ONLINE SUPPLEMENT

The Interaction between Bronchoconstriction and Cough in Asthma

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ABBREVIATIONS USED:

ATP, adenosine triphosphate; BHR, 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; FEV1, Forced expiratory volume in 1 second; GEE, generalise estimating equations; GINA, Global initiative for Asthma; ICS, inhaled corticosteroids; PC_{20}, Provocative concentration of methacholine causing a 20% drop in FEV1; SABA, salbutamol; TGF-B, transforming growth factor beta; TRPV1, Transient receptor potential vanilloid type 1

SUPPLEMENTARY 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 the cough reflex, 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.

DETAILED METHODS:

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.

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 E1).

On visit 1, subjects underwent history and examination, spirometry, and a capsaicin cough challenge, as previously described (1, 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}; we have previously shown these parameters to be highly reproducible (8). 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) (9) 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 E1). 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.

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). The GEE models assumed an exchangeable working correlation matrix structure with a robust variance estimation and were adjusted for baseline ED50 cough responses and the effects of gender, period and intervention sequence assessed. 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.

SUPPLEMENTARY RESULTS:

Subjects

Two subjects screened did not meet BHR criteria (PC20>16mg/ml), but fourteen subjects completed the first six visits (periods 1 and 2). Ten of those subjects (four males) returned to complete the additional two visits (visits 7 and 8, period 3). Table E1 shows the demographics and key baseline data of the subjects enrolled.

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 1A). The analysis was adjusted for baseline ED50 coughs and there was no significant effect of gender, period or sequence.

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 15.7-8.9) vs. 13.7% (18.7-8.7) of baseline respectively, $p=0.49$) (Figure E2).

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 1B). 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 1B). Therefore, each 10% improvement in FEV1 equated to a reduction of 2.4 coughs evoked by capsaicin.

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 2).

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 E3).

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).

SUPPLEMENTARY 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(10). 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 especially in subjects with asthma (1, 7). 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 (11-13) . 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 several 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)

(14). Furthermore TGF-β, a mediator typically associated with mechanical stress, has also been shown to sensitise nerve terminals via ATP release (15, 16). 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 (17, 18). Finally, bronchoconstriction alters the deposition of inhaled particles within the airways (19). During bronchoconstriction capsaicin may have been deposited in areas more densely innervated with cough fibres. Such as effect 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 responses, 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 some important mechanistic insights about how airway pathophysiology in asthma relates to the development of symptoms through neuronal activation. These data shows that bronchoconstriction increases the activation of capsaicin-responsive airway nerves but the mechanisms and mediators involved require further evaluation.

ACKNOWLEDGMENTS

The authors would like to thank all the subjects who participated in the study, the National Institute for Health Research (NIHR) South Manchester Clinical Research Facility (CRF) and the NIHR/Wellcome Trust Central Manchester CRF.

Author's contributions: Concept and design; IS, HB, MW, POB, SJF, JAS. Data generation; IS, and HB. 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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ONLINE TABLES AND FIGURES:

Table E1: 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.

ONLINE FIGURES

Figure E1: Flow chart summarising the study design

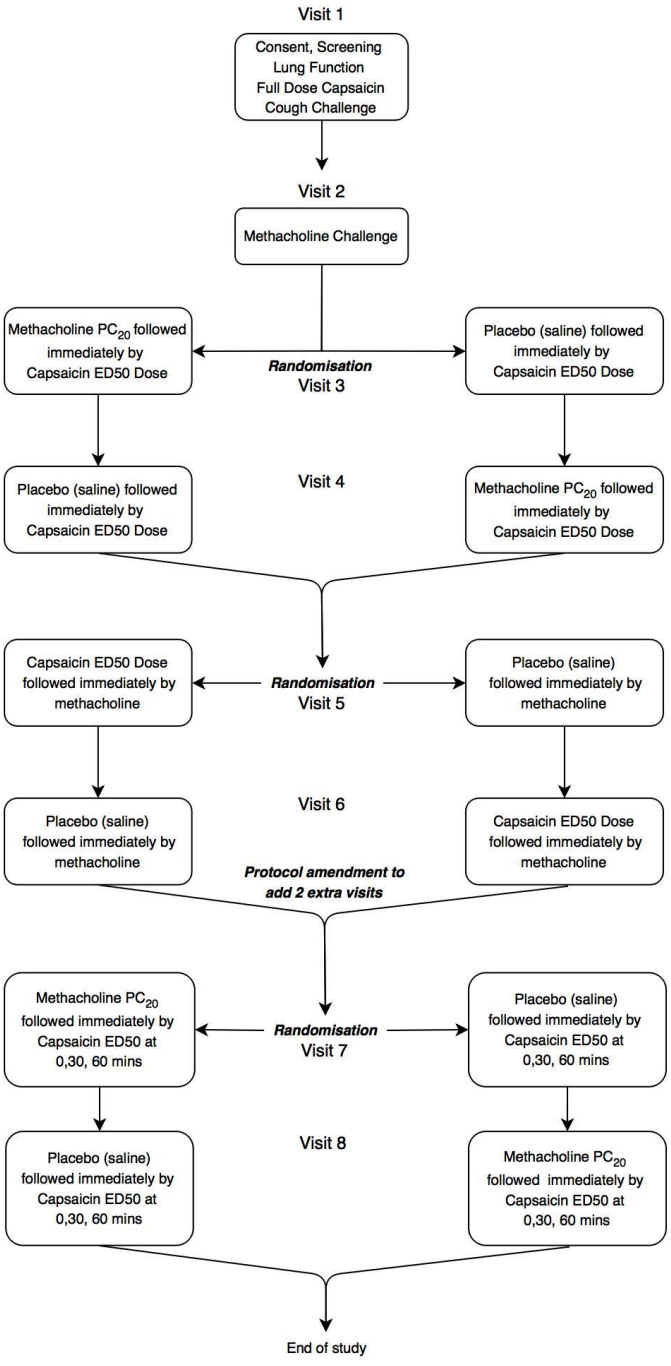
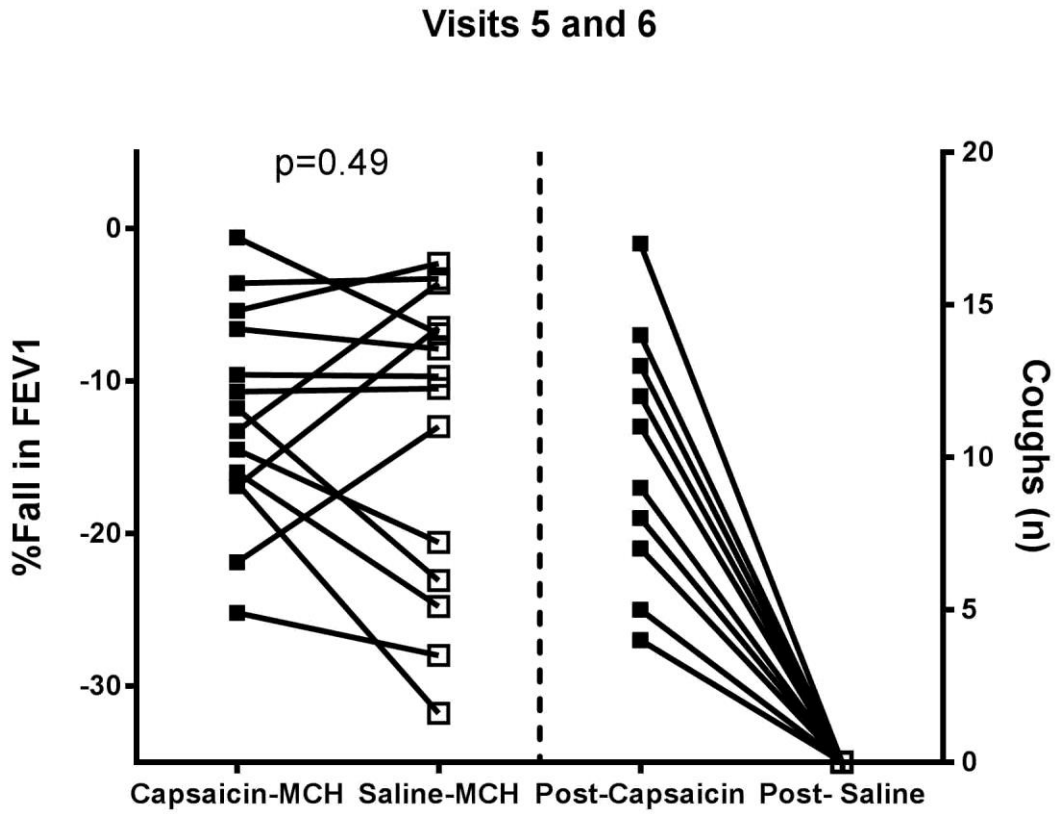
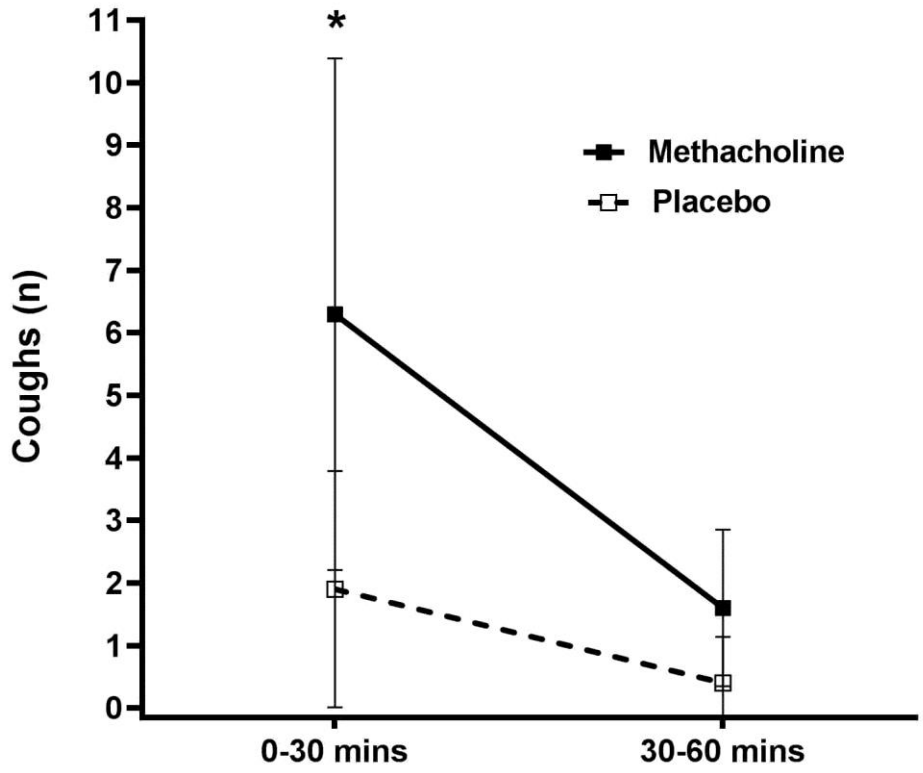


Figure E2: The effects of capsaicin evoked coughing on methacholine bronchial hyper-responsiveness. Fall in %FEV1 from baseline (left y-axis) after methacholine and saline challenge with corresponding capsaicin evoked coughs (right y-axis).



325 **Figure E3:** Spontaneous coughs during 0-30 mins and 30-60 mins after inhaling

326 methacholine and placebo. Mean and 95% CI shown.* indicates p=0.05.



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