Hospital of South Manchester, Level 2, Education and Research Centre, Manchester, M23 9LT. jacky.smith@manchester.ac.uk, +44 (0)161 291 5863

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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: 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 may 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.

Key words: atopy; transient receptor potential vanilloid type 1; vagus; pharmacodynamic modelling

ABBREVIATIONS USED:

ACQ, Asthma control questionnaire, BDNF, Brain-derived natriuretic factor; BHR, Bronchial hyper-responsiveness; BMI, Body mass index; C2, Concentration of capsaicin inducing at least 2 coughs; C5, Concentration of capsaicin inducing at least 5 coughs; ED50, Capsaicin dose inducing half-maximal response; Emax, Maximum cough response evoked by any concentration of capsaicin; FENO, Fractional exhaled nitric oxide; FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; GINA, Global initiative for Asthma; HV, Healthy volunteers; LCQ, Leicester cough questionnaire; NGF, Nerve growth factor; PC_{20}, Provocative concentration of methacholine causing a 20% drop in FEV1; TRPV1, Transient receptor potential vanilloid type 1

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[10-12, 19]. 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[15]. 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[13, 14].

METHODS:

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.

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[15], 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.

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[16, 17]. 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[15]; 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.

RESULTS:

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.

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.

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.

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.

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).

Safety of full dose capsaicin challenge

Transient bronchoconstriction after inhaling capsaicin has been reported in patients with asthma[18]. 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.

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 C2and 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).

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[19]. 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 that 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[15, 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[15]. 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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353 **TABLES AND FIGURES:**

354 **Table 1:** Comparison of subjects with asthma and healthy volunteers. Data quoted as

355 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

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357

358 **Table 2:** Description of the key characteristics of patients with asthma. Data quoted as

359 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	210	175

	(58-470)	(70.0-460)	(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: $\sqrt{(e^{\omega^2}-1)} \cdot 100$

382 **Table 4: Emax and ED50 values for healthy volunteers and asthmatics** Values below
383 represent the E_{max} and ED_{50} values for a typical healthy and asthmatic male/female (i.e. with
384 median values for all model-incorporated covariates).

385

	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

386

FIGURE LEGENDS:

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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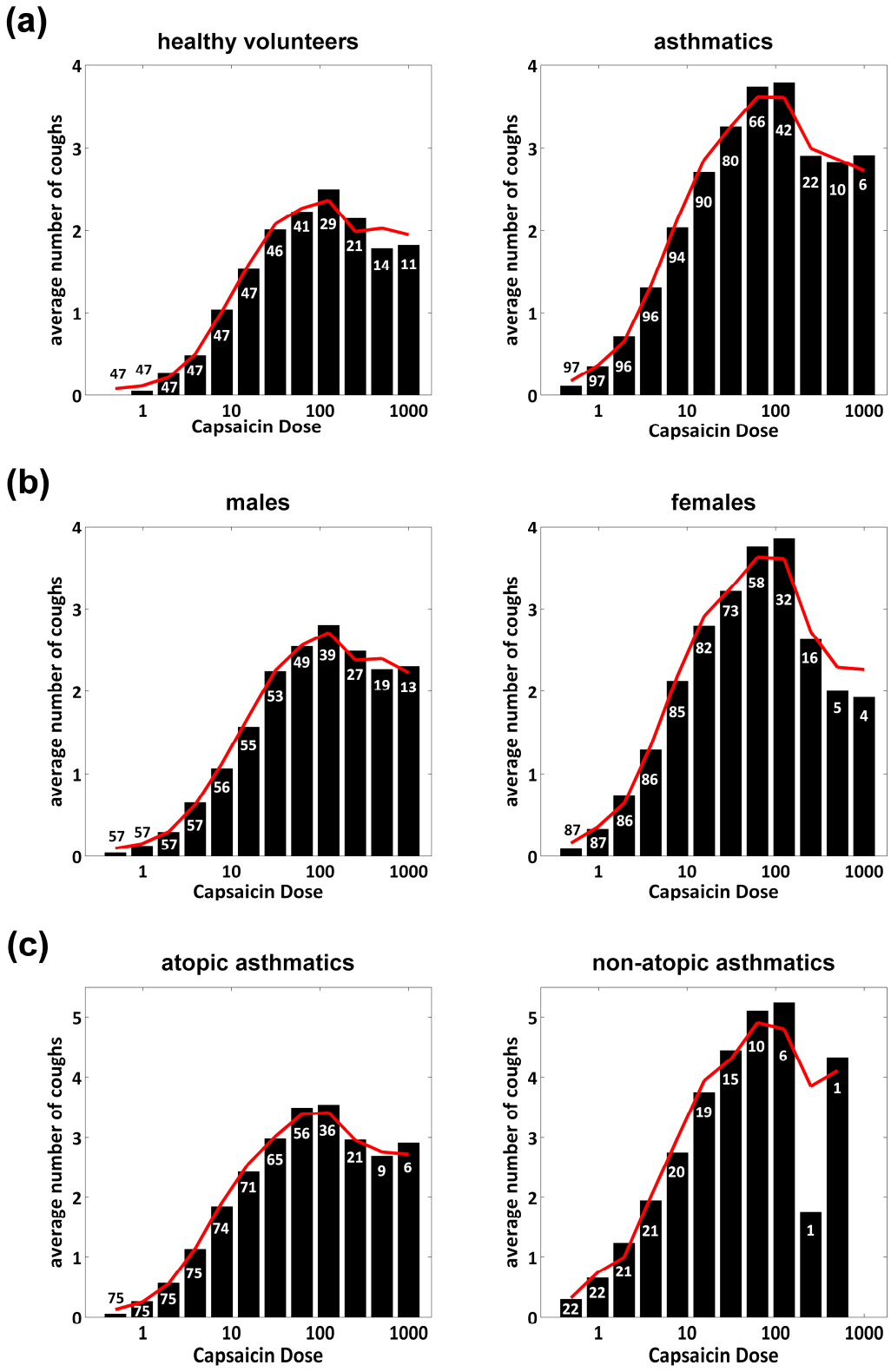
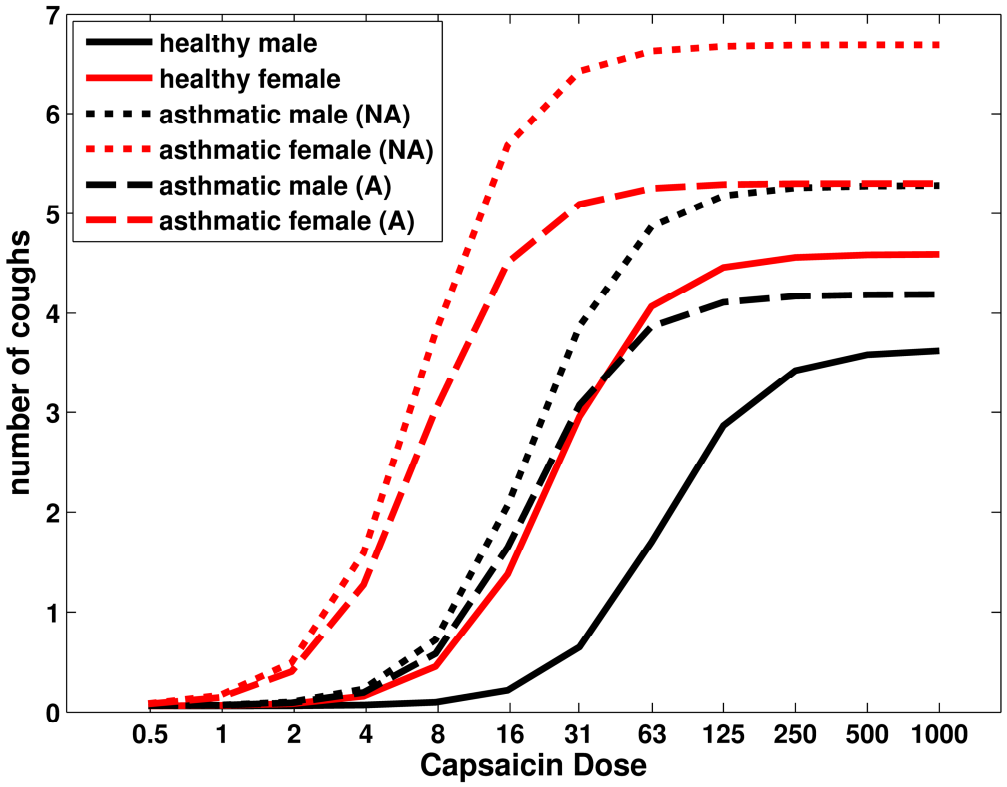
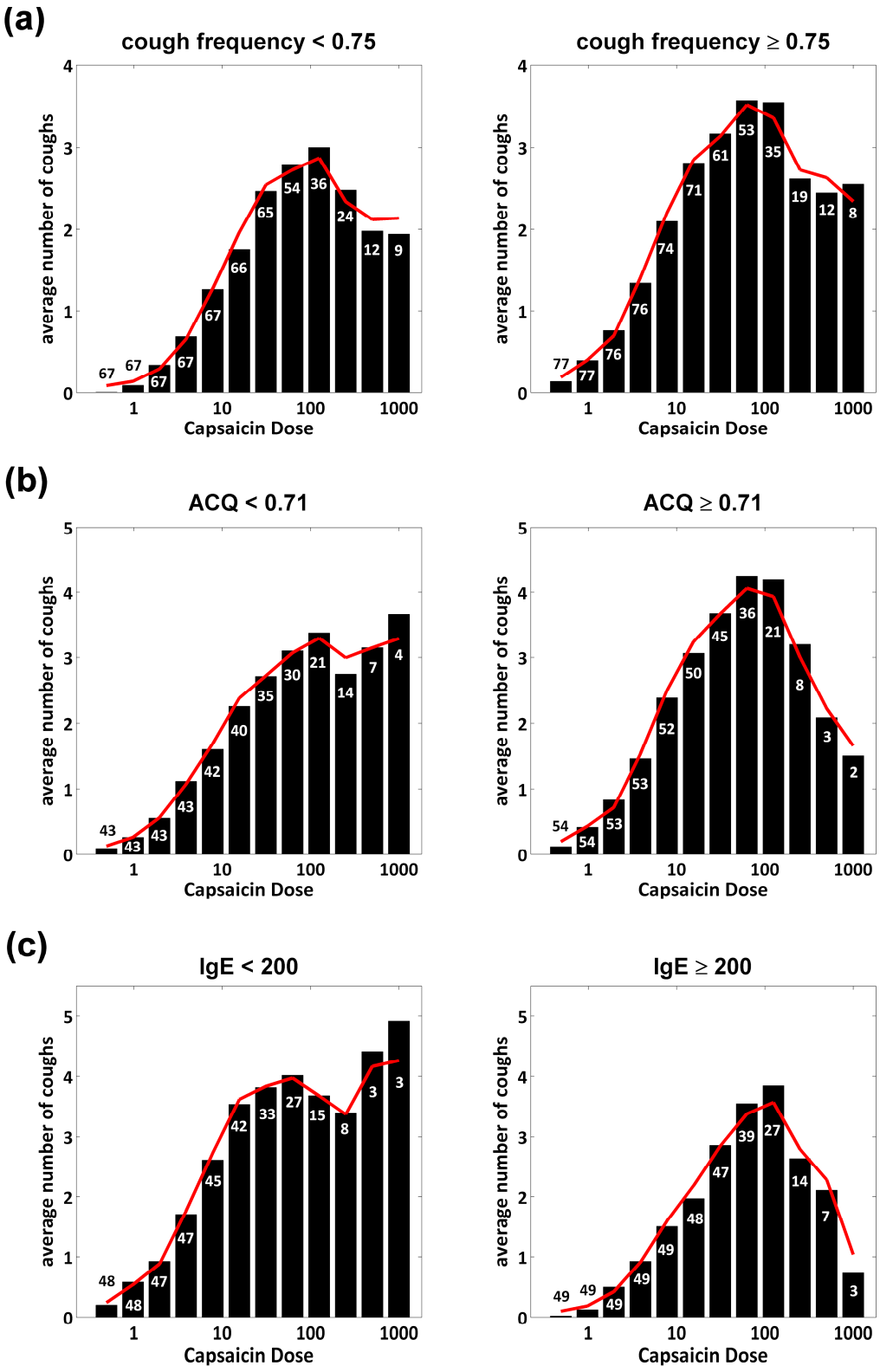
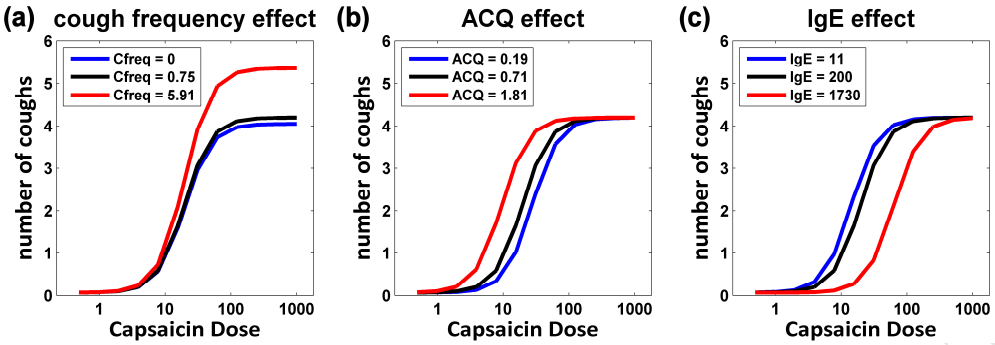

(a)
healthy volunteers
asthmatics
average number of coughs
Capsaicin Dose
47 47 47 47 47 46 41 29 21 14 11
97 97 96 96 94 90 80 66 42 22 10 6
(b)
males
females
57 57 57 56 55 53 49 39 27 19 13
87 87 86 86 85 82 73 58 32 16 5 4
(c)
atopic asthmatics
non-atopic asthmatics
75 75 75 75 74 71 65 56 36 21 9 6
22 22 21 21 20 19 15 10 6 1 1
1
10
100
1000







ONLINE DATA SUPPLEMENT

Capsaicin Cough Responses in Asthma: Evidence for Neuronal Dysfunction

Imran Satia[1,2], Nikolaos Tsamandouras [3], Kimberley Holt [1], Huda Badri [1], Mark Woodhead [1,4], Kayode Ogungbenro [3], Timothy W Felton [1,2], Paul M O'Byrne[1], Stephen J Fowler [1], Jaclyn A Smith[1,2]

1. Centre for Respiratory Medicine and Allergy, University of Manchester and Manchester Academic Health Science Centre, Manchester, UK
2. University Hospital of South Manchester, Manchester, UK
3. Centre for Applied Pharmacokinetic Research, Manchester Pharmacy School, University of Manchester, UK
4. Central Manchester NHS Foundation Trust, Manchester, UK

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 Duse 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 atleast 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.

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 ($Emax, ED50, \gamma$) 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.

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.

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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TABLES

Table E1

	Group	Geometric Mean, (95% CI) Capsaicin µmol/L	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)	

FIGURE LEGENDS

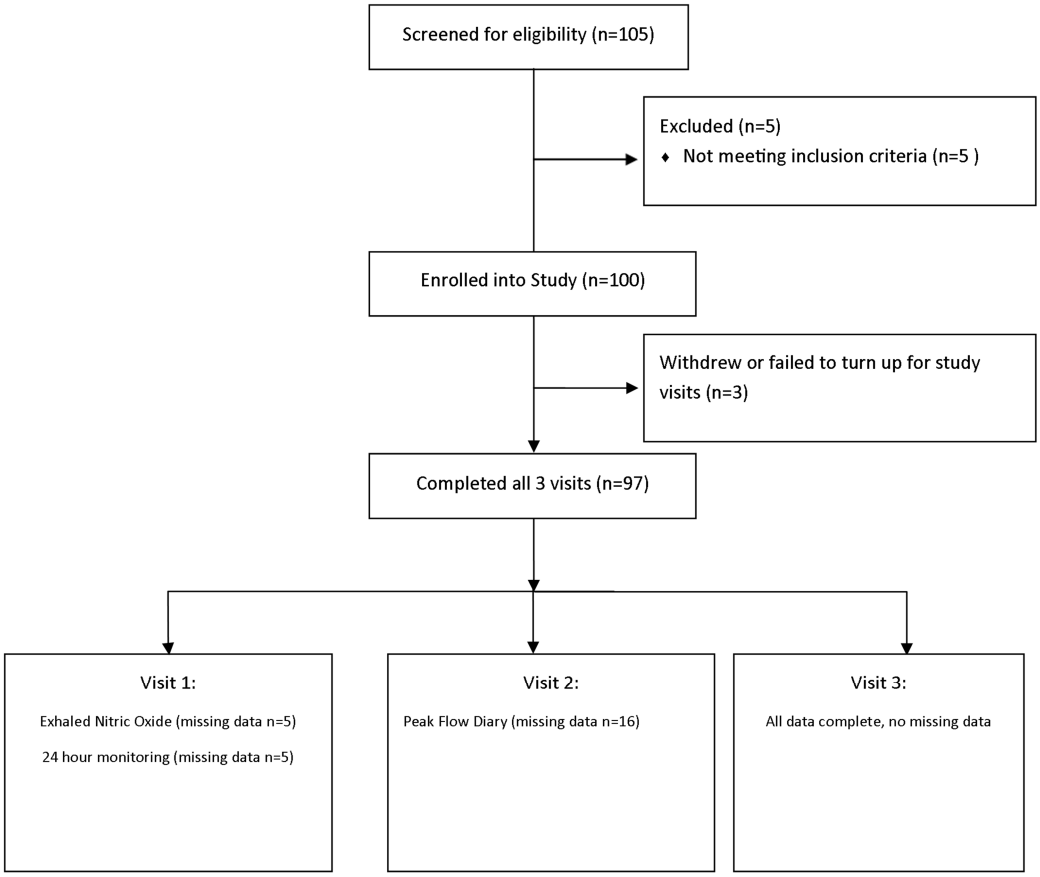
Figure E1: Patient flow diagram illustrating number of patients screened, withdrawals and missing data.

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.


Screened for eligibility (n=105)
Excluded (n=5)
♦ Not meeting inclusion criteria (n=5)
Enrolled into Study (n=100)
Withdrew or failed to turn up for study visits (n=3)
Completed all 3 visits (n=97)
Visit 1:
Exhaled Nitric Oxide (missing data n=5)
24 hour monitoring (missing data n=5)
Visit 2:
Peak Flow Diary (missing data n=16)
Visit 3:
All data complete, no missing data