Genomic, serologic, and clinical case-control study of *Chlamydia pneumoniae* and peripheral artery occlusive disease

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Objectives: **Chlamydia pneumoniae has been related to atherosclerotic disease in both seroepidemiologic and genomic studies. We performed a case-control study to determine seropositivity and DNA detection in arteries of patients with peripheral artery occlusive disease and of healthy subjects.**

Methods: **The study included 64 patients with peripheral artery occlusive disease, and 50 control subjects who underwent varicose vein surgery, matched to the patient group for age, sex, and tobacco use. The fibrinogen level in all study subjects was measured as a marker of inflammation. Blood samples were taken from all subjects for determination of immunoglobulin (Ig) G elementary bodies (EB) against *C pneumoniae* with microimmunofluorescence (MIF) and enzyme-linked immunosorbent assay (ELISA), and of IgA EB with ELISA. The cutoff titers were 1:32 for MIF and 1.1 for ELISA. Biopsy specimens of arterial atheromatous plaque were obtained from patients, and of pudendal artery and saphenous vein from control subjects, and were studied with hemi-nested polymerase chain reaction.**

Results: **There were no differences in fibrinogen level between patients and controls. The prevalence of IgG anti-EB with MIF was 78% in patients and 24% in control subjects ($P = .0001$; odds ratio [OR], 11.3; 95% confidence interval [CI], 4.7-27.2). Prevalence of IgG anti-EB with ELISA was 75% in patients and 16% in control subjects ($P = .0001$; OR, 15.7; 95% CI, 6.1-40). There were no differences in IgA anti-EB titers. Bacterial DNA was detected in 67% of atheromatous plaques versus 12% of pudendal arteries ($P = .0001$) and 4% of saphenous veins. A weak correlation was found between seropositivity and the presence of intravascular DNA.**

Conclusions: **Our results support the hypothesis that *C pneumoniae* is related to the pathogenesis of atherosclerotic peripheral artery occlusive disease. (J Vasc Surg 2004;40:359-66.)**

Clinical Relevance: **This study explored the infectious hypothesis in the context of the pathogenesis of atherosclerosis. This hypothesis has been supported by findings that certain infectious agents can cause or accelerate the course of diseases in which the possibility of a microbial cause was not previously proposed, as in the case of peptic ulcer and spongiform encephalopathy. The present study demonstrated the presence of *Chlamydia pneumoniae* and seropositivity in atheromatous plaques in patients with peripheral artery occlusive disease. These results contribute to a body of research that is opening up the possibility of treating atherosclerotic disease with antibiotic agents, and preventing it with immunization.**

The infectious pathogenesis of atherosclerosis was originally proposed by Osler in 1908, but was rapidly discarded after failure to isolate any pathogen responsible.[1] Atherosclerosis was subsequently associated with herpes simplex virus and cytomegalovirus.[2,3] Not until 1988 did Saikku et al[4] publish the first seroepidemiologic study that reported high titers of circulating antibodies against *Chlamydia pneumoniae*[5] in patients with coronary atherosclerosis. These findings were corroborated by Thom et al,[6] who discovered the bacteria in 1991, and Saikku et al[7] repeated their own results in a wider study in 1992. Later studies also demonstrated the presence of circulating immunocomplexes against *C pneumoniae* in patients with ischemic heart disease.[8] In 1992 C pneumoniae in atherosclerotic vascular tissue was first reported,[9] detected with immunocytochemistry. This microorganism has also been detected with electronic microscopy and polymerase chain reaction (PCR) studies, and at culture of biopsy specimens of the atherosclerotic aortic, coronary, femoral, and carotid artery walls.[10] Several subsequent studies have suggested that chronic infection with *C pneumoniae* may contribute to the pathogenesis of atherosclerosis.[11,12]

The objective of the present case-control study was to assess the relationship between *C pneumoniae* and peripheral artery occlusive disease (PAOD) involving the carotid, femoral, and aortoiliac arteries. The study comprised 3 parts: clinical study (inflammation markers), study of immune response, and detection of *C pneumoniae* DNA in samples of arterial wall from patients and control subjects.

METHODS

An observational, analytical case-control study was conducted in 114 patients treated in the Vascular Surgery Department at our center. All laboratory determinations,

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Competition of interest: none.

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Table I. Anatomic-clinical subgroups of patients with peripheral artery occlusive disease, and criteria for inclusion in study

Clinical subgroups	*Case subjects*	*Clinical status*
Cerebrovascular disease	26	21, stroke plus ICA stenosis >70% 5, ICA stenosis >99%
Aortoiliac disease	18	10, G-II (Fontaine) 8, G-III (Fontaine)
Femoropopliteal disease	20	5, G-II (Fontaine) 15, G-III (Fontaine)

ICA, Internal carotid artery.

including antibodies, lipids, fibrinogen, and determination of *C pneumoniae* DNA, were processed and interpreted in blinded fashion.

Patients. One hundred twenty-six consecutive patients who required revascularization surgery to treat PAOD at various sites were recruited for the study. Thirty-nine patients with active inflammatory or infectious processes were excluded, as were 23 patients with Fontaine grade IV lower limb ischemia. Therefore there were 64 patients, all with Fontaine grade II or III disease. Further information on anatomic subgroups and clinical characteristics of these patients is given in Table I. The control group comprised 50 subjects with chronic superficial venous insufficiency.

It should be borne in mind that the control subjects were recruited from among patients who underwent surgery to treat chronic venous insufficiency, consisting of saphenectomy under regional or general anesthesia, and all had C_2 or C_3 disease, according to the CEAP classification system. Consequently all control subjects underwent general examination in the Anesthesia Department, including electrocardiography, chest x-ray studies, blood and biochemical analyses, and circulatory examination. In all control subjects anesthetic risk was American Society of Anesthesiologists (ASA) I or II. Control subjects with coronary, pulmonary, or renal disease, either previously known or discovered during the preoperative examination, were excluded from the study. The absence of posterior tibial pulse or dorsal pedal pulse was an exclusion criterion, but no patients were excluded for this reason. All control subjects underwent carotid duplex ultrasound scanning, and none demonstrated stenosis greater than 20%.

Patients and control subjects enrolled in the study were interviewed regarding previous genitourinary infections, to avert possible serologic cross-reactions between *C pneumoniae* and *Chlamydia trachomatis*. No history of these infections was reported by any subject. For the same reason, patients with chronic respiratory disease or pneumonia in the previous 3 months were excluded, to avert cross-reactions with *Chlamydia psittaci*. We also excluded patients with any chronic inflammatory or infectious diseases other than atherosclerosis. To prevent the possible elimination of *C pneumoniae* with use of antibiotic agents, patients who received antibiotic therapy for any reason in the previous 3 months were excluded, as were subjects allergic to β-lactam, because our antibiotic prophylaxis for these patients is with erythromycin, an effective antibiotic against *C pneumoniae*.

All subjects selected for the study signed informed consent forms to participate. The study was approved by the ethics committee at our center.

Clinical determinations. Clinical and analytical data considered included sex, exposure to tobacco, obesity (>30% above theoretical weight), systolic arterial blood pressure, hypertension, cholesterol concentration, hypercholesterolemia, triglyceride concentration, and hypertriglyceridemia. Current smokers and ex-smokers who had smoked for more than 10 years were considered exposed to tobacco.

Marker of inflammation. Fibrinogen levels were always determined (P-T Fibrinogen HS) before surgery, with the derived fibrinogen assay.

Antibody determinations. Immunoglobulin (Ig) G to elementary body (EB) of *C pneumoniae* was studied with two analytic techniques: indirect microimmunofluorescence (MIF; MRL *Chlamydia pneumoniae* IgG MIF), performed as described,[13,14] and enzyme-linked immunosorbent assay (ELISA; *Chlamydia pneumoniae* IgG Vircell), as described.[15] For greater precision, the process was automated with the use of a sample dilutor (TECAN Megaflex) and plate processor for ELISA (BEP III; Dade Behring). A single experienced researcher made the MIF observations, which were carried out in duplicate to confirm their validity. Before evaluation of the results, 10% of the samples were retested, and similar results were obtained.

For the MIF technique we considered all titers 1:32 or greater as positive. IgA to EB was also studied with ELISA (C pneumoniae IgA Vircell), with use of *Chlamydia* outer membrane complexes. Absorbance was measured at 450 or 620 nm. Results were expressed as indexes by dividing the absorbance of the sample by that of the cutoff. Indexes less than 0.9 were scored as negative, 0.9 to 1.1 as uncertain, and greater than 1.1 as positive. Studies with uncertain results were repeated, and the new result was taken as valid. For greater precision the process was automated with the use of a sample dilutor (TECAN Megaflex) and plate processor (BEP III; Dade Behring). Before evaluation of the results, 10% of the samples were retested, and similar results were obtained.

Determination of *C pneumoniae* DNA in biopsy specimens. In the patient group, histologic samples were obtained from the atheromatous plaques collected during carotid thromboendarterectomy, femoropopliteal bypass, or aortoiliac bypass surgery. In the control subjects a 1-cm segment of the pudendal artery was collected during the varicose vein surgery. The presence of *C pneumoniae* DNA was compared between samples from patients and control subjects. A segment of varicose saphenous vein from the control subjects was also obtained and processed. The biopsy specimens were immediately washed with physiologic saline solution to remove any remaining blood or

perivascular tissue, and were immersed in sterile sucrose-phosphate-glutamic acid buffer transport and preservation medium. They were refrigerated at −4°C to −10°C for 12 to 16 hours, and then maintained at −70°C until analysis with hemi-nested PCR.

A personal modification[16] of the technique initially described by Campbell et al[17] was used. The samples were homogenized, and DNA was extracted for enzymatic digestion with proteinase K. The amount of DNA was matched among samples (50-100 ng/mL). The first PCR was carried out with the primers HL-1 (GTTGTTCATGAAGGCCTACT) and HR-1 (TGCATAACCTACGGTGTGTT) with a Perkin-Elmer 9600 thermocycler, and produced a fragment of 437 base pairs. A second PCR was performed on this product, with primers HR-1 and HM-1 (GTGTCATTCGCCAAGGTTAA), and produced a shorter fragment of only 229 base pairs. All primers were of the Pst-1 segment. The reading was performed with electrophoresis in 2% agarose gel. The β-actin gene served as internal control for DNA amplification and extraction.

To prevent false positive amplifications, procedures recommended to prevent contamination were strictly observed and all reactions were performed under stringent conditions. All reagents were aliquoted and stored in different locations. PCR reagents were prepared before each assay in a master mixture, which was then aliquoted. Preparation of the master mixture, extraction of the DNA and addition of the template to the PCR mixture, and thermal cycling were performed in 3 different, well-separated rooms, each with its own dedicated set of micropipettes and gowns. Only aerosol-resistant barrier pipette tips were used. Meticulous laboratory techniques and adherence to standard PCR anti-contamination procedures were the norm, including frequent glove changes and decontamination of surfaces with ultraviolet (UV) light and sodium hypochlorite. All tubes, pipette tips, and reagents, except for the primers and Taq polymerase, were exposed to 254 nm of UV light in a nucleic acid linker oven (Stratalinker UV Crosslinker; Stratagene) before use. A number of negative controls (numerous negative water and PCR reagent-only samples) were included in each PCR assay.

Statistical analysis. Statistical analysis was carried out with the SPSS statistical package (version 10, 2001; SPSS Inc). Exact logistic regression was performed with the LogXact program (version 2.1, 1996; Civel Software Corp). Continuous variables were compared with the Student *t* test. Discrete variables were analyzed with the χ^2 test or Fischer exact test; the Pearson correlation was used to calculate the correlation among the variables. Exact logistic regression was used to fit a multivariate model explaining the presence of *C pneumoniae*, and the Hosmer-Lemeshow test was applied to the adjusted model.

RESULTS

Adequate samples for analysis were obtained from all 114 study patients, with no accidental losses or destruction. Patient baseline characteristics are shown in Table II. There were no significant differences in the distribution of sex, tobacco exposure, or age above the 25th percentile between the two groups. The mean age of the patients was 6 years older than that of the control subjects.

Table II. Baseline clinical characteristics of 64 subjects with PAOD (cases) compared with 50 subjects without PAOD (controls)

	Cases (N = 64)	*Controls (N = 50)*	P
Age (y) (mean ± SD)	66.06 ± 7.46	60.12 ± 3.49	<.0001*
Age >59 y (first quartile) (%)	84.4	76	.261†
Male sex (%)	79.7	80	.967†
Smoking (past or current) (%)	71.9	60	.182†
Diabetes mellitus (%)	31.3	4	<.0001‡
Hypertension (%)	53.1	8	<.0001†
Hypercholesterolemia (%)	29.7	4	<.0001‡
Hypertriglyceridemia (%)	28.1	8	.007†
Obesity (%)	26.6	28.2	.864†

PAOD, Peripheral artery occlusive disease.
*Student *t* test.
†χ^2 test.
‡Fischer exact test.

Comparability. There were differences between the groups in other covariables studied. Diabetes, hypertension, hypercholesterolemia, and hypertriglyceridemia occurred much more frequently in patients compared with control subjects. There was no significant difference in obesity between the groups.

To fit a multivariate model explaining the presence or absence of *C pneumoniae* between study groups, and taking into account the sample size, exact logistic regression was applied (Table III, online only). The only factor identified as clearly and strongly significant was the effect of belonging to the patient or control group (odds ratio [OR], 18.055; 95% confidence interval [CI], 4.260-76.527). The high value of the OR estimated in this case may be explained by overestimation due to the sample size and strong relationship between risk factors. The goodness of fit of the adjusted model was verified with the Hosmer-Lemeshow test, and was not significant (χ^2 = 12.742; P = .121; 8 degrees of freedom). In addition, outliers and weighted data were looked for; none were detected.

Marker of inflammation. Fibrinogen levels were 367.69 ± 96.81 in patients versus 355 ± 79.11 in control subjects. This small difference was not statistically significant (P = .454, Student *t* test). The value of the third quartile was 422.25 mg/dL; 26.6% of patients had levels above this value, versus 22% of control subjects. This difference was not statistically significant (P =.574, χ^2 test). The OR for fibrinogen above the 75th percentile was 1.282 (95% CI, 0.538-3.058).

Anti–*C pneumoniae* antibodies. These results are shown in two ways: not grouped, either for MIF or ELISA (Table IV; Fig 1), and analyzed as positive or negative (Tables V and VI). With MIF and ELISA, titers for IgG were 75% and 78%, respectively, in patients, versus 16% and 24%, respectively, in control subjects. This difference was

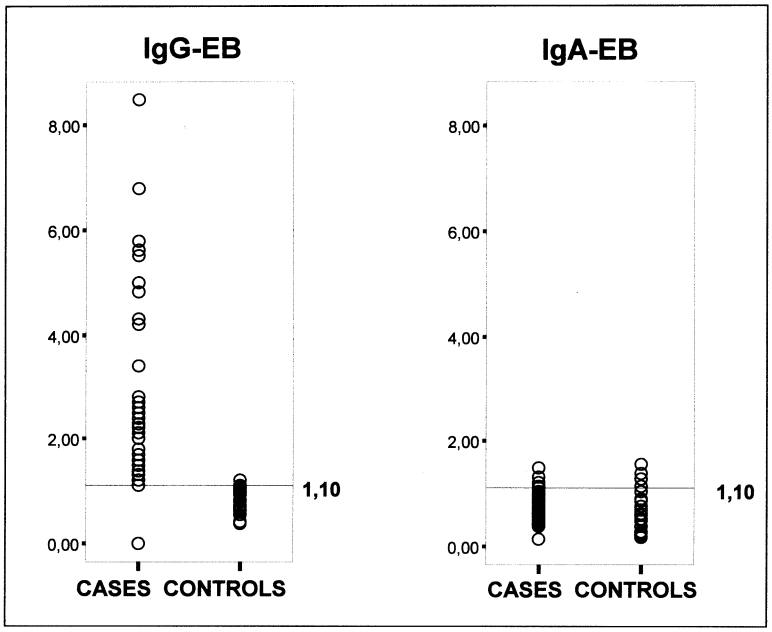

Fig 1. Distribution of IgG and IgG antibody titers with enzyme-linked immunosorbent assay Technique (cutoff point, 1.1). IgG-EB, Immunoglobulin G elementary bodies; IgA-EB, immunoglobulin A elementary bodies.

Table IV. Distribution of IgG-EB titers with MIF technique

	<1/16		*1/16*		*1/32*		*1/64*		*1/128*	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Cases	4	6.25	10	15.6	11	17.2	21	32.8	18	28.1
Controls	30	60	8	16	8	16	4	8	0	0
									P <.0001*	

IgG, Immunoglobulin G; *EB,* elementary body; *MIF,* microimmunofluoroescence.
*χ^2 test.

statistically significant with both methods ($P < .0001$, χ^2 test), with an OR of 11.31 (95% CI, 4.696-27.236) for MIF and 15.75 (95% CI, 6.126-40.495) for ELISA.

IgA seropositivity was 7.8% in patients, versus 16% in control subjects. This difference was not statistically significant ($P = .172$, χ^2 test), with an OR of 0.445 (95% CI, 0.136-1.458).

Results for clinical subgroups considered, showing a uniform distribution, are shown in Table VI.

***C pneumoniae* DNA.** *C pneumoniae* DNA was detected with PCR in 43 atheromatous plaques from patients (67.2%) and 6 external pudendal arteries from control subjects (12%; Figs 2 and 3), a statistically significant difference ($P < .0001$, χ^2 test). Table VI shows that the presence of *C pneumoniae* DNA had a uniform distribution among subgroups.

Detection of *C pneumoniae* DNA in saphenous vein samples was considered separately. It was detected in 2 samples (4%) from 2 control subjects with positive DNA in the external pudendal artery.

Correlation between antibodies and intraplaque infection. The relationship among the diagnostic capabilities of the 3 serologic determinations was studied. Comparison between the 2 IgG antibody techniques showed that both were positive in 47 patients and both were negative in 43 patients (r = 0.582, Pearson correlation; $P = .001$, χ^2). The results for intraplaque infection are exhibited in Table VII. Although the correlation rates for IgG were low, they were statistically significant. IgA antibodies were not correlated with IgG or genomic detection.

DISCUSSION

The findings of the present case-control study support the hypothesis that *C pneumoniae* has a role in atherosclerosis, because the rates of seropositivity and DNA detection were higher in patients than in matched control subjects. The strengths of this study are that an adjusted case-control design was followed, and serologic, clinical, and genomic studies were performed.

Table V. Antibodies against elementary body of *Chlamydia pneumoniae*

Antibody	*Technique*	*Seropositivity (%)*		*Odds ratio*	*95% Confidence interval*
		Cases	*Controls*		
IgG-EB	MIF	78.1	24	11.31	4.696–27.236
IgG-EB	ELISA	75	16	15.75	6.126–40.495
IgA-EB	ELISA	7.8	16	0.445	0.136–1.456

Ig, Immunoglobulin; *EB,* elementary body; *MIF,* microimmunofluorescence; *ELISA,* enzyme-linked immunosorbent assay.

Table VI. Seropositivity of different antibodies against elementary bodies of *Chlamydia pneumoniae* and DNA detection in anatomic-clinical subgroups studied

		Aortic (%)	*Femoral (%)*	*Carotid (%)*	P*
MIF	IgG-EB	77.8	90	69.2	.240
ELISA	IgG-EB	61.1	85	76.9	.227
ELISA	IgA-EB	11.1	0	15.0	.142
DNA	Positive	66.7	65	69.2	.954

MIF, Microimmunofluorescence; *ELISA*, enzyme-linked immunosorbent assay; *Ig*, immunoglobulin; *EB*, elementary body.
*χ^2 test.

Table VII. Correlation between seropositivity according to different serologic techniques and intraplaque infection determined by presence of DNA of *Chlamydia pneumoniae*

Positive antibodies	*Positive DNA*	*Correlation*		*Odds ratio*	*95% Confidence interval*
		*r**	P†		
IgG-EB (MIF)	37	0.368	.0001	4.03	2.17–11.21
IgG-EB (ELISA)	36	0.423	.0001	6.23	2.73–14.21
IgA-EB (ELISA)	3	−0.144	.112	0.36	0.09–1.38

Ig, Immunoglobulin; *EB*, elementary body; *MIF*, microimmunofluorescence; *ELISA*, enzyme-linked immunosorbent assay.
*Pearson coefficient.
†χ^2 test.

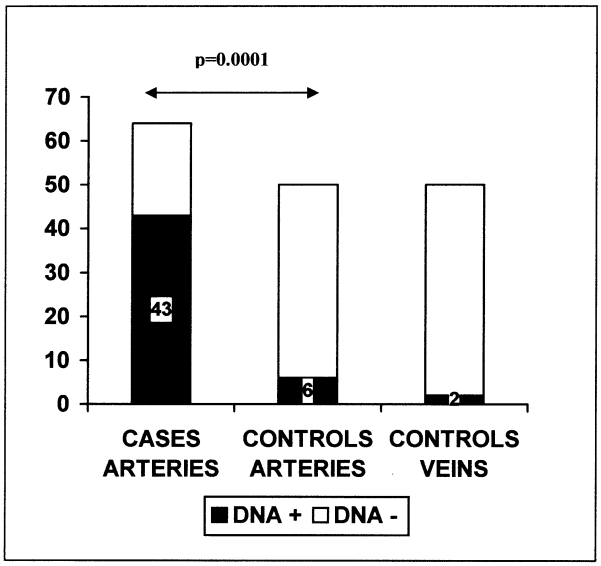

Fig 2. Detection of *Chlamydia pneumoniae* DNA in samples of vascular tissue detected with polymerase chain reaction in 43 of 64 atheromatous plaques from patients, 6 of 50 external pudendal arteries from control subjects, and 2 of 50 saphenous veins.

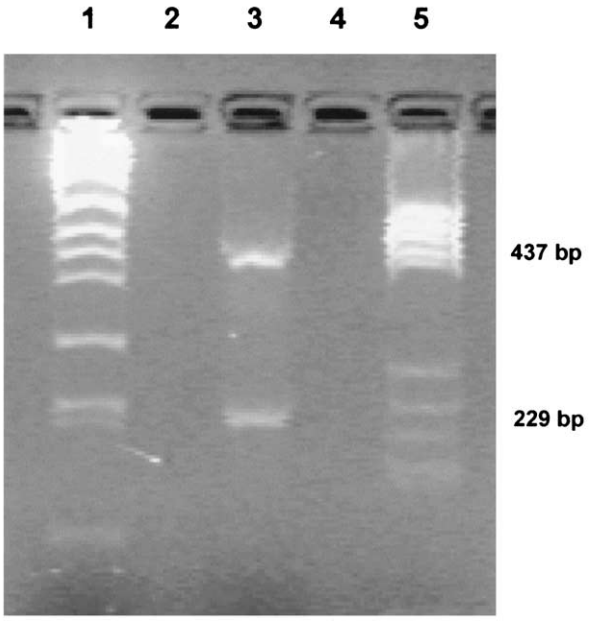

Fig 3. Amplification of *Chlamydia pneumoniae* DNA (agarose gel). *Lane 1,* MW 154-2176 bp; *lane 2,* negative control; *lane 3,* positive sample; *lane 4,* negative sample; *lane 5,* MW 8-578 bp. *bp,* Base pairs.

It was not easy to find sufficient adults older than 65 years who could supply an artery during a surgical intervention and form a comparable control group. The control subjects were selected after recruitment of the patients, to control for the 2 main risk factors, age and sex. Although patients with PAOD were significantly older, by 6 years,) than control subjects, the model was comparable (Table III). The control subjects were also matched for tobacco use, to avert a possible bias reported in the literature.[18]

The extraction of a segment of the external pudendal artery during conventional varicose vein surgery is a minimal procedure, because this artery can normally be ligated if required. The novelty of this work was the extraction of arteries for analysis. There were no postoperative complications attributable to extraction of biopsy specimens of external pudendal artery.

Fibrinogen levels, as a marker of inflammation and chronic infection, were slightly higher in the PAOD group,

although the difference did not reach statistical significance. Variable data have been reported by other authors.[19,20]

Seropositivity for IgG-EB with MIF was defined at a high level of greater than 1:32 titers, because in a recent workshop on standardization of *C pneumoniae* diagnostic methods the Centers for Disease Control and Prevention regarded an IgG titer greater than 16 as indicating past exposure.[21] We believe that we increased the specificity of the test by using a cutoff titer greater than 1:32. The ELISA technique, which obviates this problem, showed similar seropositivity values as the MIF results.

The OR ratios are higher than in previous reports, with only a few studies[22-24] showing ORs within this range; however, bear in mind that thresholds for seropositivity are highly variable. There have been no reports of ORs above 10 with ELISA, although this remains a little-used technique.[15,22,25]

Our findings for IgA-EB are comparable with recent studies, which also found low rates of seropositivity.[3,24,26] Results of published seroepidemiologic case-control studies are conflicting, with some[27-29] reporting that seropositivity to *C pneumoniae* is associated with arterial disease and others[26,30] reporting that it is not. Even case-control studies published by the Atherosclerosis Risk in Communities study researchers came to divergent conclusions, with an OR of 2.00 for seropositivity in patients with stroke[27] but no association in patients with coronary heart disease.[31]

An issue that is yet to be resolved is the type of antigen to be used in serologic testing. Traditionally lipopolysaccharide has been used, but tests are now available against EB and other epitopes, promising improved sensitivity and specificity. The genomic study found *C pneumoniae* DNA in 67.2% of samples of diseased arteries versus 12% of atheroma-free arteries. PCR is a highly specific technique, and provides a reliable diagnosis.[17] We used a hemi-nested PCR, thereby achieving virtually total certainty that the DNA fragments found were of *C pneumoniae*.[18-20,22-32] However, the histologic structure of the pudendal artery is different from that of the femoral or carotid arteries, and it rarely is associated with atherosclerosis. We believe that the artery of a live person always offers better evidence than that of a cadaveric artery, used in other studies.[33-35] The main advantage of our approach is that a real case-control study can be performed with arterial tissue from comparable subjects.

Twenty-two case-control studies that examined the presence of *C pneumoniae* in non-coronary atheromatous plaques with PCR have been published. In 8 studies the controls were apparently healthy arteries from patients; the DNA was detected in 0% to 76% of patients and 0% to 53% of control samples[33,36] (Table VIII, online only). We detected the DNA in 67% of patients versus 12% of control subjects. We have no explanation for our finding of *C pneumoniae* DNA in 6 control arteries, especially when 4 of them demonstrated negative serologic findings. This situation has been reported by other authors.[31,36,37] We are also unable to explain the presence of *C pneumoniae* DNA in 2 of 50 samples of varicose saphenous vein. Varicose vein walls can have a certain degree of inflammation,[38] and we could not determine whether the DNA came from vascular wall cells or from macrophages.

Our study shows poor correlation between seropositivity and *C pneumoniae* DNA detection, and only for IgG-EB. Few studies have found any relationship, and always with a larger sample size than ours.[24,37]

For infection to be a risk factor,[10,11] independent of other risk factors such as hyperlipidemia, tobacco use, hypertension, diabetes, or family history of the disease, it is necessary to address the action mechanism by which *C pneumoniae* produces atherosclerosis and to determine whether *C pneumoniae* causes the initial damage, triggering the atherosclerosis, or accelerates and aggravates previous atheromatous lesions. Even atherosclerotic plaques, with their high leukocyte content, may be a site where *C pneumoniae* is merely present without participating in the pathogenesis of atherosclerosis.

It is not possible to deduce from our study the causal relationship between chronic *C pneumoniae* infection and PAOD. However, the high seropositivity rate in the patients and the frequent detection of viable *C pneumoniae* in the atheromatous plaques suggest that *C pneumoniae* infection has some role in the pathogenesis of atherosclerosis. Our finding of DNA in healthy arteries and varicose veins in patients without clinical PAOD can be variously interpreted as evidence for and against the participation of *C pneumoniae* in atherosclerosis.

In conclusion, the findings of our matched case-control study further support the relationship between *C pneumoniae* infection and PAOD. Wider case-control studies of comparable subjects are warranted to definitively establish whether some chronic infections can induce atherosclerosis.

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