ABSTRACT: Background: Seroepidemiological studies have shown an association between raised antibody titres against *Chlamydia pneumoniae*, and carotid atherosclerosis or stroke. However, direct evidence for a causal link between arterial infection with *C. pneumoniae* and carotid disease remains weak. We hypothesized that long-term follow-up of patients with pathologically-proven arterial *C. pneumoniae* infection might provide further insight into the role of *C. pneumoniae* in carotid atherosclerosis. Methods: We followed a cohort of 70 carotid endarterectomy patients for ipsilateral restenosis, contralateral progression, and all-cause mortality (four year median follow-up period). All patients had presence or absence of *C. pneumoniae* in their carotid plaques documented by immunohistochemistry after endarterectomy. A survival function was generated and the log-rank test was used to assess the difference in survival between subjects with and without documented chlamydial infection in their plaque. Results: Baseline demographic and cardiovascular risk factors were similar between the two groups, and survival analysis demonstrated no difference (p>0.05) in all-cause mortality, or all-cause mortality combined with restenosis and progression. Conclusions: Our data finds no causal role for *C. pneumoniae* in restenosis or progression of carotid disease or mortality in this patient population with advanced carotid atherosclerosis.

RÉSUMÉ: *Chlamydia pneumoniae* et athérosclérose après endartérectomie carotidienne. Introduction: Des études séréroépidémologiques ont montré une association entre un taux élevé d’anticorps contre *C. pneumoniae* et l’athérosclérose carotidienne ou l’accident vasculaire cérébral. Cependant, les preuves directes qu’il existe un lien causal entre l’infection artérielle à *C. pneumoniae* et la maladie carotidienne demeurent faibles. Nous avons émis l’hypothèse que le suivi à long terme de patients porteurs d’une infection artérielle à *C. pneumoniae* prouvée en anatomo-pathologie pourrait fournir d’autres indices sur le rôle de *C. pneumoniae* dans l’athérosclérose carotidienne. Méthodes: Nous avons suivi une cohorte de 70 patients ayant subi une endartérectomie carotidienne, pour resténose ipsilatérale, progression contralatérale et mortalité toute cause (suivi médian de quatre ans). Chez tous les patients, la présence ou l’absence de *C. pneumoniae* dans les plaques carotidiennes a été documenté par immunohistochimie après l’endartérectomie. La différence dans la survie entre les sujets avec et sans infection à *C. pneumoniae* dans leurs plaques a été évaluée en générant une fonction de survie et en utilisant le test du log-rank. Résultats: Les données démographiques et les facteurs de risque étaient similaires entre les deux groupes et l’analyse de survie n’a pas montré de différence (p > 0,05) dans la mortalité toute cause ou la mortalité toute cause combinée à la resténose et à la progression. Conclusions: Nos données ne sont pas en faveur d’un rôle causal de *C. pneumoniae* dans la resténose ou la progression de la maladie carotidienne ou dans la mortalité dans cette population de patients porteurs d’athérosclérose carotidienne sévère.

Atherosclerosis is increasingly viewed as a disease with a strong inflammatory component. Following an initial report of higher antibody titers in patients with acute or chronic coronary artery disease,1 *Chlamydia pneumoniae* has been shown to be commonly present in the human arterial tree, preferentially localized within atheromatous vascular tissue.2,3 Evidence exists for an association between infection with *C. pneumoniae* and carotid atherosclerosis: seroepidemiological studies have linked
antibody titers to both ultrasonographic\textsuperscript{4–9} and clinical\textsuperscript{10–14} manifestations of carotid disease, and \textit{C. pneumoniae} has been detected in carotid artery specimens by molecular biological,\textsuperscript{1,4–21} immunohistochemical,\textsuperscript{2,15,19,22–26} and cell culture\textsuperscript{23,27} methods. However, no direct evidence exists to show that \textit{C. pneumoniae} causes atherosclerosis. Moreover, the observed associations have been disputed by other investigators who have not detected \textit{C. pneumoniae} in carotid arteries\textsuperscript{20} or have not observed an association between \textit{C. pneumoniae} antibody titers and cerebrovascular ischemia\textsuperscript{20} or carotid atherosclerosis on ultrasound.\textsuperscript{11,30,31}

Seroepidemiological studies investigating the link between \textit{C. pneumoniae} infection and future risk of carotid atherosclerosis over time have been contradictory.\textsuperscript{8,9,12,29} Given the high proportion of positive antibody titers found in study sample populations, serology may be a less specific indicator of vascular (versus widespread pulmonary) infection with \textit{C. pneumoniae} in the general population.\textsuperscript{19} Indeed, there is no correlation between the serological inference of vascular \textit{C. pneumoniae} infection via raised antibody titres, and the direct demonstration of \textit{C. pneumoniae} within atherosclerotic plaques as detected by polymerase chain reaction\textsuperscript{14,19,32,33} or immunohistochemical methods.\textsuperscript{19,23}

We followed a cohort of patients with previously demonstrated \textit{C. pneumoniae} infection of their carotid plaque,\textsuperscript{23} to evaluate whether direct evidence of the existence of \textit{C. pneumoniae} within the carotid artery could predict future development of clinically significant atherosclerotic carotid disease or death within this group over time.

\textbf{Methods}

\textbf{Study Subjects and Investigations}

Study subjects were enrolled from patients undergoing carotid endarterectomy by a single surgeon at a university-affiliated hospital between Feb. 1994 – Feb. 1996. The study was approved by the Research Ethics Board at St. Michael’s Hospital, and all participating patients provided informed consent. A previous report described the baseline clinical and pathological characteristics of this cohort but did not provide long-term follow-up.\textsuperscript{23} The patients were followed for a median of four years to assess mortality, recurrence of ipsilateral disease, progression of contralateral disease, and occurrence of new neurological events. New neurological events were defined as the occurrence of any new transient or permanent neurological deficit as determined by patient history and/or physical exam. Patients were contacted by telephone and reassessed in clinic by repeat carotid duplex ultrasonography and baseline biochemistry. Between endarterectomy and subsequent participation in the study, patients were followed with routine care, and all interval carotid ultrasound studies were retrieved and examined for evidence of restenosis or progression of carotid atherosclerotic disease. Demographic data and cardiovascular risk factors were verified by patient interview, chart review, and contact with family and/or attending physician. All new neurological events, regardless of duration or presumed cause, were assessed at clinical follow-up. All outcomes were collected by investigators blinded to patient \textit{C. pneumoniae} status.

\textbf{Pathological Examination}

Pathological specimens were examined by a single pathologist as previously described.\textsuperscript{25} Briefly, all specimens were fixed in 10% buffered formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination. Movat’s pentachrome stain was also used in the examination of plaque morphology. Immunohistochemical staining was performed on paraffin-embedded sections according to the avidin-biotin-peroxidase method. Chlamydial antigens were detected by reacting arterial tissue sections with the \textit{C. pneumoniae} specific monoclonal antibodies TT-401 (IgG; Washington Research Foundation, at dilution 1:100) and RR-402 (IgG; Dako, at dilution 1:100) and the genus-specific monoclonal antibody CF-2 (IgG; Washington Research Foundation, at dilution 1:600). The Chlamydia-Cel-Pn antibody was used in our previous study\textsuperscript{23} but is no longer available or used in literature. Specimens were thus considered positive for this study only when staining could be unambiguously verified using existing antibodies as described. Our current staining protocol is now consistent with the majority of publications describing immunohistochemical detection of \textit{C. pneumoniae}, and uses a panel of antibodies recently recommended by a consensus report of the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada).\textsuperscript{34} Results using this methodology (51% positive samples) compare favourably with those of our previous paper (54% positive samples, using the TT-401 antibody). Statistical analysis of \textit{C. pneumoniae} staining beyond that of a binary variable (present or absent) was precluded by our limited sample size and thus was not attempted. Positive controls consisted of human vascular tissues. Negative controls used mouse ascitic fluid (IgG) or phosphate-buffered saline instead of the primary antibody.

\textbf{Biochemical Examination}

Patients were asked to fast overnight prior to donating a blood sample from which fasting serum glucose, homocysteine, hemoglobin A1C, and fasting lipid profile were determined.

\textbf{Ultrasound Examination}

Carotid duplex doppler ultrasound studies (baseline and follow-up) were performed by a single operator in an ICAVL-accredited laboratory (Intersocietal Commission for the Accreditation of Vascular Laboratories), using a color duplex scanner (128XP, Acuson Inc., Mountainview, CA). The common, internal and external carotid arteries were examined bilaterally using an angle of insonation of 60\textdegree{} and measurements taken at proximal, mid, and distal sites in the internal and common carotids in accordance with published criteria.\textsuperscript{35} Ultrasonographic stenoses were grouped into the clinically relevant categories of minimal (<50%), moderate (50-69%), severe (70-99%) and occluded (100%). “Restenosis” was defined as an increase by at least one ultrasound stenosis category between baseline (postoperative) and follow-up ultrasound of the operated (ipsilateral) internal carotid artery. “Progression” was defined as an increase by at least one ultrasound stenosis category between baseline (peri-operative) and follow-up ultrasound of the contralateral internal carotid artery. If the contralateral carotid artery was occluded at study
entry (the time of ipsilateral carotid endarterectomy) progression was considered negative.

**Data Analysis**

Our sample was restricted to those patients initially described in our previous paper and is, therefore, a sample of convenience. Data are reported in frequency tables. We examined the incidence of ipsilateral recurrence of carotid atherosclerosis (“restenosis”) and progression of contralateral carotid atherosclerosis (“progression”) within both groups as revealed by duplex ultrasound, all-cause mortality, the occurrence of new neurological events and a combined outcome of all-cause mortality, restenosis, and progression of carotid atherosclerosis. A survival function was generated using the date of carotid endarterectomy as the index event. All patients who had not suffered an outcome event were censored at the date of final clinical follow-up. The log-rank test was used to assess the difference between patients with and without *C. pneumoniae* positive carotid plaques. A Cox proportional hazards model was developed to adjust for baseline hypertensive status and age. Proportions were assessed using chi-square analysis or Fisher’s exact test and continuous variables were assessed using the Mann-Whitney rank sum test for unequal variances.

**Figure 1:** Immunohistochemistry for *C. pneumoniae* elementary bodies (RR402 antibody, Dako). A: Carotid plaque with thick fibrous cap showing negative staining for *C. pneumoniae* along with fewer foam cell macrophages (hematoxylin counterstain, original magnification 100X), B: Higher power view of boxed region in A (original magnification 200X), C: *C. pneumoniae* antigens (arrow) within foam cell macrophages in vulnerable area of carotid plaque (hematoxylin counterstain, original magnification 100X), D: Higher power view of boxed region in C (original magnification 250X). (CP = C. pneumoniae)
Six patients from our initial cohort were lost to follow-up, leaving 70 patients (92%) who were followed for a mean of 4.0 ± 1.1 years (mean ± standard deviation). There were 48 men and 22 women in this group.

*C. pneumonia* staining, verified using an antibody panel including TT-401, RR-402, and CF-2 antibodies, was absent from plaques from 34 patients, and present in plaques from 36 patients. All patients showed severe atherosclerosis on histopathology, irrespective of *C. pneumonia* staining (Figure 1). A trend towards increased plaque thrombus (64% vs. 53%) and ulceration (25% vs. 12%) in patients positive for *C. pneumonia* did not reach statistical significance.

Baseline demographics and incidence of cardiovascular risk factors were similar between the two groups (Table 1). Current serum biochemistry in a subset from each group showed total cholesterol, HDL, LDL, homocysteine, and fasting blood sugar.

### Table 1: Demographic data for patients with and without *C. pneumonia* in carotid plaques

<table>
<thead>
<tr>
<th></th>
<th><em>C. pneumonia</em> negative (n=34)</th>
<th><em>C. pneumonia</em> positive (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death or last follow-up (mean ± SD, years)</td>
<td>71.1 ± 9.7</td>
<td>72.9 ± 5.8</td>
<td>0.67</td>
</tr>
<tr>
<td>Age at carotid endarterectomy (mean ± SD, years)</td>
<td>67.4 ± 10.1</td>
<td>69.1 ± 5.5</td>
<td>0.66</td>
</tr>
<tr>
<td>Symptomatic carotid endarterectomy</td>
<td>85.3%</td>
<td>77.8%</td>
<td>0.62</td>
</tr>
<tr>
<td>Hypertension</td>
<td>64.7%</td>
<td>66.7%</td>
<td>0.94</td>
</tr>
<tr>
<td>Diabetes</td>
<td>23.5%</td>
<td>19.4%</td>
<td>0.90</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>52.9%</td>
<td>69.4%</td>
<td>0.24</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>8.8%</td>
<td>5.6%</td>
<td>0.67</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>29.4%</td>
<td>25.0%</td>
<td>0.88</td>
</tr>
</tbody>
</table>
| History of ischemic heart disease:  
  none or asymptomatic | 44.1% | 55.6% | 0.47 |
  angina | 17.6% | 25.0% | 0.65 |
  myocardial infarction | 38.2% | 19.4% | 0.14 |
| History of smoking:  
  none | 20.6% | 11.1% | 0.48 |
  former | 64.7% | 69.4% | 0.87 |
  current | 14.7% | 19.4% | 0.84 |
| Length of follow-up (median, years) | 3.9 | 4.1 | 0.41 |

### Table 2: Serum biochemistry data for patients with and without *C. pneumonia* in carotid plaques

<table>
<thead>
<tr>
<th></th>
<th><em>C. pneumonia</em> negative (mean ± SD, n=11)</th>
<th><em>C. pneumonia</em> positive (mean ± SD, n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (normal* &lt; 2.20 mmol/L)</td>
<td>1.83 ± 0.61</td>
<td>1.68 ± 0.78</td>
<td>0.45</td>
</tr>
<tr>
<td>Cholesterol (normal &lt; 5.20 mmol/L)</td>
<td>5.35 ± 1.28</td>
<td>5.23 ± 0.76</td>
<td>0.99</td>
</tr>
<tr>
<td>HDL (normal &gt; 0.90 mmol/L)</td>
<td>1.04 ± 0.44</td>
<td>1.12 ± 0.37</td>
<td>0.54</td>
</tr>
<tr>
<td>LDL (normal &lt; 3.90 mmol/L)</td>
<td>3.47 ± 1.15</td>
<td>3.34 ± 0.75</td>
<td>0.99</td>
</tr>
<tr>
<td>HgA1c (normal = 0.035-0.065)</td>
<td>0.056 ± 0.005</td>
<td>0.061 ± 0.012</td>
<td>0.04</td>
</tr>
<tr>
<td>Homocysteine (normal = 4-19 µmol/L)</td>
<td>13.0 ± 5.3</td>
<td>13.8 ± 5.1</td>
<td>0.67</td>
</tr>
<tr>
<td>Fasting blood glucose (normal &lt; 7 mmol/L)</td>
<td>5.79 ± 0.88</td>
<td>5.91 ± 1.68</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* normal values determined by laboratory standards

### Table 3: Neurological events in patients with and without *C. pneumonia* in carotid plaques

<table>
<thead>
<tr>
<th></th>
<th><em>C. pneumonia</em> negative</th>
<th><em>C. pneumonia</em> positive</th>
</tr>
</thead>
</table>
| TIA:  
  ipsilateral carotid | 2 (1)* | 1 |
  other | 2 | 1 (1) |
| Stroke:  
  ipsilateral carotid | 1 | 0 |
  other | 4 (2) | 1 |

* values in parentheses denote number of cases associated with changes on bilateral carotid ultrasonography

TIA = transient ischemic attack

Results

Six patients from our initial cohort were lost to follow-up, leaving 70 patients (92%) who were followed for a mean of 4.0 ± 1.1 years (mean ± standard deviation). There were 48 men and 22 women in this group. *C. pneumoniae* staining, verified using an antibody panel including TT-401, RR-402, and CF-2 antibodies, was absent from plaques from 34 patients, and present in plaques from 36 patients. All patients showed severe atherosclerosis on histopathology, irrespective of *C. pneumoniae* staining (Figure 1). A trend towards increased plaque thrombus (64% vs. 53%) and ulceration (25% vs. 12%) in patients positive for *C. pneumoniae* did not reach statistical significance.

Baseline demographics and incidence of cardiovascular risk factors were similar between the two groups (Table 1). Current serum biochemistry in a subset from each group showed total cholesterol, HDL, LDL, homocysteine, and fasting blood sugar.
to be similar between the two groups and within normal limits (Table 2). HgA1c, while within normal limits in both groups, was higher in patients positive for \textit{C. pneumoniae} on univariate statistical analysis. It is unlikely that this would bias our results towards rejection of a significant effect of \textit{C. pneumoniae} on patient outcome.

Among 34 patients negative for \textit{C. pneumoniae}, there were eight deaths (three from cardiovascular causes), five cases of restenosis, five cases of progression, and three new neurological events over 4.1 years median follow-up. Approximately one third of all new neurological events were associated with ultrasonographically demonstrated restenosis or progression of carotid atherosclerosis on follow-up (Table 3).

There was no difference between the proportion of patients in each group who died, or suffered restenosis, progression, or neurological events (p = 0.68, 0.31, 0.43, and 0.09 respectively). Survival analysis showed no statistical difference between the group with positive \textit{C. pneumoniae} staining and the group without. This was true with all-cause mortality as the endpoint (including cardiovascular deaths) or with the combined outcome of death, restenosis or progression (Figures 2, 3). Multivariable adjustment using Cox proportional hazards modeling did not change these results.

**DISCUSSION**

The presence of \textit{C. pneumoniae} within the atherosclerotic plaque resected at carotid endarterectomy did not predict the subsequent combined risk of mortality, restenosis, or progression in a defined cohort of high-risk patients over a four-year follow-up period. This report is novel in its examination of the future risk of carotid atherosclerosis over time as it relates to the immunohistochemically proven presence of \textit{C. pneumoniae} within arterial walls rather than that inferred via serology. Our results are consistent with others showing no association between the presence of \textit{C. pneumoniae} in carotid endarterectomy samples and plaque instability as assessed by transcranial doppler embolization rates or computed tomographic evidence of ipsilateral middle cerebral artery strokes.20

\textit{C. pneumoniae} infection is common in the general community, and raised antibody titers are frequently found in the general population (IgG: 58-83%, IgA: 30-68%)5,13,19,30 and among patients suffering from stroke or TIA (IgG: 45-81%, IgA: 30-47%).9,10,13,29 Little correlation has been found between general serology and the specific presence or absence of \textit{C. pneumoniae} in atherosclerotic plaques in most investigations,14,19,32,33 including patients in this study.23 The bulk of evidence linking \textit{C. pneumoniae} to carotid atherosclerosis stems from the association observed in cross-sectional studies between raised serum antibody titers and ultrasound evidence of subclinical atherosclerosis in the general population,4,5,7,8 among patients presenting with symptomatic cerebrovascular ischemia,10,11,13,14 or in groups with specific atherosclerosis risk factors.6,9,36 However, the published evidence is not unanimous.11,29-31 The interpretation of cross-sectional studies is prone to the fallacy of reverse causality. Instead of concluding that \textit{C. pneumoniae} is a causative factor in atherosclerosis, an alternate conclusion is that atherosclerotic plaques are prone to infection by \textit{C. pneumoniae}.

Few laboratories have succeeded in culturing viable \textit{C. pneumoniae} from carotid samples,15,27 and studies that have employed reverse transcription-polymerase chain reaction to detect bacterial RNA as a sign of viable pathogen have published contradictory results.17,21,28,37 It thus remains possible that much of the detected \textit{C. pneumoniae} in atherosclerotic plaques represents either antigen deposition38 or nonviable or
nonmetabolically active bacteria.21 *C. pneumoniae* could thus be an “innocent bystander” which reaches atherosclerotic plaques via infected circulating macrophages and monocytes following initial pulmonary inoculation.

Clinical studies examining the future risk of stroke stratified by *C. pneumoniae* serology have also been conflicting. Among hypertensive men, increased serum titers of antibodies against *C. pneumoniae* were associated with an increased risk of stroke during 6.5 years of follow-up.12 Increased *C. pneumoniae* IgA titers were associated with early (but not late) carotid atherosclerosis.8 Seropositivity to *C. pneumoniae* predicted a faster increase in carotid intima-media thickness on ultrasound among patients presenting with their first ischemic cerebrovascular event;9 however, seropositive patients had a significantly higher baseline intima-media thickness. Others have failed to confirm these findings. Adjustment for other cardiovascular risk factors in one study resulted in an association between positive *C. pneumoniae* IgG titers and a paradoxically decreased risk of cerebral infarction.29 Similar negative results have been reported in the cardiac literature.30 While smaller trials initially suggested a therapeutic benefit to macrolide antibiotic therapy aimed at elimination of *C. pneumoniae*21 a larger more recent randomized trial of 300 patients seropositive for *C. pneumoniae* failed to show any difference in incidence of cardiovascular events following six months of treatment with azithromycin.42

Our study has notable limitations and may not be generalizable. The study sample was highly selected because patients who have undergone carotid endarterectomy are liable to have advanced atherosclerotic disease and are at the highest risk of recurrent vascular events. Although all were operated upon by a single surgeon for significant carotid stenosis according to NASCET and ACAS criteria, the rates of restenosis and a single surgeon for significant carotid stenosis according to NASCET and ACAS criteria, the rates of restenosis and progression in our study correspond well with other reports.33,41 We restricted our analysis to the clinically relevant endpoints of death, restenosis, and progression. *C. pneumoniae* may have differentially affected each endpoint. The power of the study to detect a small effect size was low.

This study adds to the body of knowledge probing the association between *C. pneumoniae* and atherosclerotic disease. Our data do not support a large effect size for *C. pneumoniae* being a causal agent in carotid artery restenosis, progression, or mortality after carotid endarterectomy.

**Acknowledgements and Funding**

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