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Published in:
Biomarkers

DOI:
[10.1080/1354750X.2017.1375559](https://doi.org/10.1080/1354750X.2017.1375559)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Stenberg, H., Wadelius, E., Moitra, S., Aberg, I., Ankerst, J., Diamant, Z., Bjermer, L., & Tufvesson, E. (2018). Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics. *Biomarkers*, 23(1), 51-60. <https://doi.org/10.1080/1354750X.2017.1375559>

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To cite this article: Henning Stenberg, Erik Wadelius, Subhabrata Moitra, Ida Åberg, Jaro Ankerst, Zuzana Diamant, Leif Bjerner & Ellen Tufvesson (2018) Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics, Biomarkers, 23:1, 51-60, DOI: [10.1080/1354750X.2017.1375559](https://doi.org/10.1080/1354750X.2017.1375559)

To link to this article: <https://doi.org/10.1080/1354750X.2017.1375559>

Accepted author version posted online: 01 Sep 2017.
Published online: 18 Sep 2017.

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RESEARCH ARTICLE

Club cell protein (CC16) in plasma, bronchial brushes, BAL and urine following an inhaled allergen challenge in allergic asthmatics

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ABSTRACT

Background: Club cell protein (CC16) is a pneumoprotein secreted by epithelial club cells. CC16 possesses anti-inflammatory properties and is a potential biomarker for airway epithelial damage. We studied the effect of inhaled allergen on pulmonary and systemic CC16 levels.

Methods: Thirty-four subjects with allergic asthma underwent an inhaled allergen challenge. Bronchoscopy with bronchoalveolar lavage (BAL) and brushings was performed before and 24 h after the challenge. CC16 was quantified in BAL and CC16 positive cells and CC16 mRNA in bronchial brushings. CC16 was measured in plasma and urine before and repeatedly after the challenge. Thirty subjects performed a mannitol inhalation challenge prior to the allergen challenge.

Results: Compared to baseline, CC16 in plasma was significantly increased in all subjects 0–1 h after the allergen challenge, while CC16 in BAL was only increased in subjects without a late allergic response. Levels of CC16 in plasma and in the alveolar fraction of BAL correlated significantly after the challenge. There was no increase in urinary levels of CC16 post-challenge. Mannitol responsiveness was greater in subjects with lower baseline levels of CC16 in plasma.

Conclusions: The increase in plasma CC16 following inhaled allergen supports the notion of CC16 as a biomarker of epithelial dysfunction.

ARTICLE HISTORY

Received 27 June 2017
Revised 16 August 2017
Accepted 30 August 2017

KEYWORDS

Asthma; club cell protein (CC16); inhaled allergen challenge; bronchoalveolar lavage; airway epithelium; mannitol challenge

Introduction

Club cell 16 kDa secretory protein (CC16) is primarily produced by the non-ciliated club cells found in the epithelium of bronchi and bronchioles (Singh *et al.* 1988). The exact function of CC16 is unknown, although evidence points towards an anti-inflammatory and immunomodulatory role within the airways (Levin *et al.* 1986, Miele *et al.* 1987, Dierynck *et al.* 1995). A polymorphism in the CC16 gene has been linked to an increased risk of developing asthma during childhood (Laing *et al.* 1998) and is associated with lower plasma levels of CC16 (Laing *et al.* 2000). Lower circulating levels are also seen in asthmatic subjects compared to healthy controls (Shijubo *et al.* 1999b), as well as fewer CC16-positive epithelial cells in small airways (Shijubo *et al.* 1999a). Lower levels of CC16 have also been linked to a more rapid decline of lung function in patients with COPD (Park *et al.* 2013) and in the general population (Guerra *et al.* 2015).

CC16 is mainly secreted into the lumen of the respiratory tract, and can thus be measured in high concentrations in bronchoalveolar lavage (BAL) fluid (Bernard *et al.* 1992). However, CC16 is also present in plasma, assumed to be a result of passive diffusion over the bronchoalveolar/blood barrier and due to increased leakage during epithelial stress (Hermans *et al.* 1999). A transient increase in CC16 plasma

levels could also be due to changes in production and/or secretion by club cells, acute inflammation has however been shown to decrease synthesis in a lung injury rat model using intratracheal lipopolysaccharides (Arsalane *et al.* 2000). While lower levels of CC16 have been found in serum and BAL fluid of smokers (Bernard *et al.* 1992, Shijubo *et al.* 1997), increases in circulating CC16 have been found in animal models and humans following acute exposure to substances believed to cause epithelial damage with increased airway permeability (Bernard *et al.* 1997, Hermans *et al.* 1999, Broeckaert *et al.* 2000b). CC16 could therefore be a biomarker of airway epithelial integrity (Broeckaert *et al.* 2000a), an important factor of asthma development (Heijink *et al.* 2014).

Exercise, mannitol and eucapnic voluntary hyperventilation (EVH) are stimuli believed to exert their effect by dehydration of the epithelial lining fluid, causing pro-inflammatory mediator release with subsequent bronchoconstriction in susceptible subjects (Anderson, 2016). Levels of CC16 have been shown to increase in both plasma and urine after exercise (Romberg *et al.* 2011, Tufvesson *et al.* 2013), and in urine after mannitol inhalation challenge and EVH (Bolger *et al.* 2011b, Kippelen *et al.* 2013). An epithelial injury would also follow seemingly regardless of the integrity of the epithelium pre-challenge, considering there were no differences in

increases of CC16 levels between asthmatics and healthy controls, or between those who responded with bronchoconstriction and those who did not.

Exposure to house dust mite (HDM) allergen Der p 1 has been shown to cleave essential portions of tight junctions *in vitro*, disrupting the epithelial barrier function (Wan *et al.* 1999). Epithelial impairment with facilitated access of allergen to dendritic cells is believed to be a driving factor in allergic sensitization, but little is known about the effect of inhaled allergens on the human airway epithelium *in vivo* (Lambrecht and Hammad, 2014). To our knowledge, the effect of allergen inhalation on the airway epithelium in the context of CC16 has not been studied so far. We therefore aimed to assess CC16 in plasma, urine and in the respiratory compartment before and after an inhaled allergen challenge.

Clinical significance

- CC16 is an anti-inflammatory pneumoprotein and a potential biomarker of airway epithelial dysfunction, a driving factor in development of allergic asthma. Knowledge about effects of different airway challenges on CC16 is however limited.
- CC16 increases in plasma after an inhaled allergen challenge, indicating increased secretion and/or leakage over a dysfunctional epithelium.
- CC16 synthesis is not affected by acute inflammation, but a correlation between lower systemic CC16 levels and increased airway hyper-responsiveness to mannitol indicates that long-term chronic inflammation leads to decreased CC16 levels.

Methods

Subjects

Thirty-four subjects with allergic asthma according to GINA guidelines (Global initiative for asthma 2017) were included (Figure 1 and Table 1). None were previous or current smokers. All subjects were clinically stable either on a daily dose of 100–400 µg budesonide ($n=16$) or without any ICS ($n=18$), and were instructed not to change their regular ICS dosing regimen during participation. Apart from asthma, all subjects were otherwise in good general health. None of the subjects were treated with oral corticosteroids, anti-IgE, allergen-specific immunotherapy, phosphodiesterase inhibitors, muscarinic or leukotriene receptor antagonists for at least six months before inclusion. All subjects signed a written informed consent, and the study was approved by the Regional Ethics Review Board in Lund, Sweden (2012/800).

Study design

The study consisted of: (1) screening visit, with a methacholine inhalation challenge, a skin prick test and determination of IgE levels in serum (all 34 subjects), (2) baseline bronchoscopy ($n=21$), (3) mannitol inhalation challenge ($n=30$), (4) inhaled allergen challenge (all 34 subjects) and (5) second

Figure 1. Study flow diagram. The diagram illustrates the selection of subjects, showing the number of subjects selected for screening and the number of subjects excluded for reasons defined within each box.

bronchoscopy 24 h after the allergen challenge ($n=19$). All subjects were presented with the option of completing all visits apart from bronchoscopies (i.e. completing visits 1, 3 and 4), and 13 subjects chose this option. Two of the 21 subjects who completed visit 2 opted not to participate in the second bronchoscopy due to discomfort experienced during the first one, but did complete the rest of the study including the allergen challenge and blood sampling. The baseline bronchoscopy and the second bronchoscopy were performed within 3–14 weeks [median 4 (IQR 3–4) weeks]. At least 72 h passed between each visit from 1 to 4 to avoid any interference between the tests. Four subjects failed to complete the mannitol inhalation challenge due to scheduling reasons. Data were collected between February 2013 and March 2016. All subjects sensitized to any pollen were tested outside of the relevant pollen season. At screening, subjects completed an Asthma Control Test (ACT) questionnaire (Jia *et al.* 2013), a skin prick test (ALK-Abello, Hørsholm, Denmark) was performed and serum IgE levels (RAST) were analysed to determine sensitizations to 10 allergens, including HDM (*D pteronyssinus* and *D farinae*), cat, horse, dog, *alternaria alternata*, *cladosporium herbarum*, grass, birch and ragweed pollen. All subjects had a positive skin prick test (wheal diameter ≥ 3 mm) (Heinzerling *et al.* 2013) and confirmed

Table 1. Subject characteristics.

	Single responders (n = 19)	Dual responders (n = 15)
Sex, F/M (n)	9/10	8/7
Age (years)	27 (27–41)*	24 (22–31)*
Duration of asthma (years)	20 (10–24)	15 (13–21)
ACT (score)	22 (20–24)	22 (21–25)
FEV ₁ (%p)	96.5 (88.7–103.6)	94.8 (92.5–103.2)
Methacholine PD ₂₀ (μg)	208.1 (104.8–549.9)	228.0 (174.7–917.0)
Mannitol challenge, Pos/Neg (n)	9/7	6/8
Mannitol PD ₁₅ (mg)	288 (145–369)	401 (327–422)
Regular use of ICS (n)	6	10
Total IgE (kU/l)	126.0 (58.2–430.0)	86.8 (52.2–127.5)
Number of sensitizations (n)	5 (4–6)	4 (3–6)
Allergen used in challenge (n) (Cat/Horse/HDM/Birch/Grass)	10/4/2/2/1	8/2/1/2/2
Specific IgE for allergen used in challenge (kU/l)	3.8 (2.6–10.1)	6.6 (2.0–26.2)
SPT wheal diameter for allergen used in challenge (mm)	8 (7–11)	9 (8–11)
Allergen dose given (SQ-U)	250.3 (118.7–626.3)	386.6 (250.3–1303.1)
CC16 in plasma at baseline (ng/ml)	5.6 (4.3–7.1)	6.2 (4.0–8.2)
CC16 in urine at baseline (ng/μmol creatinine)	0.08 (0.03–0.2)	0.1 (0.01–0.2)
Underwent bronchoscopy, first/second (n)	11/11	10/8

ACT: asthma control test; FEV₁%p: forced expiratory volume in 1 s; %p: percent of predicted value; PD₂₀: provocative dose required to decrease forced expiratory volume in 1 s (FEV₁) by 20%; PD₁₅: provocative dose required to decrease FEV₁ by 15%; ICS: inhaled corticosteroids; HDM: house dust mite; SPT: skin prick test; SQ-U: standardized quality units; CC16: club cell protein.

Data presented as median (IQR), where applicable.

**p* < 0.05: significant difference between groups.

lower airway symptoms to the specific allergen used for the inhaled allergen challenge.

Spirometry

Spirometry (Jaeger MasterScope, Erich Jaeger GmbH, Würzburg, Germany) was measured according to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines (Miller *et al.* 2005), generating the forced expiratory volume in 1 s (FEV₁). Per cent of predicted values (%p) were calculated from reference spirometric values by Crapo *et al.* (Crapo *et al.* 1981). Spirometry was measured before methacholine, mannitol and allergen challenges, between inhalations and repeatedly after the allergen challenge as described below.

Methacholine inhalation challenge

The methacholine challenge was carried out to screen for airway hyper-responsiveness, using a tidal-volume-triggered device (Aerosol Provocation System, APS, Erich Jaeger GmbH, Würzburg, Germany). Pre-challenge spirometry was performed in triplicate, with the highest value defined as baseline. An inhalation of 9 mg/ml NaCl was performed as a negative control, and if FEV₁ measured after 2 min dropped $\geq 5\%$ from baseline, the subject was excluded from further testing. Subsequently, five inhalations with increasing methacholine doses (50, 150, 300, 600 and 900 μg, maximal cumulative dose 2000 μg) were conducted. FEV₁ was measured 2 min after each inhalation and the test was completed whenever FEV₁ decreased $\geq 20\%$ from baseline. Subjects without a decrease of $\geq 20\%$ in FEV₁ were regarded as non-responders and were excluded from further testing.

Mannitol inhalation challenge

A mannitol powder kit (Aridol™, Pharmaxis, Frenchs Forest, Australia) was used, administering eight incremental steps to

a maximal cumulative dose of 635 mg of mannitol, according to the manufacturer's instructions. Spirometry was performed in triplicate before the challenge and the highest value was chosen as baseline. Spirometry was measured 60 s after each inhalation, followed immediately by the next inhalation. The challenge was completed and considered positive if/when FEV₁ decreased $\geq 15\%$ from baseline.

Inhaled allergen challenge

An automatic, inhalation-synchronized dosimeter jet-nebulizer (Spira Elektro 2, Respiratory Care Center, Hämeenlinna, Finland) was used for the allergen challenge. FEV₁ was measured in triplicate before the challenge, with the highest value chosen as baseline. A single diluted allergen extract (cat, horse, HDM, birch or grass pollen; ALK-Abelló, Hørsholm, Denmark, chosen based on maximal response according primarily to subject's history, secondarily to skin prick test and/or serum IgE levels) was administered by counted deep breaths over several steps with gradually increasing doses. 1.2 standardized quality units (SQ-U) was given as the first step. FEV₁ was measured 5 and 10 min after completion of each inhalation step. If FEV₁ had not dropped $\geq 10\%$ from baseline, the next inhalation (starting directly after the last spirometry) contained a four-fold increase in dose. If FEV₁ dropped by 10–15% from baseline, the next dose was doubled, and if FEV₁ dropped 15–20%, FEV₁ was measured every 5 min for the following 30 min. If FEV₁ remained stable at a 15–20% decline from baseline, the previous dose was repeated. Whenever FEV₁ dropped $\geq 20\%$ from baseline, the challenge was considered completed (=time point 0 h) and no further allergen was inhaled. If a drop in FEV₁ $\geq 20\%$ from baseline was not achieved after a maximal cumulative dose of 20,000 SQ-U, the subject was excluded from further testing and analysis.

Additionally, spirometry was performed 4, 5, 6, 7 and 8 h post-allergen challenge. Subjects were defined as dual responders if they had a late allergic response (LAR, defined as

≥12% decrease in FEV₁ from baseline, occurring at any time point 4–8 h post-allergen challenge). Subjects without a LAR were defined as single responders.

Analysis of CC16

Plasma samples were collected in sodium heparin tubes immediately before the allergen challenge (baseline) and 0, 0.5, 1, 2, 6, 8 and 23 h post-challenge. Samples were stored at –80 °C pending analysis. Baseline samples were collected at 08.30 AM ±30 min in all subjects. The 0 h post-challenge sample was collected at 11.30 AM ±60 min, with subsequent samples at the predetermined time intervals.

Upon arrival on the day of the allergen challenge, subjects were instructed to empty their bladder and discard the urine sample. Urine was collected two hours later, just before the start of the allergen challenge (baseline), and 0.5, 2, 6, 8 and 23 h post-challenge. Male subjects were instructed to discard the first 100 ml before each sampling, in order to avoid contamination of CC16 from the prostate. Samples were stored at –80 °C pending analysis. All samples were analysed for creatinine using a COBAS 6000 System analyser (Roche Diagnostics, Basel, Switzerland) and CC16 results in urine are normalized to urinary creatinine to compensate for differences in dilution.

CC16 in plasma, urine and BAL was measured using the Human Club Cell Protein ELISA kit from BioVendor (Modrice, Czech Republic) according to the manufacturer's protocol. The detection limit for CC16 was 0.020 ng/ml. Analysis was run in duplicate and the mean value was used for statistical calculations. Concentrations of CC16 in BAL were normalized to total protein content to compensate for differences in dilution, and are presented as CC16 divided by total protein.

Bronchoscopy

Bronchoscopies were performed according to clinical routine, and 30 minutes before the bronchoscopy, subjects inhaled a nebulized mixture of salbutamol 0.5 mg/ml and ipratropium 0.2 mg/ml. A flexible bronchoscope (Olympus IT60, Tokyo, Japan) was inserted into the trachea and the airways were systematically examined.

Brush samples and bronchoalveolar lavage

Bronchial brushings were sampled on sub-segmental levels during bronchoscopy. BAL was then performed on the opposite side. At the second bronchoscopy, the procedure was mirrored.

From one brush, the brush cells were lysed for RNA preparation of total brush cell mRNA. From another brush, the brush cells were placed on microscope slides using a CytospinTM cytocentrifuge, fixed with 4% paraformaldehyde (PFA) and stored in phosphate buffered saline (PBS) until later staining (Tufvesson *et al.* 2017).

BAL was sampled by infusion of 150 ml (3 × 50 ml) of 0.9% PBS at room temperature, re-aspirated by gentle suction. The first 50 ml sample was defined as the bronchial fraction

while subsequent samples were pooled and defined as the alveolar fraction, as previously described (Van Vyve *et al.* 1992). The BAL was filtered through a 100 µm filter and centrifuged at 200×g for 5 min (+4 °C), and the BAL supernatant was analysed for CC16 as described above.

For CC16 staining, the cells were permeabilized using 0.5% Triton in 1× PBS for five minutes, and thereafter subjected to blocking for 30 min (Protein Block Serum Free, Ready-to-use (Dako Inc., Carpinteria, CA)), primary antibody (mouse monoclonal anti-human CC16/Uteroglobin/SCGB1A1, 0.5 µg/µl, R&D Systems, Abingdon, UK, diluted 1:100 in 1% BSA in PBS) for 60 minutes, washing and secondary antibody (goat polyclonal anti-rat IgG HRP-conjugated antibody, 0.5 µg/µl, Thermo Fisher Scientific, Rockford, IL) diluted 1:200 in block buffer for 60 min. Negative controls were stained through omitting the primary antibody. Liquid DAB + Substrate Chromogen System (Dako Inc., Carpinteria, CA) was used for brown colour development. The samples were subsequently dyed with haematoxylin for 40 seconds, and dehydrated stepwise with ethanol (70–99.9%) and xylene, and mounted with Pertex[®] (Histolab, Gothenburg, Sweden).

All slides were assessed by the same person, who was blinded, using a Nikon Eclipse 80i microscope with a built-in Olympus DP80 camera, CellSens Dimension, v. 11.1 (Olympus Corporation A software). A minimum of 200 cells was counted in each sample and the number of CC16 positive cells was quantified. Failed brush samplings containing too few total cells to count were excluded from analysis.

Semi-quantitative real-time PCR

RNA preparation was performed using RNeasy Mini kit with DNase treatment (from Qiagen GmbH, Hilden, Germany) and cDNA was synthesized using iScriptTM cDNA Synthesis Kit from Bio-Rad Laboratories (Hercules, CA). The extracted RNA was quantified and checked using 260:230 nm absorbance spectra of a NanoDrop (Thermo Fisher Scientific, Rockford, IL), and cDNA from 29 ng RNA was used in each PCR reaction. Semi-quantitative real-time PCR was performed on an Applied Biosystem (Foster City, CA) 7900 thermocycler (95 °C for 15 s, 60 °C for 30 s and 74 °C for 30 s, during 45 cycles) using iTaqTM SYBR Green Supermix with ROX from Bio-Rad Laboratories (Hercules, CA). Primers (from InvitrogenTM), were used at 300 nM. The sequences (from 5' to 3') for the CC16 primers were; forward: CTT TCA GCG TGT CAT CGA AA and reverse: TGA TGC TTT CTC TGG GCT TT, Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes with primers as previously presented (Tufvesson *et al.* 2011). The mean of the housekeeping genes was used as an internal standard, and the 2^{ΔCt}-model was used for quantification of CC16.

Statistical analyses

SPSS Statistics version 23.0 was used for statistical analysis (Chicago, IL). The Mann–Whitney test was used for comparisons between single and dual responders. Paired

comparisons were performed using the Wilcoxon matched-pairs signed rank test. Spearman's rank test was used for correlation analyses. A p value of <0.05 (two-tailed) was considered statistically significant. Data are presented as median (IQR), where applicable.

Results

Nineteen subjects were single responders while 15 subjects were dual responders (Table 1).

CC16 in plasma

Levels of CC16 in plasma in all subjects were significantly increased at 0, 0.5 and 1 h post-allergen challenge compared to baseline (Figure 2(A)). There were no differences in CC16 plasma levels between single and dual responders at baseline (Table 1) or after the allergen challenge at any time (Figure 2(B)). There were no significant differences in CC16 plasma levels between subjects treated with ICS and subjects not treated with ICS at any time (data not shown).

CC16 positive cells and mRNA

The proportion of CC16 positive cells (Figure 3(A–D)) in brush samples was significantly higher in dual responders compared to single responders at 24 h post-allergen challenge, but was not different at baseline and did not change significantly after the allergen challenge in any of the groups (Figure 3(E)).

There were no differences in mRNA levels between single and dual responders before or at 24 h post-challenge, and there was no significant change in mRNA levels post-challenge compared to baseline, neither in all subjects nor when analysing single and dual responders separately (Figure 3(F)).

CC16 in BAL fluid

The level of CC16 in the bronchial fraction of BAL was significantly increased after the allergen challenge compared to baseline levels in single responders (Figure 4(A)), but not in dual responders or when analysing all subjects. CC16 was significantly higher in dual responders compared to single responders at baseline in bronchial BAL. In the alveolar fraction, there was also a tendency towards an increase of CC16 in the single responders after the allergen challenge (Figure 4(B)), but there were no significant differences in absolute levels of CC16 between single and dual responders at baseline or post-challenge.

Investigating whether normalizing CC16 levels for total protein concentration in BAL was suitable, we found no difference between single and dual responders in total protein concentration in alveolar or bronchial fractions of BAL pre- or post-challenge, and no significant change in total protein concentration from baseline to post-challenge in any fraction. Total protein concentration did not correlate with CC16 concentration in any fraction of the BAL fluid at any time point

Figure 2. CC16 in plasma. Absolute levels of plasma CC16 in all subjects (A) and relative values compared to baseline in single and dual responders (B). Results are presented as median (and IQR). (●) single responders, (□) dual responders. ** $p < 0.01$, *** $p < 0.001$: significant difference compared to baseline.

(r -values ranging from 0.042 to 0.16 and p values ranging from 0.53 to 0.87).

Correlations between different compartments

Post-challenge alveolar BAL fluid levels of CC16 correlated significantly with plasma levels of CC16 collected approximately at the same time (23 h post-challenge) (Figure 5(A)). A significant correlation was also seen when analysing single responders separately ($r = 0.65$, $p = 0.046$), but not for dual responders only ($r = 0.77$, $p = 0.10$). No significant correlation was seen at baseline ($r = -0.020$, $p = 0.94$). When comparing the change of CC16 levels in plasma from baseline to 23 h with the change in alveolar BAL levels of CC16, there was a significant correlation in all subjects (Figure 5(B)), but not when analysing single responders or dual responders separately ($r = 0.52$, $p = 0.16$ and $r = 0.46$, $p = 0.37$, respectively). Absolute levels of CC16 in plasma at 23 h post-challenge correlated significantly with the change in alveolar BAL levels of CC16 ($r = 0.56$, $p = 0.029$). In contrast, there were no significant correlations between absolute or relative bronchial BAL fluid levels and plasma levels at any time (r -values ranging from -0.16 to 0.26 and p values ranging from 0.41 to 0.78).

At baseline, there was a tendency towards inverse correlations between CC16 mRNA levels in brushings and CC16 in BAL fluid, but correlations were not significant ($r = -0.53$, $p = 0.064$ for brush mRNA and bronchial BAL fluid, $r = -0.52$,

Figure 3. CC16 positive cells and mRNA. (A–D) Representative pictures of brush samples with immunocytochemical staining for CC16. CC16+ = cells with positive brown staining for CC16, i.e. club cells. Arrows indicate ciliated epithelial cells, not stained by DAB + chromogen. Samples were taken from (A) single responder at baseline, (B) single responder post-challenge, (C) dual responder at baseline and (D) dual responder post-challenge. (E) Percentage of CC16 positive cells and (F) mRNA levels in brush samples, before (baseline) and 24 h after (post-challenge) the allergen challenge, in single and dual responders. Lines denote medians and p values are presented for each comparison. A.U.: arbitrary units; (●) single responders, (□) dual responders.

$p = 0.071$ for brush mRNA and alveolar BAL fluid). There were no correlations between post-challenge brush CC16 mRNA levels and CC16 in any fraction of the BAL fluid ($r = -0.38$, $p = 0.16$ for bronchial BAL; $r = 0.30$, $p = 0.28$ for alveolar BAL). Baseline CC16 mRNA levels correlated significantly with post-challenge CC16 mRNA levels ($r = 0.71$, $p = 0.005$).

There was a tendency towards a significant correlation between post-challenge brush CC16 mRNA and CC16 plasma levels at 23 h post-challenge ($r = 0.45$, $p = 0.08$). Apart from that, CC16 levels in plasma did not correlate with CC16 levels in urine, levels of brush CC16 mRNA or proportion of CC16 positive cells in brushings at any time point (r -values ranging

Figure 4. CC16 in bronchoalveolar lavage (BAL). Levels of CC16 in bronchial (A) and alveolar (B) fractions of BAL, before (baseline) and 24 h after (post-challenge) the allergen challenge, in single and dual responders. All results are normalized for total protein concentration, and p values are presented for each comparison. (●) single responders, (□) dual responders.

Figure 5. Association between CC16 in plasma and CC16 in bronchoalveolar lavage (BAL). Correlation in all subjects between CC16 in the alveolar fraction of BAL and CC16 in plasma at 24 and 23 h post-challenge, respectively (A), and the correlation in all subjects between changes in levels of CC16 in alveolar fraction of BAL from baseline to 24 h post-challenge and changes in levels of CC16 in plasma from baseline to 23 h post-challenge (Δ -values) (B). Spearman's rho and p values are presented. (●) single responders, (□) dual responders.

Figure 6. Plasma CC16 and reactivity to mannitol. Correlation between baseline CC16 levels in plasma and the PD₁₅Mannitol. (●) single responders, (□) dual responders.

from -0.034 to 0.32 , p values ranging from 0.25 to 0.98). Neither were there any significant correlations between the proportion of CC16 positive cells in brushings and CC16 levels in any fraction of the BAL fluid at any time (r -values ranging from -0.50 to 0.38 , p values ranging from 0.17 to 0.83).

Airway hyper-responsiveness and CC16 in plasma

In subjects with a positive mannitol inhalation challenge, the dose of mannitol required to decrease FEV₁ by 15% (PD₁₅Mannitol) correlated significantly with levels of CC16 in plasma both at baseline (Figure 6) and at 23 h post-allergen challenge ($r = 0.64$, $p = 0.013$). Correlations were not significant when analysing single responders or dual responders separately, neither at baseline ($r = 0.38$, $p = 0.32$ and $r = 0.70$, $p = 0.23$, respectively) nor 23 h post-challenge ($r = 0.57$,

Figure 7. CC16 in urine. Absolute levels of CC16 in urine in all subjects (A), and relative values compared to baseline in single and dual responders (B). Results are presented as median (and IQR), and are normalized for creatinine concentration. (●) single responders, (□) dual responders. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significant difference compared to baseline.

$p = 0.11$ and $r = 0.56$, $p = 0.40$, respectively). There were no significant differences between subjects with a positive and a negative mannitol challenge regarding CC16 levels in plasma, urine or BAL at any time. The PD₁₅Mannitol did not correlate with CC16 levels in BAL, brush CC16 mRNA levels or proportion of CC16 positive brush sample cells (r -values ranging from -0.35 to 0.30 , p values ranging from 0.35 to 0.80).

CC16 levels in plasma did not correlate at any time with the provocative dose required to decrease FEV₁ by 20% during the methacholine challenge (PD₂₀Methacholine), or with the dose of allergen given (r -values ranging from -0.20 to 0.21 , p values ranging from 0.24 to 0.99). There were no correlations between plasma levels of CC16 and FEV₁% p or ACT score at baseline ($r = 0.19$, $p = 0.30$ and $r = -0.14$, $p = 0.50$, respectively).

CC16 in urine

Urinary levels of CC16 corrected for creatinine were significantly decreased in all subjects at 2, 6, 8 and 23 h post-allergen challenge compared to baseline (Figure 7(A)). There were no differences in CC16 urine levels between single and dual responders at baseline (Table 1) or after the challenge (Figure 7(B)). Urine creatinine levels were significantly increased 23 h post-challenge compared to baseline [12.9

(2.8–18) vs. 6.4 (2.2–11.6) mmol/L, $p = 0.041$], but were unchanged compared to baseline at all other time points.

Discussion

We have shown that CC16 levels in plasma of asthmatic subjects are increased directly after an inhaled allergen challenge, and that levels of CC16 in BAL fluid were increased in the subgroup of single responders while the proportion of CC16 positive cells was higher in brush samples from the dual responders 24 h post-allergen challenge. CC16 mRNA levels were however unaffected, indicating a constitutive synthesis regardless of acute inflammation. CC16 levels in plasma correlated with CC16 levels in the alveolar fraction of BAL, and with the reactivity to inhaled mannitol. In contrast to previously reported increases after an exercise challenge (Bolger *et al.* 2011a, Romberg *et al.* 2011, Tufvesson *et al.* 2013), the inhaled allergen challenge did not lead to increased urinary levels of CC16.

The allergen challenge was performed with repeated inhalations of incremental doses, meaning that the duration of the challenge itself was usually approximately 1–1.5 h from the first dose to the drop in FEV₁ of $\geq 20\%$. During this time, there was probably a gradually increasing leakage of CC16 over the bronchoalveolar/blood barrier, resulting in higher CC16 plasma levels already after completing the allergen inhalations (=time point 0 h). This increase might reflect the anti-inflammatory properties of CC16. It may in fact be even more pronounced considering that CC16 levels generally are lower during the time of the day when the post-allergen challenge samples were collected (Helleday *et al.* 2006). If our allergen challenge would be designed as a bolus dose instead of slowly increasing doses over an extended time period, it is possible that we would see differing results regarding the peak of CC16 increase in plasma.

A substantial CC16 concentration gradient of about 10,000 to 1 between the epithelial lining fluid and plasma is believed to drive the passive diffusion of CC16 over the epithelium (Broeckeaert *et al.* 2000a). In the BAL fluid of our subjects, concentrations of CC16 were approximately 100 times higher than in plasma, which is reasonable considering that the BAL technique results in approximately a 100-fold dilution of the epithelial lining fluid (Broeckeaert *et al.* 2000a). Given the overwhelming amount of CC16 in BAL compared to circulating levels, it can be assumed that the increase in plasma CC16 is completely derived from the respiratory compartment. Our findings of significant correlations between CC16 in plasma and CC16 in the alveolar fraction of BAL post-allergen challenge (but not pre-challenge) support the hypothesis that epithelial damage leads to increased leakage over the bronchoalveolar/blood barrier. The size of the aerosols produced by our nebulizer (1.6 μm) (Nieminen *et al.* 1988) would result in a more peripheral deposition (Horváth *et al.* 2011), explaining why no correlations were seen with CC16 in the bronchial fraction. CC16 plasma levels did not differ between subjects with ICS treatment and subjects without ICS treatment. Although ICS has been shown to protect the airway epithelial integrity of

healthy subjects, it was also revealed that epithelial cells from asthmatic subjects were less responsive to that protective effect and were more easily affected by oxidative stress (Heijink *et al.* 2014). Furthermore, our subjects were treated with only low to moderate doses of ICS, which also could explain why no inhibiting effect was seen on the increase in CC16 plasma levels.

In a previous study, repeated low dose allergen challenge over seven consecutive days led to decreased concentrations of CC16 in BAL of subjects with allergic asthma (Lensmar *et al.* 2000). In our study, CC16 levels in BAL were increased in single responders only, while the dual responders had significantly higher levels already at baseline. The allergen exposure in our study was higher and the time of sampling was different compared to the study by Lensmar *et al.* (Lensmar *et al.* 2000), with our results reflecting more of an acute inflammatory response. The absence of an increase in CC16 levels in BAL among the dual responders could be due to some form of desensitization of their club cells, thus reducing the anti-inflammatory response and the capacity of released CC16 to inhibit the LAR.

Surprisingly, urinary levels of CC16 were decreased after 2–23 h compared to baseline, although with large variations. CC16 was normalized for creatinine, which was significantly increased in the whole group at 23 h post-challenge, and when not correcting for creatinine the CC16 levels were significantly decreased only at 6 and 8 h post-challenge (data not shown). The increase in urinary creatinine was probably due to some degree of dehydration in subjects not drinking or eating anything prior to the bronchoscopy, as per protocol. We have previously shown that CC16 is increased in urine following an exercise challenge test (Tufvesson *et al.* 2013). Exercise increases glomerular permeability (Axelsson *et al.* 2011), and we also found that both albumin and protein HC leaked into the urine along with CC16. Mannitol and EVH has also been shown to lead to a minor increase in urinary CC16 levels (Bolger *et al.* 2011b, Kippelen *et al.* 2013), although these challenges should not affect glomerular permeability. If CC16 is subject to tubular reabsorption, like e.g. the similarly sized protein β 2-microglobulin (Wibell *et al.* 1973), the divergent results after different types of airway challenges could be explained by the fact that exercise, mannitol and EVH cause more epithelial damage and lead to higher CC16 plasma levels. This would exceed the threshold for tubular reabsorption capacity, while more chronic low-grade changes in plasma, i.e. after allergen exposure, would not. Another possible explanation for the more subtle response (compared to post-exercise) is that some allergens could exert a proteolytic effect on CC16, partially inhibiting the measured increase in CC16 plasma levels.

CC16 mRNA levels were unchanged, indicating that CC16 is constitutively expressed in the respiratory compartment and not affected by acute inflammation. Despite this, there were some changes in CC16 levels in BAL, which indicates increased leakage and/or secretion from granules since neither of these would affect mRNA levels. The proportion of CC16 positive cells was however higher in brush samples from dual responders, i.e. subjects with a higher degree of airway inflammation. This might be due to increased club

cell proliferation in accordance with its anti-inflammatory role. Another explanation might be epithelial damage, meaning that more cells lose their attachment to the epithelium and are caught in the brushings. These potential mechanisms have however not been investigated previously, and further studies will be required to explore the club cell response to allergen exposure. A limitation of the present study is that BAL and brush samples were collected at 24 h post-challenge. Results may have differed if samples were collected e.g. one hour post-challenge when a clear increase in CC16 plasma levels was observed, but this would be associated with practical difficulties and safety issues.

Our finding of lower circulating levels of CC16 in subjects with a higher degree of airway hyper-responsiveness to mannitol is consistent with previous results of decreased CC16 levels in asthmatic subjects (Shijubo *et al.* 1999b) and with the idea of CC16 as an anti-inflammatory pneumoprotein (Broeckaert and Bernard, 2000). This does however make the interpretation of CC16 levels in plasma more complex, if it was to be used as a biomarker of airway inflammation or epithelial dysfunction.

Conclusions

In summary, we have demonstrated that an inhaled allergen challenge in asthmatic subjects leads to an increase in plasma CC16, with increasing CC16 levels in BAL fluid of some subjects but without any signs of altered CC16 mRNA expression in brush samples. We believe that the increase in plasma CC16 might be a result of increased secretion and/or leakage of constitutively expressed CC16 in the respiratory compartment, and that CC16 in plasma could potentially be used as a biomarker of airway epithelial dysfunction following pro-inflammatory stimuli. However, we also found an association between low circulating CC16 levels and increased airway hyper-responsiveness to mannitol. This is consistent with previous reports of lower CC16 levels in asthmatic subjects (Shijubo *et al.* 1999b, Laing *et al.* 2000), and long-term effects of airway inflammation on CC16 should therefore be taken into account.

Acknowledgements

The authors would like to thank Anton Degerskär for assisting with laboratory analyses, and the staff at the Research Unit, Respiratory Medicine and Allergology, Skåne University Hospital, and especially Jonas Olsson, for clinical assistance and collection of data.

Disclosure statement

Subhabrata Moitra has received honoraria from Current Respiratory Medicine Reviews, Lancet Respiratory Medicine and ERS. The other authors report no conflicts of interest.

Funding

This work was supported by independent grants from the Swedish Asthma and Allergy Association's Research Foundation, Swedish Heart and Lung Foundation, Crafoord Foundation, Evy and Gunnar Sandberg's Foundation and Österlund Foundation.

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