Chlamydia pneumoniae and Atherosclerosis. Identification of Bacterial DNA in the Arterial Wall

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Objective - *The intracellular Gram-negative bacterium Chlamydia pneumoniae has been associated with atherosclerosis. The presence of Chlamydia pneumoniae has been investigated in fragments of the arterial wall with a technique for DNA identification.*

Methods - *Arterial fragments obtained from vascular surgical procedures in 58 patients were analyzed. From these patients, 39 were males and the mean age was 65±6 years. The polymerase chain reaction was used to identify the bacterial DNA with a pair of primers that codify the major outer membrane protein (MOMP) of Chlamydia pneumoniae. The amplified product was visualized by electrophoresis in the 2% agarose gel stained with ethidium bromide, and it was considered positive when migrating in the band of molecular weight of the positive controls.*

Results - *Seven (12%) out of the 58 patients showed positive results for Chlamydia pneumoniae.*

Conclusion - *DNA from Chlamydia pneumoniae was identified in the arterial wall of a substantial number of patients with atherosclerosis. This association, which has already been described in other countries, corroborates the evidence favoring a role played by Chlamydia pneumoniae in atherogenesis.*

Keywords: Chlamydia pneumoniae, atherosclerosis, polymerase chain reaction

Atherosclerosis and its associated diseases (acute myocardial infarction and ischemic stroke) are important causes of morbidity and mortality in industrialized and developing countries. Differences in the prevalence of conventional risk factors (smoking, hypertension, and dyslipidemia), however, do not explain temporal and geographic variations in the prevalence and severity of these diseases. About one third of the individuals with atherosclerosis are estimated not to have any of these major risk factors. This resulted in the search for new risk factors, such as coagulation factors, inflammatory factors, and infectious agents.

Atherosclerosis is an inflammatory disease [1]. According to the current theory of atherogenesis, this process begins with an "initial injury" that triggers an inflammatory response involving lymphocytes, macrophages, smooth muscle cells, and endothelial cells. Some of the triggers proposed for the initial injury in atherosclerosis include the following: carbon monoxide of cigarettes, hemodynamic friction upon the endothelium, hypercholesterolemia, oxidized LDL, and more recently, infectious agents such as herpesvirus, cytomegalovirus, *Helicobacter pylori*, and *Chlamydia pneumoniae*.

The association of *Chlamydia pneumoniae* and coronary, carotid, and peripheral arterial disease has been described in several countries in diverse types of studies as follows: a) serum-epidemiological studies [2-6]; b) anatomico-pathological studies [7-17]; and c) experimental studies [18-22]. Reports on cultivation of *Chlamydia pneumoniae* in fragments of arteries exist, showing the viability of the bacterium and its multiplication capacity in that type of environment [10,12]. In vitro studies have shown the ability of *Chlamydia pneumoniae* to infect smooth muscle cells, endothelial cells, and human macrophages [23-25]. Experiments in rabbits infected with *Chlamydia pneumoniae* showed the development of fatty streaks and lesions similar to atherosclerosis in the aorta [18]. When the animals were treated with macrolides, however, these lesions did not develop [26]. Preliminary clinical randomized trials [27,28] that still await confirmation support a causal relation between *Chlamydia pneumoniae* and coronary artery disease.

Chlamydia pneumoniae is an intracellular Gram-negative bacterium with a wide distribution [29]. It accounts for 10% of the pneumonias in the community and serum-positivity is high in adults age [29]. Most of the individuals will have been exposed to *Chlamydia pneumoniae* at least once during their lives. As efficient antibiotics against *Chlamydia pneumoniae* are available, the confirmation of a causal relation between *Chlamydia pneumoniae* and atherosclerosis may drastically change the approach to atherosclerotic disease.

As Brazilian data about the association of *Chlamydia pneumoniae* and atherosclerosis did not exist, we carried out this preliminary study aiming to investigate the presence of *Chlamydia pneumoniae* in the arterial wall of individuals with atherosclerotic disease using techniques of DNA detection.

Methods

This is a descriptive study of patients with atherosclerotic disease who underwent vascular surgical procedures. Fragments of arterial tissues excised from 58 patients operated upon (53 myocardial revascularizations and 5 vascular surgeries) were the object of this study. The fragments originated from the following sites: ascending aorta (50), abdominal aorta (4), coronary artery (3), and femoral artery (1). Thirty-nine patients were males and 19 were females, and their ages ranged from 45 to 79 years (mean of 65 years). The surgical procedures were performed at the Serviço de Cirurgia Cardíaca of the Instituto de Cardiologia (INCA) and at the Serviço de Cirurgia Vascular of the Hospital Regional (São José, SC), which are public hospitals in Santa Catarina state, during the period of March to November '98. The study was approved by the Committee on Ethics in Research of the Instituto de Cardiologia de Santa Catarina, and the patients signed a consent form for the use of the surgical material in the research.

The arterial fragments encompassed the three tunicae (media, intima, and adventitia). The fragments obtained in myocardial revascularizations corresponded to the orifices cut in the ascending aorta and coronary arteries for placement of saphenous venous or internal thoracic artery graft bypasses. In the surgeries of the abdominal aorta and femoral artery, the fragments were resected from grossly damaged regions.

These fragments were then cut into smaller pieces (± 1mm), which were put into sterilized flasks with 0.9% saline solution and stored at -20°C to be processed within 2 months.

After thawing, the tissue was macerated and put into suspension in a buffer solution for protein digestion composed of the following items: 0.1mg/ml of proteinase K (Sigma®), 100mM of NaCl; 10mM of TrisHCl in pH 8; 25mM of ethylenediaminetetraacetic acid (EDTA); and 0.5% of sodium dodecyl sulfate. The solution was then incubated at 50°C for 12-18 hours. DNA extraction was performed through standard techniques [30] using a solution of phenol/chloroform/isoamyl alcohol (25:24:1) mixed with the centri-fuged liquid phase of the product minus the protein. The DNA pellet was suspended in the buffer solution and agitated for a few hours at room temperature to facilitate solubilization. Then, a mixture of 200µM of each oligonucleotide (dATP, dCTP, dGTP, and dTTP – Sigma®), 1µM of each primer, and 1U of the *Taq* DNA polymerase (Sigma®) were added to the DNA in the solution.

The two primers used codify the major outer mem–brane protein (MOMP) of *Chlamydia pneumoniae* and their sequences are as follows [16]: a) TGC CAA CAG ACG CTG GCG TAG CAA (bases 1053 to 1076 of the MOMP gene); b) TAA CTG CAT GGA ACC CTT CTT TAC TAG (bases 1254 to 1280 of the MOMP gene). DNA amplification using the polymerase chain reaction technique was performed in a Perkin-Elmer® thermocycler model 9600, using 25 to 30 cycles (50°C for 2 min, 95°C for 5 min, and 72°C for 5 min). The amplification products were qualitatively analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide, and they were considered positive when the band migrated to the molecular weight corresponding to the positive controls. Negative controls with distilled water were also used. The gel was photographed with a Polaroid® camera under ultraviolet light. Routine care to avoid contamination of the material was taken.

The patients' records were revised and data about their risk factors were recorded. The definitions for the risk factors were as follows: tobacco smoking (current smoker or ex-smoker for no longer than 3 months); hypertension (systolic pressure at rest >140mmHg or diastolic pressure of 90mmHg, or both; or treatment with antihypertensive drugs, or both); diabetes mellitus (fasting glycemia >126 mg/dL or treatment with oral drugs for treating hyperglycemia, or insulin, or both); obesity (body mass index >30 kg/m^2).

Results

The arterial fragments of 7 out of 58 patients were positive for the presence of *Chlamydia pneumoniae* DNA. From the positive fragments, 5 (71.4%) were removed from the ascending thoracic aorta, one (14.3%) from the abdominal aorta, and one (14.3%) from the femoral artery (table I).

Distribution of the general characteristics, site of removal of the arterial fragments, and the risk factors of the positive and negative patients for *Chlamydia pneumoniae* are shown in table II.

Table I - Distribution of the sites of excision of the arterial fragments in patients with positive and negative results polymerase chain reaction for *Chlamydia pneumoniae*.

Location	Positive (n = 7)	Negative (n = 51)
Ascending thoracic aorta	5	45
Abdominal aorta	1	3
Coronary artery	-	3
Femoral artery	1	-

Table II - Prevalence of the classical risk factors for atherosclerosis in the group of patients with positive and negative results for the presence of *Chlamydia pneumoniae* using the polymerase chain reaction technique.

Risk factors	Positive(N=7) n (%)	Negative (N=51) n (%)
Age (mean)	65,3	65,4
Male sex	5 (71)	34 (67)
Smoking	7 (71)	30 (59)
Hypertension	6 (86)	31 (61)
Diabetes mellitus	1 (14)	10 (20)
Hypercholesterolemia	2 (29)	9 (18)
Obesity	2 (29)	43 (22)

Discussion

Our study showed 12% (7/58) of the results positive for *Chlamydia pneumoniae* detected with the PCR technique in fragments of arteries with atherosclerosis. In the international medical literature positivity for *Chlamydia pneumoniae* in arteries varies from 0 to 100%.

In 1993, Kuo et al[7] in South Africa detected *Chlamydia pneumoniae* for the first time in atherosclerotic lesions originated from autopsies using the PCR technique and found a positivity of 43%. Grayston et al[31] showed, using the same technique, the presence of *Chlamydia pneumoniae* in 60% of the fragments of carotid arteries obtained through endarterectomy. Campbell et al[9] showed the presence of the microorganism in 32% of the fragments of endarterectomy of coronary arteries in American patients. Kuo et al[8], using the PCR technique, detected 14% positivity for *Chlamydia pneumoniae* in autopsy samples from atherosclerotic coronary arteries of American individuals, whose ages ranged from 15 to 34 years. Ong et al[16], in the United Kingdom, showed the presence of *Chlamydia pneumoniae* in 44% of the fragments of aortic aneurysms and in 55% of the iliac arteries. Blasi et al[15] found positivity for *Chlamydia pneumoniae* in 51% of the fragments of the atherosclerotic aortas studied. Wong et al[32] found DNA of *Chlamydia pneumoniae* in grafts of the saphenous vein (38%), of the native coronary arteries (38%), and of the internal thoracic artery (30%). Juvonen et al[33] showed 100% (12/12) positivity for *Chlamydia pneumoniae* in aneurysms of the abdominal aorta with PCR and immunohistochemical techniques.

Maass et al[14] found 21% (51/238) positivity for *Chlamydia pneumoniae* in atheromatous tissues of various parts of the body (thoracic aorta during revascularization procedures, carotid endarterectomy, correction of aortic aneurysms, and revascularization of lower limbs). In addition, the authors showed the viability of the microorganism in 16% of the arterial samples examined.

Conflicting studies, however, exist. Weiss et al[34] found only one PCR-positive sample in a total of 58 analyzed specimens. Similarly, Paterson et al[35] found positivity in none of the 49 arterial samples removed in endarterectomies and autopsies.

In a review of 13 pathology studies[36] carried out at Oxford University, 52% of the atheromatous lesions showed the presence of *Chlamydia pneumoniae*, using either immunohistochemistry, PCR, or electron microscopy. The controls of these studies showed positivity of only 5%, resulting in an odds ratio of 10 (95% CI = 5-22), and, therefore, proving to be a very strong statistical association.

Another more recent review[37] of 17 studies on *Chlamydia pneumoniae* in the vascular tissue concludes that the presence of that microorganism in atherosclerotic lesions is currently unquestionable, but no conclusive evidence exists in regard to its causal role in atherosclerosis.

The low positivity of *Chlamydia pneumoniae* in our study and the variability between the different studies may be explained as follows: a) lack of uniformity in the distribution of *Chlamydia pneumoniae* in the arterial wall; b) differences in techniques of detection; c) geographical differences in the prevalence of infection by *Chlamydia pneumoniae*; and d) publication bias. Nevertheless, once the presence of the microorganism is detected in a damaged area, questioning its role in the process is quite natural. What role does *Chlamydia pneumoniae* play in atherogenesis? The three following possibilities exist: a) *Chlamydia pneumoniae* is a mere observer of the process, i.e., it only colonizes the damaged arterial wall without taking part in atherogenesis; b) *Chlamydia pneumoniae* accelerates atherogenesis, working with other risk factors; c) *Chlamydia pneumoniae* is the cause of the arterial inflammation.

Based on evidence currently available, we can say that *Chlamydia pneumoniae* is present in a high percentage of atherosclerotic plaques, but the meaning of this finding is still controversial. Data are compatible with the theory of participation of *Chlamydia pneumoniae* in atherogenesis; the evidence of causality, however, is still lacking.

The causal relation between *Chlamydia pneumoniae* and atherosclerosis is yet to be demonstrated. Some clinical randomized trials, however, have been published[27,28,38,39], in which two macrolides (roxithromycin and azithromycin) were used. Combined clinical events (myocardial infarction, ischemic death, and recurrent angina) decreased in a statistically significant way in 2 studies[28,38], but not in another[39]. In this latter study, a reduction in the levels of inflammatory markers (C-reactive protein and interleukin-6) was shown after the use of the antibiotics. Because these are preliminary studies, the results of the ongoing clinical trials should be awaited[40,41]. These trials have enough statistical power to test the hypothesis of the benefit of the use of antichlamydial antibiotics in the prevention of cardiovascular events.

The importance of defining the role of *Chlamydia pneumoniae* in atherogenesis is fundamental, because it may drastically change the paradigm of the disease and its therapeutical approach, as well.

The recent example of peptic ulcer and the change of its paradigm make us consider the possibility of an infectious cause for atherosclerosis. For a long time, peptic ulcer was believed to be caused by gastric hyperacidity and that an acid environment would be hostile to any type of micro-

organism. Nevertheless, several studies confirmed the role of the helical bacterium *Helicobacter pylori* in determining that disease. Clinical randomized trials have shown the efficacy of antibiotics in healing gastroduodenal ulcerated lesions, confirming the causal relation between *Helicobacter pylori* and peptic ulcer.

The study of the role of an infectious agent in determining such a widely distributed disease as atherosclerosis has important clinical and public health implications. If a causal relation exists, the treatment of individuals with *Chlamydia pneumoniae* may modify the high incidence and prevalence of a disease that affects hundreds of thousands of people annually, and accounts for at least one fourth of the deaths of the adult population. Our study, due to its transversal characteristic, does not establish any causal relation between *Chlamydia pneumoniae* and atherosclerosis. It supports, however, the set of data currently available, which favor the infectious hypothesis of atherosclerosis.

Acknowledgments

References

1. Ross R. Atherosclerosis. An inflammatory disease. New Engl J Med 1999; 340:115-26.

2. Saikku PLM, Matilla K, Elkman MR, et al. Serologic evidence of an association of a novel Chlamydia, TWAR, with coronary artery disease and acute myocardial infarction. Lancet 1988; 2: 983-6.

3. Melnick SL, Shahar E, Folsom AR, et al. Past infection by *Chlamydia pneumoniae* strain TWAR and asymptomatic carotid atherosclerosis. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Am J Med 1993; 95: 499-504.

4. Davidson M, Kuo CC, Middaugh JP, et al. Confirmed previous infection with *Chlamydia pneumoniae* (TWAR) and its presence in early coronary atherosclerosis. Circulation 1998; 98: 628-33.

5. Ossewaarde JM, Feskens EJ, De Vries A, Vallinga CE, Kromhout D. *Chlamydia pneumoniae* is a risk factor for coronary heart disease in symptom-free elderly men, but Helicobacter pylori and cytomegalovirus are not. Epidemiol Infect 1998; 120: 93-9.

6. Cook PJ, Honeybourne D, Lip GY, Beevers DG, Wise R, Davies P. *Chlamydia pneumoniae* antibody titers are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. Stroke 1998; 29: 404-10.

7. Kuo CC, Gown AM, Benditt EP, Grayston JT. Detection of *Chlamydia pneumoniae* in aortic lesions of atherosclerosis by immunocytochemical stain. Arterioscler Thromb 1993; 13: 1501-4.

8. Kuo CC, Grayston JT, Campbell LA, Goo YA, Wissler RW, Benditt EP. *Chlamydia pneumoniae* (TWAR) in coronary arteries of young adults (15-34 years old). Proc Natl Acad Sci USA 1995; 92: 6911-4.

9. Campbell LA, O'Brien ER, Cappucio AL, et al. Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. J Infect Dis 1995; 172: 585-8.

10. Ramirez J, Ahkee A, Ganzel BL, et al. Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. The *Chlamydia pneumoniae*/Atherosclerosis Study Group Ann Intern Med 1996; 125: 979-82.

11. Muhlestein JB, Hammond EH, Carlquist JF, et al. Increased incidence of Chlamydia species within the coronary arteries of patients with symptomatic atherosclerotic versus other forms of cardiovascular disease. J Am Coll Cardiol 1996; 27: 1555-61.

12. Jackson LA, Campbell LA, Kuo CC, Rodriguez DI, Lee A, Grayston JT. Isolation of *Chlamydia pneumoniae* from a carotid endarterectomy specimen. J Infect Dis 1997; 176: 292-5.

13. Maass M, Krause E, Engel PM, Kruger S. Endovascular presence of *Chlamydia pneumoniae* in patients with hemodynamically effective carotid artery stenosis. Angiology 1997; 48: 699-706.

14. Maass M, Bartels C, Kruger S, Krause E, Engel PM, Dalhoff K. Endovascular presence of *Chlamydia pneumoniae* DNA is a generalized phenomenon in atherosclerotic vascular disease. Atherosclerosis 1998; 140(suppl 1): S25-30.

15. Blasi F, Denti F, Erba M, et al. Detection of *Chlamydia pneumoniae* but not Helicobacter pylori in atherosclerotic plaques of aortic aneurysms. J Clin Microbiol 1996; 34: 2766-9.

16. Ong G, Thomas BJ, Mansfield AO, Davidson BR, Taylor-Robinson D. Detection and widespread distribution of *Chlamydia pneumoniae* in the vascular system and its possible implications. J Clin Pathol 1996; 49: 102-6.

17. Kuo CC, Coulson AS, Campbell LA, et al. Detection of *Chlamydia pneumoniae* in atherosclerotic plaques in the walls of arteries of lower extremities from patients undergoing bypass operation for arterial obstruction. J Vasc Surg 1997; 26: 29-31.

18. Fong IW, Chiu B, Viira E, Fong MW, Jang D, Mahony J. Rabbit model for *Chlamydia pneumoniae* infection. J Clin Microbiol 1997 35: 48-52.

19. Laitinen K, Laurila A, Pyhala L, Leinonen M, Saikku P. *Chlamydia pneumoniae* infection induces inflammatory changes in the aortas of rabbits. Infect Immun 1997; 65: 4832-5.

20. Moazed TC, Kuo C, Patton DL, Grayston JT, Campbell LA. Experimental rabbit models of *Chlamydia pneumoniae* infection. Am J Pathol 1996; 148: 667-76.

21. Moazed TC, Kuo C, Grayston JT, Campbell LA. Murine models of *Chlamydia pneumoniae* infection and atherosclerosis. J Infect Dis 1997; 175: 883-90.

22. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. *Chlamydia pneumoniae* infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. J Infect Dis 1999; 180: 238-41.

23. Godzik KL, O'Brien ER, Wang SK, Kuo CC. In vitro susceptibility of human vascular wall cells to infection with *Chlamydia pneumoniae*. J Clin Microbiol 1995; 33: 2411-4.

24. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic smooth muscle cells. Infect Immunity 1996; 64: 1614-20.

25. Kalayoglu MV, Byrne GI. Induction of macrophage foam cell formation by *Chlamydia pneumoniae*. J Infect Dis 1998; 177: 725-9.

26. Muhlestein JB, Anderson JL, Hammond EH, et al. Infection with *Chlamydia pneumoniae* accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 1998; 97: 633-6.

27. Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B. Treatment with the antibiotic roxithromycin in patients with acute non- Q-wave coronary syndromes. The final report of the ROXIS Study. Eur Heart J 1999; 20: 121-7.

28. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azythromycin in males survivors of myocardial infarction. Circulation 1997; 96: 404-07.

29. Grayston JT. *Chlamydia pneumoniae* (TWAR). In: Mandell GL BJ, Dolin R, ed. Pinciples and Practice of Infectious Diseases. 4ᵗʰ ed. Vol. 2. New York: Churchill Livingstone, 1995: 1696-1701.

30. Pai J, Knoop FC, Hunter WJ, Agrawal DK. *Chlamydia pneumoniae* and occlusive vascular disease: Identification and characterization. J Pharmacol Toxicol Methods 1998; 39: 51-61.

31. Grayston JT, Kuo CC, Coulson AS, et al. *Chlamydia pneumoniae* (TWAR) in atherosclerosis of the carotid artery. Circulation 1995; 92: 3397-400.

32. Wong YK, Thomas M, Tsang V, Gallagher PJ, Ward ME. The prevalence of *Chlamydia pneumoniae* in athrsclerotic and nonatherosclerotic blood vessels of patients attending for redo and first time coronary artery bypass graft surgery. J Am Coll Cardiol 1999; 33: 152-6.

33. Juvonen J, Juvonen T, Laurila A, et al. Demonstration of *Chlamydia pneumoniae* in the walls of abdominal aortic aneurysms. J Vasc Surg 1997; 25: 499-505.

34. Weiss SM, Roblin PM, Gaydos CA, al. Failure to detect *Chlamydia pneumoniae* in coronary atheromas of patients undergoing atherectomy. J Infect Dis 1996; 173: 957-62.

35. Paterson DL, Hall J, Rasmussen SJ, Timms P. Failure to detect *Chlamydia pneu-*

moniae in atherosclerotic plaques of Australian patients. Pathology 1998; 30:169-72.

36. Danesh J, Collins R, Peto R. Chronic infections and coronary artery disease: is there a link? Lancet 1997; 350: 430-6.

37. Taylor-Robinson D. *Chlamydia pneumoniae* in vascular tissue. Atherosclerosis 1998; 140(suppl 1): S21-4.

38. Gurfinkel EBG, Beck E, Testa E, Livellara B, Mautner B for the ROXIS Study Group. Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. Eur Heart J 1999; 20: 121-7.

39. Anderson JL, Muhlestein JB, Carlquist J, et al. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for Chlamydia pneumoniae infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study. Circulation 1999; 99: 1540-7.

40. Dunne M. WIZARD and the design of trials for secondary prevention of atherosclerosis with antibiotics. Am Heart J 1999; 138(5 Pt 2): S542-4.

41. Gupta S. Chronic infection in the aetiology of atherosclerosis: focus on *Chlamydia pneumoniae*. Atherosclerosis 1999; 143: 1-6.