PROCEEDINGS OF SCIENCE

Etiology of vascular related infection

Alessandra Ciervo

Istituto Superiore di Sanità
Department of Infectious, Parasitic and Immune-mediated Diseases
viale Regina Elena 299- 00161 Rome, Italy

E-mail: ciervo@iss.it

Fabiola Mancini

Istituto Superiore di Sanità
Department of Infectious, Parasitic and Immune-mediated Diseases
viale Regina Elena 299- 00161 Rome, Italy

E-mail: fabiola.mancini@iss.it

Patrizio Sale

La Sapienza University

Department of Experimental Medicine and Pathology,

viale Regina Elena, 324- 00161 Rome, Italy

E-mail: patrizio.sale@tin.it

Matteo A. Russo

La Sapienza University

Department of Experimental Medicine and Pathology,

viale Regina Elena, 324- 00161 Rome, Italy

E-mail: matteoantonio.russo@uniroma1.it

Antonio Cassone[1]

Istituto Superiore di Sanità
Department of Infectious, Parasitic and Immune-mediated Diseases
viale Regina Elena 299- 00161 Rome, Italy

E-mail: cassone@iss.it

Frontiers in Imaging Science: High Performance Nuclear Medicine Imagers for Vascular Disease Imaging (Brain and Heart)
Istituto Superiore di Sanita', Rome, Italy
13-14 November, 2006

---

[1]Speaker

PoS(FISBH2006)006

ABSTRACT

Atherosclerosis is probably generated and its progress certainly sustained by chronic inflammation process. In this connection, several studies are actually considering infection as another possible co-factor of atherosclerosis development. Viral and bacterial pathogens have long been suspected to affect atherogenesis directly.. *Helicobacter pylori*, some viruses and, particularly, *Chlamydia pneumoniae* are infection agents most often considered in these studies. Serological, epidemiological, histological and immunological studies are in progress to test the above hypothesis. Overall, the role of *C.pneumoniae* in favoring the atherogenesis and/or its adverse acute events appears to be strengthened by recent evidence, which includes a clear-cut demonstration and quantification of the bacterial agent in the atherosclerotic plaque, as we conclusively show in this paper.

1. Introduction

Inflammation is a key process in the development of atherosclerosis, and one of the rate-limiting steps in the development of atherosclerotic lesions is the production of inflammatory chemokines by endothelial cells and vascular smooth muscle cells [1]

Atherosclerosis and cardiovascular diseases are highly complex pathogenic events with numerous factors simultaneously and sequentially collaborating in subtle ways to induce lesion development and progression.

The lesion, or atheroma, is an inflammatory site composed of a necrotic lipid-rich core, modified vascular endothelium, smooth muscle cells, foamy monocyte/macrophages, lymphocytes and a variety of inflammatory mediators [2].

One of the the most involved mediator of inflammation is the Heat Shock Protein 60 (HSP60) that has been detected in atherosclerotic lesions from both human and experimental animals but not in normal arteries, suggesting that they may play a significant role in the pathogenesis of atherosclerosis [2]. In keeping with this, HSP60-specific T cell clones have been detected infiltrating the atheromatous plaque [3[.

Because traditional risk factors such as hyperlipidemia, hypertension, diabetes, age, sex, smoking, and familial history cannot fully explain the occurrence of atherosclerosis in a number of subjects, other factors could be involved [4]. Infectious agents including bacteria such as *Chlamydia pneumoniae* (CP) and *Helicobacter pylori* or viruses such as human cytomegalovirus have long been suspected to initiate or contribute to the disease [5, 6]. Here, we focused on the putative role of CP in the atherosclerosis and its implications in the development of atheromatous lesions.

2. *Chlamydia pneumoniae*: the most important pathogen-player

Chlamydia pneumoniae (CP), an obligate intracellular human pathogen, causes both lower and upper acute respiratory illnesses, including pneumonia, bronchitis, pharyngitis and sinusitis. CP can cause prolonged or chronic infections which may be due to persistence for months or years [7]. These persistent infections have long been suspected to be implicated in the development of a number of chronic diseases including atherosclerosis, mostly on the basis of seroepidemiological studies [8].

CP can infect and survive in a wide number of host cell types, including lung epithelium, resident macrophages (alveolar and monocyte derived), circulating monocytes, arterial smooth muscle cells and vascular endothelium [9]. Once infected, the physiology of the various cell types present in the lung, circulatory tree and atheroma itself may be profoundly modified , and facilitation to atheroma formation via circulating monocytes and lymphocytes can occur[10].

Similarly to other chlamydiae, CP is an obligate intracellular parasite , with a rather characteristic intracellular developmental cycle , very different from oither bacteria. In fact, .chlamydial growth is biphasic, consisting of two alternating functional and morphological forms (Fig. 1). The elementary body (EB) is the metabolically inert, infectious form of the organism that is capable of transient extracellular survival. EBs bind to as yet undefined host cell receptors, are internalized via a pathogen-specified process and are detectable within a membrane-bound vesicle immediately after entry. Soon after, chlamydiae differentiate from infectious EB to the intracellular replicative form of the organism, referred to as the reticulate body or RB. This differentiation,

which is dramatic in terms of altered chlamydial morphology, must reflect an orchestrated sequence of differential gene expression. RB multiplication results in the formation of an intracellular microcolony (termed the inclusion) of chlamydiae. The final stages of chlamydial growth during a productive infection involve differentiation of RB back to EB. This is accompanied by lysis of the host cell or direct release of EB, which restart the infectious cycle both in the same or a different host via inhalation ..

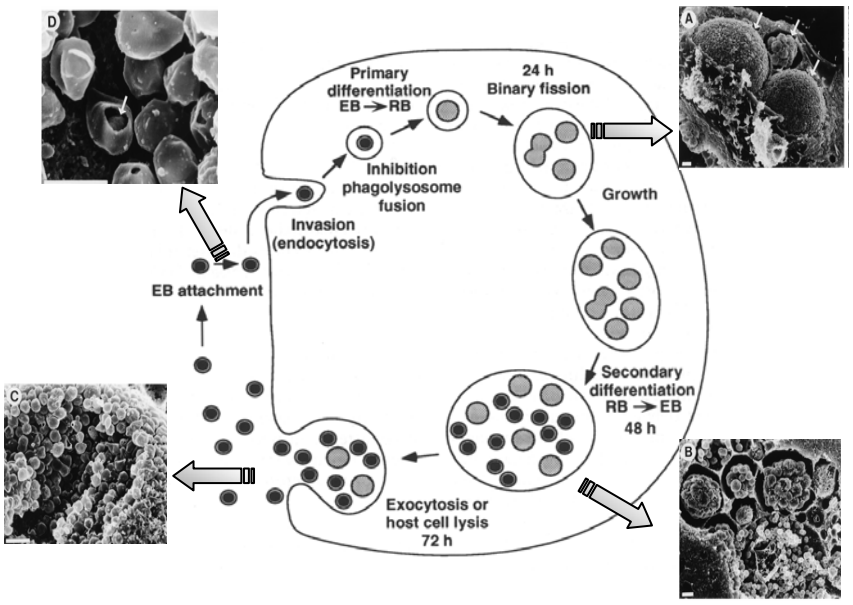

Figure 1: the developmental cycle of *C. pneumoniae*. A, B, C, D micronographs of CP in the different stages of replication.

Productive infection is not the sole outcome, in that CP may respond to treatment by forming inclusions containing atypical non-infectious RB larger in diameter termed aberrant form (AF). In general, it is likely that this aberrant developmental step leads to the persistence of viable but non-culturable chlamydiae within infected cells over long periods, establishing a chronic, persistent infection. This outcome must be rather

frequent when we consider the seroprevalence of IgG and IgA in most populations. AF forms are, however, reversible. Removal of several stress factors allow the production of viable and infectious EB. A direct example of the induction of AFs forms is the therapeutic treatment in patients with coronary artery disease with certain antibiotics. The therapy fails to eradicate CP but, instead, results in a chronic or persistent non-productive infection [9]. In in vitro experiments, Interferons cause persistence of CP infection.

Most of the major sequelae of chlamydial disease are thought to arise from either persistent or recurrent chlamydial infections . The most logical hypothesis is that persistence would allow constant antigen presentation and stimulation of deleterious host responses including autoimmunity [7].

3.The evidence: *Chlamydia pneumoniae* is not a innocent bystander

Exposure to CP is extremely common, and infections occur repeatedly among most people. The prevalence of antibody titers continues to rise in populations of adults, reaching a peak of 80% of males and 70% of females, and the population prevalence studies demonstrate that infections are not restricted geographically and that re-infections occur frequently [10].

Evidence for the presence of the organism in atherosclerotic lesions has emerged from several different groups of investigators using immuno-histochemistry, electron microscopy, *in situ* hybridization or amplification of chlamydial DNA by polymerase chain reaction (PCR). Shor et al. were the first to demonstrate that CP was present in atherosclerotic lesions. These investigators used transmission electron microscopy (TEM) to detect CP in macrophage foam cells, followed by confirmatory studies on the

same tissue using immunohistochemistry to detect CP antigens and PCR to detect CP-specific DNA [13]. In a subsequent study histological and PCR-based evidence for CP in atherosclerotic lesions was found by evaluating autopsy tissue taken from individuals between the ages of 15 and 34 years [14,15].

In a recent study . it was investigated whether CP infection occurred beyond the coronary plaques, namely in the myocardium of individuals who died of acute myocardial infarct (AMI). The presence of CP cell wall antigen (OMP-2) and CP-HSP60 was investigated in the myocardium and coronary plaques of 10 AMI and 10 age-matched control patients by immunohistochemistry, electron microscopy, and molecular biology. OMP-2 and CP-HSP60 were detected in the whole coronary tree. CP presence was strongly associated with a T-cell inflammatory infiltrate. These results suggest that CP may underlie both coronary and myocardial vulnerabilities in patients who died of AMI and corroborate the notion that CP may act by reducing cardiac reserves, thus worsening the ischemic burden of myocardium [16].

Interestingly is also the antibody detection of CP-HSP60 an inflammatory antigen abundantly expressed by persistent CP infection. The persistent infection of the lesion results in the chronic production of HSP60 with subsequent inflammation and lesion progression [17, 18].

4.Human and CP HSP60 are found in patients with stable coronary disease.

In previously studies, we measured the levels of anti-CP-HSP60 and anti-CP immunoglobulin G (IgG) in 179 patients with unstable angina, 40 with acute myocardial infarction, and 40 with stable angina (SA), as well as 100 control subjects. Forty-one patients with acute coronary syndromes (ACS) were also studied at follow-up. We also measured plasma levels of high-sensitivity C-reactive protein (hs-CRP) and troponin T

(TnT). Seropositivity to Cp-HSP60 was found in 99% of ACS patients but in only 20% of SA patients and none of the control subjects. Seropositivity to Cp was detected in 67% of ACS patients, 60% of SA patients, and 30% of the control subjects. No differences in Cp-HSP60 IgG and in Cp IgG were observed between patients with myocardial infarction and patients with unstable angina. No correlation was found between Cp-HSP60 IgG, TnT, and hs-CRP or between IgG against Cp and hs-CRP. In ACS patients at follow-up, Cp-HSP60 IgG decreased from 0.88+/-0.25 to 0.45+/-0.14 arbitrary units ($P<0.0001$), becoming negative in 12 patients. In conclusion the seropositivity for Cp-HSP60 appears to be a very sensitive and specific marker of ACS, unrelated to Cp IgG antibody titers or hs-CRP and TnT levels [19, 20].

We have also developed a new PCR tool for the detection of CP DNA in atheromatous plaques. It is real-time PCR LightCycler assay with FRET probes technology. The assay proved particularly suitable for the specific and quantitative detection of a low DNA copy number in conventional PCR-negative samples Among fifteen nested-PCR negative atherosclerotic plaques examined, our method detected three positive plaques containing 50(+/-3), 37(+/-2) and 24(+/-2) DNA copy number+/-SD in three independent experiments. Real-time PCR holds promise for CP quantitation in human atherosclerotic plaques [21]. However, not in all studies CP has been found in atheromatous plaques.

5. Real-Time polymerase chain reaction and laser capture microdissection for detection and localization of CP in atherosclerotic plaques

Laser capture microdissection (LCM) has been proposed as potential application in human pathology to isolate material of interest for molecular evaluation, and it has been successfully applied in cancer and cardiovascular research [22]. As shown above, detection of CP in atheromatous plaques has become a critical, yet still unresolved, issue. We considered that combination of LCM with the quantitative real time PCR assay could be a potential tool to detect and quantify CP DNA in the atherosclerotic plaques. We analyzed 16 patients who underwenrt endo-arterectomy, aged 55-70. All plaques presented lynpho-monocytic infiltrate with nercrosis and various degree calcification. Formalin-fixed, paraffin-embedded biopsy samples were subjected to LCM (Fig. 2).

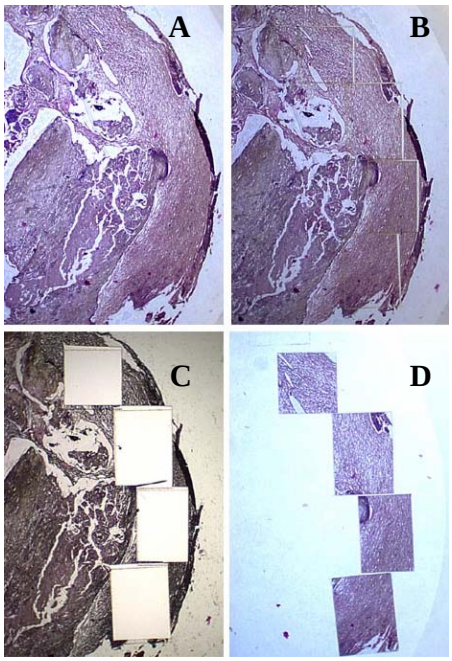

Figure 2: LMD of atheroma biopsy tissue using the laser microbeam microdissection system. *A*: plaque with an extensive inflammatory infiltrate represented by lymphocytes, histiocytes, eosinophils, and multinuclear giant cells associated with cell

necrosis. *B*: The same plaque has been traced before the laser activation. *C*: The same field after microdissection of the selected cell. Four holes are visible in the remaining tissue. *D*: The excised cell were transferred on the adhesive cap of a nanotube.

The microdissected tissue areas were measured, documented, and collected on an adhesive cap of nanotubes for nucleic acid extraction. For each biopsy, the DNA was extracted from the section of the internal region and the distal end of the plaque. DNA samples was subjected to the quantitative real-time PCR as previously described [21] (Fig. 3).

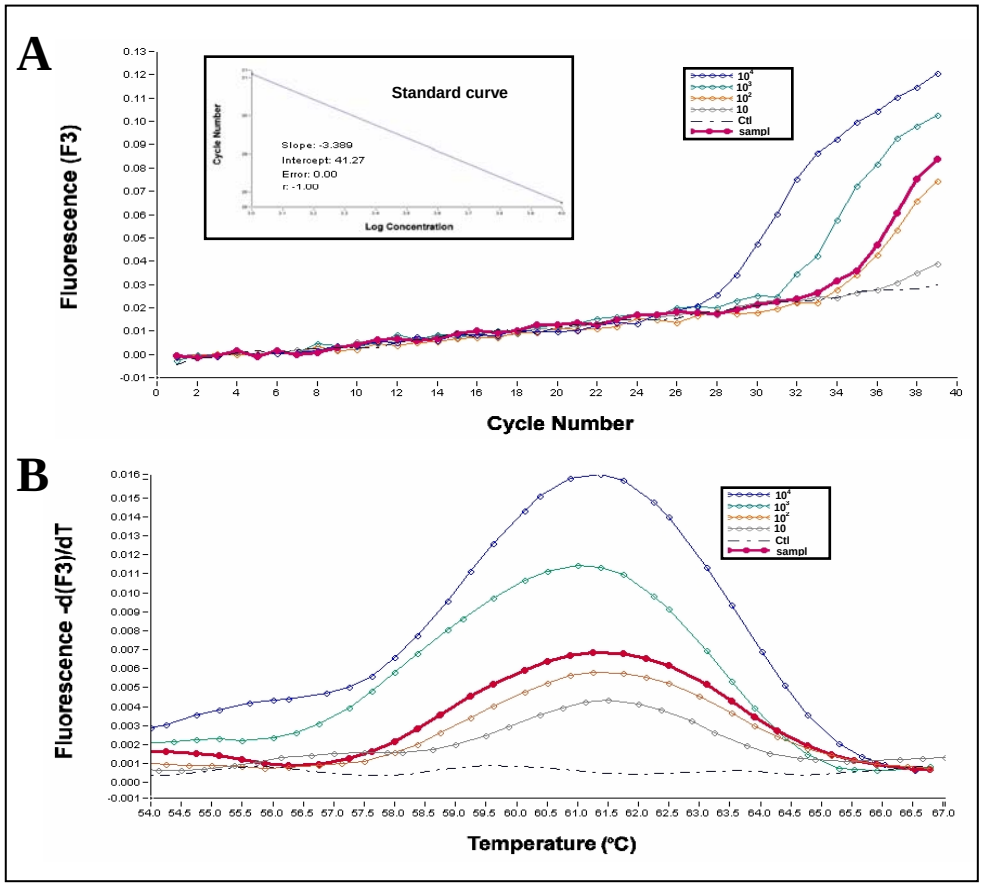

Figure 3: Real- time PCR assay for a sample biopsy. *A*: The CP DNA quantification was determined by the CP-DNA standard curve. *B*: specific melting curve analysis for CP [21].

The results are cumulatively shown in table1. A total of 44% (7/16) plaques were positive for CP DNA.

Table 1

Plaques	**Portion of lesion**	**CP DNA quantification (total DNA copie number)**
N° 1	**Plaque**	**900**
N° 2	**Plaque/Near the Plaque**	**2200/50**
N° 3	**Plaque**	**1600**
N° 4	**Plaque**	**800**
N° 5	**Plaque/Near the Plaque**	**2000/80**
N° 6	**Plaque**	**700**
N° 7	**Plaque/Near the Plaque**	**1900/20**

In 3 plaques (n° 2, 5 and 7) CP DNA was detected in the plaque and the nearby tissue The others 4 plaques presented CP DNA just in the plaque portion. Remarkably, the bacterial DNA was detected in the portion near the plaque exclusively in the biopsy samples witch presented an higher number of DNA copies. In conclusion the microdissections of carotid plaques analyzed in this study with the combination of the two advanced technologies LCM/real-time PCR have shown:

i) CP-DNA is present within the plaque of a high number of subjects,

ii) CP-DNA is localized almost always within the plaque,

iii) in some subjects, CP-DNA is present in the plaque to a high copy number, compatible with CP infection.

6. Conclusions

There are many issues which require further investigations for a full assessment of the role played by C.pneumoniae in atherosclerosis and its acute events. Nonetheless, the evidence here provided couples to previous studies by ourselves and others in demonstrating the CP is not an ephemeral inhabitant of atherosclerotic plaques, and , in some cases, of the myocardium itself. It is unrealistic that this sort of bacterial presence in a critical lesion my go unnoticed by the host , as CP were only an innocent bystander. We believe that at least in a number of subjects, CP may induce inflammation and recruitment of inflammatory cells as to cause or contribute to plaque destabilization or other events. Overall, there is promise that the unravelling of the role of this agent , new preventive and/or therapeutic approaches may be devised to help control a primary cause on pathological events in heart and brain such as atherosclerosis.

References

[1] P. Libby, Inflammation in atherosclerosis, *Nature* **420** (2002) 868–874

[2] R. Ross, Atherosclerosis: an inflammatory disease, *N Engl J Med* **340** (1999) 115–126.

[3] M. S. Nieminen, K. Mattila., and V. Valtonen, Infection and inflammation as risk factors for myocardial infarction, *Eur Heart J* **14,** (**Suppl. K)** (1993) 12–16.

[4] J. T. Kuvin, and C. D. Kimmelstiel, Infectious causes of atherosclerosis, *Am Heart J* **137** (1999) 216–226.

[5] D. N. Streblow, S. L. Orloff and J. A. Nelson, Do pathogens accelerate atherosclerosis? *J Nutr* **131** (2001) 2798S–2804S

[6] H.U Jahn, M. Krull, F.N. Wuppermann, A.C. Klucken, S. Rosseau, J. Seybold, J. H. Hegemann, C. A. Jantos, and N. Suttorp, Infection and activation of airway epithelial cells by *Chlamydia pneumoniae, J Infect Dis* **182** (2000) 1678–1687.

[7] C.A. Gaydos, J.T. Summersgill, N.A. Sahney, J.A. Ramirez, and T.C. Quinn, Replication of *Chlamydia pneumoniae in vitro* in human macrophages, endothelial cells and aortic artery smooth muscle cells, *Infect Immun* **64** (1996) 1614–1620.

[8] T.M. Lin, L.A. Campbell, M.E. Rosenfeld, and C.C. Kuo, Monocyte-endothelial cell coculture enhances infection of endothelial cells with *Chlamydia pneumoniae, J Infect Dis* **181** (2000) 1096–1100.

[9] J.B. Muhlestein, J.L. Anderson, J.F. Carlquist, T. E. Madsen, T. L. Bair, R. R. Pearson, and J. L. Anderson, Randomized secondary prevention trial of azithromycin in patients

with coronary artery disease: primary clinical results of the ACADEMIC study, *Circulation* **102** (2000) 1755–1760.

[10] J.T. Grayston, Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis, *J Infect Dis* **181** (Suppl. 3) (2000) S401–S410.

[11] A. Shor, C.C. Kuo, and D.L. Patton, Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques, *S Afr Med J* **82** (1992) 158–161.

[12] C.C. Kuo, A. Shor, L.A. Campbell, H. Fukushi, D.L. Patton, and J.T. Grayston, Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries, *J Infect Dis* **167** (1993) 841–849.

[13] C.C. Kuo, A.M. Gown, E.P. Benditt, and J.T. Grayston, Detection of *Chlamydia pneumoniae* in aortic lesions of atherosclerosis by immunocytochemical stain, *Arterioscler Thromb* **13** (1993) 1501–1504.

[14] G. Spagnoli, S. Pucci, E. Bonanno, A. Cassone, F. Sesti, A. Ciervo, and A. Mauriello, Persistent Chlamydia pneumoniae infection of cardiomyocytes is correlated with fatal myocardial infarction, *Am J Pathol* **170** (2007) 33-42

[15] W.L. Beatty, R.P. Morrison, and G.I. Byrne, Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis, *Microbiol Rev* **58** (1994) 686–699.

[16] M. Mayr, B. Metzler, S. Kiechl, J. Willeit, G. Schett, Q. Xu, and G. Wick, Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of *Escherichia coli* and *Chlamydia pneumoniae*: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis, *Circulation*; **99** (1999) 1560–1566.

[17] A. Ciervo, P. Visca, A. Petrucca, L. M. Biasucci, A. Maseri , and A. Cassone, Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of *Chlamydia pneumoniae* in patients with coronary heart disease, *Clin Diagn Lab Immunol* **9** (2002) 66–74.

[18] L.M. ,Biasucci G. Liuzzo, A. Ciervo, A. Petrucca, M. Piro, D.J. Angiolillo, F. Crea, A. Cassone, and A. Maseri, Antibody response to chlamydial heat shock protein 60 is strongly associated with acute coronary syndromes, *Circulation* **107** (2003) 3015–3017.

[19] A. Ciervo, A. Petrucca and A. Cassone, Identification and quantification of *Chlamydia pneumoniae* in human atherosclerotic plaques by LightCycler real-time-PCR, *Mol Cell Probes* **17** (2003) 107-111.

[20] C. Cimenti, M. Pieroni. A. Russo, P. Sale, M. A. Russo, A. Maseri, and A. Frustaci, Laser microdissection in clinical cardiovascular research, *Chest* **128** (2005) 2876-2881.