Plasma substance P levels in patients with persistent cough

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Short title: Plasma SP in persistent cough

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26 responsiveness, plasma

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Abstract (250 words)

Background: Substance P (SP) is involved in the pathogenesis of cough in animal models. However, few studies of humans have been reported and the roles of SP in clinical cough remain obscure.

Objectives: To clarify the relevance of plasma levels of SP in patients with persistent cough.

Methods: We studied 82 patients with cough persisting for at least 3 weeks and 15 healthy controls. Patients were classified as having asthmatic cough (cough variant asthma and cough-predominant asthma; n = 61) or non-asthmatic cough (n = 21; post-infectious cough, n = 6; gastroesophageal reflux disease, n = 5; idiopathic cough, n = 5; others, n = 5). Correlations were evaluated between plasma SP levels as measured with ELISA and each of methacholine airway hyperresponsiveness (airway sensitivity and airway reactivity), capsaicin cough sensitivity, sputum eosinophil and neutrophil counts, and pulmonary function.

Results: Plasma SP levels were significantly elevated in patients with both asthmatic and non-asthmatic cough compared with controls (31.1 (18.0-52.2) and 30.0 (15.1-50.3) vs. 15.4 (11.3-23.7) pg/ml; p = 0.003 and p = 0.038, respectively), but did not differ between the two patient groups (p = 0.90). Plasma SP levels correlated with airway sensitivity (threshold dose of methacholine) in the patients with asthmatic cough (r = -0.37, p = 0.005), but not with airway reactivity, cough sensitivity, FEV_1 values or sputum eosinophil and neutrophil counts in either group.

Conclusions: Increased levels of SP in plasma are associated with persistent cough in

humans, and might be related to airway sensitivity in asthmatic cough.

Abbreviation list

AHR: airway hyperresponsiveness

CGRP: calcitonin gene-related peptide

CVA: cough variant asthma

$FEF_{25-75\%}$: forced mid-expiratory flow

GERD: gastroesophageal reflux disease

RARs: rapidly adapting receptors

SP: substance P

Introduction

Substance P (SP) is one of several neuropeptides that are widely distributed in sensory peripheral nerves [1] and in the central nervous system [2]. Ample evidence supports a role for SP in the mechanism of cough in animal models [3-5]. Although its activity in cough induction is controversial [3,6], SP elicits a sensitizing effect on the cough reflex [4,5], while conflicting results also exist [7].

A few studies have examined the relationship between SP and cough in humans. Patients with attenuated cough sensitivity associated with advanced Parkinson's disease and aspiration pneumonia have reduced SP levels in sputum [8,9]. Substance P-immunoreactive nerve densities of the bronchial epithelium are increased in patients with cough variant asthma (CVA), compared with those of patients with classic asthma and healthy controls [10]. Levels of SP are also increased in nasal lavage fluid [11] and sputum [12] from patients with non-asthmatic cough. Furthermore, elevated numbers of calcitonin gene-related peptide (CGRP)-immunoreactive nerves in the bronchial epithelium of patients with idiopathic persistent cough correlate with cough sensitivity to inhaled capsaicin, whereas levels of SP are not elevated or correlate with cough sensitivity [13]. Yoshihara et al. have shown that plasma SP levels are elevated in patients with paroxysmal cough due to pertussis [14]. However, further information about plasma SP levels in persistent cough of other etiologies has not been reported.

Cough is attributed to various causes [15-18]. Persistent cough due to asthmatic and non-asthmatic causes might involve different mechanisms, since asthmatic cough is elicited

by bronchoconstriction whereas non-asthmatic cough might be primarily ascribed to increased cough sensitivity. Although cough and bronchoconstriction often occur simultaneously, they are regarded as separate reflexes 19]. Bronchoconstrictors including methacholine provoke cough without altering the cough reflex 20].

Other than cough, evidence shows that SP functions in bronchoconstriction and airway hyperresponsiveness (AHR) [21,22]. Therefore, SP might be differently involved in the mechanisms of both asthmatic and non-asthmatic cough. Indeed, SP contents in the nasal lavage fluid of patients with non-asthmatic cough are associated with increased cough sensitivity [11], whereas in asthma, sputum levels of SP correlate with airflow obstruction [23].

Here, we compared plasma SP levels in patients with combined subacute cough (duration of 3 to 8 weeks) and chronic cough (> 8 weeks) as defined by a guideline [15] with those of healthy controls. We also examined the relationship between plasma SP levels and various clinical and functional indices to determine the roles of SP in patients with asthmatic and non-asthmatic cough.

Material and Methods

Participants

We studied 82 consecutive patients who were referred to the outpatient asthma and cough clinic of Kyoto University Hospital between October 2007 and August 2009 because of cough that had persisted for at least 3 weeks. None of the patients had abnormal chest radiographic findings, or had been prescribed with angiotensin-converting enzyme inhibitors, oral or inhaled corticosteroids, leukotriene receptor antagonists, or other anti-allergic drugs. None had been taking drugs that may interfere with circulating substance P levels, such as anti-histamines, angiotensin- converting enzyme inhibitors, or centrally acting drugs such as dopamine receptor agonists. All patients were either never smokers or former smokers who had smoked less than 10 pack-years and had quitted smoking for more than one year.

Causes of cough were determined according to the Japanese cough guidelines [16]. In brief, patients with AHR to methacholine or reversible airflow obstruction and improvement of coughing with β_2-agonists were considered as having chronic cough due to asthma. Patients with cough as the sole or predominant symptom (cough-variant or cough-predominant asthma) were included, and were categorized as having asthmatic cough. The others were categorized as having non-asthmatic cough caused by the following [16]: sinobronchial syndrome (chronic sinusitis complicated by neutrophilic airway inflammation of the lower airways) [17,24] diagnosed based on positive sinus images, and symptoms related to chronic sinusitis improved with macrolides; gastroesophageal reflux disease (GERD) based on

response to treatment with proton-pump inhibitors; post-infectious cough, based on a history of upper respiratory tract infection followed by cough that spontaneously subsided; atopic cough, based on findings suggesting an atopic predisposition or induced sputum eosinophilia as well as a response to anti-histamines [16,25]; cough due to pertussis based on the typical clinical course of pertussis and a positive antipertussis toxin antibody reaction; and idiopathic cough, for which extensive examinations and intensive therapeutic trials were negative or failed to reveal any conclusive findings. The numbers of patients who underwent more specialized and detailed examinations or assessment were as follows: 9 for thoracic CT, 4 for sinus CT, 3 for esophageal endoscopy, 3 for bronchoscopy, and 3 for ENT consultations. To compare the plasma SP levels among different causes of subacute and chronic cough, patients with cough due to multiple causes were not included in the cohort, who had asthma and GERD (n=4), postinfectious cough and GERD (n=2), asthma and sinobronchial syndrome (n=2), or sinobronchial syndrome and GERD (n=1).

We also studied 15 healthy controls recruited from our hospital staff who had no history of respiratory disease. We excluded individuals with atopic dermatitis in this study, because its presence positively affects the plasma SP levels [26]. The Ethics Committee of Kyoto University approved the research protocol (approval number E-300) and written informed consent was obtained from all participants.

Measurement of plasma levels of substance P

Blood was sampled at presentation in all subjects. Samples were immediately centrifuged

and plasma was mixed with an equal volume of a stabilizer (Kyowa Medex Co., Ltd. KM Assay Center, Nagaizumi-cho, Shizuoka, Japan) that inhibits neutral endopeptidase [27]. The samples were frozen at -20°C. Investigators who were blinded to the clinical conditions of the patients measured plasma SP levels using a competitive ELISA method [27,28]. The sensitivity of this assay is 4.1 pg/ml. The specificity of the assay for SP measurement is 100%, and the assay does not significantly cross-react with neurokinin A or neurokinin B.

Sputum induction and processing

Sputum was induced and processed as described by Pin [29] with slight modifications [30]. In brief, after pretreatment with salbutamol, sputum was induced by inhaling hypertonic saline (3%) solution for 15 min from an ultrasonic nebulizer. Adequate plugs of sputum were treated with 0.1% dithiothreitol (Sputasol, Oxoid Ltd., Hampshire, UK), followed by Dulbecco's phosphate-buffered saline (PBS). Eosinophil and neutrophil percentages were determined by counting at least 400 non-squamous cells on centrifuged preparations visualized by May-Grünwald-Giemsa staining.

Our primary purpose of evaluating sputum cells was to investigate the association of cellular inflammation of the airways with plasma levels of SP. Sputum cell differentials were also used for the diagnosis of disease, e.g., atopic cough.

Pulmonary function test

We measured forced vital capacity (FVC), FEV_1, and forced mid-expiratory flow ($FEF_{25-75\%}$)

157 with a use of Chestac-65V (Chest MI Corp., Tokyo, Japan), as described [31].

158

159 **Methacholine challenge test**

160 We determined AHR by measuring respiratory resistance (Rrs; $cmH_20/L/sec$) (Astograph^{TM};
161 Chest, Tokyo, Japan) under continuous methacholine inhalation as described [32,33]. The
162 index of airway sensitivity was Dmin, namely, the cumulative dose of inhaled methacholine
163 at the inflection point where Rrs started to continuously increase. One unit of Dmin is
164 equivalent to a dose of 1 mg/ml of methacholine inhalation for 1 min. When the respiratory
165 resistance did not increase despite methacholine inhalation at the highest concentration,
166 Dmin was assigned a value of 50 units, which was the total cumulative dose of
167 methacholine. The slope of the respiratory dose-response curve (SRrs) was used as the
168 measure of airway reactivity [33]. Fifty-nine patients with asthmatic cough and all 21 with
169 non-asthmatic cough underwent the test.

170

171 **Capsaicin cough sensitivity test**

172 Cough sensitivity was tested by continuous inhalation of capsaicin as described [34] with a
173 slight modification of capsaicin concentrations [24]. Ten doubling concentrations of
174 capsaicin solution (0.61 - 312 μM) were inhaled until ≥5 coughs were induced. Each
175 concentration of capsaicin was inhaled for 15 sec during tidal breathing every 60 sec. The
176 concentration of capsaicin causing ≥5 coughs is referred to as C5 [24,34]. Fifty-nine patients
177 with asthmatic cough and all 21 with non-asthmatic cough underwent the test.

178

179 **Statistical analysis**

180 Data are expressed as median values (25^{th} to 75^{th} percentiles) except when noted otherwise
181 and were analyzed using JMP 6.0 (SAS Campus Drive, Cary, NC, USA). Comparisons of
182 two and three groups were achieved using the Mann-Whitney and Kruskal-Wallis tests,
183 respectively, and the latter were analyzed post hoc using the Steel-Dwass test [35-37].
184 Categorical data were compared using the χ^2 test. Correlations between data were analyzed
185 using Spearman's rank correlation test. P values of <0.05 were considered statistically
186 significant.

187

Results

Characteristics of the three groups

Table 1 shows the characteristics of the 61 patients with asthmatic cough, 21 with non-asthmatic cough and 15 controls. Only the ratios of sputum eosinophils significantly differed among the three groups.

Cough in the non-asthmatic cough group was due to post-infection (n = 6), GERD (5), atopic cough (2), pertussis (2), sinobronchial syndrome (1) but was idiopathic in 5 patients.

Outcomes in patients with asthmatic and non-asthmatic cough

Table 2 shows the outcomes of the two patient groups. Two of the patients with asthmatic cough were ineligible for AHR analysis since the inflection point where Rrs increased could not be determined because severe coughing was elicited. Patients with asthmatic cough were significantly more sensitive to methacholine as determined by Dmin than those with non-asthmatic cough. C5 was marginally lower in patients with non-asthmatic cough than those with asthmatic cough.

Comparison of plasma SP levels among the three groups

Plasma SP levels were significantly higher in patients with asthmatic and non-asthmatic cough compared with healthy controls (31.1 (18.0-52.2) and 30.0 (15.1-50.3) vs. 15.4 (11.3-23.7) pg/ml), but did not significantly differ between the two patient groups (Fig. 1).

208

209 **Relationships between plasma SP levels and clinical indices**

210 Plasma SP levels significantly correlated with airway sensitivity determined by Dmin only in
211 patients with asthmatic cough (Table 3, Fig. 2). Plasma SP levels did not correlate with
212 cough duration, airway reactivity, capsaicin cough sensitivity, spirometric indices or sputum
213 neutrophil and eosinophil counts in either patient group (Table 3).

214

Discussion

We measured plasma level of SP in patients with subacute and chronic cough of various origins and healthy subjects. We discovered that plasma SP levels are elevated in patients with cough of asthmatic and non-asthmatic origins. We also found that plasma SP levels correlate with airway sensitivity in patients with asthmatic cough. These results indicate that SP is involved in both asthmatic and non-asthmatic cough, and its role in the mechanisms of these types of cough might differ.

Substance P synthesized in the cell body of C-fibers is transported along axons towards the peripheral and central terminals, where it is stored in large-granular vesicles. C-fiber activation evokes SP release into the airway dependent on the axon reflex in guinea pigs. Airway SP causes bronchospasm, vasodilation, edema and mucus secretion, which secondarily evokes the activation of rapidly adapting receptors (RARs) in the airway, resulting in an enhanced cough reflex [5,6]. The sensitizing effect of SP on RARs has also been demonstrated in the central nervous system, especially in the nucleus tractus solitarius, which also results in an enhanced cough reflex [38,39]. While airway SP levels or expression in patients with persistent cough are conflicting [10-13,40,41], we found elevated plasma SP levels in patients with persistent asthmatic and non-asthmatic cough.

We found that SP may be associated with airway sensitivity in asthmatic cough. Airway sensitivity and airway reactivity are two major components of AHR, and might have different underlying mechanisms [33,42]. Both airway sensitivity and reactivity are associated with SP. Umeno et al. reported that intravenous SP increases airway sensitivity to

histamine in guinea pigs [22] and Cheung et al. showed that inhaled SP enhances maximal airway narrowing to methacholine in patients with asthma [21]. Airway sensitivity may be determined by the strength of the stimulus that triggers the airways to constrict, such as epithelial damage, neural control and inflammatory cell numbers, while airway reactivity may be caused by the responsiveness of airways to applied stimuli such as smooth muscle contractility, viscous and elastic loads, and airway swelling [42]. Our results suggest that SP plays a role in the pathophysiology of asthmatic cough by affecting airway sensitivity.

Although levels of plasma SP were higher in patients with non-asthmatic cough than in healthy controls, we found no correlation between plasma SP levels and various indices including capsaicin cough sensitivity. This is in conflict with the findings of Cho et al., who showed that SP levels in nasal lavage fluid correlate with capsaicin cough sensitivity in patients with non-asthmatic cough [11]. This discrepancy might be attributed to the different sample sources, or smaller sample size in our study. Indeed, although Cho et al. found a correlation between SP in nasal lavage fluid and cough sensitivity in all 38 of their patients with non-asthmatic cough, no correlation was evident when the patients were separated into two equal, separately analyzed groups (n = 19 for each) with increased and normal cough sensitivity [11].

The two phenotypes of asthma might involve different pathophysiological mechanisms: CVA or cough-predominant asthma and classic asthma that predominantly presents with wheezing. De Diego et al. have reported that although classic asthma and CVA have similar profiles of airway inflammatory markers, their relationships with AHR and

cough sensitivity differ [43]. Lee et al. found increased SP-immunoreactive nerve densities in patients with CVA but not in those with classic asthma [10]. Substance P is associated with neurogenic inflammation and subsequent airflow obstruction in asthma [23,44]. We found no correlation between plasma SP levels and spirometric indices, which contradicts the findings of Tomaki et al., who found a negative correlation between sputum SP levels and FEV_1/FVC in patients with classic asthma [23]. This discrepancy might be attributed to the different sample sources, and less prominent airflow obstruction in our patients (FEV1/FVC of 80.3% by average) compared with those of Tomaki et al. (71.3%).

Airway inflammation stimulates receptors of unmyelinated C-fibers of the vagus nerve, thus causing the release of tachykinins such as SP from the C-fibers [11,45,46]. A correlation between sputum SP levels and sputum eosinophil count has been reported in asthma [23]. Since patients with CVA show evidence of airway inflammation with increased eosinophils [47] and since non-asthmatic cough might be associated with increased neutrophils [48], we evaluated the correlation between plasma SP levels and sputum counts of eosinophils and neutrophils in each patient group. However, we found no correlation between plasma SP levels and these sputum cells in either patient group. Circulating levels of SP might not be related to cellular inflammation of the airways in patients with persistent cough.

Our study has some limitations. Firstly, we could not determine whether SP levels in plasma reflect those in the airways. Moreover, although SP is widely distributed in the central and peripheral nervous system, there is increasing evidence that it may be

277 synthesized and released from inflammatory cells such as eosinophils, monocytes and
278 macrophages, lymphocytes and dendritic cells [49-52]. Therefore elevated levels of SP in
279 plasma may reflect over expression of SP in inflammatory cells as well as those in sensory
280 nerves. Secondly, atopic dermatitis might have influenced the levels of SP in plasma [53].
281 Atopic dermatitis was present in eight patients with asthmatic cough but none in those with
282 non-asthmatic cough. However, we found no difference in plasma SP levels between patients
283 with and without atopic dermatitis ($p = 0.51$). Thirdly, the sample size of patients with
284 non-asthmatic cough was small, and the etiology of their cough was diverse. We found no
285 differences in plasma SP levels among the three most common diagnostic subgroups of
286 non-asthmatic cough (post-infectious cough [n = 6], GERD [n = 5], and idiopathic cough [n
287 = 5]; $p = 0.196$ by Kruskal-Wallis test). Future larger studies might clarify the roles of SP,
288 especially in non-asthmatic cough. Fourthly, while success rate of sputum induction in our
289 previous series of 407 patients with classic asthma was 73.0% [54], the success rate in the
290 present study was lower (57/97, 59%). This may be because cough due to cough variant
291 asthma or non-asthmatic origin such as GERD is more dry or non-productive in nature as
292 compared with cough of classic asthma [55], and “healthy” controls do not present with
293 sputum by definition. Fifthly, patients diagnosed as not having GERD might actually have
294 had GERD, because the diagnosis of GERD solely depended on the response to specific
295 therapy, not involving the gold standard for the diagnosis. The same is true for more
296 uncommon causes of cough such as obstructive sleep apnea and tracheal collapse.

297 Despite these limitations, the elevated plasma SP levels in patients with asthmatic

298 and non-asthmatic cough are notable. Substance P in plasma is associated with persistent
299 cough and it might be related to airway sensitivity in asthmatic cough.

References

1. Groneberg DA, Harrison S, Dinh QT, Geppetti P, Fischer A. Tachykinins in the respiratory tract. *Curr Drug Targets.* 2006;7(8):1005-1010.
2. Bonham AC, Sekizawa SI, Joad JP. Plasticity of central mechanisms for cough. *Pulm Pharmacol Ther.* 2004;17(6):453-457; discussion 469-470.
3. Kohrogi H, Graf PD, Sekizawa K, Borson DB, Nadel JA. Neutral endopeptidase inhibitors potentiate substance P- and capsaicin-induced cough in awake guinea pigs. *J Clin Invest.* 1988;82(6):2063-2068.
4. Fox AJ. Modulation of cough and airway sensory fibres. *Pulm Pharmacol.* 1996;9(5-6):335-342.
5. Moreaux B, Nemmar A, Vincke G, Halloy D, Beerens D, Advenier C, Gustin P. Role of substance P and tachykinin receptor antagonists in citric acid-induced cough in pigs. *Eur J Pharmacol.* 2000;408(3):305-312.
6. Takahama K, Fuchikami J, Isohama Y, Kai H, Miyata T. Neurokinin A, but not neurokinin B and substance P, induces codeine-resistant coughs in awake guinea-pigs. *Regul Pept.* 1993;46(1-2):236-237.
7. El-Hashim AZ, Amine SA: The role of substance P and bradykinin in the cough reflex and bronchoconstriction in guinea-pigs. *Eur J Pharmacol* 2005, 513:125-133.
8. Ebihara S, Saito H, Kanda A, Nakajoh M, Takahashi H, Arai H, Sasaki H. Impaired efficacy of cough in patients with Parkinson disease. *Chest.* 2003;124(3):1009-1015.

9. Nakagawa T, Ohrui T, Sekizawa K, Sasaki H. Sputum substance P in aspiration pneumonia. *Lancet.* 1995;345(8962):1447.

10. Lee SY, Kim MK, Shin C, Shim JJ, Kim HK, Kang KH, Yoo SH, In KH. Substance P-immunoreactive nerves in endobronchial biopsies in cough-variant asthma and classic asthma. *Respiration.* 2003;70(1):49-53.

11. Cho YS, Park SY, Lee CK, Yoo B, Moon HB. Elevated substance P levels in nasal lavage fluids from patients with chronic nonproductive cough and increased cough sensitivity to inhaled capsaicin. *J Allergy Clin Immunol.* 2003;112(4):695-701.

12. Pizzichini MM, Pizzichini E, Parameswaran K, Clelland L, Efthimiadis A, Dolovich J, Hargreave FE. Nonasthmatic chronic cough: No effect of treatment with an inhaled corticosteroid in patients without sputum eosinophilia. *Can Respir J.* 1999;6(4):323-330.

13. O'Connell F, Springall DR, Moradoghli-Haftvani A, Krausz T, Price D, Fuller RW, Polak JM, Pride NB. Abnormal intraepithelial airway nerves in persistent unexplained cough? *Am J Respir Crit Care Med.* 1995;152(6 Pt 1):2068-2075.

14. Yoshihara S, Kanno N, Ando T, Fukuda N, Abe T, Ichimura T, Yanaihara N. Involvement of substance P in the paroxysmal cough of pertussis. *Regul Pept.* 1993;46(1-2):238-240.

15. Pratter MR, Brightling CE, Boulet LP, Irwin RS. An empiric integrative approach to the management of cough: ACCP evidence-based clinical practice guidelines. *Chest* 2006: 129: 222S-231S

16. Kohno S, Ishida T, Uchida Y, Kishimoto H, Sasaki H, Shioya T, Tokuyama K, Niimi A, Nishi K, Fujimura M *et al*: The Japanese Respiratory Society guidelines for management of cough. *Respirology* 2006, 11 Suppl 4:S135-186.
17. Niimi A. Geography and cough aetiology. *Pulm Pharmacol Ther.* 2007;20(4):383-387.
18. Wei W, Yu L, Lu H, Wang L, Shi C, Ma W, Huang Y, Qiu Z. Comparison of cause distribution between elderly and non-elderly patients with chronic cough. Respiration 2009;77:259-64.
19. Karlsson JA, Sant'Ambrogio G, Widdicombe J. Afferent neural pathways in cough and reflex bronchoconstriction. *J Appl Physiol.* 1988;65(3):1007-1023.
20. Ohkura N, Fujimura M, Hara J, Ohsawa M, Kamei J, Nakao S: Bronchoconstriction-triggered cough in conscious guinea pigs. *Exp Lung Res* 2009, 35:296-306.
21. Cheung D, van der Veen H, den Hartigh J, Dijkman JH, Sterk PJ. Effects of inhaled substance P on airway responsiveness to methacholine in asthmatic subjects in vivo. *J Appl Physiol.* 1994;77(3):1325-1332.
22. Umeno E, Hirose T, Nishima S. Pretreatment with aerosolized capsaicin potentiates histamine-induced bronchoconstriction in guinea pigs. *Am Rev Respir Dis.* 1992;146(1):159-162.
23. Tomaki M, Ichinose M, Miura M, Hirayama Y, Yamauchi H, Nakajima N, Shirato K. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am J Respir Crit Care Med.* 1995;151(3 Pt 1):613-617.

24. Matsumoto H, Niimi A, Tabuena RP; Takemura M, Ueda T, Yamaguchi M, Matsuoka H, Jinnai M, Chin K, Mishima M. Airway wall thickening in patients with cough variant asthma and nonasthmatic chronic cough. *Chest.* 2007;131(4):1042-1049.

25. Fujimura M, Ogawa H, Yasui M, Matsuda T. Eosinophilic tracheobronchitis and airway cough hypersensitivity in chronic non-productive cough. Clin Exp Allergy 2000;30:41-7.

26. Toyoda M, Nakamura M, Makino T, Hino T, Kagoura M, Morohashi M. Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br J Dermatol* 2002: 147: 71-79

27. Hosokawa C, Takeuchi S, Furue M. Severity scores, itch scores and plasma substance P levels in atopic dermatitis treated with standard topical therapy with oral olopatadine hydrochloride. *J Dermatol* 2009: 36: 185-190

28. Fehder WP, Campbell DE, Tourtellotte WW, Michaels L, Cutilli JR, Uvaydova M Douglas SD. Development and Evaluation of Chromatographic Procedure for Partial Purification of Substance P with Quantitation by Enzyme Immunoassay. Clin Diagn Lab Immunol 1998*;* 5*:* 303–307.

29. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax.* 1992;47(1):25-29.

30. Takemura M, Niimi A, Minakuchi M, Matsumoto H, Ueda T, Chin K, Mishima M. Bronchial dilatation in asthma: relation to clinical and sputum indices. *Chest.* 2004;125(4):1352-1358.

31. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J; ATS/ERS Task Force. Standardisation of spirometry. *Eur Respir J.* 2005;26(2):319-338.

32. Takishima T, Hida W, Sasaki H, Suzuki S, Sasaki T. Direct-writing recorder of the dose-response curves of the airway to methacholine. Clinical application. *Chest.* 1981;80(5):600-606.

33. Niimi A, Matsumoto H, Takemura M, Ueda T, Chin K, Mishima M. Relationship of airway wall thickness to airway sensitivity and airway reactivity in asthma. *Am J Respir Crit Care Med.* 2003;168(8):983-988.

34. Niimi A, Matsumoto H, Ueda T, Takemura M, Suzuki K, Tanaka E, Chin K, Mishima M, Amitani R. Impaired cough reflex in patients with recurrent pneumonia. *Thorax.* 2003;58(2):152-153.

35. Steel RGD: A rank sum test for comparing all pairs of treatments. Technometrics 1960;2:197-207.

36. Dwass M: Some k-sample rank-order tests, Contributions to Probability and Statistics(Eds. I. Olkin et al), Stanford University Press, 1960, pp. 198-202.

401 37. Neuhauser M, Bretz F. Nonparametric all-pairs multiple comparisons. Biometrical J.
402 2001;43:571-580
403 38. Joad JP, Munch PA, Bric JM, Evans SJ, Pinkerton KE, Chen CY, Bonham AC. Passive
404 smoke effects on cough and airways in young guinea pigs: role of brainstem substance
405 P. *Am J Respir Crit Care Med.* 2004;169(4):499-504.
406 39. Sekizawa S, Joad JP, Bonham AC. Substance P presynaptically depresses the
407 transmission of sensory input to bronchopulmonary neurons in the guinea pig nucleus
408 tractus solitarii. *J Physiol.* 2003;552(Pt 2):547-559.
409 40. Patterson RN, Johnston BT, Ardill JE, Heaney LG, McGarvey LP. Increased tachykinin
410 levels in induced sputum from asthmatic and cough patients with acid reflux. *Thorax.*
411 2007;62(6):491-495.
412 41. Chang AB, Gibson PG, Ardill J, McGarvey LP. Calcitonin gene-related peptide relates
413 to cough sensitivity in children with chronic cough. *Eur Respir J.* 2007;30(1):66-72
414 42. Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between
415 hypersensitivity and excessive airway narrowing. *Eur Respir J.* 1989;2(3):267-274.
416 43. De Diego A, Martínez E, Perpiñá M, Nieto L, Compte L, Macián V, Senent L. Airway
417 inflammation and cough sensitivity in cough-variant asthma. *Allergy.*
418 2005;60(11):1407-1411.
419 44. Boomsma JD, Said SI. The role of neuropeptides in asthma. *Chest.* 1992;101(6
420 Suppl):389S-392S.

45. Bonham AC, Kott KS, Ravi K, Kappagoda CT, Joad JP. Substance P contributes to rapidly adapting receptor responses to pulmonary venous congestion in rabbits. *J Physiol.* 1996;493 (Pt 1)229-238.

46. Widdicombe JG. Neurophysiology of the cough reflex. *Eur Respir J.* 1995;8(7):1193-1202.

47. Niimi A, Amitani R, Suzuki K, Tanaka E, Murayama T, Kuze F. Eosinophilic inflammation in cough variant asthma. *Eur Respir J.* 1998;11(5):1064-1069.

48. Jatakanon A, Lalloo UG, Lim S, Chung KF, Barnes PJ. Increased neutrophils and cytokines, TNF-alpha and IL-8, in induced sputum of non-asthmatic patients with chronic dry cough. *Thorax.* 1999;54(3):234-237.

49. Aliakbari J, Sreedharan SP, Turck CW, Goetzl EJ: Selective localization of vasoactive intestinal peptide and substance P in human eosinophils. *Biochem Biophys Res Commun* 1987, 148:1440-1445.

50. Germonpre PR, Bullock GR, Lambrecht BN, Van De Velde V, Luyten WH, Joos GF, Pauwels RA: Presence of substance P and neurokinin 1 receptors in human sputum macrophages and U-937 cells. *Eur Respir J* 1999, 14:776-782.

51. Lai JP, Douglas SD, Ho WZ: Human lymphocytes express substance P and its receptor. *J Neuroimmunol* 1998, 86:80-86.

52. Lambrecht BN, Germonpre PR, Everaert EG, Carro-Muino I, De Veerman M, de Felipe C, Hunt SP, Thielemans K, Joos GF, Pauwels RA: Endogenously produced substance P

contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. *Eur J Immunol* 1999, 29:3815-3825.

53. Toyoda M, Nakamura M, Makino T, Hino T, Kagoura M, Morohashi M. Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br J Dermatol.* 2002;147(1):71-79.

54. Matsuoka H, Niimi A, Matsumoto H, Ueda T, Takemura M, Yamaguchi M, Jinnai M, Chang L, Otsuka K, Oguma T, Takeda T, Chin K, Mishima M. Patients' characteristics associated with unsuccessful sputum induction in asthma. J Allergy Clin Immunol 2008;121:774-6

55. Jinnai M, Niimi A, Ueda T, Matsuoka H, Takemura M, Yamaguchi M, Otsuka K, Oguma T, Takeda T, Ito I, Matsumoto H, Mishima M. Induced sputum concentrations of mucin in patients with asthma and chronic cough. Chest. 2010;137:1122-9

Figure legends

Figure 1. Comparison of plasma SP levels among groups.

Plasma SP levels are elevated in patients with asthmatic and non-asthmatic cough compared with controls, while the two patient groups do not significantly differ.

Figure 2. Correlation between plasma SP levels and airway sensitivity.

Plasma SP levels negatively correlate with Dmin in asthmatic, but not in non-asthmatic cough. Logarithmic data are presented for Dmin.

462 **Table 1. Characteristics of the three subject groups**

	Asthmatic cough (n = 61)	Non-asthmatic cough (n = 21)	Healthy controls (n = 15)	p values*
Male, n	16 [26%]	5 [24%]	8 [53%]	0.10
Age, y	52 (33-65)	49 (34-65)	43 (38-60)	0.9989
Former smokers, n	12 [20%]	3 [14%]	2 [13%]	0.77
FEV_1/FVC, %	80.3 (74.5-84.8)	80.0(75.7-85.4)	80.6 (78.8-84.0)	0.75
FEV_1, %predicted	101.2 (90.4-111.4)	101.5 (89.1-106.9)	100.6 (90.6-108.6)	0.92
FEF_{25-75}, %predicted	83.7 (67.5-105.7)	93.0 (74.2-108.9)	78.3 (73.9-100.0)	0.74
Sputum eosinophil, %†	1.1 (0.3-4.3)	0.4 (0.2-0.9)	0.0 (0.0-0.8)	0.040‡
Sputum neutrophil, %†	59.6 (43.3-79.3)	58.8 (45.9-84.6)	60.0 (42.8-84.1)	0.94

463 Data are expressed as medians (25^{th} to 75^{th} percentile) or numbers [%].

464 NA, not applicable.

465 *Kruskal-Wallis or χ^2 test.

466 †Sputum induction was successful in 38 patients with asthmatic cough, 10 with non-asthmatic cough,

467 and nine healthy controls.

468 ‡Asthmatic cough vs. controls, p = 0.07; asthmatic cough vs. non-asthmatic cough, p = 0.27.

469 and non-asthmatic cough vs. controls, p = 0.51 by Steel-Dwass test.

470

Table 2. Characteristics of the two patient groups

	Asthmatic Cough (n = 61)	Non-asthmatic cough (n = 21)	p values*
Cough duration	12 (7-24)	8 (4-40)	0.18
Atopy[†], n	38 [63%]	11 [58%]	0.67
Serum IgE, U/ml[‡]	80.0 (28.0-190.0)	77.0 (10.0-400.0)	0.71
Dmin, units[§]	2.8 (1.4-6.6)	9.8 (2.9-29.0)	0.007
SRrs, $cmH_20/L/s/min$[§]	1.3 (0.8-2.4)	1.2 (0.5-2.2)	0.54
C5, μM[‖]	9.8 (2.4-19.5)	2.4 (0.9-14.6)	0.07

Data are expressed as medians (25^{th} to 75^{th} percentiles) or number [%]. NA, not applicable.

*Mann-Whitney U-test or χ^2 test.

[†]Atopy was determined based on presence of specific serum IgE antibodies to at least one common inhalant allergen, including cat dander, dog dander, weed pollens, grass pollens, molds or house dust mites. Data are missing for one patient with asthmatic cough and two with non-asthmatic cough.

[‡]Data are missing for two patients each with asthmatic and non-asthmatic cough.

[§]Data are missing for two patients with asthmatic cough. Two patients among those with asthmatic cough were not eligible for analysis of AHR.

[‖]Data are missing for two patients with asthmatic cough.

481 **Table 3. Correlations between plasma SP levels and clinical indices in the two patient**

482 **groups**

	Asthmatic cough		Non-asthmatic cough	
	r	p	r	p
Cough duration	0.10	0.42	-0.24	0.29
Serum IgE, U/ml	0.18	0.19	0.35	0.14
FEV_1/FVC, %	-0.18	0.17	0.04	0.88
FEV_1, %predicted	-0.02	0.88	0.12	0.62
FEF_{25-75}, %predicted	-0.05	0.73	0.05	0.84
Dmin, Units	-0.37	0.005	0.08	0.73
SRrs, $cmH_2O/L/s/min$	0.01	0.93	0.05	0.85
C5, μM	0.16	0.23	0.01	0.95
Sputum eosinophil, %	0.15	0.36	0.09	0.80
Sputum neutrophil, %	0.18	0.28	-0.39	0.26

483

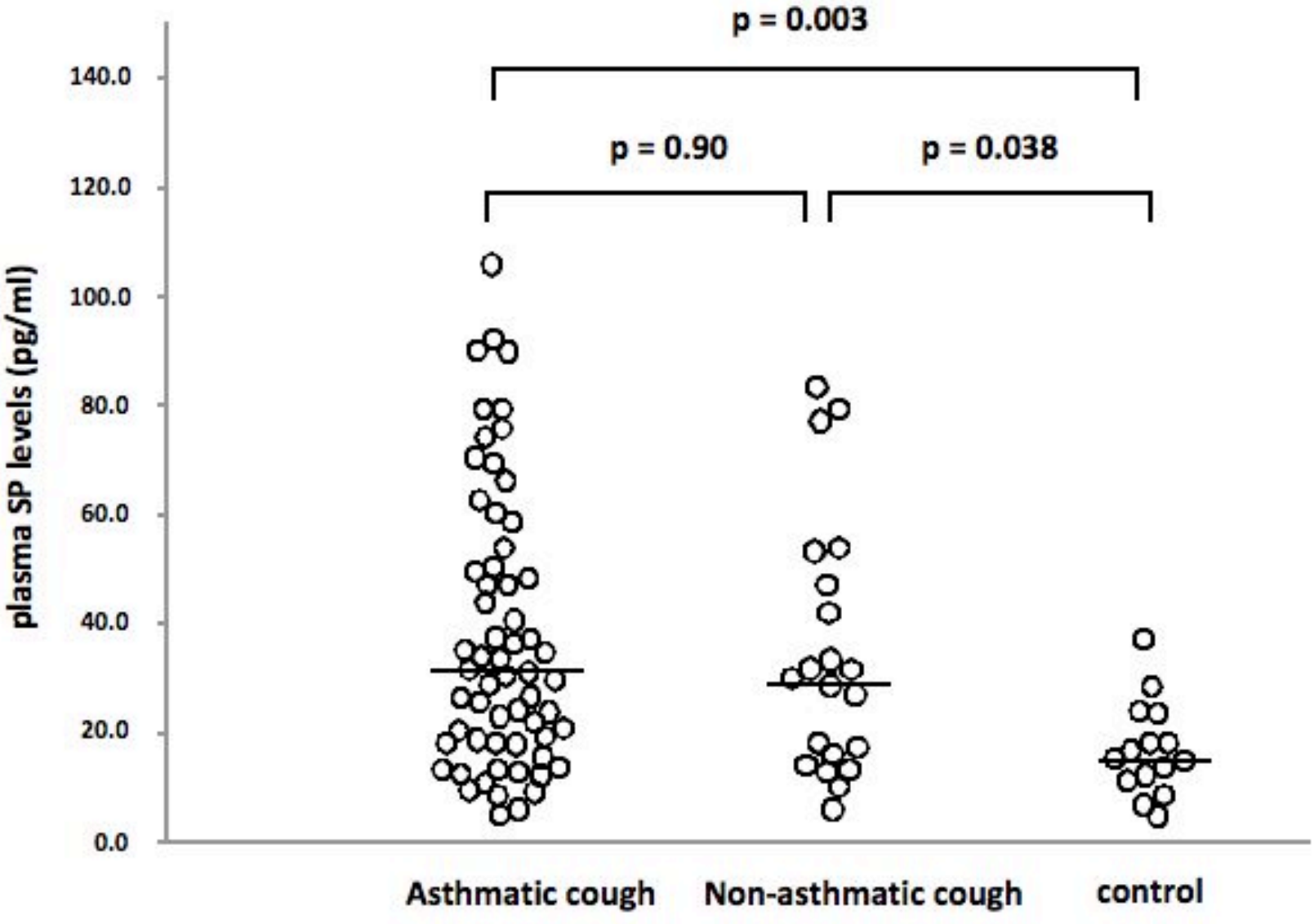
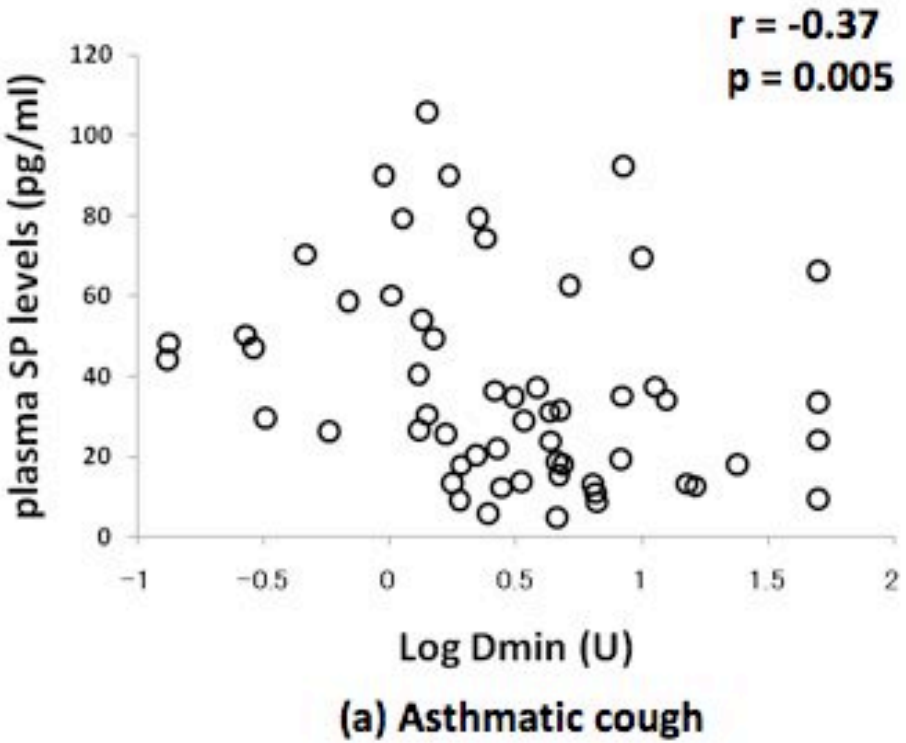
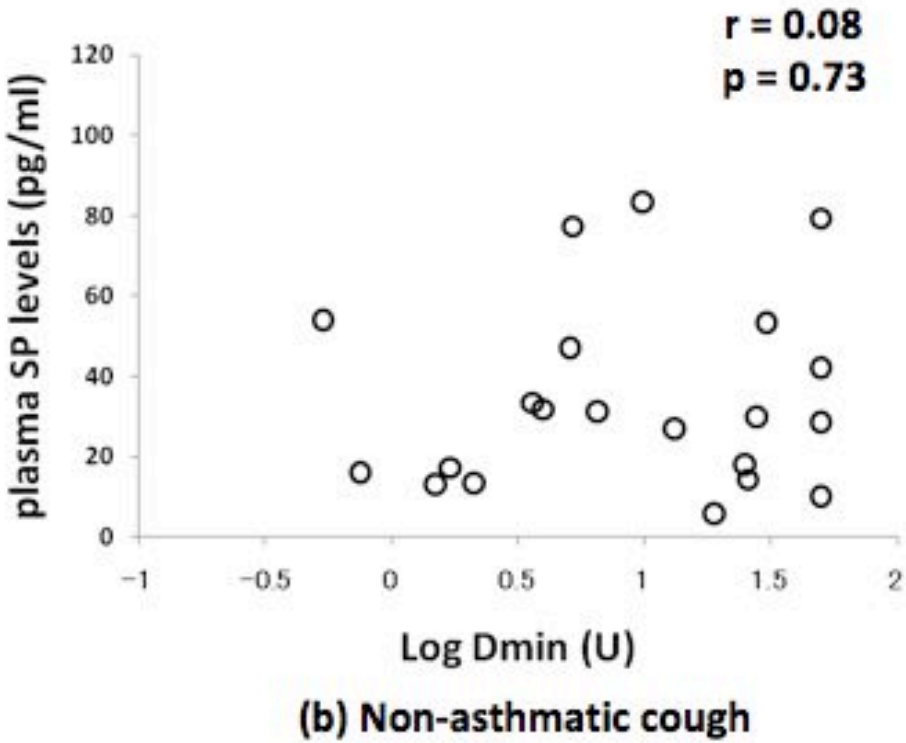

p = 0.003
p = 0.90
p = 0.038
plasma SP levels (pg/ml)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Asthmatic cough
Non-asthmatic cough
control

Figure 1



(a) Asthmatic cough



(b) Non-asthmatic cough

Figure 2