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DIRECT AND INDIRECT EVIDENCE OF *CHLAMYDIA PNEUMONIAE* IN PATIENTS WITH SIGNIFICANT STENOSIS OF *A. CAROTIS* OF ATHEROSCLEROTIC ORIGIN

ZEMAN K.[1], POSPÍŠIL L.[2], ČANDERLE J.[2], ŠTROBLOVÁ H.[3], LEYPOLD J.[4], GREGOR Z.[4], MEDKOVÁ Z.[5], ŠERÝ O.[6]

[1] First Department of Internal Medicine – Cardioangiology, St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University Brno,
[2] Veterinary Research Institute, Brno
[3] Department of Clinical Microbiology, Faculty Hospital Brno
[4] Second Department of Surgery, St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno
[5] BIO-PLUS Laboratories Ltd., Brno
[6] Laboratory of Neurobiology and Molecular Psychiatry, Faculty of Science, Masaryk University, Brno

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A b s t r a c t

The aim of this study was to ascertain the role of *Chlamydia pneumoniae* in the pathogenesis of significant carotid atherosclerotic stenosis in thirty patients (25 male and 5 female, 67.5 years old on average) through both direct and indirect methods. Direct evidence of polymerase chain reaction (PCR) in the DNA product *Chlamydia pneumoniae* in the atherosclerotic plaque obtained during the operation of *a. carotis* was positive in 56.7 % of the patients. Blood sera were analysed using enzyme immunoassay (ELISA) for the detection of species-specific antibodies in the immunoglobulin classes IgA, IgG and IgM against *Chlamydia pneumoniae*, and ELISA for the detection of antibodies in the class IgG to chlamydial heat shock protein (cHSP60). Species-specific antibodies of *Chlamydia pneumoniae* in the IgA class were detected in 60 % of the patients, in the IgG class in 86.7 %, and in the IgM class in 3.3 %. Chlamydial heat shock protein antibodies were detected in 56.7 % of the patients. The results of the direct evidence of *Chlamydia pneumoniae* in the atherosclerotic plaques using the PCR method and the detection of specific IgA chlamydial antibodies in the sera lead us to the conclusion that *Chlamydia pneumoniae* can be a significant participant in the origin and progression of atherosclerosis as well as in the strategy of the consequent treatment and remission prevention after carotid stenosis surgery.

K e y w o r d s

Nested PCR, DNA *Chlamydia pneumoniae*, Specific antibodies, Chlamydial heat shock protein

INTRODUCTION

Human pathology distinguishes two very significant species in the *Chlamydia* gram-negative intracellular bacteria genus: *Chlamydia pneumoniae (C. pneumoniae)*

and *Chlamydia trachomatis (C. trachomatis)*. The former is an important respiratory pathogen and the latter is known as the cause of the most common sexually transmitted infection –urogenital chlamydiosis. Both of them can also participate in the origin and progression of a number of diseases, most commonly asthma, arthritis, atherosclerosis (3A syndrome), and others (*1–5*).

Recent research into *C. pneumoniae* has concentrated primarily on its possible role in the pathogenesis of vascular diseases whose consequences are mostly more significant than those of respiratory diseases, traditionally caused by this infection. Based on numerous seroepidemiological studies as well as experimental *in vitro* tests and tests on animals, *C. pneumoniae* is very likely to participate in or even initiate the atherosclerotic process (*6*). The relationship between *C. pneumoniae* and the ischaemic heart disease was first studied in 1988 in Finland (*7*). The authors of the present work have also repeatedly conducted research into the relationship between *C. pneumoniae* and ischaemic heart disease (*8, 9*). Persistent *C. pneumoniae* infections in the lungs and atherosclerotic plaques have been shown (*10, 11, 12*). According to most studies, chronic chlamydial infection is a very important factor in atherogenesis, as it induces system inflammation and autoimmune processes in the course of its pathophysiological process. The cultivation evidence of *C. pneumoniae* is very difficult and molecular biological tests for a direct proof have not yet become a standard part of common diagnostics; therefore most infections are detected indirectly through the detection of specific antibodies for the antigens of this gram-negative microbe. The antibodies' dynamics proves persistent infection or reinfection.

The aim of this study is to ascertain the participation of *Chlamydia pneumoniae* in the pathogenesis of significant carotid atherosclerotic stenosis using both direct and indirect methods.

MATERIALS AND METHODS

Thirty patients (25 male and 5 female, 67.5 years old on average) with significant *a. carotis* stenosis were repeatedly examined and tested prior to their operations on the afflicted carotid in the Second Department of Surgery of St. Anne's Faculty Hospital of the Faculty of Medicine, Masaryk University in Brno. The clinical examination included electrocardiography, sonography, and angiography of *aa. carotidis*. The patients' blood sera were tested for the concentration of glucose, lipid spectrum elements (total cholesterol – TC, low-density lipoprotein cholesterol fraction – LDL-C, high-density lipoprotein cholesterol fraction HDL-C, triglyceride – TG), fibrinogen, wide-range C-reactive protein (CRPwr), homocysteine, and uric acid in accordance with the manual of the Central Laboratory of St. Anne's Faculty Hospital. The known biochemical danger factors of atherosclerosis in the blood sera (*Table 1*) showed the following figures: high total cholesterol level in 26.7 % of patients, LDL-C in 46.7 %, TG in 30 %, and lower HDL-C in 53.3 %. A higher level of glucoses was ascertained in 70 % of patients, of fibrinogen in 70 %, of homocysteine in 23.3 %, of CRPwr in 23.3 %, and of uric acid in 16.7 % of the patients. In addition to the diagnosed *a. carotis* stenosis, the patients' history also revealed other serious diseases. Medical records of the patients operated on for carotid stenosis showed hypertension in 83.3 %, ischaemic heart or lower extremities disease in 43.3 % and 13.3 % respectively, diabetes mellitus in 43.3 %, and stroke in 36.7 %. Moreover, 63.3 % of patients were overweight with a body mass index (BMI; body weight in kg/height in m^2) of 25–29.9 and 23.3 % were obese (BMI >30).

Table 1

Some characteristics of patients before surgery for significant stenosis of *a. carotis* of atherosclerotic origin

n = 30	(mean ± S.D.)
age (years)	67.5 ± 6.4
body mass index (kg/m^2)	28.4 ± 3.4
glucose (mmol/l)	6.97 ± 2.05
TC (mmol/l)	4.69 ± 0.93
TG (mmol/l)	1.81 ± 0.80
LDL-C (mmol/l)	2.87 ± 0.82
HDL-C (mmol/l)	1.03 ± 0.17
TC/HDL-C	4.68 ± 1.18
fibrinogen (mmol/l)	4.21 ± 0.64
homocysteine (mmol/l)	11.73 ± 5.54
uric acid (mmol/l)	343.67 ± 60.57
C-reactive protein (wide range) (mmol/l)	10.16 ± 20.97

The surgery – direct endarterectomy or everse endarterectomy, is indicated as a preventive intervention in asymptomatic patients with a stenosis of *a. carotis interna* greater than 75–80 %. The operation will be mostly performed in regional anaesthetisation after a preceding application of heparin. The principle of the surgery is in enclosing by clamps the *a. carotis interna* and *a. carotis communis* respectively, and in their longitudinal incision– arteriotomy, under a permanent control of the patient's consciousness and the motility of their extremities. In case of signs of neurological inadequacy after enclosing *a. carotis* an internal shunt will be inserted. The removal of the sclerotic plaques (endarterectomy) follows. The arteriotomy will be closed with the aid of an autologous venous graft or with the help of an artificial patch (PTFE). The surgery and the examination of biological material will be performed with the explicit agreement of the patients.

C. pneumoniae was directly detected through qualitative polymerase chain reaction (nested PCR) in the atherosclerotic material obtained in the endarterectomy of *a. carotis*. Within 48 hours, genomic DNA was isolated from the atherosclerotic plaque, using an UltraClean Tissue DNA Kit (MoBio, USA). For the PCR *C. pneumoniae* detection we used a MONOTEST Chlamydia pneumoniae DNA amplification kit (Amplimedical, Bioline, Italy), which uses a modified method of nested PCR *C. pneumoniae* detection (*13*). The primers were selected to amplify the sequence of the gene encoding the outer membrane protein (OMP) of *C. pneumoniae*. The amplification reaction was conducted in a total volume of 50 µl consisting of mastermix, 5 µl of genomic DNA, and DNA polymerase. One microlitre of the PCR product from the first amplification was used for the second amplification. The PCR reaction took place in the Touchgene Gradient thermal cycler (Techne, UK). The PCR products were visualised on the 2 % agarose gel EliPhore (Elisabeth Pharmacon, Czech Republic) coloured with ethidium bromide.

The patients' sera were also analysed using enzymatic immunoanalysis (ELISA) tests for the detection of *Chlamydia pneumoniae* species-specific antibodies in the immunoglobulin classes IgA, IgG and IgM, and for the detection of chlamydial heat shock protein (cHSP60) antibodies in the class IgG. The ELISA tests were conducted using MEDAC (Germany) diagnostic sets.

RESULTS

Direct evidence from the PCR of the C. pneumoniae DNA product in the atherosclerotic material obtained during the operation of a. carotis was positive in 56.7 % of the examined patients. Species-specific antibodies of C pneumoniae in the IgA class were detected in 60 % of the patients, in 86.7 % in the IgG class, and in 3.3 % in the IgM class. Chlamydial heat shock protein antibodies were detected in 56.7 % of the patients (*Fig. 1*).

DISCUSSION

The direct evidence of C. pneumoniae in 56.7 % of the samples of atherosclerotic material obtained during the operation of patients with acute carotid stenosis and the evidence of species-specific C. pneumoniae antibodies, namely in the IgA class (60 %), provide a proof of an active C. pneumoniae infection and its probable participation in the atherosclerotic process. From the point of view of template DNA amplification, the conventional polymerase chain reaction (nested PCR) used has a smaller dynamic scale compared to quantitative real-time PCR, as it may not detect small amounts of bacterial DNA. Therefore a direct evidence of C. pneumoniae may not correspond with the detection of specific antibodies. Of the 17 patients with carotid atherosclerosis, DNA C. pneumoniae was demonstrated only in 3, whereas antibodies were detected in 70.6 % of them (*14*). In contrast, *Ciervo et al. (12)* detected DNA C. pneumoniae in a further 20 % of the nested PCR negative atherosclerotic plaques using the real-time PCR.

Comparing the serological findings, we may also note the 69.6 % (*8, 9*) of patients with unstable angina pectoris positively tested for specific IgA C. pneumoniae antibodies. This figure is statistically highly significant compared to findings in patients without clinically apparent vascular disease (36.6 %). The interpretation of the detection of species-specific C. pneumoniae antibodies in classes IgM and IgG in relation to atherosclerosis is more complicated, as the IgM antibodies are only present for a short term and therefore rarely detected, while the IgG antibodies have a long-term, even lifelong, persistence. The results of a study conducted by Greek cardiologists (*15*) show that high titres of IgA antibodies in patients with unstable coronary syndrome are closely related to the development of other clinical manifestations of advanced coronary stenosis (angina pectoris attacks, myocardial infarction and heart failure).

Special attention should also be paid to chlamydial heat shock protein antibodies (anti-cHSP 60) in the blood sera, whose frequency of 56.7 % was higher in patients with carotid stenosis than in healthy blood donors (49 male and 51 female) in a previous study (*16*), in which anti-cHSP 60 antibodies were detected only in 23 %. This detection is not a part of standard diagnostics, despite the cHSP 60 protein being an important factor in the pathogenesis and immunopathogenesis of chlamydial infection and atherosclerosis. This belief is also supported by the results of research

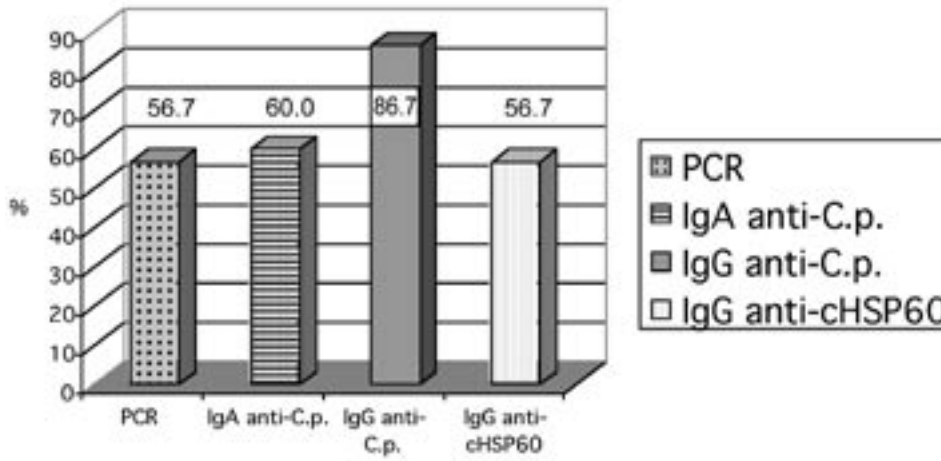

Fig. 1
Direct (PCR) and indirect (serological) evidence of *C. pneumoniae* in 30 patients with significant stenosis of *a. carotis* of atherosclerotic origin

showing the correlation between cHSP 60 in the blood sera and atherosclerosis (*17*). The soluble chlamydial heat shock protein evolves during an infection and an inflammatory process in the endothelium and activates the immune system in the course of the development of the atherosclerotic plaque, hence it is regarded as an independent danger factor detectable in the blood sera, in atherosclerotic plaques or in the arterial intima together with human HSP 60 (*16–20*). The detection of this protein's antibodies can be seen as an enrichment of the serological diagnostics of chlamydial infections.

As mentioned above, there are numerous studies on the participation of C. pneumoniae in diseases related to atherosclerosis and its complications, such as acute myocardial infarction, transient ischaemic attacks, unstable angina pectoris, valve disorders, and aortal aneurysm (*8–11*). One of the leading studies in this field, the Bruneck study, focused on the pathogenesis of atherosclerosis in a group of 828 men and women (*21*). The results of the study can be taken as evidence of chronic infection playing an apparent role in atherosclerosis. The induction of system inflammation and autoimmunity can serve as a pathophysiological element. A. carotis stenosis is one of the serious clinical atherosclerotic disorders, in which infection, namely a C. pneumoniae infection, is an important cofactor (*22*).

Nevertheless, not all authors reached the same conclusions. One study (*23*) failed to demonstrate its hypothesis that the simple presence of C. pneumoniae antibodies can be associated with the infection of coronary artery walls in patients

suffering from heart ischaemia, despite the evidence that these patients had higher antibody titres than people with negative results for heart ischaemia.

Atherosclerosis is, however, a multifactorial disease, and there are several danger factors involved in its pathogenesis. Some of them may relate to chronic C. pneumoniae infection. In the course of their lives most people are, some of them repeatedly, exposed to C. pneumoniae infections. The key factors seem to be the size of the infectious dose, the contagion path, the genetic disposition of the affected individual, and the pathogen of the infection progression. C. pneumoniae is one of the most common microbial agents connected with infectious origin of atherosclerosis, which has also been shown experimentally in animals (*24, 25*), and yet we cannot exclude other possible agents causing local inflammation, such as herpes viruses, cytomegalovirus and Helicobacter pylori (*26, 27),* despite disapproval from some experts (*28*).

Other danger factors must also be taken into consideration, for instance lipid metabolism disorders (variations in the concentrations of lipoprotein cholesterol fractions, leptin, etc.), genetic predispositions, diabetes, hypertonia, age, sex and smoking habits which, directly or indirectly, with varying influence contribute to the origin of significant stenosis as the results of atherosclerotic changes (*10, 29*).

The results of the direct evidence of Chlamydia pneumoniae in atherosclerotic plaques using the PCR method and the detection of specific IgA chlamydial antibodies in the sera bring us to the conclusion that Chlamydia pneumoniae can be a significant participant in the origin and progression of atherosclerosis as well as in the strategy of consequent treatment and remission prevention following a carotid stenosis surgery. In this respect, the detection of cHSP 60 antibodies or a direct evidence of cHSP 60 in the atherosclerotic plaque or in the artery wall can also be of diagnostic and prognostic value.

A c k n o w l e d g e m e n t

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Zeman K., Pospíšil L., Čanderle J., Štroblová H., Leypold J., Gregor Z., Medková Z., Šerý O.

PŘÍMÝ A NEPŘÍMÝ PRŮKAZ *CHLAMYDIA PNEUMONIAE* U PACIENTŮ S VÝZNAMNOU STENÓZOU *A. CAROTIS* ATEROSKLEROTICKÉHO PŮVODU

S o u h r n

Cílem této studie bylo posoudit přímými a nepřímými metodami účast *C. pneumoniae* v patogenezi významné aterosklerotické stenózy karotid u třiceti nemocných (25 mužů a 5 žen, průměrný věk 67,5 roku). Přímý průkaz PCR produktu DNA *C. pneumoniae* v aterosklerotickém materiálu získaném při operaci *a. carotis* byl pozitivní u 56,7 % vyšetřených pacientů. Krevní séra nemocných byla vyšetřena enzymatickými imunoanalýzami (ELISA) pro detekci protilátek proti druhově specifickému proteinu vnější membrány *C. pneumoniae* v globulinových třídách IgA, IgG a IgM a proti proteinu tepelného šoku chlamydií (cHSP60) v globulinové třídě IgG. Protilátky proti druhově specifickým antigenům

C. pneumoniae v globulinové třídě séra IgA byly prokázány u 60 %, IgG u 86,7 % a IgM u 3,3 % pacientů. Protilátky proti proteinu tepelného šoku chlamydií byly prokázány u 56,7 %. Na základě výsledků předkládané práce považujeme především přímý průkaz *C. pneumoniae* v aterosklerotických plátech metodou PCR a specifických protilátek proti *C. pneumoniae* v IgA globulinové třídě séra za významné ve smyslu její možné účasti na vzniku a progresi aterosklerózy a pro doplnění následné léčby a prevence remise po operaci stenózy karotid.

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