[Frontiers in Bioscience E4, 2423-2432, June 1, 2012]

Early atherosclerotic plaques show evidence of infection by *Chlamydia pneumoniae*

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1. ABSTRACT

*Chlamydia pneumoniae (*Cpn) could play an important role in the development of atherosclerosis. Cpn interferes with HIF-1alpha regulation in infected host cells during intracellular replication in hypoxia. We obtained carotid artery specimens with low (n=38), high (n=25) levels of stenosis and 10 control middle cerebral arteries. Fifty eight percent of the carotids with low levels of stenosis showed evidence of the viable Cpn. Ninety one percent of the positive results were derived from pre-atheromatous lesions. Only 12 percent of plaques removed at endarterectomy showed the presence of Cpn DNA. All control middle cerebral arteries failed to show evidence of live Cpn. Ninety one percent of sera from 22 endarterectomy patients showws the presence of Cpn antibodies. Immunohistology of carotid arteries with low levels of stenosis was used to confirm the presence of HIF-1alpha in infected specimens and showed a correlation between the over-expression of HIF-1alpha and Cpn in the plaque (p less than 0.05). Cpn might play an important role in activation and development of the initial stages of atherosclerotic lesions.

2. INTRODUCTION

The development of cardiovascular disease has been attributed to the presence of pathogens such as *Cytomegalovirus, Helicobacter pylori, Herpes simplex virus,* or *Chlamydia pneumoniae* (Cpn) (1-4). *Chlamydia pneumoniae*, an obligate intracellular Gram-negative bacterium, was first implicated in 1992 in the pathogenesis of atherosclerosis (5). This seroepidemiological study showed a higher number of detectable Cpn-antibodies in patients with coronary heart disease than in controls. However, contradictory reports exist regarding the importance of this infectious agent in the development and progression of atherosclerosis. Studies have described findings of between 0% and more than 60% Cpn-positive tissue in carotid arterial lesions (6-10). The most recent reviews about this issue showed very poor reproducibility of the results depending on which laboratory analyzed the samples (11,12). The type of test that had been used to detect the presence of *C. pneumoniae* in atheroma specimens was also criticized because of the high number of false positive results presented (13,14). It seems that polymerase chain reaction (PCR) is a better technique to detect *C. pneumoniae*

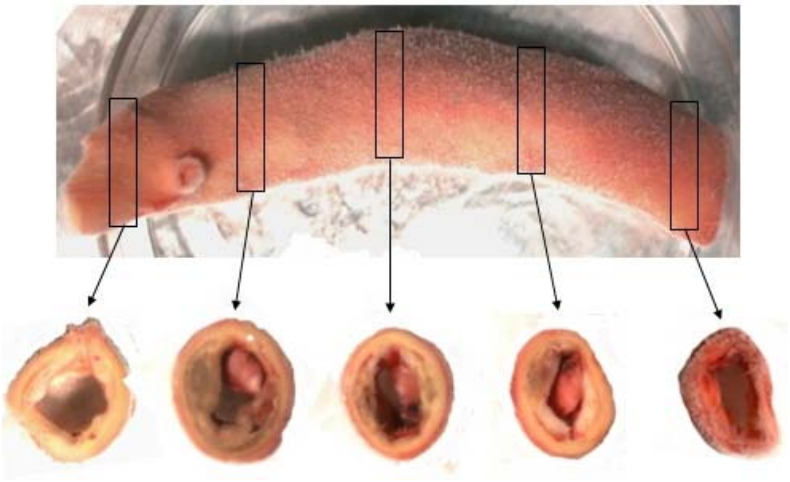

Figure 1. Representative areas cut in a carotid artery with less than 50% stenosis. The greatest thickness of the artery was designated as "the plaque area".

than for example immunohistochemistry or electron microscopy (15-17). *In vitro* and *in vivo* studies demonstrated how this common respiratory pathogen is capable of infecting endothelial cells (18-20), smooth muscle cells (21,22) or monocytes (23) inducing proatherogenic processes like foam cell formation (24-26) or expression of chemokines and adhesion molecules (27). Other studies showed that the inactivated bacterium, without infectious capacity, is still able to activate both an inflammatory and immune response (28,29). Given that atherosclerosis is considered a chronic inflammatory disease (30,31), interaction of *C. pneumoniae* with host cells of the vessel wall may provoke a cellular response to infection. This cellular response might have an important role in the development of atheroma independently of other typical cardiovascular risk factors like hypercholesterolemia or diabetes.

During bacterial infection hypoxia is a characteristic feature of the tissue microenvironment. Inflammatory reactions to bacterial infections usually reduce tissue oxygenation and induce cellular responses to hypoxia (32,33). The adaptive response of mammalian cells to the stress of oxygen depletion is coordinated by the action of hypoxia-inducible factor 1 (HIF-1). HIF-1 comprises an oxygen-dependent expressed alpha subunit and the constitutively expressed beta subunit. In normoxic conditions, HIF-1alpha is translated and rapidly degraded by the proteosome. In hypoxia, the degradation of HIF-1alpha is blocked resulting in a rapid increase of HIF-1alpha levels. After stabilization, HIF-1alpha translocates to the nucleus where it heterodimerizes with HIF-1beta to activate transcription of target genes (34-37). Recent studies reported that *C. pneumoniae* directly interferes with HIF-1alpha regulation in infected host cells during intracellular replication in hypoxia (38).

Progression of carotid artery atherosclerosis leads to ischemic brain stroke. Different seroepidemiological studies showed a relationship between infection with *C. pneumoniae* and cerebrovascular disease. Chronic infection with this bacterium was associated with an increased risk of stroke and transient ischemic events (39-42). However, PCR studies with large and small cerebral vessels showed low presence of this pathogen in the atheroma tissue (43-45). Several issues, including *C. pneumoniae*'s direct involvement in lesion progression are still unsolved. The aim of the present study was to investigate the presence of *C. pneumoniae* infection in different vascular territories related to the risk of ischaemic stroke and to identify any differences between early and advanced lesions. Furthermore, we studied the relationship between the presence of *C. pneumoniae* and over-expression of the HIF-1alpha in carotid lesions to confirm that in our specimens, there was an association between the hypoxic tissue environment and the bacterial infection.

3. MATERIALS AND METHODS

3.1. Patients and sample collection

We included 38 human carotid arteries with low to moderate stenosis (less than 50% by EcoDoppler ultrasonography) obtained as vascular transplants from organ donors or post-mortem autopsies. Carotid arteries, including common carotid artery, and a large portion of both the internal and external carotid arteries were excised by a vascular surgeon as part of a standard procedure for organ transplantation. There was no time delay as the other organs were removed simultaneously. Post-mortem samples were processed immediately according to the current bank of human samples protocol. Human carotid endarterectomy (CEA) specimens (n = 25) were obtained from patients with a high-grade degree of carotid stenosis (>70% on EcoDoppler imaging confirmed by angio-RM or angio-TC). Control human middle cerebral arteries (MCA) (n = 10) were obtained from post-mortem autopsies. These patients had no symptomatic atherosclerotic or cerebrovascular disease. Five different areas from each carotid artery with stenosis < 50% and four different areas of endarterectomies and MCA, due to their smaller size, were cut. An example of different areas cut in a carotid artery is represented in Figure 1. An additional area was fixed for 24 hours in buffered formalin, briefly decalcified to remove excess calcium and embedded in paraffin wax. Sections (5µm) were cut on a microtome. Plaque morphology was assessed by analysis of haematoxylin and eosin stained sections. Carotid plaques with stenosis < 50%

Table 1. AHA classification and anatomo-pathological characteristics of plaques

	Carotids low-moderate stenosis (n=38)	MCA[1] (n=10)	Carotids high-grade stenosis from CEA[2] (n=25)	
AHA Classification			*UNC*[3]	50
Early (I-III)	55.3	60	*UC*[4]	22.7
Intermediate (IV-Va)	44.7	40	*F*[5]	27.3
Necrotic core	44.7	20.0		
Calcification	44.7	0.0		
Inflammation	57.9	10.0		
Intraplaque hemorrhage	0.0	0.0		
Cholesterol clefts	18.4	0.0		

Values are %; Abbreviations: [1]Middle Cerebral Arteries; [2]Endarterectomies; [3]Ulcerated noncomplicated; [4]Ulcerated complicated; [5]Fibrous.

Table 2. Patient's clinical data

Feature	Carotids low-moderate stenosis (n=38)	MCA[1] (n=10)	Carotids high-grade stenosis from CEA[2] (n=25)	p-value
Age (Mean±SD)	67 ± 15	65 ± 19	70 ± 7	NS
Sex (♂/♀)	25/13	8/2	20/5	NS
WBC[3] x10^9/L (Mean ± SD)	12.3 ± 10.0	11.1 ± 5.0	7.6 ± 2.2	NS
Glucose (Mean ± SD) (mmol/l)	6.7 ± 2.0*	10.8 ± 7*†	7.7 ± 2.6†	0.009
Total cholesterol (Mean ± SD) (mmol/l)	3.4 ± 1.0*	4.2 ± 1.0	4.1 ± 0.8*	0.010
HTA[4] n, (%)	15 (39.5)	7 (70)	17 (68)	NS
DM[5] n, (%)	8 (21.1)*	3 (30)	13 (52)*	0.037
DLP[6] n, (%)	9 (23.7)*	3 (30)	15 (60)*	0.022
Smoking n, (%)	9 (23.7)*†	7 (70)*	17 (68)†	0.000
Alcohol abuse n, (%)	8 (21.1)	3 (30)	2 (8)	NS
CAD[7] n, (%)	8 (22.2)*	3 (30)	12 (48)*	0.000
Statins n, (%)	5 (20.8)	0 (0)*	11 (44)*	0.030
Antiplatelets n, (%)	4 (16.7)†	3 (30)*	24 (96)*†	0.000
Rasb[8] n, (%)	9 (37.5)*	5 (50)†	5 (20)*†	NS
Symptomatic n, (%)	-	-	19 (79)	-

*†Statistically significant between these groups; NS, not significant. Abbreviations: [1]Middle cerebral arteries; [2]Endarterectomies; [3]White blood cells; [4]Hypertension; [5]Diabetes mellitus; [6]Dyslipemia; [7]Coronary artery disease; [8]Renin angiotensin system blockers.

and MCA were classified according to the American Heart Association guidelines (AHA) with some modifications (46). Carotid plaques from endarterectomies were classified as ulcerated non-complicated (UNC), ulcerated complicated (UC) exhibiting hemorrhagic transformation, or fibrous (F) (47). Classification and anatomo-pathological characteristics of plaques are presented in Table 1. The other parts of the arteries were snap-frozen in liquid nitrogen and stored at -80ºC and used for PCR analysis. Serum from the patients who underwent endarterectomy surgery was obtained to study the presence of C. pneumoniae antibodies in these patients. The study was approved by the local ethical committee in accordance with institutional guidelines, and the family's written informed consent was obtained. Patients' basic clinical data and vascular risk factors are summarized in Table 2.

3.2. PCR protocol

DNA extraction was performed from ~25mg of tissue with the QIAamp DNA Mini Kit (Quiagen) according to the manufacturer's instructions. Different laboratories and working areas were used to avoid contamination between DNA extraction and different PCR reactions (15). The sequence of the 474-bp PstI fragment was used as the basis for this PCR to detect C. pneumoniae. The specificity of the fragment was analyzed through BLAST (Basic Local Alignment Search Tool) to confirm that only C. pneumoniae family members had this sequence. To detect C. pneumoniae DNA with a high level of sensitivity, a 2-step nested PCR protocol was implemented. Primers were designed with the Lasergene software (DNAstar). The primers for the primary PCR were designated Chp-1L (5'-CACCGTCGTACAGCAGAAATC-3') and Chp-2R (5'- GGGGTTCAGGGATCATTTGTA-3') producing a 398-bp sequence. Nested primers and the thermal cycling conditions described by Nadareishvil *et al* (48) were used. As a result of the nested PCR, a fragment of 214bp was obtained. Each run contained a negative control, in which sterile water replaced the specimen sample, and a positive control consisting of C. pneumoniae DNA TW-183 (a kind gift from Dr. Essig of the Institut für Med, Mikrobiologie und Higiene Universitatsklinikum Ulm Robert-Koch-Str., Germany). If contamination was observed, the results of the PCR assay were rejected, all laboratories and equipment were carefully cleaned, different working areas were used, and reagents were replaced by unopened ones. All PCRs were carried out using a 9700 Gene Amp PCR System (Applied Biosystems). PCR products were visualized under UV light after electrophoresis in 2% ultra-PURE-agarose (Invitrogen) stained with ethidium bromide. An example of nested PCR products with a negative and a positive control is represented in Figure 2.

3.3. Serological testing

Sera from 22 patients who underwent carotid endarterectomy were tested at serial dilutions from 1/32 to 1/512 for IgG, from 1:8 to 1:64 for IgA, and 1:16 for IgM-antibody titre determination. The commercial

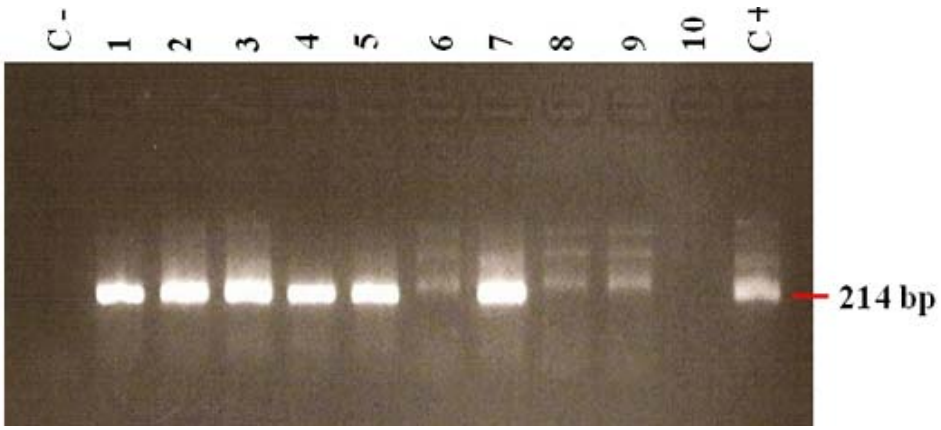

Figure 2. Shows an example of nested PCR products, separated by electrophoresis using 2% ultraPUREagarose. Negative and positive controls for *C. pneumoniae* are shown. Lanes 1, 2, 3, 4, 5 and 7 were positive whilst 6, 8 and 9 were negative. The positive nestedPCR resulted in production of a 214 bp fragment.

microimmunofluorescence (MIF) test is an indirect fluorescent antibody technique for measurement of IgG-, IgM- and IgA antibodies to *C. pneumoniae.* The test utilizes purified, formalinized whole elementary bodies (EB) of *C. pneumoniae*, fixed onto microscope slides as distinct dots of antigen, leading to detection of surface exposed protein-reactive antibodies. The MIF test was performed according to the manufacturer's instructions (Labsystems, Helsinki, Finland). Briefly, serial serum dilutions were incubated with the antigen on a microscope slide for 30 min (for IgG and IgA tests), and for 3 hours (for the IgM test). Subsequently, the slides were rinsed and dried, fluorescein isothiocyanate (FITC)-labeled anti-IgG, IgM or IgA conjugates were applied and incubated for 30 min. A coverslip was mounted with appropriate mounting fluid over the antigen spot and the microscope slides were again rinsed and dried. The microscopic examination was done with an Olympus Vanox AHBT3 microscope at X1000 magnification. Two experienced investigators evaluated the slides simultaneously.

3.4. Immunohistochemical analysis

Immunohistochemical analysis in 15 of the 38 human carotid arteries with low to moderate stenosis was performed to detect the presence of hypoxia-inducible factor-1α. These 15 arteries were randomly selected. The analyses were performed in the highest thickness area of the artery (the plaque area). Paraffin-processed sections (5 μm) were deparaffinized in xylene and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and antigen retrieval was carried out (Tris/HCl 10mM, pH10, 20 min at 95-99ºC). Then the slides were treated with 10% hydrogen peroxide in methanol (30 minutes at room temperature, RT) to remove endogenous peroxidase activity. Sections were blocked using 10% normal serum in PBS-Tween 0.1% (30 minutes at RT) and stained with the specified dilution of primary antibody, anti-HIF-1α (1:2000, mouse monoclonal antibody, Abcam, Cambridge, UK) overnight at 4ºC. After washing in PBS, sections were incubated with biotinylated secondary antibody (Vector Laboratories; 1:200), and incubated at RT for 1 hour.

After rinsing in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories) was applied, and the slides were incubated at RT for 30 minutes. Colour was developed using 3, 3'-diaminobenzidine (DAB) and sections were counterstained with haematoxylin before dehydration, clearing, and mounting. Negative controls in which the primary antibody was replaced with PBS were used to test for non-specific binding (data not included). All immunostaining was assessed by 2 investigators simultaneously using a double-headed light microscope.

3.5. Statistical analysis

All statistical analysis was performed with the SPSS for Windows statistical software program (version 15.0, SPSS Inc.). One-way ANOVA was used to assess the statistical significance of patients' clinical data, differences between the three groups of arteries and the relationship between the different risk factors and the presence of C. pneumoniae to quantitative variables. The Chi-square test was used for measurement of differences in qualitative variables. The Chi-square test was also used to study the relationship between seropositive samples, HIF-1alpha and the presence of C. pneumoniae. Statistical significance was assumed when P was less than or equal to 0.05.

4. RESULTS

4.1. PCR detection of C. pneumoniae

4.1.1. Detection of C. pneumoniae in carotid arteries with low-moderate stenosis (lower than 50%)

Twenty-two of the 38 (58%) carotid specimens with low and moderate stenosis were positive for the presence of C. Pneumoniae DNA. However, not all the areas studied of each sample were positive. Only 2 of the 22 positive results ($P<0.05$) were from regions containing atheroma plaque tissue (type IV-Va lesion). 91% of the positive results came from pre-atheroma graded areas (grade less than IV) whereas only 9% came from atheroma graded areas. Results are presented in Figure 3. There was no relationship between the presence of C. pneumoniae and the

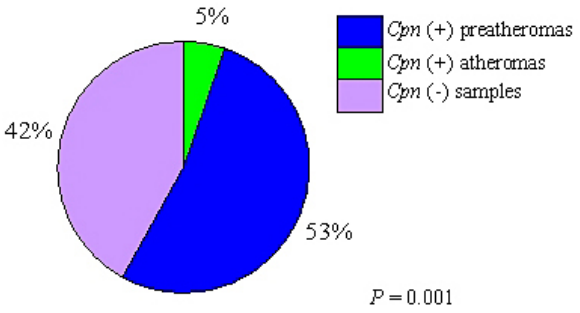

Figure 3. Shows the presence of *C. pneumoniae* DNA in carotid arteries with less than 50% stenosis. 20 preatheromatous and 2 atheromatous samples were positive whilst 1 atheromatous and 15 preatheromatous samples were negative.

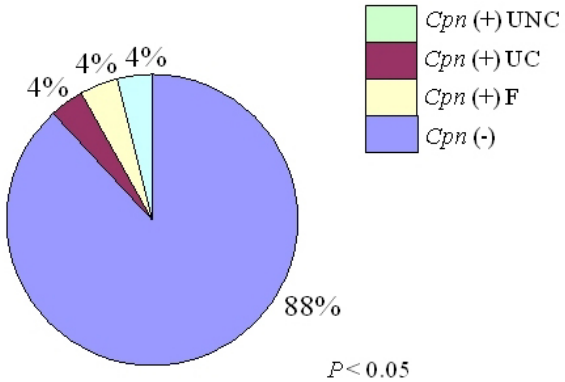

Figure 4. Shows the presence of *C. pneumoniae* DNA in endarterectomy specimens. 88% were negative whilst 12% were positive.

different cardiovascular risk factors, treatments, or the anatomo-pathological characteristics.

4.1.2. Detection of *C. pneumoniae* in carotid arteries with high-grade stenosis (higher than 70%)

Only 3 of the 25 (12%) endarterectomy specimens analyzed by PCR were *C. pneumoniae* positive, the remaining 88% being negative ($P<0.05$). A positive result was considered when at least some of the plaque parts analyzed were positive. Results are presented in Figure 4. These 3 positive samples were from symptomatic patients. Negative results were obtained from 16 symptomatic and 6 asymptomatic patients. 50% of the endarterectomy samples studied were classified as ulcerated non-complicated, 22.7% were ulcerated and complicated and 27.3% were fibrous. However, there was no relationship between the presence of the bacterium and these characteristics. There was also no relationship between Cpn (+) and cardiovascular risk factors or treatments.

4.1.3. Detection of *C. pneumoniae* in middle cerebral arteries

Six middle cerebral arteries were classified as early (type I-III lesion) and the other 4 arteries were classified as intermediate (type IV-Va) according to the AHA classification. The four different areas of the 10 middle cerebral arteries analyzed were negative for the presence of *C. pneumoniae* DNA.

4.2. Serological detection of *C. pneumoniae*

Of the 22 serum samples studied, a total of 20 results were positive (91%) and 2 were negative (9%). IgG antibodies against *C. pneumoniae* were found in 16/22 cases. IgM antibodies were present in 5/22 cases and IgA antibodies in 12/22 cases. Two of the patients who had positive results in the PCR analysis were also positive in the serological study. Serological studies of anti-chlamydial IgG, IgA, and IgM revealed no association between seropositive samples and the presence of *C. pneumoniae* within the plaque as measured by PCR.

4.3. Immunohistochemistry of HIF-1alpha

In type IV/Va lesions taken from patients with low to moderate carotid artery stenosis, there was highly selective staining with HIF-1alpha. There was a strong staining in neovessels surrounding the lipidic core and in the neovessels of the neointima. Furthermore, inflammatory cells were strongly stained with anti-HIF-alpha antibody. Representative immunostaining taken from a fibroatheromatous lesion is presented in Figure 5. The extent of staining was graded according to a semi-quantitative scale of 0 to + + +: 0, no staining detected; +, weak staining; + +, moderate staining; + + +, extensive staining. There was a significant correlation between the over-expression of HIF-1alpha in the plaque area and the presence of *C. pneumoniae* in some parts of the artery ($P<0.05$). Results are presented in Table 3.

5. DISCUSSION

Our study of atherosclerotic carotid arteries suggests that infection with *C. pneumoniae* is common in early lesions. 58% of the carotid artery samples from subjects with less than 50% stenosis showed evidence of the viable organism. Ninety one percent of the positive results came from pre-atheromatous lesions (grade less than IV) whereas only 9% came from atheromatous lesions. In addition, only 12% of plaques removed at endarterectomy, i.e. carotid arteries with stenosis of more than 70%, showed the presence of *C. pneumoniae* DNA. A relationship between infection and cardiovascular risk factors could not be demonstrated.

Some studies have showed more than 60% positive results for the presence of *C. pneumoniae* on analysis of carotid plaques following endarterectomy (7,9,49), whilst others demonstrated a high variability in positive results in the same group of specimens studied depending on whether they had been analysed by immunohistochemistry, from cultured cells, by PCR or other techniques (13). There were many false positive results (50). Normally, a lower percentage of samples demonstrated the presence of *C. pneumoniae* if PCR was the technique employed (51). Nested PCR increases the reliability of the results but it is also associated with higher risk of contamination (15,16,52, 53). Initially, we

Table 3. Relationship between HIF-1alpha immunostaining and the presence of *C. pneumoniae* in carotid lesions with stenosis <50%

n = 15		**Cpn**		**Total**
		Neg	**Pos**	
HIF-1alpha	0	4	0	4
	+	3	0	3
	++	0	4	4
	+++	1	3	4
	Total	8	7	15
			p value	0.007

0, no staining detected; +, weak staining; ++, moderate staining; +++, extensive staining.

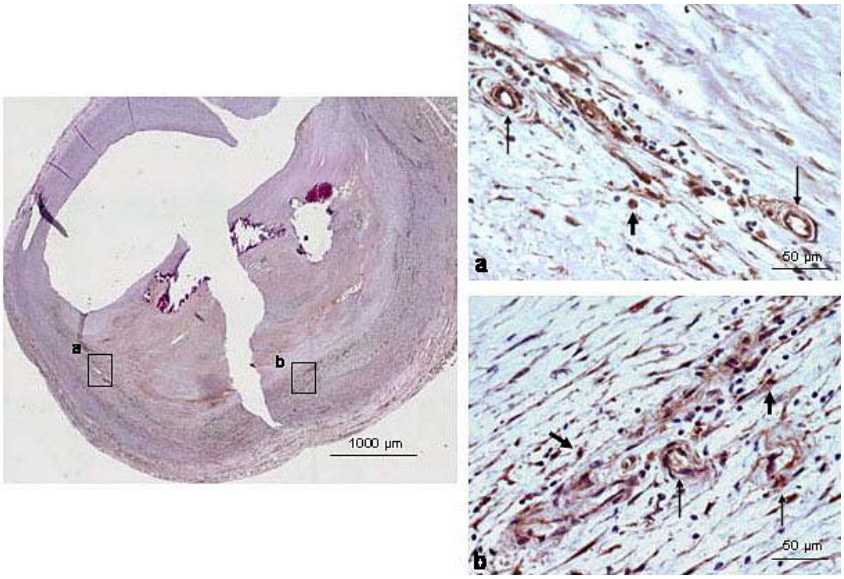

Figure 5. Immunohistochemistry of HIF-1alpha in a fibroatheromatous carotid artery. There was strong staining in neovessels surrounding the lipid core and in neointimal neovessels (Thin arrows) and also strong staining in inflammatory cells (Thick arrows)

experienced contamination problems, and therefore we had to use different laboratories for the extraction of DNA, PCR processes and for running agarose gels. We always cleaned thoroughly all the material necessary for the experimental processes and we used negative and positive *C. pneumoniae* controls. The last reviews have demonstrated, like us, that there was little presence of *C. pneumoniae* in endarterectomy plaques. However, this is the first report demonstrating that early to moderate carotid lesions and preatheromatous areas are highly infected with *C pneumoniae*. Bacterial infections induce HIF-1alpha expression. Some reports demonstrated that HIF-1alpha is involved directly or indirectly in the regulation of specific immune effector molecules during infection, such as antimicrobial peptides and tumour necrosis factor-alpha (37,54). A recent study showed for the first time that *C. pneumoniae* is able to replicate within infected host cells and to maintain infectivity under hypoxic conditions.

Therefore, to directly manipulate host cell metabolism and function in hypoxia, *C. pneumoniae* may somehow take control of the central oxygen-sensing mechanism of HIF-1alpha (38). When atherosclerotic lesions develop, the arterial wall thickness increases and oxygen diffusion capacity is impaired. At the same time, oxygen consumption is augmented, and an energy imbalance may occur. These local metabolic disturbances result in progression of atherosclerosis, plaque growth and remodelling, with the formation of a necrotic core. We confirmed that in our specimen there was a notable correlation between expression of HIF-1alpha and the presence of *C. pneumoniae* in carotid atherosclerotic lesions. We observed an over-expression of HIF-1alpha in the atheromatous areas of infected arteries. This pathogen may play an important role in activation and development during the initial stages of carotid atherosclerosis. Probably, the infection of cells could lead to an immune response, and sunsequently, the bacterium would be eradicated, migrate to other parts of the artery, or survive in a persistent state. If the bacterium was eradicated, the remaining elements such as membrane components would still be able to maintain signal activation, such as that of the toll like receptors, therefore resulting in induction or maintenance of a chronic inflammatory stage. This theory would explain the positive results that different studies have shown using inmunohistochemical processes that were negative using PCR. It is possible that we did not find the presence of the bacterium in advanced lesions due to the fact that *C. pneumoniae* is an obligate intracellular bacterium that needs live cells to replicate, which may indicate that it prefers to infect and replicate in normal arteries rather than in advanced carotid arterial lesions which are normally

calcified, ulcerated and containing small numbers of cells. Different seroepidemiological studies showed a high prevalence of *C. pneumoniae* antibodies in the population, which increases with age (55,56). These studies demonstrated that most people were infected and re-infected throughout life (57). Higher numbers of patients with coronary artery diseases seem to have *C. pneumoniae* antibodies than control patients. However, in this study, there was no correlation with the presence of the bacterium in atheromatous tissue (10). Our results support these studies. We showed that 91% of patients had positive serological results however there was no relationship between seropositive samples and the presence of *C. pneumoniae* within the atheromatous plaque. As mentioned above, previous studies have demonstrated that the inactivated bacterium can produce many of the same effects as that of the viable pathogen. The inactivated bacterium is able to activate different signal responses including the NFKB pathway in endothelial cells (28,29,44). The presence of the bacterium in circulating cells could induce immune and inflammatory response that contribute to the development of atherosclerosis.

Some previous studies have found the presence of *C. pneumoniae* in middle cerebral arteries (58). In these studies, as in many seroepidemiological studies, it was suggested that this bacterium may be involved in development of acute and chronic cerebrovascular disease (39). However, the presence of C. pneumoniae was always detected in only a few of the studied middle cerebral arteries or was not detected at all (43-45). In our case, the presence of the pathogen was not detected despite having studied several areas of the artery including the atherosclerotic region. Our results are consistent with other studies that could not demonstrate a correlation between the presence of Chlamydial DNA in cerebral arteries and stroke (44). Although the present study demonstrates that *Chlamydia pneumoniae* can frequently be detected in early atherosclerotic stages of carotid arteries, future studies should investigate the mechanisms through which this pathogen may have a causative role in the initiation and development of carotid atherosclerosis, which is a risk factor for clinical stroke.

6. ACKNOWLEDGEMENTS

All authors contributed to the manuscript. AL performed most of the studies, including histology, PCR, antibody testing and prepared the manuscript. MMT helped with sample preparation from human material. NR helped with histology, PCR work and antibody testing. OJB did histology work. MS helped with manuscript preparation and design of the study. JK designed the work, helped with sample collection and manuscript preparation.

This work was supported by grant from Plan Nacional SAF2009-12130 and Beca Fundación Mútua Terrassa. M Slevin was supported by the BBVA.

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Abbreviations: Cpn: *Chlamydia pneumoniae*, HIF-1: Hypoxia Inducible Factor-1, PCR: polymerase chain reaction, CEA: carotid endarterectomy, MCA: middle cerebral artery, AHA: American Heart Association, UNC: ulcerated non-complicated, UC: ulcerated complicated, BLAST: Basic Local Alignment Search Tool, MIF: microimmunofluorescence, EB: elementary body, FITC: fluorescein isothyocianate, NFKB: nuclear factor kappa beta, DNA: deoxyribonucleic acid

Key Words: *Chlamydia pneumoniae*, Atherosclerosis, Carotid artery, Stroke

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