

A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects

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Abstract

Biomarkers associated with asthma aetiology and exacerbation have been sought to shed light on this multifactorial disease. One candidate is the serum concentration of the Clara cell secretory protein (CC16, sometimes referred to as CC10 or uteroglobin). In this review, we examine serum CC16's relation to asthma aetiology and exacerbation. There is evidence that acute exposures to certain pulmonary irritants can cause a transient increase in serum CC16 levels, and limited evidence also suggests that a transient increase in serum CC16 levels can be caused by a localized pulmonary inflammation. Research also indicates that a transient increase in serum CC16 is not associated with measurable pulmonary damage or impairment of pulmonary function. The biological interpretation of chronic changes in serum CC16 is less clear. Changes in serum CC16 concentrations (either transient or chronic) are not specific to any one agent, disease state, or aetiology. This lack of specificity limits the use of serum CC16 as a biomarker of specific exposures. To date, many of the critical issues that must be understood before serum CC16 levels can have an application as a biomarker of effect or exposure have not been adequately addressed.

Keywords: *Asthma, biomarker, Clara cell secretory protein, CC16, CC10, environmental exposures*

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Introduction

Over the past 20 years, considerable effort has been expended to identify the exact role of 16 kDa Clara cell secretory protein (CC16) in respiratory tract inflammatory diseases, including asthma. While the exact function of CC16 is not known, it is

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believed to play a role in reducing inflammation of the airways. Because of its presumed relationship to inflammation, CC16 has been proposed as a biomarker in the study of asthma. In this review, we examine the current knowledge about serum CC16 and its relationship to asthma aetiology and exacerbation. In order to evaluate the utility of serum CC16 as a biomarker of exposure and effect, we provide background information on CC16 and then focus on several aspects of the exposure–effect continuum (Figure 1) by reviewing the literature on changes in levels of CC16 (both short- and long-term) and acute and chronic changes to the respiratory system.

A useful biomarker should be validated and have toxicological relevance such that it can be used for risk assessment (Bernard & Hermans 1997). Criteria for evaluating the utility of a biomarker include (Needham 2005): (i) Does the biomarker have high sensitivity? (ii) Does the biomarker have high specificity? (iii) Is it biologically relevant? The biomarker should also be stable in the biological sample, specific with respect to the target tissue, and sensitive to levels of exposure to agents of concern (Bernard & Hermans 1997). In addition, there must be sufficient information such that changes in the concentrations of the biomarker can be used to determine health significance and clinical relevance. A biomarker's utility is further complicated by the need to differentiate between adaptive and toxic effects, reversible and irreversible effects, and adverse versus non-adverse effects (Bernard & Hermans 1997). These criteria will be discussed in the context of serum CC16 and asthma.

Literature search

The information in this review was obtained from literature located by an electronic search of PubMed using the following key words in various combinations: asthma, Clara cell, CC16, exercise, ozone, smoking, asthma phenotype, polymorphism,

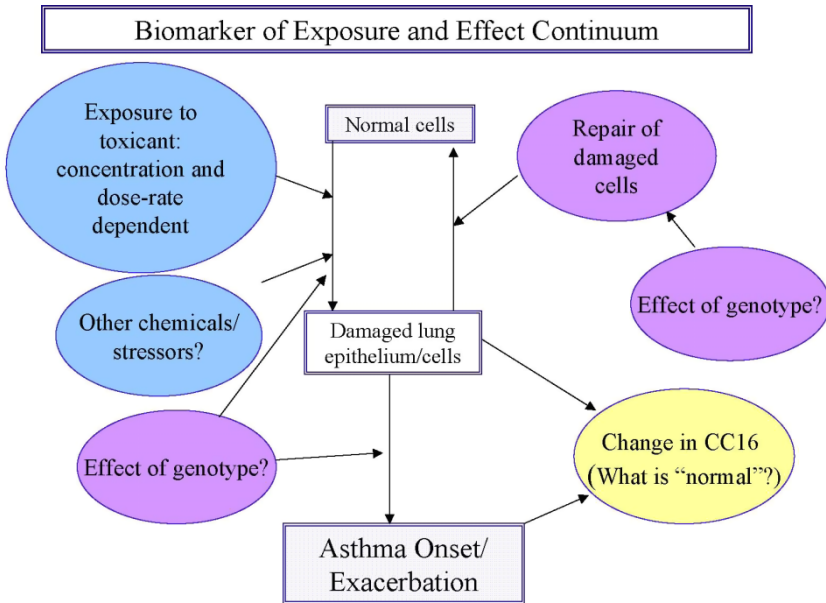


Figure 1. Exposure-effect continuum for progression from normal lung epithelium cells to asthma onset/exacerbation.

indoor swimming pool, disinfection byproducts, DBP, trihalomethanes, trichloramine. The PubMed link 'Related articles' was utilized for many of the relevant publications. Finally, the bibliographies of references were reviewed to identify additional references not appearing in the PubMed or other electronic search results. This review is limited to English language publications and is inclusive of published literature on serum CC16 from 1978 to March 2007. In addition, this review is focused on serum CC16 (with some reference to pulmonary fluids); it is premature at this time to evaluate critically the utility of urinary CC16 because the literature on urinary CC16 is limited (Andersson et al. 2007).

Background: Clara cell secretory protein or CC16

What cells produce CC16?

Clara cell secretory protein (hereafter referred to as CC16) is a small protein secreted by the non-ciliated bronchiolar Clara cells which are found predominantly in the respiratory bronchioles of humans; Clara cells are still abundant, but found in fewer numbers, in the terminal bronchioles (Boers et al. 1999). CC16 is expressed at high levels in the human respiratory epithelium by Clara cells, which are unique to the lung (Yoneda 1978), and in non-ciliated columnar cells of the large and small bronchi and bronchioles (van Vyve et al. 1995). CC16 is produced by nasal mucosal epithelial cells (Benson et al. 2005), the male urogenital tract (Bernard et al. 1997), the endometrium, fetal lung and kidney (Hermans & Bernard 1999), amniotic fluid (Bernard et al. 1994a), and in the female urogenital tract (Laing et al. 1998). CC16 is found in airways in increasing concentrations from the trachea to bronchi and to terminal bronchioles, with levels found in the prostate, endometrium, and kidney about 20 times lower than those present in the lung (Broeckaert et al. 2000a). Most of these data are based on studies of adults; few data on children are available.

A significant correlation between bronchoalveolar lavage fluid (BALF) CC16 levels and serum CC16 levels has been found by Shijubo et al. (1997). Despite being produced in organs other than the lungs, some researchers have concluded that 'serum [CC16] levels appear to reflect [CC16] levels in the lower respiratory tract, and serum [CC16] levels are not influenced by the release of uteroglobin or hUP-1 from urogenital organs' (Shijubo et al. 1997). Conversely, Halatek et al. (1998) found no correlation between BALF and serum levels of CC16 in rodents.

What is the function of CC16?

The exact physiological function of CC16 in the lung is not known (Singh & Katyal 1997), but it is believed to play a role in reducing inflammation of the airways (Jorens et al. 1995) and protecting the respiratory tract from oxidative stress (Broeckaert & Bernard 2000). The amino acid sequence of CC16 has been well conserved evolutionarily in mammals, suggesting an important role in pulmonary physiology (Hashimoto et al. 1996, Candelaria et al. 2005). CC16 may also have an immunosuppressive role (Jorens et al. 1995, Shijubo et al. 2003) and anti-tumour qualities (Broeckaert & Bernard 2000). Animal studies have demonstrated that deficiencies in CC16 are associated with increased susceptibility to damage due to oxidative stress (Mango et al. 1998, Johnston et al. 1999). A detailed review on the function of this protein is available (Broeckaert & Bernard 2000).

Why has serum CC16 been proposed as a biomarker?

Historically, in order to assess lung damage, investigators have used bronchoalveolar lavage (BAL) (yielding BALF) as a means of collecting cells and seeking evidence of cellular damage. While CC16 is secreted from the Clara cells into BALF (Bernard et al. 1993, Shijubo et al. 1997), CC16 is thought to be small enough to diffuse into serum from the airways along a concentration gradient (Bernard et al. 2005). Even though BALF concentrations of CC16 are greater than in the serum (Bernard et al. 1993, Shijubo et al. 1997, Broeckaert & Bernard 2000), normal serum levels are sufficiently high to measure using techniques such as enzyme-linked immunosorbent assay (ELISA). Therefore, serum CC16 could potentially provide a less-invasive method for assessing airway damage.

What are normal physiological levels and what natural factors influence those levels?

Measured levels of CC16 have been reported to vary widely across individuals, indicating interindividual variation in synthesis and secretion of CC16 (Broeckaert et al. 2000a). In healthy individuals, CC16 levels can vary by a factor of 10 or more (Hermans & Bernard 1999) (data for healthy, non-smokers are shown in Figure 2). As shown in Figure 2, healthy non-smoking population data are fairly limited and the range of values of CC16 is over an order of magnitude. Bernard et al. (1997) measured serum CC16 levels in seven healthy subjects three times over the course of one day. The coefficient of variation for each subject ranged from 5.5% to 23.9% (Bernard et al. 1997).

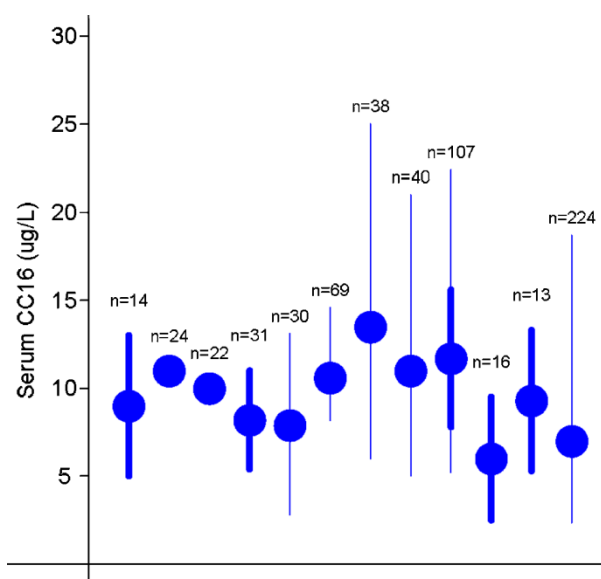


Figure 2. Mean serum CC16 (g l^{-1}) levels in healthy non-smokers with no exposures to specific factors affecting serum CC16 reported. Data from Nanson et al. 2001, Broeckaert et al. 2000b (pre-ride data), Blomberg et al. 2003, Lagerkvist et al. 2004, Michel et al. 2005, Robin et al. 2002, Shijubo et al. 1997, 2000. Line represents standard deviation (thick line) or range (thin line).

A number of factors have been explored for associations with serum CC16 levels, some of which may reduce the value of serum CC16 levels as a biomarker of exposure or effect (data summarized in Table I).

Gender. The only report on the influence of gender in healthy subjects (Shijubo et al. 1997) found no effect of gender on serum CC16 levels. While Hermans et al. (2003) reported a gender effect on serum CC16 levels, the study involved individuals with renal failure. Serum CC16 has a half-life of approximately 2–3 h due to rapid clearance through the kidney (Broeckaert et al. 2000a) and serum CC16 levels are strongly predicted by creatinine clearance (Hermans et al. 2003); thus, this apparent gender difference is more likely due to differences in renal function.

Diurnal variation. There is conflicting information on the effects of diurnal variation on serum CC16. Helleday et al. (2003) observed diurnal variations in serum CC16 in adults. Bernard et al. (1997) studied seven healthy subjects with serum CC16 levels measured over 1 day and found no significant difference among the three sets of means [SDs] (16.7 [5.4], 14.3 [3.7] and 14.5 [3.9] $\mu\text{g l}^{-1}$), suggesting lack of a diurnal variation. However, Helleday et al. (2006) observed a time-dependent diurnal variation in serum CC16 in 18 healthy subjects and proposed a method for mathematically compensating for this observed variation.

Body mass index (BMI). While Broeckaert et al. (2000a) reported serum CC16 to be independent of BMI, Nomori et al. (1996) found a positive correlation between hyperlipidaemia and obesity and serum CC16. Serum CC16 levels were positively correlated with triglyceride, total cholesterol, free cholesterol, apoproteins A-I, A-II and B and BMI.

Age. Serum CC16 levels have been reported to increase with age-related declines in glomerular filtration rate (Broeckaert et al. 2000a). Age-related changes in CC16 have been postulated to be related to increased alveolar capillary leakage and non-specific deterioration of the lung (Hermans et al. 2003). Carbone et al. (2002) found slightly lower concentrations of serum CC16 in children than adults (children 6.0 ± 3.5 ; adults 9.3 ± 4.0 $\mu\text{g l}^{-1}$).

Genotype/phenotype. The human CC16 gene maps to an important genetic region (11q12–13) which has been linked by various studies to asthma and its related phenotypes (Cookson et al. 1989, van Herwerden et al. 1995). Several polymorphisms have been identified in the gene encoding for CC16, with the best characterized being an adenine to guanine substitution at position 38 (A38G). The relationship between polymorphic expression of CC16 and asthma has been equivocal, with some studies finding a positive association between the incidence of asthma and a polymorphism in the CC16 gene (Laing et al. 2000, Saadat et al. 2004, Sharma & Ghosh 2004, Candelaria et al. 2005) and other studies finding no such association (Gao et al. 1998, Mao et al. 1998, Mansur et al. 2002, Sengler et al. 2003, Sharma & Ghosh 2004).

Exercise. Studies have provided conflicting results regarding the effect of exercise on serum CC16 levels. Nanson et al. (2001) demonstrated that exercise (in the form of firefighting tasks and treadmill exercise) is associated with increased serum CC16

Table I. Natural factors affecting levels of CC16 in serum ($\mu\text{g l}^{-1}$).

Gender	Age (years)	Factor	Control	<i>n</i>	Smoker	Subject CC16, mean (range)	Control CC16, mean (range)	Reference
1 woman/6 men	24–43	Diurnal variation		7	6: never, 1: yes	–	16.7 (morning), 14.3 (midday), 14.5 (night)	Bernard et al. 1997
–	–	Obesity	Yes	44	–	–	14.1 (5.6–29.6)	Summarized in Hermans & Bernard 1999
–	–		No	34	–	14.4 (5.2–34.4)	–	Summarized in Hermans & Bernard 1999
12 men/2 women	–	No exercise	Yes	14	No	–	9 ± 4	Nanson et al. 2001
	–	Exercise (firefighting exercises)		14	No	15 ± 13	–	
	–	Exercise (treadmill)		10	No	15 ± 8	–	
Men		Obesity (BMI > 25)	No	47	–	139.2 ± 98.2		Nomori et al. 1996
Men		Normal BMI (21–23)		70		90.3 ± 57.1		Nomori et al. 1996 ^a
Men		Lean BMI (< 20)		17		65.6 ± 40.8		Nomori et al. 1996
Men		Diabetes		69		104 ± 92.5 (19– 419)	–	Nomori et al. 1996
Men		Healthy		138		–	125.4 ± 79.9 (20–409)	Nomori et al. 1996
		Artherosclerosis		24		149.0 ± 71.1 (84–398)	–	Nomori et al. 1996
		Healthy		48		–	128.5 ± 73.3 (28–410)	Nomori et al. 1996

^aThe nephelometric latex immunoassay (LIA) method used by Nomori et al. is seen as particularly susceptible to optical aberration from other molecules such as triglycerides which increases serum turbidity leading to positive interference with different immunoassay (Haughton & Mason 1992). Such interference might have been responsible for the reported high serum levels of CC16 by Nomori et al., a concept which is supported by their finding of close correlation between CC16, triglycerides and interdependent parameters such as cholesterol, different apolipoproteins, and body mass index (BMI).

levels, but possibly only in those individuals who do not regularly exercise or only after high levels of exertion. Nanson et al. are uncertain as to whether the increase in serum CC16 is due to increased lung epithelium leakage limited to small molecules or to increased production of CC16 or 'other unexplained causes'; however, because no changes in sputum tumour necrosis factor (TNF)- α were observed, they hypothesize that exercise did not have an effect on lung permeability. Nanson et al. recommend that in future studies of serum CC16, subjects refrain from exercise for at least 24 h before collection of serum. Lagerkvist et al. (2004) did not find significant changes in serum CC16 levels in healthy children before and after 2 h of outdoor exercise. Carbonnelle et al. (2002) found a statistically significant increase in serum CC16 levels 10–20 min, but not 11 h, after a 45-min swim training session among swimming athletes, regardless of the method of pool disinfection.

A critical question is whether and how serum CC16 concentrations may change as a result of how the lungs are used. It may be that the breathing changes associated with exercise vary with the type of exercise. For example, swimmers hold their breath momentarily between each breath as their faces become submerged which is a different pattern of breathing compared to other forms of exercise. In competitive swimming, people strain their muscles while they are breath holding which may significantly increase the intraluminal pressure in the airways. The conflicting results found thus far make it difficult to render definitive conclusions about the effect of exercise on serum CC16 levels.

Utility of CC16 as a biomarker of effect

It has been suggested that chronic and transient changes in serum CC16 concentrations can be used as biomarkers for pulmonary disease states and pulmonary stress/inflammatory response, respectively. Chronic versus transient changes in serum CC16 concentrations have been hypothesized to have different biological mechanisms, implications for pulmonary effects, and potential value as a biomarker of effect.

The pulmonary epithelial barrier undergoes continuous repair and regeneration processes following exposure to the broad range of pulmonary irritants encountered in everyday life. Irritant environmental exposures may result in transient or permanent changes in function of different cell types in the pulmonary airways. There is no clear distinction between challenge and damage, but rather a continuum between the two. At one end of the continuum, cell repair occurs as rapidly as cell damage, while at the other end of the continuum, the rate of cell damage exceeds the repair capacity resulting in damage to the lung. The critical question is where observed changes in serum CC16 fit into this continuum.

Biomarker of acute pulmonary effects: transient changes in serum CC16

Transient increases in serum CC16 could be due to (i) increased CC16 transport by concentration gradient from BALF into serum due to a transient increase in pulmonary epithelium 'leakiness' (Arsalane et al. 2000), (ii) increased production of CC16, or (iii) decreased renal clearance. An experiment in rodents using inhaled lipopolysaccharide (LPS) demonstrated that the increase in serum CC16 was not a result of increased CC16 production or synthesis by Clara cells (Arsalane et al. 2000). Therefore, a transient increase in serum CC16 can at least in part be a result of

increased 'leakiness' rather than an increase in CC16 production by Clara cells. However, this is the only careful experiment to test this hypothesis and LPS is known to cause pulmonary inflammation without causing epithelial cell damage (Brigham & Meyrick 1986, Johnston et al. 1999).

Several human exposure and laboratory animal studies have been conducted to identify transient changes in serum CC16 concentrations from exposure to pulmonary irritants. These are summarized below and in Table II.

Smoke. Bernard et al. (1997) examined six voluntary firefighters (a combination of smokers, ex-smokers and non-smokers) who were exposed to fumes from a polypropylene fire for approximately 20 min. Acute exposure to smoke resulted in a statistically significant increase in serum CC16 ($54.4 \mu\text{g l}^{-1}$ exposed vs. $19.5 \mu\text{g l}^{-1}$ for control subjects) measured immediately after the fire. The increase in serum CC16 levels was not accompanied by functional signs of lung impairment. By 10 days post-exposure, CC16 levels had decreased to levels similar to those in six age-matched controls. As noted by Nanson et al. (2001), firefighting involves physically demanding tasks (even in the absence of smoke exposure) that are associated with increases in serum CC16 levels.

Ozone. Exposures to ozone have been found by some researchers to cause a transient increase in serum CC16 while others found no effect. Broeckaert et al. (2000b) studied the effect of exposure to ambient ozone on serum CC16 by examining 24 non-smoking cyclists. While the effects of ozone exposure and exercise cannot be completely separated in this study, the authors report that serum CC16 levels increased in participants both before and after their rides in an ozone dose-dependent manner. Changes in lung function were not correlated with serum CC16 levels. Blomberg et al. (2003) conducted a single-blinded, crossover control study in which volunteers were exposed to filtered air and ozone (0.2 ppm) challenges on two separate occasions, each separated by at least 3 weeks. Exposures were 2 h long and subjects alternated cycles of exercise and rest each lasting 15 min. Serum CC16 concentrations were elevated at 2 and 4 h post-exposure to ozone and returned to baseline within 18 h post-exposure. Serum CC16 increases were reported as occurring without increases in traditional markers of epithelial permeability (albumin and total protein concentrations in lavage fluid). No association was found between increase in serum CC16 and lung function change, at least in the short term. Interestingly, the control group had a statistically significant decrease in serum CC16 following exposures to filtered air. While the crossover design of the study with a control group of air versus O_3 both exercising should have accounted for exercise-related changes, based on the hypothesis that exercise is associated with increased serum CC16, it is unclear why no post-exercise increase in serum CC16 was observed in the air-exposed group. These inconsistent results highlight the difficulties of using CC16 as a biomarker for irritant exposure.

Some investigators have observed no effects or marginal effects of ozone exposure on CC16 levels in serum or BALF (Lagerkvist et al. 2004, Bernard et al. 2005). Bernard et al. (2005) measured serum CC16 concentrations among children exposed to ambient ozone levels of $48\text{--}221 \mu\text{g m}^{-3}$ while attending summer camp and found no increase in serum CC16 concentrations.

Table II. Levels of CC16 in serum ($\mu\text{g l}^{-1}$) in subjects exposed to various pulmonary toxicants and controls.

Gender	Age (years)	Exposure	<i>n</i>	Smoker	Subject CC16, mean (range)	Control CC16, mean (range)	Reference
–	27–49, mean: 38.2	Smoke from combustion of polypropylene	6	3: no, 1: never	~20–95 (12 for smoker)	–	Bernard et al. 1997*
1 woman/6 men	30–53, mean: 46	–	6	5: never, 1: yes	–	10–41 (20–30 for smoker)	
12 women/10 men	21–43, mean: 24	0.2 ppm ozone for 2 h (2 h post ozone exposure)	22	No	12.0 \pm 4.5	–	Blomberg et al. 2003 (controlled ozone exposure; study included cycling)
	21–43, mean: 24	2 h post filtered air exposure			–	8.4 \pm 3.1	
	21–43, mean: 24	4 h post ozone exposure			11.7 \pm 5.0	–	
	21–43, mean: 24	4 h post air exposure				7.9 \pm 2.6	
	21–43 mean: 24	Pre-exposure level				~10*	
–	36 \pm 9 (19–57)	Silica	86	–	–	16.3 (2.2–53)	Bernard et al. 1994
–	34 \pm 8 (20–57)	No silica	86	–	12.3 (4–40)	–	
43 male	Mean: 11.6	House cleaned with chlorine bleach	78	–	9.9 (1.4–25.5)	–	Nickmilder et al. 2007
81 male	Mean: 11.5	No use of chlorine bleach	156	–	–	9.5 (1.6–25.6)	
15 women/9 men	28.5 \pm 3.4	Ozone 0.033–0.103 ppm (mean 0.076 ppm) plus exercise (cycling)	24	No	(After cycling) men: 12.3 \pm 0.9, women: 11.9 \pm 1.3	(Before cycling) men: 11.2 \pm 0.8, women: 11.1 \pm 0.6	Broeckaert et al. 2000b (ambient exposure)
39 men/15 women	Median: 42	Tobacco smoke	54	Yes	7.6 (6.0–11.2)	–	Robin et al. 2002
55 men/14 women	Median: 51		69	No	–	10.6 (8.17–14.6)	Robin et al. 2002
–	–	Tobacco smoke	104	Yes	7.89 \pm 2.8 (1.6– 15.30)	–	Shijubo et al. 1997
–	–	Control	107	No	–	11.7 \pm 3.9 (5.2–22.4)	
324 men	47 (30–61)	Bioaerosols	325	No	11.0 (5.6–23.0)	–	Steiner et al. 2005 (data for never smokers shown; values median and 5th and 95 th percentiles)

Table II (Continued)

Gender	Age (years)	Exposure	<i>n</i>	Smoker	Subject CC16, mean (range)	Control CC16, mean (range)	Reference
341 men	41 (22–58)	Control	369	No	–	9.4 (4.3–18.0)	Halatek et al. 2005b
92 men	41.5	Aluminum	50	Some	11.9	–	
	38.9	Control	42	Some	–	15.4	
5 male/5 female	6–12	Chlorine	10	–	23.5 ± 2.5	–	Bonetto et al. 2006
5 male/5 female	6–12	control	10	–	–	9.5 ± 0.5	

*Estimated values from figures.

Broeckaert et al. (2000b) exposed 2-month old C57Bl/6 mice to 0.08 ppm ozone for 4 or 8 h. The authors found a statistically significant increase in serum CC16 concentrations immediately post-exposure. Concentrations of albumin in the BALF were increased (an indication that pulmonary inflammation occurred) but CC16 concentrations in BALF did not change (Broeckaert et al. 2000b).

Lipopolysaccharide. Arsalane et al. (2000) exposed rats intratracheally to LPS and determined the time-course of changes in serum CC16 concentrations. Serum CC16 concentrations were elevated within 3 h post-installation and remained elevated for at least 72 h post-administration, but returned to pre-exposure levels by 7 days post-dosing. CC16 in BALF decreased substantially after dosing.

Humans exposed to LPS have exhibited qualitatively similar results (Michel et al. 2005). Human volunteers were exposed via inhalation to 0.5, 5.0 and 50 μg LPS (Michel et al. 2005). Following the 50 μg LPS exposure, serum CC16 concentrations remained unchanged 3 h post-exposure, were elevated at 6 and 8 h post-exposure to 5.0 and 50 μg LPS, and were back to pre-exposure levels at 24 h post-exposure. A dose-response increase in peak serum CC16 levels was found.

Trichloramine (nitrogen trichloride, NCl_3). In mice exposed to 11.9 mg m^{-3} NCl_3 for 8 h (a concentration known to cause a 50% reduction in ventilation (Gagnaire et al. 1994)), serum CC16 exhibited no change through the first 2 h into the exposure; levels were elevated within 4 h and returned to within pre-exposure levels 16 h after the exposure ended (the only time point measured after the exposure) (Carbonnelle et al. 2002). It is not clear if these observed effects on serum CC16 were a result of direct toxic damage to the pulmonary epithelium or secondary to pulmonary hypoxia resulting from the hypoventilation. No pulmonary toxicity in the mice (as assessed by light microscopy and based on unchanged LDH levels in BALF throughout the experiment) was noted (Carbonnelle et al. 2002). There were no changes in serum CC16 concentrations in mice exposed for 4 h to lower concentrations of NCl_3 (0.53, 0.8 or 3.45 mg m^{-3}).

Cigarette smoke. van Miert et al. (2005) exposed female Sprague–Dawley rats to cigarette smoke for two 1-h exposures, separated by a 30 min break with exposure to clean air. A dose-dependent increase in serum CC16 levels was observed, with no noticeable changes in CC16 BALF levels. Serum CC16 levels peaked within 2–4 h and returned to baseline within 24 h.

Swimming pool disinfection byproducts. Carbonnelle et al. (2002) measured serum pneumoproteins, including CC16, in three groups of individuals in order to examine the use of these proteins as biomarkers of exposure (to NCl_3) and effect (asthma). The first group was comprised of 29 healthy swimmers (16 children and 13 adults) who provided blood samples before entering the pool and after swimming in a chlorinated pool with a mean air NCl_3 level of 490 $\mu\text{g m}^{-3}$. Children undertook 'free' activities for an average of 2 h, with the youngest children playing and the older children swimming. Adults were asked to stay poolside without swimming for 1 h, and then to swim, with swimming times ranging from 15–45 min. The second group included 14 trained, healthy swimmers who swam for 45 min in a chlorinated pool (mean NCl_3 air level of 355 $\mu\text{g m}^{-3}$) and, 1 week later, for 45 min in a non-chlorinated pool treated

with copper/silver (NCl_3 not detected). The participants were asked to refrain from intense exercise for 48 h prior to the study. Blood samples were taken before entering the pool, immediately after each swimming period, and 11 h after the second blood draw. The third group was three young adults attending a pool disinfected with chlorine gas (mean NCl_3 air level of $440 \mu\text{g m}^{-3}$). These individuals stayed poolside for 1 h and then swam for 45 min. All participants in the three groups were healthy and all except one were non-smokers. Carbonnelle et al. did not find significant changes in serum CC16 in recreational child swimmers. For adults, they found a decrease in serum CC16 after 1 h without swimming, with levels returning toward ‘normal’ after swimming. They reported a transient increase in serum CC16 in the trained swimmers immediately after exercise in both chlorinated and non-chlorinated pools and in the three young adults immediately after exercise in the chlorinated pool. In the trained swimmers, serum CC16 levels had returned to pre-exposure levels after 11 h. They postulated that the increase is due to mechanical stress on the epithelial barrier caused by intense exercise; the ‘stress-induced alterations in airway barriers might present a link with the relatively high incidence of airways diseases found in elite athletes’. A synopsis of studies related to CC16, swimming pools, and asthma is given in Table III.

Chlorine. In a study of 10 children accidentally exposed to chlorine gas at a swimming pool (Bonetto et al. 2006), respiratory distress and reduced lung function were observed in all children, as well as increased serum CC16 levels ($23.5 \pm 2.5 \mu\text{g l}^{-1}$) as compared with healthy controls ($n = 10$, $9.5 \pm 0.5 \mu\text{g l}^{-1}$). Both lung function and eNO improved within a few weeks. No evaluations of CC16 were conducted after lung function recovery.

Table III. Summary of three studies on serum CC16, indoor swimming pools and asthma.

Study	$[\text{NCl}_3]$ in pool air	Change in serum CC16	Mean serum CC16 levels in trained, regular swimmers ($\mu\text{g l}^{-1}$)	Mean serum CC16 levels in others ($\mu\text{g l}^{-1}$)
Carbonelle et al. 2002	355–490 $\mu\text{g m}^{-3}$	Child swimmers – no change. Adults – decrease during poolside time; back to ‘normal’ after swimming. Trained swimmers; Transient increase	9.0 ± 2.8 (pre-swim), 13.2 ± 5.1 (post-swim). Weak negative association with CC16 and cumulative pool attendance in trained swimmers	Child swimmers: 6.0 ± 3.5 (pre-swim), 5.7 ± 3.9 (post-swim). Adults: 9.3 ± 4.0 (pre-swim), 7.2 ± 2.8 (post-swim)
Bernard et al. 2003	No data	Positive association with cumulative pool attendance but no clear ‘dose’-response		Children: mean 7.08
Lagerkvist et al. 2004	No data	Lower CC16 in pool visitors as compared to non-pool visitors		Child pool visitors: 5.7 ± 2.4 (pre-exercise); 5.3 ± 1.7 (post-exercise). Non-pool visitors: 8.2 ± 2.8 (pre-exercise), 8.0 ± 2.6 (post-exercise)

Other. Hermans et al. (1999) administered a single intraperitoneal injection of various chemicals known to selectively damage Clara cells, including 4-ipomeanol and methylcyclopentadienyl manganese tricarbonyl (MMT), to female Sprague–Dawley rats and measured CC16 levels in the lung and serum. These chemicals caused a reduction in levels of CC16 in BALF, a decrease in CC16-positive Clara cells in BALF, and an increase in serum CC16 and albumin levels in BALF, which was interpreted to indicate that permeability changes had occurred in the lung (Hermans et al. 1999).

Biomarker of permanent pulmonary effects: chronic changes in serum CC16

Chronic changes in serum CC16 concentrations have been explored in relation to specific disease states and chronic exposures to pulmonary irritants and toxicants. We describe human survey studies here (no human controlled exposure studies are available).

Human survey studies of pulmonary toxicants

Several studies surveyed humans with exposure to known pulmonary toxicants to determine if there is a common pathology with levels of serum CC16. Studies associated with various pulmonary toxicants are reviewed below (summary data are given in Table II).

Tobacco smoke. An approximately 30% reduction in levels of serum CC16 has been found for smokers (Shijubo et al. 2000). In addition, CC16-positive bronchiolar cells were reduced in smokers ($16 \pm 5\%$) as compared to lifelong non-smokers ($27 \pm 7\%$) (Shijubo et al. 1997, 2000) (Figure 3). However, a dose-response relationship between smoking and serum CC16 levels (as well as levels of CC16 in BALF) has not been reported nor has a significant correlation been found between the proportion of

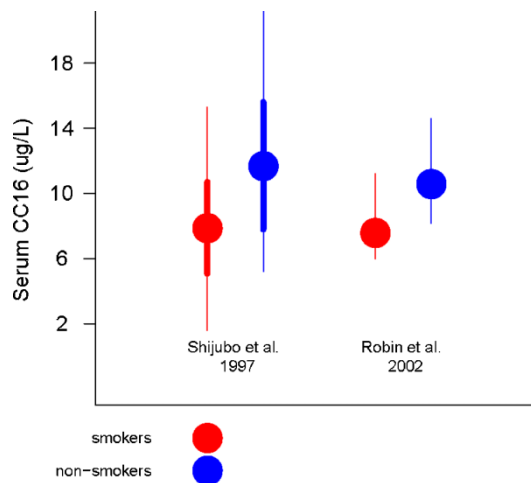


Figure 3. Serum CC16 levels in smokers and controls (data from Shijubo et al. 1997, Robin et al. 2002). Data for Robin et al.: mean and range; data for Shijubo et al.: mean, standard deviation and range.

CC16-positive bronchiolar epithelial cells and cumulative cigarette smoking (Shijubo et al. 1997).

Robin et al. (2002) found that smokers had lower levels of serum CC16 compared to non-smokers (Figure 3), which the authors ascribe to smoke-induced Clara cell toxicity. Female smokers had lower serum CC16 levels than male smokers. Robin et al. also found an increase in serum protein (SP)-B, which has been related to increased leakage of the epithelium. However, there was no increase in serum SP-A in smokers compared with non-smokers. These results appear contradictory, since increased leakage should also result in increased levels of serum CC16, unless the toxicity to Clara cells was the dominant effect. Robin et al. conclude that 'larger studies are needed to determine normal variance before the utility of pneumoproteinaemia can be fully exploited'.

Bioaerosols. Wastewater workers exposed to bioaerosols had somewhat higher levels of serum CC16 compared with controls (with only a small explained variance), but no consistent increase in SP-B. No work-related effects on the respiratory systems of workers were observed (Steiner et al. 2005).

Particulate matter. Exposure to crystalline silica (Bernard et al. 1994b) and foundry dust (Broeckaert & Bernard 2000 – no data given) has been associated with lower serum CC16 levels. There were no differences found in respiratory symptoms, chest radiographs, or lung function tests between silica-exposed workers and controls (Bernard et al. 1994b).

Nitrogen oxides. Sixty workers in a nitric acid production plant and 61 non-exposed people were studied for changes in the respiratory system and glomerular kidney function (based on animal models, nitric oxide (NOx) exposure has been shown to affect significantly Clara cells in laboratory animals) (Halatek et al. 2005a). The study group was a combination of smokers, ex-smokers and non-smokers. NOx-exposed workers had lower levels of serum CC16 compared to controls with the exception of the 75th to 90th percentile NOx-exposed participants, who had higher serum CC16 levels. NOx also caused renal effects, which could in turn affect the clearance of CC16 (Broeckaert et al. 2000a). The authors refer to serum CC16 as a non-specific marker and observe that the most sensitive biomarkers of response were β_2 -microglobulin in serum and α -tocopherol.

Swimming pool disinfection byproducts. Carbonnelle et al. (2002) examined cumulative pool attendance (ranging from 111 to 2740 h) and levels of serum pneumoproteins and found a weak negative correlation between serum CC16 in 16 trained swimmers and cumulative pool attendance (Table III). Carbonnelle et al. concluded that at exposure levels found in indoor pools, NCl₃ causes permeability changes affecting the deep lung (evidenced by changes in SP-A and SP-B, which are mostly produced in the alveolar epithelium), but mostly sparing the larger airways (evidenced by lack of change in CC16 levels, secreted by the Clara cells along the tracheobronchial tree).

Bernard et al. (2003) examined the relationship between exposure to NCl₃ in indoor swimming pools and serum CC16 concentrations, hypothesizing that the CC16 levels would be related to the potential effects of NCl₃ on the respiratory epithelium and to the risk of lung diseases including asthma. The first group studied

was 226 healthy children from seven schools with compulsory pool attendance (79 from two primary schools in Brussels and 147 from five primary schools in the Ardenne). Pool attendance was determined by information provided by school directors. The five pools were treated with sodium hypochlorite and concentrations were within recommended limits for Belgium ($0.5\text{--}1.5\text{ mg l}^{-1}$). Cumulative school pool attendance ranged from less than 0.55 to 6.42 h per week times years. Levels of NCl_3 in pool air or other environmental chemicals were not reported. The mean CC16 serum level in the children was $7.08\text{ }\mu\text{g l}^{-1}$ ($2.35\text{--}18.7\text{ }\mu\text{g l}^{-1}$; $n=224$) (Table III). While the authors state that the association between pool attendance and serum levels of serum pneumoproteins was 'systematically positive, showing that regular pool attendance increases the permeability of the lung epithelium barrier to these proteins', they found 'no clear dose-effect relation (between pool exposure) ... and serum CC16'. The authors postulated that lack of a dose-response relationship was because CC16 serum levels reflect both epithelial permeability and integrity of the Clara cells.

Lagerkvist et al. (2004) studied the effects of pool attendance on levels of serum CC16 and change in forced expiratory volume in 1 s (FEV_1) post-exercise in healthy children after outdoor exercise by measuring these variables in pool visitors (20 children) and in a control group (31 children) before and after outdoor exercise. Forty per cent of the children were regular pool visitors (defined as visiting an indoor pool for at least 1 h per month over 6 months or longer), with a median frequency of 4 h per month (range 1–35) and swimming exposures since the age of 6 months to 10 years. Levels of chlorination byproducts in indoor swimming pool air were not measured. Lagerkvist et al. found no significant differences between pool and non-pool visitors for predicted FEV_1 before or after exercise. They reported significantly lower average serum CC16 levels in pool visitors (Table III) but the pre- and post-exercise CC16 levels were not significantly different.

Chlorine bleach. Nickmilder et al. (2007) studied the association between regular household cleaning with bleach and risk of respiratory and allergic disease in children. The authors examined 234 children, 78 of whom lived in houses cleaned at least once weekly with chlorine bleach. They observed lower risk of asthma and eczema, and sensitization to indoor aeroallergens in children living in bleach-cleaned houses. They further found no effect of cleaning with bleach on serum CC16 levels (mean levels of $9.9\text{ }\mu\text{g l}^{-1}$ vs. $9.5\text{ }\mu\text{g l}^{-1}$ in houses with and without use of chlorine bleach, respectively). No air measurements of chlorine were given.

Human survey studies with respiratory and other diseases

There have been several investigations into the relationship between serum or BALF levels of CC16 and various respiratory diseases. Part of the interest lies in attempts to understand the role that CC16 might play in the pathogenesis of the disease and partly in characterizing the clinical utility of CC16 as a diagnostic or prognostic indicator for the disease in question. Following is a synopsis of reported associations of various respiratory diseases with serum CC16 levels (Table IV). For some diseases for which no information on serum CC16 levels was found, information on BALF or nasal lavage fluids was included.

Table IV. Levels of CC16 in serum ($\mu\text{g l}^{-1}$) in subjects with respiratory health conditions and controls.

Gender	Age (years)	Health Condition	Smoker (yes/no)	<i>n</i>	CC16 subject, mean [SD] (range)	CC16 control mean [SD] (range)	Reference
Male	20–50	Healthy	No	38	–	13.5 (6–25)	Shijubo et al. 2000
Female	20–50	Healthy	No	40	–	11 (5–21)	Shijubo et al. 2000
Male	44 ± 10	Healthy	No	51	–	11.5 [3.6]	Shijubo et al. 1997
Female	42 ± 8	Healthy	No	56	–	11.9 [4.2]	Shijubo et al. 1997
Male	43 ± 7	Healthy	Yes	58	7.8 [2.9]	–	Shijubo et al. 1997
Female	44 ± 8	Healthy	Yes	48	8.0 [2.7]	–	Shijubo et al. 1997
Male	–	Healthy	Yes	69	15.7 (3.4–51)	–	As summarized in Shijubo et al. 2003
Female	–	Healthy	Yes	65	15.9 (5–63.5)	–	As summarized in Shijubo et al. 2003
–	–	Lung fibrosis	–	23	29	–	As summarized in Shijubo et al. 2003
–	–	Control	–	33	–	18	
–	–	Chronic bronchitis	–	6	29	–	As summarized in Shijubo et al. 2003
–	–	Control	–	25	–	82	
–	–	Lung cancer	–	18	50	–	As summarized in Shijubo et al. 2003
–	–	Control	–	25	–	82	
–	–	Sarcoidosis	–	31	20	–	As summarized in Shijubo et al. 2003
–	–	Control	–	64	–	12	
–	0–14	Asthma	–	24	13.2 ± 8.4 (2.6–35.9)	–	Gioldassi et al. 2004
–	0–14	Control	–	27	–	27.5 ± 17.6 (1.22–95.6)	Gioldassi et al. 2004
Female	28–83 median 53	Chronic renal failure	No	17	73.8	–	Hermans et al. 2003
Male	16–87 median 51		No	37	43.2	–	Hermans et al. 2003
12 men/51 women	Mean 46.8 ± 15.5)	Asthma	No	63	7.02 ± 3.05 (1.52–15.8)	–	Shijubo et al. 1999 ^a
15 men/39 women	Mean 42.0 ± 9.0)	Control	No	64	–	11.7 ± 3.9 (5.16–22.4)	
Male	–	Healthy	–	9	–	11.8 (3.0–19.1)	As summarized in Hermans & Bernard 1999
Female	–	Healthy	–	9	–	13.4 (8.3–19.1)	Summarized in Hermans & Bernard 1999

Table IV (Continued)

Gender	Age (years)	Health Condition	Smoker (yes/no)	<i>n</i>	CC16 subject, mean [SD] (range)	CC16 control mean [SD] (range)	Reference
Male	–	Healthy	–	55	–	21.7 (7.1–50.8)	Summarized in Hermans & Bernard 1999
Female	–	Healthy	–	59	–	27.9 (4.0–77.4)	Summarized in Hermans & Bernard 1999
–	–	IPF	–	29	33	–	Summarized in Hermans & Bernard 1999
Male	43–70 (mean 55)	Healthy	–	23	–	18	
		COPD	Yes	6	29	82.0 (<i>n</i> = 25)	Bernard et al. 1992 ^b
10 men/7 women	25–74 (mean 47)	Sarcoidosis	No	17	134.0	82.0 (<i>n</i> = 25)	Bernard et al. 1992 ^b
Female and male	18–66 (mean 42)	Healthy	Yes	35	10.4 (Geo mean) (4–24) ^c	–	Hermans et al. 1998
			No	61	–	15.4 (Geo mean) (5–35) ^c	

–, not given.

^aPatients with asthma ≥ 10 years CC16 = 6.4; with asthma < 10 years CC16 = 7.9 and no different from controls.

^bAccording to Hermans & Bernard (1999), these serum CC16 values should be divided by 3.23 to account for the use of different standards.

^cEstimated values from figures.

Acute respiratory distress syndrome (ARDS). ARDS is characterized by major lung inflammation. BALF CC16 levels were found to be elevated in people with ARDS compared with controls (544 vs. 161 $\mu\text{g l}^{-1}$) (Jorens et al. 1995). Lesur et al. (2006) examined the relationship between serum CC16 levels and outcome, mechanical ventilation duration, and incidence of non-pulmonary organ failure in 78 patients with ARDS. Median serum CC16 were higher in non-survivors from days 2–14 of the study and higher CC16 levels were associated with fewer days free of the ventilator, and increased frequency and severity of non-pulmonary organ failure. The authors propose the use of serum CC16 as a predictor of outcome in ARDS patients at high risk for mortality.

Allergic rhinitis. Intermittent allergic rhinitis is associated with decreased levels of CC16 in nasal lavage fluids compared with healthy controls [5.43 ± 1.53 vs. 12.93 ± 2.53 ng ml^{-1} (Benson et al. 2005); 12.9 vs. 22.0 $\mu\text{g l}^{-1}$, median levels during birch pollen season (Johansson et al. 2005, Benson et al. 2007)]. It was hypothesized that decreased expression of CC16 could be related to the pathogenesis of intermittent allergic rhinitis.

Asthma. Fewer CC16-positive epithelial cells have been found in the airways of asthmatics compared with controls (Shijubo et al. 2000). This may explain why investigators have observed lower serum CC16 levels in asthmatic children compared with healthy children (Gioldassi et al. 2004) and adults (Shijubo et al. 2000, Ye et al. 2004). Shijubo et al. (1999) found no significant difference in serum CC16 between atopic and non-atopic asthmatics. In addition, asthmatics with a long duration of the illness (>10 years) had lower levels of serum CC16 than those with a less than 10-year history of asthma (Shijubo et al. 1999). A correlation between lung function (FEV_1/FVC) and serum CC16 levels has been observed in asthmatics (Ye et al. 2004). More recently, de Burbure et al. (2007) found no difference in sputum CC16 levels in individuals with atopic asthma ($n=32$), atopic rhinitis ($n=20$) and non-smoking, non-atopic controls ($n=19$).

Bronchitis obliterans (BO). Low levels of CC16 have been observed in patients with BO after lung transplants, and BO is associated with high mortality after allogeneic stem cell transplantation (Mattson et al. 2005). Mattson et al. (2005) measured serum CC16 levels in eight patients with BO, eight with chronic graft-versus-host disease (GVHD), and eight controls, and observed that low or decreasing levels of serum CC16 were associated with the development of BO. The results suggest that measurements of serum CC16 after stem cell transplantation may be useful as an early marker for BO (Mattson et al. 2005).

Chronic obstructive pulmonary disease (COPD). CC16 in BALF has been reported to be lower in people with COPD compared with controls (Bernard et al. 1992, Gioldassi et al. 2004). Contradictory reports on serum CC16 levels in COPD patients compared with controls have been published, with Ye et al. (2004) finding levels to be similar in the two groups, and Bernard et al. (1992) reporting lower levels in COPD patients than in controls.

Sarcoidosis. CC16 levels were reported to be elevated in serum (Bernard et al. 1992) in individuals with sarcoidosis compared with healthy controls (Shijubo et al. 2000, Ye et al. 2004). However, for BALF, the results are contradictory with one study showing similar CC16 levels (3.6 mg l^{-1} in controls vs. 3.5 mg l^{-1} in patients) (Bernard et al. 1992) and a second study showing higher levels in sarcoidosis patients (Shijubo et al. 2000).

Diabetes and atherosclerosis. Serum CC16 was not significantly different in patients with diabetes and atherosclerosis compared with healthy controls (Nomori et al. 1996).

Research needs and conclusions

Research needs

Many basic questions remain unanswered with respect to the use of serum CC16 as a biomarker of acute or chronic pulmonary damage. Several underlying factors affecting serum CC16 levels are not yet sufficiently characterized to allow the use of serum CC16 as a biomarker of effect. These include the 'normal' variation in serum CC16 in humans as a function of gender, age, BMI, circadian rhythm, ethnicity, temperature, humidity (Barbet et al. 1988, Anderson, 2006), pulmonary infection and exposure to allergens (e.g. pollen, moulds, dander). In addition, exercise is known to affect serum CC16 levels, but the effects of types of exercise conditioning, intensity of physical exercise, and breathing pattern during exercise (e.g. holding breath as in swimming and weight lifting versus extended heavy breathing such as in endurance sports) are not well-defined.

Furthermore, the interactions between the biological and physical processes (i.e. CC16 production, epithelial permeability and/or clearance) that govern transient changes in serum CC16 levels have not been carefully characterized. Research is needed to determine the nature of genotype/phenotype interactions that govern the steady-state levels of serum CC16 and dictate the degree of response to a transient pulmonary challenge. Sufficiently powered studies are also required to address the relationship between steady-state serum CC16 concentrations in healthy persons and those with pulmonary diseases. Such studies need to ascertain the transient changes in serum CC16 following acute exposure to pulmonary irritants in chronic disease status (e.g. asthma, COPD).

Controlled human exposure studies are needed to better understand the dose–response relationship between acute exposure to chemicals, and changes in serum CC16 level before drawing definitive conclusions from the limited published literature. Before either alterations in steady-state serum CC16 concentrations or transient changes in serum CC16 concentrations can be reliably used as a biomarker of a challenge to the pulmonary system, as a biomarker of permanent damage to the lungs, or as a biomarker of exposure to environmental chemicals, investigators should consider factors that impact on lung epithelium permeability and/or Clara cell function [e.g. exercise (Nanson et al. 2001), exposures to tobacco smoke, formaldehyde, acetaldehyde and halogenated hydrocarbons, chloramines, infectious biological factors, dampness and moulds, parent atopy (Hery et al. 1995, Drobnic et al. 1996, Helenius & Haatela 2000, Thickett et al. 2002), and inhalation of *Legionella pneumophila* (Massin et al. 1998)].

Conclusions

Three main processes control serum CC16 levels: (i) CC16 production rates by Clara cells into the alveolar lavage fluid; (ii) rate of diffusion from alveolar lavage fluid into the serum, which is affected by leakiness of the pulmonary epithelial barrier; and (iii) CC16 renal clearance. Combining these factors with the multitude of other factors that can affect serum CC16 levels (both known, but not fully or adequately elucidated, and potentially unknown), at present, the authors believe that CC16 is a protein of limited utility as a single biomarker of effect, at least in uncontrolled epidemiology or survey-type studies.

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