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# *Chlamydia pneumoniae* AND STROKE

## Is there a direct relationship?

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**Abstract** – **Objective:** To investigate the possible relationship between atherothrombotic stroke and *Chlamydia pneumoniae*. **Method:** 150 patients with carotid atherothrombosis were enrolled. The casuistic was divided in three groups: ischemic stroke (IS): 65 patients; transient ischemic attack (TIA): 26 patients; and control: 59. The IS or TIA onset was up to 30 days from the beginning of the study. Carotid atheromatoses was diagnosed by Doppler-ultrasonography. Patients with cardioembolic risk or non-atherothrombotic origin were excluded. Comparisons were done between the three groups, and within each group according to the different age subgroups, to the main arteries affected, and to the atherogenic risk factors. Bacteria detection was done using polimerase chain reaction. **Results:** Only one patient tested positive for *C. pneumoniae* belonging to the control group. **Conclusion:** These results do not suggest that *C. pneumoniae* participated in the onset of IS or TIA or that it has a role in carotid plaque destabilization.

KEY WORDS: stroke, chlamydia, atherosclerosis, risk factors, cerebral infarct.

**_Chlamydia pneumoniae_ e acidente vascular cerebral aterotrombótico: existe relação direta?**

**Resumo** – **Objetivo:** Investigar a possível relação entre *Chlamydia pneumoniae* e acidente vascular cerebral aterotrombótico (AVC). **Método:** 150 pacientes com aterotrombose carotídea foram estudados. A casuística foi dividida em 3 grupos: AVC: 65 pacientes; ataque isquêmico transitório (AIT): 26 pacientes e controles: 59. O início do AVC ou AIT era até 30 dias da inclusão no estudo. A ateromatose carotídea foi diagnosticada por ultrassonografia com Doppler. Os pacientes com risco cárdio-embólico ou sem evidência de aterotrombose foram excluídos. Foram estabelecidas comparações entre os 3 grupos e dentro de cada grupo, formado subgrupos de acordo com diferentes idades, território arterial comprometido e fatores de risco. A detecção da bactéria foi feita por reação de polimerização em cadeia. **Resultados:** Somente um paciente, pertencente ao grupo controle, teve resultado positivo. **Conclusão:** Estes achados não sugerem que a *C. pneumoniae* participe no desencadeamento do AVC ou AIT ou que tenha papel na desestabilização da placa.

PALAVRAS-CHAVE: acidente cerebral vascular, aterosclerose, fatores de risco, infarto cerebral, chlamydia.

Several studies have reported infections as a risk factor for stroke (AVC)[1,2] which could act in the unleashing, the progression or the destabilization of the atherosclerotic plaque[1]. The confirmation of such hypothesis would open an important space, both for an eventual specific therapeutic approach in the acute phase of the stroke and for its prevention. There is an increasing volume of evidences suggesting the association between inflammation, infection, and atherosclerosis[3,4], although that relation is still not well established[5]. There are authors that defend that the inflammation is the key for the development and the progression of atherothrombosis[3,6]. The most frequent infectious agent implicated to atherosclerosis is *Chlamydia pneumoniae*, an intracellular bacteria. Several mechanisms are known by which *pneumoniae* could mediate an atherogenic process in the arteries; most of these show the capacity of *pneumoniae* to trigger an inflammatory reaction on the vascular wall that would lead to the activation and progression of the atheroma, and the start of thrombotic complications[4-6].

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There is a lot of speculation regarding the possible role of *C. pneumoniae* in atherosclerosis, which could act in the unleashing, the evolution, worsening or destabilization of the plaque, making it vulnerable to thromboembolic complications. The infection caused by *C. pneumoniae* has been mentioned as an independent risk factor for vascular diseases, including cerebrovascular ones[5-7]; however that point of view is still much questionable. Opinions vary from one extreme to the other[6], from the ones that defend the microorganism would be the causal agent of atherosclerosis[7,8] to the ones that believe it would merely be an innocent bystander[9]. Despite the strong association found through several epidemiologic and experimental studies, the causality of the facts cannot be simply established. There are still no safe data on the fact that *pneumoniae* would be either related or not to atherosclerosis, and in case it is positive, how that relation would be[9]. It could act in the beginning of the process (unleashing the histological profile), during its course, either aggravating it or in its final phases, destabilizing the plaque. Most of the early studies used immunofluorescence assays, dosing serum levels of IgA, IgG, or IgM, as a diagnostic method for *pneumoniae*. The results, however, are conflicting, with some positive[10] and other negative findings[8,11-13], with the methodology employed being questioned. More recently, *pneumoniae* DNA detection by the polymerase chain reaction (PCR) has been shown to be a valid diagnostic method, and apparently more reliable than other techniques[11]. The presence of circulating DNA of *pneumoniae*, diagnosed through the PCR in humans, may represent a persistent systemic infection by the bacteria.

The factors that leave the transformation of the asymptomatic plaque to symptomatic have been the target of many studies and is very important in the development of ischemic stroke. This study was performed in order to analyze the possible role of *pneumonia* in this stage of atherosclerotic plaque.

## METHOD

This is a prospective study with patients admitted to the Santa Casa de São Paulo Hospital, in the period between March 2004 to August 2006; 150 patients (age range: 40–85 years), with carotid atherothrombosis, were enrolled. The patients were divided into three groups: ischemic stroke (IS): 65 patients (40 men); transient ischemic attack (TIA): 26 patients (11 men) and control: 59 patients (22 men). The IS or TIA onset was established up to 30 days from the beginning of the study. Stroke and TIA were diagnosed by the symptoms presented with confirmation obtained by computed tomography (CT-scan) or magnetic resonance imaging (MRI). Carotid atheromatous plaque was diagnosed by Doppler ultrasonography; IS and TIA by clinical and tomographic evaluation. Patients that presented cardioembolic risk (atrial fibrillation, recent myocardial infarction, intramural thrombus, arrhythmias), lacunar infarction, cerebral hemorrhage, or other with non-atherothrombotic origin were excluded. Age, gender, and risk factor for stroke were not considered for patient exclusion. The control group was formed by patients that presented atherosclerotic plaques in the carotid arteries and that had not undergone a ischemic event, thus being considered to be a asymptomatic plaque, different from the others with symptomatic plaque. Comparisons were done between the three groups, and within each group for the different age groups, the main arteries affected, and the atherogenic risk factors were detected.

Thirth patients were between 40-55 years old (18 with IS, 4 TIA, 8 controls), 49 between 56–70 years old (21, 11, 17 respectively) and 71 between 71–85 years old (27, 11, 33 respectively). The artery with the highest stenosis degree was considered: 87- internal carotid artery (37, 16, 34); 37- common carotid artery (16, 6 ,15); and 26- carotid bulb (12, 4, 10). Regarding risk factors, 87 had blood hypertension (43, 17, 27); 33 were diabetic (16, 8, 9); 45 had hypercholesteremia (21, 5, 19); 22 were smokers (13, 3, 6), and 44 (13, 5, 26) did not have these classic risk factors.

Bacteria detection was performed by PCR in the venous blood.

To each 1.5 ml Eppendorf tube containing blood, 1000 μ/L of the LYSES SOLUTION was added, vortexed vigorously for 15 sec., centrifuged for 2 min. at 13,000 g, and the supernatant was discarded by aspiration with a Pasteur pipette. Then, to the pellet, 500 μ/L of TEN, 5 μ/L of SDS (10%), and 3 μ/L of proteinase K (125 mg/ml) was added, mixed and incubated overnight in a 56°C block heater. After that, 150 μ/L 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 was transferred to a new 1.5 ml Eppendorf tube and 650 μ/L of cold isopropanol was added. DNA was precipitated in a freezer at –20°C overnight. After that period, the supernatant was discarded and the pellet was washed twice with cold 70% ethanol and air-dried for 30 min. The DNA pellet was dissolved in 200 μ/L of TE and stored at –20°C until use. Five microliters (5 ul) of this DNA was used as template for PCR amplification and 1 μ/L per tube in our "in house" Internal Control PCR (Human β-Globin gene) for each sample studied.

The *C. pneumoniae* PCR was performed as follows:

Single PCR was done in a final volume of 20 μ/L 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 94°C for 5 minutes, followed by 40 cycles of 95°C for 45 sec, 62°C for 45 sec, and 72°C for 1 minute. A final extension step was done at 72°C for 7 minutes. The *C. 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 94°C as pre-denaturation step, followed by 35 cycles of 1 min at 94°C, 1 min 30 sec at 62°C, and 2 min at 72°C. A final extension step was done at 72°C 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 *C. 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 the Ethical Committee of Santa Casa de São Paulo (project 045/00, approved in 26.06.00).

## RESULTS

Only one patient tested positive for *C. pneumoniae*; that patient belonged to the control group. The remaining results were negative.

The results by different sub-groups, are presented in the Tables (Tables 1–4).

## DISCUSSION

The factors that lead to the transformation of one asymptomatic plaque into a symptomatic one, including the infectious and inflammatory agents, are still controversial. Why does a sick person in a specific part of his/her life becomes symptomatic, and why do sick people with the same degree of stenosis, either symptomatic or asymptomatic, heave really different risk degrees for a stroke[16], are questions that really matter and that still have nor appropriate answer. The investigation herein seeks to help in this task, by analyzing a possible relation of with the occurrence of a new stroke and a probable destabilization of the atherosclerotic plaque. Considering that the inclusion criteria for the study herein have been developed in order to only allow for the admission of patients with atherothrombotic manifestations in the study group, with a good

*Table 1. Distribution by age.*

| | | IS | | TIA | | Control | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| Age (y) | Sex | N | +CP | N | +CP | N | +CP | N | +CP |
| 40–55 | Men | 10 | 0 | 2 | 0 | 3 | 0 | 30 | 0 |
| | Women | 8 | 0 | 2 | 0 | 5 | 0 | | |
| 56–70 | Men | 14 | 0 | 5 | 0 | 7 | 0 | 49 | 0 |
| | Women | 7 | 0 | 6 | 0 | 10 | 0 | | |
| >71 | Men | 15 | 0 | 6 | 0 | 14 | 1 | 71 | 1 |
| | Women | 12 | 0 | 7 | 0 | 17 | 0 | | |

Y: years; IS: ischemic stroke; TIA: ischemic transient attack; N: number of patients; +CP: positivity for *Chlamydia pneumoniae.*

*Table 2. Distribution by main affected artery.*

| | IS | | TIA | | Control | | Total | |
|---|---|---|---|---|---|---|---|---|
| Artery | N | +CP | N | +CP | N | +CP | N | +CP |
| ICA | 37 | 0 | 16 | 0 | 34 | 0 | 87 | 1 |
| CCA | 16 | 0 | 6 | 0 | 15 | 0 | 37 | 0 |
| CB | 12 | 0 | 4 | 0 | 10 | 0 | 26 | 0 |

ICA: internal carotid artery; CCA: common carotid artery; CB: carotid bulb; IS: ischemic stroke; TIA: ischemic transient attack N: number of patients; +CP: positivity for *Chlamydia pneumoniae.*

*Table 3. Distribution by main risk factors.*

| | IS | | TIA | | Control | | Total | |
|---|---|---|---|---|---|---|---|---|
| Risk factors | N | +CP | N | +CP | N | +CP | N | +CP |
| Hypertension | 43 | 0 | 17 | 0 | 27 | 1 | 87 | 1 |
| Diabetes | 16 | 0 | 8 | 0 | 9 | 0 | 33 | 0 |
| Hypercholesteremia | 21 | 0 | 5 | 0 | 19 | 0 | 45 | 0 |
| Smoker | 13 | 0 | 3 | 0 | 6 | 0 | 22 | 0 |
| No classic risk factor | 13 | 0 | 5 | 0 | 26 | 0 | 44 | 0 |

IS: ischemic stroke; TIA: ischemic transient attack N: number of patients; +CP: positivity for Chlamydia pneumoniae

*Table 4. Distribution according to the carotid stenoses degree.*

| Stenoses degree | 1–40% | 41–59% | 60–99% | 100% |
|---|---|---|---|---|
| *C. pneumonia* positivity | 0 | 1 | 0 | 0 |
| Number of patients | 11 | 35 | 13 | 0 |

safety margin we could say that the elements in this study that had a stroke or a TIA had a symptomatic plaque.

In the current study we used PCR for detection of *pneumoniae* for a diagnostic research; this laboratorial method allows the active infection diagnosis, being negative for the patients that were previously contaminated and who are already healed. Most part of the studies are being performed using diagnosis by measuring IgG, IgA ou IgM antibodies, and that deserves some considerations. Seroepidemiological studies have important limitations, and several of them might be taken into consideration when interpreting the results obtained with that kind of study[17,18]. The presence of IgG antibodies simply translates a primary infection and does not allow for the distinction between a chronic case, a persisting case, a reinfection or an old isolated infection[17]. The definition of seropositivity has been varying a lot from study to study, and there is no straight definition for the subject. The positivity criterion varies according to different authors, with titles of IgG as ≥1:8; ≥1:16, ≥ 1:64, ≥1:128, ≥1:256, which makes comparisons and conclusions difficult. Despite the fact that the IgM antibody is an important marker for acute infection, it can provide a crossed reaction, with a false positive for the rheumatoid factor[17,18] or other infections[18]. A few studies have investigated a possible relation between the stages of atherosclerosis and the antibody classification. Laboratorial variation is another important aspect that many times makes it very difficult to interpret the results.

Our results (Tables 1–3) do not suggest that *pneumoniae* participated in the destabilization of the plaque, for there was no positivity in patient groups with recent stroke or TIA. If that bacteria contributed for the destabilization of the plaque, that would make it have a greater symptomatic probability, and we would expect to find a larger amount of positive diagnosis in the stroke or TIA group when compared to the control group, which did not happen. The work of Ong and associates[19] corroborates that point of view, where by studying a largely prevalent population for *pneumoniae* and atherosclerosis, they found high indexes of IgG and negativity when searching for PCR, in carotid atheroma. Such authors suggest that if there is any relation between atherogenesis and infection by *pneumoniae*, it must be indirect and a non-essential variable for the development of thrombosis[19]. Tondella et al.[20], did not find any positivity for that research after evaluating 30 carotid arteries atherosclerotic plaques using the PCR technique. Also searching for carotid arteries plaques obtained by endarterectomy, LaBiche et al.[21] have compared 37 pieces extracted from symptomatic patients to 57 from asymptomatic patients, and when the diagnosis was established due to PCR, IgM and IgG, they did not find a significant difference between the two groups. However, they could observe there was a relation between the symptoms that occurred and the infection, when the research was performed by IgA, with the patients that have showed very high levels (>1:128)[21]. Such authors remember that the single positivity for *pneumoniae* is not enough to explain the event of the cerebrovascular process, and conclude that the bacteria has an insignificant role in converting a atherosclerotic plaque into the symptomatic state[21]. In an analysis performed with the serum level of IgA and IgG in the carotid plaque withdrawn by endarterectomy, Vainas et al.[22] conclude, in a similar way to our casuistic, that *pneumoniae* is not related to plaque destabilization. Such authors[22] state that this infectious agent would be related to atherosclerotic phenomena, probably in the first stages of its development, but would not have any role in plaque destabilization. In a recent study, by means of the analysis of carotid plaques obtained through endarterectomy in patients with atherothrombotic stroke, no relation to *pneumoniae* was detected[12]. Konya et al.[13] did not find any correlation between the presence of *pneumoniae* in the carotid plaque and the severity of the stroke. A negativity finding in the PCR diagnosis, as in the essay herein, suggests that *pneumoniae* does not play any role in complications nor in the destabilization of the plaque.

In relation to age and gender (Table 1) no difference was detected between the groups. Such data corroborate the previous reports as *"The Northern Manhattan Stroke Study"*[8], which did not find any difference between young people and the elderly, men and women.

The association of *pneumoniae* to the classic risk factors (RF) for vascular diseases is another aspect that deserved special attention. Considering the atherogenic RFs might be metabolic related and that there are reports on the interrelation of *pneumoniae* to the classic RFs[2], that possible issue was also a target in the investigation. We tried to identify if there would be the influence of any RF or of an interrelation between a particular RF to the infection caused by the bacteria and the evolution of the medical profile. Several authors[2,7] have been highlighting that possibility and developed specific researches on the subject, a fact that justifies the separate analysis in this casuistic. Arterial hypertension (Table 4) did not show any relation between the active infection by *pneumoniae* and the occurrence of symptoms, that could translate into an instability of the atherosclerotic plaque. Similar results were described in literature[7,23], that support the

idea that arterial hypertension has no influence over a possible atherogenic role related to the infection caused by that agent. Smoking has been frequently mentioned in literature as a related RF to *pneumoniae*[2]; though in our casuistic (Table 4) the results did not corroborate that hypothesis, for there was no larger positivity among smokers related to the diagnosis for *pneumoniae.* We should point out that most reports of that positive association were established by setting the antibodies level, which could show an old infection. Hypercholesterolemia is another atherogenic RF widely investigated, and there are several reports of a possible role linking the infection by *pneumoniae* and/or contributing for the severity of the medical profile, when associated to the infection. In the present study there was no correlation found between hypercholesterolemia, infection by *pneumonia*, and clinical signs of destabilization of the atherosclerotic plaque. That finding is similar to other reports[7,23]. Diabetes mellitus was also separately analyzed; the results (Table 4), did not show any relation between the agents analyzed, similarly to the other analyses of RFs. That finding is also corroborated by other authors[7,23].

The possibility of *pneumoniae* acting selectively or preferably among the main brain arteries was also studied, and was not corroborated. (Table 2).

A possible relation between the stenosis level and the active infection by *pneumoniae* was also checked. That analysis was only carried out in the control group, in order to allow for an evaluation on the evolution of the stenosis in the stable plate. With that model we sought to evaluate a possible severing role before the ischemic event occurred, with a consequent plaque destabilization. The results presented in Table 4 have once again not detected any relation. That fact, which by our point of view is not being sufficiently discussed in literature, reinforces the idea that the active infection by *pneumoniae* is not a risk factor for plaque destabilization.

In conclusion, our casuistic does not suggest that *pneumoniae* participates in the onset of IS or TIA, and does not suggest that this bacteria plays a role in plaque destabilization.

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