ORIGINAL ARTICLE

No evidence for a direct role of *Helicobacter pylori* and *Mycoplasma pneumoniae* in carotid artery atherosclerosis

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Background: That infections with certain pathogens, by initiating an inflammatory response, may contribute to the development of atherosclerosis is suggested by clinical and experimental evidence.

Aim: To analyse atherosclerotic plaques of the carotid artery, samples of apparently healthy greater saphenous veins and circulating leucocytes from the same individual patients for the presence of *Helicobacter pylori* and *Mycoplasma pneumoniae*.

Methods: Samples from 36 patients undergoing carotid endarterectomy for symptomatic carotid artery stenosis were analysed by polymerase chain reaction for the presence of DNA specific for *H pylori* and *M pneumoniae*. IgG antibody titres against *H pylori* and *M pneumoniae* and plasma levels of soluble E-selectin, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1 were determined.

Results: *M pneumoniae*-specific DNA was detected in the atherosclerotic plaques of 13 of 36 (36.1%) patients, in the saphenous veins of 9 of 36 (25%) patients and in the leucocytes of 27 of 36 (75%) patients. No salient association was observed between the presence of *M pneumoniae*-specific DNA in leucocytes and atherosclerotic plaques or veins. A marked correlation between the presence of *M pneumoniae* in the respective specimens and the studied inflammatory markers or the presence of anti-*M pneumoniae* antibodies was not observed. *H pylori*-specific DNA could not be detected in the specimens tested.

Conclusions: The absence of *H pylori* and the random distribution of *M pneumoniae* in tissue samples obtained from patients with symptomatic carotid artery stenosis do not support a role for these pathogens in the development of atherosclerosis due to a direct interaction of the bacteria with the vasculature.

G iven their association with inflammation, which is now seen as a key event in the development and progression of atherosclerosis, infectious agents have been studied extensively as a possible cause of this vascular disease.[1] In particular, infectious agents such as *Chlamydia pneumoniae*, *Helicobacter pylori*, *Mycoplasma pneumoniae*, herpes simplex virus, Epstein–Barr virus and cytomegalovirus have been implicated in the initiation and progression of atherosclerosis.[2] Among the bacterial pathogens associated with atherosclerosis, a possible role of *C pneumoniae* in the development and progression of this disease has been extensively studied.[3] In addition to seroepidemiological data linking the bacterium to atherosclerosis, the presence of *C pneumoniae* in atherosclerotic lesions has been shown by immunohistochemistry, identification of genomic material by polymerase chain reaction (PCR), in situ hybridisation and electron microscopy, and by the isolation of viable bacteria from plaque material.[3] In contrast with *C pneumoniae*, a role of *H pylori* infection in atherosclerosis is more elusive. *H pylori* has been recognised as the primary cause of peptic ulcers. Features in common in the epidemiology of peptic ulcers and coronary artery disease (CAD) have prompted seroepidemiological studies to test the association between infection with this agent and atherosclerosis. Early reports showed that seropositivity to *H pylori* is a risk factor for CAD.[4] It was also suggested that infection with this bacterium may increase C reactive protein (CRP) and other inflammatory mediators associated with atherosclerosis.[5 6] Later studies could not, however, find a correlation between *H pylori* infection and CAD or inflammatory markers.[7] *H pylori* DNA was found in

the coronary arteries of patients who had died of myocardial infarction and in carotid endarterectomy specimens, but these findings could not be confirmed in studies of aortic aneurysm and carotid atherosclerotic plaques.[8–11] *M pneumoniae* is a commonly occurring respiratory pathogen that has been shown to cause chronic infections similar to *C pneumoniae*. The findings on the presence of *M pneumoniae* in atherosclerotic lesions, however, are highly controversial and the need for studies on the presence of *M pneumoniae* in vascular tissue has been postulated.[9 12–14]

A limitation of most investigations on the presence of pathogenic organisms directly in the vasculature is the fact that only material from vascular lesions was used to detect the pathogen.[9 15–17] In some of these studies, control vessels without atherosclerosis were obtained from a different group of people who were mostly not age matched, which led to the concern that differences in prevalence of the respective microorganism were due to the differences in age between the study group and the younger control group.[14] Thus, on the basis of these studies, it is not possible to evaluate whether the presence of certain microorganisms is restricted to the vascular lesion or whether the pathogen is also found in healthy parts of the vasculature. We recently dealt with this issue and used atherosclerotic plaques, apparently healthy veins and leucocytes, all obtained from the same people, to

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Abbreviations: CAD, coronary artery disease; CRP, C reactive protein; ELISA, enzyme-linked immunosorbent assay; GSV, greater saphenous vein; PCR, polymerase chain reaction; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1

detect *C pneumoniae*. We were able to show that *C pneumoniae* DNA was present in more than 80% of leucocytes and atherosclerotic plaques but only in 6.5% of apparently healthy veins in these patients.[18]

Given the controversial results described above, on the possible presence of two other bacterial pathogens that may be associated with atherosclerosis, namely *H pylori* and *M pneumoniae*, in vascular tissue, we analysed by PCR atherosclerotic plaques of the carotid artery, samples of apparently healthy greater saphenous veins (GSVs) and circulating leucocytes collected from the same 36 patients who had carotid artery stenosis, for the presence of DNA specific for these bacteria.

SUBJECTS AND METHODS
Subjects
Between December 1999 and December 2001, a total of 36 patients (22 men, mean age 74 years, range 54–86 years; 14 women, mean age 73 years, range 57–88 years) underwent carotid endarterectomy for symptomatic carotid artery stenosis of ⩾80%. Colour duplex imaging estimated an average degree of stenosis of 85% (range 80–99%). The stenoses were haemodynamically important and there was no perioperative antibiotic treatment. Of these patients, 31 were smokers or had stopped smoking about ⩽4 years ago, 24 had hypertension, 21 dyslipidaemia, 9 diabetes mellitus and 25 a combination of risk factors. Table 1 shows other details of patients and symptoms. Minor strokes were defined as morphologically positive strokes, as diagnosed by magnetic resonance, whose clinical and neurological symptoms disappeared within 4 weeks. Data on the presence of *C pneumoniae* DNA in this group of patients have been published by our group recently.[18] A control group consisted of 25 people (17 men, mean age 63 years, range 45–79 years; 8 women, mean age 60 years, range 48–76 years) without evidence of marked carotid artery stenosis (<25%) as determined by colour duplex imaging. Of these people, 1 was a smoker, 7 had hypertension, 7 dyslipidemia, 5 diabetes mellitus and 12 a combination of risk factors.

Endarterectomy was carried out by removing the plaque in the adventitia–media sheath, with additional eversion endarterectomy of the external carotid artery. A proximal GSV patch harvested from the groin before carotid artery dissection was used for closure of the carotid artery incision. Tissue specimens and blood samples were treated as described.[18] All human material was obtained and processed, and informed consent was obtained, according to the recommendations of the hospital's Ethics Committee and Security Board.

Table 1 Details of patients and symptoms (n = 36)

	n (%)
Age in years (mean (SD))	73 (10)
Symptom	
Amaurosis fugax	9 (25)
Transient ischaemic attacks	28 (78)
Minor stroke	9 (25)
Combination of symptoms	5 (14)
Inflammatory markers	
CRP in mg/dl (mean (SD))	0.7 (1.2)
sE-selectin in ng/ml (mean (SD))	10.7 (9.0)
sICAM-1 in ng/ml (mean (SD))	47.8 (21.1)
sVCAM-1 in ng/ml (mean (SD))	577.4 (178.0)

CRP, C reactive protein; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1.

DNA isolation
DNA from circulating leucocytes was prepared according to Miller *et al*.[15] Alternatively, DNA was isolated from the same blood samples with a commercially available DNA extraction and purification kit, using FTA cards for blood collection and storage (Life Technologies, Paisley, UK). No difference was seen when DNA analysed by either method was further processed. DNA was isolated from about 100 mm^3 of either the carotid atherosclerotic plaque or pieces of healthy GSV by digestion with 1 ml of a solution containing 50 mmol/l Tris-HCl, 100 mmol/l EDTA, 100 mmol/l NaCl, 1% sodium dodecyl sulphate, 0.5 mg/ml proteinase K, pH = 7.2 at 55℃, overnight. After addition of 410 μl of 5 mol/l NaCl and centrifugation for 10 min at 13 000 rpm, the DNA was precipitated with twice the volume of 96% ethanol. The DNA was washed four times with 70% ethanol and resuspended in 1 ml of a buffer containing 10 mmol/l Tris, 1 mmol/l EDTA, pH = 8.0 at 4℃.[18–20] Care was taken to maintain aseptic handling of the blood and tissue samples during the DNA isolation procedure. Patient samples and PCR reaction assembly were kept in separate laboratories to prevent contamination of the samples by PCR products. Each sample was analysed in duplicate and gave identical results. A positive and a negative control per run consisting of six samples (leucocytes, plaques and veins from two patients) were included. Positive and negative controls were processed exactly in the same manner as the clinical samples and contained all the components used in the clinical samples. Positive controls were spiked with target DNA (*H pylori* or *M pneumoniae*) and negative controls contained all the PCR reagents and sterile water. In all cases, positive controls were positive whereas negative controls gave negative results.

Polymerase chain reaction
To detect *H pylori* at a required level of sensitivity, a portion of the *glmM* gene was amplified with a PCR protocol published by Lu *et al*.[21] *M pneumoniae* was detected according to a PCR protocol published by Ursi *et al*.[22] The PCR protocols were modified slightly. Briefly, the amplification conditions consisted of an initial denaturation step at 95℃ for 5 min, 30 cycles at 95℃ for 1 min, the respective annealing temperature for 1 min and 72℃ for 1 min, a final elongation step at 72℃ for 7 min, followed by cooling to 4℃. For details, see table 2.

The PCR protocol for determination of *C pneumoniae* and the detection of DNA specific for this pathogen in these samples was recently published.[18]

Serology and ELISA
IgG antibodies against *M pneumoniae* and *H pylori* were determined by using commercially available enzyme-linked immunosorbent assays (ELISAs; SeroMP-IgG Kit, Savyon Diagnostics, Ashdod, Israel; Meridian Diagnostics, Milan, Italy). Plasma levels of sE-selectin (soluble E-selectin), sICAM-1 (soluble intercellular adhesion molecule-1) and sVCAM-1 (soluble vascular cell adhesion molecule-1) were measured with specific ELISAs (R&D Systems, Minneapolis, Minnesota, USA). CRP was determined in the plasma samples of the patients with a highly sensitive assay obtained from Dade Behring (Deerfield, Illinois, USA).

Statistical analysis
Data are represented as mean and SD. The significance of any differences in proportions was tested by using the χ^2 test or Fisher's exact test, as appropriate. After determination of the distribution pattern, statistical differences between groups were determined either by the unpaired Student's t test or by the unpaired Student's t test after log transformation. Multiple comparisons were accounted for by the

Table 2 Primers

Target	Sequence (5′→3′)	Location	Annealing temperature (°C)	Product size (bp)
HP sense	aag ctt tta ggg gtg tta ggg gtt t	784–809 (glu M)	55	294
HP antisense	aag ctt act ttc taa cac taa cgc	1077–1053 (glu M)		
MP sense	gcc acc ctg ggg ggc agt cag	2599–2619	65	209

HP, *Helicobacter pylori*; MP, *Mycoplasma pneumoniae*.

Bonferroni–Holm method. All probability values were two tailed, and the confidence intervals were calculated at the 95% level. All calculations were carried out with SPSS V.11.0.1 for Windows.

RESULTS

H pylori-specific DNA could not be detected in any sample of leucocytes, veins or atherosclerotic plaques tested. *H pylori* IgG positivity was found in 21 of 36 patients. *H pylori*-specific DNA was, however, detected in samples of gastric tissue used as a control and known to contain the bacterium (data not shown). In contrast with the results obtained for *H pylori*, through PCR, *M pneumoniae*-specific DNA was detected in the atherosclerotic plaques of 13 of 36 (36.1%) patients, in the saphenous veins of 9 of 36 (25%) patients and in the leucocytes of 27 of 36 (75%) patients, and in 12 of 25 (48%) patients without evidence of marked carotid artery stenosis. No noticeable association was found, however, between the presence of *M pneumoniae*-specific DNA in leucocytes and in atherosclerotic plaques or veins (table 3).

Furthermore, the presence of *M pneumoniae* in either of the investigated specimens was not associated with the presence of *C pneumoniae* as determined in these specimens previously (data not shown).[18]

When plasma levels of the soluble adhesion molecules sE-selectin, sICAM-1, and sVCAM-1 and CRP were determined as markers of endothelial and platelet activation or inflammation, no differences were observed between the groups tested (table 4).

Positive IgG titres to *M pneumoniae* were found in 12 of 36 patients. We could not, however, show an association between seropositivity for *M pneumoniae* IgG and presence of the bacterium in the atherosclerotic plaque, saphenous veins or leucocytes (data not shown).

DISCUSSION

Early reports of an association between an infection with *H pylori* and CAD have not been substantiated in more recent studies.[4] Similarly, reports on the presence of the bacterium in atherosclerotic tissue are contradicting. *H pylori* DNA has been found in atherosclerotic plaques from carotid and coronary arteries and from the aorta, whereas other studies were able to detect such DNA only in a very small number of specimens or failed to detect *H pylori* DNA in atherosclerotic plaques from carotid arteries or from aortic aneurysms.[8 10 11 23] A recent study in which immunohistochemistry was used found no evidence for the presence of *H pylori* in atherosclerotic carotids.[24] In agreement with these findings, we could not detect *H pylori*-specific DNA in carotid atherosclerotic plaques from 36 patients with symptomatic carotid artery stenosis. Furthermore, saphenous veins or leucocytes obtained from the same patients were also negative for *H pylori*-specific DNA. Similar to results reported by Blasi *et al*,[10] however, 58% of the patients were seropositive for *H pylori*. In this respect, it should be emphasised that recent clinical studies found an association between seropositivity to *H pylori* and atherosclerosis, ischaemic stroke and endothelial dysfunction, supporting a role of the bacterium in the development of vascular disease.[23 25 26] On the basis of our results, however, we speculate that such a role is not due to a direct interaction of the pathogen with the vasculature.

Similar to *H pylori*, the role of *M pneumoniae* in the pathophysiology of cardiovascular disease remains elusive. An association between an infection with *M pneumoniae* and myocarditis, pericarditis, cerebral stroke and vasculitis has been reported.[27–30] In a review, Taylor and Thomas have emphasised the need to study the presence of *M pneumoniae* in vascular tissue to clarify its possible role in cardiovascular disease processes.[14] In several studies, *M pneumoniae*-specific DNA was found in calcified aortic valves and in atherosclerotic plaques, whereas in other studies no evidence for the presence of the bacterium in atherosclerotic plaques and leucocytes was detected.[9 12 31] We showed *M pneumoniae*-specific DNA in 36.1% of the atherosclerotic plaques, in 25% of the saphenous veins and in 75% of the leucocytes of the patients studied. In contrast with our earlier paper, however, in which we reported an association between the presence of *C pneumoniae* in leucocytes and its presence in atherosclerotic plaques, there was no marked association between the presence of *M pneumoniae*-specific DNA in leucocytes and atherosclerotic plaques or veins.[18] Furthermore, the presence of *M pneumoniae* was not associated with the presence of *C pneumoniae* in the investigated specimens, when retrospectively compared with our previous data.[18] In addition, we found *M pneumoniae*-specific DNA in 48% of the leucocytes

Table 3 Distribution of *Mcycoplasma pneumoniae* DNA in atherosclerotic plaques of the carotid arteries and in saphenous veins obtained from patients whose leucocytes tested either positive or negative for the presence of *M pneumoniae*-specific DNA as determined by PCR

	Atherosclerotic plaques pos*		Atherosclerotic plaques neg†	
	Veins pos, n (%)	Veins neg, n (%)	Veins pos, n (%)	Veins neg, n (%)
Leucocytes pos (n = 27)	4 (15)	6 (22)	3 (11)	14 (52)
Leucocytes neg (n = 9)	1 (11)	2 (22)	1 (11)	5 (56)

neg, negative; PCR, polymerase chain reaction; pos, positive.
*Tested positive for the presence of *M pneumoniae*-specific DNA by PCR.
†Tested negative for the presence of *M pneumoniae*-specific DNA by PCR.

Table 4 Plasma levels of sE-selectin, sICAM-1, sVCAM-1 and CRP in patients undergoing carotid endarterectomy for symptomatic carotid artery stenosis

	Leucoctyes		Atherosclerotic plaques		Saphenous veins	
	pos* (n = 27)	neg† (n = 9)	pos (n = 13)	neg (n = 23)	pos (n = 9)	neg (n = 27)
sE-selectin	10.6 (9.3)	11.3 (8.1)	8.2 (6.8)	11.8 (9.7)	9.0 (6.9)	11.3 (9.7)
sICAM-1	46.4 (15.0)	64.1 (21.3)	52.0 (18.7)	49.8 (18.0)	48.8 (10.9)	51.1 (19.9)
sVCAM-1	578.7 (187.4)	573.6 (156.5)	643.5 (233.8)	540.1 (128.7)	526.8 (158.1)	594.2 (183.8)
CRP	0.74 (1.33)	0.51 (0.74)	0.37 (0.57)	0.86 (1.42)	0.27 (0.31)	0.82 (1.35)

CRP, C reactive protein; neg, negative; pos, positive; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1.
sE-selectin, sICAM-1 and sVCAM-1 are given in ng/ml; CRP is given in mg/dl; values represent mean (SD).
*Tested positive for the presence of *Mcycoplasma pneumoniae*-specific DNA by polymerase chain reaction (PCR).
†Tested negative for the presence of *M pneumoniae*-specific DNA by PCR.

obtained from people without evidence of marked carotid artery stenosis. This difference, however, could be compromised by the lower age of these people and by the lower number of smokers in this group.[32]

No considerable differences in plasma levels of markers of endothelial and platelet activation such as sE-selectin, sICAM-1 and sVCAM-1, or of the inflammatory marker CRP, was found in patients whose plaques, leucocytes or veins either tested positive or negative for the presence of *M pneumoniae*-specific DNA. Positive IgG titres to *M pneumoniae* were found in 30% of patients. No association was found, however, between seropositivity for *M pneumoniae* IgG and presence of the bacterium in the atherosclerotic plaques, saphenous veins or leucocytes. This may be because *M pneumonia* is not known to provoke a persistent infection, although it induces a lifelong persistence of antibodies.[33] We show here the presence of *M pneumoniae*-specific DNA in atherosclerotic plaques, apparently healthy veins and leucocytes from patients with symptomatic carotid artery stenosis. The random distribution of the DNA in these tissue samples and the lack of a correlation of its presence with markers of endothelial and platelet activation and inflammation, however, do not support the hypothesis of a direct implication of *M pneumoniae* in the pathogenesis of cardiovascular disease. In this respect, it should be noted that two recent studies have shown an association between seropositivity to *M pneumoniae* and CAD or stroke, in patients who were also seropositive for antibodies against *C pneumoniae*, emphasising a likely role of the infectious burden by multiple infections with various pathogens in the pathophysiology of these diseases.[34] [35]

In conclusion, the data presented in our paper showing the absence of *H pylori* and the random distribution of *M pneumoniae* in tissue samples obtained from patients with symptomatic carotid artery stenosis do not provide evidence for a role of these common pathogens in the development of atherosclerosis owing a direct interaction of the bacteria with the vasculature.

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