

A relationship between serological markers of chronic *C. pneumoniae* and CMV infection and hsp60 in patients with atherosclerotic carotid stenosis

Maciej Rabczyński¹, Żanna Fiodorenko-Dumas^{2✉}, Krzysztof Mastej¹, Ilias Dumas², Rajmund Adamiec¹ and Małgorzata Paprocka-Borowicz²

¹Department of Angiology, Hypertension and Diabetology, Wrocław Medical University, Wrocław, Poland; ²Department of Physiotherapy, Faculty of Health Science, Wrocław Medical University, Wrocław, Poland

A number of epidemiological studies conducted over the last decade indicate a relationship between specific pathogen infections and the development of atherosclerosis, although no pathogenetic pathways connecting these two have been determined. Recent reports support the role of heat shock proteins (HSPs) in atherogenesis. The HSPs are also believed to be a link between the infection and the development of atherosclerotic lesions. **The aims of study:** Immunohistochemical evaluation of carotid artery segments to show the relationship between the presence of heat shock proteins and the serum levels of anti-hsp60 antibodies. An attempt to demonstrate a relationship between an expression of chronic *C. pneumoniae* and CMV antigens. **Material and Methods:** The study included 41 patients qualified for carotid artery endarterectomy and 18 healthy volunteers of corresponding age. Levels of anti-hsp60, anti-*C. pneumoniae* IgA and IgG, anti-CMV IgG antibodies as well as hsCRP were determined. **Results:** The mean serum levels of anti-hsp60 antibodies were higher in patients with advanced atherosclerosis as compared to healthy volunteers (55.3±64.1 vs 32.8±29.8; $p < 0.05$). There was a strong correlation between anti-hsp60 antibodies and the expression of hsp60 in carotid arterial wall, as confirmed by immunohistochemical evaluation. The study group showed statistically significant higher levels of hsCRP. Furthermore, statistically significant higher serum levels of anti-*C. pneumoniae* IgG and IgA as well as anti-CMV IgG antibodies were found in the study group as compared to controls. No correlation was shown between the markers of chronic infection induced by the tested pathogens and serum levels of anti-HSP and hsCRP. **Conclusions:** Higher protein expression in vascular walls is closely correlated with the level of anti-hsp60. At the same time, no significant relationship between anti-hsp60 antibodies and serological markers of infection was observed, which may only indicate an indirect role of infection in the assessment of breaking the immunological tolerance against autologous HSPs.

Key words: anti-hsp60, atherogenesis, hsCRP, *C. pneumoniae*, CMV, carotid artery

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INTRODUCTION

The recent world literature indicates a positive correlation between the presence of serological markers of infections caused by some pathogens and the aggravation of atherosclerotic lesions in individual vascular areas. Most data relate to the relationship between *Chlamydia pneumoniae* infection and the risk of cardiovascular complications, including vascular brain diseases. *C. pneumoniae* was isolated from aortic, coronary, carotid and lower limb atherosclerotic lesions (Mussa *et al.*, 2006). Literature data indicates that an infection caused by these bacteria is a vascular disease risk factor comparable to smoking, LDL cholesterol or arterial hypertension (Grayston, 2000). Statistics for such infections relate to as many as 80% of men and 70% of women over 65 years old, whereas the incidence in younger age groups is lower and concerns about 50% of population aged 20 years (Grayston, 2000). A number of epidemiological, serological and histopathological studies indicate the involvement of *C. pneumoniae* in the carotid atherosclerotic process (Kazmierski & Kozubski, 2002). Using the PCR method, Polish researchers have managed to show the presence of bacterial DNA in atherosclerotic plaques obtained from 67% of patients qualified for carotid endarterectomy due to carotid artery stenosis. In contrast, no evidence of pathogen's genetic material was found in carotid artery samples from the control group, i.e. organ donors showing the signs of brain death (Janczak, 2004). The presence of *C. pneumoniae* in carotid atherosclerotic lesions was further confirmed in 64% of patients who underwent the surgery, based on immunohistochemical method or *in situ* hybridization, as reported by Mosorin and coworkers (Mosorin *et al.*, 2000). Serological examination has shown the presence of *C. pneumoniae* and cytomegalovirus antigens in immune complexes circulating in the serum of patients with recent stroke (Tarnacka *et al.*, 2002). The latest data indicate their involvement in the process of endothelial cell damage due to an autoimmune reaction against heat shock proteins 60 (Hsp60), which are expressed on the endothelial cells (Mandal *et al.*, 2004).

Heat shock proteins (Hsp) belong to a homogenous group of proteins, characterized by a high amino acid structure homology between species. Their function is

✉ e-mail: z.fiodorenko@poczta.onet.pl

Abbreviations: Hsp, Heat shock proteins

to protect other intercellular proteins against proteolysis caused by negative external environmental factors, such as increased temperature, oxygen deficiency or infection. Wick *et al.* (Wick *et al.*, 1995) have already discussed the possibility of cross-reaction between exogenous material (mycobacterial mHsp65 proteins or *Escherichia coli* GroEL and GroES) and the corresponding human hsp60 exposed on the endothelium. There are literature reports showing that the cHsp60 molecule (*Chlamydia pneumoniae* HSP) shows some structural similarity to human hsp60 (Mayr *et al.*, 1999) and that antibodies against cytomegalic virus are able to recognize some human hsp epitopes (Bason *et al.*, 2003). Thus, one of the oldest systems for cell protection against unfavourable external environmental factors could initiate the (auto)immune process, which consequently leads to endothelial cell damage and the initiation of atherosclerotic plaque formation.

An experiment conducted in an animal model and described in 1992 by Xu *et al.* provided the first evidence supporting the involvement of HSPs in atherogenesis (Xu *et al.*, 1992).

Another evidence was based on an epidemiological study involving a group of 867 inhabitants of South Tyrol (Xu *et al.*, 1993). The study analyzed the correlation between anti-hsp65 titres and the presence of atherosclerotic lesions in carotid arteries assessed by Duplex-Doppler scanning.

Significantly higher titres of antibodies were observed in patients with atherosclerotic lesions identified by ultrasonographic examination. Another study, which was conducted within 48 hours after stroke onset, has shown that the stroke is associated with statistically higher titres of serum anti-hsp65 antibodies. The study confirmed that the presence of high serum titres of these antibodies is a risk factor independent of stroke occurrence (Gromadzka *et al.*, 2001).

Aims of the study

Determination of the role of heat shock proteins in the development of atherosclerosis based on serum levels of anti-hsp60 antibodies.

Immunohistochemical evaluation of carotid artery samples to show the relationship between the presence of hsp60 in vascular wall cells and the serum levels of anti-hsp60 antibodies.

An attempt to demonstrate the connection between increased levels of anti-hsp60 antibodies and markers of chronic *C. pneumoniae* and CMV infection expressed as levels of certain immunoglobulins.

METHODS AND MATERIALS

Consent of Wrocław Medical University Research Ethics Committee was obtained for the purpose of this study. All persons involved in the research have been informed of its aim and have given their written consent to participate in the study. The research included 41 individuals (12 women and 29 men) aged between 46 and 64 years (mean age 55.83 ± 3.9) qualified for carotid endarterectomy in the Department of Angiology, Hypertension and Diabetology, Wrocław Medical University. The group included patients with symptomatic internal carotid artery stenosis of $>70\%$ in colour-coded Doppler imaging. Tissue material obtained from the internal carotid artery, fixed in 10% Formalin Buffered Solution and embedded in paraffin blocks was used for immunohistochemical analysis.

Samples were obtained from 20 patients (7 women and 13 men) aged 47–64 years (mean age 55.35 ± 3.49 years) during the procedure of internal carotid artery endarterectomy.

Control group ($n=18$) included healthy volunteers (9 women and 9 men), aged 40–59 years (mean age 50.38 ± 5.29). A detailed medical history was collected for all patients, none of whom was diagnosed with diabetes, hypertension or symptoms of coronary, peripheral or carotid atherosclerosis. The patients received medical examination and additional tests, such as carotid artery Doppler Sonography. Venous blood was sampled from all subjects in order to assess blood cell count, inflammatory parameters (WBC, ESR, fibrinogen) and lipid profile. HbA1c was additionally measured in diabetic patients.

The above mentioned procedures were performed in a laboratory setting, using standard methods. Additionally, blood was sampled to evaluate the level of anti-hsp60, anti-*C. pneumoniae* IgA and IgG and anti-CMV IgG antibodies as well as hs-CRP levels. Assays were performed using the following kits: Anti-Human Hsp60 (total) ELISA kit (Cat. No. EKS-650) from Stressgen, Anti-*Chlamydia pneumoniae* ELISA (IgG) (Cat. No. EI-2192-9601G) from Euroimmun and Anti-*Chlamydia pneumoniae* ELISA (IgA) (Cat. No. EI-2192-9601A), High Sensitivity C-Reactive Protein Enzyme Immunoassay (Cat. No. EIA-3954) from DRG International Inc. and AxSYM CMV IgG reagent set (Cat. No. B4B47P) from Abbott.

Immunohistochemical tests were performed in order to detect hsp60 in carotid artery preparations. Tissue material obtained during carotid endarterectomy, fixed in 10% Formalin Buffered Solution and embedded in paraffin blocks was used in the study. Next, the paraffin blocks were used to prepare 4 μm sections using a microtome. In a similar manner, immunohistochemical preparations were prepared for the control group with breast cancer tissue. Three-stage immunohistochemical ABC method based on avidin-biotin-peroxidase complex was used in order to detect the analyzed protein. Murine monoclonal anti-hsp60 antibody was the primary antibody used in the research (anti-h/m/r HSP60, Purified Mouse Monoclonal IgG, clone 264233, cat. No. MAB 1800, by R&D System). Protein expression was evaluated using a semi-quantitative method. A 200-times magnified microscope image of one preparation was transferred to AnalySIS docu and the presence of selected protein was then analyzed in 5 randomly chosen fields of vision within the analyzed lesions areas. The semiquantitative method for the evaluation of hsp60 expression in the epithelial cells used authors' plus-scale including the following ranges of protein occurrence:

- 0 — no expression
- + — weak expression, observed in few cells
- ++ — moderate expression, observed in quite many cells, but in less than half of cells
- +++ — strong expression, observed in more than half of all cells
- ++++ — very strong expression, observed in all cells

The collected data was analysed statistically in order to obtain descriptive statistics such as: the arithmetic mean, standard deviation, median, correlation coefficient, regression line equation. Statistical significance level of $p < 0.05$ was accepted for all tests. The U Mann-Whitney test was used due to a nonparametric distribution of the analyzed characteristics in the groups. Spearman rank correlation test was used to verify the correlation between the characteristics. Correlation coefficient (r) was

Table 1. Levels of inflammatory parameters together with the lipid profile.

	WBC (10 ³ /mm ³)	ESR (mm/h)	Fibrinogen (g/l)	Total cholesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)
Study group n = 41	7.58±1.8	18.7±14.4	3.98±1.14	195.5±48.9	107.1±41.3	51.0±15.6	187.2±105.1
Control group n = 19	5.9±1.2	8.2±5.4	3.0±0.76	186.7±27.2	102.1±24.7	65.6±15.2	95.0±9.3
Significance of differences	P<0.05	P<0.05	P<0.05	ns	ns	P<0.05	P<0.05

Results are expressed as mean values ± standard deviation; ns — statistically insignificant.

Table 2. Serum levels of antibodies.

	anti-HSP 60/65 (ng/mL)	anti- <i>C. pneumoniae</i> IgA (index)	anti- <i>C. pneumoniae</i> IgG (RU/mL)	anti-CMV IgG (AU/mL)	hsCRP (ng/mL)
Study group n = 41	55.3±64.1	0.78±0.42	56.3±39.9	337.3±275.4	5.1±4.4
Control group n = 19	32.8±29.8	0.34±0.25	27.0±27.4	133.8±143.4	2.5±3.3
Significance of differences	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

Results are expressed as mean values ± standard deviation; ns — statistically insignificant.

used to express the strength of the relationship. A positive correlation is indicated by a positive value, while a negative value indicates a negative correlation. STATISTICA 7.1 was used for all statistical calculations.

RESULTS

Tables 1 and 2 as well as the accompanying Figures show the results for the individual groups. The study group showed higher levels of inflammatory parameters (hs-CRP, WBC, ESR, fibrinogen) as compared to the control group. There were significant differences between patient populations in terms of lipid profiles, with significantly lower HDL cholesterol levels and higher triglyceride levels in the study group. The lack of significant difference in LDL levels is associated with a common use of statins in patients with atherosclerotic carotid stenosis. Results are shown in Table 1. Analyzing mean anti-hsp60 serum levels in patients with advanced atherosclerosis, it can be observed that they were higher than in healthy volunteers, and the difference is statistically significant (55.3 ± 64.1 vs 32.8 ± 29.8 ; $p < 0.05$) (Rabczynski *et al.*, 2012). The results are shown in Table 2.

Table 3. Serum levels of anti-CMV IgG antibodies.

	Beta	Beta error	t(38)	p
<i>C. pneumoniae</i> IgA	-0.091	0.157	-0.582	0.563771
CMV IgG	0.246	0.157	1.569	0.124951
No significant correlation. R=0.26199638 R2=0.06864210; F(2,38)=1.4003 p<0.25895. Estimate standard error: 65.174				

Table 4. Level of HSP 60/65 expression in immunohistochemical preparations

Expression	none (0)	weak (+)	moderate (++)	strong (+++)	very strong (++++)
n (20)	1	6	6	5	2

Also, statistically significant higher levels of anti-*C. pneumoniae* IgA and IgG as well as anti-CMV IgG antibodies were observed in patients with atherosclerotic carotid stenosis, as compared to the control group. The differences were statistically significant. The study showed no significant association between the analyzed parameters, particularly between anti-hsp60 levels and the serological markers of *C. pneumoniae* or CMV infection. No significant correlation was found between anti-hsp60 antibodies and the markers of simultaneous infection with both pathogens. Results for the correlations of the examined immunoglobulins are presented below (Table 3).

Material fixed in 10% Buffered Formalin Solution and embedded in paraffin blocks was used for immunohistochemical research. Samples were obtained from 20 patients (7 women and 13 men) aged 47–64 years (mean age 55.35 ± 3.49). The group included 8 patients with type-2 diabetes, the other 12 did not suffer from this endocrinopathy.

The expression of hsp60 was evaluated using a semi-quantitative method and a plus-scale created by the authors of the article. Hsp60 was identified in 19 preparations (95% of all cases). The analyzed protein was not expressed in only one specimen. In 6 cases, protein expression was considered weak, i.e. observed in a few cells (+); in the other 6 cases – moderate, i.e. observed in quite many cells, but less than half (++) . Five preparations showed strong expression, i.e. observed in more than half of all cells (+++), and 4 cases showed a very strong expression, i.e. observed in every cell. The results are shown in Table 4. Immunohistochemical examination supported the expression of hsp60 in *vasa vasorum* endothelial cells (Fig. 1, 1a, 1b) and in carotid artery endothelial cells (Fig. 2). Within the *tunica media*, the presence of the protein has been detected mainly in macrophages (Fig. 3, 3a). In patients, whose carotid artery samples were taken for histopathological evaluation, a correlation between anti-hsp60 serum levels and the immunohistochemically assessed hsp60 expression was observed. A strong positive correlation was found (R=0.660; $p < 0.005$).

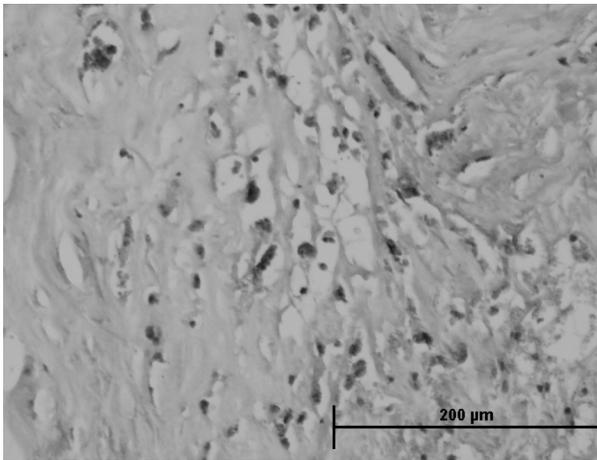


Figure 1. Considerable HSP 60 expression in macrophages contiguous with atherosclerotic lesions (+++). In adipose tissue, HSP expression in endothelium cells *vasa vasorum*

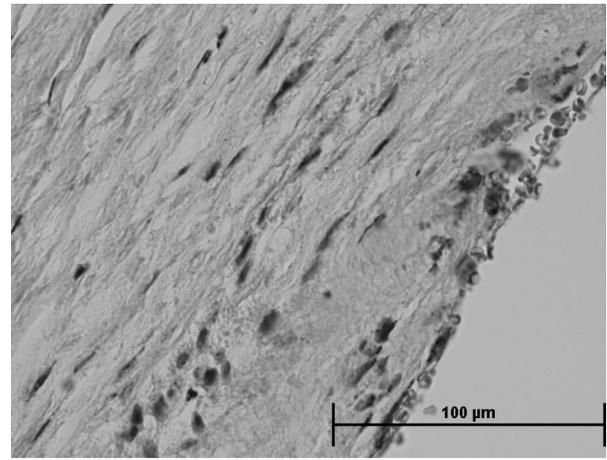


Figure 2. Expression in endothelium cells and in *tunica media*, clear HSP protein expression (++)

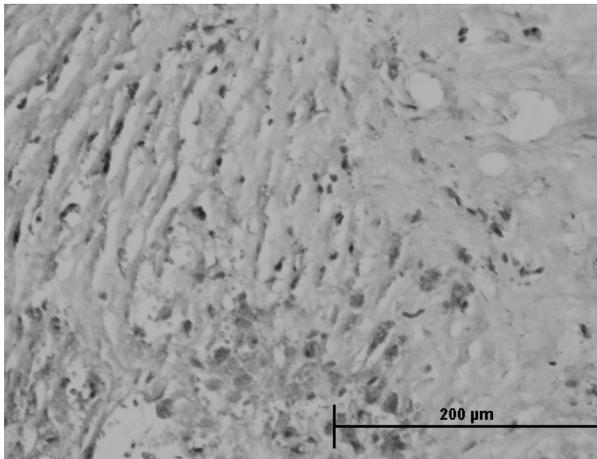


Figure 1a. Considerable HSP 60 expression in macrophages contiguous with atherosclerotic lesions (+++). In adipose tissue, HSP expression in endothelium cells *vasa vasorum*

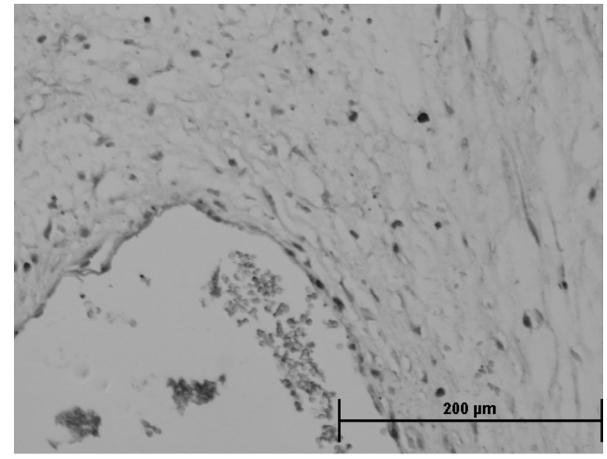


Figure 3. HSP expression in carotid artery endothelium cells and *tunica media* macrophages (+++)

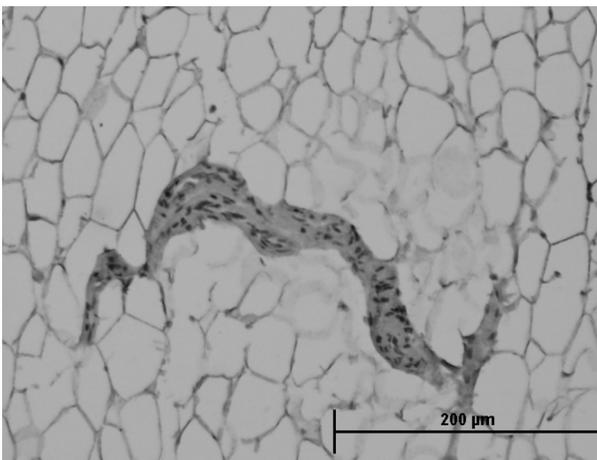


Figure 1b. Considerable HSP 60 expression in macrophages contiguous with atherosclerotic lesions (+++). In adipose tissue, HSP expression in endothelium cells *vasa vasorum*

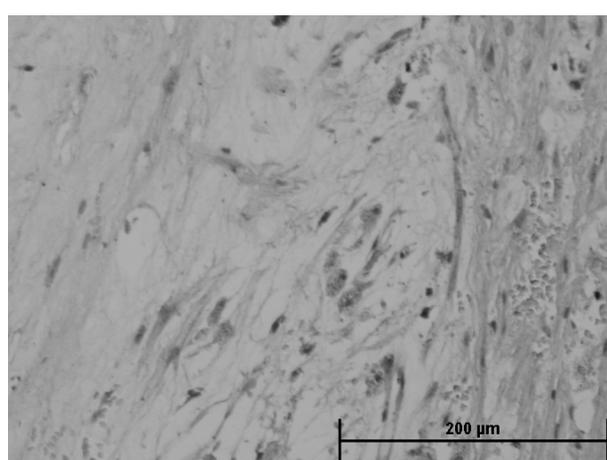


Figure 3a. HSP expression in carotid artery endothelium cells and *tunica media* macrophages (+++)

DISCUSSION

The involvement of hsp60 in carotid artery atherogenesis was confirmed in the study. Investigations on the potential pro-atherosclerotic properties of heat shock proteins have been continued since it was experimentally shown that intravascular administration of mycobacterial hsp65 antigen (mHSP65) induces the development of arterial atherosclerotic lesions in an animal model (Mandal *et al.*, 2004). Maron *et al.* (Maron *et al.*, 2002) experimentally confirmed the role of hsp60 in atherogenesis and suggested the possibility to control the atherosclerotic progression by induction of the immune tolerance against these proteins. One of the considered mechanisms involves the stimulation of the immune response by proteins, with simultaneous production of anti-hsp60 antibodies. The autologous heat shock protein might become an immunogenic antigen as a result of structural changes and potential modifications induced by oxidative stress or metabolic disorders (Mandal *et al.*, 2005) by forming complexes with other antigens.

Some authors claim that intercellular protein (soluble HSP, sHSP), when located in extracellular space, might not be recognized by the immune system as belonging to this cell (Xu *et al.*, 2000). It is also suggested that there might be some genetic factors contributing to the increased expression of anti-hsp60 antibodies as evidenced by a correlation between the levels of these antibodies and IL-6 gene polymorphism (Veres *et al.*, 2002). Multi-directional interactions between the heat shock proteins and the immune system, which may induce pathological processes, are summarized by Macario *et al.* (Macario *et al.*, 2010).

According to the leading hypothesis, stimulation of the immune system with the subsequent production of anti-hsp60 antibodies occurs as a result of chronic infection with pathogens, among which *Chlamydia pneumoniae* and CMV are given the most attention (Wick *et al.*, 2004). Structural conservatism and high amino acid sequence homology between heat shock proteins in different species, which allow for a cross-reaction between pathogenic and human proteins, are of central importance in this process. It was experimentally shown that T cells sampled from atherosclerotic plaques are able to recognize autologous hHsp60 and cHSP60 (*Chlamydia pneumoniae*) (Benagiano *et al.*, 2005). This phenomenon, referred to as an 'antigenic mimicry', became a link between the infection and the immune-mediated process of arterial atherosclerosis.

Furthermore, latest reports indicate the presence of T lymphocytes, which are able to recognise human hsp60 epitopes and thus to initiate the atherosclerotic process, already in the initial atherosclerotic lesions in patients (Almanzar *et al.*, 2012). Serological markers of *C. pneumoniae* and CMV infection were detected in the study population, however no correlation between these immunoglobulins and anti-hsp60 levels was found. According to the current view, the atherosclerotic process is more likely to be associated with a simultaneous infection with several pathogens (pathogen burden) rather than with a single pathogen. Therefore, attempts have been made to determine the connection between the analyzed anti-hsp60 antibodies and the markers of a simultaneous infection with two pathogens, however again with no statistically significant results. Also, it has not been confirmed whether a reaction occurs between chronic *C. pneumoniae* or CMV infection and the induction of immune response connected with the expression of anti-hsp60 antibodies. The obtained results are in line

with the findings by Haider *et al.* (Haider *et al.*, 2002), who did not provide a support for the connection between serological markers of chronic *H. pylori*, *C. pneumoniae* and CMV infection and an increased cardiovascular risk in their prospective study. Sessa *et al.* (Sessa *et al.*, 2006) did not show a significant relationship between the serum levels of anti-*C. pneumoniae* antibodies and the presence of these bacteria within the atherosclerotic plaque (determined by the PCR method) in patients with asymptomatic carotid atherosclerosis. In patients with atherosclerotic lower limb ischaemia, the presence of pathogens in the atherosclerotic lesions was confirmed in 4 out of 9 cases (Cuffini *et al.*, 2006) and in 28.6% of cases in another study, however this analysis showed a connection between the presence of bacteria and serological markers of infection in the serum (Kaklikkaya *et al.*, 2006). More frequent occurrence of immunoglobulins against *Chlamydia pneumoniae* was not supported by Drożdż *et al.* (Drożdż *et al.*, 2003) study, which involved patients with critical lower limb ischaemia. No correlation was found between the presence of antibodies in the serum and the occurrence of pathogens in arterial walls, which might support the 'antigenic mimicry' hypothesis, i.e. an indirect role of infection as a factor that 'breaks' the tolerance against endogenous heat shock proteins. The above mentioned findings may partially result from difficulties imposed by attempts to explicitly determine serological markers of chronic *C. pneumoniae* infection. Some authors are of the opinion that the diagnosis of chronic infection based solely on IgG results is insufficient. They believe that the chronic nature of infection may be indicated by low titres of antibodies in patients unable to completely eliminate the pathogen as a result of immune system impairment due to dietary deficiency or substance abuse (Sessa *et al.*, 2006). A number of controversies is also raised in relation to methods used in the research: it is emphasised that the results of serological, immunohistochemical or PCR studies may vary depending on the assay method used and may not always be specific for *C. pneumoniae* (Apfalter, 2006). Controversies related to methods used in the diagnosis of chronic infection with *Chlamydia pneumoniae* have been collected by Ieven MM (Ieven & Hoymans, 2005). Although the role of contagious pathogens in atherogenesis was already suggested in 1850 (Lamb *et al.*, 2003) and have gained in importance during the last decade, following the implementation of new research methods, the therapeutic ineffectiveness of antibiotics in the prevention of atherosclerosis and its complications remains a strong argument against infectious theory of atherosclerosis. Neither the first promising experiments in animal models (Fong, 2000) nor the attempts to use macrolides in secondary prevention of peripheral ischaemia (Wiesli *et al.*, 2002) and heart attack (Gurfinkel *et al.*, 1999) were supported in ACADEMIC (Muhlestein *et al.*, 2000), WIZARD (O'Connor *et al.*, 2003) or ACES (Grayston *et al.*, 2005) randomized clinical trials. Antibiotic therapy does not provide any benefits in the form of decreased mortality or a reduction in other cardiovascular events in patients with atherosclerosis. Higher incidence of antibodies against the analyzed pathogens in patients with carotid atherosclerotic stenosis compared to healthy individuals implies that chronic infection may be a factor that "breaks" the immune tolerance against the autologous HSPs 60. Immunohistochemical evaluation of samples obtained during endarterectomy of the internal carotid arteries revealed the presence of hsp60 in the atherosclerotic wall. Expression was observed after reaction with anti-hHsp60 murine monoclonal antibody, mainly

in macrophages of the *tunica media*, *vasa vasorum* epithelial cells and in the vascular endothelium. In only one case hsp was not detected in the arterial wall.

Lack of hsp expression in the internal carotid artery endothelium in some of the preparations might have resulted from the surgical technique, which removed the endothelium surface from the arterial walls. The data is in line with the findings by Kol *et al.* (Kol *et al.*, 1998), which support the presence of hHsp60 in 89% of the evaluated atherosclerotic plaques. Furthermore, the authors observed that in 47% of cases human hsp60 occurs simultaneously with its bacterial equivalent cHsp60 (*Chlamydia pneumoniae* hsp60). The presence of hsp60 on the surface of HUVEC cells was also observed by Pfister *et al.* (Pfister *et al.*, 2005). However only this study has shown a strong, positive correlation between the presence of hsp60 on the surface of vascular endothelial cells (EC) and the serum levels of anti-hsp60 antibodies. Therefore, the levels of anti-hsp60 antibodies reflect the expression of this protein in the vascular wall. CRP is a widely recognized predictor of cardiovascular diseases, coronary disease and acute coronary syndromes (ACS) in particular. According to some authors, CRP is associated with endothelial dysfunction, which is manifested by impaired vasorelaxation (Fichtlscherer *et al.*, 2000). Although an increase in hsCRP levels was observed in the study group, no significant correlation between CRP level and other evaluated parameters was found.

CONCLUSIONS

Atherosclerosis proceeds with ongoing statistically significant increase of the concentration of antibodies anti-*C. pneumoniae* IgA and IgG, as well as antibodies anti-CMV IgG.

Increase in the concentration of antibodies anti-HSP 60 in blood serum, stands for their participation in pathogenic trail of atherogenesis. At the same time, no significant connection was found between anti-HP 60 antibodies and serological factors of infection, which may serve as a proof of its indirect role in the process of "breaking" of the tolerance towards autologous heat shock proteins.

High expression of HSP 60 protein on the surface of the arterial wall undergoing atherosclerotic changes, remains closely correlated with the increase in the concentration of antibodies in blood serum, independently of co-occurring diabetes.

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