Research Article

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Interleukin-4, hemopexin, and lipoprotein-associated phospholipase A2 are significantly increased in patients with unstable carotid plaque

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Abstract: Objective: This study aimed to compare the plasma levels of lipoprotein-associated phospholipase A2 (Lp-PLA2), hemopexin (Hpx), and interleukin-4 (IL-4) in patients with carotid artery atherosclerosis based on neurological symptoms and plaque histopathology and to find association between plaque stability and neurological symptoms. This single-center study included patients treated surgically for significant stenosis of the internal carotid artery. Serum levels of biomarkers

were determined, and a histopathological analysis of the carotid plaques was performed. Within 70 patients, 40 asymptomatic and 30 symptomatic; 38 patients (54.3%) were diagnosed with unstable carotid plaque and 32 patients (45.7%) had a stable carotid plaque. Significantly higher incidence of unstable carotid plaque was detected in symptomatic patients ($p < 0.001$). Compared to asymptomatic patients, higher expression of Lp-PLA2 ($285.30 \pm 2.05 \mu\text{g/l}$), Hpx ($0.38 \pm 0.01 \text{ ng/l}$), and IL-4 ($65.77 \pm 3.78 \text{ ng/l}$) in plasma were detected in symptomatic patients. Subsequently, higher expression of Lp-PLA2 ($297.34 \pm 2.3 \mu\text{g/l}$), Hpx ($0.41 \pm 0.02 \text{ ng/l}$), and IL-4 ($64.74 \pm 4.47 \text{ ng/l}$) in plasma was observed in patients with unstable plaques ($n=38$). Statistically significant ($p < 0.001$) differences in expression of Lp-PLA2, Hpx, and IL-4 between patients with unstable and stable plaques were detected. Moreover, only the differences between symptomatic and asymptomatic patients in the expression of Lp-PLA2 and IL-4 in plasma were statistically significant ($p < 0.001$). This study showed that Lp-PLA2, IL-4, and Hpx levels are significantly increased in patients with an unstable carotid plaque.

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1 Introduction

Stroke is considered the primary cause of acquired disability in the elderly population and the second most common cause of mortality in developed countries. Carotid artery stenosis is a well-established cause of brain ischemia, accounting for up to 20% of stroke cases [1]. The degree of intraluminal narrowing of the internal carotid artery is the main criterion for surgical treatment. Recently, carotid endarterectomy has been indicated for

asymptomatic patients with more than 70% stenosis of the internal carotid artery and for symptomatic patients with more than 50% stenosis of the internal carotid artery [2]. The current treatment decision is based only on the degree of stenosis and not on the biological features of the carotid plaque. However, recent studies have suggested that not merely the degree of carotid artery stenosis but also plaque features could contribute to brain ischemia [3]. The term “vulnerable plaque” is well-established [4] and responsible for the majority of the strokes in patients with moderate to severe carotid stenosis [5]. Vulnerable, unstable carotid plaques have some of the following features: thinned or ruptured cap, large lipid core, intraplaque hemorrhage, surface thrombus, cap or plaque inflammation, and marked neovascularization [6,7]. The irregular surface on the unstable plaque can lead to thrombus formation. Subsequent detachment of the embolic material from the plaque surface and its entry into the brain circulation can cause transient ischemic attacks or strokes. Another possibility that an unstable plaque causes stroke is plaque or cap rupture with embolization of lipid core and plaque detritus to brain circulation [8]. Thus, plaque morphology, besides the degree of internal carotid artery stenosis, is a significant risk factor for brain ischemia [9]. A nine-fold increase in the risk of stroke in patients with features of unstable plaque has been reported [7]. The detection of a vulnerable carotid plaque can help in identifying patients who are at a high risk for stroke, but they are not candidates for surgery according to the current guidelines. Such patients might be candidates for surgery or stenting even if they have a lesser degree of carotid artery stenosis, that is, below 70% in asymptomatic and below 50% in symptomatic patients. Moreover, evidence suggests that an unstable carotid plaque might be a predictor of the presence and severity of coronary artery disease and other forms of systemic atherosclerosis [10].

The detection of vulnerable, unstable carotid plaques is of high importance and is based on imaging studies or on the concentration of specific biomarkers such as lipoprotein-associated phospholipase A2 (Lp-PLA2). Contrast-enhanced ultrasonography, multi-detector computed tomography angiography, or magnetic resonance angiography has been evaluated for their ability to detect vulnerable carotid plaques [3].

Several markers have been evaluated to identify patients with an unstable carotid plaque; these include high-sensitivity C-reactive protein (Hs-CRP), hemopexin (Hpx), metalloproteinase-2, and metalloproteinase-9. However, currently, Lp-PLA2 is considered an independent biomarker, not only for brain ischemia, but also for

coronary artery disease and peripheral arterial occlusive disease [11,12].

Lp-PLA2 is an enzyme also known as acetylhydrolase because of its ability to hydrolyze the platelet-activating factor. Under the conditions of the hydrolysis of membrane phospholipids, Lp-PLA2 participates. It is produced by inflammatory cells in the atherosclerotic plaque and then leached into the blood [13,14]. Lp-PLA2 activity leads to the formation of pro-atherogenic substances (lysophosphatidylcholine and oxidized fatty acids). Oxidized **low-density lipoprotein** (LDL) particles are the leading cause of atherosclerosis and play an essential role in the formation of necrotic nucleus, followed by the migration of other inflammatory cells into the atherosclerotic plaque, thus creating a vicious cycle [15]. After modification of the atherosclerotic plaque affected by Lp-PLA2, this plaque is highly susceptible to rupture; hence, increased Lp-PLA2 concentration or activity can accurately identify patients with a high risk for stroke. One of the essential properties of the Lp-PLA2 is its high vascular specificity, while its increased expression level was not induced by other types of inflammation unlike most other inflammatory markers, e.g., CRP [16]. Lp-PLA2 is secreted by inflammatory plaque cells and is therefore involved in the development of vulnerable plaques. The association between increased expression of Lp-PLA2 and incidence of stroke is well known [11,17-19]. However, higher levels of Lp-PLA2 are associated not only with a higher risk for stroke, but also with a higher risk for coronary artery disease and cardiovascular mortality [17-19].

Hpx is a protein primarily synthesized in the liver and is considered a reactant of the acute phase of inflammation. The role of Hpx is also considered in the process of atherosclerosis along with the protective function of **high-density lipoprotein** (HDL) particles and its protective role in inflammatory diseases. Hpx is probably responsible for reducing the oxidation of lipids in the atherosclerotic plaque; thus, a vulnerable plaque with a high level of embolization is stabilized. It binds free hemoglobin (Hb) and heme, which are responsible for high oxidizing stress, and it is involved in the formation of oxygen radicals, followed by high cytotoxic effect on the endothelium. Hpx appears to be a novel biomarker for atherosclerotic diseases. Recent studies have evaluated the associations between Hpx and atherosclerosis mainly in animal models [20]; however, only a few studies assessed the association between Hpx and carotid artery disease in humans [21].

Interleukin-4 (IL-4) is considered an anti-inflammatory cytokine. IL-4 regulates macrophage polarization to

M2 phenotype, which protects against atherosclerosis. Results of studies assessing the concentration of plasma levels of IL-4 in patients with atherosclerosis have been controversial [22,23].

The objective of our study was to compare the plasma levels of biomarkers such as Lp-PLA2, Hpx, and IL-4 in patients with significant stenosis of the internal carotid artery, based on their neurological symptoms and plaque histopathology. Lp-PLA2 is a well-established biomarker of the unstable carotid plaque and was chosen for a correlation. This study was considered a pilot study in our institute regarding biomarkers of unstable carotid plaque. We intend to investigate more intensively the iron metabolism and the immune mechanism in unstable carotid plaques. M2 macrophages were found in the peripheral plaque regions with increased production of IL-4. These macrophages have high phagocytic activity and are located in plaque regions rich in iron. They are considered to phagocytize and utilize Hb deposits. These macrophages seem to be athero-protective [24]. Thus, we decided first to observe the relationship between plasma levels of Lp-PLA2, Hpx, IL-4, and plaque stability in a pilot study.

2 Material and Methods

2.1 Patients

This single-center prospective study was performed at the Department of Vascular Surgery, Eastern Slovak Institute of Cardiovascular Diseases and Faculty of Medicine, University of Pavol Jozef Safarik, Kosice. Seventy patients with significant stenosis of the internal carotid artery, who were referred to our department from January 2016 to September 2016, were included in the study. Symptomatic patients were defined as patients who had suffered an ischemic stroke or transient ischemic attack (TIA) within the last six months, while asymptomatic patients were defined as those who did not suffer any stroke or TIA within the previous six months. Significant stenosis of the internal carotid artery was defined as stenosis of more than 50% of the lumen in symptomatic patients and more than 70% in asymptomatic patients. The degree of stenosis was determined using angiography. Carotid endarterectomy was performed in all patients, and the excised carotid plaque was sent for histopathological analysis.

Exclusion criteria were recent heart failure, heart ischemia, uncontrolled hypertension, atrial fibrillation, peripheral arterial occlusive disease, acute and

chronic liver diseases, acute and chronic renal failure, decompensated metabolic disease, and acute and chronic infection. Patients with history of cancer and autoimmune disease were also excluded from the study. Those patients were excluded as such diseases might lead to elevation of respective biomarkers and might influence the outcomes of our research.

Heart failure, heart ischemia, and atrial fibrillation were evaluated by electrocardiography and echocardiography. When echocardiography was insufficient, cardiac diseases were furthermore evaluated by coronary angiography. Peripheral arterial occlusive disease was defined as a presence of claudication and limb rest pain and confirmed by ultrasonography. Liver, kidney, decompensated metabolic, and autoimmune diseases were confirmed by patient's history and laboratory findings. Acute and chronic infections were evaluated by the CRP level and infection-related symptoms.

2.2 Biomarker measurements

Blood samples of all patients were taken one day before the surgery, and the concentration of specific markers, namely, Hpx, Lp-PLA2, and IL-4, were determined. The levels of these parameters were determined using the ELISA method (human Lipoprotein-associated phospholipase A2, Cusabio, USA; Human Hpx, Abcam, UK), and the results were evaluated with the Tukey test. The plasma concentration of IL-4 was measured using an ELISA kit Human IL-4 Platinum ELISA (eBioscience, San Diego, USA). Blood samples for the measurement of specific markers were centrifuged after collection at 2500 rpm for 10 min. Serum samples were then frozen at -80°C until analysis. Samples were processed using Synergy H4 multiplate reader (BioTek Vermont, USA).

2.3 Histopathology of carotid plaques

The plaques collected during carotid endarterectomy were fixed in 4% neutral buffered formalin for 24 hours, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Subsequently, serial five μ m thickness blocks were sliced for hematoxylin-eosin staining. The samples from the area of maximum stenosis were examined. Histopathological characteristics of the retrieved carotid plaques were reported according to the updated American Heart Association classification of advanced atherosclerosis [25] and a previously well-validated scoring system published by Lovett et

al. [6]. For each plaque, the following features were recorded: rupture of the fibrous cap, lipid core size, nodular calcification, neovascularization, inflammatory infiltrates, infiltration of the fibrous cap, proportions of fibrous tissue, intraplaque hemorrhage, presence of foam cells, and surface thrombus.

A large lipid core was considered as one that occupied at least 50% of the thickness of the plaque. Calcification was deemed to be present when nodular deposits were seen [26]. Intraplaque hemorrhage included recent or old bleeding and was defined as evidence of red blood cell within the plaque-causing disruption of plaque architecture [27]. Rupture was recorded when there was clear communication between the lipid core and the lumen with a break in the fibrous cap. Thrombus was recorded when there was an organized collection of fibrin and red blood cells in the lumen [28]. A thin cap was defined as fibrous tissue interposed between the lumen and the lipid core, which had a minimum measured thickness of $\leq 200 \mu\text{m}$ [6].

An observer who was experienced in vascular pathology assessed all histological specimens was blinded to clinical characteristics. Based on the presence of these features in each plaque, an overall stability rating was given by the histopathologist as “unstable” or “stable.” Unstable plaques demonstrated many or all features, and “stable” plaques showed none of them.

2.4 Statistical analysis

Results of clinical characteristics of the study population and plasma level of biomarkers were evaluated using the one-way analysis of variance for independent measures (GraphPad Prism 5.0, GraphPad Software Inc., La Jolla, CA), where values of $p \leq 0.1$ were considered statistically significant. Correlation of the plaque stability and symptoms were calculated using the chi-square test (GraphPad Prism 5.0) with equal statistical significance, as mentioned above. Boxplots were composed by Microsoft Office Excel 2007.

3 Results

A total of 70 patients (26 women and 44 men) were included in the study. The mean age of patients was 70.0 ± 8.1 years. Forty patients (57%) were asymptomatic. Neurological symptoms were present in 30 symptomatic patients (43%), where 20 patients (66.6%) had a history of ischemic stroke

and 10 (33.3%) had a history of TIA. At hospital admission, all patients were on antiplatelet therapy. Carotid endarterectomy was performed under general anesthesia in all 70 patients. Transcranial cerebral oximetry was used for neuro-monitoring during carotid artery clamping in all patients. Two patients (2.8%) required shunt placement during carotid endarterectomy; these patients were treated with conventional carotid endarterectomy with a patch. The remaining 68 patients (97.2%) underwent an eversion carotid endarterectomy. All patients were prescribed with antiplatelet therapy postoperatively and were clinically evaluated by an independent neurologist after surgery. Table 1 provides the clinical characteristics of the study populations. There were no significant differences in age, sex, and underlying diseases when the study population was divided according to neurological symptoms or plaque stability (Table 1).

Based on the histopathology of carotid plaques, the patients were divided into two groups: 38 patients (54.3%) had an unstable carotid plaque and 32 patients (45.7%) had a stable carotid plaque. Of the 30 symptomatic patients (those who suffered ischemic stroke or TIA), 23 patients (76.6%) had an unstable plaque and only 7 patients (23.3%) had stable plaque. Symptomatic patients had 3.28 times higher incidence of unstable plaques than stable plaques. Of the 40 asymptomatic patients, 25 patients (62.5%) had stable plaques and 15 patients (37.5%) had unstable plaques. Asymptomatic patients had 1.66 times higher incidence of stable plaques than unstable plaques. The difference was significant at $p < 0.0001$ (Table 2).

3.1 Plasma levels of Lp-PLA2, Hpx, IL-4, and neurological symptoms

The symptomatic group had a higher mean plasma level of Lp-PLA2 ($285.30 \pm 2.05 \mu\text{g/l}$) than the asymptomatic group ($274.35 \pm 3.38 \mu\text{g/l}$). The difference in the Lp-PLA2 levels between the symptomatic and asymptomatic groups was significant ($p < 0.1$). No significant differences were noted in the plasma levels of Lp-PLA2 between men and women within the two groups.

The mean plasma Hpx level in the symptomatic group was $0.38 \pm 0.01 \text{ ng/l}$, while the mean plasma Hpx level in the asymptomatic group was $0.351 \pm 0.012 \text{ ng/l}$, and the difference in Hpx levels between the symptomatic and asymptomatic groups was not significant. No significant differences were found in the plasma levels of Hpx between men and women within the two groups.

The symptomatic group had a higher mean plasma level of IL-4 ($65.77 \pm 3.78 \text{ ng/l}$) than the asymptomatic

Table 1: Clinical characteristic of the study population.

	Total	Neurological symptoms			Plaque stability		
		Asymptom	Symptom	p-value	Stable	Unstable	p-value
No.	70	40 (57%)	30 (43%)		32 (46%)	38 (54%)	
Age	70	69	72	0.264	69	71	0.625
Men	44 (62%)	22 (55%)	22 (71%)	0.116	20 (63%)	24 (63%)	0.954
Heart disease	47 (67%)	29 (72%)	18 (60%)	0.27	21 (67%)	26 (68%)	0.804
Diabetes	16 (23%)	9 (22%)	7 (23%)	0.948	6 (19%)	10 (26%)	0.55
Pulmonary disease	4 (6%)	2 (5%)	2 (7%)	0.766	2 (6%)	2 (5%)	0.859
Smoker	13 (19%)	6 (15%)	7 (23%)	0.374	6 (19%)	7 (18%)	0.971
Renal insufficiency	7 (10%)	5 (12%)	2 (7%)	0.927	3 (9%)	4 (11%)	0.872
Hypertension	67 (95%)	38 (95%)	29 (97%)	0.733	32 (100%)	35 (92%)	0.786

Asymptom: asymptomatic patients

Symptom: symptomatic patients, those who suffered ischemic stroke or transient ischemic attack

p-value ≤ 0.1 was considered as statistically significant

Table 2: Correlation between plaque stability and symptoms.

	Total	Stable	Unstable
Symptomatic	30	7 (23.3%)	23 (76.6%)
Asymptomatic	40	25 (62.5%)	15 (37.5%)

This result is observed with high significance at $p < 0.0001$.

Table 3: Plasma levels of biomarkers in correlation with the neurological symptoms.

	Symptomatic	Asymptomatic	p-value
Lipoprotein-associated phospholipase A2	285.30 $\pm$ 2.05	274.35 $\pm$ 3.38	*
Hemopexin	0.38 $\pm$ 0.01	0.351 $\pm$ 0.012	non-significant
Interleukin-4	65.77 $\pm$ 3.78	42.69 $\pm$ 1.73	***

*** mean $p < 0.0001$, ** mean $p < 0.05$, * mean $p < 0.1$

group (42.69 $\pm$ 1.73 ng/l), and the difference in IL-4 levels between the symptomatic and asymptomatic groups was significant ($p < 0.001$). There were no significant differences in the plasma levels of IL-4 between men and women within the two groups. The correlation between plasma levels of biomarkers and neurological symptoms are presented in Table 3 and Figure 1.

3.2 Plasma levels of Lp-PLA2, Hpx, IL-4, and plaque morphology

We also analyzed the correlation between plasma levels and plaque morphology. The mean plasma concentration of Lp-PLA2 in the group with unstable plaque was 293.90 $\pm$ 1.5 μ g/l, and in those with stable plaque was 261.40 $\pm$ 1.3 μ g/l. Increased of the expression of Lp-PLA2 was detected statistically significant in the group of the unstable plaque samples ($p < 0.001$). Sex-based correlation regarding differences in plasma concentration of Lp-PLA2 was not statistically significant.

The mean plasma Hpx level (0.399 $\pm$ 0.012 ng/l) in the group with unstable plaque was higher than that (0.324 $\pm$ 0.0033 ng/l) in patients with stable plaque. The difference in levels between patients with unstable plaque and stable plaque was significant ($p < 0.001$). Sex-based correlation regarding differences in plasma concentration of Hpx was not statistically significant.

The mean plasma level of IL-4 in the group with unstable plaques was 65.0 $\pm$ 2.7 ng/l, which was significantly lower than that of patients with stable plaque (37.8 $\pm$ 1.6 ng/l, $p < 0.001$), showing no significant differences in the plasma levels of IL-4 between men and women within the groups. The correlations of plasma levels of biomarkers with plaque stability are shown in Table 4 and Figure 2.

Figure 1: Correlation between plasma levels of biomarkers and neurological symptoms.

Figure 2: Correlation between plasma levels of biomarkers and plaque stability.

Table 4: Correlation between plasma levels of biomarkers and plaque stability.

	Stable	Unstable	p-value
Lipoprotein-associated phospholipase A2 (µg/l)	261.4 ± 1.3	293.9 ± 1.5	***
Hemopexin (ng/l)	0.324 ± 0.0033	0.399 ± 0.012	***
Interleukin-4 (ng/l)	37.8 ± 1.6	65.0 ± 2.7	***

*** mean $p < 0.0001$, ** mean $p < 0.05$, * mean $p < 0.1$

4 Discussion

Our research showed that patients with symptomatic stenosis of the internal carotid artery had significantly higher plasma levels of Lp-PLA2 and IL-4 compared to patients with asymptomatic stenosis. Also, plasma levels of Lp-PLA2, Hpx, and IL-4 were significantly higher in patients with unstable plaque compared to patients with stable plaque. We confirmed the correlation between plaque morphology and neurological symptoms as well. We found that 76.6% of symptomatic patients have unstable carotid plaques and only 23.4% of patients have stable plaques. The difference was statistically significant ($p < 0.0001$). The presence of neurological symptoms was significantly associated with unstable carotid plaque, and

patients with previous neurological symptoms had a 3.28-fold higher incidence of unstable plaques.

4.1 Unstable plaques

Randomized studies have shown that most patients with carotid artery stenosis were never actually destined to suffer a stroke, 88% of patients with asymptomatic stenosis of 60-99% were stroke-free during five years, and 84% of symptomatic patients with 70-99% stenosis were stroke-free for five years [5]. Thus, there has been interest in the identification of the marker specific for “high risk for stroke.” Conversely, if it could be possible to identify “low risk for stroke” protein marker in patient’s blood, there might be better medical treatment applied even when the patient is indicated for invasive therapy according to the current guidelines.

Vulnerable, unstable plaques are characterized by thinned or ruptured cap, large lipid core, intraplaque hemorrhage, surface thrombus, cap or plaque inflammation, and marked neovascularization [6]. Marnane et al. observed a nine-fold increase in the risk of stroke recurrence before carotid endarterectomy in patients with symptomatic carotid artery stenosis associated with abundant macrophage content in carotid plaques. High plaque macrophage content independently predicted the risk of early stroke recurrence [7]. Experimental

studies have shown a crucial role of inflammation in destabilization and rupture of atherosclerotic plaque, mediated also by matrix metalloproteinases leading to fibrous cap rupture [7]. The same author identified low fibrous tissue content in unstable plaque to be associated with a six-fold increased risk of recurrent stroke. Fibrous cap disruption and neovascularization were also significantly associated with increased risk of recurrent brain ischemia [7]. Rupture of an advanced unstable plaque appears to be the reason for the formation of the thrombus on the unstable plaque that can occlude the carotid artery or can lead to cerebral embolization, both associated with neurological symptoms [8].

Unstable carotid plaques are associated with increased risk of stroke not only in symptomatic but also in asymptomatic patients. A meta-analysis of eight prospective studies, with a total of 7,557 patients, observing patients with asymptomatic carotid stenosis found that patients with unstable, echolucent plaques had a 2.31-fold increased risk of stroke compared to patients with stable plaque based on ultrasound assessment [29].

4.2 Lp-PLA2

Our results strongly confirm the role of Lp-PLA2 in the pathophysiology and clinical presentation of an unstable carotid plaque. Similar to our findings, some studies have reported the association of increased plasma levels of Lp-PLA2 in patients with unstable atherosclerotic plaque [11,14,15,30]. The plasma level of Lp-PLA2 correlates more with the composition of atherosclerotic plaque rather than its size [31]. The Rotterdam Study showed a higher activity of Lp-PLA2 in patients after stroke [32].

Within the last 15 years, Lp-PLA2 is considered as the specific biomarker not only for stroke but also for coronary heart disease. West of Scotland Coronary Prevention Study (WOSCOPS) firstly associated higher plasma levels of Lp-PLA2 with cardiovascular events [17]. The JUPITER trial also confirmed that patients with high Lp-PLA2 activity had a more than twofold higher risk of developing cardiovascular events compared to Lp-PLA2 detected in negative controls [33]. Additionally, the Bruneck Study found that the population in the third tertile of Lp-PLA2 activity had a higher risk of cardiovascular events (HR=2.2, 95% CI: 1.1-4.8) compared to those in the first tertile [34]. Further, a meta-analysis published by Thompson et al. in 2010, including patients from 32 prospective studies on Lp-PLA2, confirmed the relationship between Lp-PLA2 mass and activity, and the incidence of stroke, coronary artery disease, and cardiovascular mortality

[35]. Nowadays, a meta-analysis performed by Li et al. in 2017, and another 12 studies before June 2016, confirmed the specific increase of a mass and activity of Lp-PLA2 correlated with a higher risk of cardiovascular events [19]. The pooled HR for coronary heart disease was 1.46 (95% CI: 1.2-1.78; $p < 0.001$) and was 1.58 for stroke (95% CI: 1.21-2.07; $p < 0.001$).

Based on the evidence, the guidelines of major international societies, such as the European Society of Cardiology and the American College of Cardiology, stated measurement of the level of Lp-PLA2 as one of the biomarkers playing a key role in the identification of cardiovascular risk in adult patients [36].

Up to date, detailed pathophysiological mechanisms revealing the correlations between expression of Lp-PLA2 in plasma and stability of carotid plaques remain unknown. In the proinflammatory immune response to the stimuli and through the process of atherosclerosis, Lp-PLA2 was detected playing a pivotal role. Lp-PLA2 induces aggregation of the matrix peptidoglycans through activation and modification of LDL and its sub-sequential increased affinity to bind these matrix peptidoglycans. Collection of modified LDL in the subendothelial space of the arterial wall is a crucial step in the initiation of endothelial activation and consequent plaque rupture. Detection of Lp-PLA2 was specifically located to the carotid plaques, while its expression in the surrounding physiologically healthy arterial wall was negative. In the process of destabilization of the atherosclerotic plaque, attended with massive lipid accumulation, leukocyte infiltration, and cell necrosis, Lp-PLA2 was detected highly expressed. These findings indicate that Lp-PLA2 is a marker for an unstable plaque. As Lp-PLA2 is produced by foam cells and macrophages in unstable plaques, excessive Lp-PLA2 might be released into the circulation and become a predictor of vulnerable plaques [11].

We found significantly increased level of circulating Lp-PLA2 in patients with neurological symptoms and those with unstable carotid plaques. Our results confirm Lp-PLA2 as a biomarker of plaque stability. Similar results were reported by Mannheim et al. in 2008, who studied 167 patients after carotid endarterectomy and found increased local expression of Lp-PLA2 in symptomatic patients and in those with vulnerable carotid plaque [14]. In 2012, Sarlon-Bartoli et al. analyzed 42 patients with high-grade carotid artery stenosis and confirmed a significant correlation between plaque stability and neurological symptoms [11].

4.3 Hpx

Plaque neovascularization was considered to be a harmless feature of advanced atherosclerotic plaques; however, recently, there is growing evidence that neovascularization might trigger plaque progression and vulnerability. Because of inappropriate angiogenesis, new vessels are weak and tend to rupture, causing extravasation of red blood cells into the plaque. Red blood cells in the oxidative environment of the plaque can undergo dissolution [37]. Hb outside the protective environment of red blood cells is prone to oxidation. The generated high-valence iron compounds are highly reactive intermediates. The red blood cell membrane and the released Hb have vigorous pro-inflammatory and pro-atherogenic activities and might contribute to the further development of a vulnerable plaque [38]. The defense and adaptation mechanisms in response to the deleterious effects of cell-free Hb and heme include plasma proteins haptoglobin (Hp) and Hpx. These proteins can scavenge and eliminate free Hb and heme from circulation [39]. Hpx is an acute-phase plasma protein with a high affinity to bind with heme. Hpx-heme complexes are phagocytosed mainly by hepatocytes and macrophages. Hpx plays a protective role in reducing heme-induced oxidative stress [39].

Compared to numerous studies on the association of Lp-PLA2 with stroke and cardiovascular diseases, the association between Hpx and atherosclerosis has not been extensively studied. Watanabe et al. reported that Hb scavenger proteins, Hp, and Hpx are significantly increased in apolipoprotein A1-containing particles of HDL in mouse models of atherosclerosis and in coronary heart disease patients [40]. Watanabe found a novel protective role of Hpx in the inflammatory properties of HDL in atherogenic mice. He suggested that Hb/Hpx/ Hp pathway modulates the inflammatory properties of HDL in both mice and humans [41].

Free heme is also released from methemoglobin following reperfusion after cerebral ischemia and is a toxic component in the peripheral blood. Hpx was considered to be neuroprotective because of its ability to bind with heme. Dong et al. reported that Hpx is mainly expressed in the neurons following a short-duration middle cerebral artery occlusion followed by reperfusion in the rat brain. He also found that the administration of exogenous Hpx during the onset of reperfusion reduced the infarct volumes and improved the outcomes of reperfusion injury after cerebral ischemia [42].

Our outcomes confirm increased plasma levels of Hpx as a marker of carotid plaque instability. When analyzing

our patients according to plaque morphology, we found unstable plaque to be significantly associated with an increased Hpx plasma level ($p < 0.001$). Our analysis of patients based on symptomatology revealed a higher plasma level of Hpx in the symptomatic group than that in the asymptomatic group, but the difference was not significant. This may be due to the limited number of patients in our research, and studies with higher number of patients are necessary to confirm the significant difference between symptomatic and asymptomatic patients

4.4 IL-4

It is well known that IL-4 has a pro-inflammatory effect [43]. M0 macrophages can polarize as a pro-inflammatory M1 subset after stimulation with interferon- γ and lipopolysaccharide. These macrophages produce a range of pro-inflammatory cytokines. The alternative M2 type can be induced from M0 macrophage by stimulation with IL-4. M2 macrophages produce anti-inflammatory cytokines IL-10 and transforming growth factor- β [24]. In plaques, pro-inflammatory M1 macrophages were discovered decades earlier, while M2 macrophages with anti-inflammatory effects were found only recently [44]. Opinions on the impact of IL-4 in the process of development of a vulnerable atherosclerotic plaque were conflicting. In 2015, Zhao et al. showed on animal models that IL-4 might induce macrophages to take on an M2 phenotype to resolve inflammation, thereby protecting against atherosclerosis [45]. In 2013, Profumo found no significant difference in IL-4 levels in patients with carotid atherosclerosis compared to healthy subjects [23]. Recent studies found M1 macrophages to be associated with unstable plaques and M2 macrophages with stable plaques. Stoeger et al. found that M1 macrophages dominated the rupture-prone regions of the plaque over M2 type, while the fibrous caps of lesions showed no significant differences between subsets. Vascular adventitial tissue showed an increased M2 macrophage activation profile [46]. M1 macrophages secrete pro-inflammatory cytokines such as tumor necrosis factor- α , IL-6, and IL-12.

In contrast to M1, M2 macrophages secrete anti-inflammatory factors, and they can promote angiogenesis and tissue remodeling and repair [47]. In the advanced stage of atherosclerosis, Th2 cytokines are present in the plasma of patients. These cytokines can activate M2 macrophages and promote the formation of a fibrous cap, thereby enhancing plaque stability. Increased level of IL-4

appears to be the defense mechanism to an advanced stage of atherosclerosis and can contribute to plaque stabilization.

Phagocytosis seems to be the other anti-inflammatory function of M2 macrophages. In the progression of atherosclerosis, cellular apoptosis and its detritus are resolved by M2 phagocytosis. Phagocytosis of debris, damaged, dead, and apoptotic cells inhibits the production of pro-inflammatory cytokines and stimulates the production of anti-inflammatory IL-10 cytokine. Phagocytosis also protects the tissue from exposure to harmful pro-inflammatory responses, immunogenic content of dying, damaged, and dead cells. In the advanced stage of atherosclerosis, phagocytosis of apoptotic cells is severely impaired. This results in the accumulation of dead macrophages that block the final resolution of inflammation and thus increase local necrosis and plaque vulnerability [48].

In our study, a higher mean plasma level of IL-4 was seen in the symptomatic group than that in asymptomatic group. The difference between the symptomatic and asymptomatic groups was significant ($p < 0.001$). Similarly, patients with unstable plaques had significantly higher plasma level of IL-4 than those with stable plaques ($p < 0.001$). The explanation for this is not clear. IL-4 might be a biomarker of plaque instability, and its increased levels might predict neurological symptoms in patients with carotid atherosclerosis or can be considered a biomarker of activated defense mechanisms of organism against advanced atherosclerosis, and increased plasma level of IL-4 might be associated with the increased athero-protective activity of M2 macrophages, trying to stabilize a vulnerable carotid plaque.

5 Limitations of the study

The main limitation of our research is the small study size, which has likely reduced the statistical power to detect some correlations. The difference between the mean plasma levels of Hpx between symptomatic and asymptomatic patients was not significant, but when the entire cohort of patients was divided according to plaque stability, the difference was significant at $p < 0.001$. A study with more patients would find the difference in Hpx level between symptomatic and asymptomatic patients at a significant level. Another limitation is the lack of functional insight into the iron metabolism and athero-protective mechanisms in unstable, vulnerable plaques. This is a pivotal study, and for better understanding of

these processes, a study with more patients, more markers of Hb metabolism, and more cytokines and immune cells is required. That would give better functional insight at the athero-protective functions of M2 macrophages, IL-4, and other anti-inflammatory cytokines as IL-10 and transforming growth factor- β . A better understanding of athero-protective mechanisms in unstable plaques that lead to tissue repair and stabilization of unstable plaques could lead to better therapy of those patients.

6 Conclusion

In this study, we identified that the presence of neurological symptoms is significantly associated with the formation of unstable carotid plaque. Furthermore, our results showed increased plasma levels of Hpx and IL-4 to be significantly associated with plaque instability and with more neurological symptoms compared to those in asymptomatic patients. We confirmed Lp-PLA2 as a biomarker of plaque instability in patients with carotid atherosclerosis. Presence of unstable plaques and incidence of neurological symptoms was observed as highly statistically correlated with increased expression of Lp-PLA2. Higher levels of biomarkers like Lp-PLA2, Hpx or IL-4 might be indicators of a clinically more severe form of carotid atherosclerosis and might indicate the need for surgical or endovascular treatment at an earlier stage.

Ethics approval and informed consent (Ethics approval and consent to participate): The study protocol has been approved by the Ethical Committee of the East Slovak Institute of Cardiovascular Diseases on 7th December 2015.

All patients included in the study provided informed consent, and the copies are available for review by the Editor-in-Chief of this journal.

Availability of data and material: Datasets generated during the study are available for review by the Editor-in-Chief of this journal.

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All authors read and approved the final manuscript.

Authors' contributions: PS collected the data, wrote the draft and revised the manuscript, VS performed surgeries, participated in drafting the article, revised the manuscript and submitted the paper, ZH performed laboratory

measurements, collected data and participated in drafting the article, IK collected data, completed literature search and designed the paper, AJM participated in drafting the article and conducted English editing of the paper, ST, PS and DP designed the paper, MK and VP performed surgeries, literature search and designed the paper, RM performed statistical analysis and participated in drafting the article. MF performed surgeries and coordinated with all authors.

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