

***Chlamydia pneumoniae* and *Mycoplasma pneumoniae* in Calcified Nodes of Stenosed Aortic Valves**

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Objective

We investigated whether *Chlamydia pneumoniae* (CP) and *Mycoplasma pneumoniae* (MP) are present in aortic valve stenosis (AS).

Methods

Immunohistochemistry was utilized to identify CP antigens, *in situ* hybridization to identify MP DNA, and electron microscopy was used to evaluate the following three groups: Normal - 11 normal autopsy valves; Atherosclerosis - 10 autopsy valves from patients with systemic atherosclerosis and no AS; and AS - 14 surgical specimens of AS analyzed in 3 sub-regions: AS-Preserved - peripheral, preserved regions; AS-Fibrosis - peri-calcified fibrotic tissue; and AS-Calcification - calcified nodules.

Results

The positive area fraction of CP antigen median values were 0.09, 0.30, 0.18, 1.33, and 3.3 in groups Normal, Atherosclerosis, AS-Preserved, AS-Fibrosis, and AS-Calcification, respectively. CP density was significantly greater in Atherosclerosis and AS-Calcification than in Normal ($P<0.05$). Within the AS group, the amount of CP was greater in the Calcification and Fibrosis regions ($P<0.05$). MP-DNA positive area fraction (median values) were 0.12, 0.44, 0.07, 0.36, and 1.52 in groups Normal, Atherosclerosis, AS-Preserved, AS-Fibrosis, and AS-Calcification, respectively. The amount of MP-DNA was greater in AS-Calcification than in Normal ($P<0.05$). Within the AS group, MP-DNA was in larger quantity in the Calcification and Fibrosis regions ($P<0.05$).

Conclusion

AS Calcified nodes present higher concentration of CP and MP suggesting that these bacteria may be associated with the development of calcification and inflammation. This adds novel similarities between AS and the atherosclerosis process, which may have infection mechanisms involved.

Key words

Mycoplasma pneumoniae; *Chlamydia pneumoniae*; Aortic Valve Stenosis; Atherosclerosis; Elderly

The process of aortic degeneration associated with aortic valve stenosis (AS) in the elderly patient shares many similarities with that of coronary artery atherosclerosis¹. Atheromatous plaques and calcification of nonrheumatic aortic valves seem to have a similar pathogenesis². Valves with AS often exhibit inflammatory changes with infiltration of macrophages and T lymphocytes around calcified nodules, which resemble the inflammatory response seen in coronary atherosclerosis². Furthermore, cholesterol deposits precede the calcified nodes in AS. The same clinical risk factors usually associated with coronary artery disease are associated with nonrheumatic calcific aortic stenosis²⁻⁷. These include older age, male sex, increased serum levels of low-density lipoprotein (LDL) and Lp (a) lipoprotein⁸, smoking, hypertension, diabetes, hypercholesterolemia⁹. Gene polymorphism could also be relevant in the development of atherosclerosis¹⁰ and in aortic valve disease¹¹. Osteopontin, a phosphorylated glycoprotein that can be actively synthesized by macrophages has been linked to calcification and has been related to the severity of coronary artery disease¹² and to advanced lesions of aortic stenosis¹³. However, identification of the causes and factors associated with the development of calcification in AS is still a challenge.

Chlamydia pneumoniae (CP) has been suggested to be involved in the pathogenesis of atherosclerosis, related with gene polymorphisms¹⁴ and inflammation¹⁵ but this still is a controversial matter¹⁶⁻¹⁸. CP has also been associated with the inflammatory processes of aortic valve lesions¹⁹. We have previously demonstrated that *Mycoplasma pneumoniae* (MP) is present in association with CP in atheromatous plaques of coronary arteries with unstable coronary syndromes, usually associated with wall inflammation and frequently having calcification^{15,17}. Given the similarities between coronary artery atherosclerosis and AS, we hypothesized that MP and CP could be involved in the development of calcific aortic stenosis in the elderly.

Methods

Three groups of aortic valves were analyzed: group Normal, 11 valves obtained from autopsies of patients aged 52 ± 16 years (7 female, 4 male) without systemic atherosclerosis or aortic stenosis; group Atherosclerosis, 10 valves obtained from autopsies of patients aged 69 ± 9 years (5 female, 5 male) with severe systemic atherosclerosis but without aortic stenosis; and group Aortic Valve Stenosis (AS), 14 surgical specimens from patients aged 71 ± 8

years (4 female, 10 male) undergoing aortic valve replacement for AS. Severe AS was defined as the valve containing areas of calcification and diffuse fibrotic thickening and clinical evidence of severe obstruction. Exclusion criteria were evidence of rheumatic disease, endocarditis, Marfan's syndrome or coexisting valvar disease in the other valves (except mitral annular calcification). The valve leaflets of the group AS were analyzed in 3 sub-regions: areas distant from the calcified nodules (AS-Preserved), peri-calcified fibrous tissue (AS-Fibrosis), and calcified foci (AS-Calcification).

A representative section of the central portion of the leaflets was fixed in formalin 10%. Decalcification was performed when necessary in formic acid 25%. Serial 5- μ m thick sections were obtained for hematoxylin and eosin staining, the immunohistochemical technique (IHC) for detecting *Chlamydia pneumoniae* (CP), and the *in situ* hybridization technique (ISH) for detecting *Mycoplasma pneumoniae* (MP). The specificity of the CP-IHC reaction was confirmed by *in situ* hybridization in the 3 most positive cases at IHC.

A quantitative analysis was performed with the image analysis system Quantimet 500-Leica to detect the area fraction occupied by CP antigens (CP-Ags) and MP-DNA.

Adjacent valvular fragments of each case were also collected for electron microscopy analysis.

Immunohistochemistry technique (IHC) - Five- μ m thick sections of each specimen were submitted to heat antigen recovery (microwave) and incubation with pure monoclonal anti-CP antibody (clone RR-402, Dako, Carpinteria, CA, USA, antimajor outer external membrane of CP), overnight at 4° C. This antibody does not cross react with the following agents: adenovirus, respiratory virus, A and B flu viruses, *Chlamydia trachomatis*, and some *Chlamydia psittaci* serotypes. We confirmed such specificity by simultaneously performing reactions in histological sections positive for cytomegalovirus and herpes virus. The secondary antibody was antimice immunoglobulin produced in rabbits, conjugated with biotin (Dako, Carpinteria, CA, USA) diluted 1:2000, for 1 hour at 37° C. The reaction was detected with streptavidin peroxidase conjugate (Amersham International, England), diluted 1:1000, for 1 hour at 37° C, and 3,3-diaminobenzidine (Sigma Chemical Corporation, St. Louis, MO). Sections were later counterstained with Harri's hematoxylin, dehydrated, cleared, and mounted with Entellan (Merck, Germany).

In situ hybridization technique (ISH) for MP and CP - Cell permeabilization of the histological sections was made using 0.01 M citrate buffered solution, pH 6.0 \pm 0.1, in the microwave oven. Endogenous peroxidase was blocked with 3% H₂O₂. Hybridization mixture containing the following ingredients was applied: the probe, deionized formamide, 50% dextran sulfate, 20 X SSC, Denhardt solution, salmon sperm DNA, yeast tRNA, poly A and C, and diethylpyrocarbonate pure water. DNA double-strands were denatured at 95 \pm 5° C. The signal was amplified using the catalyzed signal amplification system for ISH, Dakogen Point (Dako, Carpinteria, CA, USA). The reaction was visualized with the chromogen 3,3-diaminobenzidine (Dako, Carpinteria, CA, USA). Sections were counterstained with Harri's hematoxylin.

Controls: negative controls for ISH and IHC were carried out omitting the probe or the primary antibody. Human histological sections previously diagnosed as positive for MP and CP were used for control of both reactions. In addition, as positive control for *in situ*

hybridization, we used a repetitive Alu sequence probe (alu1/alu2) (Genetic Research, AL, USA) and for negative control, we used a plasmid DNA, labeled with biotin (Dako, Carpinteria, CA, USA).

Probes: *Mycoplasma pneumoniae* labeled probe was prepared by nick translation of a highly specific clone for *M. pneumoniae*. The clone was specifically isolated and characterized to be highly specific for MP to the exclusion of hybridization with other species of Mycobacteria and other bacterial species (Enzo Diagnostics, Farmingdale, NY, USA). *Chlamydia pneumoniae* probe was the biotinylated oligonucleotide sequence (20 bp) synthesized by GIBCO/BRL, Rockville, MD, USA.

Electron microscopy - The fragments of the valves collected for electron microscopy were previously decalcified as described above when necessary, fixed in 3% glutaraldehyde, and postfixed with 1% osmium tetroxide. The fragments were contrasted with 0.5% uranyl acetate. Fragments were blocked in araldite resin. Ultra-thin sections (60 to 90 nm) were obtained and contrasted with 5% lead citrate solution²⁰.

Statistical analysis - The variable area fraction was described through the observation of the minimum and maximum values, and the calculation of means, medians, and standard deviations.

As the assumption of normality or equality of variance was not met, a nonparametric test (distribution-free) was used to compare the 3 independent groups of sampled data (groups Normal, Atherosclerosis, and AS). The Kruskal-Wallis test was used to test the hypothesis of equality between the 3 groups, with multiple comparisons, and the different group was determined by the Dunn test.

To test the mean equality in different regions of the same valve in the group with aortic stenosis (AS-Preserved, AS-Fibrosis, and AS-Calcification), the Friedman nonparametric test was used²¹.

The significance level used for all the tests was 5%.

Results

Analysis of *C. pneumoniae* antigens in calcified and noncalcified valves - The immunohistochemistry demonstrated several positive granules into the calcified foci and in the fibrous tissue surrounding them represented by dark brown points (Figure 1B). The normal and noncalcified valves from atherosclerotic patients exhibited a much lower number of CP antigens (Figure 1A).

Median, mean, and standard deviation (SD) of area fraction positive for CP-Ags in the different valve groups are shown in Table I.

Comparative analysis of median area fraction positive for CP-Ags revealed a significantly greater number of CP-Ags only in AS-Calcification as compared to Normal (p<0.05).

Comparison of the different valvular regions of group AS showed that AS-Preserved had a significantly lower number of CP-Ags than did groups AS-Fibrosis and AS-Calcification (P<0.05).

Analysis of *M. pneumoniae*-DNA in calcified and noncalcified valves - *In situ* hybridization revealed concentrations of MP-DNA (in brown) in the calcified foci (Figure 1C). Some areas of valves from the Atherosclerotic group also exhibited many positive granules but little CP.

Median, mean, and standard deviation (SD) of area fraction of *Mycoplasma pneumoniae*-DNA (MP-DNA) in the different groups are shown in Table II.

Comparison of groups Normal, Atherosclerosis, and AS-Preserved revealed that group Atherosclerosis has significantly higher

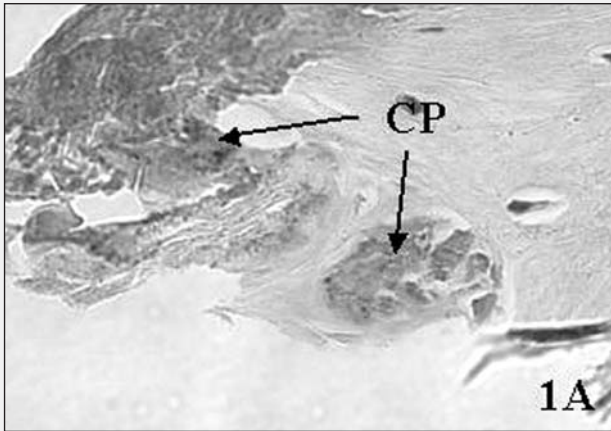


Fig. 1A - Calcified focus *in situ* in a stenotic aortic valve exhibiting many *Chlamydia pneumoniae* DNA positive granules (in brown) by the *in situ* hybridization technique - original magnification 40x.

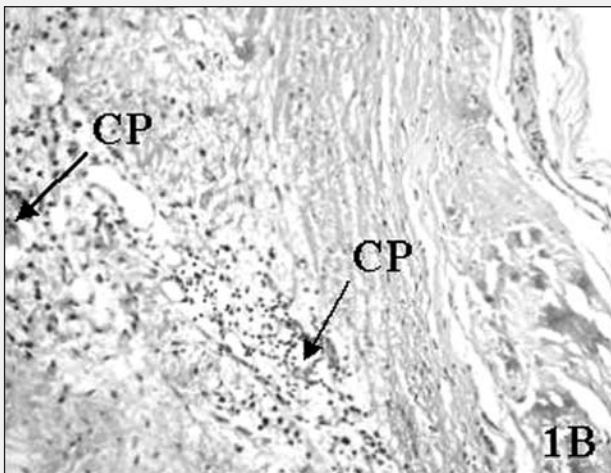


Fig. 1B - Severe inflammatory infiltrate nearby calcified foci showing diffuse positive granules of *Chlamydia pneumoniae* antigen (in brown) into the cytoplasm of inflammatory cells and in the interstitial matrix (immunohistochemistry technique - original magnification - 20x).

Table I - Area fraction of CP-Ags

Group	N	Median	Mean±SD
Normal	11	0.09	1.26±1.90
Atherosclerosis	11	0.30	1.48±2.02
AS-Preserved	13	0.18	0.21±0.17
AS-Fibrosis	14	1.33 [§]	2.60±3.27
AS-Calcification	14	3.36 ^{*†}	5.82±5.41

*P<0.05 AS-Calcification vs. Normal and Atherosclerosis; †P<0.05 AS-Calcification and AS-Fibrosis vs. AS-preserved.

values than AS-Preserved (P<0.05). The group AS-Calcification (calcified foci) had a statistically significant greater amount of MP-DNA than did groups Normal and Atherosclerosis (P<0.05). No difference existed among groups Normal, Atherosclerosis, and AS-Fibrosis (P=0.16).

Analysis of different regions of group AS revealed that more preserved valvular regions (AS-Preserved) had lower numbers of MP than did the calcified nodules (AS-Calcification) and the pericalcified fibrotic region (AS-Fibrosis) (P<0.05).

Density of *C. pneumoniae* and of *M. pneumoniae* in the valves - The density of CP-Ags and MP-DNA were similar in valves from Normal and Atherosclerosis groups. In the group with aortic ste-

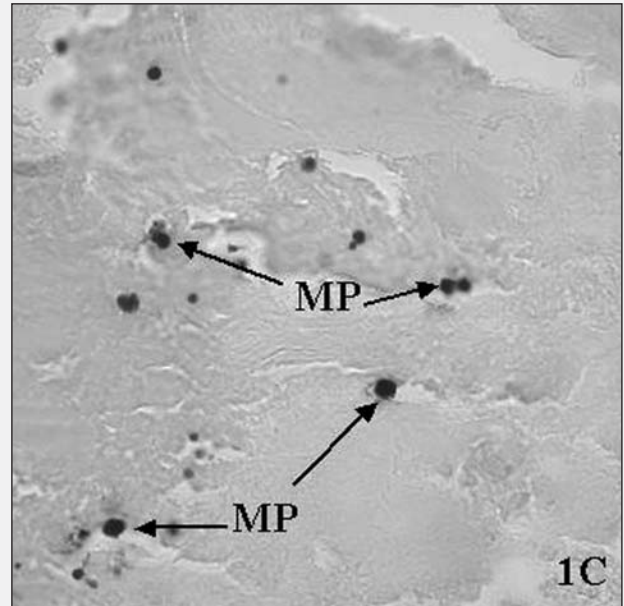


Fig. 1C - Focus of calcification in stenotic aortic valve exhibiting many positive *Mycoplasma pneumoniae* (MP) DNA granules in brown by the *in situ* hybridization technique. Original magnification - 40x.

Table II - Area fraction of MP-DNA

Group	N	Median	Mean±SD
Normal	11	0,12	0,38±0,58
Atherosclerosis	10	0,44*	0,67±0,66
AS-Preserved	12	0,08	0,12±0,13
AS-Fibrosis	13	0,36 [§]	1,78±3,63
AS-Calcification	13	1,52 ^{*†}	2,27±2,06

*P<0.05 Atherosclerosis vs. Normal and AS-preserved; †P<0.05 AS-Calcification vs. Normal and Atherosclerosis vs. AS-Preserved; §P<0.05 AS-Calcification vs. AS-Fibrosis and AS-Preserved.

nosis, the density of both CP-Ags and MP-DNA in the calcified region were higher than the density in the Preserved region of the valve and than that in the Normal group. In the fibrotic region, the density of CP-Ags and MP-DNA were also greater than that in the Preserved region (Figure 2).

Discussion

Many similarities between the process of atherosclerosis and that of AS suggest that common causative agents may underlie both diseases. Mohler et al²² found that 88% of surgically excised heart valves from patients who underwent cardiac valve replacement surgery contained atherosclerotic plaques, 83% had dystrophic calcification, and mast cells were present in calcified and ossified valves and most prominently in atheromatous regions.

We have previously reported that *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are present in atherosclerotic plaques^{15,17,18}. Given the similarity between atherosclerosis and aortic valve stenosis, we hypothesized that such agents may also be present in AS and play an important role in the pathogenesis of the disease. In the present study, we searched for CP and MP in aortic valves with and without stenosis and in nonstenotic aortic valves from atherosclerotic patients, using different anatomopathological techniques.

The probe used against MP is very specific, as is the monoclonal antibody against the external wall of CP. The results showed that both bacteria are frequently present in all aortic valves, independently of calcification. However, a significantly greater amount of both is present in the calcified foci. The surrounding dense fibrotic tissue also exhibits large amounts of CP-Ags.

MP and CP were found in 100% of the studied valves. Aortic valves of patients without AS present scarce amount, regardless of whether they had systemic atherosclerosis or not. On the other hand, patients with AS presented higher amounts of both agents. These results suggest that MP and CP may play a role in the development of calcification in AS. The detection of both bacteria in the calcified nodules was obtained by all 3 techniques – *in situ* hybridization, immunohistochemistry, and electron microscopy – a demonstration that the results are consistent and reliable. The ultra-structural analysis demonstrated that in the calcified nodules these bacteria seem to be degenerated, but in many of them we recognized the double external membrane and the pearl shape that characterize elementary bodies of *Chlamydia pneumoniae* (Figure 3A and 3B).

A significant difference exists in the reported incidence of CP in valves of patients with AS in the literature. Andreasen et al⁹ found 0% using PCR (polymerase chain reaction) and culture, while Juvonen et al (16) reported 53% using immunohistochemistry and 18% by PCR in surgical specimens of patients undergoing aortic valve replacement due to severe nonrheumatic stenosis.

PCR was the most often used method for detection of *C.pneumoniae* in previous studies. This may explain in part the wide variation of positivity in the results. Studies have demonstrated that there is a significant variation in the ability of assays among clinical laboratories to reliably detect infectious agents^{23,24}.

On the other hand, most of the studies, did not quantify the amount of infectious agents present in the tissue in order to correlate with the intensity of the lesion and inflammation. PCR is a technique that is only capable to detecting the presence or not of the DNA sequence searched.

In the present work we preferred to use the *in situ* hybridization technique that provides information not only about the presence, but also localization and quantity of the searched DNA sequence.

In order to check the reliability of these results, we simultaneously searched for the presence of specific antigens using immunohistochemistry technique with the most commonly used monoclonal antibody against *C.pneumoniae*. Finally, we searched by electron microscopy for the presence of structures that were compatible with *C.pneumoniae* forms and found large numbers of them, mainly surrounding the calcified nodules.

The demonstration that *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* bacteria are present in significantly higher concentrations in aortic valves of patients with aortic stenosis does not appear to be a coincidental finding. From a pathophysiological point of view, these bacteria have the potential to actively participate in the genesis of the pathological findings of this disease.

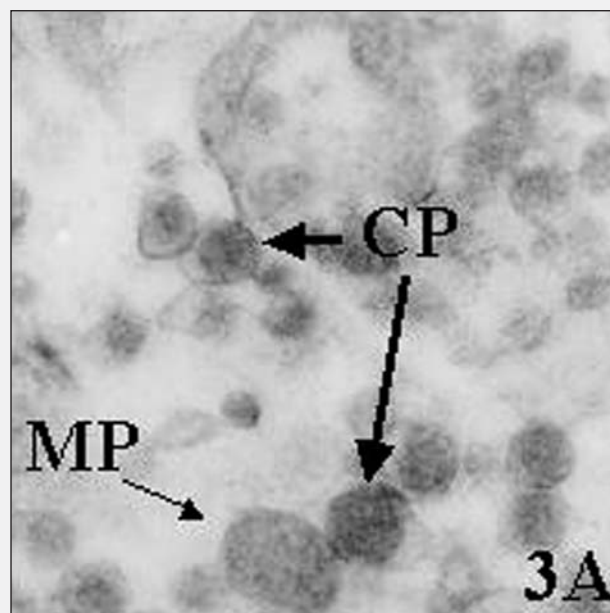


Fig. 3A - Concentration of *Chlamydia pneumoniae* elementary bodies (CP) and *Mycoplasma pneumoniae* (MP) round structures involved in only one membrane into a focus of calcification. Original magnification - 13.000x.

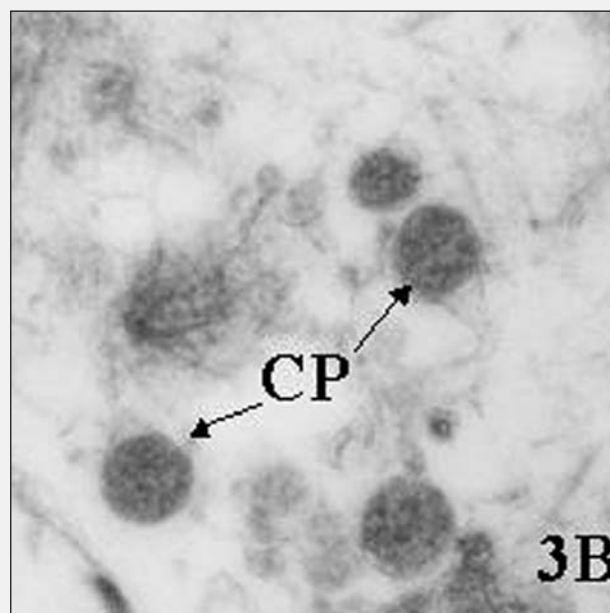


Fig. 3B - Elementary bodies of *Chlamydia pneumoniae* (CP) represented by round electron dense structures with a pear-shaped external wall expansion in the extracellular matrix. Original magnification - 10.000x.

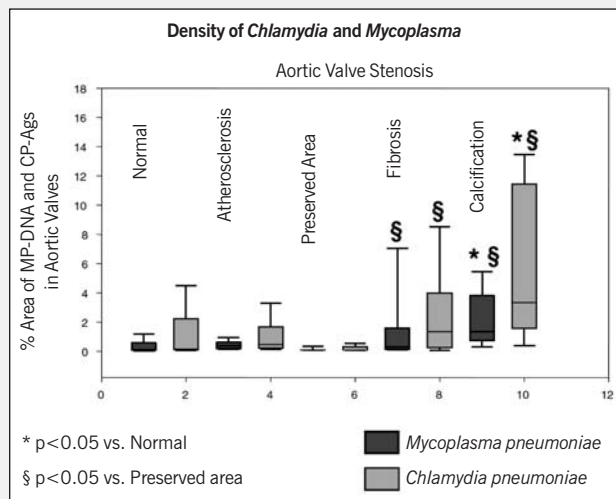


Fig. 2 - Density of *Chlamydia pneumoniae*-antigens and *Mycoplasma pneumoniae*-DNA in three groups of valves and in aortic valve stenosis subregions.



The demonstration of these two agents in aortic valves has never been shown before and opens another approach in the research on aortic valve stenosis.

Chlamydia pneumoniae are gram-negative bacteria that dwell exclusively within other cells such as macrophages. They are capable of inducing the production of enzymes by the host cell such as metalloproteinases and 92-kDa gelatinase. These enzymes can damage components of the extracellular matrix and result in changes that are similar to those observed in chronic inflammatory conditions, such as atherosclerosis and aortic stenosis^{25,26}.

Mycoplasma pneumoniae are small, simple bacteria and the only ones capable of utilizing cholesterol for their reproduction. They are frequently associated with respiratory infections but may also be involved in vascular disease and are capable of inducing chronic inflammatory states. Their external membrane is rich in phospholipids, which can induce the oxidation of the host's cellular membrane. Additionally, they can induce cytokines in the host's cells and can boost the virulence of other bacteria²⁷.

It is therefore plausible that these two bacteria actively participate in the process of aortic valve disease. *Mycoplasma pneu-*

moniae may stimulate the virulence of *Chlamydia pneumoniae* leading to an intense inflammatory process, resulting in debris of cellular membranes of the bacteria and host cells, with accumulation of oxidized LDL-cholesterol²⁸ and Lp(a)⁸ and a local inflammatory process²⁹. The presence of a large number of phospholipids from degraded membranes of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* is an adequate substrate for calcium deposition, culminating in calcification of the valve. In early lesions, microscopic calcification is present in areas with lipoprotein accumulation^{8,28}. As the disease progresses, the abnormal areas become confluent. The microenvironment of the damaged valve with the presence of the infectious agents, cholesterol deposition, and inflammation results in favorable conditions for the development of calcification and hardening of the valve.

In conclusion, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are present in high concentrations in calcific aortic valve stenosis. Our results suggest that these bacteria are associated with the development of calcification and inflammation, adding novel similarities between aortic valve stenosis and the atherosclerotic processes, both of which may have infection involved in their basic pathogenesis.

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