

## ORIGINAL PAPER

**HSP60, SP110 AND TNF- $\alpha$  EXPRESSION IN *CHLAMYDIA PNEUMONIAE*-POSITIVE VERSUS *CHLAMYDIA PNEUMONIAE*-NEGATIVE ATHEROSCLEROTIC PLAQUES**NEMANJA RADOJEVIC<sup>1,2</sup>, BATRIC VUKCEVIC<sup>1,2</sup>, ANDREA JOVOVIC<sup>2</sup>, FILIP VUKMIROVIC<sup>1,2</sup><sup>1</sup>Clinical Centre of Montenegro, Montenegro<sup>2</sup>Faculty of Medicine, University of Montenegro, Montenegro

---

Traditionally recognized risk factors for atherosclerosis are not presented in 50% of patients with ischemic heart disease. Chronic inflammation with low pathogenic agents with slightly, or no signs of inflammation is the mainstay of atherosclerosis and could be triggered by an infectious agent, most commonly by *Chlamydia pneumoniae*.

Immunostaining of 33 *Chlamydia pneumoniae*-positive and 30 *Chlamydia pneumoniae*-negative quadruple arterial sets were examined for protective Sp110, and atherogenic HSP60 markers, as well as for TNF- $\alpha$  which is inflammatory marker affected by both of them.

The *Chlamydia pneumoniae*-negative deceased subjects were statistically significantly older and their BMI was significantly lower. The results showed that age, hypercholesterolemia, diabetes, arterial hypertension and BMI were negatively correlated with *Chlamydia pneumoniae*-positivity, while no significant relationship was found between *Chlamydia pneumoniae*-positivity and a positive family history of cardiovascular diseases, as well as smoking. Significantly higher presence of Sp110 in *Chlamydia pneumoniae*-negative group versus significantly higher presence of HSP60 in *Chlamydia pneumoniae*-positive group. *Chlamydia pneumoniae*-negative plaques showed higher TNF- $\alpha$  expression; difference is present for all arteries examined except the Willis circle.

This study may provide a model for further understanding the mechanisms of *Chlamydia pneumoniae* atherogenesis and evaluating chlamydial intervention strategies for preventing the advancement of atherosclerotic lesions enhanced by bacterial infections.

**Key words:** atherosclerosis, *Chlamydia pneumoniae*, cardiovascular disease, SP110, HSP60, TNF- $\alpha$ .

---

**Introduction**

Inflammatory cells and the appearance of mediators are proof of the chronic inflammatory character of atherosclerosis. Atherosclerosis is actually a response to a noxa that damages the endothelium. The so-called "injurious agent" is any chemical sub-

stance or microorganism that causes an endothelial lesion and consequently an inflammatory reaction.

Monocyte and T lymphocyte chemotaxis with cytokine secretion initiates an inflammatory cascade and remodelling of the diseased part of a blood vessel's wall. Regardless of the origin of the agent, the host response is not sufficient because atherosclerosis pro-

gressively spreads and the lumen of the blood vessel narrows [1].

When it comes to the infectious genesis of atherosclerosis, a large amount of attention has been dedicated to bacterial and viral agents. The determined presence of microorganisms in atherosclerotic plaque and lipid-laden cells has resulted in the hypothesis that microorganisms stimulate scavenger receptor expression to use lipids as a source of energy [2].

*Chlamydia pneumoniae* (CP) is an obligatory intracellular, gram-negative bacterium from the Chlamydiae family, recognized as a cause of sinusitis, laryngitis, pharyngitis, bronchitis and out-of-hospital pneumonia. CP is often present in the blood vessels of smokers and it is believed that they also have a higher rate of lung colonization with this microorganism [3]. The presence of CP has been proved in 40% to 50% of atherosclerotic plaques by immunohistochemical tests, PCR tests, electron microscopy, as well as with cell cultures [4]. Laboratory tests on animals have shown that CP causes atherosclerosis in LDL receptor knockout mice that have been fed on a diet with a high concentration of cholesterol. The presence of antibodies against CP was found in about 50% of the examined patients with atherosclerosis [4]. It is thought that CP passes from macrophages to endothelial cells, causing them to dysfunction and increasing permeability for LDL. At the same time, the lipopolysaccharide of the bacterial wall causes a reduction in LCAT activity, the consequence of which is an increase in levels of LDL and a decrease in levels of HDL.

The presence of CP in endothelial cells causes the proliferation of smooth muscle cells and the secretion of IL-8 and IL-16, and the expression of ICAM, VCAM and E selectin [5].

The occurrence of atherosclerosis in a younger population without traditional factors of risk has awakened the suspicion that there is an infectious agent that serves as a trigger in the atherosclerotic process. Examination for SP110 and HSP60 can be of some importance in the molecular examination of atherosclerotic plaques.

The bacterium performs dysregulation of LDL receptors and this additionally burdens the cells with lipids and favours the appearance of foam cells [6]. It is possible that the bacteria do not enter the intima of a blood vessel's wall like a "Trojan horse" inside the macrophage, but that inflammation has been stimulated by the autoimmune nature of the disease caused by molecular mimicry of HSP60 (virulence factor of CP) with proteins of the intima of the blood vessel. Heat shock proteins (HSP), normally localized intracellularly, become expressed on the cell surface in stressful conditions, such as an infection, and consequently activate antigen-presenting cells and start a cascade of the immune response [7]. The molecular

mimicry hypothesis has gained in importance given the fact that human HSP60 (hHSP60) shows an 85% compatibility with chlamydial HSP60 (cHSP60) [11]. In addition to a number of the other effects of HSP60, Koi *et al.* have demonstrated cHSP60 and hHSP60 in plaques on carotid atherosclerotic plaque samples, as well as their joint action in promoting TNF- $\alpha$  secretion.

Unlike HSP60, SP110 has a protective role in atherosclerosis. SP110, a human analogue of the Ipr1 gene, may be a constitutional defence factor against CP, and therefore against infectious atherosclerosis [12]. Namely, it is believed that predisposition to atherosclerosis is inherited as a complex of genetic characteristics of the organism by polygenic inheritance. The sst1 gene locus was found on chromosome 1 of a mouse, which contains genes whose expression leads to increased susceptibility to tuberculosis and infection with other intracellular pathogenic bacteria. Within the sst1 locus is a group of Ifi-75 genes, whose most important representative is the Ipr1 gene. Ipr1 is over-expressed in mice resistant to infection, and its expression does not occur in the population of mice susceptible to infection [13]. The closest human homologue of this gene is the SP110 gene, which is located on chromosome 2, and is thought to encode transcription cofactors as part of the action of hormone receptors in the cell nucleus. In this way, Ipr1 and SP110 would affect the action of numerous hormones, including corticosteroids, vitamin D and retinoic acid, with consequences for the life cycle of immune cells and the inflammatory response of the host [14]. The SP110 protein, which encodes the gene of the same name, has been observed in nuclear bodies (multiprotein complexes that regulate gene transcription and are localized in the nucleus) in acute viral infections and numerous autoimmune and malignant diseases [15]. Leu *et al.* demonstrated the protective role of SP110/Ipr1 in intracellular infections in a mouse model of *M. tuberculosis* infection by modulating NF- $\kappa$ B activity in the form of promotion of anti-apoptotic mechanisms and down-regulation of TNF- $\alpha$  secretion. Thus, in contrast to HSP60, SP110 has a protective role in the processes of atherosclerosis and, in addition, they have the opposite effect on the expression of TNF- $\alpha$ , namely HSP60, by increasing the concentration of it, and vice versa with SP110.

The aim of the study is to hypothesize the correlation of CP-positive and CP-negative atherosclerotic plaques with the presence of HSP60 and SP110 expression in plaques, as well as TNF- $\alpha$  as a connected inflammatory marker.

## Material and methods

The research was performed on fragments of blood vessel tissue obtained by forensic autopsies

at the Department of Pathology and Forensic Medicine of the Clinical Centre of Montenegro. All the deceased subjects had a cardiovascular-related cause of death.

The study included 124 sets of samples, of which 63 sets were considered for interpretation, as this number of subjects met the criteria for declaring the presence or absence of CP in plaques. The subjects were divided into two groups on the basis of criteria for the presence (33 subjects) or absence (30 subjects) of CP. A total of 252 artery samples were examined (63 quadruple sets of samples consisted of coronary artery, carotid artery, Willis' circle artery and abdominal aorta samples).

The presence of classical risk factors for atherosclerosis (hyperlipoproteinemia, diabetes, arterial hypertension, smoking, a family history of cardiovascular disease) was determined by a post-mortem interview with the family and by consulting medical records. The BMI was calculated at autopsy.

Samples were taken from the part of the blood vessel where atherosclerotic plaque was visible macroscopically at the site of the fatty stripe stage (identified as an area of yellowish discolouration at or above the level of the intima). Every set of samples consisted of the carotid artery, coronary artery, Willis' circle artery, and abdominal aorta. After sampling, the tissue was fixed in 4% formalin and paraffin moulds were formed. Routine haematoxylin and eosin staining, as well as immunohistochemical staining, were performed.

For the purpose of deparaffinization and rehydration, tissue sections were immersed in xylene for 3 min twice, followed by 3 min immersion in 1:1 solution of xylene and 100% ethanol and 3 min immersion in ethanol solutions of decreasing concentrations (100% twice, 95%, 70% and 50%, respectively), after which they were rinsed under running cold tap water. Antigen retrieval was performed by treating the sections with 10mM citrate buffer in the microwave for 10 minutes twice, after which they were rinsed with deionized water. Endogenous peroxidase was blocked using 3% H<sub>2</sub>O<sub>2</sub> solution for 10 minutes at room temperature. The sections were then incubated with primary antibodies using the protocols instructed by the manufacturer.

The following primary antibodies were used: OMP1 (Polyclonal, Medac Inc, Hamburg, Germany, 1:10), CP-RR-402 (Monoclonal, Washington Research Foundation, Seattle, US, 1:1000), SP110 (Polyclonal Rabbit Anti-SP110, Abcam, Cambridge, UK, 1:200), HSP60 (Polyclonal Rabbit Anti-HSP60, Abcam, Cambridge, UK, 1:100) and TNF- $\alpha$  (Polyclonal Rabbit Anti-TNF- $\alpha$ , Abcam, Cambridge, UK, 1:100).

Standard LSAB+ (streptavidin-biotin-peroxidase) technique was used for immunohistochemical iden-

tification of the antigens, with 3-amino-9-ethylcarbazole as chromogenic substrate. Each incubation was followed by rinsing the sections in Tris Buffered Saline (0.05M, 7.6 pH) and contrasting with hematoxylin.

Assessing of CP presence in plaques was performed by two immunobodies: OMP1 (Polyclonal, Medac Inc, Hamburg, Germany, 1:10) and CP-RR-402 (Monoclonal, Washington Research Foundation, Seattle, US, 1:1000). A positive result for both markers was interpreted as a reliable indicator of the presence of CP in the plaque, and a negative finding for both markers was a reliable finding of the absence of CP.

Statistical analyses were performed using IBM Statistics (SPSS) 10, as well as methods of descriptive statistics,  $\chi^2$ -test, ANOVA test and logistic regression. For microphotografies we used digital Zeiss AxioCam 105 Color (5 megapixel resolution camera) with white filter on Zeiss Axio Scope.A1 microscope. Digitalisation of slides was done by ZEN 2.3 lite software (blue edition, Carl Zeiss Microscopy, GmbH, 2011), using magnification 40 $\times$ /0.75. Immunohistochemical slides were analysed by Plug-in IHC profiler on ImageJ software. Three randomly selected cross-sections were analysed, and mean values were set as results for each sample.

## Results

The mean age at the time of death was 59 years (ranging from 32 to 90 years, with a standard deviation of 15.77). The mean age of the CP-positive deceased subjects was 55.45  $\pm$  15.41, while the mean age of CP-negative deceased subjects was 62.67  $\pm$  15.36. The CP-negative deceased subjects were statistically significantly older ( $p < 0.001$ , t-test). The BMI of CP-positive deceased subjects was 27.69  $\pm$  4.72, while the BMI of CP-negative subjects was 32.67  $\pm$  4.40, which is significantly higher ( $p < 0.001$ , t-test).

The presence of classical risk factors was analysed using a  $\chi^2$ -test. Hypercholesterolemia, diabetes and a positive family history of cardiovascular diseases were significantly frequent in CP-negative samples. There was no significant difference in the incidence of arterial hypertension and smoking between the groups (Table I).

Logistical regression was performed as a method of simultaneous investigation of the relationship of all the examined factors with CP-positivity (unlike the previous analysis in which the risk factors were examined separately) (Table II). For an odds ratio (OR), as a product of logistical regression, less than 1, the result shows that a particular test factor is negatively related to the test result. The results showed that age, hypercholesterolemia, diabetes, arterial hypertension and BMI were negatively correlated with

**Table I.** Classic risk factors between the groups

RISK FACTORS		CP-NEGATIVE	CP-POSITIVE	P-VALUE
Hypercholesterolemia	-	13 (43.3%)	23 (69.7%)	< 0.001
	+	17 (56.7%)	10 (30.3%)	
Diabetes mellitus	-	21 (70%)	28 (84.8%)	0.005
	+	9 (30%)	5 (15.2%)	
Arterial hypertension	-	15 (50%)	18 (54.5%)	0.471
	+	15 (50%)	15 (45.5%)	
Family history of cardiovascular disease	-	10 (33.3%)	15 (45.5%)	0.049
	+	20 (66.7%)	18 (54.5%)	
Smoking	-	11 (36.7%)	11 (33.3%)	0.579
	+	19 (63.3%)	22 (66.7%)	
In total		30 (100%)	33 (100%)	

**Table II.** Logistic regression of the relationship of classical risk factors with CP-positivity

FACTORS EXAMINED	LOGISTIC REGRESSION PARAMETERS		
	ODDS RATIO	P-VALUE	95% CI
Age	0.965	0.001	0.944-0.986
Hypercholesterolemia	0.195	< 0.001	0.096-0.397
Diabetes mellitus	0.371	0.013	0.169-0.811
Arterial hypertension	0.448	0.030	0.217-0.925
Positive family history for cardiovascular disease	0.929	0.832	0.471-1.832
Smoking	0.755	0.446	0.366-1.556
BMI	0.773	< 0.001	0.718-0.832

**Table III.** TNF- $\alpha$  expression in CP-positive vs. CP-negative samples

	MIN	MAX	MEAN VALUE	STANDARD DEVIATION	CP-POSITIVE	CP-NEGATIVE	P-VALUE
Carotid artery	7	25	15.19	5.29	12.48 $\pm$ 4.64	18.17 $\pm$ 4.29	< 0.001
Willis circle	5	15	9.57	2.87	9.58 $\pm$ 2.72	9.57 $\pm$ 3.07	0.99
Aorta	7	25	15.49	5.07	12.94 $\pm$ 4.23	18.30 $\pm$ 4.43	< 0.001
Coronary artery	7	25	15.79	5.54	13.61 $\pm$ 5.02	18.20 $\pm$ 5.13	0.001
In total	5	25	14.01	5.43	12.15 $\pm$ 4.47	16.06 $\pm$ 5.67	< 0.001

ANOVA for comparison between arteries:  $p < 0.001$

CP-positivity. The most significant negative correlation (the lowest OR) was shown by hypercholesterolemia. No significant relationship was found between CP-positivity and a positive family history of cardiovascular diseases, as well as smoking.

Immunotesting for SP110 showed that this marker is significantly more expressed in CP-negative plaques in all the examined arteries (Table III). The HSP60 marker was significantly more expressed in CP-positive plaques in all the examined arteries (Table IV). CP-negative plaques showed a higher TNF- $\alpha$  expression. The difference is present in all arteries except the Willis' circle (Table V).

## Discussion

The results of the presented study showed that classical risk factors for atherosclerosis were more present in deceased subjects with CP-negative plaques, suggesting that CP may have been the cause of their atherosclerosis. The subjects with CP-negative plaques were significantly older and had a higher BMI at the time of death compared with CP-negative subjects. A significant difference between the CP-positive and CP-negative groups was found for the presence of hypercholesterolemia, diabetes, and a positive family history of cardiovascular disease,

**Table IV.** HSP60 expression in CP-positive vs. CP-negative samples

	MIN	MAX	MEAN VALUE	STANDARD DEVIATION	CP-POSITIVE	CP-NEGATIVE	P-VALUE
Carotid artery	5	25	14.67	4.99	17.21 ± 3.82	11.87 ± 4.65	< 0.001
Willis circle	6	24	13.36	4.84	16.33 ± 4.28	10.10 ± 2.96	< 0.001
Aorta	7	24	13.91	4.49	16.72 ± 4.14	10.80 ± 2.28	< 0.001
Coronary artery	6	25	14.08	4.60	16.45 ± 4.37	11.47 ± 3.28	< 0.001
In total	5	24	14.00	4.73	16.68 ± 4.12	11.05 ± 3.43	< 0.001

*ANOVA for comparison between arteries: p = 0.49*

**Table V.** TNF- $\alpha$  expression in CP-positive vs. CP-negative samples

	MIN	MAX	MEAN VALUE	STANDARD DEVIATION	CP-POSITIVE	CP-NEGATIVE	P-VALUE
Carotid artery	7	25	15.19	5.29	12.48 ± 4.64	18.17 ± 4.29	< 0.001
Willis circle	5	15	9.57	2.87	9.58 ± 2.72	9.57 ± 3.07	0.99
Aorta	7	25	15.49	5.07	12.94 ± 4.23	18.30 ± 4.43	< 0.001
Coronary artery	7	25	15.79	5.54	13.61 ± 5.02	18.20 ± 5.13	0.001
In total	5	25	14.01	5.43	12.15 ± 4.47	16.06 ± 5.67	< 0.001

*ANOVA for comparison between arteries: p < 0.001*

while there was no significant difference for arterial hypertension or smoking. Also, logistical regression showed that hypercholesterolemia had the most pronounced negative correlation with CP-positivity. The results indicate that the absence of classical risk factors for atherosclerosis in early CP-positive plaques suggest that bacterial infection may be a pathogenic trigger of the atherosclerosis process in the younger population without a significant load of risk factors. Izadi et al. demonstrated the presence of CP DNA in the plaques of 21.9% of patients with coronary atherosclerosis treated with an aorto-coronary bypass graft. Positive IgG on CP was present in 45.7% of patients, while IgM was positive in 4.8%; which suggests that it is a combination of long-term chronic infection and advanced plaque [16]. Sessa et al. examined the presence of CP DNA in atherosclerotic plaques, macrophages, and pericarotid lymph nodes in patients who had undergone carotid endarterectomy and found their presence to be higher than 70%. Their results indicate that the presence of CP in macrophages was more noticeable in the group of patients who had symptomatic disease, compared to asymptomatic patients, and they conclude that bacterial infection potentially contributes to plaque instability with previous significant blood vessel stenosis [16]. It is thought that PCR testing of macrophages on CP DNA could represent a significant diagnostic step in assessing the risk of atherosclerotic plaque complications [17], primarily because of the proven importance of macrophage positivity for CP and the ease of taking macrophages for analysis compared to histological analysis of plaque, which is carried out only after interventions that are intended for therapeutic

purposes [18]. Patients who are seropositive for CP have a faster progression of subclinical, non-manifest atherosclerotic plaques, confirmed by Player et al. in which coronary artery calcification was monitored on CT as an indicator of the plaque stage [19]. Wong et al. published a review of the relevant literature stating that CP is found in 15-100% of atherosclerotic plaques. The authors of this review found that the bacterium is predominantly found in advanced plaques, so they take the position that the bacterium does not start, but rather supports, the atherosclerotic process [20]. On the other hand, Assar et al. showed that CP DNA was present in 29.4% of thoracic aortic atherosclerotic plaques and in 5.9% of internal thoracic artery samples taken as a control group without atherosclerotic plaques. Also, their results indicate that there is a significant positive correlation between the presence of CP in advanced atherosclerotic plaque and in a healthy blood vessel without atherosclerosis within the same organism, which contradicts the view that the presence of bacteria in plaque is actually contamination due to a damaged endothelium [21]. El Yazouli et al. demonstrated in a sample of 115 patients with cardiovascular disease that dyslipidaemia was less common in the group of patients with the presence of CP DNA in their atherosclerotic plaques. Statistical analysis in their study showed that about 60% of patients had CP infection as an associated factor with other, classical risk factors for cardiovascular disease; 25% showed an independent role of classical risk factors; while 12% showed CP infection as a unique risk factor for atherosclerosis [22].

Our study showed a significantly higher expression of HSP60 in those infected, compared to the uninfected

ed plaques. Seropositivity for cHSP60 (c-chlamydial) is a sensitive and specific marker of acute coronary syndrome, independent of anti-CP IgG titre, levels of highly sensitive CRP or troponin T (7). Proven chronic infection with CP and anti-hHSP60 positivity are predictive factors for a cardiovascular event, especially if associated with high CRP [23] values. Risk factors for atherosclerosis (hypertension, smoking, oxLDL, free radicals, etc.) which act as a stress factor to the endothelium, favouring the release of hHSP60 in the cytosol and expression on the endothelial surface, thus helping with an autoimmune reaction initiated by cHSP60 [24]. It is Proven that mitogenic potential exerted on CP exerts mitogenic potential in the smooth muscle cells whose dominant effect through cHSP60 [24] and by increasing the expression of transcription factors EGR1 [24] and NF- $\kappa$ B [25]. It has been experimentally proven that formalin-inactivated CP cannot enter cells and that cHSP60 does not come into contact with host cells due to the intracellular localization of this protein within the bacterium. Also, in such an experimental model, there is no increase in the expression of E selectin, ICAM-1 and VCAM-1 on the host endothelium. This proves that the membrane antigens of CP do not play a role in the expression of these adhesive molecules, but that it is the role of cHSP60 [26]. Koi *et al.* demonstrated the co-localization of cHSP60 and hHSP60 in plaques on carotid atherosclerotic plaque samples, as well as their joint action in promoting the secretion of TNF- $\alpha$  and MMP by macrophages [27]. cHSP60 also stimulates the specific proliferation of T lymphocytes [28], as well as B lymphocytes, with a consequent increase in the secretion of IL-10 and IL-6 [29]. T lymphocytes in early, asymptomatic plaques have been shown to exhibit autoreactivity to hHSP60 [11]. Konflach *et al.* demonstrated by multiple linear regression that the concentration of hHSP60-specific T lymphocytes was significantly associated with intima-media thickness in the male population aged 17-18 years, while there was no significant association in the male population aged 50-69 years, which suggests that T-cell activation by hHSP60 is more significantly associated with wounds than with advanced atherosclerosis [30]. Exogenous intake of cHSP60 experimentally causes endothelial dysfunction in rabbits on a high cholesterol diet [31]. Hypercholesterolemic rabbits, in which exogenous intake of cHSP60 was performed, developed a fat stripe more rapidly, and macrophage infiltration was also more pronounced in more developed plaques, which suggests that this virulence factor supports both the early and late stages of atherosclerosis [32]. Oral administration of immunosuppressive doses of HSP60 in ApoE to mice leads to a reduction of atherosclerotic plaque, while subcutaneous administration has the opposite effect [33]. Also, Zhong *et al.*

described that intranasal application of small doses of HSP60 leads to a significant 33.6% reduction in plaque in the aortic root [34]. These results may be useful in the process of developing a possible "atherosclerosis vaccine" based on HSP60. Kuroda *et al.* demonstrated on carotid plaque samples obtained by endarterectomy that the most significant co-localization of cHSP60 and the CP antigen occurs within plaque macrophages; they conclude that altering macrophage function is a significant step in exhibiting the atherogenic effect of cHSP60 [35]. Knoflach *et al.* emphasize the importance of T lymphocytes, through results that indicate that reactivity against HSP60 is more present in T lymphocytes within developed plaque compared to T lymphocytes in circulating blood. Their conclusion is that HSP60-specific T lymphocytes circulate in the blood, and then their accumulation in advanced plaque occurs [36]. It is thought that testing the plasma concentration of HSP60-specific T lymphocytes in the future could be a screening method for early atherosclerosis [37]. Also, in a study by Bodolay *et al.* on patients with mixed systemic connective tissue disease, antibodies to HSP60 were shown to be more prevalent in a subgroup of patients who had cardiovascular comorbidities [38].

Significantly higher expression of SP110 in CP-negative plaques was observed, suggesting a protective role of this factor. SP110, a human analogue of the *Ipr1* gene, may be a constituent protective factor against CP and against infectious atherosclerosis. Leo *et al.* demonstrated a protective role of SP110/*Ipr1* in intracellular infections in a mouse model of *M. tuberculosis* infection by modulating NF- $\kappa$ B activity in the form of promotion of anti-apoptotic mechanisms and down-regulation of TNF- $\alpha$  secretion. Infected mice that showed SP110/*Ipr1* activity had developed lung lesions and better survival [12]. The lower expression of TNF- $\alpha$  in CP-positive plaques can potentially be attributed to SP110 activity, more precisely and it is attempted to suppress the inflammatory response to the presence of bacteria in the blood vessel wall. There are no human studies examining the expression of SP110 in atherosclerosis, nor the effect of this factor on CP, and in this domain this study can be considered pioneering. It is necessary to conduct additional genetic and molecular research in order to clarify its role in the infectious process of atherosclerosis, as well as to examine its potential in the prevention and treatment of atherosclerosis.

Although the influence of TNF- $\alpha$  in the infectious process of atherosclerosis is experimentally verified, mice with a genetic defect of TNF- $\alpha$  infected with CP had a significantly lower incidence of atherosclerosis [39]. Results show that the expression of TNF- $\alpha$  was more noticeable in CP-negative plaques. There is a possibility that early forms of plaque do not

show a pronounced effect of bacterial infection on the growth of this inflammatory marker. Janczak *et al.* analysed advanced, stenotic carotid plaques in 100 patients, detecting the presence of CP DNA in 68% of plaques, and infected plaques had a high expression of bFGF and TNF- $\alpha$  [40]. On the other hand, a meta-analysis by Filard *et al.* showed that patients with atherosclerosis had elevated systemic values of hsCRP, fibrinogen, IL-6, TNF- $\alpha$  and IFN- $\gamma$ , while patients seropositive for CP had elevated hsCRP, fibrinogen, and IL-6 values, with no significant effect of seropositivity on the values of other biochemical inflammatory markers [41]. Oxahar *et al.* showed that infection of a human mast cell culture with CP results in an early increase in IL-8 and MCP-1 secretion, while an increase in TNF- $\alpha$  secretion was observed later in the development of the infection process [42].

Finally, our study showed most of the classical risk factors for atherosclerosis do not correlate with CP-positivity, pointing out very possible infective genesis of atherosclerosis. Protective SP110 and potentially linked TNF- $\alpha$  are significantly less expressed in CP-positive plaques, contrary to HSP60.

*The authors declare no conflict of interest.*

## References

- Libby P. Coronary artery injury and the biology of atherosclerosis: inflammation, thrombosis, and stabilization. *Am J Cardiol* 2000; 86 (8B): 3J-8J.
- Renko J Bacterial DNA signatures in arterial inflammation [academic dissertation], University of Tampere, 2008.
- Dabiri H, Rezadehbashi M, Badami N, et al. Detection of Chlamydia pneumoniae in atherosclerotic plaques of patients in Tehran, Iran. *Jpn J Infect Dis* 2009; 62: 195-197.
- Epstein SE. The multiple mechanisms by which infection may contribute to atherosclerosis development and course. *Circ Res* 2002; 90: 2-4.
- Honarmand H. Atherosclerosis Induced by Chlamydia pneumoniae: A Controversial Theory. *Interdiscip Perspect Infect Dis* 2013; 2013: 941392.
- Di Pietro M, Filardo S, De Santis F, Sessa R. Chlamydia pneumoniae infection in atherosclerotic lesion development through oxidative stress: a brief overview. *Int J Mol Sci* 2013; 14: 15105-15120.
- Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation* 1999; 100: e20-8.
- Pothineni NVK, Subramany S, Kuriakose K, et al. Infections, atherosclerosis, and coronary heart disease. *Eur Heart J* 2017; 38: 3195-3201.
- Lalla E, Lamster IB, Hofmann MA, et al. Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 2003; 23: 1405-1411.
- Shah PK. Inflammation, infection and atherosclerosis. *Trends Cardiovasc Med* 2019; 29: 468-472.
- Bachmaier K, Neu N, de la Maza LM, Pal S, et al. Chlamydia infections and heart disease linked through antigenic mimicry. *Science* 1999; 283: 1335-1339.
- Leu JS, Chen ML, Chang SY, et al. SP110b Controls Host Immunity and Susceptibility to Tuberculosis. *Am J Respir Crit Care Med* 2017; 195: 369-382.
- Kramnik I, Dietrich WF, Demant P, Bloom BR. Genetic control of resistance to experimental infection with virulent Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* 2000; 97: 8560-8565.
- Pan H, Yan BS, Rojas M, et al. Ipr1 gene mediates innate immunity to tuberculosis. *Nature* 2005; 434: 767-772.
- Bloch DB, Nakajima A, Gulick T, et al. Sp110 localizes to the PML-Sp100 nuclear body and may function as a nuclear hormone receptor transcriptional coactivator. *Mol Cell Biol* 2000; 20: 6138-6146.
- Izadi M, Fazel M, Akrami M, et al. Chlamydia pneumoniae in the atherosclerotic plaques of coronary artery disease patients. *Acta Med Iran* 2013; 51: 864-870.
- Sessa R, Di Pietro M, Schiavoni G, et al. Chlamydia pneumoniae DNA in patients with symptomatic carotid atherosclerotic disease. *J Vasc Surg* 2003; 37: 1027-1031.
- Frutos MC, Monetti MS, Mosmann J, et al. Molecular characterization of Chlamydia pneumoniae in animals and humans from Argentina: Genetic characterization of Chlamydia pneumoniae. *Infect Genet Evol* 2016; 44: 43-45.
- Sessa R, Di Pietro M, Schiavoni G, et al. Chlamydia pneumoniae in asymptomatic carotid atherosclerosis. *Int J Immunopathol Pharmacol* 2006; 19: 111-118.
- Player MS, Mainous AG 3rd, Everett CJ, Diaz VA, et al. Chlamydia pneumoniae and progression of subclinical atherosclerosis. *Eur J Prev Cardiol* 2014; 21: 559-565.
- Wong YK, Gallagher PJ, Ward ME. Chlamydia pneumoniae and atherosclerosis. *Heart* 1999; 81: 232-238.
- Assar O, Nejatizadeh A, Dehghan F, et al. Association of Chlamydia pneumoniae Infection With Atherosclerotic Plaque Formation. *Glob J Health Sci* 2015; 8 (4): 260-267.
- Yazouli LE, Hejaji H, Elmdaghri N, et al. Investigation of Chlamydia pneumoniae infection in Moroccan patients suffering from cardiovascular diseases. *J Infect Public Health* 2018; 11: 246-249.
- Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002; 22: 1547-1559.
- Deniset JF, Hedley TE, Hlaváčková M, et al. Heat shock protein 60 involvement in vascular smooth muscle cell proliferation. *Cell Signal* 2018; 47: 44-51.
- Kern JM, Maass V, Maass M. Chlamydia pneumoniae-induced pathological signaling in the vasculature. *FEMS Immunol Med Microbiol* 2009; 55: 131-139.
- Rupp J, Hellwig-Burgel T, Wobbe V, et al. Chlamydia pneumoniae infection promotes a proliferative phenotype in the vasculature through Egr-1 activation in vitro and in vivo. *Proc Natl Acad Sci U S A* 2005; 102: 3447-3452.
- Kol A, Bourcier T, Lichtman AH, Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J Clin Invest* 1999; 103: 571-577.
- Mosorin M, Surcel HM, Laurila A, et al. Detection of Chlamydia pneumoniae-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol* 2000; 20: 1061-1067.
- Wick C. Tolerization against atherosclerosis using heat shock protein 60. *Cell Stress Chaperones* 2016; 21: 201-211.
- Knoflach M, Kiechl S, Mayrl B, et al. T-cell reactivity against HSP60 relates to early but not advanced atherosclerosis. *Atherosclerosis* 2007; 195: 333-338.
- Mussa FF, Chai H, Wang X, et al. Chlamydia pneumoniae and vascular disease: an update. *J Vasc Surg* 2006; 43: 1301-1307.
- Hu Y, Chen Z, Jiang L, Chen F, Jin R, Cheng L. Effects of oral and subcutaneous administration of HSP60 on myeloid-de-

- rived suppressor cells and atherosclerosis in ApoE<sup>-/-</sup> mice. *Biochem Biophys Res Commun* 2018; 498 (4): 701-706.
34. Bahamondez-Canas TF, Cui Z. Intranasal immunization with dry powder vaccines. *Eur J Pharm Biopharm* 2018; 122: 167-175.
  35. Kuroda S, Kobayashi T, Ishii N, et al. Role of Chlamydia pneumoniae-infected macrophages in atherosclerosis developments of the carotid artery. *Neuropathology* 2003; 23: 1-8.
  36. Knoflach M, Bernhard D, Wick G. Anti-HSP60 immunity is already associated with atherosclerosis early in life. *Ann N Y Acad Sci* 2005; 1051: 323-331.
  37. Wick G, Knoflach M, Xu Q. Autoimmune and inflammatory mechanisms in atherosclerosis. *Annu Rev Immunol* 2004; 22: 361-403.
  38. Bodolay E, Prohászka Z, Paragh G, et al. Increased levels of anti-heat-shock protein 60 (anti-Hsp60) indicate endothelial dysfunction, atherosclerosis and cardiovascular diseases in patients with mixed connective tissue disease. *Immunol Res* 2014; 60: 50-59.
  39. Zafiratos MT, Cottrell JT, Manam S, et al. Tumor necrosis factor receptor superfamily members 1a and 1b contribute to exacerbation of atherosclerosis by Chlamydia pneumoniae in mice. *Microbes Infect* 2019; 21: 104-108.
  40. Janczak D, Ziolkowski P, Szydelko T, et al. The presence of some cytokines and Chlamydia pneumoniae in the atherosclerotic carotid plaque in patients with carotid artery stenosis. *Postepy Hig Med Dosw (Online)* 2015; 69: 227-232.
  41. Filardo S, Di Pietro M, Farcomeni A, et al. Chlamydia pneumoniae-Mediated Inflammation in Atherosclerosis: A Meta-Analysis. *Mediators Inflamm* 2015; 2015: 378658.
  42. Oksaharju A, Lappalainen J, Tuomainen AM, et al. Pro-atherogenic lung and oral pathogens induce an inflammatory response in human and mouse mast cells. *J Cell Mol Med* 2009; 13: 103-113.

### Address for correspondence

**Andrea Jovovic**  
Faculty of Medicine  
University of Montenegro  
81000 Podgorica, Montenegro  
Tel. +38267814560  
e-mail: jovovicandrea33@gmail.com