Chlamydia pneumoniae Infection and Early Asymptomatic Carotid Atherosclerosis

Hugh S. Markus, DM; Matthias Sitzer, MD; David Carrington, PhD; Michael A Mendall, MD; Helmuth Steinmetz, MD

Background—Chronic Chlamydia pneumoniae infection has been implicated in the pathogenesis of atherosclerosis but whether it plays a role at an early stage in the disease is uncertain. An early estimate of atherosclerosis can be obtained by ultrasonic imaging of the carotid artery to determine intima-media thickness (IMT) and the thickness of any atheroma plaques.

Methods and Results—in 983 normal population individuals aged 30 to 70 years, we measured common carotid artery (CCA) and carotid bulb IMT, and also carotid plaque thickness and the degree of internal carotid artery (ICA) stenosis. C. pneumoniae IgA titers of ≥16 and IgG titers of ≥64 were taken as positive. There was no association between C. pneumoniae IgA or IgG seropositivity with right, left, or mean CCA or bulb IMT, or with the presence of carotid plaques. There was a significant association between IgA seropositivity and >50% mean carotid stenosis with an odds ratio of 5.24 (95% CI 1.24 to 22.21, \( P = 0.0245 \)) after controlling for age and sex; after controlling for other cardiovascular risk factors, this was not significant 3.96 (95% CI 0.84 to 18.78, \( P = 0.082 \)). No association was found between IgA or IgG seropositivity and markers of fibrinogen, log C-reactive protein, or leukocyte count.

Conclusions—We found no evidence that serological evidence of C. pneumoniae infection is associated with early atherosclerosis. It is possible that IgA seropositivity is associated with more advanced disease but this hypothesis needs to be examined in a population with a higher prevalence of advanced atherosclerosis. We found no evidence that C. pneumoniae results in a chronic systemic inflammatory state. (Circulation. 1999;100:832-837.)

Key Words: Chlamydia pneumoniae ■ ultrasonics ■ atheroma ■ carotid arteries ■ bacterial infection ■ inflammation

E stablished cardiovascular risk factors do not fully explain the risk of both ischemic heart disease and stroke. Epidemiological studies have implicated bacterial infection in the pathogenesis of both conditions. In particular, Chlamydia pneumoniae has been suggested as a causative pathogen.1 C. pneumoniae, an obligate intracellular pathogen, is a common cause of respiratory tract infections, which are usually subclinical and self-limiting. Because C. pneumoniae is difficult to culture, confirmation of infection requires identification of systemic antibody responses. About half of the general population is seropositive to C. pneumoniae by the age of 50, suggesting that reinfection is common.3 An association between antibodies to C. pneumoniae and ischemic heart disease was first reported in a case-control study in 1988 from Finland.3 The association was confirmed on samples from the prospective Helsinki Heart Study; elevated IgA titers, or the presence of immune complexes containing C. pneumoniae antigen, were found to confer a doubling of risk of a cardiac event within the following 6 months.3 This increased risk was independent of age, hypertension, and smoking status. Several other case-control studies have also found C. pneumoniae seropositivity to be an independent risk factor for both ischemic heart disease and stroke.4

Further evidence implicating C. pneumoniae in the pathogenesis of atherosclerosis is provided by examination of plaques from both coronary and other arteries using both immunohistochemical and polymerase chain reaction techniques,4 and more recently by direct culture.5,6 These have demonstrated C. pneumoniae antigen within macrophages and smooth muscle cells in atheromatous plaques but not in normal tissue adjacent to the sclerotic lesions or in control normal arteries.

A recent review of the epidemiological evidence in a total of 2700 subjects concluded that there does seem to be both a real independent association between C. pneumoniae seropositivity and ischemic heart disease and a real association between C. pneumoniae and atheromatous lesions.4 Despite this, many questions remain.4 If C. pneumoniae does contribute to pathogenesis, this could be by a number of mechanisms and at several stages in the atherogenic process. Possible
mechanisms include triggering and continuation of chronic inflammatory changes which may act via direct chronic endothelial damage, other pro-atherogenic effects, or a prothrombotic effect. There is only limited data relating \( C. pneumoniae \) seropositivity to markers of chronic inflammation in community samples. Alternatively, it is possible that the organism is merely an innocent bystander which does not contribute to the pathogenesis of cardiovascular disease.

We used high-resolution Duplex ultrasound to determine the relationship between \( C. pneumoniae \) seropositivity and early changes of carotid atherosclerosis in a community sample. Specifically, we measured carotid artery intima-media thickness (IMT) and determined the presence or absence and extent of any carotid bifurcation atheroma plaques.

Methods

Subjects

All members of a medium-sized German health insurance company who lived within a radius of 50 km from the examination institute \((n=12,824)\) were invited to participate. Within a predefined time limit, 1,837 (mean age 53 ± 12 years, 50% men) agreed to participate. The first 100 subjects aged 30 to 75, in whom there was no past history of stroke or transient ischemic attack, were included in this study. In 17 cases, cross-reactions with other chlamydial species occurred and these cases were excluded from analysis. Therefore, 983 subjects were studied.

Informed consent was obtained from all participants before examination. Participants attended for a single visit. Cardiovascular risk factors were assessed using a standardized computer-assisted interview technique. Risk factors included the following: duration of smoking (sum of all years smoked by both smokers and ex-smokers), history of arterial hypertension (treatment with antihypertensive medication or blood pressure > 160 systolic or 95 diastolic), history of diabetes mellitus, and body mass index.

Baseline demographic characteristics of the population were as follows: 272 (27.7%) were hypertensive, 49 (5.0%) had diabetes mellitus, 183 (18.6%) were current smokers, and 339 (34.5%) were ex-smokers. Among smokers, mean ± SD duration of smoking was 28 ± 11.2 years. Mean body mass index was 27.15 ± 4.05 kg/m²; mean total cholesterol, 226.4 ± 40.3 mg/dL; and mean fibrinogen, 252 ± 61.5 mg/dL. There was a past history of angina in 29 (3.0%), myocardial infarction in 7 (0.7%), and intermittent claudication in 12 (1.2%).

Nonfasting blood samples were drawn from each subject, serum was separated, and analyses were performed within 4 hours. Serum total cholesterol was determined enzymatically using a commercial kit (Boehringer Mannheim). Fibrinogen was determined accordingly to the Clauss method (Multifibren, Behringwerke AG). Full blood count was determined. In addition, 6 mL of serum from each participant was frozen on dry ice and stored at 28° for subsequent analysis. After each 300 samples, a standard serum (standard human plasma; Behringwerke AG, Marburg, Germany) was processed and analyzed. After each 300 samples, a standard serum (standard human plasma; Behringwerke AG, Marburg, Germany) was processed and analyzed.

\( C. pneumoniae \) titers were measured by microimmunofluorescence, as described previously, using the stored frozen samples. Elementary bodies of \( C. pneumoniae \) strain IOL 207 were used as a representative \( C. pneumoniae \) strain type. Cross-reactions with \( Chlamydia psittaci \) and \( Chlamydia trachomatis \) were assessed. IgA titers ≥ 16 and IgG titers ≥ 64 were taken as positive in view of the results of our previous studies using the same assay to determine the relationship with cardiovascular disease. The same positive and negative control samples used in our previous studies were included in each batch and in all cases were correctly identified as seropositive or seronegative. C-reactive protein concentration was measured by an in-house enzyme linked immunosorbent ELISA assay. The interassay and intra-assay coefficients of variation were 4% and 8%, respectively.

Ultrasound Imaging

A standard carotid Duplex examination was performed to determine the presence and extent of carotid plaques and intima-media thickness. A 7.5- to 10-MHz linear array transducer (P700SE, Phillips Medical System) was used. Settings for depth-gain compensation, preprocessing, persistence and postprocessing were held constant. The gain was adjusted so that the least dense arterial wall interface was just visible. The vertical and horizontal calibration measurements were performed every 100th measurement using an ultrasound assurance phantom. Measurements were performed in a blinded fashion on images captured during the systole of a single heartbeat.

All ultrasonic examinations were stored on an S-VHS video system for offline analysis. Common carotid artery (CCA) IMT was measured at points 2.5 and 3.5 cm proximal from the flow divider. From this, a mean of common carotid IMT was determined for both right and left sides in each individual. IMT measurements were also made at a single point on the posterior wall of the carotid bulb. Interobserver retest reliability for 4 observers was determined for 54 vessel segments; linear regression gave values of \( r = 0.82 \) to 0.88 while using the method of Bland and Altman; ± 2SD of the mean of the difference between the 2 examinations ranged from 0.08 to 0.12 mm. Intraobserver reliability for 4 observers for 102 vessel segments was also determined (linear regression \( r = 0.76 \) to 0.84, ± 2SD of the mean of the difference between the means of 0.10 to 0.16 mm).

At the same time, the extent of any carotid plaque was measured using a method we have previously published. Carotid plaque was defined as any obscuration of the free luminal vessel surface with a distance between the luminal-intimal interface and the medial-adventitial interface > 1.7 mm. In the presence of plaque (as defined by us), the degree of ICA stenosis was determined as the maximum cross-sectional luminal area reduction. Interobserver reliability for 4 different observers for 30 carotid plaques was determined (linear regression \( r = 0.76 \) to 0.90; ± 2SD of the mean of the difference between the 2 observers was 5 to 10%). Of the 983 subjects analyzed serologically, imaging was of sufficient quality for analysis of the ultrasound images in the following number of cases: right CCA IMT (980), Left CCA IMT (982), R bulb (969), left bulb (968), any plaque (981).

Statistical Analysis

All biochemical and serological assays were performed blinded to clinical details and the results of the ultrasound examinations. Three sections of analysis were performed. Firstly, the relationship between \( C. pneumoniae \) seropositivity and carotid IMT and plaques was determined. Second, the relation between conventional cardiovascular risk factors and carotid IMT and plaques was performed. Third, the relationship between \( C. pneumoniae \) seropositivity and both conventional cardiovascular risk factors and markers of inflammation (C-reactive protein, fibrinogen, white cell count) was determined. The C-reactive protein distribution was skewed but approximated well to a normal distribution following logarithmic transformation. For each section, univariate analysis was performed, followed by multivariate analysis using either multiple regression or logistic regression, as appropriate, to allow controlling for other cardiovascular risk factors. Because of the few patients with significant carotid plaque, analyses were performed to determine whether there was any association with the presence of carotid plaque per se and also with the presence of extensive carotid disease defined as a mean right and left ICA stenosis ≥ 50%.

Results

\( C. pneumoniae \), Carotid IMT, and Plaque

There was no association between \( C. pneumoniae \) IgA or IgG seropositivity and right, left, or mean CCA or bulb IMT (Table 1). These results were not altered after controlling for

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age and sex, or after controlling for other cardiovascular risk factors. To determine whether *C. pneumoniae* seropositivity was only associated with a markedly increased IMT, an analysis was performed by quartile of mean CCA IMT distribution. Again, there was no association between IgA or IgG seropositivity and mean CCA IMT (Table 2).

There was no association between *C. pneumoniae* IgA or IgG seropositivity and the presence or absence of carotid plaques (Table 3). However, there was a significant association between IgA seropositivity and >50% mean carotid stenosis with an odds ratio (OR) of 4.18. This association persisted after logistic regression to control for age and sex (OR 5.24, 95% CI 1.24 to 22.21, \( P = 0.0245 \)). After also controlling for other cardiovascular risk factors (hypertension, cholesterol, smoking, cholesterol, fibrinogen, BMI), the association was no longer significant (OR 3.96, 95% CI 0.84 to 18.78, \( P = 0.082 \)). The wide CIs reflect the small number of subjects (9) in whom there was a mean of >50% stenosis. There was no association between IgG seropositivity and mean carotid stenosis >50% both on univariate analysis (Table 3) or after controlling for age and sex.

### Carotid IMT, Plaque, and Other Cardiovascular Risk Factors

On univariate analysis, there were significant associations between common carotid IMT and male sex (\( P = 0.0001 \)), history of hypertension (\( P = 0.0001 \)), diabetes (\( P = 0.03 \)), age (\( P = 0.0001 \)), serum cholesterol (\( P = 0.0001 \)), duration of smoking (\( P = 0.0001 \)), fibrinogen (\( P = 0.0001 \)), and body mass index (\( P = 0.0001 \)). On multivariate analysis using multiple regression, independent relationship persisted between mean common carotid IMT and age, sex, hypertension, smoking, cholesterol, and fibrinogen (Table 4).

On univariate analysis, there were significant associations between the presence of carotid plaque and male sex (\( P = 0.03 \)), history of hypertension (\( P = 0.0001 \)), age (\( P = 0.0001 \)), serum cholesterol (\( P = 0.005 \)), duration of smoking (\( P = 0.0001 \)), and fibrinogen (\( P = 0.0001 \)). On multivariate analysis using logistic regression, independent relationships persisted between the presence of carotid plaque and age, sex, hypertension, smoking, cholesterol, and fibrinogen (Table 5).

### Chlamydia Seropositivity and Other Cardiovascular Risk Factors, Including Inflammatory Markers

On univariate analysis, *C. pneumoniae* IgA seropositivity was significantly associated with younger age, male sex, and duration of smoking. There was no association with hypertension, diabetes, body mass index, or total cholesterol. The associations with younger age, male sex, and duration of smoking remained significant after controlling for other cardiovascular risk factors: age in years OR 0.976 (95% CI 0.954 to 0.998), male sex OR 2.24 (95% CI 1.02 to 4.91), and duration of smoking OR 1.71 (95% CI 1.04 to 2.79) (Table 6).
In this large cross-sectional study we found no association between carotid artery IMT and *C. pneumoniae* seropositivity and mean carotid stenosis as determined by multiple regression. In contrast, there was a significant increase in IgA seropositivity in individuals with advanced carotid atherosclerosis, defined as a mean stenosis ≥50%. This persisted after controlling for age and sex but not after controlling for other cardiovascular risk factors. However, in this asymptomatic population, there were few individuals with advanced carotid atheroma; the confidence intervals of this association were therefore wide, and no firm conclusion can be drawn about the association between *C. pneumoniae* and advanced atherosclerosis. This association can only been seen as hypothesis generating and need reexamining in studies in populations with a higher prevalence of carotid stenosis. Interestingly, however, this association was only found with IgA antibodies. We have previously found an association between IgA seropositivity and risk of ischemic heart disease but no association with IgG seropositivity.\(^8\) IgA is believed to provide a better marker of chronicity of chlamydial infection than IgG.

Our results imply that *C. pneumoniae* infection is not associated with the earlier stages of carotid atherosclerosis. It may be associated with the later stages of the disease, but studies using similar technology in populations of patients with a higher prevalence of severe stenosis are required to determine whether this is a real association. If this were the case, *C. pneumoniae* could either be an innocent bystander in advanced atheromatous plaques or play a pathogenic role in the progression of established plaques.

In this study we used a microimmunofluorescent technique to determine *C. pneumoniae* seropositivity. This has the disadvantage of requiring interpretation by an expert microscopist but avoids criticism of tests based on chlamydial immune complexes or chlamydial lipopolysaccharide for detection of *C. pneumoniae* infection which can produce spurious associations due to cross-reactions with antigens, such as cardiolipin, that may be associated with cardiovascular disease. Using this same technique, we have previously found an association between *C. pneumoniae* IgA titers, but not IgG titers, and the risk of ischemic heart disease with an OR of 1.91 (95% CI 0.95 to 3.83).\(^8\) The risk was little

### TABLE 3. Association Between *C. pneumoniae* Seropositivity and the Presence of Any Carotid Plaque and Mean Carotid Stenosis >50%

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Plaque</th>
<th>No Plaque</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA 1 in 16</td>
<td>15 (19.5%)</td>
<td>145 (16.0%)</td>
<td>1.26 (0.70-2.29)</td>
<td>0.43</td>
</tr>
<tr>
<td>IgG 1 in 64</td>
<td>5 (6.5%)</td>
<td>83 (9.2%)</td>
<td>0.69 (0.27-1.75)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Percentages in parentheses are for the proportion of patients who were seropositive at the indicated titre level of all those with plaque, no plaque, stenosis, or no stenosis, as indicated.

### TABLE 4. Relationship Between Conventional Cardiovascular Risk Factors and Mean CCA IMT as Determined By Multiple Regression

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>B</th>
<th>95% CI of B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>0.00658</td>
<td>0.00578–0.00738</td>
<td>0.00001</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.0411</td>
<td>0.0241–0.0581</td>
<td>0.00001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.0385</td>
<td>0.0364–0.0406</td>
<td>0.00001</td>
</tr>
<tr>
<td>Duration of smoking, y</td>
<td>0.00134</td>
<td>0.00074–0.00193</td>
<td>0.00001</td>
</tr>
<tr>
<td>Diabete mellitus</td>
<td>-0.0163</td>
<td>-0.0542–0.1249</td>
<td>0.391</td>
</tr>
<tr>
<td>Serum cholesterol, mg/dL</td>
<td>0.000235</td>
<td>0.000031–0.000439</td>
<td>0.021</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>0.000174</td>
<td>0.000032–0.000317</td>
<td>0.015</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.00111</td>
<td>-0.00098–0.00320</td>
<td>0.290</td>
</tr>
</tbody>
</table>

BMI indicates body mass index.

### TABLE 5. Relationship Between Conventional Cardiovascular Risk Factors and the Presence of Carotid Plaque as Determined By Logistic Regression

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Exp(B)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>1.120</td>
<td>1.082–1.160</td>
<td>0.0001</td>
</tr>
<tr>
<td>Male sex</td>
<td>2.792</td>
<td>1.524–5.115</td>
<td>0.0009</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.441</td>
<td>1.400–4.254</td>
<td>0.0013</td>
</tr>
<tr>
<td>Duration of smoking, y</td>
<td>1.018</td>
<td>1.001–1.034</td>
<td>0.0331</td>
</tr>
<tr>
<td>Diabete mellitus</td>
<td>0.804</td>
<td>0.311–2.080</td>
<td>0.653</td>
</tr>
<tr>
<td>Serum cholesterol, mg/dL</td>
<td>1.008</td>
<td>1.001–1.014</td>
<td>0.017</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>1.004</td>
<td>1.000–1.008</td>
<td>0.041</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>1.010</td>
<td>0.944–1.080</td>
<td>0.773</td>
</tr>
</tbody>
</table>

BMI indicates body mass index.
changed (OR 2.09, 95% CI 1.01 to 4.32) after adjustment for multiple potential confounding variables including age, obesity, smoking history, blood pressure, cholesterol, and socioeconomic status.

There is considerable evidence suggesting that ultrasonic measurements of early atherosclerosis are clinically meaningful. Both IMT and the presence and size of any atheromatous plaques can be determined in the common carotid artery and bifurcation. There is a close correlation between common carotid artery IMT determined ultrasonically and IMT measured histologically.\textsuperscript{12,13} IMT can be measured with a high degree of inter- and intraobserver reproducibility.\textsuperscript{14} Strong associations have been found between increased IMT and conventional cardiovascular risk factors, and independent associations with age, sex, hypertension, smoking, serum cholesterol, and fibrinogen were confirmed in this study.\textsuperscript{15} In cross-sectional studies, a correlation between IMT and both carotid atherosclerosis and coronary artery disease has been demonstrated.\textsuperscript{16} In prospective studies, increased IMT has been related to an increased risk of cardiovascular endpoints.\textsuperscript{17} Carotid ultrasound also allows visualization and measurement of the extent of carotid plaques.\textsuperscript{18} These are a more direct reflection of the atherosclerotic process and again their presence, determined using ultrasound, has been correlated with a wide variety of cardiovascular risk factors as well as coronary artery disease measured angiographically. In this study, we confirmed that age, male sex, hypertension, smoking, cholesterol, and fibrinogen were independent risk factors for carotid plaque.

Intima-media thickness can be measured by several methods. It can be measured at a single point in each vessel, or at a number of standard points as in our study. The number of points has varied from 1 to 10 or more in different studies, and both the maximal thickness at 1 point and the mean thickness have been determined.\textsuperscript{19} The different methods used can make comparisons between the results of different studies difficult.\textsuperscript{19} It is important to establish not only which method of measurement is the most reproducible, but also which is the best independent predictor of future cardiovascular events. A recent prospective study reported the association with future events was greatest when a single measurement was taken at the point of maximal thickness.\textsuperscript{20} More recently, semi-automated computer programs have been written which average the IMT over a segment of the CCA, and both the maximal thickness at 2 or more sites, or bilateral thickening, can be examined in a population with a higher prevalence of advanced atherosclerosis. We found no evidence that \textit{C. pneumoniae} results in a chronic systemic inflammatory state.

One previous study has examined the relationship between \textit{C. pneumoniae} seropositivity and IMT.\textsuperscript{22} The prevalence of IgG \textit{C. pneumoniae} seropositivity, determined by microimmunofluorescence, was determined in 326 individuals with marked increased IMT and compared with paired controls with IMT in the lower 75th percentile range. A significant association was found with an OR of 1.76 (95% CI 1.21 to 2.57) which remained significant after controlling for other risk factors at 2.00 (1.19 to 3.35). The cases studied had well developed atherosclerosis with entry criteria being severe CCA thickening >2.5 mm thickness at 2 or more sites in 1 CCA, or bilateral thickening >1.7 mm. Usually, such marked IMT is associated with significant internal carotid stenosis, although no measurement of this was given in the paper. In our study, none of the 983 subjects would have fulfilled these entry criteria, although of the 8 subjects with the greatest degree of IMT in our study, 5 had >60% mean ICA stenosis. Therefore, this previous study\textsuperscript{22} essentially found an association with severe asymptomatic atherosclerosis which would be consistent with the possible association we found with >50% stenosis.

There is increasing evidence that one of the primary mechanisms in atherogenesis may be inflammation. C-reactive protein has been found to predict the risk of future myocardial infarction and stroke.\textsuperscript{23} It has been suggested that \textit{C. pneumoniae} may contribute to the pathogenesis of atherosclerosis by causing chronic systemic inflammation.\textsuperscript{1} Associations have been reported with fibrinogen\textsuperscript{24} and C-reactive protein,\textsuperscript{8} but previous studies investigating the relationship of markers of chronic inflammation to seropositivity have been small.\textsuperscript{4} In this large community population, we found no association between \textit{C. pneumoniae} seropositivity and C-reactive protein, fibrinogen, or white cell count. These results would argue against \textit{C. pneumoniae} predisposing to atherosclerosis via chronic systemic inflammation. In contrast, we found associations between \textit{C. pneumoniae} seropositivity and both male sex and smoking as previously reported.\textsuperscript{8}

In conclusion, we found no evidence that \textit{C. pneumoniae} positive serology is associated with very early atherosclerosis. It is possible that IgA seropositivity is associated with more advanced disease, but this hypothesis needs to be examined in a population with a higher prevalence of advanced atherosclerosis. We found no evidence that \textit{C. pneumoniae} results in a chronic systemic inflammatory state.

Acknowledgments

This work was funded by a British Heart Foundation Project Grant (PG97070). Clinical and imaging data collection was funded by the Stiftung Deutsche Schlaganfall-Hilfe.

References


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Circulation. 1999;100:832-837
doi: 10.1161/01.CIR.100.8.832

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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