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## A positive association of peripheral arterial occlusive disease (PAD) and *Chlamydomphila (Clamylidia) pneumoniae*

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The relationship between *Chlamydomphila pneumoniae* (*Cp*) infection and peripheral arterial occlusive disease (PAD) was studied by analyzing clinical samples from 95 patients with PAD and 100 controls. The following investigations were conducted: IgG and IgA against lipopolysaccharide (LPS) and against purified *Cp*-specific antigens from elementary bodies (EB) with ELISA; anti-EB IgG, with MIF; *Cp* DNA in arterial biopsy and peripheral blood mononuclear cells with heminested PCR; LPS with ELISA; and bacteria culture in HEp-2 cells from arterial biopsy. A significantly higher ratio of anti-EB IgG was detected in patients. There were no significant differences in anti-LPS IgG, anti-LPS IgA and anti-EB IgA between cases and controls. *Cp* DNA findings in the vascular wall biopsy showed significant differences between cases and controls. We obtained results that significantly involve *Cp* infection with PAD through the detection of anti-EB IgG from serum and bacterial DNA from arterial biopsy.

Classical risk factors for atherosclerosis (AT) only explain 60% of cases. Microorganisms such as *Chlamydomphila pneumoniae* (*Cp*) have been associated with this disease (CAMPBELL and KUO 2003). *Cp* is a respiratory pathogen (MONNO *et al.* 2002) that can spread through the organism from this localization through the monocyte-macrophage system (MOAZED *et al.* 1998). The relationship between *Cp* infection and AT has been demonstrated in observational and experimental studies. Since 1988, more than 70 prospective and cross-sectional studies of serum samples have studied anti-*Cp* antibodies in patients with AT. These works showed higher circulating anti-*Cp* antibodies and immune complexes with lipopolysaccharides (LPS) (LINNANMAKI *et al.* 1993, SAIKKU *et al.* 1988) in patients with symptomatic AT, with or without surgical treatment (TIRAN *et al.* 1999). Studies presenting conflicting results have also been published (ANDERSON *et al.* 1998, GERDES *et al.* 2003, WEISS *et al.* 1996).

The presence of the microorganism has also been investigated in coronary and peripheral atherosclerotic vascular tissues, using immunohistochemistry, PCR, *in situ* hybridization, electron microscopy, and cell culture. More than 50 studies detected *Cp* in atherosclerotic tissues using at least one of these methods, although the ranges detected were highly variable (CAMPBELL and KUO 2003). On the other hand, some authors detected no DNA of the bacteria or did not recover it in cell culture (WEISS *et al.* 1996, BISHARA *et al.* 2003, LINDHOLT *et al.* 1998, ONG *et al.* 2001). The microorganism has also been detected in peripheral blood mononuclear cells (PBMCs), which would explain its dissemination from pulmonary to vascular localization (BLASI *et al.* 1999, MUHLESTEIN 2000). Animal studies have confirmed the latter and may explain its initiating and/or adjuvant action in the development of AT (Muhlestein *et al.* 1998). Clinical trials (ACADEMIC, WIZARD, MARBLE and ACES, among others) have attempted to prevent secondary events in association with the infection

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from its vascular localization by means of different antibiotic treatments (DUNNE *et al.* 1999, JACKSON *et al.* 1997, MUHLESTEIN *et al.* 2000).

Most of the above studies described associations between *Cp* infection and atherosclerotic coronary artery disease using isolated parameters that do not allow the results to be interpreted or applied to peripheral arterial occlusive disease (PAD).

For these reasons, patients with PAD were included in the present work, in which several simultaneous parameters were analyzed in order to establish a possible relationship with the bacteria through the presence of specific antibodies, antigens and DNA, and by their culture in a cell line. All of these parameters serve to improve our knowledge of the association between *Cp* infection and PAD.

## Materials and methods

A case-control study design was adopted: group 1 comprised patients with PAD, potentially susceptible to infection by the bacteria, and group 2 were controls. Both groups of subjects, who presented no respiratory or genital diseases, were recruited at our hospital and underwent various tests.

**Description of patients and controls: Group 1.** Formed by 95 patients with PAD (diagnosed by basal electrocardiogram, echo-Doppler and clinical examinations according to established criteria of intermittent claudication and Fontaine stage III) and with absence of other diseases (LABS *et al.* 1999). They comprised 41 subjects with severe carotid stenosis, 18 with occlusive disease of infrarenal or iliac abdominal aorta, 20 with femoropopliteal occlusive disease and 16 with aneurism of infrarenal abdominal aorta of more than 5 cm diameter (total of 70 males and 25 females; mean age of  $66 \pm 8$  years). Samples of serum and vascular tissue with atherosclerosis were obtained from all patients in the group, and a PBMCs sample was also obtained from 15 of them.

**Group 2:** Formed by 100 patients with chronic superficial venous insufficiency and without PAD, which was ruled out by clinical, carotid echo-Doppler and basal electrocardiogram examinations, or any other disease (45 males and 55 females, mean age  $60 \pm 3$  years). This group was comparable with group 1 in terms of tobacco use, social situation, and number of patients over 60 years old. During the varicose vein resection, 1 cm of the external pudendal artery was removed from 50 patients, too. This artery was tested in the controls. A serum sample was also obtained from all patients.

**Tests performed:** In all subjects, IgG anti-elementary bodies (EB) were investigated with MIF, and anti-EB IgG and IgA and anti-LPS IgG and IgA were studied with ELISA. In 15 subjects with PAD and 130 other subjects (80 with PAD and 50 with chronic superficial venous insufficiency), *Cp* DNA was analyzed with heminested PCR in PBMCs and arterial biopsies, respectively. LPS was investigated with ELISA in 80 arterial biopsies of PAD patients; and bacteria were cultured in HEp-2 cells from 15 arterial biopsies of PAD patients.

**Methods of collection and preservation of samples:** Arterial biopsies were washed with physiologic serum and placed in 2-SPG medium. They were refrigerated at 4 °C until their collection (for maximum of 4 h) or were immediately frozen at -20 °C for 12–16 h and then at -80 °C until their analysis. PBMCs samples were preserved in the same way after their separation from other blood components. Serum samples were frozen at -20 °C until their analysis.

The Ethics Committee of the hospital approved the study and all subjects signed their informed consent. No patient suffered active infections before arterial surgery that required antibiotic treatment. The processing and interpretation of all laboratory determinations were performed in a blinded fashion.

**Investigation of anti-*Cp* antibodies:** IgA and IgG against LPS of *Chlamydiaceae* were investigated with an ELISA test (Chlamydien-rELISA, MEDAC, Germany) (GUTIÉRREZ-FERNÁNDEZ *et al.* 2001). IgA and IgG against purified *Cp*-specific antigens from EB were analyzed using MIF (*C. pneumoniae*, MRL Diagnostic, USA) (BENNESEN *et al.* 2002) and ELISA tests (*C. pneumoniae*, VIRCELL S.L., Spain) (GUTIÉRREZ *et al.* 2002).

**Detection of *Cp* DNA in biopsy of vascular wall using heminested PCR:** A variation of PCR developed by CAMPBELL *et al.* (1992) was used for these determinations. This methodology was previously reported by our group (GUTIÉRREZ *et al.* 2001). The amount of DNA was matched among samples (50–100 ng/ml).

**Detection of *Cp* DNA in PBMCs:** A methodology previously published by our group was used to separate PBMCs from plasma and red blood cells (GUTIÉRREZ *et al.* 1989). The method to detect the DNA was the same as for the biopsy samples, except that lysis buffer was not used in the DNA extraction.

**Investigation of LPS antigen of *Chlamydiaceae* in atheromous plaque:** A previously described ELISA (MicroTrack II, DADEBEHRING Diagnostics Inc., Germany) was used (DEAN *et al.* 1998), in which samples and controls are additionally left in the elution solution at room temperature for 2 h to facilitate the release of the bacterial LPS. The validation data for LPS detection by MicroTrack II in arterial specimens were not provided.

**Cell culture of *Cp*:** The *shell-vial* method proposed by MAASS and HARIG (1995) was used, with some modifications. Three samples from each patient were cultivated in a different way: one was incubated 96 h at 37 °C; another was centrifuged three times on alternate days (45 min to 800 g), changing the culture medium after each centrifugation; and the third was subcultured 11 times. Infected cell monolayers were observed using a direct MIF test with mouse monoclonal anti-LPS antibody to detect *Cp* infection (IMAGEN *Chlamydia*, DAKO, Germany).

**Statistical analysis:** The SPSS version 11.5.1 (SPSS Inc. 1989–2002) program was used. The comparison of discrete variables was performed with the chi-square ( $\chi^2$ ) test. The ability of anti-*Cp* antibodies to predict atheromous plaque infection was determined by calculating the predictive value in samples from patient and control groups. The exclusion of the association between *Cp* infection and the development of PAD is the null hypotheses of this analysis.

## Results

### *Antibodies against Cp*

Table 1 shows the samples in which antibodies were detected. A significantly ( $p < 0.001$ ) higher ratio of anti-EB IgG was detected in group 1 (by MIF and ELISA). There were no significant differences in anti-LPS IgG, anti-LPS IgA and anti-EB IgA between cases and controls.

Table 1  
Samples with positive results in each laboratory serologic test and study group

Groups (samples)	PADa (95)		Controls (100)	
	N <sup>d</sup>	%	N	%
ANTI-EB <sup>b</sup> IgG (MIF)	68	71.6	34	34
ANTI-EB IgG (ELISA)	70	73.7	31	31
ANTI-EB IgA (ELISA)	11	11.6	11	11
ANTI-LPS <sup>c</sup> IgG (ELISA)	10	21.1	14	14
ANTI-LPS IgA (ELISA)	21	22.1	21	21

<sup>a</sup>PAD – peripheral arterial occlusive disease. <sup>b</sup>EB – elementary bodies of *Cp*. <sup>c</sup>LPS – lipopolysaccharide. N<sup>d</sup> – number of positive samples

*Cp* DNA in vascular wall biopsy and PBMCs

*Cp* DNA findings in the vascular wall biopsy showed significant differences between all group 1 subgroups and group 2 ( $p < 0.001$ ) (Table 2). No *Cp* DNA was detected in 15 PBMCs samples from group 1 patients. Table 3 displays the positive (PPV) and negative (NPV) predictive values for tests that predicted infection of atheromous plaque by detection of anti-*Cp* antibodies; infection was confirmed by presence of *Cp* DNA. The anti-EB IgG ELISA and MIF tests presented the highest PPV. No test had a noteworthy NPV.

LPS antigen of *Chlamydiaceae* and cell culture of atheromous plaque *Cp*

Only one sample in group 1 showed a positive result for LPS of *Chlamydiaceae* by ELISA (1.25 %). This sample came from a patient with no anti-*Cp* antibodies or *Cp* DNA, and no *Cp* strain could be recovered by cell culture.

**Discussion**

The present study aimed to make an additional and novel contribution to published studies on the relationship between *Cp* infection and PAD. The results obtained appear to support the conclusions of authors who consider that *Cp* infection may participate in the processes studied.

*Antibodies against Cp*

Previous reports using serologic studies have positively (BLANCHARD *et al.* 2000) or negatively (GERDES *et al.* 2003) related *Cp* infection to peripheral AT. We found a positive relationship only for anti-EB IgG and not for anti-LPS IgG and IgA or anti-EB IgA, which may

Table 2  
Presence of DNA of *Chlamydomphila pneumoniae* in groups 1 and 2

Localization of samples	N° of samples with DNA/total samples (%)
Group 1	52/80 (65)
CS <sup>a</sup>	18/26 (69.2)
IIAOD <sup>b</sup>	12/18 (66.7)
FPO <sup>c</sup>	13/20 (65)
IAAA <sup>d</sup>	9/16 (56.3)
Group 2	6/50 (12)

<sup>a</sup>CS – carotid stenosis. <sup>b</sup>IIAOD – infrarenal or iliac abdominal aortic occlusive disease. <sup>c</sup>FPO – femoropopliteal occlusion. <sup>d</sup>IAAA – infrarenal abdominal aortic aneurysm

Table 3  
Predictive values of assays to detect anti-*Chlamydomphila pneumoniae* antibodies infection in atheromous plaque

	anti-EB <sup>c</sup> IgG (MIF)	anti-EB IgG (ELISA)	anti-EB IgA (ELISA)	anti-LPS <sup>d</sup> IgG (ELISA)	Anti-LPS IgA (ELISA)
PPV <sup>a</sup>	0.724	0.724	0.052	0.172	0.259
NPV <sup>b</sup>	0.417	0.333	0.153	0.236	0.306

<sup>a</sup>PPV – positive predictive value. <sup>b</sup>NPV – negative predictive value. <sup>c</sup>EB – elementary bodies of *Chlamydomphila pneumoniae*. <sup>d</sup>LPS – lipopolysaccharide

be explained by various biological phenomena. There may be a persistent *Cp* infection in the PAD patients with bacteria that replicate little and express less LPS, as observed with *Chlamydia trachomatis* (GEISLER *et al.* 2001). On the other hand, the short half-life of anti-LPS antibodies in relation to anti-EB antibodies may limit its detection, especially in chronic infections such as AT (TIRAN *et al.* 1999, VEERKOYEN *et al.* 1997). We believe that EB may be recognized by the immune system before their arrival at the vascular endothelium. This may result from the expulsion of EB at certain points of their biological cycle during their transport through the monocyte-macrophage system, and small stimuli would be sufficient to induce antibody synthesis. Nevertheless, our results may show some biases: a) although the presence of major respiratory infection was an exclusion criterion for group 1 and 2 subjects, the presence of minor respiratory illnesses can increase IgG and IgA titers when associated with arterial disease (TIRAN *et al.* 1999); b) infection without seroconversion has been previously reported (EMRE *et al.* 1994); and c) the detected signal may be related to heterotypical immunologic responses (due to antigenic similarities with other *Chlamydiaceae* species, gram negative bacilli or human heat shock proteins) rather than to anti-*Cp* antibodies. This may be due to the presence of poorly characterized epitopes of the bacteria into the employed kits. Moreover, if LPS remain in the EB, it could be interpreted as an increase in specific anti-*Cp* antibodies (MAURIN *et al.* 1997).

#### *Cp* DNA in vascular wall biopsy and PBMCs

We found a higher proportion of positive results compared with previous findings in patients with stenosis of carotid arteries or infrarenal or femoropopliteal aorta. Our findings were similar to reports in patients with stenosis of iliac artery or abdominal aortic aneurysm (DOBRILOVIC *et al.* 2001).

However, we obtained no positive PCR results in PBMCs. This finding agrees only with that of ONG *et al.* (2001) and differs from that reported by other authors, who detected *Cp* DNA in up to 60% of isolates (SMIEJA *et al.* 2002). This discrepancy may be explained by variability in PBMCs separation methods and difficulties of sample preservation. A recent study concluded that preservation problems and a scant DNA presence led to negative findings in replicas of samples previously found to be positive (BOMAN *et al.* 2000, SMIEJA *et al.* 2001).

#### LPS of *Chlamydiaceae* and cell culture of atheromous plaque *Cp*

Given the LPS and the anti-LPS antibodies detection results, the positive LPS result by ELISA may be a false positive. Considering the antibodies detected and the above-reported hypothesis regarding the absence of their synthesis, the scant LPS presence in these samples is comprehensible, because its expression may be limited by immunocomplexes, hampering LPS detection. Moreover, the extraction of chlamydial LPS is a difficult procedure, especially from calcified tissues. This is the first report of the application of this kit test to arterial wall biopsies, precluding any comparison with other results.

In relation to cell culture, authors have isolated *Cp* in peripheral artery but they did not study a control group, so that a definitive association cannot be established (APFALTER *et al.* 2000). At any rate, the scant isolation rates demonstrate the complexity of the technique and the difficulty of growing the bacteria, especially in initial cultures (APFALTER *et al.* 2000, JACKSON *et al.* 1997).

The contribution of this study to the existing literature on the association between *Cp* infection and PAD derives from the simultaneous performance of multiple tests that detect different targets. Data were obtained on *Cp* from its recognition by the immunologic system, during its transport through monocyte-macrophage system, and up to the determination of its DNA in atheromous plaque. To summarize, we obtained results that significantly involve *Cp* infection with peripheral AT through the detection of anti-EB IgG and bacterial DNA.

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