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Multimarker Approach in Discriminating Patients with Symptomatic and Asymptomatic Atherosclerotic Carotid Artery Stenosis

Piotr Musialek,[a,c] Wieslawa Tracz,[a,c] Lukasz Tekieli,[a,c] Piotr Pieniazek,[a,c] Anna Kablak-Ziembicka,[a,c] Tadeusz Przewlocki,[a,c] Ewa Stepien,[d] Przemyslaw Kapusta,[c] Rafal Motyl,[c,e] Jakub Stepniewski,[a,c] Anetta Undas,[b,c] Piotr Podolec[a,c]

[a]Jagiellonian University Department of Cardiac and Vascular Diseases, Krakow, Poland
[b]Jagiellonian University Department of Cardiac and Vascular Surgery and Transplantation-Division of Experimental Cardiology, Krakow, Poland
[c]John Paul II Hospital, Krakow, Poland
[d]Jagiellonian University Department of Clinical Biochemistry, Krakow, Poland
[e]Center for Clinical Neurology, Krakow, Poland

Background and Purpose Several circulating biomarkers have been implicated in carotid atherosclerotic plaque rupture and thrombosis; however, their clinical utility remains unknown. The aim of this study was to determine the role of a large biomarker panel in the discrimination of symptomatic (S) vs. asymptomatic (A/S) subjects in a contemporary population with carotid artery stenosis (CS).

Methods Prospective sampling of circulating cytokines and blood lipids was performed in 300 unselected, consecutive patients with ≥50% CS, as assessed by duplex ultrasound (age 47-83 years; 110 with A/S and 190 with S) who were referred for potential CS revascularization.

Results CS severity and pharmacotherapy did not differ between the A/S and S patients. The median values of total cholesterol, low-density lipoprotein cholesterol, and lipoprotein(a) did not differ, but high-density lipoprotein (HDL) cholesterol was significantly higher (p<0.001) and triglycerides were lower (p=0.03) in the A/S-CS group than in the S-CS group. Interleukin-6 (IL-6) and high-sensitivity C-reactive protein were higher (p=0.04 and p=0.07, respectively) in the S-CS group. Circulating visfatin, soluble CD 40 receptor ligand, soluble vascular cell adhesion molecule, leptin, adiponectin, IL-1β, IL-8, IL-18, monocyte chemoattractant protein-1, myeloperoxidase, matrix metalloproteinases-8, -9, and -10, and fibrinogen were similar, but tissue inhibitor of matrix metalloproteinases-1 (TIMP) was reduced in S-CS compared to A/S-CS (p=0.02). Nevertheless, incorporation of TIMP and IL-6 did not improve the HDL-cholesterol receiver operating characteristics for S-CS status prediction. S-CS status was unrelated to angiographic stenosis severity or plaque burden, as assessed by intravascular ultrasound (p=0.16 and p=0.67, respectively). Multivariate logistic regression analysis revealed low HDL-cholesterol to be the only independent predictor of CS symptoms, with an odds ratio of 1.81 (95% confidence interval=1.15-2.84, p=0.01) for HDL <1.00 mmol/L (first quartile) vs. >1.37 (third quartile). In S-CS, osteoprotegerin and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) were elevated in those with recent vs. remote symptoms (p=0.01 and p=0.02, respectively).

Conclusions In an all-comer CS population on contemporary pharmacotherapy, low HDL-cholesterol (but not other previously implicated or several novel circulating biomarkers) is an independent predictor of S-CS status. In addition, an increase in circulating osteoprotegerin and Lp-PLA_2 may transiently indicate S transformation of the carotid atherosclerotic plaque.

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Correspondence
Piotr Musialek, MD, DPhil
Jagiellonian University
Department of Cardiac and Vascular Diseases, John Paul II Hospital,
ul. Pradnicka 80, Krakow 31-202, Poland
Tel +48-12-6142287
Fax +48-12-4234376
E-mail pmusialek@szpitaljp2.krakow.pl

Introduction

Risk stratification in asymptomatic subjects with atherosclerotic extracranial internal carotid artery (ICA) stenosis (CS) is a major challenge in contemporary neurology and vascular medicine. In the general population, as many as 10-15% individuals aged over 55-60 years have significant (≥50%) CS.[1] Carotid plaque destabilization and rupture with thrombus formation is associated with 20-25% of ischemic strokes through embolization to the ipsilateral intracranial arteries and/or an increase in stenosis severity resulting in hemodynamic compromise.[1,2] Although CS is a well-documented and modifiable risk factor for ischemic stroke,[1] population screening for CS is not recommended[1] because of the difficulty identifying those asymptomatic individuals who would benefit from carotid plaque removal (endarterectomy) or plaque sealing (stent) to reduce the stroke risk.[1] For lesions with stenosis of ≥50%, two large randomized trials (Asymptomatic Carotid Atherosclerosis Study, and Asymptomatic Carotid Surgery Trial) found no relationship between the stenosis severity and the risk of CS-associated stroke.[1,3] Although the conversion of asymptomatic CS to symptomatic CS occurs relatively infrequently (≈0.3-2.0% per year),[1,3] ≈80% of disabling strokes occur without any warning sign,[1] indicating that for stroke-affected patients with CS, any mechanical revascularization of CS (if offered) is already "too late". Finally, routine diagnostic tools (such as duplex ultrasound, magnetic resonance angiography, or computed tomography angiography) remain largely ineffective for stratifying asymptomatic CS subjects according to stroke risk.[1] Therefore, at present, interventional treatment of asymptomatic CS remains a statistical risk (with the number-needed-to-treat to prevent one stroke over 10 years being as high as 20-100)[1,3] rather than a risk-assessment-based treatment of those who are likely to have a stroke despite currently optimal medical management.

Several circulating biomarkers have been implicated in symptomatic transformation of the atherosclerotic carotid plaque through their association with plaque erosion, rupture, and thrombosis,[4-10] and it has been proposed that such biomarkers could play an important part in identifying those asymptomatic subjects with CS who would benefit from carotid plaque removal or sealing.[11,12] Since individual biomarkers may lack a sufficient discriminating power to impact clinical decision-making, it has been suggested that a "multimarker approach" will provide more powerful and clinically useful information.[12,13] The value of multimarker analysis involving blood lipids and a large panel of circulating cytokines in patient discrimination according to CS-symptomatic status was evaluated in an all-comer population of CS subjects on contemporary pharmacotherapy.

Methods

Study subjects

This study involved an all-comer population of 300 consecutive subjects with CS referred to a tertiary referral center[14] for carotid artery revascularization decision-making during 2008-2011. CS was at least 50%, as assessed by duplex ultrasound velocity; this was further confirmed by conventional angiography. The patients consulted by an independent neurologist were classified either as "symptomatic" if they had a history of symptoms attributable to atherosclerotic CS, or "asymptomatic" in the absence of such neurological symptoms. Based on the last occurrence of neurological symptoms, the symptomatic subjects were further classified as those with last-symptom occurrence during the preceding 6 months (labeled "recently" symptomatic) or those with a last-symptom episode more than 6 months prior (labeled "remotely" symptomatic).

Patients with restenotic or nonatherosclerotic carotid disease (e.g., Takayasu arteritis), known or suspected infection, chronic inflammatory disease, congestive heart failure (New York Heart Association class III/IV), or on renal replacement therapy were excluded. The following additional exclusion criteria were also applied: stroke or acute coronary syndrome during the preceding 2 weeks (to minimize the confounding effect of a temporary biomarker elevation as a result of the ischemic event), critical limb ischemia, inability to evaluate CS plaque burden with intravascular ultrasound (e.g., string-sign ICA stenosis on noninvasive imaging), and a potential cause for past or future neurological symptoms other than atherosclerotic carotid disease[2] (e.g., atrial fibrillation, thrombophilia, or documented intracranial atherosclerosis). The distributions of classic risk factors (diabetes, hyperlipidemia, arterial hypertension, and smoking) were evaluated, and detailed medical treatment was recorded on admission.

The study protocol was approved by the institutional Ethical Committee, and the patients gave informed written consent to participate.

Laboratory data

Fasting venous blood was drawn between 7 and 9 a.m. from the antecubital vein with minimal stasis. Ethylenediaminetetraacetic acid-anticoagulated plasma and serum samples were centrifuged at 1600×g (at 4°C for 20 minutes for plasma and at 20°C for 10 minutes for serum), and the obtained aliquots were stored at -80°C before being analyzed. Lipid profile and creatinine were assayed by routine laboratory techniques. High-sensitivity C-reactive protein (hsCRP) was determined by an immunoturbidimetric assay (Roche). Fibrinogen was measured according to the Clauss method (Instrumentation Laboratory). Biomarkers were evaluated according to manu-

facturer's reagents and standards by using commercially available high-sensitivity ELISA kits from the following manufacturers: total adiponectin and total leptin-ALPCO Diagnostics; soluble CD 40 receptor ligand (CD 40L), interleukin (IL)-1β, IL-6, IL-8, and IL-18, matrix metalloproteinase (MMP)-8, MMP-9, and MMP-10-R&D Systems; lipoprotein-associated phospholipase A_2 (Lp-PLA_2)-diaDexus; monocyte chemoattractant protein (MCP), myeloperoxidase (MPO), tissue inhibitor of matrix metalloproteinases-1 (TIMP), and soluble vascular cell adhesion molecule (sVCAM)-Bender MedSystems; and total human lipoprotein a [Lp(a)]-BIOTEK; osteoprotegerin (OPG) and visfatin-MBL International. All measurements were performed in duplicate by technicians blinded to the sample status, and the average value was used for analysis. The intra- and interassay coefficients of variation were ≤6.4% and ≤8.1%, respectively.

Imaging

A CS severity of ≥50% was determined by duplex sonogram (Toshiba Aplio PowerVision ultrasound machine equipped with a 4- to 11-MHz linear-array transducer) using the classic velocity criteria of peak-systolic velocity ≥125 cm/s, end-diastolic velocity ≥40 cm/s, and a visible plaque,[1,14-16] and was further confirmed by catheter angiography.[14] Of the initial 303 screened patients, 3 were excluded because the catheter angiogram did not confirm a carotid lesion severity with a diameter stenosis of at least 50%. The presence of symptomatic peripheral arterial occlusive disease (PAD) was verified by noninvasive imaging (duplex ultrasound or computed tomography angiography) or a history of surgical or endovascular PAD interventions. Consistent with our previously reported protocol,[14] coronary angiography was performed routinely, and coronary artery disease was diagnosed from a history of coronary revascularization or the presence of at least one significant stenosis in a major branch on a coronary angiogram. To evaluate the burden of CS atheroma, intravascular ultrasound (IVUS) images were acquired with a commercially available rapid-exchange phased-array scanner (Eagle Eye, ChromaFlo application from Volcano Corp for improved vessel lumen/plaque interface determination)[17] in 293 subjects (97.7%). In seven patients (2.3%) the angiographically detected stenosis was considered too severe to attempt lesion crossing with an IVUS probe without predilatating the lesion.

Statistical analysis

Data were evaluated with Statistica 10.0. The distributions of all continuous variables were assessed using the Shapiro-Wilk test. Continuous data are expressed as median (first quartile-third quartile) values and differences between groups were analyzed using a parametric *t*-test or Mann-Whitney test, as applicable. The categorical data are presented as the percentage and number of patients in the groups, and were compared using the χ^2 test or the Fisher exact test. Biomarkers that were not interrelated were entered into receiver operating characteristics (ROC) analysis for CS-symptomatic status prediction. The biomarker cutoff values were calculated by using the Youden index. In addition, univariate and multivariate logistic regression analyses (including data log-transformation as necessary) were performed to identify the independent biomarkers with a discriminating power. All tests were two-tailed, and the significance level was defined as $p<0.05$.

Results

Clinical presentation, carotid stenosis severity, and medical treatment

Patients with symptomatic ICA stenosis (*n*=190, 63.3%) had a history of cerebral stroke (*n*=127), retinal embolization (*n*=5), cerebral transient ischemic attack (TIA, *n*=79), or transient ocular blindness (*n*=23). The asymptomatic and symptomatic patient groups did not differ with respect to clinical characteristics and carotid stenosis severity, as assessed by duplex sonogram (Table 1). Medical treatment on index hospital admission included the use of angiotensin-converting enzyme inhibitors or angiotensin receptor inhibitors (89.1% vs. 90.5% in asymptomatic vs. symptomatic patients, respectively), β-blockers (70.1% vs. 67.8%), calcium-channel blockers (35.5% vs. 32.1%), and diuretics (38.1% vs. 35.7%; $p>0.1$ for all). Nearly all patients were on a statin (asymptomatic, 99.1%; symptomatic, 100%). The groups were similar with respect to atorvastatin/simvastatin use (65.5%/34.5% and 71.1%/28.9%) and the proportion of patients on different statin doses (20-80 mg, $p=0.26$ for asymptomatic vs. symptomatic, and $p=0.45$ for recently vs. remotely symptomatic). Concomitant fibrate use also did not differ between asymptomatic (4.5%) and symptomatic (3.7%) patients. All subjects were receiving antiplatelet treatment with aspirin and/or thienopyridine. The absence of intergroup differences in clinical characteristics and medical therapy enabled biomarker profile comparisons with respect to CS symptoms.

The degree of angiographic ICA diameter stenosis did not differ between asymptomatic and symptomatic CS (medians of 67.4% vs. 65.7%, $p=0.18$). Within the group of symptomatic lesions, median angiographic CS severity by diameter stenosis was 66.5% in those with symptoms last symptom occurrence ≤6 months and 63.9% in those with last symptom occurrence >6 months ($p=0.08$). As measured by IVUS, there was no overall difference in the atheroma burden between the asymptomatic and symptomatic subjects, with median (range) values of 83.0% (77.1-87.6%) vs. 82.7% (76.2-

Table 1. Demographic and clinical characteristics of the study group, and index internal carotid artery (ICA) Doppler velocities

	Asymptomatic patients (*n*=110)	Symptomatic patients (*n*=190)	*p* value	Symptomatic patients		*p* value
				≤6 months (*n*=119)	>6 months (*n*=71)	
Age, years	67 (60-71)	66 (60-72)	0.96	67 (61-73)	64 (59-69)	0.09
Gender: men, % (*n*)	59.1 (65)	67.4 (128)	0.15	68.1 (81)	66.2 (47)	0.79
Arterial hypertension, % (*n*)	86.4 (95)	90.5 (172)	0.20	89.9 (107)	91.5 (65)	0.84
Diabetes, % (*n*)	27.3 (30)	35.3 (67)	0.15	37.8 (45)	30.9 (22)	0.34
On insulin, % (*n*)	8.2 (9)	11.6 (22)	0.35	10.9 (13)	12.7 (9)	0.72
h/o MI	29.1 (32)	23.2 (44)	0.24	17.6 (21)	32.4 (23)	0.02
Smoking (current or past), % (*n*)	56.4 (62)	55.3 (105)	0.89	53.8 (64)	57.8 (41)	0.64
CAD, % (*n*)	67.3 (74)	66.3 (126)	0.87	61.3 (73)	74.7 (53)	0.09
PAD, % (*n*)	17.3 (19)	11.6 (22)	0.17	10.9 (13)	12.7 (9)	0.70
BMI	27.8 (25.9-30.2)	27.7 (25.5-30.1)	0.95	27.7 (25.5-30.1)	27.7 (25.5-30.1)	0.95
BMI ≥30 kg/m^2, % (*n*)	28.2 (31)	27.9 (53)	0.98	28.6 (34)	26.7 (19)	0.89
Creatinine (μmol/L)	87.0 (74-103)	85.0 (74-99)	0.66	83 (74-97)	87 (76-104)	0.21
eGFR <60 mL/min (MDRD), % (n)	24.5 (27)	20.5 (39)	0.47	19.5 (23)	22.5 (16)	0.36
Index ICA peak systolic velocity, m/s	2.64 (2.02-3.5)	2.55 (1.9-3.3)	0.22	2.62 (1.9-3.5)	2.44 (1.9-3.2)	0.61
Index ICA end-diastolic velocity, m/s	0.87 (0.7-1.2)	0.86 (0.7-1.2)	0.86	0.87 (0.7-1.3)	0.84 (0.6-1.2)	0.56

Continuous data are median (Q_1-Q_3); categorical data are % (*n*).
BMI: Body Mass Index, CAD: coronary artery disease, eGFR: estimated Glomerular filtration rate, h/o MI: history of myocardial infarct, MDRD: modification of died in renal disease formula, PAD: peripheral arterial occlusive disease.

88.8%; *p*=0.87). However, the atheroma burden was significantly (*p*=0.03) higher in those with recent symptoms of CS (84.6%; 76.9-89.8%) than in those with remote symptoms of CS (80.1%; 73.1-86.0%), consistent with inward plaque remodeling following its symptomatic rupture.[18]

Circulating biomarkers in asymptomatic and symptomatic patients with carotid stenosis

Circulating cytokines and blood lipid levels in the study groups are given in Table 2. While the levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, and Lp(a) did not differ, those of high-density lipoprotein (HDL) cholesterol were significantly higher in the CS-asymptomatic subjects [1.30 mmol/L (range=1.1-1.5 mmol/L) vs. 1.08 mmol/L (range=0.9-1.3 mmol/L), *p*<0.001]. The level of triglycerides (TGs) was significantly higher in the CS-symptomatic patients [1.31 mmol/L (range=0.9-1.7 mmol/L) vs. 1.42 mmol/L (range=1.1-1.9 mmol/L), *p*=0.03]. The distribution of HDL-cholesterol levels (Fig. 1A) indicates a clear shift toward lower HDL-cholesterol values in those with symptomatic CS. In addition, the LDL-/HDL-cholesterol ratio was significantly higher in the CS-symptomatic patients [2.35 (range=1.7-2.9) vs. 2.05 (range=1.6-2.6), *p*=0.008]. However, there were no differences in the lipid profile between those with last symptom occurrence ≤6 months and those with the last episode >6 months (Table 2), consistent with the concept that lipids are associated with a long-term (chronic) rather than acute risk of CS-symptomatic transformation.

The IL-6 level was significantly higher in the symptomatic patients [3.69 pg/mL (range=1.44-6.81 pg/mL) vs. 2.44 pg/mL (range=1.07-5.41 pg/mL, *p*=0.04)], but no between-group difference was found for the other studied interleukins (IL-1β, IL-8, and IL-18) (Table 2). Although the median level of hsCRP was higher in the symptomatic subjects [2.11 mg/L (range=1.4-6.1 mg/L) vs. 2.08 mg/L (range=1.2-3.4 mg/L)], the overall difference did not reach statistical significance (*p*=0.07). Exclusion of subjects with an hsCRP exceeding 10 mg/L[19] (*n*=2, 1.8%, in the asymptomatic group; *n*=8, 6.7% in the symptomatic ≤6 months group; *n*=6, 8.4%, in the symptomatic >6 months group; in all cases, hsCRP values were ≤15 mg/L) had no effect on the hsCRP analysis outcome (Table 2). Nevertheless, analysis of hsCRP distribution (Fig. 1B) indicated that while both asymptomatic and symptomatic subjects exhibited a prevalence peak at ≈2 mg/L, the symptomatic subjects exhibited a bimodal distribution with a nadir at ≈5.2 mg/L and a second, smaller prevalence peak at ≈7 mg/L. When this nadir was taken as a cutoff, there were 11.2% asymptomatic subjects vs. 27.7% symptomatic subjects with hsCRP ≥5.2 mg/L [odds ratio (OR)=3.06, 95% confidence interval (95%CI)=1.51-6.22, *p*=0.002], indicating that hsCRP levels exceeding 5.2 mg/L may be associated with a significant increase in the likelihood of CS-symptomatic status. However, hsCRP ≥5.2 mg/L could not differentiate between those with recent vs. remote symptoms of CS (*p*=0.727), consistent with the potential chronic rather than acute association between hsCRP and the risk of CS-symptomatic transforma-

Table 2. Laboratory characteristics of the study group

	Asymptomatic* patients (*n*=110)	Symptomatic* patients (*n*=190)	*p* value	Symptomatic* patients		*p* value
				≤6 months (*n*=119)	>6 months (*n*=71)	
Total cholesterol (mmol/L)	4.5 (3.8-5.3)	4.4 (3.9-5.0)	0.31	4.38 (3.9-4.9)	4.43 (3.9-5.2)	0.42
LDL-cholesterol (mmol/L)	2.58 (2.0-3.2)	2.51 (2.1-3.0)	0.37	2.46 (2.0-2.9)	2.54 (2.1-3.1)	0.46
HDL-cholesterol (mmol/L)	1.30 (1.1-1.5)	1.08 (0.9-1.3)	<0.001	1.07 (0.9-1.3)	1.11 (0.9-1.3)	0.74
LDL/HDL ratio	2.05 (1.6-2.6)	2.35 (1.7-2.9)	0.008	2.24 (1.7-2.8)	2.37 (1.9-3.0)	0.41
Triglycerides (mmol/L)	1.31 (0.9-1.7)	1.42 (1.1-1.9)	0.03	1.41 (0.9-1.8)	1.42 (1.2-2.0)	0.12
Lp(a) (mg/dL)	9.27 (4.1-17.3)	9.79 (4.1-23.6)	0.25	10.11 (4.1-27.1)	9.48 (4.0-19.4)	0.75
hs-CRP (mg/L)	2.08 (1.2-3.4)	2.11 (1.4-6.1)	0.07	2.37 (1.4-5.5)	1.93 (1.2-6.4)	0.47
hs-CRP (≤10)† (mg/L)	1.99 (1.2-3.3)	2.0 (1.2-3.9)	0.28	2.17 (1.3-3.9)	1.77 (1.1-2.8)	0.27
Fibrinogen (g/L)	4.44 (3.6-5.0)	4.11 (3.3-4.9)	0.19	3.96 (3.4-5.2)	4.13 (3.3-4.8)	0.62
IL-1β (pg/mL)	0.14 (0.1-0.2)	0.13 (0.1-0.2)	0.87	0.13 (0.1-0.2)	0.12 (0.1-0.2)	0.68
IL-6 (pg/mL)	2.44 (1.1-5.4)	3.69 (1.4-6.8)	0.04	3.41 (1.5-6.7)	4.16 (1.3-7.3)	0.94
IL-8 (pg/mL)	8.21 (5.3-11.4)	8.73 (5.8-11.9)	0.49	8.73 (6.3-12.0)	8.73 (4.9-13.3)	0.67
IL-18 (μg/mL)	0.33 (0.3-0.4)	0.32 (0.2-0.4)	0.78	0.33 (0.2-0.4)	0.32 (0.3-0.4)	0.47
sVCAM (mg/mL)	0.87 (0.7-1.1)	0.91 (0.7-1.2)	0.28	0.86 (0.7-1.1)	0.94 (0.7-1.2)	0.43
CD40L (ng/mL)	0.31 (0.2-0.5)	0.35 (0.2-0.5)	0.63	0.37 (0.2-0.5)	0.31 (0.2-0.4)	0.67
Lp-PLA_2 (ng/mL)	326.9 (260-381)	319.7 (266-373)	0.65	328.8 (274-407)	310.4 (247-338)	0.02
Visfatin (ng/mL)	0.32 (0.2-0.6)	0.25 (0.1-0.5)	0.25	0.26 (0.2-0.6)	0.22 (0.1-0.5)	0.39
MCP-1 (ng/mL)	0.22 (0.2-0.3)	0.25 (0.2-0.3)	0.25	0.26 (0.2-0.3)	0.24 (0.2-0.3)	0.34
MPO (ng/mL)	45.4 (28.9-79.4)	48.3 (29.6-77.8)	0.82	49.1 (29.6-78.6)	47.5 (29.0-76.9)	0.88
MMP-8 (ng/mL)	20.0 (10.6-32.3)	20.2 (11.3-37.5)	0.42	19.7 (9.9-35.0)	24.5 (12.4-39.1)	0.40
MMP-9 (μg/mL)	0.13 (0.1-0.2)	0.14 (0.1-0.2)	0.36	0.13 (0.1-0.2)	0.16 (0.1-0.2)	0.29
MMP-10 (μg/mL)	0.66 (0.4-0.8)	0.58 (0.5-0.8)	0.63	0.56 (0.5-0.8)	0.66 (0.5-0.8)	0.12
TIMP (ng/mL)	146.2 (115-178)	130.7 (108-176)	0.02	130.6 (101-166)	131 (112-165)	0.75
Leptin (ng/mL)	12.15 (4.9-27.2)	11.37 (5.1-19.5)	0.57	11.06 (5.1-19.8)	12.18 (5.8-19.2)	0.70
Adiopnectin (μg/mL)	4.13 (2.9-5.9)	3.96 (2.7-6.6)	0.64	3.56 (2.3-6.7)	4.42 (3.0-6.5)	0.24
Leptin/adiponectin ratio	3.01 (1.0-6.3)	1.91 (0.9-5.6)	0.36	1.81 (0.9-5.4)	2.19 (0.9-7.3)	0.61
OPG (pmol/L)	4.28 (2.9-5.0)	4.45 (3.3-5.9)	0.21	4.71 (3.6-6.3)	4.13 (2.8-5.4)	0.01

Data are shown as median (Q_1-Q_3).
*The terms "symptomatic" or "asymptomatic" refer to neurological symptoms attributable to carotid artery stenosis by an independent neurologist, †Subjects with hsCRP ≥10 mg/L excluded (*n*=2 with asymptomatic carotid stenosis, *n*=8 symptomatic ≤6 months and *n*=6 with last symptoms >6 months; NB. the peak hsCRP level was 13.86 mg/L).
CD40L: soluble CD 40 receptor ligand, HDL: high-density lipoprotein, hs-CRP: high sensitivity C-reactive protein, IL: interleukin, LDL: low-density lipoprotein, Lp(a): lipoprotein a, Lp-PLA_2: lipoprotein-associated phospholipase A_2, MCP: monocyte chemoattractant protein, MMP: matrix metalloproteinase, MPO: myeloperoxidase, OPG: osteoprotegerin, sVCAM: soluble vascular cell adhesion molecule, TIMP: tissue inhibitor of matrix metalloproteinases-1.

tion. In addition, there was a weak but statistically significant negative correlation between HDL and hsCRP (r=-0.32, p< 0.0001).

The plasma levels of the MMPs (MMP-8, MMP-9, and MMP-10) did not differ between the study groups (Table 2). In contrast, circulating TIMP was significantly lower in the CS-symptomatic patients [130.0 ng/mL (range=7.3-166.5 ng/mL) vs. 146.2 ng/mL (range=115.3-177.9 ng/mL), p=0.02], indicating a shift toward reduced metalloproteinase inhibition in subjects with symptomatic CS. The overall levels of fibrinogen, visfatin, CD40L, sVCAM, leptin, adiponectin (including leptin/adiponectin ratio), LP-PLA_2, MCP-1, MPO, and OPG also did not differ between the symptomatic and asymptomatic patients. However, when the symptomatic patients were divided into those with recent vs. remote symptoms, the levels of LP-PLA_2 and OPG were significantly higher in the recently CS-symptomatic subjects [0.33 μg/mL (range=0.3-0.4 μg/mL) vs. 0.31 μg/mL (range=0.2-0.4 μg/mL), p=0.02; 4.70 pg/mL (range=3.6-6.3 pg/mL) vs. 4.14 pg/mL (range=2.8-5.5 pmol/L), p=0.02].

Since patients for whom >6 months has elapsed since their last episode of CS-attributable neurological symptoms (i.e., those "remotely" symptomatic by the current study definition) are often classified as "asymptomatic",[1,16,20] the effect of merging this subset (n=71) with the a *priori* asymptomatic group (n=190) on the biomarker data was tested. Incorporation in

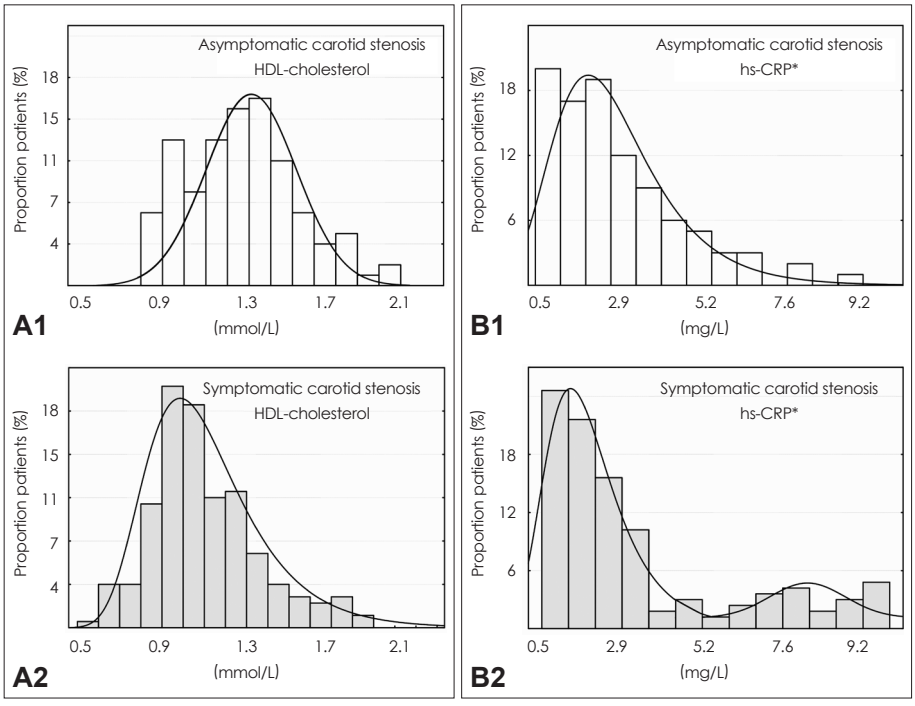


Fig. 1. Distributions of HDL-cholesterol and hsCRP* in patients with carotid stenosis according to the presence or absence of a history of carotid stenosis-associated symptoms. A: Displays HDL-cholesterol level distribution (asymptomatic carotid stenosis top, A1; symptomatic carotid stenosis bottom, A2). B: hsCRP level distribution (asymptomatic carotid stenosis top, B1; symptomatic carotid stenosis bottom, B2). HDL: high-density lipoprotein, hsCRP: high sensitivity C-reactive protein.

the asymptomatic cohort of the patients with the last neurological symptoms occurring >6 months prior to biomarker sampling blunted the asymptomatic vs. symptomatic difference in HDL-cholesterol and removed the differences in LDL/HDL-cholesterol, TGs, TIMP, and IL-6, indicating that subjects with prior symptoms of CS remain distinct from the asymptomatic group.

ROC analysis for CS-symptomatic status prediction

Receiver operating characteristics analysis included HDL, TIMP, and IL-6 (but not TGs, which showed a weak though significant negative correlation with the HDL level; r=-0.28, p<0.001). The highest area under the curve (AUC) was found for HDL-cholesterol (AUC=0.70, 95%CI=0.63-0.76, cutoff=1.16 mmol/L, positive predictive value=0.78, negative predictive value=0.52) (Fig. 2A). IL-6 paired with TIMP performed similarly to HDL-cholesterol taken alone (Fig. 2B), but combining all three biomarkers (i.e., HDL+IL-6+TIMP) did not surpass the diagnostic accuracy of HDL-cholesterol (Fig. 2C).

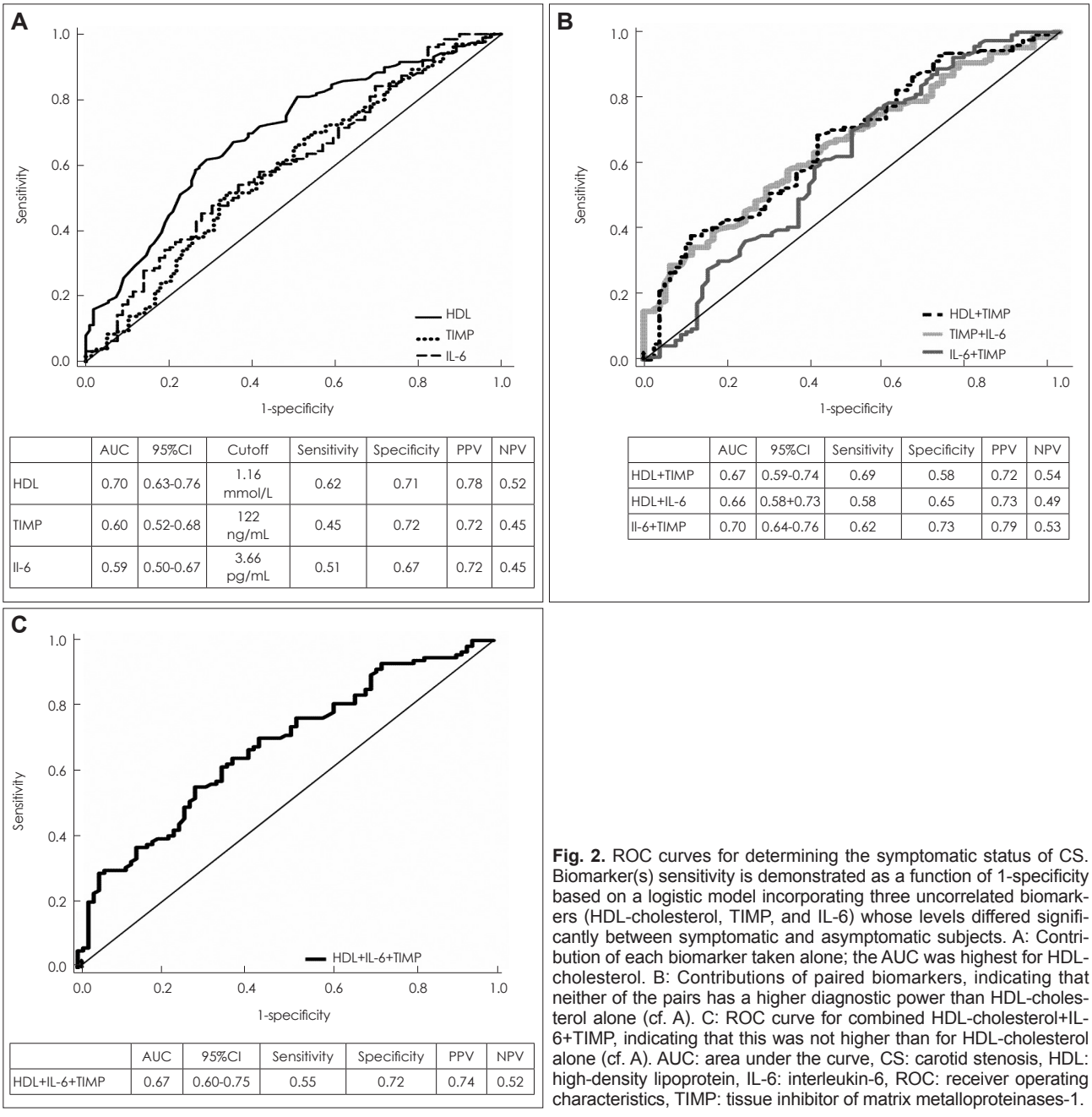
Lipoprotein-associated phospholipase A_2 and OPG were significantly higher in those with CS symptoms occurring ≤6 months vs. >6 months (Table 2) and were not interrelated (r=-0.09, p=0.32), and so their individual vs. combined power in the prediction of CS-recently vs. CS-remotely symptomatic status was tested. A greater power was found for Lp-PLA_2+OPG (AUC=0.70, 95%CI=0.60-0.78, p<0.01) when compared to the ROC for Lp-PLA_2 alone (AUC=0.63, 95%CI=0.53-0.72, p=0.019, cutoff=361 ng/mL) or OPG alone (AUC=0.62, 95% CI=0.52-0.72, p=0.02, cutoff=3.73 pmol/L), consistent with the idea that Lp-PLA_2 and OPG may affect plaque biology via different mechanisms.

Univariate and multivariate logistic regression models

Univariate and multivariate logistic regression analyses were performed to assess the role of angiographic stenosis severity and atheroma burden on intravascular ultrasound, and of HDL-cholesterol, TIMP, IL-6, and TG level in predicting CS-symptomatic status. In the univariate model, HDL-cholesterol and TIMP (but not IL-6 or TG) predicted the CS-symptomatic status (OR=2.51, 95%CI=1.73-3.65, p<0.001 for HDL-cholesterol <1 mmol/L vs. >1.37 mmol/L; and OR=1.59, 95%CI=1.06-2.41, p=0.027 for TIMP <111 ng/mL vs. >204 ng/mL) (Table 3). The angiographic stenosis severity and atheroma burden as assessed by IVUS were not predictive of CS symptoms (p=0.16 and p=0.68, respectively). In a multivariate model, a low HDL-cholesterol level was the sole predictor of CS-symptomatic status (OR=1.81, 95%CI=1.15-2.84 for HDL-cholesterol <1 mmol/L vs. >1.37 mmol/L, p=0.01) (Table 3).

Discussion

The key finding of this study, which evaluated the largest up-to-date panel of circulating biomarkers in subjects with CS, is that low HDL-cholesterol was the sole independent predictor of CS-symptomatic status in a contemporary CS population. In particular, we found that the likelihood of CS-associated



	AUC	95%CI	Cutoff	Sensitivity	Specificity	PPV	NPV
HDL	0.70	0.63-0.76	1.16 mmol/L	0.62	0.71	0.78	0.52
TIMP	0.60	0.52-0.68	122 ng/mL	0.45	0.72	0.72	0.45
Il-6	0.59	0.50-0.67	3.66 pg/mL	0.51	0.67	0.72	0.45

	AUC	95%CI	Sensitivity	Specificity	PPV	NPV
HDL+TIMP	0.67	0.59-0.74	0.69	0.58	0.72	0.54
HDL+IL-6	0.66	0.58+0.73	0.58	0.65	0.73	0.49
Il-6+TIMP	0.70	0.64-0.76	0.62	0.73	0.79	0.53

	AUC	95%CI	Sensitivity	Specificity	PPV	NPV
HDL+IL-6+TIMP	0.67	0.60-0.75	0.55	0.72	0.74	0.52

Fig. 2. ROC curves for determining the symptomatic status of CS. Biomarker(s) sensitivity is demonstrated as a function of 1-specificity based on a logistic model incorporating three uncorrelated biomarkers (HDL-cholesterol, TIMP, and IL-6) whose levels differed significantly between symptomatic and asymptomatic subjects. A: Contribution of each biomarker taken alone; the AUC was highest for HDL-cholesterol. B: Contributions of paired biomarkers, indicating that neither of the pairs has a higher diagnostic power than HDL-cholesterol alone (cf. A). C: ROC curve for combined HDL-cholesterol+IL-6+TIMP, indicating that this was not higher than for HDL-cholesterol alone (cf. A). AUC: area under the curve, CS: carotid stenosis, HDL: high-density lipoprotein, IL-6: interleukin-6, ROC: receiver operating characteristics, TIMP: tissue inhibitor of matrix metalloproteinases-1.

symptoms increased by a factor of 1.81 (95%CI=1.15-2.84) between the first and fourth HDL-cholesterol quartiles. Despite statistically significant differences in IL-6, TIMP, and TG level between the CS-symptomatic and CS-asymptomatic subjects, in the multivariable model these biomarkers were not independent predictors of CS symptoms. Moreover, their addition to HDL-cholesterol failed to provide any incremental value over HDL-cholesterol alone in the ROC analysis (Fig. 2). Thus, the findings of the present study in an unselected population of CS subjects on contemporary pharmacotherapy are unable to confirm the clinical utility of several circulating biomarkers that have previously been suggested to indicate the symptomatic status of CS, including hsCRP,[4,5] IL-6,[4,21] Lp(a),[4] and MMPs.[4,6] Moreover, this analysis did not demonstrate the utility of several other classic (e.g., CD40L, sVCAM, fibrinogen, IL-1β, IL-8, IL-18, MPO, and MCP-1)[4,11] and novel (e.g., visfatin and leptin/adiponectin)[7,9,22] circulating biomarkers of atherosclerotic plaque destabilization and rupture.

Subjects with symptomatic CS demonstrated a reduced level of circulating TIMP, consistent with dysregulation of the MMP/TIMP balance in patients with symptomatic CS, resulting in decreased endogenous inhibition of MMPs.[23] Although

Table 3. Univariate and multivariate logistic regression analysis for the prediction of carotid artery stenosis symptomatic status

Marker	Model	<1st quartile vs. 3rd quartile	OR	95% CI	*p* value
HDL-cholesterol (mmol/L)	Univariate	<1.00 vs. >1.37	2.51	1.73-3.65	<0.001
TIMP (ng/mL)	Univariate	<111 vs. >204	1.59	1.06-2.41	0.027
IL-6 (pg/mL)	Univariate	<1.28 vs. >6.15	0.73	0.49-1.09	0.106
Triglycerides (mmol/L)	Univariate	<1.05 vs. >1.85	0.73	0.52-1.03	0.072
HDL-cholesterol (mmol/L)	Multivariate	<1.00 vs. >1.37	1.81	1.15-2.84	0.010
TIMP (ng/mL)	Multivariate	<111 vs. >204	1.49	0.96-2.31	0.073

HDL: high-density lipoprotein, IL: Interleukin, OR: odds ratio, TIMP: tissue inhibitor of matrix metalloproteinases-1.

the overall levels of Lp-PLA_2 and OPG did not differ between those with and without CS symptoms, these two biomarkers were significantly elevated in subjects with recent vs. remote CS symptoms. Both Lp-PLA_2 and OPG promote plaque instability and rupture.[4,8] The present finding is in line with recent histological evidence of significantly increased Lp-PLA_2 and OPG expression in carotid plaques in patients neurologically symptomatic during the preceding 1.5-4 months,[4,8] and OPG prediction of premature atherosclerosis progression in asymptomatic normotensive individuals.[24] Furthermore, the absence of a correlation between levels of Lp-PLA_2 and OPG (r=-0.09, p=0.32) is consistent with their independent action in promoting plaque instability. Incorporation of both Lp-PLA_2 and OPG in the ROC analysis increased their individual AUC in the determination of recently symptomatic CS status (AUC=0.63 for Lp-PLA_2, 0.62 for OPG, and 0.70 for Lp-PLA_2+OPG).

"Symptomatic" status of the carotid stenosis

There is currently no single, generally applied definition of symptomatic CS with respect to the time elapsed since the last symptom episode. For example, the term "asymptomatic" has been used not only to label patients with no history of ipsilateral symptoms,[10,25] but also to those with prior symptoms who have been free of neurological events for a period from 1-4 months[8] to 12 months,[6] with the cutoff for the CS symptom-free period frequently taken as 6 months.[1,16,20] With these varying definitions, subjects with a history of CS symptoms during the previous 1-12 months have been classified in different previous studies either as "symptomatic" or "asymptomatic."[8,20,26,27] Data from these studies,[8,20,26,27] when considered on aggregate, suggest that the use of different definitions of CS "symptomatic" status could have a significant impact on the findings. For clarity of analysis, in the present study all patients with history of CS symptoms were considered "symptomatic". Moreover, the applied cutoff of 6 months for distinguishing those with recent vs. remote symptoms is consistent with recent histological evidence that most carotid plaques stabilize within 6 months after the neurological event.[25]

There is experimental evidence[4,10,25] that certain biomarkers have a chronic effect on carotid plaque stability (e.g., low HDL, low TIMP, and elevated IL-6), whereas others (possibly Lp-PLA_2 and OPG) have a more transient impact or acutely reflect plaque destabilization. Several previous studies[23,28] have interpreted the serum biomarker level in the context of plaque histology rather than of the neurological symptoms of carotid plaque rupture and thrombosis. Indeed, it is well known that carotid plaques can undergo several episodes of rupture and thrombosis that may remain clinically silent, although they usually lead to plaque progression.[29,30] Our finding that inclusion of the remotely symptomatic subjects in the asymptomatic group reduced or abolished the between-group differences in circulating biomarkers is consistent with the concept that the circulating biomarker profile of subjects with remote symptoms of CS may remain at least partially different from that of never-symptomatic subjects.

Systemic biomarker concentration vs. *in-situ* plaque destabilization and rupture

In an ideal setting of the assessment of circulating biomarkers in relation to CS clinical symptoms, either the release of the biomarker from the index lesion should be sufficiently high to affect its systemic level or a "causative" circulating biomarker released elsewhere should exclusively affect stability of the index-in this case carotid-lesion under consideration. This is not necessarily the case because instability of the atherosclerotic lesion(s) in one vascular bed (e.g., carotid) is often associated with instability of atherosclerotic lesion(s) in other beds (e.g., coronary)[30] through "vulnerable blood" mechanisms.[31,32] The available data suggest that atherosclerotic plaque destabilization and rupture in one specific vascular territory involves an interplay between the local (*in situ*) factors[7,33-35] that make a particular plaque prone to erosion or rupture ("vulnerable plaque") and systemic factors ("vulnerable blood").[31,32] Thus, the circulating biomarker level actually reflects the net effect of 1) biomarker production and release in the "target" atherosclerotic lesion, with a local release possibly too small to be detected systemically[6,27,34] and/or occurring only transiently;[25] 2) the contribution of biomarker release from atherosclerotic plaques in other vascular territories; and 3) for some biomarkers, their production elsewhere (e.g., the liver in the case of fibrinogen or hsCRP).[22,26,36]

Several previous studies of the relationship between circulating biomarkers and carotid atherosclerosis have either excluded patients with atherosclerosis in other vascular territories[35] or have used combined end points (e.g., death/TIA/stroke/myocardial infarction, or any revascularization and/or carotid plaque progression) to identify a biomarker utility in relation to CS-symptomatic status.[9,13,15,26,32] In addition, contemporary pharmacotherapy, which involves a high proportion of statin and angiotensin-converting enzyme inhibitor use, may play an important role in reducing the level of inflammatory markers and/or weaken the relationship between biomarkers and the risk of symptomatic plaque transformation.[37-39]

Low HDL-cholesterol as an independent predictor of CS-symptomatic status

High-density lipoprotein-cholesterol protects against atherosclerosis via several anti-inflammatory, antioxidant, and antithrombotic effects, including reverse cholesterol transport in the liver, prostacyclin release, and the inhibition of endothelial adhesion molecule expression, monocyte chemotactic activity, and LDL oxidation.[40] Our novel finding of HDL-cholesterol as an independent predictor of CS-symptomatic status in patients with established carotid atherosclerosis is consistent with the association between low HDL-cholesterol with the unstable carotid plaque phenotype on conventional histology.[30] It is also consistent with the finding of low HDL-cholesterol more frequent in stroke and TIA patients with atherosclerotic large-vessel stenosis than in those with stroke/TIA in the absence of atherosclerotic large-vessel stenosis.[41] The failure of previous studies to identify a link between low HDL-cholesterol and the neurological symptoms of carotid atherosclerosis may have been due to the inclusion of relatively small samples and/or the classification of the patients with last symptoms of CS >3-6 months as asymptomatic,[8] which would blunt any potential differences between the truly asymptomatic vs. those with prior symptoms of CS. Previous work suggested a protective role for high HDL-cholesterol levels against the progression of carotid atherosclerosis rather than its symptomatic conversion;[4] there is also recent prospective evidence that increased HDL-cholesterol protects against the progression of intracranial atherosclerosis.[43]

The CS severity was similar in the symptomatic and asymptomatic subjects included in the present study, which supports the concept of a protective role played by high HDL-cholesterol through reducing the risk of symptomatic transformation of the carotid plaque. A recent study of left main coronary artery atherosclerosis[44] indicated that a low HDL/LDL-cholesterol ratio may be related to an increased lipid content and smaller fibrous content observed on the plaque radiofrequency IVUS imaging, possibly rendering the atherosclerotic plaque more amenable to symptomatic transformation. Such an association is yet to be evaluated for carotid bifurcation atherosclerotic disease.

Limitations

While this study employed the most extensive up-to-date panel of biomarkers in patients with carotid atherosclerosis, the discriminative power of remnant lipoprotein cholesterol,[43,45] which was shown recently to be a risk factor for large-artery atherosclerotic stroke,[46] was not evaluated. Secondly, due to the natural history of carotid atherosclerosis, the CS cohort labeled "asymptomatic" is known to include up to ≈10-15% of subjects whose CS is likely to turn symptomatic over the subsequent 10 years.[3] This natural presence of future-symptomatic subjects in the thus-far-asymptomatic group (both labeled "asymptomatic") may obscure biomarker profile differences in a cross-sectional study. Moreover, in search of patient characteristics that might aid clinical decision-making in an all-comer population with CS, we deliberately avoided any preselection of study subjects.[47] Thus, the present study cohort included a sizeable proportion of patients with established (and/or symptomatic) atherosclerosis in other vascular territories (Table 1). This is likely to blunt any differences in the circulating biomarker profiles between the study groups. Finally, the finding that circulating OPG and Lp-PLA_2 might transiently label symptomatic transformation of the carotid atherosclerotic plaque warrants further, prospective[48] validation in a large "natural history" study with 1) repeated (serial) biomarker sampling to enable a change in biomarker level "just" prior to symptom occurrence, and 2) ideally, repeated carotid plaque imaging. However, due to the relatively low event rate in subjects with asymptomatic carotid stenosis on contemporary pharmacotherapy and the unavoidable crossover of some patients to mechanical revascularization, such a large longitudinal study is unlikely to be conducted.

Conclusions

The present analysis in patients with CS subjected to contemporary pharmacotherapy found that among several circulating biomarkers that have previously been implicated in indicating carotid plaque destabilization and symptomatic transformation, low HDL-cholesterol was the only independent predictor of neurological symptoms. This finding is consistent with the detrimental effect of low HDL-cholesterol in patients with clinical manifestation of atherosclerosis in some other vascular beds,[39] indicating that the HDL-cholesterol level should be considered in the routine risk stratification of asymptomatic carotid stenosis. The findings also suggest that low HDL-cholesterol could constitute an important therapeutic target in subjects with carotid stenosis. Nevertheless, the ex-

tents to which a low HDL-cholesterol level is a causal factor versus an epiphenomenon have not been determined.[40] Moreover, there is recent evidence that forced elevation of defective HDL-cholesterol is clinically ineffective.[40] Therefore, whether (and which) pharmacologic interventions aimed at increasing HDL-cholesterol would actually reduce the risk of carotid plaque symptomatic transformation has to be demonstrated.

Conflicts of Interest

The authors have no financial conflicts of interest.

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