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Calcitonin Gene-Related Peptide relates with cough sensitivity in children with chronic cough

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Short title: BAL neuropeptides in children

ABSTRACT

Objectives: Airway neuropeptides, in particular calcitonin gene-related peptide (CGRP), are likely important in the pathogenesis of chronic cough. We evaluated (1) the relationship between cough sensitivity and bronchoalveolar lavage (BAL) neuropeptides, and (2) effect of reflux esophagitis (RE) on cough, cough sensitivity, and BAL neuropeptides in children not selected for cough. We hypothesised that CGRP would be increased in children with chronic cough and relate to cough sensitivity.

Methods: Capsaicin cough sensitivity was performed in children undergoing gastro-duodenal endoscopy. CGRP, substance-P (SP) and neurokinin-A (NKA) were measured in BAL obtained non-bronchoscopically. Children were defined as 'coughers' if chronic cough was present.

Results: Coughers (n=21) had significantly reduced cough sensitivity but were just as likely as non-coughers (n=19) to have RE. Median CGRP was significantly (p=0.02) higher in coughers with esophagitis (5.0, IQR 10.1) than in non-coughers with esophagitis (1.4, 4.0). CGRP significantly negatively correlated to cough sensitivity in coughers but not in non-coughers.

Conclusion: Elevated CGRP, but not SP or NKA, is associated with chronic cough in children only when esophagitis coexists. CGRP in BAL relates to cough sensitivity and is likely important in the pathophysiology of chronic cough.

Keywords: cough, children, sensory neuropeptides, CGRP, capsaicin cough sensitivity

INTRODUCTION

Chronic cough is a commonly encountered symptom and can result from a wide variety of pulmonary and non pulmonary conditions. Irrespective of its aetiology, chronic cough causes significant morbidity[1,2] and is associated with enhanced cough sensitivity in children[3,4] and adults.[5,6,7] Cough sensitivity is commonly measured using capsaicin cough challenge.[7,8]

Understanding mechanisms of disease processes is an important step in the advance of medicine. In the area of cough, the majority of such data have been obtained from animal studies whereby significant inter-species differences exist.[9] The mechanisms associated with chronic cough in humans include neurogenic inflammation through the sensory pathway[10,11,12] and vanilloid receptor (VR) activation.[13] VRs (such as transient receptor potential vanniloid-1 (TRPV-1) receptors) have been found to be likely contributors to an enhanced cough response in chronic persistent cough.[13,14] In 29 adults with chronic persistent cough from diverse causes, Gronberg et al described a significant correlation between capsaicin tussive response and the number of TRPV-1 positive nerves of airway mucosal biopsy specimens.[13] In animals, the cellular distribution of TRPV-1 neurons are in sensory neurons.[15] Sensory nerve stimulation primarily involves neurogenic inflammation whereby neuropeptides such as CGRP and SP play a key role.[9,12]

In the airways, neuropeptides thought to be particularly important include substance P (SP), neurokinin-A (NKA) and calcitonin gene-related peptide (CGRP).[9,16] SP, NKA and CGRP are pro-inflammatory sensory neuropeptides[9] although the pulmonary effects of CGRP are also anti-inflammatory.[16] CGRP in particular has been shown to be important in the mechanism of chronic cough.[16,17] In human studies, increased CGRP but not SP

immunoreactive nerves were found in biopsies of adults with chronic cough.[18] These adults also had significantly increased cough sensitivity to capsaicin (ie a reduced threshold to elicit a cough response)[18] In animal models, gastroesophageal reflux (GER) is associated with sensory nerve stimulation.[19]

Whether data in airway mucosal biopsies can be related to bronchoalveolar lavage (BAL) in the pathophysiology of chronic cough is unknown. To date, there are no studies that have related cough sensitivity to these key neuropeptides (CGRP, SP, NKA) in BAL. Indeed, there is currently no data on BAL quantification of these neuropeptides in children with or without chronic cough. BAL provides different and complementary information to mucosal biopsy, with the advantage that a broader range of airways are sampled, both in terms of the numbers and size of airways sampled.

There are several unique and important features to the population we studied. Prior studies have used selected populations of adults presenting with cough,[13,18] and this may select individuals with an abnormal airway response. In order to avoid this potential bias, we have studied children presenting for upper gastrointestinal endoscopy where there was no selection bias for coughing.[20,21] In addition, we have evaluated neuropeptides in children where data are scant. GERD (symptoms or complications of GER[22]) is associated with chronic cough but the mechanism and 'cause and effect' remains controversial in humans.[23] We conducted a study to evaluate 1) the relationship between cough sensitivity and BAL neuropeptides, and 2) effect of reflux esophagitis on cough, capsaicin cough sensitivity, and BAL neuropeptides, in children unselected for the presence of chronic cough. We hypothesised that CGRP would be increased in children with chronic cough and relate to capsaicin cough sensitivity.

METHODS

Children aged under 14 years undergoing elective flexible upper gastrointestinal endoscopy were invited to participate in the study as previously described.[20,21] Briefly, children were enrolled for the study on the morning of their procedure (flexible upper endoscopy performed under general anaesthesia including endotracheal intubation). Medical history was obtained from a parent on a standardised proforma for all children. Parent(s) also scored their child's cough on a validated cough visual analog scale of 1 (no cough) to 10 (most severe cough).[24] Exclusion criteria were; children with neuro-developmental abnormalities, known underlying cardiorespiratory disease other then asthma and those with a clinical history of primary aspiration (coughs and chokes with feeds at least twice a week). Children were defined as 'coughers' (C+) if the parents or consultant (gastroenterologist or respiratory paediatrician) had elicited a history of cough in association with GERD symptoms.[20] Reflux esophagitis (RE) was considered present if histology of oesophageal biopsy showed reflux esophagitis determined by pathologists blinded to the child's respiratory history. Written consent was obtained and the study approved by our institution's human ethics committee.

Capsaicin cough sensitivity test and spirometry were performed on those aged ≥6 years prior to endoscopy. Cough sensitivity test described specifically for children was performed using a single inhalation dosimeter technique.[8] Outcomes of cough sensitivity test were C2, defined as the concentration of capsaicin that stimulated ≥2 coughs and C5, defined as that associated with ≥5 coughs. Increased cough sensitivity is represented by lower C2 and/or lower C5 values ie lower concentration of capsaicin was required to elicit coughs. Spirometry was performed using a turbine spirometer (Kit, Cosmed, Italy) and Australian values were used for predictive values.[25]

BAL and BAL examination

BAL was obtained using a standardised and repeatable[26] non-bronchoscopic technique. Briefly, with the child's head turned to the left, a catheter was passed as far as possible through the endotracheal tube, ensuring that it went beyond the estimated carina site. Two aliquots of sterile normal saline (1ml/kg to maximum of 20mls) were instilled and suctioned into separate mucus traps. The second aliquot was used for BAL cellularity and neuropeptide analysis. The first aliquot was used for microbiology and the results previously described.[20] Cell count was performed (by 'blinded' cytologists) on the cell suspension, cytocentrifuge slides were prepared and stained (modified Wright's stain, Diff Quik, Lab Aids, Narrabeen, NSW, Australia) for cell differential profile. Protease inhibitor (See supplementary file) was added to the BAL supernatant that was immediately frozen and kept at -80C until thawed for analysis.

Analysis of CGRP, NKA and SP

The BAL specimens were prepared for assay of neuropeptides using an established technique using Sep-paks. Specimens were applied to a sep-pak (C18 Sep-pak; Waters, Milford Mass. USA) washed, and eluted with an acetonitrile/trifluoro-acetic acid 1% solutinn (60:40). The eluate was dried using a rotary evaporator and the residue reconstituted in buffer prior to assay. The specimen was reconstituted to concentrate the sample by two-fold. Recovery of peptides through this extraction procedure is >90%.

NKA was measured using an in-house (Belfast, N Ireland) radioimmunoassay. The antibody was raised in guinea pig to synthetic human NKA (Bachem, St Helens, UK). The antibody was directed to the N-terminal and cross reacts fully with NKB and Neuropeptide K. Cross

reaction with SP is <0.1%. Radiolabel NKA is purchased from GE Healthcare (St Giles, UK). The limit of the assay is 2ng/l. There is no external quality control system available for NKA however three internal controls were added to each assay batch. Co-efficient of inter- and intra- assay variation was 9.8% and 6.2% respectively. CGRP was measured by radioimmunoassay using a commercial antiserum to human CGRP. (Antibody code RAS-6012N, Peninsula Laboratories, San Carlos Calif USA). The antibody cross-reacts with Human CGRP I and II. Radiolabel was purchased from GE Healthcare (St Giles, UK). Three internal quality control specimens were included in each assay batch. The limit of detection was 2ng/l and inter- and intra- assay variation 11.5% and 7.5% respectively. Substance P was measured by ELISA using a commercial kit (Catalogue no. DE1400, R&D Systems, Minneapolis, USA). The assay does not cross react with NKA, NKB or neuropeptide K. The limit of detection of the assay was 8ng/l.

Statistical analysis

Children were categorised into coughers (C+) and non coughers (C-) with RE (RE+) and without RE (RE-). Chi square was used to compare categorical variables between groups with Continuity correction utilised when the table cell had <5. Data were not normally distributed and thus non parametric analyses were used; Mann-Whitney for comparisons between 2 groups and Kruskal Wallis when >2 groups were compared. Medians and inter-quartile range (IQR) were used for all descriptive data. Kendall's tau-b was used to examine correlation among variables as the sample size was small. Two tailed p value of <0.05 was considered significant. SPSS ver 13 was utilised for all statistical calculation.

RESULTS

The median age of the 40 children (26 boys and 14 girls) recruited was 9.6 years (IQR 6.4). Most (n=36, 90%) children were clinically suspected of having GERD and esophagitis was present in 23 (57.5%) children. Coughers (n=21) were just as likely as non-coughers (n=19) to have GERD, p=0.538. Spirometry was normal in all children who could perform spirometry (n=27), median FEV_1 99.8% predicted, FVC 100.3 % predicted. In the C+ group, cough was present for a median length of 160 (IQR 186) weeks. Median cough score was significantly higher in C+ (5, IQR 3.5) than in C- (median score of 1 ie no cough), $p < 0.0001$. There was no difference in cough score or length of cough between RE+ and RE- groups (p=0.57 and 0.96 respectively).

There was no significant difference in BAL cellular profile (percentages of neutrophils, macrophages, lymphocytes, eosinophils or total cell count) between C+ and C- groups (Table 1). However the RE- group had a significant but small increase in BAL neutrophil % when compared to the RE+ group, p=0.01. There was no difference in BAL percentages of macrophages, lymphocytes and eosinophils in those with and without RE. There was no difference in CGRP, SP or NK-A in between C+ and C- or between RE+ and RE- groups. Coughers had lower C2 but not C5. Capsaicin cough sensitivity was similar between RE+ and RE- groups (Table 1).

When grouped by presence of cough and RE, C2 was significantly different among the groups (p=0.013) but there was no significant difference in BAL cellularity, SP, NK-A (table 2). C2 was lowest in C+RE+ and highest in C-RE-group. C5 and CGRP values were just outside the significant value (p=0.06 and 0.07 respectively). In two group comparisons, CGRP was significantly different between C+RE+ and C-RE+ (p=0.02, figure 1) but not between the

other groups (p range from 0.07 to 0.74). Similarly C5 was significantly lower in C+RE+ compared to C-RE+ (p=0.04) but not in other 2 group comparisons (p range from 0.09 to 0.63).

C2 and C5 significantly (p = 0.031 and 0.020 respectively) negatively correlated (r = -0.51 and -0.55 respectively) to CGRP in coughers (figure 2a) but not in non-coughers (r = 0.28, p = 0.28 for C2 and 0.29, 0.324 respectively for C5, figure 2b). Cough sensitivity did not relate to any other parameter in coughers and non coughers. Cough sensitivity also did not correlate to CGRP, NK-A, SP or to any BAL cellularity in the total group (p ranged from 0.06 to 1.0).

DISCUSSION

We have previously shown that in most children, chronic cough was not associated with RE alone[20] and that airway neutrophilia is absent in children with RE.[21] The present paper adds to this information in showing that in the BAL of 40 children, CGRP, NKA and SP in children with chronic cough were not significantly different from those without cough. However children with cough and biopsy proven reflux esophagitis had significantly higher CGRP than children with reflux but without cough. Furthermore CGRP, but not NKA or SP, significantly related to capsaicin cough sensitivity in children with chronic cough but not in those without cough.

This is the first study that has examined these neuropeptides in airways of children with and without chronic cough. Neurogenic inflammatory mediators, in particular CGRP have been implicated in the pathogenesis of chronic cough.[12,18] Our finding of CGRP in the BAL also relating to cough sensitivity in coughers but not in non-coughers, is perhaps not surprising. In biopsy specimens CGRP immunoreactivity have been found to be related to cough sensitivity in adults with chronic cough.[18] This likely relates to plasticity of the peripheral nervous system with neurotrophins altering the pattern of innervation changes in inflammatory disease as SP and CGRP-immunoreactive nerves are sparse in normal human airways.[27] CGRP-containing airway innervating nerves originate from the nodose-jugular ganglia,[16] (among other ganglia) which is also the ganglia found to be central in the neurophysiology of the cough reflex.[28] It is thus biologically possible that up-regulation of these nerves causes a spill-over in the airways. CGRP can be released by noxious stimuli and plasma extravasation may occur,[16] and thus CGRP in BAL may occur from or lead to plasma extravasation. Increased pulmonary blood flow has been found to be associated with elevated levels of plasma CGRP[29] and high BAL levels may reflect circulating levels when

airway disease is present such as in circumstances when cough is reflective of airway disease. In an animal model, Daoui and colleagues demonstrated that protein extravasation in the airways can be induced by HCl infusion into the oesophagus, and that extravasation into the airways is mainly dependent on the release of tachykinins.[19]

Using the animal model of acid GERD, two groups have demonstrated that oesophageal stimulation by HCl causes neurogenic inflammation in the major airways.[19,30] In support of this, we found that CGRP was significant different between coughers and non coughers within the group that also had RE. This suggests that RE is a necessary factor for this difference and in the absence of RE, CGRP may be less important. The possible reasons for this finding are at least threefold. Firstly, the same sensory nerves supply the oesophagus and the airways, by a long axon route. Canning argues that gastric “refluxate can directly activate nociceptive nerves in the mucosa of the oesophagus, the pharynx, and the lower airways”.[31] Secondly, afferents from the oesophagus in some way link up with and modify (up-regulate) afferents from the airways, at ganglionic or brainstem levels. Up-regulation of the peripheral sensory neurones and brainstem regions has been described in animal studies.[32,33] A third possibility is that patients with RE also have aspiration which in some cases, but not all, causes 'chronic' cough. There are arguments for and against each of these possibilities, and further studies are required to evaluate these possibilities.

Ideally, CGRP and cough sensitivity should be remeasured upon the resolution of the cough in our cohort of children. However, given that a general anaesthetic is necessary to obtain BAL in our setting, this was not feasible and is thus one of this study’s shortfalls. Also, an association in no way proves ‘cause and effect’ or ‘which occurred first’. Another possible limitation is that although international guidelines for BAL in children were utilised,[34] it is

unknown if neuropeptides in BAL obtained by non bronchoscopic methods differ from that obtained using a bronchoscope. Furthermore although a moderately strong correlation between cough sensitivity and CGRP was found in coughers but not in non-coughers, we did not demonstrate that CGRP was higher in total cohort of coughers compared to non coughers. However, this and other non significant findings are possibly related to the small sample size.

The lack of the role of SP or NKA in this study is consistent with other studies involving cough and the lower airways.[17,18] In Forsythe and colleagues' study, CGRP but not NKA or SP induced BAL mast cells to release histamine in chronic coughers.[17] Also, O'Connell and colleagues described increased CGRP but not SP immunoreactive nerves in airway biopsies of adults with chronic cough.[18]

Neurotrophins and VRs are important concepts in the pathophysiology of chronic cough, described in biopsy specimens and animal models.[13,35] However, we did not measure these markers in this study as in a previous study in 69 children, we did not find any difference in brain-derived neurotrophic factor (BDNF) or VR-1 mRNA in BAL cells between children with (n=36) and without cough (n=33) (unpublished). Also, Chaudhuri et al described that neurotrophins were not elevated in induced sputum or serum of adults with chronic persistent cough.[36]

In our previous paper we had also described a small but significantly reduced airway neutrophil percentage in children with RE without cough.[21] This finding is also similar to a small (n=11) study in adults with GERD and cough.[37] Also, our study's limitations with respect to other diagnostic modalities of GERD was discussed in our previous papers[20,21] and thus not repeated here. Similarly, the lack of association between cough and GERD in

children was also the main discussion point in our previous paper.[20]. Cough is rarely solely attributed to RE in otherwise well children,[20,38,39] in contrast to adults.[40] Cough, symptoms of GERD and asthma are common, an association does not imply cause and effect,[41] and these symptoms co-existing merely by chance is high.[23]

Larger studies and one that includes follow-up of children with cough and BAL is necessary to confirm our findings. Nevertheless, our unique findings in this study were twofold. Firstly, children with cough and biopsy proven RE had significantly higher CGRP levels in their BAL compared to children with RE but without cough. The question about plausible mechanisms of cough in RE is thus raised and it is likely that other factors are required to be present for RE to elicit cough in humans. These factors are likely to include persistent neurogenic airway inflammation[12] and our data support this. Secondly, CGRP but not SP or NKA significantly related to capsaicin cough sensitivity in children with chronic cough but not in those without cough. We conclude that, elevated CGRP, but not SP or NKA, is associated with chronic cough in children when esophagitis coexists. CGRP is likely an important neuropeptide in chronic cough when reflux esophagitis coexists. The role of CGRP in BAL and serum deserves further evaluation.

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Table 1: Cellular profile, sensory neuropeptides and capsaicin cough sensitivity of children with (+) and without (-) cough and with and without reflux esophagitis (RE)

	Cough+ N=21	**Cough-** N=19	p*	**RE+** N=23	**RE-** N=17	p+
Demographics						
Age (median, IQR) yr	9.1, 4.5	9.8, 7.8	0.46	9.7, 4.3	8.9, 8	0.33
Male:female, n	12:9	14:5	0.45	18:5	8:9	0.09
Airway cellularity (median, IQR)						
Lymphocyte %	3.0, 3.5	2.5, 13.8	0.77	3.5, 7.5	2.5, 2.3	0.60
Neutrophil %	6.5, 4.3	4.0, 8.3	0.41	4.5, 3.8	7.0, 6.3	**0.01**
Macrophage %	90, 8.1	80, 67	0.67	86, 33	88, 9.7	0.41
Eosinophil %	0,0	0,0	0.33	0, 0	0.0. 0.8	0.77
Total cell count (x10E6/1)	86, 60	46, 169	0.21	59, 108	83, 62.5	0.25
Sensory neuropeptides (median, IQR)						
CGRP, ng/l	2.7, 4.5	0, 6.3	0.16	2.3, 6.7	2.7, 3.8	0.76
Neurokinin A, ng/l	16.2, 8.6	17.5, 9.83	0.88	18.9, 9.0	16.2, 6.2	0.11
Substance P, ng/l	57.8, 43.6	49.3, 55.5	0.67	45.7, 50.7	57.8, 54.4	0.76
Capsaicin sensitivity (median, IQR)						
C2 (umol)	14.7, 19.5	39, 58.5	**0.018**	19.5, 9.7	14.7, 39	0.17
C5 (umol)	29.3, 68.2	58.5, 58.5	0.13	39, 29.2	29.3, 117	0.06

p* refers to comparisons between C+ and C- groups using Mann Whitney test

p+ refers to comparisons between RE+ and RE- groups using Mann Whitney test

Table 2: Cellular profile, sensory neuropeptides and capsaicin cough sensitivity of children grouped by presence (+) and absence (-) of cough (C) and reflux esophagitis (RE)

	C+RE+ N=11	**C+RE-** N=10	**C-RE+** N=12	**C-RE-** N=7	p*
Airway cellularity (median, IQR)					
Lymphocyte %	3.5, 3.3	2.5, 4.8	3.0, 4.5	3.0, 4.0	0.82
Neutrophil %	5.5, 6.3	7.0, 3.8	3.5, 7.8	9.0, 9.0	0.06
Macrophage %	91, 9.5	88, 7.8	89, 30	88, 6	0.76
Eosinophil %	0, 0	0, 2.3	0,0	0,0	0.41
Total cell count (x10E6/l)	102, 75	80, 49	78, 58	84, 50	0.46
Sensory neuropeptides (median, IQR)					
CGRP, ng/l	5.0, 10.1	2.7, 3.2	1.4, 4	4.5, 5.4	**0.06#**
Neurokinin A, ng/l	20.7, 12.2	14.0, 15.1	18.9,	17.1,	0.46
Substance P, ng/l	55.7, 58.7	39.7, 88.1	22.5, 44.9	73.7, 54.6	0.91
Capsaicin sensitivity (median, IQR)					
C2 (umol)	7.4, 28.9	19.5, 11.0	29.3, 19.5	48.8, 136.5	**0.03**
C5 (umol)	19.5, 227	39, 109.7	58.5, 39	97.5, 117	0.07

*p by Kruskal Wallis

C+RE+ was significantly different to C-RE+ (p=0.02)

LEGENDS

Figure 1: Median and inter-quartile range of CGRP in BAL of children grouped by presence (+) and absence of cough (C), and reflux esophagitis (RE). CGRP was significantly different between C+RE+ and C-RE+ (p=0.02) but not between the other groups (p range from 0.07 to 0.74).

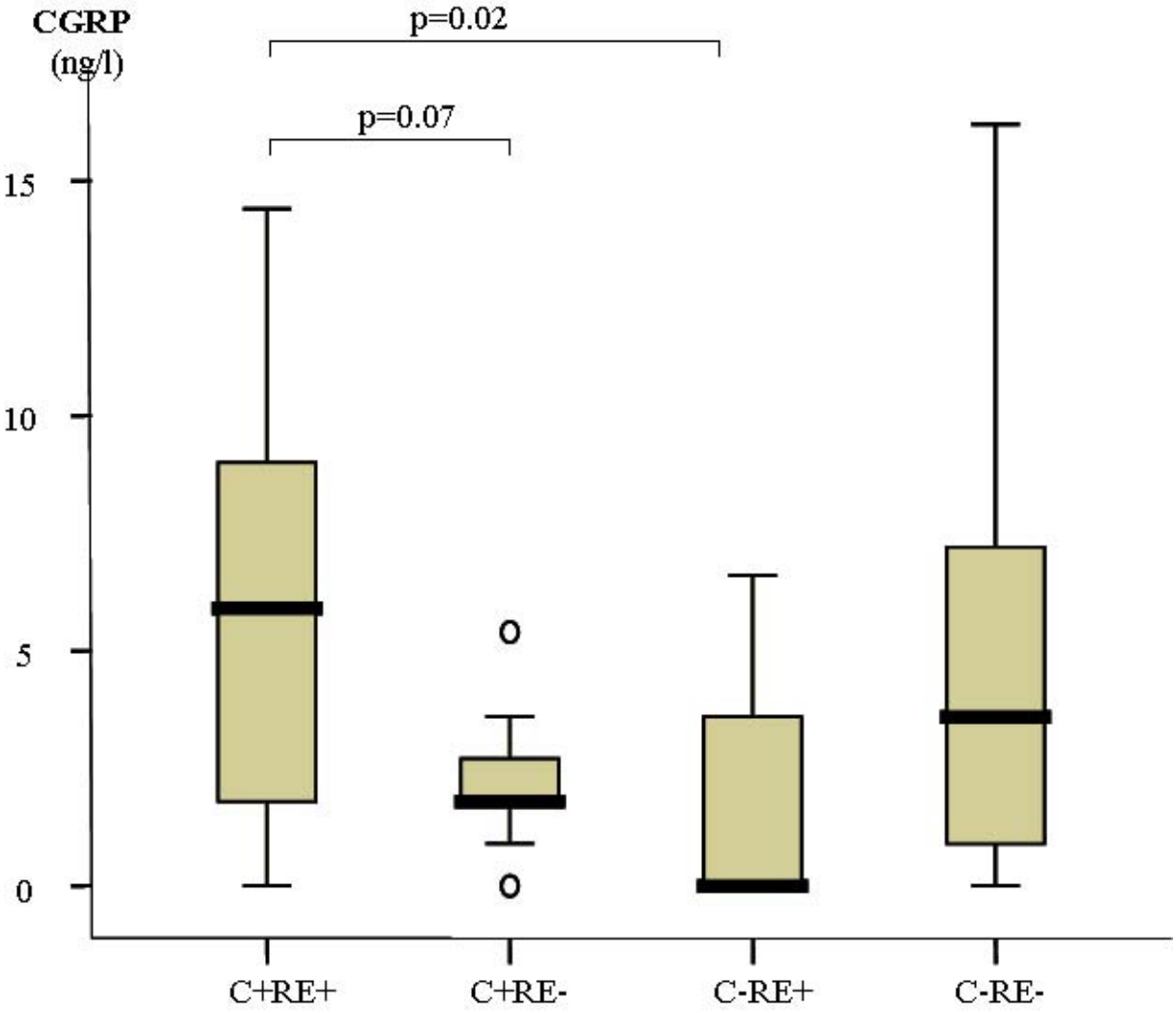
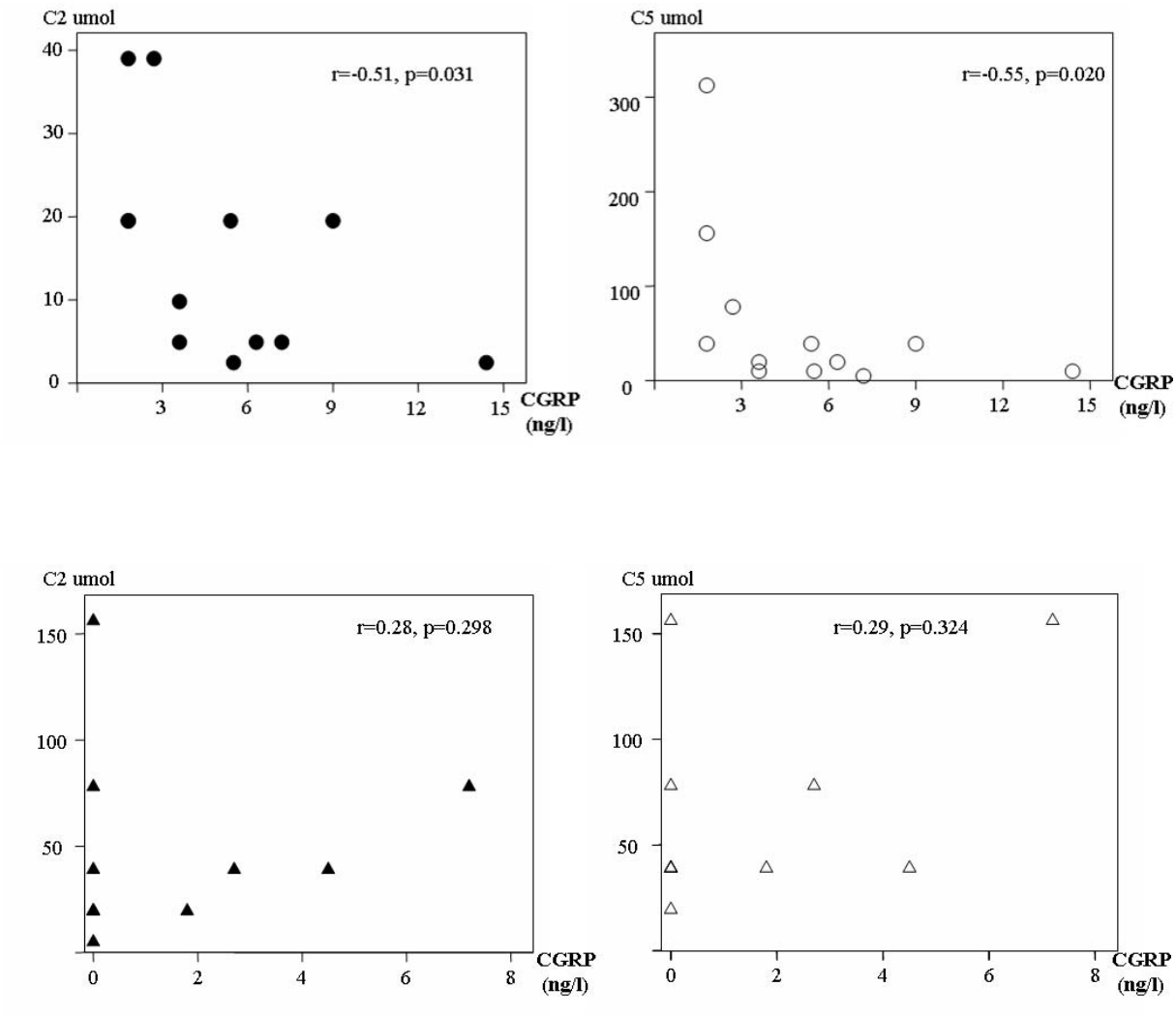


Figure 2: Scatter plot of C2 and C5 in coughers (figure 2a) and non-coughers (figure 2b)

Figure 2a: C2 and C5 significantly negatively correlated (r = -0.51 and -0.55 respectively) to CGRP in coughers.

Figure 2b: There was no significant correlation between capsaicin cough sensitivity outcomes to CGRP in non-coughers.

Footnote: The number of points in figure 2 do not add up to 40 as data on capsaicin cough sensitivity can only be obtained in older children (aged >6 years[8]).



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