**Chlamydia pneumoniae** Seropositivity Is Associated With Carotid Artery Intima-Media Thickness

Caroline Schmidt, BSc; Johannes Hulthe, MD, PhD; John Wikstrand, MD, PhD; Håkan Gnarpe, MD, PhD; Judy Gnarpe, PhD; Stefan Agewall, MD, PhD; Björn Fagerberg, MD, PhD

Background and Purpose—Infection may both augment the atherosclerotic process and contribute to later manifestations of overt clinical disease. *Chlamydia pneumoniae* elementary bodies have been detected in atherosclerotic lesions. The aim of the present study was to investigate whether elevated titers of antibodies and circulating immune complexes to *C pneumoniae* were associated with ultrasound findings indicating presence of atherosclerosis in the carotid artery.

Methods—Serum titers of antibodies to *C pneumoniae* (IgM, IgA, IgG, and circulating immune complex) were related to intima-media thickness (IMT) and plaque status measured by B-mode ultrasound in the carotid artery in 113 men with treated hypertension and at least 1 of the following risk factors: hypercholesterolemia, smoking, or diabetes.

Results—Any of the titers was elevated in 56 (50%) men, and common carotid artery IMT was thicker in this group compared with the 57 men without any elevated titers (1.00 versus 0.92 mm, *P*<0.05). There were no accompanying differences in blood pressure, lipid levels, blood glucose, or smoking. Elevation of separate antibody types and circulation immune complex were also associated with increased IMT. In the latter group, systolic blood pressure was higher among seropositive patients compared with those who had no circulating immune complex. Seropositivity was not related to plaque status.

Conclusions—Seropositivity for *C pneumoniae* was associated with an increased intima-media thickness in the common carotid artery but not plaque status in hypertensive men at high risk for cardiovascular disease. (*Stroke. 2000;31:1526-1531.)*

Key Words: carotid arteries ■ chlamydia

There is a growing body of data indicating that infections may be linked to atherosclerotic disease. Infection may both augment the atherosclerotic process and contribute to later manifestations of overt clinical disease by facilitating plaque rupture and thrombosis.1

*Chlamydia pneumoniae* (TWAR) is an intracellular Gram-negative bacterium that commonly causes respiratory infections in all age groups.2 The clinical picture varies from asymptomatic infection or mildly symptomatic disease to bronchitis and pneumonia. Humoral antibodies to *C pneumoniae* have been found in more than half of the adult population.3 Persistent infection is not uncommon after acute respiratory infection with *C pneumoniae*.4–6

*C pneumoniae* elementary bodies have been detected in atherosclerotic plaques and fatty streaks in the aorta and coronary arteries of autopsy cases, from coronary arterectomy specimens, and from carotid endarterectomy specimens.7–10 A recent meta-analysis indicated that available data support the hypothesis that *C pneumoniae* may be causative for arterial disease, although further research is needed.1

Most previous studies have examined the association between *C pneumoniae* seropositivity and coronary heart disease.1 Several recent cross-sectional studies have found a relation between *C pneumoniae* antibodies and cerebrovascular disease.7,8,11,12 We have recently reported that seropositivity for *C pneumoniae* was associated with future risk of acute cerebrovascular disease in a prospective study of male hypertensive patients.13

B-mode ultrasound enables noninvasive visualization and registration of the intima-media complex in large, superficially located arteries, such as the carotid artery. An increased intima-media thickness (IMT) is believed to be an early sign of atherosclerosis, and ultrasound measurement of IMT is increasingly used in clinical research relating to the pathophysiology of atherosclerosis.14–20

The aim of the present study was to use available data from the previously performed prospective study of male hypertensives13 to investigate whether elevated titers of antibodies and immune complexes to *C pneumoniae* were associated with ultrasound findings indicating presence of atherosclerosis in the carotid artery.

Subjects and Methods

**Study Group**

At the Hypertension Unit, Sahlgrenska University Hospital, Göteborg University, Sweden, a group of men with treated hypertension...
and (n=508) at high risk for coronary heart disease was recruited to an open, randomized, parallel-group study of the effects of a comprehensive multiple risk factor modification program, the Risk Factor Intervention Study.\textsuperscript{21} Hypertension was defined as a repeated diastolic blood pressure >100 mm Hg or if the patients already were receiving antihypertensive treatment when referred to the hypertension clinic. Other inclusion criteria were serum cholesterol $\geq 6.5$ mmol/L, and/or smoking, and/or diabetes mellitus. Only one man was treated with lipid-lowering medicine at entry. From this group of 508 men, one third of the patients were randomly selected to take part in an ultrasound study. Of 169 patients randomized to the ultrasound study, 164 patients agreed participate.\textsuperscript{15} The present report is based on the data from that ultrasound study and serological analyses of frozen serum samples obtained at the baseline examination. It was possible to analyze data from 113 patients in whom serum samples had been saved and where good-quality ultrasound recordings of the common, internal, and external carotid arteries were available.

In comparison to the examined group, the group excluded because of missing data (n=51) was characterized by being more obese (body mass index [28.3 k/m$^2$ versus 26.4 k/m$^2$; $P<0.01$]). Otherwise, anthropometric data, blood pressure, and other clinical findings (data not shown) were similar to those in the 113 subjects included in the present study.

The Ethics Committee at Sahlgrenska University Hospital approved the study, and all subjects gave informed consent to participate.

**Ultrasonography**

**Examination Procedure**

Examination was performed with an ultrasound scanner (Acuson 128) equipped with a linear 7-MHz transducer and a transducer aperture of 38 mm. The ECG signal (lead II) was simultaneously recorded to synchronize the image capture to the top of the R wave to minimize variability during the cardiac cycle. The examination included \(\approx 2\) cm of the common carotid artery, the carotid artery bulb, and 1 cm of the internal and external arteries. These regions were scanned longitudinally and transversely to assess the occurrence of plaques. If a plaque was present, a frozen B-mode image of the thickest part of the plaque in the longitudinal view was recorded on videotape. The procedure was repeated 3 times to achieve 3 separate images for analysis. A short sequence of real-time images was also recorded to assist in the interpretation of the frozen images. Pulsed Doppler was used to provide information on velocity of blood flow.\textsuperscript{16}

Images for IMT measurements were recorded from the common carotid artery. At the position of the thickest part of the far wall (visually judged), a frozen longitudinal image was captured and recorded on videotape. The procedure was repeated 3 times to achieve 3 separate images for analysis. Again, a short sequence of real-time images was recorded on videotape to assist in the interpretation of the frozen images.

**Measurement of IMT and Lumen Diameter**

The ultrasound images were analyzed in a newly developed, automated, computerized analyzing system.\textsuperscript{22} IMT was defined as the distance from the leading edge of the lumen-intima interface of the far wall to the leading edge of the media-adventitia interface of the far wall. The measurement of IMT in the carotid artery was made along a 10-mm-long segment in the common carotid artery. The computer calculated the mean and maximum thickness of the intimamedia complex (IMT$_{mean}$ and IMT$_{max}$) of the far wall. Lumen diameter was defined by the distance between the leading edges of the intima-lumen interface of the near wall and the lumen-intima interface of the far wall.\textsuperscript{23}

The coefficient of variation for recording and measurement in the common carotid artery was 10.6% for IMT$_{max}$ and 10.4% for IMT$_{mean}$.\textsuperscript{17}

**Plaque Assessment**

A semiquantitative subjective scale (visually scoring) was used to grade the size of plaques in the 4 locations in the artery region: external and internal carotid arteries, carotid bulb, and the distal part of the common carotid artery. This analysis included plaques in the near and far walls of the vessel. A plaque was defined as a distinct area with an IMT 50% thicker than neighboring sites judged visually. Plaques were graded as grade 0, no plaque; grade 1, 1 or more small plaques (each $<$10 mm); grade 2, moderate-sized plaques (the system, which is used for automated IMT measurements, cannot measure plaque area automatically; the visual plaque grading is made by 1 observer, and the differentiation between grades 1 and 2 was made subjectively in most cases; quantitative measurement of the area was made manually in the computerized analyzing system only when the size of the plaque was not obvious to the observer); and grade 3, large plaques that cause a change in blood flow defined by the pulsed-Doppler curve: peak systolic velocity $>$1.2 m/s at 60 Doppler angle.\textsuperscript{13} The variability of the plaque assessment method was examined by 1 observer who, with an interval of several months, reread the images obtained in 49 men and graded the plaque status unaware of the C pneumoniae serology. The Spearman correlation coefficient was $r=0.96$ between the first and second examinations.

**Serological Analyses**

The analysis of C pneumoniae antibodies was done with the use of a modified immunofluorescence technique, as earlier described.\textsuperscript{13} Sera were diluted 1:32 in PBS, pH 7.4, and tested for IgG, IgA, and IgM antibodies on 21-well antigen slides containing elementary body preparations of Chlamydia psittaci, C pneumoniae, and Chlamydia trachomatis in each well (Laboratory Systems Oy). Sera that were positive in screening tests for IgG were retested and tested in doubling dilutions. Sera positive in screening tests for IgA and/or IgM were absorbed with Gullsofb (Gull Laboratories) at a dilution of 1:16 to remove all IgG, then titrated in doubling dilutions with PBS. Serum dilutions were incubated with antigen slides for 14 to 16 hours at 4° to 8°C, after which slides were gently agitated in three 5-minute changes of PBS and air-dried. Fluorescein isothiocyanate–conjugated rabbit anti-human IgG, IgA, or IgM (Dakopatts) was applied to appropriate wells, and incubation was done for 30 minutes at 37°C. After a renewed washing procedure with three 5-minute changes of PBS, slides were immersed in H$_2$O for 2 minutes and air-dried. Covered slips were mounted with buffer (Gill Laboratories) and glycerol, and slides were read in a Zeiss UV microscope with a ×40 oil immersion lens and a ×10 ocular lens (total magnification ×400). All sera were tested in a blinded fashion by 1 experienced investigator. Control sera routinely used in the laboratory was included in every test run, and tests were only accepted if the control sera titers were within 1 titer step of the earlier calculated mean. The last dilution step to give specific fluorescence was reported as the reciprocal titer. On the basis of Grayston’s suggestions and on earlier experiences, a reciprocal IgG titer of $\geq 512$ and/or an IgA titer of $\geq 64$ were used as lower limits for positive serology.

All serum specimens were investigated for complex-bound IgG antibody to C pneumoniae (IC) after treatment of the serum sample with 7% polyethylene glycol 6000 (PEG) (Janssen Chimica). Equal parts of sera and PEG were mixed, left overnight at +4°C, and centrifuged the next day. Pellets obtained were resuspended and washed twice with 3.5% PEG, then finally resuspended in PBS, pH 7.2, to the same volume as the original serum aliquot. The resuspended specimens were then diluted 1:2 with PBS and tested for C pneumoniae IgG antibodies with use of the species-specific microimmunofluorescence technique. All antibody tests were read with a Zeiss UV microscope with a plane achromatic oil immersion lens at a final magnification of ×400. High titers of circulating immune complexes (cIC) were arbitrarily defined as a reciprocal titer of $\geq 8$.\textsuperscript{13}

On the basis of serological analyses, 4 groups of patients were created: (1) a group (n=57) in which no patient was seropositive to C pneumoniae according to the established cutoff limits (see points 2 to 4 below); (2) a group of patients (n=4) who had positive IgA-antibody titers ($\geq 64$) to C pneumoniae; (3) a group of patients (n=38) who had positive IgG-antibody titers ($\geq 512$) to C pneumoniae.
moniae; and (4) a group of patients (n = 26) who had positive cIC titers (≥8) to C pneumoniae.

As previously reported, these antibody titers remain high after several years of follow-up, indicating persistent infection with C pneumoniae.13

Statistical Analysis
SPSS for Windows 6.1 was used for the statistics. Means and standard deviations for differences between groups were calculated. For comparison between groups, the Mann-Whitney U test was used; for correlation analysis, calculation of Spearman’s correlation coefficient was used. A 2-sided probability value of < 0.05 was regarded as statistically significant.

Results
No patients had IgM antibodies to C pneumoniae. The relation between the different antibodies to C pneumoniae is shown in Figure 1.

Patients With Any Titer Positive to C pneumoniae
In this group, anthropometric data, serum lipids, lipoproteins, blood glucose, blood pressure, heart rate, and smoking habits were similar to the control group with negative serological findings (Table 1).

Mean and maximum far-wall IMT of the common carotid artery were significantly larger (P < 0.05) in the group with any titer positive compared with the group with all titers negative. There were no significant differences in lumen diameter or plaque status between the 2 groups (Table 2).

Patients With Positive IgA Titer to C pneumoniae
The anthropometric data, serum lipids, lipoproteins, blood glucose, blood pressure, heart rate, and smoking habits were similar to the characteristics of the control group (Table 1). Mean far-wall IMT and lumen diameter of the common carotid artery were significantly larger (P < 0.05) in the group with positive IgA titers compared with the group with all titers negative. There were no significant differences in maximum far-wall IMT or plaque status between the 2 groups (Table 2 and Figure 2).

Patients With Positive IgG Titer to C pneumoniae
The anthropometric data, serum lipids, lipoproteins, blood glucose, heart rate, and smoking habits were similar to the characteristics of the control group (Table 1). Mean far-wall IMT and lumen diameter of the common carotid artery were significantly larger (P < 0.05) in the group with positive IgG titers compared with the group with all titers negative. There were no significant differences in lumen diameter or plaque status between the 2 groups (Table 2 and Figure 2).

Patients With Positive Circulating IC-Titer to C pneumoniae
Anthropometric data, serum lipids, lipoproteins, blood glucose, diastolic blood pressure, heart rate, and smoking habits were similar to the characteristics of the control group. However, systolic blood pressure was significantly higher (P < 0.01) in the patient group with elevated cIC titers.

| TABLE 1. Characteristics at Baseline for Each Investigated Group |
|------------------|------------------|------------------|------------------|------------------|
|                  | All Titer Negative | Any Titer Positive | IgA Titer Positive | IgG Titer Positive |
|                  | (n=57)             | (n=56)            | (n=44)            | (n=38)            |
| Mean±SD          | Mean±SD            | Mean±SD           | Mean±SD           | Mean±SD           |
| Age, y           | 65.8±4.1           | 66.1±4.5          | 66.7±4.6          | 65.9±4.5          |
| Height, cm       | 175.4±6.4          | 174.4±6.8         | 174.3±7.0         | 173.8±6.2         |
| Weight, kg       | 81.5±11.1          | 80.2±11.3         | 80.8±12.1         | 80.0±11.1         |
| BMI, kg/m²       | 26.5±3.5           | 26.3±3.2          | 26.5±3.4          | 26.4±3.3          |
| S-Cholesterol, mmol/L | 6.90±1.18       | 6.83±0.89         | 6.75±0.89         | 7.07±0.72         |
| S-HDL, mmol/L    | 1.26±0.36          | 1.31±0.37         | 1.30±0.39         | 1.36±0.38         |
| S-LDL, mmol/L    | 4.75±1.09          | 4.73±0.85         | 4.64±0.85         | 4.86±0.77         |
| S-Triglycerides, mmol/L | 1.90±0.96      | 1.77±0.91         | 1.78±0.96         | 1.88±0.98         |
| B-Glucose, mmol/L | 5.2±1.3            | 5.0±1.4           | 4.9±0.6           | 5.1±1.6           |
| SBP, mm Hg       | 144.8±17.4         | 146.5±20.5        | 143.3±20.1        | 150.7±21.3        |
| DBP, mm Hg       | 78.6±8.9           | 80.5±11.0         | 79.0±11.2         | 82.9±10.2         |
| HR, mm Hg        | 59±10              | 59±10             | 60±10             | 61±10             |
| Current smoker, % | 36.8               | 39.3              | 40.9              | 34.2              |

†P<0.01 compared with the group “All Titer Negative.”
BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; and HR, heart rate.
compared with the group with seronegativity for all titers (Table 1).

Mean and maximum far-wall IMT of the common carotid artery were significantly larger \((P<0.05)\) in the group with elevated cIC titers than in the group with seronegativity for all titers. There were no significant differences in lumen diameter or plaque status between the 2 groups (Table 2 and Figure 2).

**Discussion**

The present results demonstrate that seropositivity for *C pneumoniae* is associated with an increased IMT in the common carotid artery in hypertensive men at high risk for cardiovascular disease. Various combinations of antibody fractions and various cutoff titers to define *C pneumoniae* seropositivity have been used in previous studies.1 We used predefined cutoff limits based on results from several studies,2,3,23 including studies that have related seropositivity to other evidence of active chronic *C pneumoniae* infection.24 –26 Both IgA and IgG antibodies were used because they confer complementary information on the host–*C pneumoniae* interaction. Some patients do not respond to *C pneumoniae* infection with specific antibodies of the IgG and IgA classes.12,26,27

The data from the analysis were based on a baseline examination from a previously conducted prospective study that used frozen serum samples.13,15 We have shown that IgA, IgG, and cIC titers above the suggested cutoff limits remain high over a long period of time, that they are interrelated, and that they are predictors of future cerebrovascular disease.13 We have now observed that elevated antibody titers were associated with an increased IMT in the common carotid artery. There were no differences in anthropometric data, serum lipids, lipoproteins, blood glucose, diastolic blood pressure, heart rate, and smoking habits, which might explain the differences in IMT between patients with and those without seropositivity for *C pneumoniae*. Patients with elevated titers of cIC had higher systolic blood pressure than the control group. We do not know whether this is a random finding or whether it is an expression of underlying disease mechanisms.

A large IMT may not only be caused by atherosclerosis but also by conditions affecting the smooth muscle cells in the media, such as high blood pressure. However, there is an impressive body of evidence that carotid artery IMT is related to atherosclerosis: Previous studies have shown cross-sectional associations between common carotid artery IMT and cardiovascular risk factors,16,20 prevalence of cardiovascular disease,28–30 and involvement of other arterial beds with cardiovascular disease.17,31,32 Five studies have shown that carotid artery IMT predicts cardiovascular diseases such as myocardial infarction and stroke.20,33–35

### Table 2. Arterial Wall Thickness (Intima-Media Thickness of the Far Wall), Lumen Diameter, and Cross-Sectional Intima-Media Area in the Common Carotid Artery for Each Investigated Group

<table>
<thead>
<tr>
<th></th>
<th>All Titers Negative (n=57)</th>
<th>Any Titer Positive (n=56)</th>
<th>IgA Titer Positive (n=44)</th>
<th>IgG Titer Positive (n=38)</th>
<th>cIC Titer Positive (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMT_{mean}, mm</td>
<td>0.92±0.17</td>
<td>1.00±0.22*</td>
<td>1.00±0.22*</td>
<td>1.02±0.20*</td>
<td>1.03±0.24*</td>
</tr>
<tr>
<td>IMT_{max}, mm</td>
<td>1.15±0.24</td>
<td>1.28±0.34*</td>
<td>1.27±0.35</td>
<td>1.30±0.34*</td>
<td>1.34±0.40*</td>
</tr>
<tr>
<td>LD_{mean}, mm</td>
<td>6.68±0.74</td>
<td>6.94±0.96</td>
<td>7.04±0.92*</td>
<td>6.88±0.85</td>
<td>6.67±0.81</td>
</tr>
<tr>
<td>Plaque status, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>44</td>
<td>42</td>
<td>37</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td>Small</td>
<td>24</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Moderate/large</td>
<td>32</td>
<td>42</td>
<td>47</td>
<td>38</td>
<td>40</td>
</tr>
</tbody>
</table>

*P<0.05 compared with the group “All Titers Negative.”

Figure 2. Histograms showing values for each of 4 investigated groups concerning IMT_{mean} (top), IMT_{max} (middle), and LD_{mean} (bottom). IMT_{mean} indicates mean intima-media thickness; IMT_{max}, maximum intima-media thickness; and LD_{mean}, mean lumen diameter.
The fact that no association was found between C pneumoniae seropositivity and plaque occurrence may have the following explanation: The thickness of the intima-media is a continuum with a borderline zone between a thick intima media and an established plaque. A patient with no plaque may still have a thick IMT as an indicator of atherosclerosis. Very small plaques may in addition be difficult to detect. Thus, it is difficult to obtain an exact description of plaque status. The consequence is that IMT, as a continuous variable, is associated with a higher power than plaque status, as a categorical variable, when assessing relations between the atherosclerotic process and risk factors or clinical outcome.

Our interpretations of the data are that the antibody titer levels chosen indicate persistent infection with C pneumoniae and that this is associated with atherosclerotic changes in the carotid artery and the future risk of cardiovascular disease. It has recently been reported that endothelial cytotoxicity can be caused by antibodies to the chlamydial HSP60 (heat shock protein). In agreement with our observations, one earlier study has demonstrated a positive association between seropositivity for C pneumoniae and the presence of asymptomatic carotid artery atherosclerosis determined by B-mode ultrasound.

C pneumoniae has been proposed to affect and contribute to the mechanisms involved in atherosclerotic disease process. One important phase of the process is that the organism must gain entry to the cells of the arterial wall. Initial damage to the endothelial cells in the arterial wall may be caused by an array of mechanisms, for example, blood flow shear stress, free radicals, oxidized LDL, inflammatory mediators, infection, immune complexes, or damage from smoking. In vitro work has shown that C pneumoniae is capable of infecting, surviving, and multiplying in endothelial cells, smooth muscle cells, and macrophages, all constituents of the arterial wall. Recent reports have shown that C pneumoniae infection stimulates endothelial proliferation and may cause inflammation and thrombosis.

It is thought that C pneumoniae may be phagocytosed by alveolar macrophages in the lung after a chronic infection and transported by the blood to the subendothelial region through the injured endothelium of the artery. A chronic infection of C pneumoniae in macrophages is believed to enhance the proliferative and inflammatory process of atherosclerosis by inducing production of cytokines and lipoproteins. It has recently been shown that macrophages infected with C pneumoniae will degenerate to foam cells.

Several studies have shown that C pneumoniae organisms are frequently found in atherosclerotic lesions in coronary arteries, aorta, and carotid arteries obtained from autopsy and endarterectomy specimens. However, there are also negative studies in which no relations among C pneumoniae infection, atherosclerosis, or atherosclerotic disease have been observed, although these serological analyses did not include the specific IgA titers or measurement of immune complexes.

In summary, the present study showed that seropositivity for C pneumoniae, indicating persistent infection, was associated with an increase of the IMT in the common carotid artery. The study design does not allow any conclusion of causality. There was no relationship between the occurrence of atherosclerotic plaques and elevated titers of antibodies to C pneumoniae.

Acknowledgments

This study was supported by grants from the Swedish Medical Research Council (B92-19X-00937-01A, B93-19X-00937-02B, B94-19X-00937-02B, B95-19X-00937-04B), the Swedish Heart and Lung Foundation, the Swedish Hypertension Society and King Gustaf and Queen Victoria Foundation, AstraZeneca AB, Mölndal, Sweden. We thank Jessica Nääs for excellent technical assistance.

References

38. Ward ME. The immunobiology and immunopathology of chlamydial infections. APMIS. 1995;103:769–796.


**Chlamydia pneumoniae Seropositivity Is Associated With Carotid Artery Intima-Media Thickness**

Caroline Schmidt, Johannes Hulthe, John Wikstrand, Håkan Gnarpe, Judy Gnarpe, Stefan Agewall and Björn Fagerberg

*Stroke*. 2000;31:1526-1531
doi: 10.1161/01.STR.31.7.1526

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/31/7/1526

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/