CHLAMYDIA PNEUMONIAE AND SYMPTOMATIC CAROTID ATHEROSCLEROTIC PLAQUE

A prospective study

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ABSTRACT - *Objective:* To investigate the possible link between symptomatic carotid atherosclerotic plaque and *Chlamydia pneumoniae.* *Background:* Recently, several studies have demonstrated that there may be a possible link between *Chlamydia pneumonia* and carotid atherosclerosis, however the real role of *Chlamydia pneumoniae* is not completely understood. *Method:* This is a prospective study with a total of 52 patients analyzed. All patients had been submitted to endarterectomy, and had suffered thrombotic ischemic stroke or transient ischemic attack up to 60 days prior to the surgery. Every patient presented carotid stenosis over 70%. The plaque was removed during the surgery and the laboratory exams were immediately done. Evaluation of *Chlamydia pneumoniae* DNA was done using polymerase chain reaction (PCR). *Results:* The PCR analyses of all 52 patients were negative for *Chlamydia pneumoniae.* *Conclusion:* These initial results do not show a relationship between *Chlamydia pneumoniae* and symptomatic carotid atherosclerotic plaque.

KEY WORDS: *Chlamydia pneumoniae,* atherosclerosis, carotid artery, stroke.

Chlamydia pneumoniae e placa aterosclerótica sintomática de carótida: um estudo prospectivo

RESUMO - *Objetivo:* Investigar a possível relação entre placa sintomática de carótidas e *Chlamydia pneumoniae.* *Introdução:* Vários estudos têm demonstrado uma possível relação entre *Chlamydia pneumonia* e aterosclerose carotídea, entretanto o papel definitivo da bactéria não é totalmente conhecido. Há muita especulação: poderia iniciar o processo aterosclerótico, agravá-lo ou desestabilizá-lo. *Método:* Estudo prospectivo com um total de 52 pacientes, endarterectomizados e previamente acometidos de acidente vascular cerebral isquêmico ou crise isquêmica transitória, em até 60 dias antes da cirurgia. Todos os pacientes apresentavam estenose carotídea superior a 70%. Os testes laboratoriais foram realizados imediatamente após a endarterectomia. A *Chlamydia pneumoniae* foi pesquisada através de exame de DNA com reação de polimerização em cadeia (PCR). *Resultados:* O PCR dos 52 pacientes foram negativos para *Chlamydia pneumoniae.* *Conclusão:* Estes resultados iniciais não mostram relação entre *Chlamydia pneumoniae* e desestabilização de placa aterosclerótica das carótidas.

PALAVRAS-CHAVE: *Chlamydia pneumoniae,* aterosclerose, carótida, acidente vascular cerebral.

The relationship between infection, atherosclerosis and stroke has been deeply studied and emphasized[1-3]. The stronger evidences that associate atherosclerosis with infection are upon the *Chlamydia pneumoniae* bacteria[3], an intracellular organism, identified as a cause of disease in human beings since 1983.

Several studies have been developed in the attempt to explain the basic mechanisms that could associate the *Chlamydia pneumonia* infection to atherosclerosis. The activation of endothelial cells is one of the fundamental points in atherosclerotic evolution[3]. There are different types of experimental studies that evidence endothelial cells as possible victims of *Chlamydia pneumoniae* infections[4,5] Several mechanisms are known by which *C. pneumoniae* could mediate an atherogenic process in the arteries; most of these show the capacity of *Chlamydia pneumoniae* to trigger an inflammatory reaction on the vascular wall that would lead to the activation and pro-

gression of the atheroma, and the start of thrombotic complications[6].

Most of the early studies used immunofluorescence assays, dosing serum levels of IgA, IgG, or IgM, as a diagnostic method for *Chlamydia pneumoniae*. The results, however, are conflicting, with some findings positive[7-9], and others negative[10,11], with the methodology employed questioned. Recently, *Chlamydia pneumoniae* DNA detection has been used, employing the polymerase chain reaction (PCR), which has shown to be a valid diagnostic method, and apparently more reliable than other techniques[10,11]. The presence of circulating DNA of *Chlamydia pneumoniae*, diagnosed through the PCR in humans, may represent a persistent systemic infection by the bacteria[12].

The alterations and/or atherosclerotic lesions possibly related to the infections may be acute or delayed[13]. Infection could precipitate the beginning of plaque formation and is not necessarily related with chronic state[13].

The transformation of the asymptomatic plaque to symptomatic is one of the fundamental phases for the occurrence of ischemic events; this has been the target of many studies. With the goal of investigating the possible role of *Chlamydia pneumonia* in this stage of atherosclerotic evolution, this study was developed.

METHOD

This is a prospective study, with a total of 52 patients.

The patients of the present study had follow-up at the Santa Casa of São Paulo Hospital. All of the patients, in the period between January 2002 and March 2005, who had been submitted to endarterectomy, and had suffered thrombotic ischemic stroke (IS) (42 patients) or thrombotic transient ischemic attack (TIA) (10 patients) up to 60 days prior to the surgery, were selected. Patients were excluded suspected of having cardioembolism (atrial fibrillation, recent myocardial infarction, intramural thrombus, arrhythmias), lacunar infarction and cerebral hemorrhage. Stroke and TIA were diagnosed by the symptoms presented with confirmation obtained by computed tomography (CT-scan) or magnetic resonance imaging. All of the patients were submitted to an angiographic study using digital angiography. Only patients that showed over 70% of carotid stenosis were included and considered symptomatic for carotid atherosclerosis plaque. There were no other exclusion criteria or patient selection. Age, gender and risk factor for stroke, were not considered for patient exclusion. The basic characteristics of the population are presented in the Table.

Specimens – Fragments of carotid endarterectomy were sent to the Laboratory in sterile containers immediately after surgery.

The tissues were divided into various segments and separated into three portions containing at least 18 small pieces, each representing the whole original sample fragment (both ends and middle). These portion samples were macerated and placed into three Eppendorf tubes in order to perform DNA extraction by the Proteinase K technique as described bellow:

To each 1.5 ml Eppendorf tube containing the macerated tissue samples, 1000 ul of the LYSES SOLUTION was added, vortexed vigorously for 15 sec., centrifuged for 2 min. at 13.000 rpm, and the supernatant was discarded by aspiration with a Pasteur pipette. Then, to the pellet, 500 ul of TEN, 5ul of SDS (10%) and 3ul of proteinase K (125 mg/ml) was added, mixed and incubated overnight in a 56°C block heater. After this, 150 ul of 5M NaCl was added and the tubes were vigorously agitated for 15 sec. After centrifugation at 13.000 rpm for 5 minutes the supernatant

Table. Baseline characteristics of the patients.

	Ischemic stroke N (%)	TIA N (%)
Age (y) Median ±SE	70±4.5	68±4.9
Male	22 (52.4)	6 (60)
Female	20 (47.6)	4 (40)
Ethnicity		
White	16 (38.2)	4 (40)
Black	11 (26.1)	3 (30)
Asian	8 (19)	1 (10)
Mulatto	7 (16.7)	2 (20)
Hypertension	25 (59.5)	6 (60)
Diabetes	14 (33.3)	2 (20)
Smoking	12 (28.6)	3 (30)
Hypercholesterolemia	9 (21.4)	3 (30)
Carotid stenosis (%) -median	85	80
Mean time between symptoms/surgery (days)	35±5	30±4

was transferred to a new 1.5 ml Eppendorf tube and 650ul of cold isopropanol was added. DNA was precipitated in a freezer at –20°C overnight. After this period, the supernatant was discarded and the pellet was washed two times with cold 70% ethanol and air-dried for 30 min. The DNA pellet was dissolved in 200 ul of TE and stored at –20C until use. Five microliters (5 ul) of this DNA was used as template in our "in house" *Chlamydia pneumoniae* PCR, and 1ul per tube in our "in house" Internal Control PCR (Human β-Globin gene) for each sample studied.

The *Chlamydia pneumoniae* PCR was performed as follows:

Single PCR was done in a final volume of 20ul containing 1U Taq polimerase, 1X polymerase buffer [containing 50 mMKCl, 20 mM Tris-HCl(ph8.4)], 1.5 mM $MgCl_2$, 200 uM dNTPs mixture, 0.5 uM of each primer using a Perkin Elmer 2400 (Applied Biosystems). The PCR conditions were: pre-denaturation step at 94C for 5 minutes, followed by 40 cycles of 95C for 45 sec, 62C for 45 sec, and 72C for 1 minute. A final extension step was done at 72C for 7 minutes. The *Chlamydia pneumoniae* primers used were HL-1 5'GTT GTT CAT GAA GGC CTA CT 3' as the forward and HR-1 5' TGC ATA ACC TAC GGT GTG TT 3' as the reverse according to Campbell[14]. For each sample, a quality control PCR, detecting the human beta–globin gene, was performed using the same master mix with the primers PCO-3 5'ACA CAA CTG TGT TCA CTA GC 3' and PCO-4 5'CAA CTT CAT CCA CGT TCA CC 3', according to Saiki et al[15]. Cycling conditions were: 5 min. at 94C as pre-denaturation step, followed by 35 cycles of 1 min at 94C, 1 min 30 sec at 62C, and 2 min at 72C. A final extension step was done at 72C for 7 minutes.

Ten microliters of the amplified products were electrophoresed in 1.5% agarose gel and visualized under a UV light after ethidium bromide staining. The size of the specific *Chlamydia pneumoniae* amplified product (437 bp) and the internal control beta-globin gene (110 bp) were assessed by comparison with a commercial 100 bp marker.

This study was approved by Ethical Comite of Santa Casa de São Paulo.

RESULTS

All of the 52 patients had PCR tests negative for *Chlamydia pneumoniae.*

DISCUSSION

The association between *Chlamydia pneumoniae* and atherosclerosis has been deeply discussed in recent publications, with various authors believing that this bacterium may have some active participation in the pathogenesis of this disease[3,7,8]. It is a controversial subject[16] and even though a great part of the literature supports this theory, there is still doubt as to which phase *Chlamydia pneumoniae* is linked to atherosclerotic evolution. It can act on the triggering process, in the evolution, progression, or the destabilization of the plaque.

Most of the studies that analyze the role of *Chla-mydia pneumoniae* in human atherosclerosis use, as a diagnostic method, serum doses of immunoglobulins IgA, IgM or IgG which provide information about contamination with the infectious agent, yet do not precisely define the time in which the contamination occurred. This exam, when positive, does not define, with clarity, if it is a primary or persistent infection, or a reinfection[12,17]. It is possible that a positive finding, in this situation, may translate as an old infection that has already been resolved. The positive result of this exam may reinforce the hypothesis of some role *Chlamydia pneumoniae* may play in the triggering of atherosclerosis, or in its subsequent evolution, but not necessarily in its transformation from asymptomatic to symptomatic or in plaque destabilization[17]. The DNA detection technique for *Chlamydia pneumoniae* through PCR is a valid diagnostic method that has shown to be more reliable when compared to other diagnostic procedures[1,12]. The presence of *Chlamydia pneumoniae* DNA, diagnosed using PCR, may represent a current and/or persistent infection by the bacteria[12]. Though the technique is reliable, there are controversial results in the literature with great variability as to the results, ranging from one extreme to the other, according to different authors[16]. In this study, we opted for the laboratory technique described by Dowell et al.[17], which was one of the PCR techniques recommended by the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada)[17]. Internal controls are used in PCR assays to rule out false negative results due to inefficient specimen processing or the presence of compounds in the reaction that inhibit the amplification enzyme. Inclusion of a control makes it possible to distinguish between specimen preparation or PCR failures and truly negative results. In our study, if the amplified product of the internal control (Human β-Globin gene) was not seen in the gel after staining, both PCRs (*Chlamydia pneumoniae* and Beta-globin gene) were repeated twice: (I) one time again and (II) one time with a 1/10 dilution of the original DNA extraction. Therefore positive or negative results for *Chlamydia pneumoniae* PCR were only defined if the internal control amplicon were observed in the gel electrophoresis.

The transformation of asymptomatic to symptomatic atherosclerotic plaque is an important phenomenon, which is one of the main factors responsible for the onset of clinical TIA or stroke symptoms. The precise knowledge of the elements that contribute to this transformation may allow more appropriate preventive and curative treatments.

In the present study we were interested in investigating the possible participation of *Chlamydia pneumoniae* in the transformation of the carotid atherosclerotic plaque. Patients with clinical and angiographic indication for endarterectomy, who had suffered atherothrombotic stroke or TIA up to 60 days prior to the surgery, were chosen. Considering that these patients presented an ischemic event up to 60 days prior to the surgery, and that the angiographic exam showed a stenosis level over 70%, we can say, with a good degree of precision that we were dealing with a symptomatic plaque. The surgical pieces removed were analyzed using PCR with specific primers for *Chlamydia pneumoniae*. This method is classically classified an efficient method for diagnosing a possible recent infection[1,12,16], that could, in some way, contribute to atherosclerosis worsening and plaque transformation. The direct study of the carotid arteries, obtained through the endarterectomy, has shown contradictory results. Grayston et al.[18] analyzed pieces of these arteries using immunocytochemistry and verified increased positive results for *Chlamydia pneumoniae* in patients with atherosclerosis (32 out of 56 pieces positive), in comparison to the normal controls (7 out of 13 pieces positive). Johnston et al.[19], in a similar study, using PCR as the diagnostic method, found positive results in 38% of the samples (18 out of 48 plaques verified). In contrast, Tondella et al.[20], in a recent study, in which 30 atherosclerotic plaques of the carotid arteries were also analyzed using the PCR technique, did not find any positive results for *Chlamydia pneumoniae*. This author emphasizes that the current data has not shown sufficient consistency in the relationship between *Chlamydia pneumoniae* and atherosclerosis. Similarly, Ong et al.[21] in a study of 44 human carotid artery plaques acquired through endarterectomies, did not find any positive results when the exam was done using PCR, and acquired, in these same patients, rates of 66% positively for *Chlamydia pneumoniae* in blood tests through IgG. On the other hand, Prager et al.[22] verified, using the PCR technique, 80% positivity for *Chlamydia pneumoniae* in their samples, in a study with carotids obtained through endarterectomy of symptomatic patients. Vainas et al.[23] studied 53 patients who had suffered stroke or TIA, with carotid stenosis over 70% who had undergone an endarterectomy. They used the serum dosage method for IgA and IgG and found 58% and 60% positivity for *Chlamydia pneumoniae*, respectively. They did not verify any relationship between the infectious agent and destabilization of the plaque, however, verified

an association between *Chlamydia pneumoniae* and the micro-embolization process from thrombosis, suggesting that the bacteria may have a hypercoagulability role in the atherogenic condition. LaBiche et al.[24] also investigating carotid artery plaques obtained by endarterectomy, compared 37 plaques extracted from symptomatic patients to 57 plaques extracted from asymptomatic patients, and did not find a significant difference between the two groups. They verified, using PCR, IgA, IgM, and IgG exams, positively for *Chlamydia pneumoniae* in 14.82% of the plaques (13.5% of the symptomatic, and 15.8% in the asymptomatic). There was no association between the presence or absence of the symptoms or degree of stenosis, and positive or negative results for *Chlamydia pneumoniae*. In their study, the only patients that demonstrated a relationship between the occurrence of symptoms and infection were the patients with very elevated IgA levels (>1:128).

Cochrane et al.[16], in a recent publication, disclosed that the different methods used to diagnose the bacteria may explain these conflicting results. They cite the possibility of undertaking a serried analysis in various segmented cuts of the carotid of only one patient[16]. With this procedure, the risk of false negatives would be limited, reducing the chances of investigating a segment that is not contaminated. In the present study, our procedure was based on his method.

The analysis of the results in our study, in which all of the exams were negative for *Chlamydia pneumoniae,* does not suggest an association between this bacteria and transformation to the symptomatic carotid atherosclerotic plaque. Currently, this is a much-debated topic, with many conflicting opinions[16]. It is corroborated with these initial results, as well as the cited studies with human carotids, the negative results of the main studies using specific antibiotics for *Chlamydia pneumoniae,* in the acute phase of the ischemic events, which did not verify improvement with this treatment[25,26].

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