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RESEARCH

The metabolism of big endothelin-1 axis and lipids affects carotid atherosclerotic plaque stability – the possible opposite effects of treatment with statins and aspirin

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Abstract

Background The understanding of endothelin's role in carotid plaque instability is limited. We have studied the big endothelin-1 (ET-1) axis and its role in carotid plaque stability in patients undergoing carotid endarterectomy (CEA). The interactions of endothelins with known CVD risk factors were also evaluated.

Methods We studied 77 patients, who were divided into subgroups based on the optimal cut-off for grey-scale median (GSM), a marker of plaque instability. GSM values < 46.87 were designated as unstable carotid plaque, while GSM ≥ 46.87 were assigned to stable plaque. Twelve people without carotid atherosclerosis served as controls. Big ET-1, ET-1 and ET-1 (1–31) were measured and the endothelin-converting enzyme-1 (ECE-1) and chymase activity were calculated. Clinical and laboratory parameters were also evaluated.

Results ET-1 levels and ECE-1 activity were increased in all patient groups compared to controls (all $P < 0.001$) – and were higher in patients with unstable plaque than in those with stable plaque ($P < 0.01$). ET-1 (1–31) did not differ between the groups. ET-1 levels and ECE-1 activity inversely correlated with total cholesterol, LDL-cholesterol, and GSM values, whereas GSM was positively associated with total cholesterol and LDL fractions. Detailed analysis of patients according to the pharmacotherapy used revealed that statins favored ET-1 formation independently of cholesterol-lowering properties, whereas aspirin reduced this effect.

Conclusions ET-1 formation is the main pathway of big ET-1 metabolism in patients with carotid atherosclerosis, especially in those with plaque instability. Statins and aspirin appear to have opposing effects on ET-1 formation, suggesting the greater benefit related to plaque stability in patients taking both drugs concomitantly.

Keywords Aspirin · Carotid endarterectomy · Endothelins · Grey-scale median · Statin

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Abbreviations

ASA	Aspirin
BMI	Body mass index
CCA	Calcium channel antagonists
CEA	Carotid endarterectomy
CKD	Chronic kidney disease
CRP	C-reactive protein
ECs	Endothelial cells
ECE-1	Endothelin-converting enzyme-1
ELISA	Enzyme-linked immunosorbent assay
ETB	Endothelin type B receptor
ET- 1	Endothelin-1
ET-1 (1–31)	Endothelin-1 (1–31)
EVAR	Endovascular aneurysm repair

GSM	Grey-scale median
HDL	High-density lipoprotein cholesterol
IL-1	Interleukin-1
IL-6	Interleukin-6
LDL	Low-density lipoprotein cholesterol
NF-κB	Nuclear factor of activated B-cells
PCSK9	Proprotein convertase subtilisin/kexin type 9
RAAS	Renin-angiotensin-aldosterone system
TNF-α	Tumor necrosis factor-α
TG	Triglycerides
VSMCs	Vascular smooth muscle cells

Introduction

Unstable carotid plaques are the major factors of a higher risk of ischemic stroke and transient ischemic attack [1, 2]. The stability of plaque is influenced by its composition–stable plaque consists of a high amount of fibrous tissue and calcification, whereas unstable plaques contain a lipid-rich necrotic core and inflammatory cells [3–5].

The endothelin system involving endothelins – their precursors, receptors, and endothelin-mediated signaling – plays an important role in several diseases, including cardiovascular diseases and stroke. Endothelins are secreted by different cells, mainly by endothelial cells (ECs) but also by vascular smooth muscle cells (VSMCs), fibroblasts, leukocytes, and macrophages. Endothelin-1 (ET-1) is the most abundant endothelin in the human cardiovascular system. It mainly acts on vascular smooth muscle and causes vasoconstriction by activation of its receptor ETA, whereas ET-1 secreted by inflammatory cells can stimulate the production of proinflammatory cytokines [6, 7]. Mature ET-1 is generated by enzymatic cleavage of the initial preproET-1, which is processed by a furin-type proprotein convertase into the inactive intermediate 38-amino acids, big ET-1. Next, big ET-1 can be cleaved by the endothelin-converting enzyme-1 (ECE-1) to produce the mature 21-amino acid peptide - ET-1 [8]. Moreover, big ET-1 may be cleaved by a human chymase expressed in mast cells to a 31-amino acid-length endothelin, named ET-1 (1–31) [9].

Several clinical studies have demonstrated the role of ET-1 in coronary artery atherosclerosis [10–12]. The pro-atherogenic effects of ET-1 have also been confirmed in an experimental model of atherosclerosis in mice, where the ETA receptor blockade was able to attenuate lesion development [13]. In contrast to the extensive research in the field of coronary arteries, studies on the effects of the endothelin system in carotid atherosclerosis are far more limited. A decreased circulating ET-1 level was observed 7 days after surgical endarterectomy (CEA) in the group of patients with monolateral carotid stenosis [14]. Ihling et al. [15] found that both ECE-1 and ET-1 were present in ECs, VSMCs, and macrophages of the inflammatory intimal regions of carotid plaques, indicating that the upregulation of the ECE-1/ET-1 system may contribute to the regulation of vascular tone in atherosclerotic lesions. Immunohistochemical studies of the carotid atherosclerotic plaque have also confirmed that the expression of ET-1 was greater in those areas in which the macrophages and lipid nucleus were located [16]. These authors also show the co-localization of ET-1 and arginase 2 in human carotid atherosclerotic plaques and have demonstrated that ET-1 stimulated arginase 2 expression and activity in endothelial cells, as well as arginase activity and reactive oxygen species production in macrophages, via an arginase-dependent mechanism.

Many imaging techniques are used to identify vulnerable carotid plaque. The gray-scale median (GSM), calculated based on the echogenicity of the ultrasound image, is recognized as a good marker of plaque instability. Previous studies showed numerous risk factors for atherosclerotic plaque instability. Identification of new indicators of unstable plaque is necessary to understand the pathophysiology of vascular diseases better. It has been shown that high GSM values define the more stable plaque, which is predominately rich in fibrous tissue and calcification, whereas low GSM reflects the vulnerable plaques with high lipid, inflammation, and hemorrhage content [3–5]. Particularly, LDL- cholesterol was the best lipid predictor for the extent of atherosclerosis, whereas triglyceride-rich lipoproteins seem to predict an unstable plaque [17]. It has been also demonstrated that aortic arterial stiffness was associated with unstable, echogenic plaques independently of conventional cardiovascular risk factors [18].

Recently, we showed that mean GSM values in symptomatic patients undergoing CEA were significantly lower than those in asymptomatic people. Using the receiver operating characteristic analysis we also proved that GSM can be a useful tool to distinguish between symptomatic and asymptomatic carotid artery disease [19]. In the present study, we aim to evaluate serum big ET-1 axis, and the role of individual components of this system in the instability of the atherosclerotic plaque in a group of patients who have undergone CEA. The patients were divided into subgroups based on the optimal cut-off for GSM (value 46.87) that distinguished individuals with stable and unstable carotid plaque [17]. We would also evaluate how endothelins interact with plasma lipids, inflammatory status, and other known cardiovascular risk factors in this population.

Materials and methods

Patients

The study enrolled patients who had undergone CEA in the Department of Vascular Surgery and Transplantation, Medical University of Bialystok, between January 2021 and December 2022, as was detailed presented in our previous article [19]. Seventy-seven patients who had completed laboratory examinations were included in the study. The patients were divided into 2 subgroups and analyzed based on an optimal cut-off for GSM [19]. In the present study, GSM values < 46.87 were designated as unstable carotid plaque, while GSM ≥ 46.87 were assigned to more stable plaque.

A control group for measurement of serum endothelins, lipids, and inflammation markers was also recruited. The patients enrolled in the control group were individuals qualified for endovascular aneurysm repair (EVAR) of abdominal aortic aneurysms (AAA), but had no signs of atherosclerosis. These were patients of similar age and sex, nonobese (BMI < 30 kg/m^2), without a history of malignancies and carotid atherosclerosis. No previous cerebrovascular ischemic events (transient ischemic attack, stroke), no evidence of carotid artery stenosis, and no detectable carotid plaques have been confirmed by preoperative Doppler ultrasound examination. Moreover, each of these patients underwent an angio-CT scan and Doppler ultrasound examination of the aorta and iliac arteries, as well as a Doppler ultrasound evaluation of the vertebral arteries, as part of the preoperative assessment. The exclusion criterion was the detection of atherosclerotic changes in the carotid arteries, vertebral arteries or in the aortoiliac segment in the aforementioned examinations.

The study was approved by the Ethics Committee of the Medical University of Bialystok (APK.002.390.2020), and written informed consent has been obtained from all participants in accordance with the Declaration of Helsinki. Hypertension, chronic kidney disease (CKD), diabetes, smoking status, and details of ultrasound examination of carotid arteries and carotid plaque GSM analysis have been described in the previous study [19].

Biochemical analysis

Venous blood samples were collected from participants in the morning, after at least 12 h of fasting, the day before the scheduled CEA procedure. Serum lipid profile: total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), high-density lipoprotein cholesterol (HDL-C), and triglycerides, were assayed by routine laboratory techniques using a biochemical analyzer Cobas C311, Roche, Mannheim, Germany. Non-HDL cholesterol was calculated using the formula: non-HDL cholesterol = total cholesterol – HDL-cholesterol. Serum C-reactive protein (CRP) was analyzed using a nephelometric technique (Beckman Coulter Image 800; Fullerton, CA, USA; normal range: 0–0.8 mg/dL).

Measurement of serum endothelins

Serum endothelin-1 (ET-1), big ET-1, and ET-1 (1–31) were assessed using immunoassays: Human ET-1 Quantikine ELISA, cat. no DET 100 (R&D Systems, Inc, Abingdon, UK), Human big ET-1 Platinum ELISA, cat. no BMS2266 (Invitrogen, Thermo Fisher Scientific, Warsaw, Poland) and Human ET-1 (1–31) ELISA, cat. no TK01276 (TZY Bio-Tech, Wuhan, China); respectively. All procedures were performed according to the instructions provided by the manufacturers. Analytical curves for the analyzed proteins were drawn to determine the protein concentration. The absorbance of the samples was measured using a Multiskan FC Microplate Photometer (Thermo Scientific, Waltham, USA).

Because the endothelin-converting enzyme (ECE-1) and chymase use big ET-1 as a substrate to generate the biologically active products ET-1 and ET-1 (1–31), respectively [8, 9], the activity of ECE-1 and chymase was calculated indirectly by the determination of product-to-substrate ratio [20].

Carotid ultrasound acquisition

Ultrasound examination and carotid plaque GSM analysis was described in detail previously [19]. Briefly, the examinations were performed by an experienced ultra-sonographer (A.P.), following the protocol of standard Polish clinical settings, using the ultrasound device and the VF13-5 linear transducer with a frequency band of 4.4–13 MHz (SIEMENS ACUSONX300, Siemens Healthcare, Erlangen, Germany). Obtained DICOM images with visible atherosclerotic plaque were formatted in jpg format and analyzed in the Liver Analyzer version 2.8.6.3c, evaluating the gray-scale median image based on Adobe Photoshop software Version 22.1.1 (Adobe Systems, San Jose, CA, USA).

Statistical analysis

The normality of the distribution was verified by the Shapiro–Wilk test. Categorical variables are reported as percentages (%), and continuous variables as means ± standard deviations (SDs) or medians and first quartile (QR) - third quartile (QR), depending on their distribution. The Chi-squared test was used to compare the qualitative data. The

unpaired t-test or one-way analysis of variance (ANOVA) was used for comparisons of normally distributed continuous variables. The Mann–Whitney or Kruskal-Wallis test was used for comparisons of non-normally distributed variables. We checked for correlations of variables using the Spearman test or quasi-Newton and Rosenbrock logistic regression analysis. A 2-sided *p*-value below 0.05 was considered significant. Statistica ver 13.1 computer software (StatSoft, Tulsa, OK, US) and GraphPad Prism 10.2.2 (GraphPad Software, La Jolla, CA, USA) were used in the calculations of results and presented graphically.

Table 1 Clinical characteristics of patients with Grey-scale median (GSM) < 46.87, GSM ≥ 46.87 and controls (CON)

	CON $n=12$	GSM < 46.87 $n=32$	GSM ≥ 46.87 $n=45$	p value
Age, years	72.0 ± 9.4	67.8 ± 8.2	68.1 ± 8.9	0.325[1]
Male/female, %	83.3	84.4	68.9	0.138
BMI, kg/m^2	27.5 ± 2.30	27.24 ± 4.19	26.57 ± 4.28	0.689[1]
Cholesterol, mmol/l	4.42 (3.674.63)	3.75 (3.33–4.19)	4.54 (3.52–5.38)	0.025[2]
LDL, mmol/l	2.59 (2.04–2.74)	1.98 (1.79–2.35)	2.65 (1.86–3.62)	0.046[2]
HDL, mmol/l	1.40 (1.27–1.53)	1.09 (0.96–1.27)	1.14 (0.88–1.40)	0.298[2]
Non-HDL, mmol/l	3.03 (2.40–3.61)	2.56 (2.17–2.82)	3.36 (2.46–4.24)	0.006[2]
TG, mmol/l	1.12 (0.99–1.31)	1.44 (1.12–1.84)	1.26 (1.05–1.60)	0.152[2]
CRP, mg/l	2.8 (0.9–4.9)	2.7 (1.1–5.6)	2.9 (1.2–5.5)	0.881[2]
Hypertension, %	83.3	90.6	86.7	0.972
CKD, %	16.7	6.3	20.0	0.356
Diabetes, %	25.0	25.0	33.3	0.438
Smoking, %	66.7	78.1	82.2	0.267
Diuretics, %	33.3	28.1	31.1	0.997
RAAS inhibitor, %	75.0	75.0	71.1	0.711
β-blockers, %	66.7	46.9	44.4	0.238
CCA, %	25.0	28.1	40.0	0.220
Aspirin, %	83.3	75.0	75.6	0.957
Statins, %	41.7	75.0	57.8	0.852

Categorical data are presented as percentage (%), continuous variables are presented as mean ± standard deviations if distributed normally, or as median (first quartile-third quartile) for non-normally distributed data; χ^2 test for trend was used to compare the qualitative data; [1]continuous variables normally distributed were analysed using one-way ANOVA test; [2]continuous variables non-normally distributed were analysed using Kruskal-Wallis test with Dunn's post-test

The patients group included individuals with GSM < 46.87 ($n=32$) and with GSM ≥ 46.87 ($n=45$), who had undergone carotid endarterectomy (CEA) procedure. The participants enrolled in the control group ($n=12$) were individuals qualified for endovascular aneurysm repair of abdominal aortic aneurysms, without signs of carotid atherosclerosis. The study was performed in the Department of Vascular Surgery and Transplantation, Medical University of Białystok, Poland between January 2021 and December 2022

Abbreviations: BMI, body mass index; CCA, calcium channel antagonists; CKD, chronic kidney disease; CRP, C-reactive protein; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; RAAS, renin-angiotensin-aldosterone system; TG, triglycerides

Results

The present study involved 32 patients who had undergone CEA with GSM < 46.87, 45 patients with GSM ≥ 46.87, and 12 individuals without features of carotid atherosclerosis, who served as controls. The clinical characteristics of the participants in each group are shown in Table 1. No significant differences were observed between the studied groups in terms of age, sex, BMI, inflammatory status, comorbidities, and the pharmacotherapy used, except for statins, which were more often prescribed in the group with GSM < 46.87 than in controls. The patients with GSM < 46.87 exhibited lower levels of total cholesterol and LDL-cholesterol in comparison with the controls and the group with GSM ≥ 46. Non-HDL cholesterol was higher in the group with GSM ≥ 46.87 than in the group with GSM < 46.87. Conversely, the levels of triglycerides tended to be higher in the group with GSM < 46.87 than in the controls.

Changes in the components of the big-ET-1 axis in the studied groups

The transformation of big ET-1 into biologically active products in the studied groups is schematically presented in Fig. 1A. In the whole group of patients, big-ET-1 showed a tendency to increase compared to the control group ($H=8.676$, $p=0.034$), however, a statistically significant increase in this parameter was observed between the group with GSM ≥ 46.87 in comparison to controls and patients with GSM < 46.87 (Fig. 1B). The ET-1/big ET-1 ratio, a calculated index of ECE-1 activity, was significantly higher in all patient groups than in controls ($H=20.336$, $p=0.0001$). ECE-1 value was higher in the group with GSM < 46.87 compared to the group with GSM ≥ 46.87 (Fig. 1C). Analogously to the above results, the concentration of the active product of this transformation pathway ET-1 was significantly higher in all patient groups compared to controls ($H=34.144$, $p<0.0001$), with the highest ET-1 values observed in the group with GSM < 46.87 (Fig. 1E). In contrast, calculated chymase activity tended to decrease in all patients compared to controls ($H=9.378$, $p=0.025$), with the lowest activity noted in the group of patients with GSM ≥ 46.87. In turn, patients with GSM < 46.87 showed the highest activity of this enzyme compared to all patients, especially compared to the group with GSM ≥ 46.87

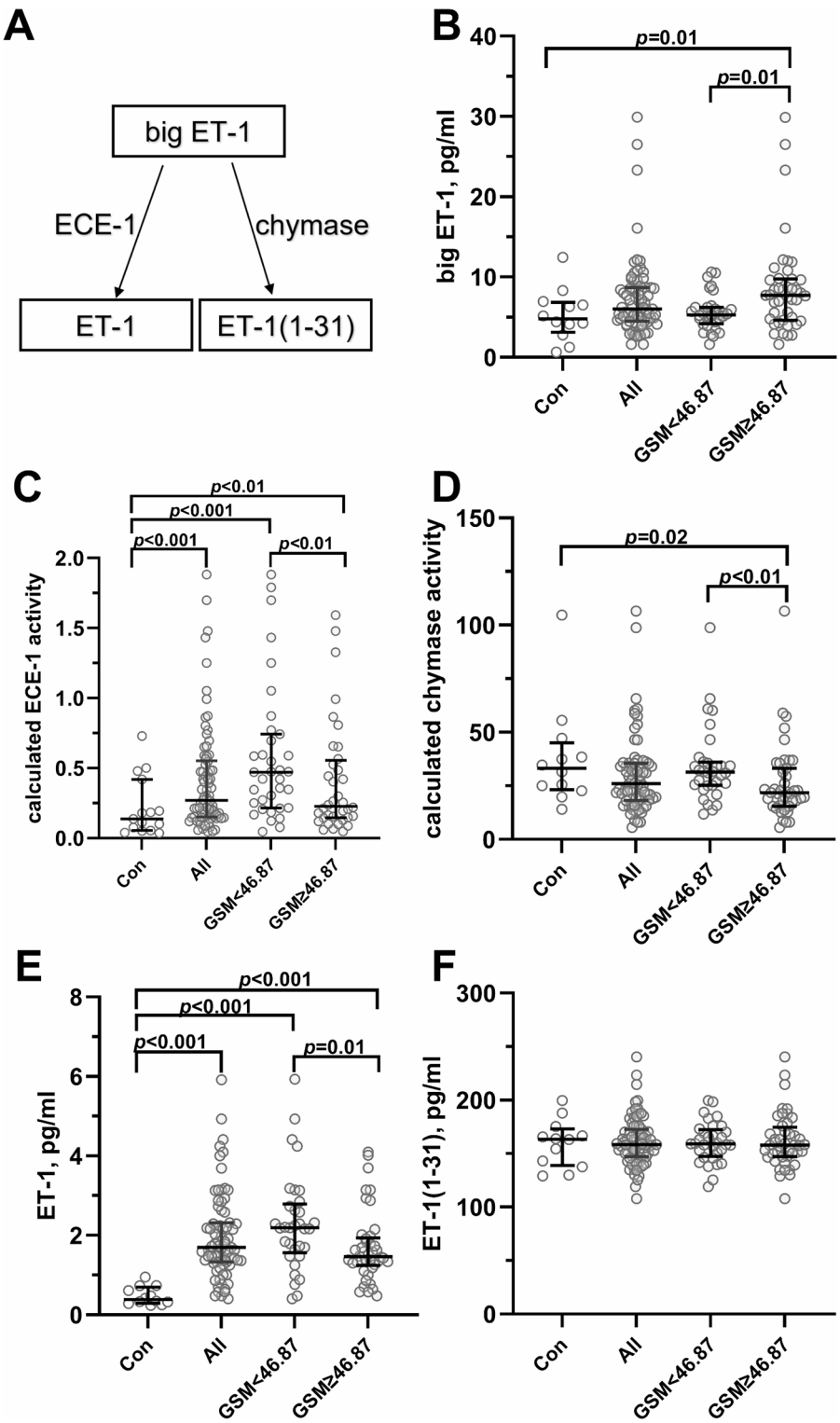

Fig. 1 The components of the big ET-1 axis in the serum of controls, all patients who have undergone carotid endarterectomy, and in subgroups with GSM < 46.87 and GSM ≥ 46.87. Points show a spread of the data. The middle line represents the median, and the lower and upper lines represent the first and third quartiles, respectively. Points beyond the quartiles represent outlier values. Panel A schematically shows the transformation of big ET-1 into ET-1 and ET-1 (1–31). Statistical analysis for data presented in panels B- F was performed using the Kruskal-Wallis test with Dunn's post-test. The patients group included individuals with GSM < 46.87 ($n = 32$) and with GSM ≥ 46.87 ($n = 45$), who had undergone carotid endarterectomy (CEA) procedure. The participants enrolled in the control group ($n = 12$) were individuals qualified for endovascular aneurysm repair of abdominal aortic aneurysms, without signs of carotid atherosclerosis. The study was performed in the Department of Vascular Surgery and Transplantation, Medical University of Białystok, Poland between January 2021 and December 2022. Abbreviations: Con, controls; ET-1, endothelin −1; ET-1 (1–31), endothelin-1 (1–31); big ET-1, big endothelin-1; ECE-1, endothelin converting enzyme-1 activity calculated as ET-1/big ET-1 ratio; GSM, grey- scale median

(Fig. 1D). The concentration of ET-1 (1–31) did not differ significantly in all groups tested (H = 0.173, $p = 0.982$), (Fig. 1F).

Associations between the endothelins system, GSM values, and clinical and laboratory parameters in the whole patient group

A significant positive correlation was observed between ET-1, big ET-1, and age ($r = 0.347$ and $r = 0.319$, both $p < 0.01$), whereas the inverse relationship was between age and the calculated chymase activity ($r = -0.307$, $p = 0.008$). Quasi-Newton and Rosenbrock logistic regression analysis showed that the male sex was associated with higher ET-1 levels ($\chi^2 = 8.496$, $p = 0.004$), whereas the presence of hypertension was associated with elevated levels of both big ET-1 ($\chi^2 = 4.483$, $p = 0.03$) and ET-1 (1–31) ($\chi^2 = 3.484$, $p = 0.05$). The comorbidity of CKD was also associated with higher levels of big ET-1 ($\chi^2 = 4.387$, $p = 0.04$). There was no association between ET-1 and ET-1 (1–31) levels ($r = 0.121$, $p = 0.31$), while a strong positive relationship was observed between ET-1 and calculated ECE-1 activity ($r = 0.736$, $p < 0.001$). Big ET-1 was inversely related to ECE-1 and chymase activities ($r = -0.970$ and $R = -0.717$, both $p < 0.001$).

Analysis of associations between the endothelins and laboratory parameters revealed an inverse correlation between total cholesterol, LDL-cholesterol, non-HDL cholesterol and ET-1 levels (Fig. 2, A-C) and the transformation of big ET-1 into ET-1 (Fig. 2D-F). As has been presented in Fig. 3, levels of total cholesterol, LDL-cholesterol and non-HDL cholesterol were positively associated with GSM values (Fig. 3, A-C), whereas ET-1 and calculated ECE-1 activity were inversely correlated with GSM (Fig. 3, D-E).

The components of the big ET-1 axis, lipids, and GSM values in patients treated with Statin and aspirin

Forty-nine patients in the whole group were treated with statins (simvastatin 20 mg/d or 40 md/d, $n = 16$; atorvastatin 20 mg/d or 40 md/d, $n = 17$ and rosuvastatin 5 mg/d or 20 mg/d, $n = 16$). The quasi-Newton and Rosenbrock logistic regression analysis revealed that statin treatment tended to be associated with higher levels of ET-1 ($\chi^2 = 2.894$, $p = 0.08$) and with calculated ECE-1 activity ($\chi^2 = 3.766$, $p = 0.05$). On the other hand, 53 patients received acetylsalicylic acid (aspirin; ASA) at a dose of 75 mg/d, and we noticed that the treatment with ASA was associated with a reduction of ET-1 levels ($\chi^2 = 4.231$, $p = 0.04$). Since 55% of patients were treated concomitantly with a statin and ASA,

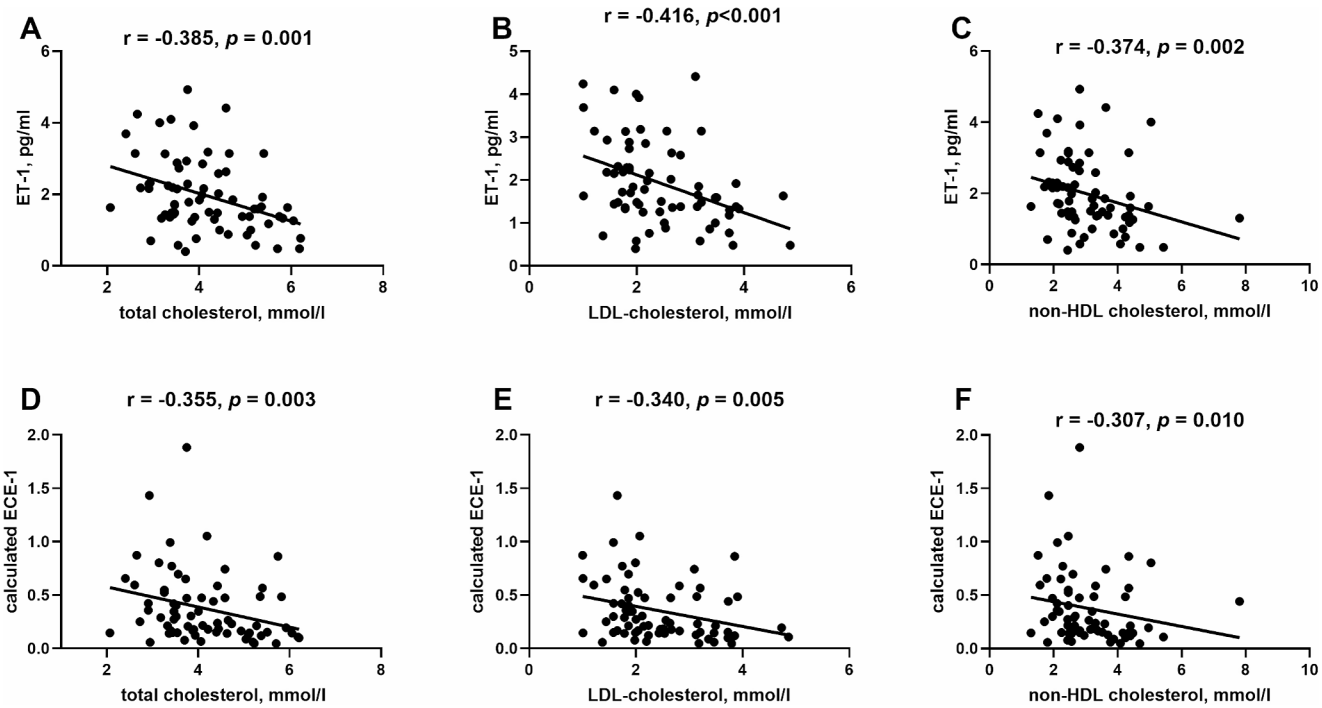

Fig. 2 Correlations between total cholesterol, LDL-cholesterol, non-HDL cholesterol levels, and endothelin-1 (ET-1; **A-C**), and between above lipid parameters and the endothelin-converting enzyme-1 activity (ECE-1; **D-F**) in all patients ($n = 77$) who have undergone carotid endarterectomy. For the correlation analysis between variables, Spearman's rank-order correlation method was used. The patients group included individuals with GSM < 46.87 ($n = 32$) and with GSM ≥ 46.87 ($n = 45$), who had undergone carotid endarterectomy (CEA) procedure in the Department of Vascular Surgery and Transplantation, Medical University of Białystok, Poland between January 2021 and December 2022

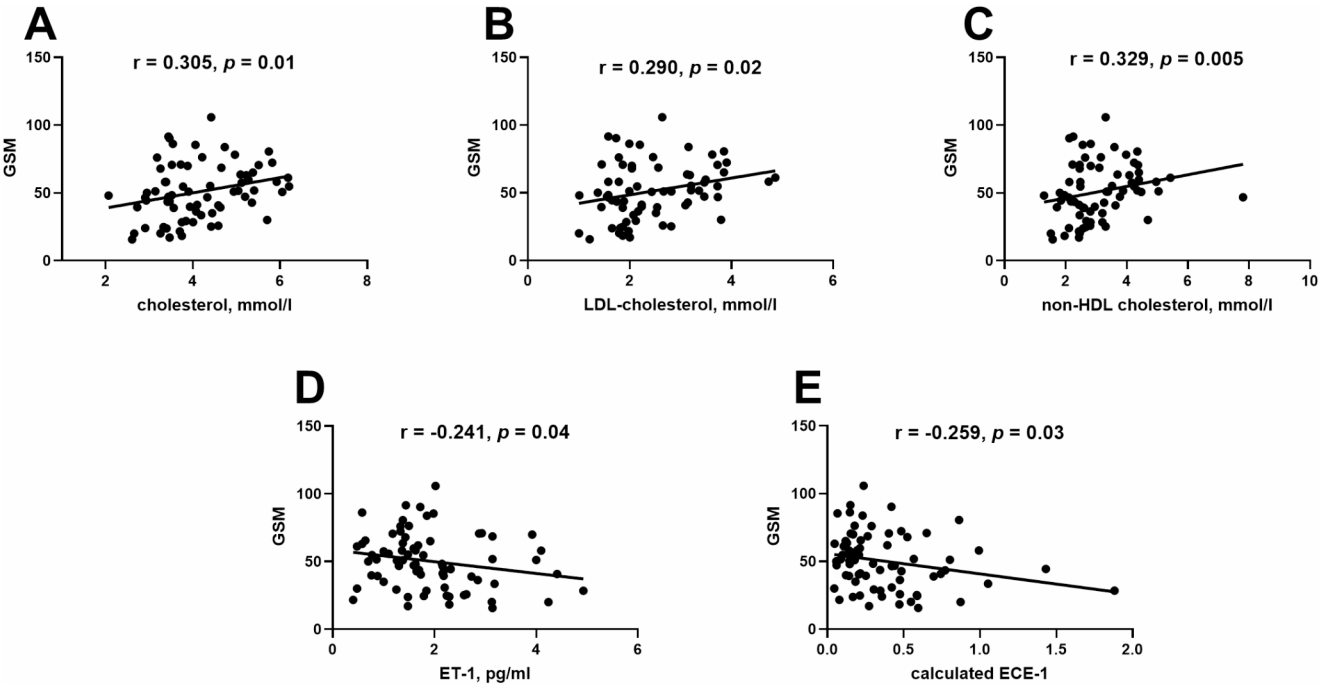

Fig. 3 Correlations between grey-scale median (GSM) values and total cholesterol (**A**), LDL-cholesterol (**B**), non-HDL cholesterol (**C**), endothelin-1 (ET-1; **D**), and the endothelin-converting enzyme-1 activity (ECE-1; E) in all patients ($n=77$) who have undergone carotid endarterectomy. For the correlation analysis between variables, Spearman's rank-order correlation method was used. The patients group included individuals with GSM < 46.87 ($n=32$) and with GSM ≥ 46.87 ($n=45$), who had undergone carotid endarterectomy (CEA) procedure in the Department of Vascular Surgery and Transplantation, Medical University of Białystok, Poland between January 2021 and December 2022

to determine the net effect of statin or aspirin on the ET-1/big ET-1 system and GSM, we distinguished 4 groups of patients: those treated simultaneously with statin and ASA ($n=42$), those treated only with a statin ($n=8$) or only with ASA ($n=12$), and those receiving none of the above drugs ($n=15$). As suspected, statin therapy was associated with a reduction in total cholesterol and LDL-cholesterol levels (Fig. 4, A-B), without affecting HDL-cholesterol and triglycerides levels (data not shown). Moreover, the patients taking only ASA had higher levels of total cholesterol and particularly LDL-cholesterol than those not taking ASA (Fig. 4, A-B). The patients treated with the statin had increased ET-1 values and the index of ECE-1 activity compared to those taking the statin and ASA simultaneously. These differences were especially seen when patients taking statin alone were compared to those taking ASA only (Fig. 4, C-D). The patients taking only statin presented lower levels of big ET-1 (Fig. 4E), and GSM values of their carotid plaque were also lower (Fig. 4F) in comparison to those who were treated with ASA only. There were no differences between the studied groups regarding age, gender, BMI, inflammatory state, comorbidities, and frequency of use of other drug groups (data not shown).

Discussion

The present study examined for the first time the transformation of big ET-1 into ET-1 and ET-1 (1–31) and the impact of this system on the stability of the atherosclerotic plaque in a population of patients with carotid atherosclerosis, who underwent the CEA procedure. We showed that the transformation of big ET-1 into ET-1 is particularly visible in the group of patients with unstable plaque and it is directly related to the GSM score, an indicator of plaque instability. We observed that serum cholesterol levels, especially the LDL fraction, were inversely associated with the ET-1 creation process, and that both the increase in ET-1 concentrations and the reduction of cholesterol levels adversely affected the stability of carotid plaque, expressed as GSM score. From a clinical point of view, the finding that the two classes of drugs commonly used in this population – statins and ASA – seem to have an opposite effect on the formation of ET-1 (which then translates into the stability of the atherosclerotic plaque) seems to be important.

The comparison of the two subgroups (GSM < 46.87 versus GSM ≥ 46.87) found no significant differences in clinical characteristics between them, indicating that the parameters included in Table 1 were not risk factors for carotid plaque instability. The cut-off difference of GSM was relatively narrow, however, this threshold was established based on

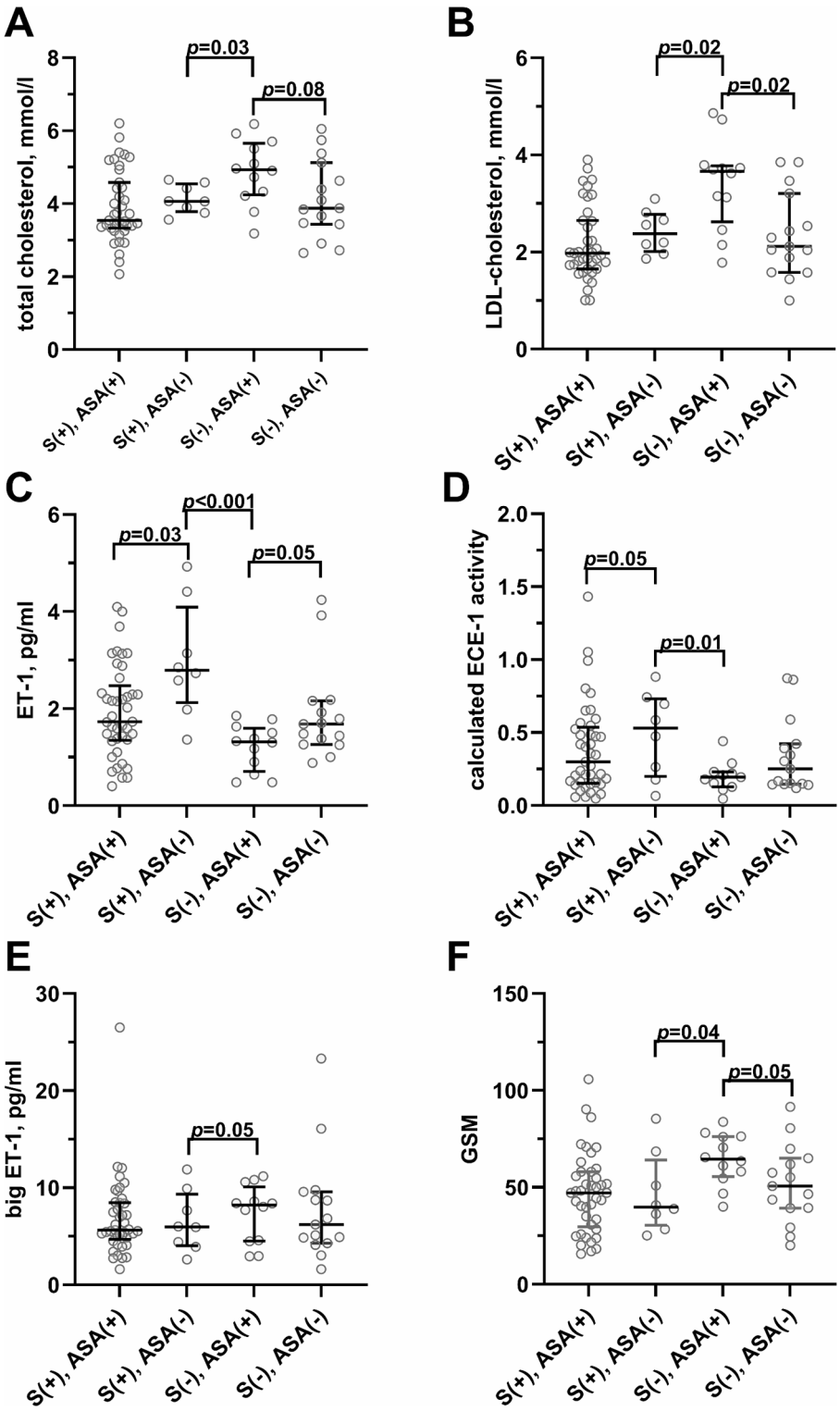

Fig. 4 The effect of statin and aspirin therapy on total cholesterol (**A**), LDL-cholesterol (**B**), endothelin-1 (ET-1; **C**) levels, endothelin-converting enzyme-1 activity (ECE-1; **D**), big endothelin-1 levels (big ET-1; E) and grey-scale median (GSM; F) values.: Points show a spread of the data. The middle line represents the median, and the lower and upper lines represent the first and third quartiles, respectively. Points beyond the quartiles represent outlier values. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post-test. Abbreviations: S(+), ASA(+), the patients treated simultaneously with statin and aspirin ($n=42$); S(+), ASA(-), the patients treated only with statin ($n=8$); S(-), ASA(+), the patients treated with aspirin alone ($n=12$); S(-), ASA(-), the patients not treated with statin or aspirin ($n=15$). The patients group included 77 individuals, who had undergone carotid endarterectomy (CEA) procedure in the Department of Vascular Surgery and Transplantation, Medical University of Białystok, Poland between January 2021 and December 2022

our previous publication [17], where we used receiver operating characteristic (ROC) analysis to determine the optimal cut-off value for distinguishing patients with symptomatic and asymptomatic carotid atherosclerosis. This cut-off was statistically validated, providing a reliable differentiation between stable and unstable plaques.

As has been presented in Fig. 1, the production of ET-1 with ECE-1 participation was the main route of transformation of big ET-1 in patients with carotid atherosclerosis, which was particularly intensified in the subgroup with unstable carotid plaque (GSM<46.87). The major source of ET-1 is the vascular endothelium; from there, ET-1 is

released towards the VSMCs, where it acts as a local hormone, promoting vasoconstriction, hypertension, inflammation, and atherosclerosis. ET-1 may also be produced by inflammatory cells, like macrophages, dendritic cells, and T cells [6, 7]. In circulation, ET-1 is present in picomolar levels, indicating that under standard physiological conditions ET-1 acts mainly as a paracrine and autocrine factor [21]. Because the ETB receptor acts as the primary clearance mechanism for circulating ET-1 [22], raised levels of ET-1 might relate to either increased production or reduced clearance. Herein, we indirectly assessed also the activity of enzymes involved in the transformation of big ET-1, by calculating the ratio of substrate (big ET-1) to enzymatic reaction products: ET-1 and ET-1 (1–31). These ratios include both conversions of big ET-1 and receptor activation by ET-1, giving an approximation of the functional enzyme activity [20]. Despite similar big ET-1 levels in the controls, in the whole patients' group and the subgroup with GSM < 46.87, ECE-1 activity and ET-1 levels were significantly higher in all studied groups compared to the control. However, in the subgroup with GSM ≥ 46.87, both ECE-1 activity and ET-1 levels were significantly lower than in the group with GSM < 46.87, and these patients had increased big ET-1 levels compared to controls and the group with GSM < 46.87. The above data suggest increased ET-1 production, especially in the group of patients with unstable atherosclerotic plaques, and are consistent with the results of the few previous studies conducted in patients with carotid atherosclerosis [15, 16].

Clinical evidence has demonstrated the efficacy of pharmacological intensive lipid-lowering therapy in inducing plaque stabilization or regression. Lipid-lowering agents, especially proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and statins, exhibit not only cholesterol-lowering effects but also anti-inflammatory and antioxidant activities, which contribute to plaque stabilization and regression [23, 24]. A recent study by Cesaro et al. [25] highlights the association between elevated lipoprotein (a) levels and features of vulnerable plaques in patients with acute coronary syndrome, indicating that this lipoprotein is critical in enhancing plaque vulnerability and acute events. Thus, the beneficial effects of lipid-lowering drugs might translate to a decrease in cardiovascular events.

The analysis of associations between the big ET-1 axis and laboratory parameters in all patients revealed inverse relations between ET-1 levels, calculating ECE-1 activity and lipids, namely total cholesterol, LDL-cholesterol and non-HDL cholesterol (Fig. 2). We also observed that the interaction between ET-1 and lipids translates into atherosclerotic plaque stability (Fig. 3). Literature data on the relationship between lipids and ET-1 are scarce, and the results are inconsistent. It is generally believed that hypercholesterolemia is linked with elevated ET-1 levels in human tissues and plasma [26, 27]. It has been also documented that plasma ET-1 may be independent of cholesterol levels [28]. Ruschitzka et al. [20] provide the first evidence that vascular ECE-1 activity and protein expression were inversely correlated with serum LDL in patients with coronary artery disease. Similarly, the inverse correlation between serum cholesterol and ET-1 levels was observed in pediatric patients with sepsis, and these biomarkers are highly valuable in predicting a patient's poor prognosis [29]. ET-1 also influences the inflammatory response by increasing the expression of proinflammatory cytokines: interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF- α) from monocytes. These cytokines, in turn, may stimulate ET-1 synthesis and release, resulting in a positive feedback system [30].

In our study, the patients did not present hypercholesterolemia, and the levels of cholesterol, LDL-cholesterol and non-HDL cholesterol were even reduced in the subgroup with unstable plaque compared to other studied groups (Table 1). Moreover, the patients represented an older population, with a mean age of 68 ± 8.6 years for the whole group. Although elevated total cholesterol and LDL-cholesterol have long been associated with worse outcomes due to atherosclerosis development and a risk factor for CVD death [31], recent studies have demonstrated that low values of these parameters are associated with worse disease outcomes and poor overall health status [32, 33]. In most people over 60 years, higher cholesterol and LDL-cholesterol levels are associated with lower cardiovascular mortality in the normal lipid reference range, a phenomenon called "the cholesterol paradox" [34]. Our results are in line with these, which showed the inverse relations between lipids and ET-1 levels, suggesting that worse general conditions of the patients may induce the alterations of these biomarkers. The positive association between GSM and cholesterol seems to confirm this hypothesis.

On the other hand, 75% of the patients in GSM < 46.87 were treated with statins, so the reduced levels of total cholesterol and LDL fraction can reflect the effect of these lipid-lowering drugs. Moreover, 75% of patients from this group were treated with ASA (Table 1). Nowadays, both statin and low-dose ASA therapy are recommended for long-term prevention of stroke [1, 35]. These two classes of drugs show beneficial pleiotropic effects on carotid plaque stability, including anti-inflammatory and antioxidant properties [2, 36]. Kadoglou et al. [36] demonstrated an increase in carotid plaque echogenicity through a mechanism related to reducing inflammation independently upon lowering cholesterol in patients with already existing carotid plaques and stroke or hypercholesterolemia. They also noticed that in patients undergoing CEA, the systemic use of statins during the

perioperative period led to reduced post-operative mortality and stroke incidence. The anti-inflammatory properties of ASA, which inhibits cyclooxygenase and other proinflammatory signaling pathways, also conferred a benefit in atherosclerosis management [1, 37].

To assess the net effect of statins and ASA on the parameters we studied, the patients were divided into subgroups, according to the pharmacotherapy regimen used (Fig. 4). Comparison of each statin group to the respective no statin and no ASA group indicated a lack of difference in total cholesterol and LDL-cholesterol, and the same trend was observed concerning GSM values. Unlike this, the patients who obtained only ASA presented the highest LDL-cholesterol compared to others, as well as better features of carotid plaque stability. These results are in line with those demonstrating the improvement in plaque stability independently of the cholesterol-lowering effect of statin [2, 36]. It can also be because the process of plaque formation and progression had been initiated before the start of statin treatment, so we were unable to assess the baseline values of lipids.

Interestingly, the patients treated with a statin alone had the highest ET-1 levels and ECE-1 activity compared to the other groups, especially to patients receiving ASA alone, while big ET-1 levels were the highest in this last group. Moreover, the patients treated simultaneously with statin and ASA presented lower ET-1 levels and ECE-1 activity than those treated with statin only. These results suggest that statin and ASA may have opposing effects on the process of transformation of big ET-1 into ET-1. Previously, it has been shown that ECE-1 mRNA expression in internal mammary arteries was higher in patients undergoing coronary artery bypass grafting surgery treated with statins than in those untreated [38]. Moreover, Rushitzka et al. [20] found that statins prevent the downregulation of ECE-1 expression by LDL in human internal mammary artery endothelial cells. Our results are in line with the above studies, suggesting that statins could intensify ET-1 formation, particularly in patients with unstable carotid plaque.

ASA has more profound pleiotropic effects on vascular function independent of its anti-platelet activity. The anti-inflammatory properties of ASA are well known – it inhibits cyclo-oxygenase and other proinflammatory signaling pathways, like the nuclear factor of activated B-cells (NF-κB), IL-6 or neutrophil elastase synthesis [39]. It has been shown that ASA can alleviate the degeneration of elastic fibers in the aorta of spontaneously hypertensive rats, which is beneficial for maintaining vascular elasticity [39]. The anti-oxidative effect of ASA on the vascular function of aged, ovariectomized rats has been also described [40]. It has been shown that ASA affects plaque stability by inhibiting endothelial dysfunction [41] or a decrease in the size of atherosclerotic lesions with fewer macrophages and lipid cores [42]. Recently conducted research shows that ASA directly inhibits cholesterol crystallization and dissolves cholesterol crystals in carotid plaque which can help prevent plaque rupture [43]. Moreover, the natural course of early carotid atherosclerosis can be slowed with ASA treatment in a dose-dependent fashion [44].

In the available literature, there are only a few studies on the effect of ASA on ET-1 levels, and their results are not unambiguous [39, 45–47]. The treatment with ASA did not significantly affect ET-1 levels during therapy with an inhibitor of angiogenesis in rodents [45]. However, the plasma levels of ET-1 were decreased and vascular remodeling was improved after long-term therapy with ASA in spontaneously hypertensive rats. Moreover, these authors observed a significant reduction in plasma renin, neutrophil elastase, and IL-6 levels after ASA therapy, concluding that the effects of aspirin on vascular remodeling may be attributed to its pleiotropic actions [39]. In humans, a slight decrease in ET-1 level was observed during the 3 months of ASA treatment in patients surviving an acute myocardial infarction [46], whereas 4 weeks of ASA monotherapy did not affect ET-1 concentrations in individuals with peripheral arterial disease [47]. The possible interaction between statin and ASA, leading to reduced ET-1 levels in our patients treated simultaneously with both drugs, might theoretically result from their anti-inflammatory effects. Although we did not find any inter-group difference in the clinical marker of inflammation – CRP, we cannot exclude such a possibility without knowing its baseline values in these persons.

Limitations

The limitation of this study is its cross-sectional design and the relatively small number of patients in subgroups. The main limitation of ultrasound techniques is their operator-dependent nature, which can reduce their accuracy, difficulties in manual delineation of hypoechoic plaques, and inter-observer agreement. Despite these limitations, the conclusion of our study might have some clinical implications. Patients were assigned to the group with stable and unstable carotid plaque based on the previously designated optimal cut-off point of GSM [19], which allowed for more accurate distinguishing of patients with stable and unstable plaques. All patients included in the study had complete information on the laboratory data, comorbidities, and medications taken. The compared groups did not differ in terms of demographic and clinical parameters, which allowed for avoiding the influence of other risk factors on the studied parameters. However, multicenter studies or larger cohorts are needed to validate the results for generalizability.

Conclusions

In summary, this study shows for the first time that ET-1 formation may be the main pathway of big ET-1 metabolism in the blood of patients with carotid atherosclerosis, especially in those with atherosclerotic plaque instability. Statins and ASA, commonly used drugs for stroke prevention, appear to have opposing effects on the ET-1/big ET-1 axis: statin treatment favors ET-1 formation, whereas ASA counteracts it. From a clinical point of view, it seems relevant that patients taking both drugs concomitantly could derive greater benefit related to ET-1-dependent carotid plaque stabilization than those treated with either drug alone. However, longitudinal studies should be conducted to assess the temporal relationship between statin/aspirin use, ET-1 axis modulation and clinical outcomes.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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