Evaluation of serum CC-16 as a biomarker for chronic obstructive pulmonary disease in the ECLIPSE cohort

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Abstract

Circulating levels of CC-16 have been linked to Clara cell toxicity and so this protein has been suggested as a marker of COPD. Serum CC-16 was measured in 2083 individuals aged 40–75 years with COPD and a smoking history of ≥10 pack years, 332 controls with a smoking history ≥10 pack years and normal lung function and 237 non-smoking controls. Serum CC-16 had a coefficient of repeatability of 2.90 over 3 months in a pilot study of 267 individuals. The median level of serum CC-16 was significantly reduced in a replication group of 1888 current and former smokers with COPD when compared to 296 current and former smokers without airflow obstruction (4.9 and 5.6 ng/mL; p<0.001) and 201 non-smokers (6.4 ng/mL; p<0.001). Serum levels of CC-16 were lower in current rather than former smokers with GOLD stage II and III COPD but were not different in individuals with stage IV disease. Former, but not current smokers, with COPD had lower serum CC-16 with increasing severity of COPD (GOLD II vs GOLD IV COPD; 5.5 and 5.0 ng/mL p=0.006; r=0.11 with FEV1, p<0.001) and had significantly higher levels if they also had reversible airflow obstruction (p=0.034). Serum CC-16 was affected by gender and age (r=0.35; p<0.001) in subjects with COPD but not by BMI or the presence of either chronic bronchitis or emphysema. These data show that serum CC-16 is reduced in individuals with COPD and that there is a weak correlation with disease severity in former smokers.

(Word count 250)
Introduction

Chronic obstructive pulmonary disease (COPD) was responsible for 3 million deaths in 2005 and is predicted to become the third leading cause of death by 2020. It is a major cause of morbidity that is estimated to affect 210 million people worldwide. The development of disease is intimately associated with the inhalation of noxious agents and in particular cigarette smoke. This causes airways disease and emphysema that result in progressive, irreversible airflow obstruction. Despite its prevalence, there are no biomarkers that can distinguish current and former smokers with and without airflow obstruction and which can be used to monitor the response to therapeutic intervention. An ideal biomarker should be lung-specific, reproducible, easy to assess in large numbers of patients and validated in a large well characterised cohort of patients and controls.

Clara cell secretory protein-16 (CC-16, CC-10 or uteroglobin) is a member of the secretoglobin family of small, secreted, disulphide-bridged dimeric proteins. It is secreted by the non-ciliated Clara cells, which are found predominantly in the respiratory bronchioles, and by non-ciliated columnar cells of the large and small airways. CC-16 is also expressed in the epithelial cells of the nose and the male and female urogenital tract. Despite being produced by other tissues, serum levels of CC-16 largely reflect protein produced by the lower respiratory tract with little contribution from protein released by the urogenital organs. Thus circulating levels of CC-16 have been suggested as a marker of respiratory disease.

CC-16 acts as an immunosuppressant and provides protection against oxidative stress and carcinogenesis. Serum levels rise following acute exposure to smoke, chlorine and lipopolysaccharide. They also rise in response to ozone but can be suppressed by inhaled fluticasone propionate. The serum level of CC-16 is low in subjects with obliterative bronchiolitis, asthma and in smokers. It is reduced in the lungs of smokers and individuals with COPD. There are conflicting data on the serum level of CC-16 in COPD but this has yet to be assessed in a large cohort of well characterised individuals. We have used the ECLISPE cohort to evaluate serum CC-16 as a potential biomarker for COPD.
Methods

The ECLIPSE cohort (SCO104960, Clinicaltrials.gov identifier NCT00292552, appendix 1).

The aims and operational aspects of the ECLIPSE cohort have been described elsewhere. Briefly, ECLIPSE is a 3 year multicentre longitudinal prospective study to identify novel endpoints in COPD. Individuals aged 40–75 years were recruited to the study if they had a smoking history of ≥10 pack-years, a post-bronchodilator ratio between forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) ≤0.7 and GOLD stage II (FEV₁ 50-80% predicted), III (FEV₁ 30-50% predicted) or IV (FEV₁ <30% predicted) COPD. Smoking (≥10 pack-years) and non-smoking (<1 pack-year) control subjects were enrolled if they were aged 40–75 years and had normal lung function (post-bronchodilator FEV₁>85% predicted and FEV₁/FVC >0.7). Individuals recruited to the study were genotyped for α₁-antitrypsin deficiency. Six PiZZ and 11 PiSZ individuals were identified and excluded from the analysis. All subjects underwent standardised spirometry following 180 mcg (2 puffs) of salbutamol with reversible airflow obstruction being defined as an increase in FEV₁ of 15% and at least 200 mL. All subjects were offered a low dose computed tomography (CT) scan of the chest to exclude non-COPD related disease and to evaluate the severity and distribution of emphysema. The CT scans were evaluated at the central imaging unit at the University of British Columbia in Vancouver. The extent of emphysema was assessed in two ways. Firstly it was independently scored by two radiologists who were blind to the individual’s lung function. Emphysema was reported as trivial, mild, moderate, severe and very severe if it affected <5%, 5-25%, 25-50%, 50-75% and >75% of the lungs respectively. A consensus reading was obtained when there was a difference of more than 1 emphysema category between the 2 observers. Otherwise the average of the 2 readings was used in the analysis. Secondly emphysema was assessed by the percentage of the lung with attenuation below -950 HU using the Pulmonary Workstation 2.0 software (VIDA Diagnostics, Inc., Iowa City, IA).

Measurement of serum CC-16

Whole blood was collected into vacutainer tubes at the beginning of the study. Serum was prepared by centrifugation at 1500g for 10-15 minutes. The serum was collected and stored at -80 °C until analyzed. Serum CC-16 was measured by operators who were blind to an individual’s lung disease using a colorimetric sandwich immunoassay method (BioVendor GmbH, Heidelberg, Germany) according to the manufacturer’s instructions. Serum samples were diluted 5- to 20-fold with the dilution buffer supplied by the manufacturer. The concentration of CC-16 was determined by comparison with a standard curve prepared with known concentrations of CC-16. The assay had a validated range of 80 pg/mL to 4000 pg/mL with an intra-assay co-efficient of variation and relative error of 1.67 to 4.10% and -9.15 to -2.01%, respectively and an inter-assay co-efficient of variation and relative error of 3.75 to 4.85% and -11.96 to -1.08%, respectively.
Statistical analysis

Reproducibility of CC-16 in the ECLIPSE cohort was assessed through Bland-Altman plots. Due to the non-normality of CC-16 values identified by Shapiro-Wilk and Kolmogorov-Smirnov tests, all CC-16 values in the ECLIPSE cohort were log-transformed prior to analysis. All comparisons between subject groups were then conducted by analysis of variance (ANOVA) based on the log-transformed values. Spearman correlation coefficients (based on ranks) were calculated for correlations between CC-16 and clinical parameters. All analyses were performed with SAS® Version 8.2 (SAS Institute, Cary, NC).

Ethics

The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines and were approved by relevant ethics and institutional review boards at the participating centres.
Results

Pilot study to test the reproducibility of serum CC-16

It is important to assess whether a putative biomarker is reproducible over time. Serum CC-16 was measured in an age-matched subgroup of 195 former smokers with COPD, 36 former smoking controls and 36 non-smoking controls selected from the ECLIPSE cohort (Table 1). It gave reproducible values in non-smokers, smokers without airflow obstruction and across all severities of COPD when measured over a period of 3 months (Fig. 1 and Table 1; coefficient of repeatability 2.90, variability 15.8%). The results for serum CC-16 were within the 95% limits of agreement for most of the subjects tested. However several subjects showed a high degree of variability between the values at baseline and at three months. The reason for this was unclear. There was no reproducible difference between median levels of CC-16 in former smokers with and without COPD in these pilot samples. The median level of CC-16 was significantly lower in COPD subjects when compared to non-smoking controls (p=0.005).

Table 1. Pilot cohort to assess the stability of serum CC-16 as a biomarker for COPD. The lung function measurements are following the administration of 180 mcg of salbutamol. All the smoking controls and individuals with COPD were former smokers. CT scans is the number of CT scans available for qualitative analysis to assess the percentage of the lungs with a density of less than -950 HU. All values are number or mean and standard deviation (in brackets) unless otherwise stated. †p values for difference between COPD subjects and smoker controls. All the parameters measured were significantly different between individuals with COPD and non-smoker controls (p≤0.005).
Assessment of serum CC-16 in individuals with COPD

Serum CC-16 was then measured in a larger replication set of 1888 individuals with COPD, 296 smoking controls with no airflow obstruction and 201 non-smoking controls (Table 2 and Fig. 2a). The median serum CC-16 was significantly lower for current and former smokers with COPD when compared to current and former smokers with no airflow obstruction (4.9 and 5.6 ng/mL respectively; p<0.001). Median CC-16 was significantly lower in current and former smokers with no airflow obstruction than in non-smoking controls (5.6 and 6.4 ng/mL; p=0.005). However there was no significant difference in serum CC-16 with increasing severity (GOLD stage) COPD when analysed in this mixed group of current and former smokers.

<table>
<thead>
<tr>
<th>COPD subjects</th>
<th>Smoker controls</th>
<th>Non-smoker controls</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1888</td>
<td>296</td>
<td>201</td>
</tr>
<tr>
<td>Age</td>
<td>63.4 (7.2)</td>
<td>54.7 (8.9)</td>
<td>53.2 (8.6)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>1222 (65)</td>
<td>161 (54)</td>
<td>74 (37)</td>
</tr>
<tr>
<td>Smoking history, pack-years</td>
<td>49.2 (27.3)</td>
<td>32.0 (22.1)</td>
<td>0.4 (0.5)</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>746 (40)</td>
<td>201 (68)</td>
<td>0</td>
</tr>
<tr>
<td>FEV(_1) (L)</td>
<td>1.4 (0.5)</td>
<td>3.4 (0.8)</td>
<td>3.3 (0.8)</td>
</tr>
<tr>
<td>FEV(_1) % pred</td>
<td>48.7 (15.5)</td>
<td>108.6 (12.1)</td>
<td>114.8 (14.0)</td>
</tr>
<tr>
<td>FEV(_1)/FVC</td>
<td>0.45 (0.11)</td>
<td>0.79 (0.05)</td>
<td>0.81 (0.05)</td>
</tr>
<tr>
<td>CT scans (n)</td>
<td>1496</td>
<td>260</td>
<td>165</td>
</tr>
<tr>
<td>% Low attenuation area (&lt;-950 HU)</td>
<td>16.9 (11.8)</td>
<td>2.2 (2.9)</td>
<td>3.9 (4.0)</td>
</tr>
<tr>
<td>CC-16 (ng/mL)</td>
<td>4.9 (3.4)</td>
<td>5.6 (3.1)</td>
<td>6.4 (3.1)</td>
</tr>
</tbody>
</table>

Table 2. Replication cohort to assess the ability of serum CC-16 to discriminate between individuals with and without COPD. The lung function measurements are following the administration of 180 mcg of salbutamol. CT scans is the number of CT scans available for qualitative analysis to assess the percentage of the lungs with a density of less than -950 HU. All values are numbers or mean and standard deviation (in brackets) unless otherwise stated. †p values for difference between COPD subjects and smoker controls. All the parameters measured were significantly different between individuals with COPD and non-smoker controls (p<0.001).

Serum CC-16 was higher in males than females with COPD (5.21 and 4.30 ng/mL; n=1222 and 666 respectively; p<0.001) and in smoker controls (6.09 and 4.66 ng/mL; n=161 and 135; p<0.001) but it was similar in men and women who were non-smokers (6.88 and 6.25 ng/mL; n=74 and n=127; p=0.385). There was a significant correlation between CC-16 and age (r=0.35; p<0.001) but serum CC-16 was not affected by BMI or the presence of either chronic bronchitis or emphysema (whether scored by the radiologist or analysis of lung density). There was a small but significant difference in serum CC-16 levels in those who did, compared to those who did not, take long acting β\(_2\)-agonists (serum CC-16 4.81 and 5.07 ng/mL; n=1280 and 608, for those who did and
did not take long acting β₂-agonists respectively; p=0.047) but no difference in serum levels of CC-16 in individuals taking inhaled corticosteroids (4.81 and 5.08 ng/mL; n=1346 and 542, for those who did and did not take inhaled corticosteroids respectively; p=0.135). Median levels of serum CC-16 were lower in current smokers than former-smokers both with (4.2 and 5.4 ng/mL; n=746 and 1142 respectively; p<0.001) and without (5.3 and 6.3 ng/mL; n=201 and 95; p<0.001) COPD. The mean pack-years smoked were 48.7 and 49.9 for former and current smokers with COPD and 31.2 and 32.4 for former and current smokers with no airflow obstruction.

Our data show a clear effect of current smoking on serum levels of CC-16. This biomarker was therefore analysed in groups divided into current and former smokers (Fig. 2b). Median serum levels of CC-16 were lower in current rather than former smokers in individuals with stage II and III COPD, but not stage IV disease. The serum levels of CC-16 were reduced in individuals with stage IV COPD irrespective of whether they were current or former smokers. There was a weak correlation between serum CC-16 with severity of COPD in former smokers (GOLD II vs GOLD IV; 5.5 and 5.0 ng/mL p=0.006; r=0.11 with FEV₁, p<0.001) but not in current smokers (GOLD II vs GOLD IV; 4.1 and 4.5 ng/mL p=0.046; r=-0.07 with FEV₁, p=0.069).

It is clear that age, gender, smoking status and lung function can all affect serum levels of CC-16. However if we adjust for these factors there is still a significant difference in median levels of serum CC-16 between individuals with COPD and the group of smoker controls (adjusted means 4.5 and 6.1 ng/mL respectively, p<0.001).

Assessment of serum CC-16 as a biomarker for reversible airflow obstruction

There was no correlation between serum CC-16 and the presence of reversible airflow obstruction (increase in FEV₁ of 15% and at least 200 mL following 180 mcg of salbutamol) in current or former smokers with COPD (CC-16 5.2 ng/mL in individuals with reversible airflow obstruction (n=393) and 4.8 ng/mL in those with no reversible airflow obstruction (n=1495)). However former smokers with COPD had a significantly higher serum CC-16 if they also had reversible airflow obstruction (6.0 and 5.2 ng/mL for former smokers with (n=222) and without (n=920) reversible airflow obstruction respectively; p=0.034). There was no effect of age but there was an effect of gender with men having higher levels of serum CC-16 if they also met the criteria for reversible airflow obstruction (5.6 and 5.1 ng/mL for men with (n=297) and without (n=925) reversible airflow obstruction respectively; p=0.046). There was no correlation between serum CC-16 and the magnitude of increase in FEV₁ following the administration of 180 mcg of salbutamol in individuals with COPD (r=0.04, p=0.062).

Discussion

Serum CC-16 is a lung derived protein that has been proposed as a biomarker for epithelial cell dysfunction. COPD is characterised by both airways disease and emphysema and thus CC-16 is an obvious candidate for assessment as a marker for this condition. It is important that any putative biomarker is stable when assessed over a short period of time. This was evaluated in a subset of 267 individuals who were either non- or former smokers. Serum levels of CC-16 had a coefficient of repeatability of 2.90 when assessed over a period of 3 months. This reflects 15.8% variability in CC-16 from
baseline measurements. Thus serum CC-16 is relatively stable and therefore worthy of further assessment as a biomarker for COPD.

Serum CC-16 was evaluated in the rest of the ECLIPSE cohort to determine whether it could distinguish individuals with COPD from current and former smokers with normal lung function. Serum CC-16 was significantly lower in individuals with COPD when compared to individuals who smoked but had normal lung function. CC-16 was lower in smoking controls than in non-smokers. However these results should be tempered by the differences in the demographics of the three groups. Individuals with COPD were significantly older, were more likely to be male than the smoker and non-smoker controls and had a greater pack-year smoking history. As a result of the recruitment criteria they had worse lung function, more emphysema on their CT scans and were more likely to be taking inhaled medication for airways disease. Thus it is important to evaluate the effect of each of these parameters on serum CC-16.

The large number of individuals with COPD who were recruited to the study made it possible to assess the effect of confounding factors on serum levels of CC-16. Serum levels of CC-16 increased with age in individuals with COPD. This was keeping with the findings of others and has been attributed to a combination of age-related-decline in glomerular filtration rate and hence reduced clearance of CC-16, and increased alveolar capillary leak. These pathologies are likely to be enhanced in individuals with COPD. Serum CC-16 was higher in men than women who were current and former smokers both with and without COPD. There was no gender effect on serum CC-16 in those individuals who were non-smokers. The absence of a gender effect is in keeping with previous reports but there has been no assessment of the relationship between gender and levels of CC-16 in individuals with COPD. There are conflicting data on the relationship between serum CC-16 and BMI, however no correlation was identified in individuals with COPD in the ECLIPSE cohort.

Smoking status has been shown previously to have a significant effect on serum CC-16. Indeed serum CC-16 was lower in current than former smokers irrespective of whether or not they had COPD. This is likely to reflect increased epithelial dysfunction as a result of toxins within cigarette smoke. However it was striking that individuals with GOLD stage IV COPD had similar levels of serum CC-16 irrespective of smoking status. This implies that epithelial damage cannot be reversed by smoking cessation in those individuals with the most severe airflow obstruction. Moreover serum CC-16 may be a useful biomarker of therapeutic strategies to repair epithelial damage in this group of individuals.

It seems likely that a biomarker for COPD will show a trend with increasing disease. This was most apparent in former smokers in whom there were significantly lower levels of serum CC-16 in individuals with GOLD stage IV disease than in those with GOLD stage II COPD. Moreover there was a weak correlation between serum CC-16 and FEV1 in this group of individuals. The relationship between serum CC-16 and disease was less clear in current smokers in whom serum CC-16 was higher in those with more severe disease (as defined by GOLD severity) but there was no correlation between serum CC-16 and FEV1. The opposing effects of levels of serum CC-16 and FEV1 in current and former smokers explains the lack of correlation between serum CC-16 and GOLD stage of COPD in the combined group of former and current smokers with COPD. It is clear that age, gender, smoking status and lung function can all affect serum levels of CC-16.
Indeed this would explain the higher serum level of CC-16 in subjects with COPD in the pilot study of reproducibility than in COPD subjects in the replication cohort. However if we adjust for all of these factors then serum CC-16 is still significantly lower in individuals with COPD than in the group of smoker controls.

It is important to determine whether the association of serum CC-16 with COPD is driven by the airways or emphysema components of the disease. There was no correlation between the presence or severity of emphysema and serum CC-16 in individuals with COPD. This was irrespective of whether emphysema was scored by a radiologist or by density mask imaging. Serum CC-16 has been proposed as a marker of airway epithelial dysfunction and so it may correlate with reversible airways disease. However there was no correlation between serum CC-16 and symptoms of chronic bronchitis and no difference in serum CC-16 in individuals taking inhaled corticosteroids when compared to individuals taking other medications. However the serum level of CC-16 was reduced in individuals with COPD receiving long acting β₂-agonists and elevated in men than women, and former rather than current smokers, with reversible airflow obstruction. Although the effects are modest the level of CC-16 in the serum may in part reflect reversible airflow obstruction.

Our data demonstrate that serum CC-16 is reduced in individuals with COPD and that there is a weak correlation with disease severity in former smokers. There is also a signal from CC-16 in former smokers with reversible airflow obstruction. Serum CC-16 did not correlate with lung function or reversible airflow obstruction in current smokers. It is important to consider the utility of CC-16 as a biomarker for COPD. The difference between the median levels of serum CC-16 in individuals with COPD, whilst statistically significant, is small. Moreover the variation between samples is 15.8%. Thus CC-16 cannot be used to screen populations of individuals for COPD and cannot be used as a surrogate for lung function testing to evaluate the presence of reversible airflow obstruction. However there is clearly a signal from CC-16 in individuals with COPD over and above that caused by smoking. Moreover the levels remain low in former smokers with GOLD stage IV COPD. Thus serum CC-16 may be useful in longitudinal studies to assess epithelial repair or may be combined with other biomarkers to diagnose or monitor the progression of COPD. Any assay based on serum CC-16 will need to correct for age, gender, smoking status and lung function.

The association of serum CC-16 with COPD reported here is from a cross sectional study of individuals with COPD. It is clearly important to assess whether CC-16 tracks with decline in lung function and progression of emphysema, airways disease, systemic features (such as BMI, fatigue, muscle wasting, systemic inflammation) and exacerbations in individuals with COPD during the 3 years of follow-up of the ECLIPSE cohort. These investigations are required to determine whether serum CC-16 can report disease progression. Finally it is important to recognise that changes in serum CC-16 are not specific to one agent, disease state or specific exposure. Thus serum CC-16 is likely to be affected by other forms of lung disease.
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Figure legends

Fig. 1. Bland-Altman plot to show the reproducibility of serum levels of CC-16. The lines represent 95% limits of agreement. Bias is -0.145 and coefficient of repeatability is 2.90.

Fig. 2. Measurement of serum CC-16 in the replication cohort of non-smokers and smokers with and without COPD. Fig. 2a. The number of individuals in each group is shown in brackets. Fig. 2b. Effect of current smoking on SP-D levels in the replication cohort. The number of individuals in each group is the same as in Fig. 2a. The number of current smokers in each group was: smoking controls 201, all COPD individuals 746, COPD GOLD stage II 344, GOLD stage III 324 and GOLD stage IV 76. The bars are median and interquartile range.
References


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Figure 1
Figure 2a

Non-smoker Controls (n=201)

Smoker Controls (n=296)

COPD Subjects (n=1888)

COPD GOLD II (n=846)

COPD GOLD III (n=811)

COPD GOLD IV (n=229)

Serum CC-16 (ng/ml)
Figure 2b

Non-smoker Controls (n=201)

Smoker Controls (n=296)

COPD Subjects (n=1888)

COPD GOLD II (n=846)

COPD GOLD III (n=811)

COPD GOLD IV (n=229)

Serum CC-16 (ng/ml)

- Total
- Current smoker
- Former smoker

p < 0.001

p < 0.001

p < 0.001

p = 0.184