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Chlamydia pneumoniae Serology Is Associated With Thrombosis-Related but Not With Plaque-Related Microembolization During Carotid Endarterectomy

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Background and Purpose—*Chlamydia pneumoniae* has repeatedly been associated with atherosclerotic disease. Our study was designed to clarify whether this association is based on *C pneumoniae*-induced transformation of a stable into an unstable atherosclerotic plaque or on stimulation of hypercoagulability leading to increased thrombotic arterial occlusions by *C pneumoniae* infection. Transcranial Doppler ultrasonographic monitoring of the middle cerebral artery during carotid endarterectomy offers the opportunity to study, before removal of the plaque, atherothrombotic emboli dislodging from an unstable carotid plaque (plaque-related emboli) and emboli related to (excessive) thrombus formation at the endarterectomy site after removal of the plaque and restoration of flow (thrombosis-related emboli).

Methods—*C pneumoniae* IgA ($\geq 1/16$) and IgG ($\geq 1/64$) seropositivity was assessed in 53 patients with symptomatic carotid artery disease undergoing carotid endarterectomy. The removed carotid plaques were studied histologically to assess plaque instability.

Results—Plaque- and thrombosis-related emboli were registered in 43 patients with an adequate transtemporal window. IgA seropositivity (58%) was associated significantly with thrombosis-related embolization ($P=0.030$) but not with plaque-related embolization or with histological plaque instability.

Conclusions—*C pneumoniae* serology is associated with microembolization after endarterectomy and restoration of flow. Since these microemboli represent platelet aggregations and are related to cerebrovascular complications, our data suggest that *C pneumoniae* infection contributes to cerebrovascular events in patients with carotid artery disease through stimulation of thrombosis. (*Stroke*. 2002;33:1249-1254.)

Key Words: carotid artery diseases ■ infection ■ intracranial embolism and thrombosis ■ thrombosis

An increasing body of evidence suggests that infections play a role in the initiation and progression of atherosclerotic disease.¹ Particularly, chronic infections with the gram-negative, intracellular bacterium *Chlamydia pneumoniae* have been linked to the development of vascular disease.² *C pneumoniae* seropositivity has been associated with acute and chronic coronary artery disease,^{3,4} early and advanced asymptomatic carotid lesions,⁵⁻⁷ and stroke.^{8,9} In addition, it has been shown that *C pneumoniae* can infect all cellular components of the vascular wall, inducing proatherogenic changes.¹⁰⁻¹⁴ These observations suggest that *C pneumoniae* infections could contribute to the development of atherosclerosis, leading to atherosclerotic plaque growth and increased arterial stenosis, and that *C pneumoniae* infection may also play a role in the formation of an unstable atherosclerotic plaque, leading to acute cardiovascular and/or cerebrovascular events.¹⁵ Plaque rupture and thrombosis are

the main mechanisms of acute arterial occlusion leading to an atherosclerotic event such as myocardial infarction or stroke.¹⁶ Although *C pneumoniae* infection could theoretically contribute to both processes, it is unclear whether the earlier reported association between *C pneumoniae* serology and acute cardiovascular and cerebrovascular events is based on stimulation of plaque- and/or thrombosis-related mechanisms by *C pneumoniae* infection.

Cerebrovascular events related to carotid artery disease are caused in the majority of cases by atherothrombotic emboli dislodging from the carotid plaque.¹⁷ Fibrous cap rupture and luminal thrombosis are the histological manifestations of carotid plaque instability¹⁸ and are the main source of cerebral microembolic signals (MES), detected by transcranial Doppler ultrasonographic (TCD) monitoring of the ipsilateral middle cerebral artery, in patients with high-grade carotid artery stenosis.¹⁹ MES not only correlate well with

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TABLE 1. Patient Characteristics

n	53
Age, mean (SD), y	67 (8)
Female/male	11/42
Current smoker	57%
Dyslipidemia*	69%
Hypertension†	60%
Diabetes	15%
TCD monitoring	81%
Intraoperative shunting	19%
Patch closure of arteriotomy	49%
Preoperative anticoagulation	98%
Discontinuation of preoperative anticoagulation	62%

*Fasting cholesterol level >6.5 mmol/L and/or triglyceride level >1.95 mmol/L and/or use of antilipemic medication.

†Systolic blood pressure >160 mm Hg and/or diastolic blood pressure >95 mm Hg and/or use of antihypertensive medication.

histological determinants of plaque instability but are also associated with clinical manifestations of plaque instability such as strokes and transient ischemic attacks (TIAs).^{20,21} Thrombus formation at the endarterectomy and clamping sites after carotid endarterectomy (CEA),²² resulting in either thrombotic occlusion of the carotid artery or downstream embolization of intracranial arteries, is the main cause of postoperative stroke and TIA.²³ Hence, MES occurring during the dissection phase of CEA are associated with carotid plaque instability, whereas MES observed after endarterectomy and restoration of flow, as well as in the early postoperative period, are related to excessive platelet aggregation and thrombus formation at the endarterectomy and clamping sites. TCD monitoring during CEA therefore offers the unique opportunity to study in vivo plaque instability and thrombosis separately by distinguishing between plaque-related MES (pMES) (during dissection) and thrombosis-related MES (tMES) (after endarterectomy and restoration of flow).

To investigate the relation between *C pneumoniae* infection and atherothrombotic disease, we studied the association between *C pneumoniae* serology and carotid plaque histology as well as perioperative microembolization in patients undergoing CEA for symptomatic carotid artery disease, with special attention to both pMES and tMES.

Subjects and Methods

Sixty patients with symptomatic carotid artery disease screened consecutively at the surgical outpatient department of Maastricht University Hospital who had been found eligible for CEA were asked to participate in this study. Patients who failed to provide informed consent (n=4) or underwent a combined CEA and coronary revascularization procedure (n=3) were excluded. Amaurosis fugax was the presenting symptom of 15 patients, 24 had TIAs, 11 had suffered a stroke, and 3 patients presented with general cerebral hypoperfusion without focal neurological symptoms. All patients had a significant (>70%), symptomatic, carotid artery stenosis.²⁴ Patient characteristics are given in Table 1. The study was approved by the Medical Ethical Committee of Maastricht University Hospital.

Carotid Artery Endarterectomy

CEA was performed with the patient under normocarbic, normotensive general anesthesia with the use of systemic heparinization (1 mg

heparin per kilogram body weight). All but 1 patient had been on preoperative anticoagulation therapy; 49 were on acetylsalicylic acid, and 3 were on coumarin derivatives. Thirty-three patients stopped the anticoagulation 3 to 10 days before surgery. The endarterectomy was performed through a longitudinal arteriotomy. During the entire procedure, TCD monitoring of blood flow velocity and of MES in the ipsilateral middle cerebral artery was performed if an adequate transtemporal window was present (n=43). A Javid shunt was used selectively in case of imminent hypoperfusion (n=10), as suggested by >70% decrease of middle cerebral artery blood flow velocity.²⁵ After completion of the endarterectomy, the arteriotomy was closed with a primary suture (n=27) or a patch (n=26; 24 venous, 1 Dacron, 1 polytetrafluoroethylene patch) at the discretion of the surgeon. The carotid atheroma harvested during operation was immediately processed for microscopic evaluation. During dissection, 10 mL venous blood was obtained for serological studies.

MES Detection During CEA

During CEA, TCD monitoring of the ipsilateral middle cerebral artery was performed through the transtemporal approach with a 2-MHz probe fixed with a metal frame (Multidop X 4, DWL). A satisfactory transtemporal window was present in 43 of the 53 patients. The Doppler signal was recorded on a 2-channel DAT recorder for additional offline analysis. No automatic MES detection system was used. The gain was set to the lowest possible value with the sweep time as fast as possible. The burst length equaled approximately 7.5 mm. MES were evaluated online during the surgical procedure by a technician and additionally offline by an experienced listener (W.H.M.). The criteria for MES used were (1) the typical sound and (2) the appearance as a short-lasting intensity increase in the fast Fourier transform in agreement with the report of a consensus committee.²⁶ pMES were counted during dissection of the neck before the carotid artery was cross-clamped, and tMES were registered after removal of the plaque and restoration of the flow (>5 minutes after clamp release). Patients with ≥ 2 pMES per hour were considered pMES+,²⁰ and patients with ≥ 6 tMES per hour were considered tMES+.²⁷

Carotid Plaque Histology

CEA specimens harvested during operation were divided into multiple macroscopic parts. A macroscopic sketch of the plaque was drawn with attention to the orientation of the different parts. The odd parts were snap-frozen in liquid nitrogen and stored at -80°C for future analysis. The even parts were formalin fixed and paraffin embedded. A representative 5- μm section of each paraffin-embedded piece of the carotid atheroma was stained with hematoxylin and eosin for characterization of plaque (in)stability. Histological plaque instability, defined as the presence of an organized luminal thrombus and/or a ruptured fibrotic cap,¹⁸ was assessed by 2 independent investigators (T.V. and R.E.) blinded for the infectious status of the patients. In case of disagreement between the 2 independent assessments, the plaque was reevaluated by the 2 investigators to reach a consensus.

C pneumoniae Serology

Venous blood drawn during operation was immediately centrifuged for 10 minutes at 1200 rpm and 4°C . Serum was stored at -20°C until determination of *C pneumoniae* serology. IgA and IgG antibodies were determined by means of an enzyme immunoassay (Labsystems). Titers were calculated from the optical density readings according to the manufacturer's instructions. *C pneumoniae* IgA and IgG seropositivity was defined as an IgA titer $\geq 1/16$ and IgG titer $\geq 1/64$, respectively.

Statistical Analysis

We used SPSS 10.0 for Windows for statistical analysis. The Fisher's exact test was used for comparison of prevalence of risk factors and patient characteristics between seropositive and seronegative patients, for comparison of tMES/pMES with plaque instabil-

ity, and for analysis of the association between tMES, pMES, or plaque instability and *C pneumoniae* serology. To identify possible confounders, the independent effect of sex, age, smoking, hypertension, dyslipidemia, diabetes, and discontinuation of preoperative anticoagulation on tMES/pMES was evaluated with univariate logistic regression analyses. Parameters that were associated significantly with tMES/pMES in these analyses were entered as potential confounders in a multivariate logistic regression model to study the association between tMES/pMES and *C pneumoniae* serology. A 2-sided probability value of <0.05 was regarded as statistically significant.

Results

Fifty-three patients with symptomatic carotid artery stenosis were included in this study. The duration of operation averaged 110 minutes (range, 73 to 189 minutes). The average clamping and/or shunting time was 35 minutes (range, 20 to 60 minutes). MES were detected during dissection and after endarterectomy and restoration of flow, with the omission of those in the first 5 minutes after cross-clamp release to avoid false-positive registration of gaseous MES. The mean pMES detection time was 64 minutes (range, 30 to 70 minutes), and the mean tMES detection time was 23 minutes (range, 15 to 42 minutes).

One patient developed neurological symptoms the day after the operation, which on reexploration of the carotid artery could be attributed to a fresh thrombus at the site of the primary arterial suture. The thrombus was removed, and the arteriotomy was closed with a venous patch. After that the patient made an uneventful recovery and did not suffer any permanent disabilities. Three more patients developed signs of peripheral facial nerve lesion and recovered fully within a month after hospital discharge.

Carotid Plaque Histology

Carotid atheroma was available for histological analysis in all patients. Signs of plaque instability, ie, an organized luminal thrombus and/or ruptured fibrotic cap, were seen in 23 patients (43%). The remaining patients had advanced but stable atherosclerotic lesions, consisting of a thick fibrous cap overlying a lipid/necrotic core with occasional intraplaque hemorrhage.

Microembolization During CEA

A transtemporal window was present in 43 patients (81%). Patients with no cranial window were older than patients with a suitable window (mean age, 73 versus 65 years; $P=0.003$), which might reflect age-related temporal bone ossification. However, prevalence of cardiovascular risk factors, preoperative diagnosis, operative techniques, plaque histology, and *C pneumoniae* serology were comparable in both groups.

Twelve patients had ≥ 2 pMES per hour and were classified as pMES+. In these patients the pMES rate varied from 2.5 to 30.8 pMES per hour. The occurrence of pMES correlated strongly with histological plaque instability (Table 2).

In 17 patients tMES were observed. Eleven patients had a tMES rate ≥ 6 tMES per hour and were designated tMES+. Thrombosis-related embolization was not related to plaque histology (Table 2).

TABLE 2. Association Between pMES or tMES and Plaque Histology

	Unstable Plaque, n (%)	Stable Plaque, n (%)	Total n	P*
pMES+	11 (55)	1 (5)	12	
pMES-	9 (45)	22 (95)	31	0.003
Total pMES	20 (100)	23 (100)	43	
tMES+	6 (30)	5 (22)	11	
tMES-	14 (70)	18 (78)	32	0.728
Total tMES	20 (100)	23 (100)	43	

pMES+ indicates ≥ 2 pMES per hour; pMES-, <2 pMES per hour; tMES+, ≥ 6 tMES per hour; and tMES-, <6 tMES per hour.

*Statistical analysis by Fisher's exact test; significance at $P<0.05$.

C pneumoniae Serology

Elevated levels of *C pneumoniae* antibody titers were a common finding in our patients. Fifty-eight percent were *C pneumoniae* IgA seropositive (IgA $\geq 1/16$), and 60% were IgG seropositive (IgG $\geq 1/64$). The distribution of cardiovascular risk factors and the use of an intraluminal shunt or a patch for arteriotomy closure were comparable in seropositive and seronegative patients (Table 3).

Influence of *C pneumoniae* Infection on Carotid Artery Disease

Table 4 shows the association between *C pneumoniae* serology and histological plaque instability, plaque-related embolization, and thrombosis-related embolization. *C pneumoniae* seropositivity was not related to histological plaque instability or plaque-related emboli. However, IgA seropositivity was associated with thrombosis-related emboli ($P=0.014$), and IgG seropositivity showed a trend toward association with tMES ($P=0.077$). After correction for confounding covariables, only IgA seropositivity was still significantly associated with tMES ($P=0.030$). Of the potential confounders sex, age, smoking, dyslipidemia, hypertension, diabetes, and discontinuation of preoperative anticoagulation, only the

TABLE 3. Distribution of Cardiovascular Risk Factors and Operative Techniques Among *Chlamydia pneumoniae*-Seropositive and -Seronegative Patients

	IgG+	IgG-	P*	IgA+	IgA-	P*
n	32	21	NS	30	23	NS
Age, y	68	65	NS	67	66	NS
Female/male	5/27	6/15	NS	5/25	6/17	NS
Current smoking	56%	57%	NS	60%	52%	NS
Dyslipidemia	63%	76%	NS	64%	74%	NS
Hypertension	63%	76%	NS	63%	57%	NS
Diabetes	13%	19%	NS	10%	22%	NS
Perioperative shunting	22%	14%	NS	27%	9%	NS
Patch closure	50%	48%	NS	50%	48%	NS
Discontinuation of preoperative anticoagulation	56%	67%	NS	55%	70%	NS

IgG+ indicates IgG seropositivity ($\geq 1/64$); IgG-, IgG seronegativity (<1/64); IgA+, IgA seropositivity ($\geq 1/16$); and IgA-, IgA seronegativity (<1/16). Other definitions are as in Table 1.

*Statistical analysis by Fisher's exact test; statistical significance at $P<0.05$.

TABLE 4. Relation Between *Chlamydia pneumoniae* Serology (IgA and IgG Seropositivity) and Histological Plaque Instability, pMES, or tMES

Dependent Variable	IgA+			IgG+		
	OR	95% CI	P	OR	95% CI	P
Plaque instability	1.0	0.3–3.0	0.994	0.8	0.2–2.8	0.616
	1.0*	0.3–3.2*	0.932*	0.8*	0.3–2.4*	0.679*
pMES+	1.6	0.4–6.6	0.483	1.9	0.5–7.5	0.376
	1.8*	0.4–8.6*	0.474*	1.9*	0.4–9.4*	0.408*
tMES+	11.3	1.3–99.1	0.014	5.1	0.95–27.4	0.058
	12.1*	1.3–114.2*	0.030*	4.8*	0.84–27.4*	0.078*

OR indicates odds ratio. Plaque instability includes fibrous cap rupture and/or organized luminal thrombosis. Other definitions are as in Tables 2 and 3. Statistical analysis by Fisher's exact test and multivariate logistic regression analysis including "discontinuation of preoperative anticoagulation" in the analysis; statistical significance at $P < 0.05$.

*Multivariate logistic regression analysis.

latter was associated with tMES ($P=0.030$) and tended toward association with pMES ($P=0.075$); it was therefore the only confounding variable introduced in the multivariate regression analysis.

Discussion

Atherosclerosis is a chronic inflammatory disease.²⁸ Chronic infections, especially *C pneumoniae* infections, may play an important role in the initiation and progression of this inflammatory process.² *C pneumoniae* can induce proatherogenic changes in endothelial cells,¹³ macrophages,¹⁴ and smooth muscle cells^{11,12}; *C pneumoniae* seropositivity has been associated with acute^{3,8,9} and chronic^{3,4} clinical atherosclerotic manifestations; and *C pneumoniae* has been detected more frequently in atherosclerotic tissue than in normal arteries.² However, the vascular presence of *C pneumoniae* has not been associated with coronary plaque morphology²⁹ or plaque-related cerebral microembolization.³⁰ Since the development of acute cardiovascular complications is associated with plaque instability and/or thrombotic occlusions of blood vessels, we wanted to study the association between *C pneumoniae* serology, plaque instability, and hypercoagulability. In the present study *C pneumoniae* serology was not associated with plaque instability but showed a strong relation with thrombosis-related microembolization during CEA, an in vivo marker of hypercoagulability.

Application of TCD monitoring of the ipsilateral middle cerebral artery during CEA offers the possibility to study the 2 basic mechanisms that contribute to cardiovascular and cerebrovascular events separately, ie, plaque destabilization and (excessive) thrombosis. MES during the dissection phase of CEA have been associated with histological determinants of plaque instability (ie, plaque rupture and/or luminal thrombosis).¹⁹ A pMES rate ≥ 2 pMES per hour has been associated with increased risk of developing cerebral ischemia.²⁰ Therefore, the occurrence of MES during dissection is an in vivo marker of plaque instability, and patients with ≥ 2 pMES per hour were defined as pMES+. After endarterectomy and restoration of flow, in the absence of an unstable plaque, MES represent thrombocyte aggregations formed at the highly thrombogenic endarterectomy and clamping sites. In a

series of 276 CEAs, the median embolic rate postoperatively was 1.33 MES per hour (interquartile range, 0 to 5.67 MES per hour).²⁷ A high embolic rate after CEA (in the upper quartile range of tMES rate, ie, ≥ 6 tMES per hour) probably identifies patients with excessive thrombus formation or inadequate thrombolysis. Therefore, for the purpose of this study, a tMES rate ≥ 6 tMES per hour was regarded as an in vivo marker of hypercoagulability, and patients with ≥ 6 tMES per hour were designated tMES+. In agreement with previous reports, pMES were associated with histological plaque instability in this study.¹⁹ However, tMES were not related to histology.

The average embolic count was related to operation time in our patients. Interestingly, only pMES correlated with this variable. Operation time was a strong predictor of embolic count during dissection ($P=0.002$) but not of embolic count after endarterectomy and restoration of flow ($P=0.873$). To avoid the confounding effect of operation time on the association between *C pneumoniae* serology and microembolization, the embolic rate (microemboli per hour) was used to define patients with clinically relevant plaque-related (≥ 2 pMES per hour) or thrombosis-related (≥ 6 tMES per hour) embolization.

C pneumoniae serology has been associated with cerebrovascular events,^{7,8} carotid intima-media thickness,^{6,7,31} and sonographically detected carotid plaques.^{6,32} Nevertheless, a number of studies have failed to show an association between *C pneumoniae* and ischemic cerebrovascular disease.^{33,34} However, the amount of positive evidence accumulated suggests that this association is material and not coincidental. Negative reports may have been hampered by biased study population and end point selection, limitations of the diagnostic assays used, or even the unfortunate coincidence of a *C pneumoniae* epidemic during sample acquisition. In a randomly selected urban population, no association existed between *C pneumoniae* serology and carotid intima-media thickness and/or the presence of sonographically detected carotid plaque.³³ However, in patients with hypertension,³¹ coronary and peripheral arterial disease,⁶ cerebrovascular disease,⁷ and end-stage renal disease,³² an association between *C pneumoniae* serology and carotid intima-media

thickness or degree of stenosis could be found. In a nested case control study, Glader et al³⁴ found no association between baseline *C pneumoniae* serology and the development of future ischemic cerebral infarction. The authors suggested that a *C pneumoniae* epidemic at the time of patient inclusion and blood sampling might have masked a possible association between *C pneumoniae* serology and cerebrovascular disease, a problem that could have been bypassed if antibody titers would have been detected in paired serum samples.

Despite numerous reports on the association between *C pneumoniae* serology and cardiovascular disease, no consensus has been reached regarding the serological criteria for chronic or persistent *C pneumoniae* infection. Various cutoff points for IgA and IgG titers or even IgA and IgG titer combinations have been used.³⁵ The heterogeneity of serological assays and of serological criteria for persistent or chronic *C pneumoniae* infections might also have contributed to conflicting results of some seroepidemiological studies. The microimmunofluorescence test is regarded as the reference method for *C pneumoniae* serology. However, Gnarpe et al³⁶ found a good correlation between enzyme immunoassay and microimmunofluorescence test in patients with hypertension or ischemic heart disease who had a low background of *C trachomatis* antibodies. The sensitivity, specificity, and positive and negative predictive values were 91, 80, 96, and 63 for IgG and 85, 88, 92, and 79 for IgA, respectively, with the microimmunofluorescence test regarded as gold standard. Likewise, in our laboratory, the sensitivity, specificity, and positive and negative predictive values of the enzyme immunoassay compared with the microimmunofluorescence test were 95, 89, 98, and 77 for IgA and 95, 73, 96, and 75, respectively, for IgG in a series of 239 samples from patients with peripheral arterial disease (n=150) and healthy controls (n=89). The intertest agreement between the enzyme immunoassay and the microimmunofluorescence test was very good for IgA ($\kappa=0.679$) and for IgG ($\kappa=0.681$) (T. Vainas, MD, et al, unpublished data, 2001). Considering the adequate performance and practical advantages of the enzyme immunoassay compared with the microimmunofluorescence test (high throughput, objective end point, cost-efficiency), we had chosen the first method to determine *C pneumoniae* serology in this study. IgA titers $\geq 1/16$ and IgG titers $\geq 1/64$ were considered positive in view of the results of previous studies showing an association between *C pneumoniae* infection and carotid artery disease.^{5,7} According to these cutoff levels, IgA seropositivity was associated with a high thrombosis-related embolic rate but not with plaque instability. Since IgA titers decline and disappear after 3 to 12 months of infection, whereas IgG antibodies may persist for years, it is believed that persistence of the short-lived IgA may be a better marker of chronic infection than IgG.³⁷ Hence, chronic *C pneumoniae* infection is associated with an in vivo marker of hypercoagulability in patients undergoing CEA for symptomatic carotid artery disease.

In this study *C pneumoniae* serology was not related to plaque instability but correlated with tMES. This suggests that the reported association between *C pneumoniae* infection and cardiovascular disease might be mediated through stim-

ulation of thrombosis-related events rather than plaque-related phenomena by *C pneumoniae*. Interestingly, immunohistochemical detection of *C pneumoniae* in carotid atheroma of 76 patients has strongly been associated with thrombosis but not with plaque ulceration, suggesting that *C pneumoniae* infection was independently associated with a greater risk of thrombosis on the plaques but not with plaque ulceration.³⁸ The separate analysis of pMES and tMES in this study showed that *C pneumoniae* seropositivity indeed is associated with tMES rather than pMES, favoring a prothrombotic and/or antithrombotic effect of *C pneumoniae* infection in carotid artery disease. Previous studies have shown that *C pneumoniae* infection of endothelial cells stimulates the nuclear factor- κ B signal transduction pathway, inducing a 4-fold increase of tissue factor¹³ and stimulating the expression of plasminogen activator inhibitor 1,¹² resulting in increased local thrombogenicity. Moreover, plasma fibrinogen levels are elevated in patients with chronic *C pneumoniae* infections³⁹ and decrease on antimicrobial treatment.⁴⁰ These observations offer a novel perspective on the association between *C pneumoniae* infection and cardiovascular events and warrant further investigation of the prothrombotic and antithrombotic effects of *C pneumoniae* infection in atherothrombotic arterial disease.⁴¹

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