

Chlamydia pneumoniae Infection and Risk for Lung Cancer

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Abstract

Background: We evaluated the relationship of *Chlamydia pneumoniae* infection with prospective lung cancer risk using traditional serologic markers [microimmunofluorescence (MIF) IgG and IgA antibodies] and *Chlamydia* heat shock protein-60 (CHSP-60) antibodies, a marker for chronic chlamydial infection.

Methods: We conducted a nested case-control study (593 lung cancers and 671 controls) within the screening arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial ($N = 77,464$). Controls were matched to cases by age, sex, randomization year, follow-up time, and smoking (pack-years of smoking, time since quitting). We assessed *C. pneumoniae* seropositivity and endpoint antibody titers (IgG and IgA against *C. pneumoniae* elementary bodies and IgG against CHSP-60).

Results: *C. pneumoniae* seropositivity by microimmunofluorescence IgG or IgA antibodies was not associated with lung cancer [odds ratio of 0.88 and 95% confidence interval (95% CI) of 0.69-1.13 for IgG; odds ratio of 0.98 and 95% CI of 0.75-1.27 for IgA]. In contrast, individuals seropositive for CHSP-60 IgG antibodies had significantly increased lung cancer risk (odds ratio, 1.30; 95% CI, 1.02-1.67), and risk increased with increasing antibody titers (P trend = 0.006). CHSP-60-related risk did not differ significantly by lung cancer histology, follow-up time, or smoking. CHSP-60 seropositivity was associated with increased risk 2 to 5 years before lung cancer diagnosis (odds ratio, 1.77; 95% CI, 1.16-2.71; P trend = 0.006), thus arguing against reverse causality.

Conclusions: CHSP-60 seropositivity and elevated antibody titers were associated with significantly increased risk for subsequent lung cancer, supporting an etiologic role for *C. pneumoniae* infection in lung carcinogenesis.

Impact: Our results highlight the potential for lung cancer risk reduction through treatments targeted toward *C. pneumoniae* infections and chronic pulmonary inflammation. *Cancer Epidemiol Biomarkers Prev*; 19(6); 1498-505. ©2010 AACR.

Background

Lung cancer is the most common cancer worldwide, with 1.35 million incident cases annually (1, 2). In addition to cigarette smoking, the major lung cancer risk factor (1, 3), recent studies underscore an etiologic role for chronic pulmonary inflammation in lung carcinogenesis (4, 5). Chronic lung infections could be one cause of pulmonary inflammation, which could act either independently or as a cofactor to tobacco smoke in increasing lung cancer risk (4).

The bacterium *Chlamydia pneumoniae*, a common cause of community-acquired pneumonia (6), has been impli-

cated in lung carcinogenesis. The association of *C. pneumoniae* infection with lung cancer risk has been variable in previous studies, with relative risk estimates ranging from 0.7 to 9.0 among seropositive individuals (7-13). This wide variability in *Chlamydia*-related lung cancer risk could reflect the retrospective nature of some studies, small sample sizes, or inadequate adjustment for confounding due to smoking (10). Importantly, previous retrospective and prospective studies have relied on serologic characterization of chronic *C. pneumoniae* infections (10). Nonetheless, modest reliability of serologic assays and the lack of a validated marker for chronic infection have precluded a precise estimation of the etiologic role of *C. pneumoniae* (10, 14, 15).

Further information on the role of *C. pneumoniae* in lung cancer could be provided by studies using additional markers of infection and inflammation. Antibodies to the *Chlamydia* heat shock protein-60 (CHSP-60) could be one such marker for identifying chronic *C. pneumoniae* infections. CHSP-60 is consistently expressed during the chronic/persistent phase of chlamydial infection and is the major immunodominant protein responsible for *Chlamydia*-induced inflammatory tissue damage (6, 16-24). No study to date has evaluated the relationship of CHSP-60 antibodies with lung cancer.

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The morbidity and mortality associated with lung cancer (1), sizeable prevalence of *C. pneumoniae* in populations (6, 10), and importantly, the ability to treat *C. pneumoniae* infections and thereby mitigate ensuing inflammation, all underscore the importance of gaining a better understanding of the possible role of *C. pneumoniae* in development of lung cancer. In the current case-control study, we investigated the relationship of serologic markers of *C. pneumoniae* infection with prospective lung cancer risk. We evaluated *C. pneumoniae* infections using assays for both traditional markers of infection [microimmunofluorescence (MIF) IgG and IgA antibodies to *C. pneumoniae* elementary bodies] as well as antibodies to CHSP-60.

Materials and Methods

Study design

We conducted a nested case-control study within the screening arm of the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. During 1992 to 2001, the trial recruited ~155,000 men and women ages 55 to 74 years into either the screening arm ($n = 77,464$) or the control arm (25). Lung cancer screening encompassed a chest x-ray at study entry (that is, baseline) followed by either three annual X-rays (for current or former smokers at enrolment) or two annual X-rays (for never smokers). At baseline, all participants in the screening arm provided questionnaire information on sociodemographic and behavioral characteristics, as well as blood specimens (26). Lung cancers were ascertained through annual questionnaires, with confirmation of positive reports through abstraction of medical charts and death certificates.

Lung cancers occurring on or before December 31, 2004 ($n = 898$), were eligible for selection into our case-control study. After exclusion of cases due to missing baseline questionnaire, previous history of any cancer, diagnosis of multiple cancers during follow-up, missing smoking information at baseline, missing consent for use of biological specimens for etiologic studies, or unavailability of serum specimens, we included $n = 593$ lung cancers in this case-control study. Controls ($n = 671$) were matched to cases by age at randomization (55-59, 60-64, 65-69, and 70-74 y), sex, year of randomization (1993-1995, 1996-1997, 1998-1999, and 2000-2001), and follow-up time in the study (1-y intervals). In addition, to tightly control for the confounding effects of cigarette smoking, we matched controls to cases based on smoking status (never, former, or current), pack-years of smoking (0-29, 30-39, 40-49, and 50+ pack-years) for current and former smokers, and time since quitting (<15 and 15+ y) for former smokers. To enhance statistical power among the never smoker group, we used a 3:1 control-case ratio, whereas a 1:1 ratio was used among former and current smokers.

Laboratory methods

Baseline serum specimens were used for all laboratory assays. We used the MIF assay using the method of Wang

et al. (27) for detection of *C. pneumoniae* IgG and IgA antibodies and determination of endpoint antibody titers (<1:16 considered as negative, 1:16, 1:32, 1:64, 1:128, up to 1:1024). The MIF assay uses purified *C. pneumoniae* elementary bodies to measure antibodies. Although this assay is considered the gold standard serologic test to test for infection, it requires visual inspection of stained cells, and issues with reproducibility have been described (14). CHSP-60 IgG antibodies were detected using an enzyme-linked immunosorbent assay (ELISA; Medac; ref. 21). ELISA optical density values were transformed into endpoint titers (<1:50, negative; 1:50; 1:100; and 1:200) as per the manufacturer's instructions.

The CHSP-60 ELISA assay uses a recombinant *C. trachomatis* HSP-60 antigen, which shares a high degree of homology (>90%) with HSP-60 antigens across other *Chlamydia* species (16). CHSPs also share homology (~50%) with human heat shock proteins (23), and elevated levels of human HSP-70 antibodies have been associated with increased lung cancer risk (28). Therefore, given the possibility that the CHSP-60 ELISA would detect cross-reacting antibodies, we conducted additional testing on a subset of 99 subjects (50 negative and 49 positive for CHSP-60 antibodies). For this group, we separately tested for IgG antibodies against 11 *C. trachomatis* serotypes using the MIF assay (27) and for IgG antibodies to human HSP-70 using an ELISA (Stressgen).

We evaluated reproducibility of *C. pneumoniae* serology assays using 90 blinded quality control samples (6 replicates each from 15 individuals). For MIF IgG, MIF IgA, and CHSP-60 IgG antibody assays, we observed good concordance across replicate samples with respect to classification as positive versus negative and antibody titers as reflected in calculated percent agreements, coefficients of variation, and intraclass r 's (Supplementary Table 1).

Statistical analyses

We assessed the relationship of *C. pneumoniae* antibodies with lung cancer risk using conditional logistic regression. In addition to the matching variables, analyses were adjusted for race, level of education, body mass index (BMI) at enrolment, regular use of aspirin/ibuprofen, family history of lung cancer, history of heart disease, and history of bronchitis/emphysema. We evaluated associations for *C. pneumoniae* seropositivity (positive defined as titers of $\geq 1:16$ for MIF IgG and IgA antibodies and $\geq 1:50$ for CHSP-60 ELISA antibodies), as well as for endpoint antibody titers in categories (IgG classified as negative, 1:16-1:64, and >1:64; IgA classified as negative, 1:16-1:32, and >1:32; and CHSP-60 antibodies classified as negative, 1:50, 1:100, and 1:200). Multiplicative statistical interactions were evaluated using product terms in logistic regression models.

Associations between *C. pneumoniae* and lung cancer were also assessed across strata defined by lung cancer histology (squamous cell carcinomas, adenocarcinomas, small cell carcinomas, large cell carcinomas, and

Table 1. Characteristics of lung cancer cases and controls from the PLCO Cancer Screening Trial

Characteristic	Controls (n = 671), n (%)	Cases (n = 593), n (%)	P*
Age at enrolment, y			
≤59	120 (17.9)	112 (18.9)	— [†]
60-64	189 (28.2)	165 (27.8)	
65-69	215 (32.0)	197 (33.2)	
70-74	147 (21.9)	119 (20.1)	
Sex			
Female	234 (34.9)	186 (31.4)	— [†]
Male	437 (65.1)	407 (68.6)	
Smoking status			
Never smoker	117 (17.4)	39 (6.6)	— [†]
Former smoker	318 (47.4)	318 (53.6)	
0-29 pack-years and quit for <15 y	30 (4.5)	30 (5.1)	
0-29 pack-years and quit for ≥15 y	65 (9.7)	65 (11.0)	
30-39 pack-years and quit for <15 y	48 (7.2)	48 (8.1)	
30-39 pack-years and quit for ≥15 y	21 (3.1)	21 (3.5)	
40-49 pack-years and quit for <15 y	18 (2.7)	18 (3.0)	
40-49 pack-years and quit for ≥15 y	13 (1.9)	13 (2.2)	
50+ pack-years and quit for <15 y	104 (15.5)	104 (17.5)	
50+ pack-years and quit for ≥15 y	19 (2.8)	19 (3.2)	
Current smoker, pack-years	236 (35.2)	236 (39.8)	
0-29	49 (7.3)	49 (8.3)	
30-39	73 (10.9)	73 (12.3)	
40-49	18 (2.7)	18 (3.0)	
50+	96 (14.3)	96 (16.2)	
Race			
White	611 (91.1)	522 (88.0)	0.10
Black	29 (4.3)	44 (7.4)	
Hispanic	6 (0.9)	10 (1.7)	
Asian/Pacific Islander	25 (3.7)	17 (2.9)	
Education			0.015
High school or less	216 (32.2)	234 (39.5)	
College or higher	455 (67.8)	359 (60.5)	
BMI at enrolment, kg/m ²			0.16
<18.5	4 (0.6)	8 (1.4)	
18.5-24.9	217 (32.3)	217 (36.6)	
25.0-29.9	313 (46.7)	253 (42.7)	
≥30	126 (18.8)	110 (18.6)	
Missing	11 (1.6)	5 (0.8)	
Regular use of aspirin or ibuprofen			0.58
No	233 (34.7)	210 (35.4)	
Yes	438 (65.3)	382 (64.4)	
Missing	0 (0.0)	1 (0.2)	
History of bronchitis/emphysema			<0.001
No	586 (87.3)	459 (77.4)	
Yes	73 (10.9)	111 (18.7)	
Missing	12 (1.8)	23 (3.9)	
History of heart disease			0.80
No	562 (83.8)	471 (79.4)	
Yes	96 (14.3)	93 (15.8)	
Missing	13 (1.9)	29 (4.9)	

(Continued on the following page)

Table 1. Characteristics of lung cancer cases and controls from the PLCO Cancer Screening Trial (Cont'd)

Characteristic	Controls (n = 671), n (%)	Cases (n = 593), n (%)	P*
Family history of lung cancer			0.003
No	569 (84.8)	451 (76.1)	
Yes	74 (11.0)	104 (17.5)	
Missing	28 (4.2)	38 (6.4)	
Time between serum sampling and subject selection in years, mean (SD)	3.3 (2.6)	3.4 (2.5)	0.65 [†]

*P values were derived from conditional logistic regression models. Models included adjustment for study matching factors: age at enrolment, sex, year of randomization, follow-up time in study in years, and smoking status (never smokers, former smokers matched on pack-years of smoking and time since quitting, and current smokers matched on pack-years of smoking). P values were calculated after exclusion of subjects with missing values.

[†]Matching variable. The distributions do not appear identical in the table because the case-control ratio varies according to smoking status.

[‡]P value calculated using a t test.

other/unspecified histologies), time between serum sampling and lung cancer diagnosis/control selection (<1, 1-2, 2-5, and 5+ y), and smoking status (never, former, and current smokers). Agreement across the three *C. pneumoniae* serology assays among control subjects was assessed using percent agreement and κ statistics. Finally, we evaluated predictors of *C. pneumoniae* seropositivity among control subjects using unconditional logistic regression. Two-sided P values < 0.05 were considered as statistically significant.

Results

Table 1 shows the characteristics of 593 cases and 671 matched controls. Because of matching, cases and controls were similar with respect to age at enrolment, sex, and smoking, including pack-years of smoking for current and former smokers and time since quitting for former smokers. Cases had significantly lower educational attainment and were more likely to have a family history of lung cancer, as well as a personal history of bronchitis/emphysema. No significant differences were observed between cases and controls for race, BMI at enrolment, regular use of aspirin/ibuprofen, or history of heart disease. The time interval between serum sampling and subject selection was similar between cases and controls.

Seroprevalence of *C. pneumoniae* among control subjects was 53.1% by MIF IgG antibodies, 30.0% by MIF IgA antibodies, and 33.8% by CHSP-60 IgG antibodies. *C. pneumoniae* seropositivity by MIF IgG or IgA antibodies was unrelated to lung cancer risk [Table 2; positive versus negative odds ratio of 0.88 and 95% confidence interval (95% CI) of 0.69-1.13 for IgG and odds ratio of 0.98 and 95% CI of 0.75-1.27 for IgA]. Likewise, titers of MIF IgG or IgA antibodies were unrelated to lung cancer risk.

In contrast, individuals positive for CHSP-60 IgG antibodies had significantly increased lung cancer risk (odds

ratio, 1.30; 95% CI, 1.02-1.67), with a significant trend for increasing risk across increasing levels of CHSP-60 antibody titers (P trend = 0.006). In a separate analysis, we found that elevated levels of high-sensitivity C-reactive protein (CRP), a marker for inflammation, were also associated with significantly increased risk for subsequent lung cancer (29). However, additional adjustment for CRP did not materially change the CHSP-60 associations (CRP-adjusted odds ratio for positive versus negative, 1.31; 95% CI, 1.02-1.70; P trend across titers = 0.006). The increased lung cancer risk associated with CHSP-60 seropositivity did not differ by MIF IgG antibody status [odds ratio, 1.18 (95% CI, 0.71-1.98) among IgG negative individuals and 1.33 (95% CI, 0.80-2.20) among IgG positive individuals; P interaction = 0.52] or by MIF IgA antibody status [odds ratio, 1.31 (95% CI, 0.91-1.88) among IgA negative individuals and 0.91 (95% CI, 0.27-3.07) among IgA positive individuals; P interaction = 0.14].

CHSP-60 seropositivity was not most strongly related to increased risk for any particular lung cancer subtype (Fig. 1A). Nonetheless, increasing CHSP-60 IgG titers were significantly associated with increased risk for lung cancers of uncommon/unspecified histologies (P trend = 0.032) and were marginally associated with increased risk for lung squamous cell carcinomas (P trend = 0.084), lung adenocarcinomas (P trend = 0.083), and large cell carcinomas (P = 0.071). The relationship of CHSP-60 with increased lung cancer risk did not vary by latency (that is, time interval between serum sampling and diagnosis of lung cancer; P interaction = 0.44), although the association was most apparent during the 2 to 5 years before lung cancer diagnosis (Fig. 1B; CHSP-60 positive versus negative odds ratio, 1.77; 95% CI, 1.16-2.71; P trend across CHSP-60 antibody titers = 0.006).

As shown in Fig. 1C, CHSP-60 antibodies were associated with increased lung cancer risk among former

Table 2. Association of *C. pneumoniae* antibodies with risk for lung cancer

Exposure	Controls (n = 671), n (%)	Cases (n = 593), n (%)	OR (95% CI)*	OR (95% CI)†
<i>C. pneumoniae</i> MIF IgG				
Negative (<1:16)	315 (46.9)	300 (50.6)	1.00	1.00
Positive (≥1:16)	356 (53.1)	293 (49.4)	0.88 (0.69-1.11)	0.88 (0.69-1.13)
<i>C. pneumoniae</i> MIF IgG titers				
<1:16	315 (46.9)	300 (50.6)	1.00	1.00
1:16-1:64	136 (20.3)	117 (19.7)	0.95 (0.68-1.32)	0.94 (0.66-1.32)
>1:64	220 (32.8)	176 (29.7)	0.85 (0.65-1.10)	0.86 (0.65-1.12)
			<i>P</i> trend [‡] = 0.22	<i>P</i> trend = 0.28
<i>C. pneumoniae</i> MIF IgA				
Negative (<1:16)	470 (70.0)	419 (70.7)	1.00	1.00
Positive (≥1:16)	201 (30.0)	174 (29.3)	0.98 (0.76-1.26)	0.98 (0.75-1.27)
<i>C. pneumoniae</i> MIF IgA titers				
<1:16	470 (70.0)	419 (70.6)	1.00	1.00
1:16-1:32	90 (13.4)	80 (13.5)	1.06 (0.75-1.50)	1.12 (0.78-1.61)
>1:32	111 (16.5)	94 (15.9)	0.91 (0.66-1.25)	0.87 (0.63-1.22)
			<i>P</i> trend = 0.69	<i>P</i> trend = 0.61
<i>Chlamydia</i> HSP-60 IgG				
Negative (<1:50)	444 (66.2)	348 (58.7)	1.00	1.00
Positive (≥1:50)	227 (33.8)	245 (41.3)	1.34 (1.06-1.69)	1.30 (1.02-1.67)
<i>Chlamydia</i> HSP-60 IgG titers				
<1:50	444 (66.2)	348 (58.7)	1.00	1.00
1:50	124 (18.5)	112 (18.9)	1.08 (0.80-1.46)	1.06 (0.77-1.45)
1:100	76 (11.3)	102 (17.2)	1.73 (1.22-2.42)	1.66 (1.16-2.38)
1:200	27 (4.0)	31 (5.2)	1.54 (0.90-2.64)	1.58 (0.89-2.81)
			<i>P</i> trend = 0.002	<i>P</i> trend = 0.006

Abbreviation: OR, odds ratio.

*Odds ratios are adjusted for matching variables: age, sex, year of randomization, follow-up time in study, smoking status (never, former, and current), pack-years of smoking and time since quitting for former smokers, and pack-years of smoking for current smokers.

†Odds ratios are adjusted for matching factors and additionally for race, level of education, body mass index at enrolment, regular use of aspirin/ibuprofen, family history of lung cancer, history of heart disease, and history of emphysema/bronchitis.

‡*P* value for trend in odds ratios across titers.

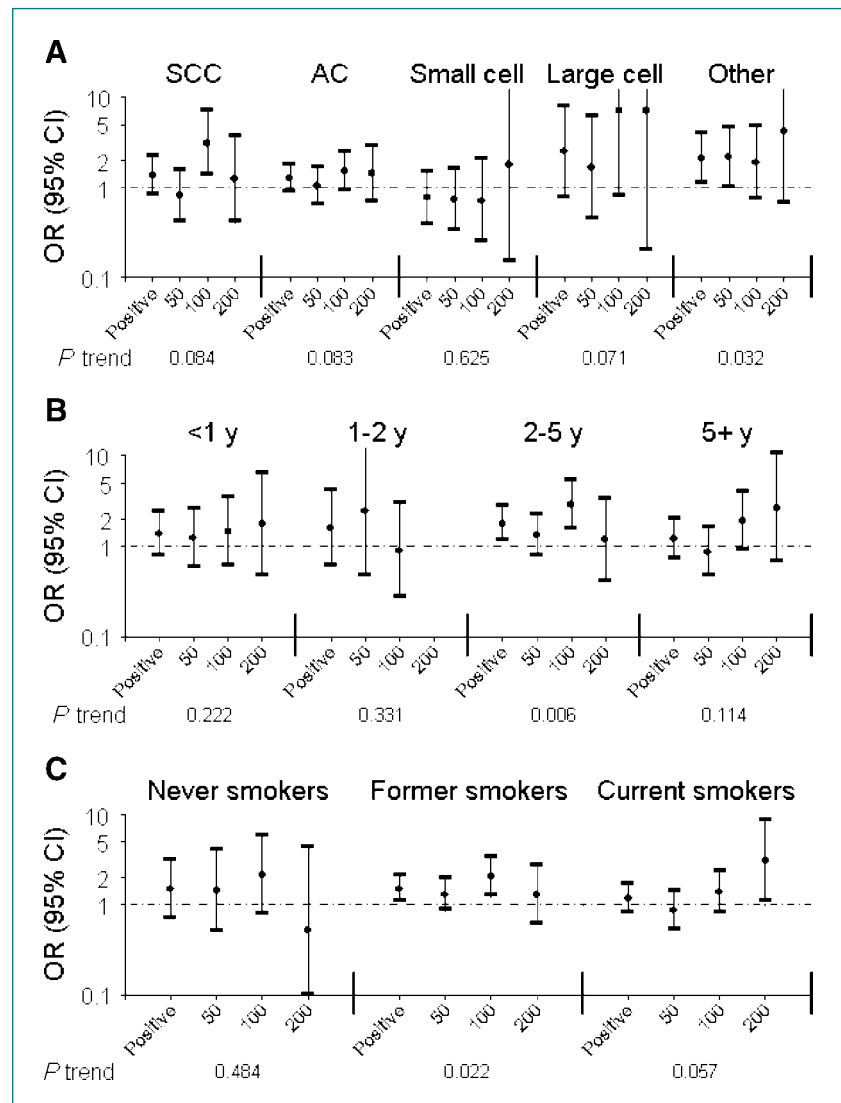
smokers (positive versus negative odds ratio, 1.48; 95% CI, 1.06-2.06; *P* trend across antibody titers = 0.022) and marginally associated among current smokers (positive versus negative odds ratio, 1.16; 95% CI, 0.81-1.67; *P* trend across antibody titers = 0.057) but were not significantly associated with lung cancer risk among never smokers. However, this difference across smoking strata was not statistically significant (*P* interaction of CHSP-60 seropositivity across smoking strata = 0.61). The association of CHSP-60 seropositivity with lung cancer risk did not differ by age at randomization (*P* interaction = 0.89), sex (*P* interaction = 0.83), or race (*P* interaction = 0.69).

Agreement across the three *C. pneumoniae* serologic assays among control subjects is shown in Table 3. We observed moderately good agreement between MIF IgG and IgA antibodies (71.8% agreement; $\kappa = 0.45$), and most individuals (91.5%) with MIF IgA antibodies were also positive for MIF IgG antibodies. Of note, however, among

CHSP-60 seropositive individuals, only 54.2% were IgG positive and 32% were IgA positive. This level of agreement was no better than chance agreement ($\kappa = 0.01$ and 0.03, respectively, with respect to CHSP-60 antibodies). Given this low degree of correlation between CHSP-60 versus MIF seropositivity, on a subset of 49 CHSP-60 seropositive and 50 seronegative subjects, we evaluated evidence for cross-reactivity with *C. trachomatis* IgG and human HSP-70 IgG antibodies. CHSP-60 seropositivity was not related to prevalence of *C. trachomatis* antibodies (seropositivity for at least 1 of 11 *C. trachomatis* serotypes, 14.3% among CHSP-60 negative individuals versus 10.0% among CHSP-60 positive individuals; $\chi^2 P = 0.51$) or levels of human HSP-70 antibodies (median, 2.62 ng/mL among CHSP-60 negative individuals versus 2.37 ng/mL among CHSP-60 positive individuals; Wilcoxon *P* = 0.70).

We evaluated demographic, medical history, and behavioral predictors of CHSP-60 seropositivity among control

Figure 1. The association of CHSP-60 antibody titers with lung cancer risk according to the following. A, lung cancer histology (squamous cell carcinomas, $n = 128$ lung cancers; adenocarcinomas, $n = 269$; small cell carcinomas, $n = 75$; large cell carcinomas, $n = 36$; and cancers of other/unknown histologies, $n = 86$); AC, adenocarcinomas; OR, odds ratios; SCC, squamous cell carcinomas. B, follow-up time (<1 y, $n = 128$ lung cancers; 1-2 y, $n = 70$; 2-5 y, $n = 238$; and 5+ y, $n = 156$). C, smoking status (never smokers, $n = 39$ lung cancers; former smokers, $n = 318$; and current smokers, $n = 236$). Odds ratios and 95% CIs are shown on a log scale. Odds ratios and confidence intervals were estimated in conditional logistic regression models. By virtue of matching, these odds ratios are adjusted for age, sex, year of randomization, follow-up time, and cigarette smoking. Odds ratios and 95% CIs are shown for CHSP-60 seropositivity (positive versus negative), as well as for CHSP-60 endpoint antibody titers 1:50, 1:100, and 1:200 as compared with CHSP-60-negative subjects (<1:50). P values for trend in odds ratios across CHSP-60 antibody titers are also shown below the X axis.



subjects. CHSP-60 seropositivity increased with age and was significantly higher among Blacks than Whites (62.1% versus 32.5%; $P = 0.001$). CHSP-60 seropositivity was not significantly associated with smoking status, level of education, BMI, regular use of aspirin/ibuprofen, history of bronchitis/emphysema or heart disease, or family history of lung cancer (data not shown). CHSP-60

seropositivity was unrelated to levels of circulating CRP among controls (median CRP levels among CHSP-60 positive individuals and negative individuals, 2.7 mg/L; Wilcoxon $P = 0.36$). Although CRP levels increased with increasing CHSP-60 antibody titers (CRP levels, 2.7, 2.3, 3.2, and 3.9 mg/L across CHSP-60 titers), this difference was not statistically significant (Kruskal-Wallis $P = 0.20$).

Table 3. Agreement across *C. pneumoniae* serologic assays among controls

	Percent agreement	Percent positive agreement	κ (95% CI)
MIF IgG vs. MIF IgA	71.8	33.0	0.45 (0.39 to 0.51)
CHSP-60 vs. MIF IgG	49.7	21.1	0.01 (-0.05 to 0.08)
CHSP-60 vs. MIF IgA	57.9	17.0	0.03 (-0.04 to 0.11)

Discussion

In a nested case-control study within the screening arm of the PLCO trial, we found that individuals with detectable CHSP-60 IgG antibodies were at significantly increased risk for subsequent lung cancer. CHSP-60 is consistently expressed by *C. pneumoniae* during chronic infection, and the host immune response to this protein contributes to inflammatory tissue damage (16). Our findings that lung cancer risk increased with CHSP-60 antibody titers and persisted for at least 2 to 5 years after detection of elevated titers suggest that chronic inflammation from *C. pneumoniae* infection acts to promote lung cancer development.

Our observation of a lack of an association between MIF IgG or IgA seropositivity and risk for lung cancer contrasts with some previous reports of increased lung cancer risk among *C. pneumoniae* MIF seropositive individuals, particularly those seropositive for MIF IgA antibodies (8-11). The modest reliability of *C. pneumoniae* MIF assays could account for the variability in published results (10, 14). Although considered the gold standard serologic test for *C. pneumoniae*, the MIF assay is difficult to perform and interpretation of results is subjective (14). For example, a recent interlaboratory reliability study reported moderate agreement for MIF IgG and IgA antibody detection (percent agreement = 71% and 79% and $\kappa = 0.53$ and 0.39, respectively; ref. 14). Although we observed good reproducibility of MIF IgG and IgA assays on our blinded replicate samples, misclassification of MIF IgG or IgA serostatus could, in part, explain the lack of association with lung cancer risk in our study.

Several lines of evidence underscore the importance of CHSP-60 antibodies as a key measurement for severity and persistence of chlamydial infections and thus *Chlamydia*-induced pathology. Studies on *C. trachomatis*-related diseases, such as pelvic inflammatory disease, tubal infertility, and ectopic pregnancy (16, 20, 21), as well as those addressing the *C. pneumoniae*-atherosclerosis relationship (23, 24, 30-32), indicate that CHSP-60 antibodies reflect a process of *Chlamydia*-induced inflammatory damage in tissues. Furthermore, these studies show that CHSP-60 antibodies are better markers than IgA antibodies for identifying chronic *Chlamydia* infections (16, 18-22). Likewise, animal models of *C. trachomatis*-related ocular trachoma and pelvic inflammatory disease show that the presence of CHSP-60 antibodies indicates chlamydial persistence (16).

The significant association of CHSP-60 antibodies with increased lung cancer risk therefore supports an etiologic role for persistent *C. pneumoniae* infections in lung carcinogenesis, while increasing lung cancer risk with increasing CHSP-60 antibody titers indicates a dose-response effect. Furthermore, the assessment of *C. pneumoniae* infections several years before a lung cancer diagnosis argues against reverse causality. Specifically, lung cancer can cause pneumonia, but it is less likely that a small tumor would lead to increased *C. pneumoniae* infection at a subclinical stage 2 to 5 years before lung cancer diagnosis.

Although *C. pneumoniae* infection is believed to increase lung cancer risk by inducing chronic pulmonary inflammation, we found that levels of CRP, a reliable marker for inflammation, did not differ by CHSP-60 seropositivity or antibody titers. It is possible that CHSP-60 seropositivity and elevated CRP levels identify different aspects or different periods of inflammatory damage, which could explain the independent associations with lung cancer risk.

Our results should be interpreted within the context of the poor correlation of CHSP-60 IgG antibodies with MIF IgG and IgA antibodies. Although unexpected, this low correlation could be related to differential expression of proteins during different phases of *C. pneumoniae* life cycle. CHSP-60 is consistently expressed throughout the chlamydial life cycle, including the chronic/persistent phase, whereas expression of several chlamydial antigens (e.g., major outer membrane protein and lipopolysaccharide) is downregulated during chronic phases (16). Therefore, it is likely that CHSP-60 antibodies identify a subset of individuals with severe or persistent infections. Cross-reactivity of the CHSP-60 assay with *C. trachomatis* or human HSP antibodies could theoretically have led to poor correlation with *C. pneumoniae* MIF results (23). However, our observations that neither *C. trachomatis* nor human HSP-70 antibodies were related to CHSP-60 seropositivity argue against this explanation.

In previous studies, *C. pneumoniae* seropositivity has been associated with increased lung cancer risk among specific subgroups such as young individuals (7, 8), men (11, 33), former smokers (8, 9), and for squamous cell carcinomas or small cell carcinomas (7, 9). We found that the elevated risk associated with CHSP-60 seropositivity or antibody titers did not differ by age, sex, smoking status, or lung cancer histology. CHSP-60 seropositivity was associated with increased risk for lung cancer among former and current smokers but not among never smokers. The lack of association among never smokers could partly reflect low statistical power to detect significant differences in this group. Alternatively, these observations could indicate that *C. pneumoniae* infection acts as a cofactor to the carcinogenic effects of tobacco smoke in increasing lung cancer risk but may lack oncogenic potential in the absence of smoking.

Our study has several strengths, including the use of prediagnostic serum specimens and careful control for important confounders such as smoking (including duration, intensity, and time since quitting). We also note the limitations of our study. As mentioned previously, the lack of concordance between CHSP-60 seropositivity and MIF IgG or IgA seropositivity was unexpected. Additional studies are needed to understand the relevance of CHSP-60 seropositivity in the natural history of *C. pneumoniae* infections and its role in lung carcinogenesis. In addition, although our study included more lung cancer cases than previous studies, some subgroup analyses were small, and our results should therefore be interpreted with caution.

In conclusion, our key observation was that individuals with antibodies directed against CHSP-60 IgG

antibodies, particularly those with higher antibody titers, were at increased risk for subsequent lung cancer. These results point to an etiologic role for chronic *C. pneumoniae* infection in lung carcinogenesis. Confirmation of these observations and additional studies are needed to further characterize the etiologic role of *C. pneumoniae* in lung carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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