

## Clara cell protein (CC16) in serum and bronchoalveolar lavage fluid of subjects exposed to asbestos

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The Clara cell protein (CC16) is a small and readily diffusible protein of 16 kDa secreted by bronchiolar Clara cells in the distal airspaces. These epithelial cells are altered in several pulmonary pathological processes induced by various lung toxicants. In the search for a new biomarker of asbestos-induced lung impairment, we used a sensitive immunoassay to determine the levels of CC16 in bronchoalveolar fluid (BALF) and serum of subjects exposed to asbestos compared with a group of healthy controls. In the BALF of asbestos-exposed subjects there was an insignificant trend towards CC16 elevation compared with controls, with a (mean  $\pm$  SD of  $0.81 \pm 0.65 \text{ mg l}^{-1}$  for asbestos-exposed subjects ( $n = 23$ ) versus  $0.39 \pm 0.19 \text{ mg l}^{-1}$  for controls ( $n = 11$ ) ( $p = 0.09$ ). In serum, CC16 concentration was significantly increased among asbestos-exposed subjects, with values of  $27.2 \pm 24.0 \mu\text{g l}^{-1}$  for asbestos-exposed subjects ( $n = 34$ ) versus  $16.1 \pm 7.6 \mu\text{g l}^{-1}$  for controls ( $n = 34$ ) ( $p = 0.01$ ). Regarding the effects of smoking, there were significant differences between generally lower CC16 levels in serum and BALF ( $p = 0.05$  and  $0.001$ , respectively) of smokers compared with the higher levels in non-smokers. Serum CC16 levels positively correlated with those in BALF, which is consistent with a diffusional transfer of CC16 from the bronchoalveolar space into the serum. No association, however, emerged between the levels of CC16 in serum or BALF and either the duration of asbestos exposure or the severity of the lung impairment as assessed by chest X-ray. These findings suggest that exposure to asbestos elicits early changes in the local and, importantly, also the systemic levels of CC16. This pneumoprotein therefore appears as a promising non-invasive biomarker of asbestos-induced lung injury and occupational disease in both smoking and non-smoking exposed subjects.

**Keywords:** Clara cell secretory protein (CC16, CC10, CCSP), lung secretory protein, asbestos, exposure, biomarker.

### Introduction

Exposure to asbestos may result in pleural and interstitial pulmonary lesions characterized by inflammation with subsequent fibrosis and possible development of malignancy (Mosman and Gee 1989). It has been long thought that these risks come to effect only after prolonged and high-dose exposure to asbestos (Doll 1955, Navratil 1982). Current data, however, warn that even short and low-dose exposure to asbestos is also harmful because there is little knowledge about the

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threshold limits of exposure in individual subjects (Becklake 1991, Teschler *et al.* 1993).

Despite the continuing decrease in asbestos manufacturing and the parallel improvement of working conditions in the factories, uncertainty persists about the presence of asbestos in the environment (Hurbankova 1999). Hence, there is a need for early indicators of health endangerment and also for markers that could be used to determine occupational risk in individual employees. The applicability of classical imaging and spirometric methods for prognostic purposes is limited; these methods are more suitable for the assessment of already manifested changes and for the evaluation of occupational disease (Navratil 1982, Majurin *et al.* 1994, Schwartz *et al.* 1994). More promising are methodical approaches enabling the characterization of early inflammatory changes, such as the gallium scan (Myslivecek *et al.* 1992), and especially the techniques for evaluating the pathological process directly where it occurs, i.e. in the lung tissue. In this context, examination of the fluid obtained by bronchoalveolar lavage (BAL) is important (Bégin *et al.* 1985, Nair *et al.* 1991, Teschler *et al.* 1996).

There have been several attempts to exploit cellular and humoral parameters determined in bronchoalveolar fluid (BALF) as possible biomarkers of changes induced in the lung by asbestos (Rom and Travis 1992, Hurbankova and Kaiglova 1993, Quinlan *et al.* 1995, Kolek *et al.* 1996). However, parameters such as serum immunoglobulins (Hurbankova and Kaiglova 1993, Trosic and Pisl 1995), CD4+ T lymphocyte count (Gellert *et al.* 1985, Peng and Wang 1993, Kolek *et al.* 1996) or cytokines (Zhang *et al.* 1993, Broser *et al.* 1996, Jagirdar *et al.* 1997) have so far had only marginal relevance for the assessment of asbestos exposure and have not been yet applied to routine examination algorithms (Wagner 1997, Mossman and Churg 1998, Rosenthal *et al.* 1999).

Recently, the respiratory community has expressed interest in the potential diagnostic usage of pneumoproteins (Hermans and Bernard 1998, 1999), i.e. lung epithelium specific proteins leaking from the pulmonary epithelial lining fluid into the systemic circulation, where they may serve as peripheral lung markers. One such promising marker is the Clara cell protein, commonly referred to as CC16 or CCSP, a low molecular weight protein of 16 kDa secreted in large amounts into the lumen of the respiratory tract by non-ciliated bronchiolar Clara cells (Bernard *et al.* 1992, Hermans and Bernard 1996). Following its diffusional transfer into the bloodstream across the air-blood barrier, CC16 is rapidly eliminated by glomerular filtration (Doyle *et al.* 1998). Serum CC16 has been shown to increase in several situations known to be associated with a disruption of the air-blood barrier, such as pulmonary fibrosis (Lesur *et al.* 1995) or lung injury caused by lung irritants such as fire smoke (Bernard *et al.* 1997) or ozone (Broeckaert *et al.* 1999). By contrast, when the barrier is intact or only slightly compromised, the assay for CC16 in serum has been shown to provide an estimate of the number of intact Clara cells. This view was supported by the diminution of CC16 in the serum of subjects who smoked, i.e. in a condition where the number of Clara cells and the level of CC16 secreted into the lung epithelial lining fluid, sampled by BAL, are markedly decreased (Bernard *et al.* 1992, 1994a, Shijubo *et al.* 1997, Hermans *et al.* 1998).

Asbestos exposure may lead to alteration of the distal airspace epithelium (Brody 1985). Moreover, the associated inflammatory and profibrotic reactions may affect the air-blood barrier and increase its permeability to solutes and

proteins (Brody 1985, Rinderknecht *et al.* 1985, Peterson *et al.* 1993). Thus, local production of CC16 in the airways and its diffusion into serum may be altered in asbestosis, which would make CC16 a possible biomarker of asbestos-induced lung pathology. We therefore investigated whether asbestos exposure is associated with changes in CC16 levels in the bronchoalveolar space and/or in the circulation. Further, we have tested the hypothesis that the levels of CC16 might be related to the duration of exposure and to the degree of lung involvement as assessed by imaging methods and cellular immunology markers.

## Methods

### Study subjects

Thirty-four subjects exposed to asbestos were recruited from the registry of the Department of Occupational Medicine, Palacky University Hospital, Olomouc, The Czech Republic. The subjects came from two asbestos-processing factories, both using the same manufacturing technology with cement and water. Approximately 10 000 tons of asbestos were processed locally each year as a mixture of chrysotile (80%), crocidolite (15%) and amosite (5%). The level of fibre dust, measured using the gravimetric method, ranged from 2.5 to 8.3 mg m<sup>-3</sup>. On counting, there were 1.0–1.7 fibres cm<sup>-3</sup>.

The characteristics of the study population are given in table 1. Occupational disease as defined by the International Labour Organisation (ILO) classification was diagnosed in 10 subjects. Malignancy was detected in seven patients; two had mesothelioma, three had epidermoid carcinoma and two had small cell lung cancer. All patients had chest X-ray abnormalities consistent with an interstitial lung process ILO 1/1 and when relevant underwent high resolution computed tomography (HRCT). The exposed subjects were categorized according to the radiological findings as follows: normal chest X-ray appearance (category 1), isolated pleural involvement (category 2), and parenchymal involvement and/or pleural lesions (category 3). The control group consisted of healthy subjects who, at the time of blood collection and lavage procedure, showed no clinical signs of lung inflammation and had no lung disease in their medical history. All the subjects who underwent bronchoalveolar lavage had normal BALF cytology, immunology and microbiology. In all control subjects there was an absence of asbestos exposure.

All subjects provided a sample of venous peripheral blood for the determination of serum CC16. For 23 asbestos-exposed subjects and 11 control subjects, BALF was available for the measurement of CC16 and albumin as well as assessment of the severity of the alveolitis on the basis of the percentage of BALF lymphocytes and the value of the CD4<sup>+</sup>/CD8<sup>+</sup> T lymphocyte ratio. BAL was performed in a standard way by infusing five successive 20 ml aliquots of 0.9% saline that were immediately aspirated by gentle suction. The first aliquot, considered representative of a bronchial wash, was discarded. The four subsequent aliquots were pooled and used for cell population determinations and CC16 and albumin measurements. Approval for the study of human subjects was obtained from the Local Ethics Committee.

Table 1. Characteristics of the study population.

	Asbestos-exposed subjects		Controls	
	Serum ( <i>n</i> = 34)	BALF ( <i>n</i> = 23)	Serum ( <i>n</i> = 34)	BALF ( <i>n</i> = 11)
Males/females	28/6	18/5	10/24	8/3
Age (years)				
Mean ± SD	55.5 ± 1.8	53.0 ± 2.4	32.5 ± 3.7	34.0 ± 5.5
Range	27–68	27–68	18–68	18–68
Non-smokers/smokers	22/12	14/9	16/18	4/7
Exposure: short/long <sup>a</sup>	10/24	10/13	NA	NA
Severity of lung impairment: category 1/2/3 <sup>b</sup>	9/11/14	8/6/9	NA	NA

NA, = not applicable.

<sup>a</sup> Exposed subjects were categorized according to the duration of exposure as follows: short exposure, 5.0 ± 1.3 years (range 3–14 years); long exposure, 24.5 ± 1.1 years (range 15–35 years).

<sup>b</sup> Severity of the lung X-ray findings was categorized as follows: category 1, no specific changes; category 2, isolated pleural involvement; category 3, parenchymal involvement.

### CC16 assay

The concentration of CC16 in serum and BALF supernatant was determined by a sensitive immunoassay relying on the agglutination of latex particles. A detailed description of this immunoassay in its application to urinary CC16 has previously been published (Bernard *et al.* 1991). The assay uses the rabbit anti-protein 1 antibody from Dakopatts (Glostrup, Denmark) and as the standard the protein purified in one of the participant laboratories. To avoid possible interference by complement, rheumatoid factor or chylomicrons, sera were pretreated by heating at 56°C for 30 min and by the addition of polyethylene glycol (16%, 1:1 v/v) and trichloroacetic acid (10%, 1:40 v/v). After overnight precipitation at 4°C, serum samples were centrifuged (3000 g for 10 min) and CC16 was determined in the supernatants. All samples were analysed in duplicate at two different dilutions. The validity and analytical performance of the CC16 latex immunoassay in different biological media have been reported previously (Bernard *et al.* 1991, 1992). Briefly, applied to serum, this assay has a detection limit of 0.5 µg l<sup>-1</sup> and an average analytical recovery of 95%. The within- and between-run coefficients of variation range from 5 to 10%. CC16 concentrations in serum and BAL fluid are in good agreement with those obtained with a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) kit recently developed by Pharmacia (Hermans *et al.* 1998).

### Other assays

BALF cell differentials were determined by counting a minimum of 400 cells in a cytocentrifuge preparation. The surface phenotype of bronchoalveolar lymphocytes was determined by indirect immunofluorescence using anti-CD4 and anti-CD8 monoclonal antibodies (Petrek 1991). Albumin was determined on the BALF supernatant by latex immunoassay, as previously reported, and was expressed per litre of lavage fluid (Bernard and Lauwerys 1983).

### Statistical analysis

All statistical analyses were performed using Statview SE software. All parameters were log-transformed before the application of parametric tests. Differences between groups were evaluated using the unpaired *t*-test or by one- or two-way analysis of variance followed by Fisher's least significance difference multiple comparison test when appropriate. Determinants significantly affecting the concentration of CC16 in serum and BALF were identified by stepwise regression analysis, testing as independent variables age, smoking status (categorized as smoker or non-smoker), and asbestos exposure using different regression models (model 1, presence or absence of exposure; model 2, duration of exposure categorized as none, short or long). Correlations between serum and BALF parameters were evaluated by the Pearson's correlation coefficient. Unless otherwise indicated, values are reported as the arithmetic mean ± SD and/or the geometric mean with range when appropriate. The level of significance was set at  $p < 0.05$ .

## Results

CC16 was significantly increased in the serum of asbestos-exposed subjects (figure 1 and table 2). Of the factors tested in stepwise regression analysis, only smoking status and asbestos exposure (model 1) emerged as significant contributors to CC16 changes in serum (partial  $r^2 = 0.19$  and  $0.054$ , respectively;  $p < 0.05$ ). No effect of the duration of exposure (model 2) on CC16 level in serum was found. There were also no differences in the serum CC16 levels between the subjects with different degrees of lung impairment as assessed by the severity of the chest X-ray and HRCT abnormalities (category 1,  $31.0 \pm 16.0 \mu\text{g l}^{-1}$ ; category 2,  $25.4 \pm 16.4 \mu\text{g l}^{-1}$ ; category 3,  $26.9 \pm 34.0 \mu\text{g l}^{-1}$ ).

None of the variables tested by stepwise regression analysis had any effect on the concentration of CC16 in BALF, although BALF levels of CC16 and albumin showed a tendency to increase in asbestos-exposed subjects compared with controls (table 3). Similarly to serum CC16, no clear relationship with length of exposure or with the degree of lung abnormalities could be detected for BALF CC16. As in serum, smoking status affected the level of CC16 in BALF, with values of  $0.52 \pm 0.50 \text{ mg l}^{-1}$  for smokers ( $n = 13$ ) and  $0.77 \pm 0.61 \text{ mg l}^{-1}$  for non-smokers ( $n = 21$ ) ( $p = 0.05$ ) when the groups of exposed and control subjects were mixed together. A positive correlation emerged between serum and BALF CC16

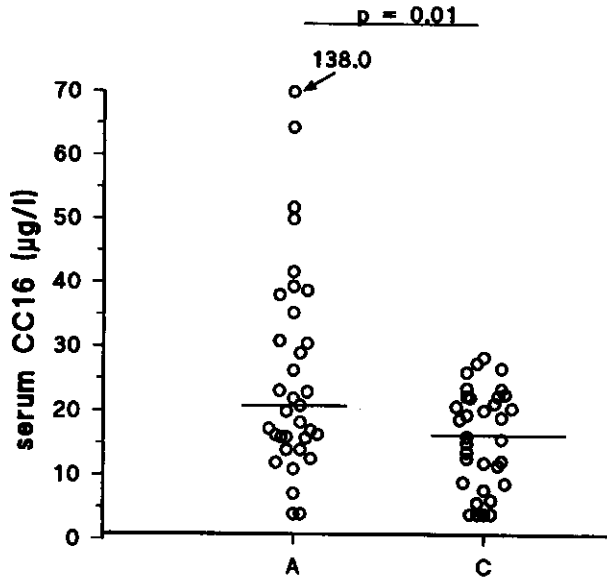


Figure 1. Serum levels of Clara cell secretory protein (CC16) in asbestos-exposed subjects (A,  $n = 34$ ) compared with CC16 serum levels in healthy control subjects (C,  $n = 34$ ). Lines represent median values.

Table 2. Serum CC16 values (in  $\mu\text{g l}^{-1}$ ) according to asbestos exposure and smoking habits.

	Controls	Asbestos-exposed subjects	Whole population	<i>p</i> value
<b>Smokers</b>				
<i>n</i>	18	12	30	
Mean $\pm$ SD	13.1 $\pm$ 8.0	18.1 $\pm$ 10.3	15.1 $\pm$ 9.11	
Geometric mean (range)	10.6 (4.0–27.5)	14.9 (4.0–38.8)	12.2 (4.0–38.8)	
<b>Non-smokers</b>				
<i>n</i>	16	22	38	
Mean $\pm$ SD	19.5 $\pm$ 5.8	32.2 $\pm$ 25.6	26.9 $\pm$ 22.2	
Geometric mean (range)	18.6 (8.8–28.4)	25.6 (7.2–138.3)	22.4 (7.2–138.3)	
<b>Whole population</b>				0.037
<i>n</i>	34	34	68	
Mean $\pm$ SD	16.1 $\pm$ 7.6	27.2 $\pm$ 24.0		
Geometric mean (range)	13.8 (4.0–28.4)	21.2 (4.0–138.3)		
<i>p</i> value				0.0007

Differences were assessed by two-way analysis of variance.

( $n = 34$ ,  $r = 0.51$ ;  $p = 0.02$ ). However, no correlation was found between serum CC16 and albumin BALF levels nor between CC16 levels and the CD4+/CD8+ T lymphocyte ratio.

## Discussion

In this study we have investigated the applicability of the Clara cell protein (CC16) as a biomarker of pathological changes induced in the lung and airways by

Table 3. Levels of CC16 and albumin in BALF according to asbestos exposure.

	Controls ( $n = 11$ )	Asbestos-exposed subjects ( $n = 23$ )	$p$ value
CC16 ( $\text{mg l}^{-1}$ )			
Mean $\pm$ SD	0.39 $\pm$ 0.19	0.81 $\pm$ 0.65	0.087
Geometric mean (range)	0.34 (0.11–0.69)	0.60 (0.07–2.72)	
Albumin ( $\text{mg l}^{-1}$ )			
Mean $\pm$ SD	23.7 $\pm$ 9.7	33.4 $\pm$ 17.0	0.082
Geometric mean (range)	22.0 (10.6–45.9)	29.7 (13.6–71.0)	
CC16/albumin ratio (%)			
Mean $\pm$ SD	1.7 $\pm$ 0.7	2.7 $\pm$ 2.4	0.42
Geometric mean (range)	1.5 (0.5–2.9)	1.9 (0.2–11.3)	

Differences between categories were assessed by analysis of variance followed by Dunnett's multiple comparison test.

the effects of asbestos fibres. We determined serum and BALF levels of CC16 in a group of well-characterized subjects exposed to asbestos and in a group of healthy control subjects. Using stepwise regression analysis, we have shown that asbestos exposure elicits a significant elevation of serum CC16 and cigarette smoking has a negative effect on CC16 levels in asbestos-exposed subjects.

Our results show that measurement of CC16 in serum yields more information than its determination at the surface of the respiratory tract in BALF. In individual patients, the serum and BALF levels of CC16 were related, but the increase in the pneumoprotein in BALF was less pronounced than in the serum. With regard to the possible use of CC16 as a biomarker of asbestos exposure, it would therefore be preferable to measure its levels in serum rather than in BALF.

The potential usefulness of serum CC16 as marker of chronic injury induced in human lung by different toxicants such as tobacco smoke (Bernard *et al.* 1997) and silica (Bernard *et al.* 1994b) has already been demonstrated. In contrast to the CC16 elevation seen in our asbestos-exposed subjects, a significant decrease in serum CC16 has been reported in workers exposed to silica dust (Bernard *et al.* 1994b). In the case of silica exposure the decrease in CC16 was ascribed to damage to the secretory cells in the bronchiolar tree, but the mechanisms accounting for the elevation in serum CC16 after asbestos exposure appear to be different. First, there is a difference between the site of action of silica and that of asbestos (Wagner 1997). Silica is deposited in the most distal airspace of the lung, damaging mainly alveolar lining cells. Asbestos, by contrast, acts more on the distal airway epithelial cells of the bronchi, which, as shown experimentally, may be stimulated to proliferate even after asbestos exposure (Quinlan *et al.* 1995). It is also possible that epithelial secretory cells are stimulated to produce and/or release CC16 by proinflammatory mediators such as cytokines, which may be induced in BAL cells by asbestos fibres or locally released by stimulated macrophages (Zhang *et al.* 1993, Broser *et al.* 1996). In addition to increased synthesis/release by pulmonary epithelial cells, we found that asbestos exposure is associated with increased intravascular leakage of CC16, which results from damage to and thus enhanced permeability of the broncho/alveolar blood barrier to lung and plasma proteins such as albumin. The conjunction of these two processes probably accounts for the observation that the elevation of CC16 is more apparent in serum than in BAL fluid.

The negative effect of cigarette smoking on CC16 levels found in our study has been repeatedly reported in different populations (Bernard *et al.* 1992, Shijubo *et al.* 1997). Reduction of Clara cell number in the airways of smokers, with an ensuing reduction in CC16 synthesis and CC16 transferable pool size, is considered to be responsible for the lower levels of CC16 in the bronchoalveolar space and blood of smokers compared to non-smokers (Bernard *et al.* 1992, 1994b, Hermans *et al.* 1998). CC16 is supposed to have an immunosuppressive and anti-inflammatory role (Lesur *et al.* 1995, Singh and Katyal 1997). In this context, the lower availability of CC16 in the airways of smokers probably increases their susceptibility to alveolar inflammation and might, therefore, be considered as an unfavourable prognostic factor. The conclusion about the negative contribution of smoking to the development of asbestosis via the CC16 pathway is in good agreement with the previous findings of Lesur *et al.* (1996), who reported decreased values of BALF CC16 in smokers compared with non-smokers. It is also in agreement with our own observation that in asbestos-exposed subjects, smoking substantially contributes to the development of chest X-ray detectable lung impairment (Kolek *et al.* 1996): in asbestos-exposed subjects, with smoking habits, there was an altered cellular immune reactivity (low CD4<sup>+</sup>/CD8<sup>+</sup> ratio) that was associated with earlier appearance of radiologically detectable changes (Kolek *et al.* 1996). Whatever the mechanisms of its adverse effects, smoking must be considered as a negative risk factor for the acceleration of asbestos-induced pulmonary pathology in exposed subjects.

The study of Lesur *et al.* (1996) is to our knowledge the only one that has dealt with pneumoproteins in asbestos-induced lung disease and addressed the possible exploitation of CC16 as well as surfactant-associated protein A as parameters for the follow-up of asbestos-exposed subjects. Both this study and our own study have reported increased levels of CC16 and the negative effect of smoking on pneumoprotein levels in asbestos-exposed subjects. Lesur *et al.* (1996) reported a more marked increase in CC16 in BALF than we did, which may be explained by the lower number of patients and control subjects in whom BALF was available in our study.

Practically, it was important to analyse whether CC16 levels are affected by the severity of the disease estimated on the basis of the imaging abnormalities or the duration of exposure. In this context it is interesting to note that, as in the study by Lesur *et al.* (1996), in our study elevated CC16 levels were early manifestations detectable after short exposure, which subsequently remained unchanged during the clinical course of disease. These findings indicate that early exposure to asbestos induces changes in CC16 persisting throughout the disease process. These observations also suggest that CC16 is probably a sensitive tool allowing the detection of early damage, but is not as suitable to follow the course of disease once it is established.

In this study we have investigated the effect of asbestos on CC16 using the qualitative (presence/absence of exposure) and semiquantitative (short/long) expressions of exposure. An alternative approach to explore the effect of asbestos on CC16 would be to use cumulative exposure, i.e. expressed as fibre-years (Green *et al.* 1997). Usage of this quantified variable may bring more information on the dose-response relationship between asbestos and CC16. We are aware that by limiting ourselves to the assessment of the presence or absence of exposure, a part of this important information could be missed. Unfortunately in our study we were

not able to trace all exposure data due to organizational changes in the Czech public health service and, therefore, more detailed investigation of the relationship between quantified asbestos exposure and CC16 remains to be addressed by a new study.

In conclusion, asbestos exposure is associated with an elevation of the pneumoprotein CC16 that can be readily detected in serum. This elevation occurs irrespective of smoking habits, although the CC16 baseline values are lower in smokers compared with non-smokers due to an adverse effect of smoking on Clara cells. This increase in CC16 in serum appears early after exposure and precedes the development of radiological pleural and parenchymal changes. Therefore, assay of CC16 appears to be a useful tool to detect early alterations in the lung and airways induced by asbestos.

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