The impact of hyperglycemia and hyperlipidemia on plasma P-selectin and platelet markers after ischemic stroke

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Abstract

Introduction: Platelet activation plays a key role in the pathogenesis of ischemic cerebrovascular diseases. Thus, it is very important to identify novel pharmacological targets for platelet inhibition to improve ischemic stroke treatment. The aim of the study was to assess the relationship between metabolic disorders and platelet activity markers in patients with acute ischemic stroke.

Material and methods: Ninety-four patients with acute ischemic stroke were divided into four groups with: normolipidemia and normoglycemia (NL/NG), $n = 25$; normolipidemia and hyperglycemia (NL/HG), $n = 21$; hyperlipidemia and normoglycemia (HL/NG), $n = 27$; hyperlipidemia and hyperglycemia (NL/NG), $n = 21$. Twenty-one healthy subjects served as controls. We assessed the CD62P expression on resting and thrombin-activated blood platelets using the flow cytometer and anti-CD61 and anti-CD62P monoclonal antibodies. CD61-positive microparticles were defined as platelet-derived microparticles. The level of sP-selectin in serum was measured by the ELISA method.

Results: We observed a significant influence of hyperlipidemia and hyperglycemia on sP-selectin concentration. A strong correlation between higher sP-selectin concentration and enhanced LDL ($p = 0.001$), total cholesterol ($p = 0.02$), HbA$_{1c}$ level ($p < 0.001$) was noticed. The level of sP-selectin and PDMPs ($p < 0.001$) were significantly higher in all groups of stroke patients compared with the controls. CD62P expression on resting and thrombin activated platelets were significantly lower in groups of patients with stroke.

Conclusions: Hyperlipidemia and hyperglycemia exert an equal stimulatory effect on tested platelet markers but with no synergistic action in stroke patients with both of the metabolic comorbidities. sP-selectin concentration in stroke patients best reflects the impact of hyperglycemia and hyperlipidemia on vascular lesions and platelet activation.

Key words: platelet activation, stroke, hyperlipidemia, hyperglycemia, P-selectin, CD61, CD62P.

Introduction

Blood platelets play a crucial role in the maintenance of normal hemostasis and pathogenesis of hemostatic disorders and atherosclerosis. Platelets' procoagulant properties are associated with procoagulant

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phospholipids, but also with specific protein receptors for the coagulation factors and proteases existing in platelet surface membranes [1–3]. Metabolic disorders, such as diabetes mellitus (DM) and hyperlipidemia, are the key factors in the development of atherosclerosis, platelet activation and other vascular diseases.

Circulating atherogenic lipoproteins have been shown to activate circulating blood platelets, and thereby contribute to the pathogenesis and progression of atherothrombosis. Platelets activated by native and oxidized low-density lipoprotein (LDL) release factors which increase the expression of LDL receptors on macrophages and stimulate LDL accumulation in these cells [4]. This interaction can further contribute to the progression of atherogenesis [5].

Diabetes mellitus is a hypercoagulable state. Several factors contribute to the prothrombotic condition which characterizes patients with DM. The most important of these factors are increased coagulation, impaired fibrinolysis, endothelial dysfunction and platelet hyperreactivity [6–8].

Platelet abnormalities in diabetes mellitus, including altered adhesion and aggregation, are the result of hypersensitivity of diabetic platelets to agonists [9]. Platelet dysfunction in diabetic patients is caused by several mechanisms such as hyperglycemia, insulin deficiency and associated metabolic conditions (obesity, dyslipidemia and enhanced systemic inflammation) [10]. Hyperglycemia may increase platelet reactivity by glycating platelet surface proteins, activating protein kinase C (a mediator of platelet activation) and increasing P-selectin expression. Insulin deficiency and endothelial dysfunction also play an important role in platelet dysfunction; however, platelets in diabetic patients are in an activated state even in the absence of vascular injury [10].

One of the most important markers of platelet activation is the adhesion molecule P-selectin [11]. This adhesion molecule is a component of the platelet α-granule membrane and of the Weibel-Palade body membrane of endothelial cells. Platelet and endothelial cell activation results in hyperexpression of P-selectin on the surface of these cells; however, the increased P-selectin expression caused by platelet activation and degranulation is short lasting. Degranulated platelets rapidly shed the surface P-selectin to the plasma pool and P-selectin circulates in plasma in a soluble form (sP-selectin) [12, 13]. The increased plasma concentration of soluble P-selectin reflects the release of this adhesion molecule from activated platelets or damaged endothelial cells. It was recently shown that an elevated concentration of sP-selectin can exert a procoagulant effect [14], and it might play an important role in thrombotic disorders. The increase of sP-selectin concentration is also associated with formation of platelet-derived microparticles (PDMPs). They influence the normal hemostatic response to vascular injury, and also express phospholipids that function as procoagulants [15].

The aim of the study was to investigate the relationship between the metabolic disorders, such as hyperlipidemia and hyperglycemia, and selected platelet activity markers (soluble P-selectin, platelet P-selectin expression and PDMPs) in patients with acute ischemic stroke. This relationships may be an important target for ischemic stroke treatment and prevention.

The study was approved by the Ethics Committee of the Medical University of Lodz, Poland (No. RNN/465/11/KB).

Material and methods

The study group consisted of 94 patients with acute ischemic stroke ($\leq$ 7 days) hospitalized in the Department of Neurology and Stroke, Medical University of Lodz, Poland. The clinical diagnosis of ischemic stroke was established using the medical history of the patients, neurological investigation and cranial computed tomography. To avoid the influence of stroke subtype on the results, only patients with stroke of large-artery atherosclerosis (LAA), based on the TOAST classification criteria [16], were included in the study. Patients with a history of infection shortly before stroke were excluded to avoid the influence of the coexisting infection on platelet activity markers. Patients with severe liver disease, renal failure, cancer, chronic inflammatory diseases, and hemorrhagic diathesis were not enrolled in the study.

The diagnosis of diabetes mellitus in patients with hyperglycemia was established before the hospitalization and diabetic patients were included in the study if glycated hemoglobin (HbA$_{1c}$) was higher than 7% in spite of hypoglycemic treatment (metformin n = 15, glimepiride n = 6, gliclazide n = 8, gliclazide + metformin n = 4, insulin therapy n = 9). The hyperlipidemia was recognized on the basis of a LDL cholesterol (LDL-C) level $\geq$ 2.6 mmol/l (100 mg/dl); however, all stroke patients received atorvastatin (20 mg/day) during hospitalization.

All studied stroke patients received a 300 mg dose of acetylsalicylic acid (ASA) on the first day, and they continued the treatment with a 75 mg ASA dose during the hospitalization. The patients in the control group received ASA 75 mg /day.

The stroke patients were divided into four groups:
1) 25 patients with normolipidemia and normoglycemia – NL/NG: LDL < 2.6 mmol/l (100 mg/dl) [17], mean: 2.20 ±0.32 mmol/l; HbA$_{1c}$ $\leq$ 7% [18], mean: 5.7 ±0.3%; 11 males, 14 females, mean age: 76.44 ±12.6 years;

2) 21 patients with normolipidemia and hyperglycemia – NL/HG: LDL < 2.6 mmol/l (100 mg/dl), mean: 2.33 ±0.34 mmol/l; HbA_{1c} > 7%, mean: 7.67 ±0.82%; 11 males, 10 females, mean age: 73.1 ±8.21 years;

3) 27 patients with hyperlipidemia and normoglycemia – HL/NG: LDL ≥ 2.6 mmol/l (100 mg/dl), mean: 3.23 ±1.28 mmol/l; HbA_{1c} ≤ 7%, mean: 5.53 ±0.33%; 9 males, 18 females, mean age: 66.07 ±12.39 years;

4) 21 patients with hyperlipidemia and hyperglycemia – HL/HG: LDL ≥ 2.6 mmol/l (100 mg/dl), mean: 3.26 ±0.56 mmol/l; HbA_{1c} > 7%, mean: 7.91 ±1.16%; 13 males, 8 females, mean age: 70.1 ±10.76 years;

Twenty-one age- and sex-matched control subjects (CS) – LDL < 2.6 mmol/l (100 mg/dl), mean: 2.43 ±0.13 mmol/l; HbA_{1c} ≤ 7%, mean: 5.22 ±0.37%; 10 males, 11 females, mean age: 60.2 ±14.6 years – were recruited from patients with discopathy or tension-type headache. Subjects were excluded from the control group if they had a history of cerebrovascular diseases. Otherwise, the exclusion criteria were identical to those applied to the patient groups.

The risk factors for ischemic stroke (arterial hypertension, ischemic heart disease and body mass index) were similar in the study groups and the controls, and patients in all groups received antihypertensive drugs, such as angiotensin convertase enzyme (ACE) inhibitors, calcium channel blockers, and β-blockers.

Complete blood cell count, HbA_{1c}, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and LDL-C levels were measured in a fasting state for all patients. Serum LDL-C levels were calculated with Friedewald's formula [19]. Biochemical determinations were performed with the Olympus AU640 Analyzer (Olympus Optical Co., Ltd., Shizuoka, Japan).

The flow cytometry (FACScan, Becton Dickinson, San Jose, USA) was used to measure the expression of P-selectin (CD62P) on resting and thrombin-activated platelets. Blood was withdrawn without stasis to avoid platelet activation and collected into two tubes. The first tube contained 0.1 ml of blood and 1 ml of a 0.5% solution of paraformaldehyde in PBS – this sample reflected the state of platelet activity *ex vivo*; the second tube containing 0.5 ml of blood and 0.5 ml of EDTA was used to assess platelet reactivity after treatment with 0.08 units of bovine thrombin for 4 min. All platelet measurements were performed within 90 min after blood withdrawal. The antibody anti-CD61-FITC (Dako) – a fluorescein-isothiocyanate-conjugated antibody to glycoprotein IIIa – was used as an activation-independent marker of platelets. A phycoerythrin-conjugated anti-CD62-PE antibody (Becton Dickinson) was used to assess α-granule degranulation. To assess the extent of the nonspecific association of protein with platelets, a control tube containing anti-CD61-FITC and nonfractionated PE conjugated IgG (Becton Dickinson) was used for each blood sample. The reaction mixture was incubated in a dark room, at room temperature, for 30 min. Then, the antibody-bound platelets were fixed with 200 µl of FACS flow liquid and analyzed.

Platelets were subtracted from other blood cells and identified by flow cytometry based on size and platelet-specific CD61 surface expression. The data were presented as the percentage of CD62P(+) platelets (%CD62) and as the median fluorescence intensity (medCD62) reflecting the density of CD62P on the platelet surface. CD61-positive microparticles were defined as platelet-derived microparticles (PDMPs). They were distinguished from other platelets on forward scatter histograms based on their size < 0.2 µm. WinMDI 2.8 was used to analyze the data collected with flow cytometry.

The level of sP-selectin was estimated by ELISA (Quantikine human sP-selectin/CD62P ELISA Kit, R&S Systems, Abingdon, UK). After 10 min of centrifugation, the blood plasma obtained from EDTA-anticoagulated samples was stored at –80°C until measurements. Measurement of sP-selectin was performed according to the manufacturer's instructions.

Statistical analysis

Since all study variable did not pass the D'Agostino normality test, differences between groups were analyzed using the Kruskal-Wallis test followed by the post-hoc Dunn's test for multiple comparisons. Spearman's correlation was performed to assess the relationship between continuous variables. All of the statistical analyses were performed using Statistica for Windows ver. 8.0. The null hypothesis was rejected if $p < 0.05$.

Results

The blood lipid profile and HbA_{1c} level in study groups are presented in Table I.

In our study we aimed to demonstrate the influence of metabolic disorders (hyperlipidemia and hyperglycemia) on the platelet activity markers. The most interesting finding was the significant positive correlation between LDL, TC, HbA_{1c} level and sP-selectin concentration (LDL : sP-selectin $p = 0.001$, TC : sP-selectin $p = 0.02$, HbA_{1c} : sP-selectin $p < 0.001$). The strongest positive correlation was observed between sP-selectin concentration and HbA_{1c} level (Figure 1). We also noted significantly higher serum concentration of sP-se-

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Table I. Blood lipid profile and HbA_{1c} level (means ± SD) in study groups

Parameters	NL/NG	NL/HG	HL/NG	HL/HG	CS
TC [mmol/l]	3.68 ±0.6	3.77 ±0.45	5.66 ±0.9	5.86 ±0.46	4.06 ±0.5
LDL [mmol/l]	2.15 ±0.33	2.33 ±0.34	4.62 ±1.27	4.28 ±0.56	2.45 ±0.13
HDL [mmol/l]	1.08 ±0.3	1.07 ±0.31	1.3 ±0.31	1.14 ±0.25	0.95 ±0.17
TG [mmol/l]	1.38 ±0.72	1.56 ±0.7	1.66 ±0.85	1.67 ±0.61	1.31 ±0.48
HbA_{1c} [%]	5.7 ±0.3	7.5 ±0.9	5.53 ±0.33	7.66 ±1.3	5.22 ±0.37

NL/NG – normolipidemic/normoglycemic group, NL/HG – normolipidemic/hyperglycemic group, HL/NG – hyperlipidemic/normoglycemic group, HL/HG – hyperlipidemic/hyperglycemic group, CS – control subjects; SD – standard deviation, TC – total cholesterol, LDL – low-density lipoprotein cholesterol, HDL – high-density lipoprotein cholesterol, TG – triglyceride, HbA_{1c} – glycosylated hemoglobin A_{1c}.
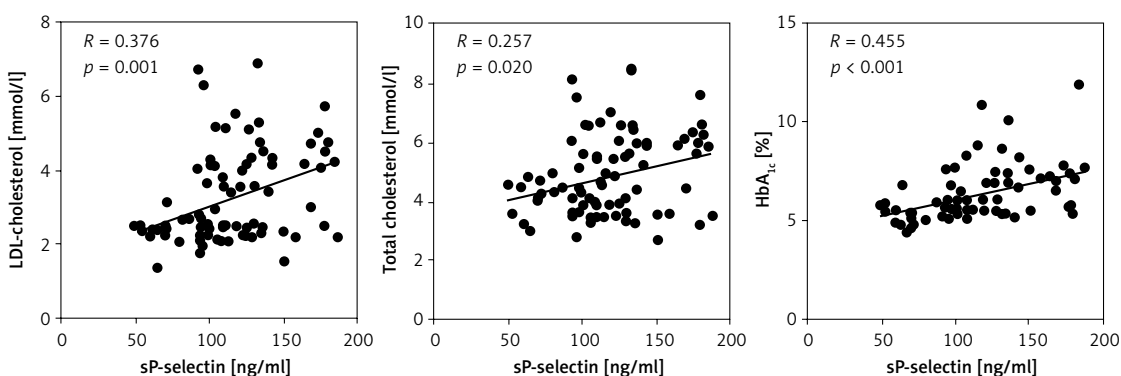

Figure 1. Correlations between sP-selectin concentration and LDL, total cholesterol, and HbA_{1c} level

lectin in all stroke patients compared with the control group (NL/NG vs. CS $p < 0.001$ and NL/HG, HL/NG, HL/HG vs. CS $p < 0.0001$). The concentration of sP-selectin was significantly higher in patients with metabolic disorders compared to NL/NG stroke patients (NL/HG vs. NL/NG $p < 0.01$; HL/NG, HL/HG vs. NL/NG $p < 0.001$) (Table II, Figure 2). We were expecting that patients with hyperlipidemia and hyperglycemia (two factors responsible for platelet activation) would present higher concentrations of sP-selectin than patients with a single risk factor. Nevertheless, we

did not observe intensification of platelet activity in the HL/HG group.

Furthermore, we noted a higher percentage of PDMPs in all groups of stroke patients compared to CS ($p < 0.001$), but we did not observe an influence of HL or HG on the percentage of circulating PDMPs (Table II, Figure 3).

The result of our study showed a significantly lower percentage of CD62(+) platelets and lower CD62P expression on resting and thrombin-activated platelets in all groups of stroke patients compared with the controls (Table II, Figure 4). An

Table II. Table presents medians of the percentages of CD62P-positive platelets (%CD62) and the median fluorescence intensity of CD62P expression (med CD62) on resting and thrombin-activated platelets, percentage of platelet-derived microparticles (PDMPs) and soluble P-selectin concentration in blood of the study patients and control subjects

Study parameters	Median				
	Control	NL/NG	NL/HG	HL/NG	HL/HG
%CD62 before thrombin	2.40	1.20*	1.40*	1.05*	1.35
%CD62 after thrombin	28.45	8.00**	3.80**	14.35*	14.40*
Med CD62 before thrombin	6.34	5.34*	4.75*	4.99*	4.90**
Med CD62 after thrombin	29.48	17.97**	14.70**	20.60*	23.88**
PDMPs [%]	8.30	12.80**	12.55**	13.20**	13.25**
sP-selectin [ng/ml]	70.75	107.66**	128.93***#	124.65***##	134.11***##

NL/NG – normolipidemic/normoglycemic group, NL/HG – normolipidemic/hyperglycemic group, HL/NG – hyperlipidemic/normoglycemic group, HL/HG – hyperlipidemic/hyperglycemic group, CS – control subjects; *p < 0.05 vs. control, **p < 0.001 vs. control, ***p < 0.0001 vs. control, #p < 0.01 vs. NL/NG, ##p < 0.001 vs. NL/NG.
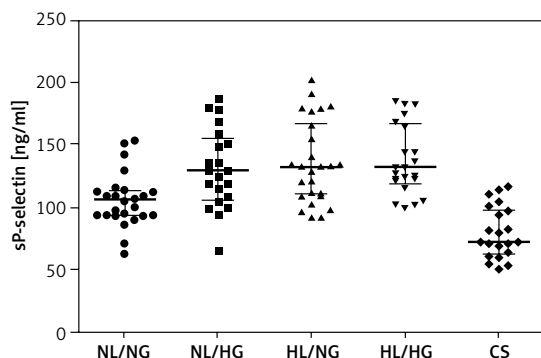
Figure 2. sP-selectin serum concentration was significantly higher in all stroke patients compared to control subjects (NL/NG vs. CS $p < 0.001$ and NL/HG, HL/NG, HL/HG vs. CS $p < 0.0001$) and in patients with metabolic disorders compared to NL/NG stroke patients (NL/HG vs. NL/NG $p < 0.01$; HL/NG, HL/HG vs. NL/NG $p < 0.001$)

NL/NG – normolipidemic/normoglycemic group, NL/HG – normolipidemic/hyperglycemic group, HL/NG – hyperlipidemic/normoglycemic group, HL/HG – hyperlipidemic/hyperglycemic group, CS – control subjects.
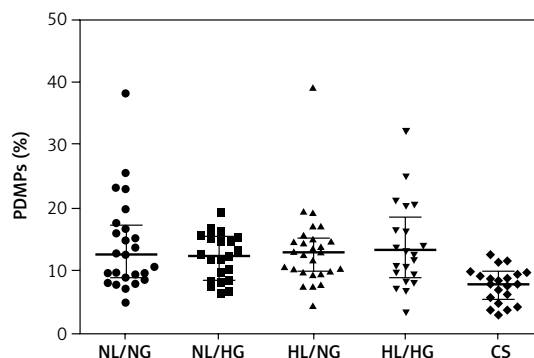

Figure 3. Percentage of PDMPs was significantly higher in all groups of stroke patients compared to CS, $p < 0.001$

NL/NG – normolipidemic/normoglycemic group, NL/HG – normolipidemic/hyperglycemic group, HL/NG – hyperlipidemic/normoglycemic group, HL/HG – hyperlipidemic/hyperglycemic group, CS – control subjects.
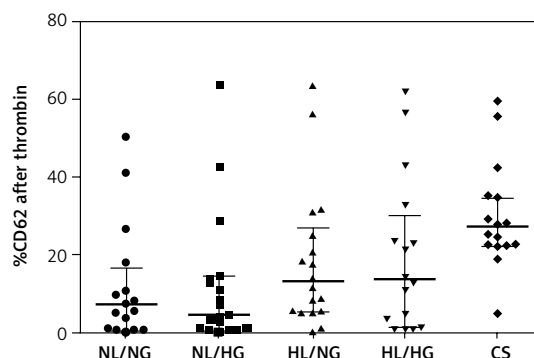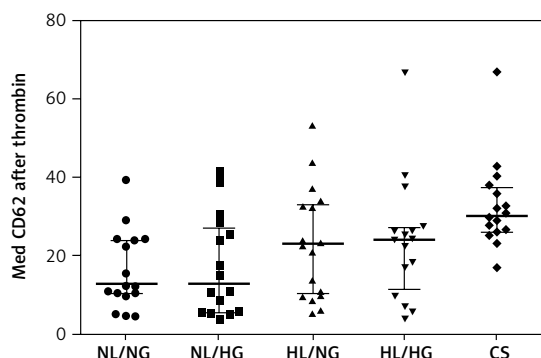

Figure 4. Median of fluorescence CD62P (med CD62) and percentage of platelets CD62P+ (%CD62) before and after stimulation by thrombin were significantly lower in all groups of stroke patients compared with CS

NL/NG – normolipidemic/normoglycemic group, NL/HG – normolipidemic/hyperglycemic group, HL/NG – hyperlipidemic/normoglycemic group, HL/HG – hyperlipidemic/hyperglycemic group, CS – control subjects.
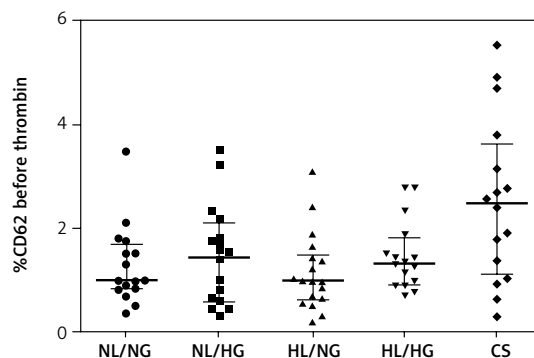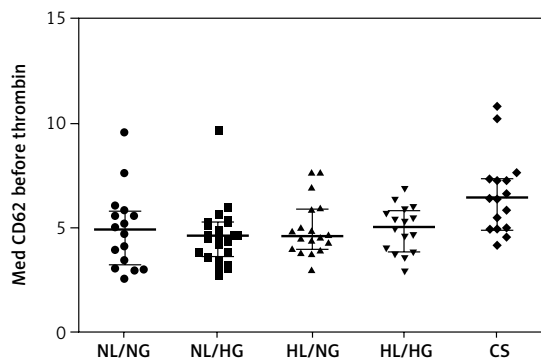
influence of metabolic disorders (hyperlipidemia and hyperglycemia) on the expression of CD62P on resting and thrombin-activated platelets was not found. There were no significant differences in the median of CD62P fluorescence and percentage of CD62P(+) platelets before and after thrombin activation between the stroke groups (Table II, Figure 4).

Discussion

Metabolic disorders such as hyperglycemia and hyperlipidemia play a crucial role in the pathogenesis of vascular events such as thrombosis, inflammation and atherosclerosis [6]. Thus, they are the main risk factors of cerebrovascular diseases.

The aim of our study was to assess the impact of these metabolic disturbances on sP-selectin

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concentration, platelet P-selectin expression and PDMPs. The results of our study demonstrate the significant influence of hyperlipidemia and hyperglycemia on sP-selectin concentration in patients with ischemic stroke. We observed a significant increase in sP-selectin plasma concentration in stroke patients with hyperlipidemia and hyperglycemia compared to normolipidemic and normoglycemic stroke patients. We also found a significant positive correlation between LDL, total cholesterol, HbA_{1c} level and sP-selectin concentration. This observation confirms the significant influence of hyperlipidemia and hyperglycemia on endothelium and platelet activation. We cannot be sure whether the higher sP-selectin levels observed in our study were released from the platelet α-granule membrane or from the Weibel-Palade body membrane of endothelial cells.

Circulating atherogenic lipoproteins have been shown to activate platelets, and hence the changes in platelet function may also be secondary to the vascular disease caused by the dyslipidemia [4]. In accordance with our results, Ferroni et al. [20] and Davi et al. [21] confirmed the relationship between increased LDL concentration and plasma sP-selectin level. They concluded that hyperlipidemia is associated with an increase of plasma sP-selectin level. Other studies have also confirmed the influence of hyperlipidemia on platelet activity [22, 23]. Data of these studies showed a significant positive correlation between platelet P-selectin expression and increased LDL level. These studies confirmed the hypothesis that hypercholesterolemia may be associated with persistent platelet activation. On the other hand, high cholesterol level is frequently associated with development of atherosclerosis. It has been observed that P-selectin expression is increased in endothelial cells overlying atherosclerotic plaques [24]. Thus high P-selectin plasma levels may also be an index of the presence of vascular lesions [21].

Hyperreactive platelets are also the major determinants of the prothrombotic state in diabetic patients [25]. The causes of the enhanced platelet activation in diabetes are multifactorial. Platelet abnormalities include altered adhesion, aggregation and hypersensitivity to agonists. Of note, glycosylation and oxidation caused by hyperglycemia increase atherogenicity of circulating LDL. All these mechanisms may potentially contribute to platelet hyperfunction and hypersensitivity [26, 27]. Changes in the cell membrane dynamics observed in diabetes result in altered exposure of surface membrane receptors [28]. P-selectin has been identified as the more important adhesion molecule. A previous study by Nomura et al. [29] confirmed these findings. They demonstrated an elevated soluble P-selectin level in hyperlipidemic patients with type 2 diabetes, and an association

with increased oxidized LDL. We also observed a significantly higher concentration of sP-selectin in all groups of stroke patients with hyperglycemia. It is important to add that the strongest positive correlation was observed between hyperglycemia (HbA_{1c}) and sP-selectin levels. This observation emphasizes the leading role of hyperglycemia in atherothrombosis progression. We could expect the intensification of platelet activity and higher sP-selectin concentration in patients with hyperlipidemia and hyperglycemia. Nevertheless, we did not observe additional effects of hyperlipidemia and hyperglycemia on platelet activity markers.

The interesting role of sP-selectin is stimulating the release of procoagulant microparticles, inducing a procoagulant state [30, 31]. A number of studies have established a clear relationship with thrombosis development in cerebrovascular ischemic diseases [32, 33]. Nomura et al. [34] reported significantly higher PDMP blood concentrations in diabetic patients with increased LDL levels than in diabetic patients with low LDL levels. This finding suggests that PDMPs may participate in the development and progression of atherosclerosis. On the other hand, our data indicated an increase of PDMP level in all stroke patients, but without an influence of hyperlipidemia or hyperglycemia on PDMP concentration. These results are concordant with the findings of Cherian et al. [35]. They observed significantly higher PDMP levels within 7 days of stroke onset in comparison to controls. Elevated levels of PDMPs observed in stroke patients may confirm the important role of PDMPs in coagulation and their thrombogenic potential. It is also possible that the local generation of PDMPs in atherosclerotic arteries may promote arterial occlusion [36].

On the other hand, an increased PDMP level in the convalescent phase after ischemic stroke was confirmed by other studies [32, 33]. Thus, it is currently difficult to determine whether the origin of the PDMPs results from acute ischemic stimulation or it is the consequence of chronic platelet activation.

Many studies have demonstrated the increase of platelet P-selectin expression in ischemic stroke [37–42], reflecting platelet hyperactivity during the acute phase of ischemia. However, our study did not demonstrate higher expression of platelet P-selectin during the first 7 days of stroke. We observed significantly lower platelet P-selectin expression in all stroke patients compared to control subjects. One explanation of this finding may be the fact that in previous studies the time point of P-selectin assessment in acute stroke was different. In some studies the assessment of platelets was carried out relatively late (after 4 weeks) [42], while in others platelet activation was assessed

within 24 h from the onset of stroke [37, 39]. However, our data are supported by the study of Marquardt *et al.* [40]. They observed an initial increase in P-selectin platelet expression only on the first day, then platelet P-selectin expression declined over time after the stroke, and the difference between the stroke group and controls was no longer significant on day 14 after the stroke. The rapid decrease in P-selectin expression may be caused by the shedding from the cell surface to the plasma pool. This hypothesis may explain our results, because we observed lower expression of platelet P-selectin but a significantly higher soluble P-selectin level in stroke patients compared to CS. These results may be evidence of rapid platelet activation in the acute phase of stroke. Thus, in view of our findings and the results of previous studies, we suggest that sP-selectin is a better marker of platelet activation than platelet P-selectin. However, we should remember that the origin of sP-selectin in blood may reflect the release of this adhesion molecule from activated platelets and also from damaged endothelial cells [43]. So, additional assessment of an endothelial injury marker could be helpful in this analysis.

It is noteworthy that we also observed decreased platelet reactivity in stroke patients. We observed lower platelet P-selectin expression after thrombin activation in all stroke patients compared to CS. This finding may be due to exhaustion of P-selectin stores in alpha granules, and this may partly explain the early hemorrhagic complication in the acute phase of stroke. Our observation is consistent with the results of Lukasik *et al.* [44]. They also confirmed lower surface expression of P-selectin under thrombin receptor activating peptide (TRAP) stimulation in patients with acute stroke.

In conclusion, plasma sP-selectin is a good marker reflecting the significant influence of metabolic disorders such as hyperglycemia and hyperlipidemia on vascular lesions and platelet activation in stroke patients. Metabolic disturbances exert an equal stimulatory effect on tested platelet markers but with no synergistic action. However, the results of our study point to the leading role of hyperglycemia in the atherothrombotic process. The enhanced sP-selectin concentration in atherosclerotic ischemic stroke is the consequence of atherogenic and platelets' procoagulant properties of oxidized LDL and hyperglycemia, but an increased level of this molecule may also reveal the effect of acute ischemic events [36]. Further studies are required to identify the main source of plasma P-selectin origin in patients with metabolic disturbances, because the increased sP-selectin level mediates a procoagulant state, potentially exacerbating the prothrombotic predisposition associated with hyperlipidemia and hyperglyce-

mia. Thus, sP-selectin is a good target for further research focusing on prevention of ischemic cerebrovascular diseases.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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