Chronic *Chlamydia pneumoniae* infection is a risk factor for the development of COPD

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**Summary**

Smoking is the major risk factor for the development of Chronic Obstructive Pulmonary Disease (COPD), but epidemiological data suggest that other etiological factors may also be involved. *Chlamydia pneumoniae* (Cpn) is an established cause of acute and chronic upper and lower respiratory tract infections. Data obtained from in vitro and in vivo studies indicate that Cpn infection can be involved in the development of both small airways disease and emphysema, the two major components of COPD. The aim of this study was to investigate the possible association between chronic Cpn infection and COPD.

The study population was comprised of 199 consecutive patients who underwent bronchoscopy due to longstanding airway symptoms and for whom spirometry and serum samples for serology were available.

Acute and convalescent sera were analysed for specific IgG and IgA Cpn antibodies using microimmunofluorescence. Chronic Cpn infection, defined as persistent elevated titres of IgA $\geq$ 1/64, was present in 85 patients. Chronic infection was associated with smoking and higher age, but no gender difference was observed. Thirty patients had COPD, defined as FEV$_1$/FVC $<70\%$ without any features of asthma. Patients with COPD were older than those without, and there was no association with gender in this group. A statistically significant association, remaining after correction for smoking, was observed between chronic Cpn infection and COPD, and there was a trend for decreasing lung function with increasing antibody titres. The results suggest that chronic Cpn infection may be an independent risk factor for the development of COPD.

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**Introduction**

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of morbidity and mortality world-wide. It is well known that there is a strong relationship between smoking and COPD,
with 10–20% of smokers developing COPD. However, lifetime non smokers can also be at risk for developing COPD, although the progression is not usually as rapid as in smokers. The increased rate of lung function deterioration seen in smoking COPD patients often levels off to that of non-smokers after smoking cessation, although some patients have a continuous rapid decline despite smoking cessation. These inconsistent findings regarding the association between smoking and declining lung function indicate that risk factors other than smoking may be of importance for the development of COPD. Chronic infections with adenovirus or *Chlamydia pneumoniae* (Cpn) have been discussed as possible additional risk factors associated with the development of COPD. Cpn is an intracellular bacterium associated with acute respiratory diseases and has a tendency to cause chronic infections. This could trigger the development of COPD by initiating release of cytokines and chemokines and thereby sustaining an inflammatory response.

The aim of this study was to evaluate the prevalence of chronic Cpn infection in patients with longstanding airway symptoms, and to correlate serological findings with COPD, smoking habits and gender. Consecutive patients referred for bronchoscopy were investigated with lung function tests and by serology for the assessment of chronic Cpn infection.

**Materials and methods**

**Patients**

In a prospective study performed February 1 1997–February 28 1999, patients referred to the Department of Respiratory Medicine, County Hospital of Gävleborg, Gävle and underwent bronchoscopy were evaluated regarding chronic Cpn infection. The study base for this analysis consists of 403 consecutive patients without lung cancer, but with long-standing airway symptoms and/or pathological chest X-rays. Results from patients with lung cancer have previously been described. All patients gave an approval to participate after oral and written information, and the Ethics Committee, Faculty of Medicine, Uppsala University approved the study.

Spirometry was not available in 70 subjects, and 125 were excluded due to abnormal chest X-ray findings that could influence the lung function (e.g. sarcoidosis, status post tuberculosis, pulmonary fibrosis, pneumonia, heart failure). This exclusion was done without knowledge of the actual lung function or antibody titres. Nine additional patients were excluded either because serology specimens were not collected before bronchoscopy (one), or convalescent sera were missing (eight). 199 subjects (110 females) remained for analysis. At the bronchoscopy no intrabronchial findings that could explain a reduction in lung function was observed in any of the patients.

Blood specimens were collected for serology and lung function tests were performed the day before bronchoscopy. Convalescent serum was obtained 32–331 (median 97) days later (50–150 days after the acute serum for 95% of the patients). During the study period no Cpn epidemic occurred in the county. Throat swabs as well as samples taken from the bronchial mucosa with a cytobrush were analysed for Cpn using polymerase chain reaction (PCR) on samples taken during the first year. During the second year immunohistochemistry was done on formalin-fixed paraffin-embedded biopsies obtained at bronchoscopy instead of PCR.

Pack-years of cigarette smoking were calculated as the product of the duration of smoking (in years) and the numbers of cigarettes smoked per day, divided by 20.

In total, 30 patients were diagnosed with COPD and 85 patients had chronic Cpn infection, as defined below.

**Serology for Cpn—Definition of chronic infection**

Blood specimens were analysed for specific antibodies to Cpn by the microimmunofluorescence (MIF) as described in detail earlier. Sera were absorbed with Gullsorb (Gull Laboratories, USA) to remove possible interference from IgG antibodies before analysis of specific IgA antibodies. Repeat blood specimens were always analysed in parallel with earlier specimens on the same antigen slide to increase the accuracy of titre level assignments for all specimens from the same patients. Endpoint titration was done in all cases, and control sera were included in each run to ensure reproducibility.

Chronic Cpn infection was defined as stable titres of specific IgA ≥ 1/64. Mean time between the samples was 104 (SD 30) days. In total 85 subjects (37% of the females and 49% of the males) had serological signs of chronic infection.

**PCR, throat specimens**

Specimens were taken with a CTA swab (*Chlamydia trachomatis* aluminum, Biohospital AB, Kopparberg, Sweden) as described earlier. Swabs were immersed in 2 SP medium, stored in the refrigerator,
and transported to the laboratory the same day. The specimens were either frozen at −70°C or immediately processed at the laboratory. Samples were prepared for PCR using a previously described method. PCR was done according to Campbell et al.8 using HL1 and HR1 as primers on all samples. All samples were run in duplicate for each PCR method; one of the replicates was spiked with Cpn DNA equivalent to 20 elementary bodies to test for inhibitors of the Taq polymerase enzyme. When inhibitors were found, specimen were diluted 1:100 with sterile deionized water and retested.

Immunohistochemistry (IHC)

IHC was done on small bronchial biopsies during the second year of the study. Biopsies obtained at bronchoscopy were formalin-fixed and embedded in paraffin, then stained using a commercially available kit employing streptavidin–biotin labelled antibodies according to an earlier report.9 A specific Cpn monoclonal antibody generously donated by Dr Kenneth Persson, Malmö, Sweden, was used after the specificity of the antibody had been ascertained. No cross-reactions were found in control samples containing Chlamydia trachomatis or Chlamydia psittaci. The monoclonal antibody used was found to react in an identical manner to RR-402, a specific Cpn monoclonal obtainable commercially from DAKOPATS, Denmark. This monoclonal is specific for protein antigen moieties. Phosphate-buffered saline, pH 7.4, fetal calf serum, mouse serum and a monoclonal antibody to CMV were used as negative controls. All analyses were done by two microbiologists with extensive experience with the methods.

Lung function test—Definition of COPD

Spirometry was performed by trained nurses according to the ATS criteria10 using a Vitalograph Compact II (Vitalograph Ltd, Buckingham, England), which was calibrated daily according to the instructions from the manufacturer, and values were calculated as percent of predicted values.11 COPD was defined as FEV1/FVC <70% without features of asthma as judged from the medical history or reversibility test (FEV1 reversibility >15%). Thirty-four patients had FEV1/FVC <70%, two of them were reversible, and two had not undergone reversibility testing or had not provided a medical history excluding the possibility of asthma. Thus, 30 patients were defined as having COPD.

Statistical analysis

Comparisons of age, pack-years, lung function and IgA titres for Cpn were performed by t-test, and comparisons of sex, smoking status, and chronic Cpn infection by the χ2 test. The association between COPD and chronic infection with Cpn was calculated by analysis of covariance with COPD as dependent factor, and current smoking status and pack-years as covariates. The association between lung function and convalescence serum titres of IgA was calculated by analysis of covariance with FEV1/FVC as a dependent factor, and current smoking status and pack-years as covariates.

Results

No differences were found between males and females regarding age, prevalence of chronic Cpn infection, or current smoking status, but males had a history of heavier smoking (pack-years), lower FVC and FEV1/FVC, and higher antibody titres to Cpn (Table 1).

Chronic infection was more common in ever smokers; the pack-years smoked in those with chronic infection was 19.3 (sd 17.7) and in those without infection 13.7 (sd 16.4), P<0.05. The mean age for those with chronic infection was 63.4 (sd 11.0) years and for those without 58.1 (sd 12.3) years (P<0.01).

Patients with COPD were significantly older than those without (P<0.01), but there was no significant relationship between COPD diagnosis and gender, Table 2.

COPD was more common among those with chronic infection than in those without (22% vs 10%; P<0.05), a statistically significant difference (P<0.05) even after correction for current smoking status, pack-years smoked or age, and patients with chronic infection had an increased incidence of COPD in all pack-year categories (Fig. 1). The degree of obstruction (FEV1% predicted) differed between the treatment groups after correction for smoking (P<0.05), and linear regression showed a tendency to association with increasing titres of specific Cpn IgA antibody titres, however this did not reach statistical significance (Fig. 2).

PCR on throat specimens taken from 191 patients was positive in 24 samples (13%). In cytobrush specimens 6/78 (8%) were positive for Cpn DNA. Three of the 6 brush positive PCR was also positive in the throat sample. IHC on biopsies from the bronchial mucous membrane was performed in 85 patients and 16 were found positive for Cpn. Two of
the IHC positive samples were positive with PCR on throat specimen.

Comparing serological signs of chronic infection with PCR on throat specimens we found that 12 of 85 (14%) with elevated IgA were positive (no throat sample was collected from 3 of the patients). Of the 114 without chronic Cpn infection 11 were positive for Cpn DNA by PCR (10%).
Discussion

This study demonstrated an association between chronic Cpn infection (stable titres of IgA ≥ 1/64) and COPD (FEV₁/FVC < 70% and no features of asthma). The association persisted after correction for the potential confounding effect of smoking or age. Lung function (FEV₁/%p predicted) differed significantly between titre classes, and showed a tendency to decline with increasing titres.

Since patients were included consecutively, and exclusion of patients with pathologic chest X-ray was performed without knowledge about their lung function or antibody titre, no selection bias was introduced. However, since the recruitment was based on a clinical requirement of bronchoscopy, a generalisation to a general COPD population should be done with caution; results need to be confirmed in other cohorts of COPD patients.

The presence of stable increased levels of serum IgA, supporting a chronic infection, and Cpn-specific circulating immune complexes have earlier been described for COPD patients. von Hertzen et al. reported that 65% of COPD patients (71% in patients with severe disease) showed evidence of suspected chronic Cpn infection. A recently published study by Falck et al. confirmed an association between Cpn IgA and decreased lung function. On the other hand, Strachan et al. found no significant association between IgA antibodies to Cpn and later treatment for chronic non-specific lung disease or decline in lung function in an epidemiological study focusing primarily on cardiovascular risk factors. A trend was seen for an increased rate of decline in lung function in the IgA antibody positive patients, but confidence intervals were wide since the study was not powered for analysis of this variable. In addition, these authors used a low cut-off level for IgA (1/16) which may not select for chronic Cpn infection.

We chose the microimmunofluorescence technique for assessment of serostatus of these patients because it is the “gold standard” for serology recommended by the Centres for Disease Control and Prevention (USA) and Laboratory Centre for Disease Control (Canada) as long as the test is performed in an experienced laboratory and is interpreted by an experienced serologist.

We attempted to confirm the presence of Cpn in swab and biopsy samples using two different methods, PCR and IHC. The correlation between identification of Cpn positive patients by serology and these methods demonstrating the presence of DNA/antigen was poor, similarly as in lung cancer patients. This is not surprising considering that the sampling size of bronchial biopsies and throat swabs is by necessity small. Cpn may have a patchy distribution in the respiratory tract and it is difficult to know where to take appropriate samples.

Smoking has earlier been demonstrated to be associated with increased levels of Cpn antibodies. An association between raised specific antibody titres to Cpn and COPD might be explained by both being associated with smoking. However, the association that we found between chronic Cpn infection and COPD remained after correction for actual smoking status and pack-years smoked, and there was a trend towards decline in lung function with increasing antibody titres. This could support a causal relationship between chronic Cpn infection and COPD.

Cpn is a ubiquitous pathogen; most individuals experience two or three Cpn infections during a lifetime. Males demonstrated higher IgA titres than females, which is in accordance with a previous study. The half-life of IgA antibodies is short, 5–6 days, and persistent elevated levels of specific IgA antibodies to Cpn have been suggested as a marker for chronic Cpn infection, and Gnarpe et al. showed that specific IgA titres ≥ 1/64 do not normally occur in healthy blood donors. For this reason, a stable titre of IgA ≥ 1/64 from at least two serum specimens was chosen for the serological definition of chronic Cpn infection used in this study.

Cpn has been established as an etiologic agent for acute upper and lower respiratory tract infections such as otitis, sinusitis, pharyngitis, bronchitis, and pneumonia, and is involved in some (4–16%) exacerbations of COPD. The organism can cause asymptomatic infections in the respiratory tract, but the long-term sequelae are unknown. In vivo and in vitro studies have demonstrated that Cpn is capable of infecting airway epithelial, phagocytic and endothelial and smooth-muscle cells. Cpn has been shown to cause ciliary dysfunction, which can contribute to an increased susceptibility to pulmonary infection. Both humoral and cellular immunity are involved in the immune response to chlamydia infections, but intracellular chlamydiae are adept at evading the immune defences, and the host does not always seem to be capable of eradicating the organism. Thus, prolonged, often sub-clinical infections can occur. The host immune response can be protective by limiting chlamydial reproduction, but this may be a double-edged sword since the same mechanisms may also contribute to the establishment of a persistent infection which could...
result in damaging long-term consequences for the host.

The pathological manifestations of COPD include signs of "small airways disease" and/or emphysema to a varying degree. The pathogenetic mechanisms differ between these two components of COPD, with cell proliferation in "small airways disease", and degradation of connective tissue in emphysema. Chronic infection with Cpn is hypothesised to have the potential of contributing to both of these manifestations.

"Small airways disease" is characterised by chronic obstructive bronchiolitis with infiltration of neutrophils, lymphocytes and macrophages, thickening of the airway wall, and increased muscle mass. Two recent studies have demonstrated the presence of Cpn in bronchioli, alveoli and alveolar macrophages in COPD patients. Infection with Cpn could lead to the stimulation of a continuous inflammatory response resulting in an increased production of IL-6 and basic fibroblast growth factor (bFGF). This could contribute to subepithelial fibrosis in small airways analogous to the scarring observed in chronic Chlamydia trachomatis infection of the eye (trachoma) or genital tract (tubal infertility). Thus, Cpn theoretically has the potential to cause tissue remodelling and the "small airways disease" seen in COPD.

Emphysema is defined by peribroncholar destruction of alveolar attachments, airway collapse, and enlargement of air spaces distal to the terminal bronchioles. Imbalance between proteinases and antiproteinases is suggested to be important in the pathogenesis of emphysema. Cpn has been identified in alveolar macrophages (AM) from patients with emphysema, and these Cpn infected phagocytes respond with a marked dose-dependent release of reactive oxygen species, TNF-α, IL-1β and IL-8, all of which may amplify a local inflammatory response to Cpn without affecting chlamydial infection and replication. Further, infected macrophages in atherosclerotic plaques have been demonstrated to produce elevated levels of TNF-α and MMP-9, and since MMP-9 is involved in connective tissue degradation, Cpn infected AM may have a role in the development of emphysema.

In conclusion, this study has demonstrated an association between chronic Cpn infection and COPD that persisted after correction for smoking. This should be confirmed in other cohorts of COPD patients. Treatment studies are also required to evaluate whether eradication of Cpn improves symptoms of COPD and reduces the accelerated decline in the lung function in these patients.

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