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# The relationship of novel adipokines, RBP4 and omentin-1, with carotid atherosclerosis severity and vulnerability

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ABSTRACT

*Objective:* We investigated the relationship of circulating novel adipokines, retinol-binding protein 4 (RBP4) and omentin-1, with advanced carotid atherosclerosis and ultrasound indexes of severity (total plaque area-TPA) and plaque echogenicity and vulnerability (Gray-Scale median – GSM score).
*Methods:* We enrolled 225 patients with high-grade carotid stenosis (HGCS) who underwent carotid revascularization (73 Symptomatic patients, 152 asymptomatic patients) and 75 age- and sex-matched, asymptomatic individuals with low-grade (<50%) carotid stenosis (LGCS). Seventy-three individuals without current manifestations of atherosclerotic disease served as control group (COG). All participants underwent carotid ultrasound with TPA and GSM score assessment. Moreover, clinical parameters, metabolic profile, and circulating levels of hsCRP and adipokines were assessed.
*Results:* RBP4 was significantly elevated in HGCS (51.44 ± 16.23 mg/L) compared to LGCS (38.39 ± 8.85 mg/L), independent of symptoms existence, whereas RBP4 levels in COG were even lower (25.74 ± 10.72 mg/L, $p < 0.001$ compared to either HGCS or LGCS). Inversely, serum omentin-1 levels were significantly lower across HGCS (490.41 ± 172 ng/ml) and LGCS (603.20 ± 202.43 ng/ml) than COG (815.3 ± 185.32, $p < 0.001$). Moreover, the considerable difference between HGCS and LGCS ($p < 0.001$) was exclusively attributed to the excessive suppression of omentin-1 concentrations in symptomatic versus asymptomatic ($p = 0.004$) patients. HGCS and LGCS did not differ in the rest of clinical and biochemical parameters. In multiple regression analysis, RBP4 (beta = 0.232, $p = 0.025$) and hsCRP (beta = 0.300, $p = 0.004$) emerged as independent determinants of TPA in patients with carotid atherosclerosis. Low serum levels of omentin-1 correlated with GSM score and symptoms but that association was lost in multivariate analysis..
*Conclusion:* RBP4 serum levels were significantly elevated in patients with established carotid atherosclerosis and were positively associated with atherosclerosis severity. The association of low serum omentin-1 with carotid plaque echolucency requires further investigation..
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## 1. Introduction

Cerebral ischemia is one of the most important causes of morbidity and mortality in industrialized countries, associated with considerable medical and socio-economic burden [1]. Carotid artery stenosis as a causative factor of ischemic strokes or transient ischemic attacks (TIAs) constitutes a major therapeutic target of primary and secondary prevention strategies [2]. Randomized

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controlled trials and subsequent meta-analyses have demonstrated the degree of carotid stenosis as the gold-standard criterion for effective carotid revascularization in combination with best medical treatment in patients with symptoms (≥50% stenosis) and some selected patients without symptoms (≥80% stenosis) [3]. In addition to the magnitude of atherosclerosis, emerging evidence supports the prognostic power of plaque vulnerability in carotid atherosclerosis evolution [4]. Thereby, insights into the quantitative evaluation of both total plaque area (TPA) and texture would help to identify potentially high risk atherosclerotic lesions that would most benefit from intervention [5].

We know from numerous clinical and experimental trials that inflammation plays a pivotal role in the development and destabilization of carotid plaques [6,7]. In vivo, the inflammatory process can easily be monitored by serum inflammatory biomarkers assay. Novel pro-inflammatory and anti-inflammatory derivatives from adipose tissue, known as adipokines, have recently been associated with carotid atherosclerosis [8]. Among them, retinol-binding protein 4 (RBP4) has emerged as a potential mediator of the interplay between obesity, insulin resistance, and inflammation [9,10]. A growing body of evidence supports the positive relationship of RBP4 with the presence of either coronary artery disease (CAD) [11,12] or subclinical carotid atherosclerosis [13]. However, the role of RBP4 in established carotid atherosclerosis is still obscure.

Another newly identified adipokine, omentin-1, has been intimately linked with protective mechanisms against obesity and insulin resistance [14]. Moreover, low plasma levels of omentin-1 have been measured in patients with extended CAD [15], or carotid atherosclerosis [16]. Nevertheless, no previous study has examined the interaction of omentin-1 with carotid atherosclerosis severity and vulnerability.

Taken together, the primary aim of the current study was to investigate the relationship of both RBP4 and omentin-1 serum levels with the presence and the ultrasonographically quantified severity of established carotid atherosclerosis. We also tested the hypothesis that these biomarkers correlate with ipsilateral neurological symptoms and valid ultrasound indices of plaque vulnerability.

## 2. Methods

### 2.1. Patients

In the present study we enrolled patients with established carotid atherosclerosis and individuals without current manifestations of atherosclerotic diseases. All participants underwent ultrasound examination of both carotids. Carotid atherosclerosis was characterized by the presence of at least one atherosclerotic plaque localized at the internal carotid artery (ICA) or the common carotid artery (CCA). Atherosclerotic plaque was defined as a focal structure encroaching into the arterial lumen by at least 50% of the surrounding IMT value or a thickness >1.2 mm [17]. Based on carotid ultrasound findings (presence of carotid plaques and quantified degree of carotid stenosis) and laboratory manifestation of generalized atherosclerosis we enrolled individuals and assigned them into the following groups:

A) High-grade carotid stenosis (HGCS) group: A total of 225 consecutive patients underwent carotid revascularization (carotid artery stenting—CAS or carotid endarterectomy—CEA) between January 2011 and May 2013. These patients were further subdivided into the following subgroups: 1) Symptomatic carotid artery stenosis ≥50% ($n = 73$), 2) Asymptomatic carotid artery stenosis ≥80% ($n = 152$). Previous (within the past six months) neurological symptoms, indicating ipsilateral to the carotid stenosis transient ischemic attack (TIA) ($n = 24$), stroke ($n = 46$) or amaurosis fugax ($n = 3$), were a prerequisite to classify patients as symptomatic. All patients of HGCS had undergone brain computed tomography (CT) scan. When CT findings were questionable brain magnetic resonance imaging (MRI) examination was additionally performed, just to limit the possibility of patients' misclassification.
B) Low-grade carotid stenosis (LGCS) group: This group was made up of 75 age- and sex-matched asymptomatic individuals with <50% carotid stenosis (3:1 HGCS/LGCS ratio). Patients assigned to LGCS were selected among patients with other vascular diseases (e.g. peripheral artery disease, abdominal aortic aneurysms etc.) either hospitalized in our department or attending our outpatient clinic. The absence of neurological symptoms and cerebral ischemic lesions was based on a complete medical history, comprehensive neurological examination, and previous brain CT and/or MRI scan.
C) Control group (COG): 73 age- and sex-matched individuals served as controls of patients with established carotid atherosclerosis (HGCS plus LGCS). These individuals were selected from a large pool-registry of our Hospital's Departments of Cardiology and Vascular Surgery. They were considered eligible if they had no current manifestations of atherosclerotic coronary or peripheral artery disease. In particular, they did not have angiographically proven CAD within the last year (non-significant stenosis of all major epicardial coronary arteries). Besides this, their peripheral arteries (carotids and lower limbs arteries) were free from atherosclerotic plaques based on ultrasound records within the last two years.

General exclusion criteria for this current analysis were cerebral hemorrhage, severe liver (AST ≥ 3 times the upper normal limit) or renal impairment (creatinine ≥ 2 mg/dl), moderate-to-severe heart failure (NYHA II—IV), atrial fibrillation or flutter, concurrent conditions/diseases interfering with the expression of inflammatory mediators, like major trauma, surgery, cardiovascular ischemic events within the previous month, malignancies, chronic inflammatory or autoimmune diseases, as well as patients with acute infection at study entrance.

Written informed consent from each participant was obtained before enrollment and all procedures were performed according to the principles of Helsinki Declaration and were approved by the hospital human ethics committee.

### 2.2. Anthropometrical and clinical data collection

At study begining body-mass index (BMI) and waist-to-hip ratio (WHR) were obtained in all participants. The percentage of body fat-mass was also measured using the body composition analyzer (Bodystat 1500, Bodystat Ltd, Isle of Man, British Isles). Blood pressure was measured twice, after keeping participants at a sitting position for 15 min. There was a 5 min interval between the two measurements and the mean value was estimated.

Using a structure questionnaire we recorded medical history, medications and co-morbidities. The latter were defined as well as: hypertension (blood pressure ≥140/90 mmHg measured on repeated occasions or presence of antihypertensive drugs), hyperlipidemia (fasting serum cholesterol levels ≥200 mg/dl or statin therapy), active smokers (current or within the previous 6 months), diabetes mellitus (fasting plasma glucose—FPG ≥120 mg/dl, or HbA1c ≥ 6.5%, or antidiabetic drugs), coronary artery disease—CAD (history of stable or unstable angina, myocardial infarction, percutaneous or surgical revascularization).

### 2.3. Carotid ultrasound examination and image-guided measurements

Carotid ultrasound examinations were performed by two experienced operators, with the use of ultrasound linear array 12 MHz transducer (General Electric LogiqE, Riverside, USA). With the patient in the supine position and the neck slightly extended, the carotid artery was investigated bilaterally in a suitable longitudinal and transverse view (the angle of insonation was less than or equal to 60°) during breath holding. In order to reach reproducibility of carotid measurements we have developed an internally validated carotid ultrasound procedure given the frequent correlative angiograms at our institution. For each subject, the carotid artery was scanned in the longitudinal direction according to a standardized protocol (imaging modality, B-mode; dynamic range, 60 dB; persistence, low; frame rate, higher than 25 frames/s) and ultrasound image recordings were stored for later offline analysis. Moreover the comparability between groups was achieved by using pre-defined anatomical areas of interest including 3 cm length of proximal ICA, the carotid bulb and 1 cm length of distal CCA. The distribution of plaques was searched at those predefined carotid arterial segments. The atherosclerotic lesions were identified in three carotid arterial segments: (a) the carotid bifurcation (50%), (b) the ICA (30%), and (c) the carotid bifurcation and the CCA (20%) (Fig. 1).

The degree of carotid stenosis was measured according to the European Society of Vascular Surgery Guidelines [18]. Peak systolic velocity (PSV) and ICA/CCA PSV ratio were calculated and thereafter the percentage of arterial stenosis was graded according to the recommendations of the Society of Radiologists in Ultrasound [19].

Morphological and textural features of the plaques were estimated from the stored ultrasound image recordings of the study subjects, using in-house software developed in Matlab (version 2012b; MathWorks, Natick, USA) [20]. The technicians doing the image analyses were unaware of clinical status of participants. In a pre-processing step, gray-scale image intensities ([0: black, 255: white]) were linearly adjusted so that the median grey level value of the blood is 0, and the median grey level value of the adventitia is 190 [21]. This standardization procedure is necessary for comparable and unbiased texture measurements over different operators or/and ultrasound scanner settings. Thereafter, we measured the Gray-Scale Median (GSM) score and the TPA of each carotid plaque [22] (Fig. 2). In case of symptomatic patients, we considered the GSM score of the culprit lesion, ipsilateral to brain infarct, whereas in asymptomatic patients we considered for statistical analysis the average value of GSM of all plaques in both carotids. In cases of multiple plaques in a single vessel, GSM and TPA were measured separately for each plaque and the average of GSM and sum of TPA measurements were considered for analysis.

### 2.4. Blood assays

For all patients, blood sampling was performed in the morning after an overnight fast, between 8.00 and 10.00am. FPG and lipid parameters were all measured in an automatic enzymatic analyzer (Olympus AU560, Hamburg, Germany). Using commercially available enzyme immunoassay kits, we assayed serum concentrations of RBP4(Immunodiagnostik AG, Bensheim, Germany) and omentin-1 (Enzo Life Sciences, Farmingdale, NY, USA). The intra-assay coefficients of variation (CVs) for RBP4 and omentin-1 were 9.7% and 5.2%, respectively, and the inter-assay CVs were 5% and 4.61%, respectively. Measurement of high-sensitivity CRP (hsCRP) was performed using a particle enhanced immunoturbidimetric assay (Hitachi 917 analyzer; Boehringer Mannheim, Germany). Samples were frozen and stored (−80 °C) until analysis in a blinded manner with respect to any clinical information.

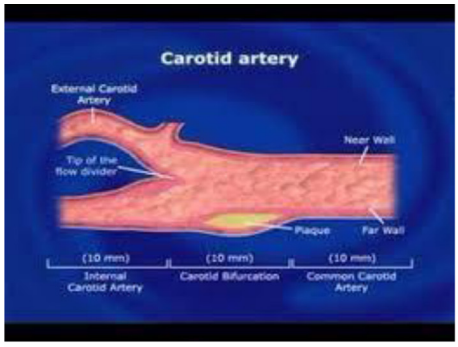
### 2.5. Statistical analysis

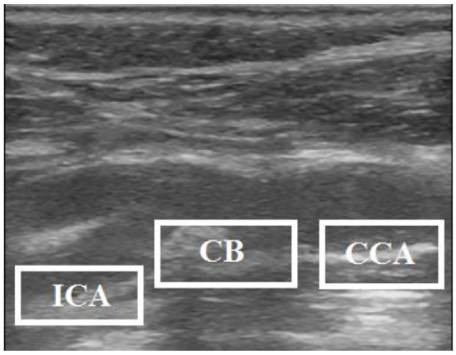
Results of normally distributed continuous variables were expressed as the mean value ± SD. Normality of distribution was assessed with Kolmogorov–Smirnov test. Comparisons of continuous and categorical variables were analyzed with the student's *t*-test and chi-square test, respectively. To test the univariate and multivariate associations of TPA (carotid stenosis severity) and GSM score (carotid atherosclerosis vulnerability) with any of the study population characteristics, we performed a Pearson correlation and multiple linear regression analysis, respectively, for normally distributed variables. Multivariate models for both TPA and GSM score were both adjusted for traditional cardiovascular risk factors (age, sex, diabetes, hypertension, dyslipidemia and BMI). A two-tailed $p$ value <0.05 was considered to be statistically significant. The computer software package SPSS (version 20.0; SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

## 3. Results

### 3.1. Comparison between established carotid atherosclerosis and controls

Results are depicted in Table 1. Patients with established carotid atherosclerosis showed significantly higher frequency of major cardiovascular risk factors, such as smoking habit, diabetes, hypertension and dyslipidemia, than individuals in the control group.




ICA, internal carotid artery; CB, carotid bifurcation; CCA: common carotid artery.

**Fig. 1.** A drawing of pre-defined anatomical carotid arterial segments and the related segmentation on carotid ultrasound image. ICA, internal carotid artery; CB, carotid bifurcation; CCA, common carotid artery.

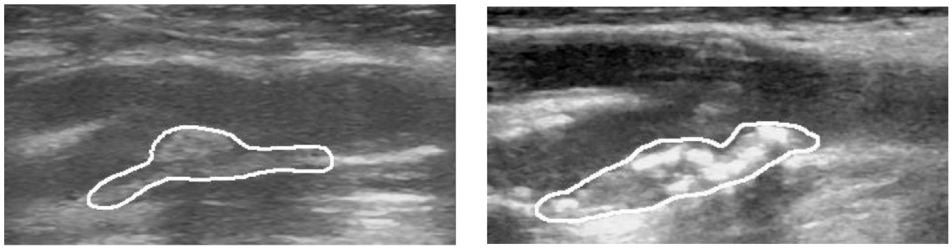



**Fig. 2.** Representative images of plaques with a high and a low GSM score.

Moreover, they appeared with higher concentrations of RBP4 and inflammatory markers (hsCRP and white blood cells count) ($p < 0.05$) and lower concentrations of omentin-1 ($p < 0.001$) than control counterparts.

Subgroup analysis revealed that among patients with established carotid atherosclerosis those with concomitant CAD had higher RBP4 levels than non-CAD counterparts (59.22 ± 15.55 mg/L vs 42.91 ± 13.44 mg/L, $p < 0.001$). Nevertheless, the latter group maintained considerably higher concentrations than controls ($p < 0.001$). Another important finding was the positive impact of male gender on RBP4 levels within the carotid atherosclerosis group (51.33 ± 16.84 mg/L vs 43.32 ± 12.11 mg/L, $p = 0.048$), but not within control group. Statins therapy improved lipid profile, but had no impact on RBP-4 serum levels. The rest of clinical parameters (e.g. diabetes, dyslipidemia, aneurysms, peripheral artery disease) had non-significant effects on RBP4 levels (data not shown).

On the other hand, the concentrations of omentin-1 in non-CAD patients with carotid atherosclerosis (576 ± 181.04 ng/ml) significantly differed from CAD-patients with carotid atherosclerosis (402.21 ± 154.21 ng/ml vs, $p < 0.001$) and controls (815.3 ± 185.32 ng/ml, $p < 0.001$). Another important finding was the significant reduction of omentin-1 serum levels in diabetic versus non-diabetic patients within both groups of carotid atherosclerosis (388.83 ± 110.19 ng/ml vs 590.56 ± 191.23 ng/ml, $p < 0.001$) and controls (788.25 ± 190.94 ng/ml vs 878.84 ± 167.84 ng/ml, $p = 0.045$). Across the whole study cohort of carotid atherosclerosis, statin-treated patients appeared with higher omentin-1 serum levels rather than statin-free counterparts (538.54 ± 197.28 ng/ml vs 458.86 ± 155.59 ng/ml, $p = 0.014$). No other confounding factors of omentin-1 were identified (e.g. hypertension, smoking, peripheral artery disease, aneurysms presence etc).

### 3.2. Comparison between high-grade and low-grade carotid stenosis groups

The clinical, biochemical, ultrasonographic, and pharmaceutical data of our age- and sex-matched HGCS and LGCS are presented in Table 2. With the exception of HDL and hsCRP, there were no significant differences in anthropometrical features, co-morbidities, medications, lipid and glycemic profile between groups ($p > 0.05$). As expected, a larger TPA was observed in HGCS than LGCS ($p < 0.001$). We observed considerably higher RBP-4 ($p < 0.001$) and lower omentin-1 ($p = 0.002$) serum levels in HGCS rather than LGCS. We then examined whether the absence of symptoms ameliorates the aforementioned differences between HGCS and LGCS. Thereby, we compared asymptomatic subgroup of patients with high-grade carotid stenosis with their counterparts following conservative therapy (LGCS). The former subgroup showed persistently elevated RBP-4 levels ($p < 0.001$) and a trend for lower omentin-1 serum levels ($p = 0.063$) than LGCS. Thus, the presence of neurological symptoms tends to affect omentin-1, but not RBP4 circulating levels.

We further proceeded to subgroup analysis as we described in the previous section. The influence of the abovementioned factors on serum levels of RBP4 (CAD, male gender) and omentin-1 (diabetes, CAD, statins therapy) remained significant within both HGCS and LGCS (data not shown).

### 3.3. Comparison between symptomatic and asymptomatic patients

Within HGCS, we comparatively evaluated symptomatic and asymptomatic subgroups. As shown in Table 3, these subgroups did not differ in anthropometrical, clinical, pharmaceutical and metabolic parameters ($p > 0.05$). Although they had similar carotid atherosclerotic burden, as assessed by the magnitude of TPA, symptomatic patients had significantly lower GSM score than asymptomatic counterparts ($p < 0.001$). In addition to this, the former subgroup appeared with lower omentin-1 serum levels ($p = 0.004$), while RBP-4 did not differ between subgroups ($p = 0.352$).

**Table 1**
Clinical and biochemical characteristics of individuals with either established carotid atherosclerosis (high and low grade carotid stenosis) or non-atherosclerotic manifestations (control group).

| Variables | Established carotid atherosclerosis ($N = 300$) | Control group ($N = 73$) | $p$ |
|---|---|---|---|
| Men/Women | 208/92 | 52/21 | 0.915 |
| Age (years) | 68 ± 8 | 65 ± 11 | 0.551 |
| Smokers ($n$) | 150 (50%) | 23 (25.27%) | <0.001 |
| Diabetes ($n$) | 107 (35.7%) | 12 (16.4%) | <0.001 |
| Hypertension ($n$) | 258 (86%) | 49 (67.1%) | 0.034 |
| Dyslipidemia ($n$) | 282 (94%) | 52 (71.2%) | <0.001 |
| CAD ($n$) | 103 (34.3%) | – | – |
| PAD ($n$) | 112 (37.3%) | – | – |
| BMI (kg/m$^2$) | 27.14 ± 3.79 | 27.44 ± 3.88 | 0.623 |
| WHR | 0.952 ± 0.083 | 0.951 ± 0.091 | 0.978 |
| Fat-mass (%) | 31.84 ± 8.20 | 33.1 ± 7.54 | 0.502 |
| Systolic BP (mmHg) | 134 ± 17 | 131 ± 17 | 0.576 |
| Diastolic BP (mmHg) | 78 ± 11 | 80 ± 12 | 0.622 |
| TChol (mg/dl) | 174 ± 44 | 192 ± 52 | 0.213 |
| HDL (mg/dl) | 47 ± 12 | 48 ± 13 | 0.801 |
| LDL (mg/dl) | 99 ± 36 | 120 ± 43 | 0.098 |
| Triglycerides (mg/dl) | 140 ± 95 | 122 ± 66 | 0.449 |
| FPG (mg/dl) | 136 ± 47 | 120 ± 25 | 0.584 |
| hsCRP (mg/L) | 2.98 ± 0.95 | 1.27 ± 0.48 | <0.001 |
| WBC (cells/μL) | 7568 ± 1885 | 7026 ± 1858 | 0.022 |
| RBP4 (mg/L) | 48.18 ± 14.12 | 25.74 ± 10.72 | <0.001 |
| Omentin-1 (ng/ml) | 518.61 ± 191.10 | 815.3 ± 185.32 | <0.001 |

Data are means ± SD. $n$, number of patients; CAD, Coronary artery disease; PAD, peripheral arterial disease; BMI, Body-mass index; BP, blood pressure; TChol, total cholesterol; FPG, Fasting Plasma Glucose; hsCRP, high sensitivity C-reactive protein; WBC, White Blood Cells; GSM score, RBP4, retinol binding protein-4.

**Table 2**
Differences in clinical and biochemical parameters in high-grade and low-grade carotid stenosis groups.

| Variables | High-grade carotid stenosis group ($N = 225$) | Low-grade carotid stenosis group ($N = 75$) | $p$ |
|---|---|---|---|
| Men/Women | 155/70 | 53/22 | 0.937 |
| Age (years) | 68 ± 9 | 66 ± 10 | 0.771 |
| Smokers ($n$) | 115 (51.1%) | 35 (46.7%) | 0.428 |
| Diabetes ($n$) | 86 (38.2%) | 21 (28%) | 0.197 |
| Hypertension ($n$) | 190 (82.4%) | 68 (90.7%) | 0.288 |
| Dyslipidemia ($n$) | 215 (95.6%) | 67 (89.3%) | 0.183 |
| CAD ($n$) | 77 (34.2%) | 26 (34.7%) | 0.980 |
| PAD ($n$) | 80 (35.6%) | 23 (30.7%) | 0.426 |
| BMI (kg/m$^2$) | 27.08 ± 3.68 | 27.32 ± 4.11 | 0.687 |
| WHR | 0.948 ± 0.083 | 0.963 ± 0.095 | 0.711 |
| Fat-mass (%) | 31.49 ± 8.23 | 32.86 ± 8.18 | 0.510 |
| Systolic BP (mmHg) | 135 ± 19 | 130 ± 14 | 0.620 |
| Diastolic BP (mmHg) | 78 ± 11 | 77 ± 11 | 0.901 |
| TChol (mg/dl) | 173 ± 41 | 176 ± 56 | 0.668 |
| HDL (mg/dl) | 48 ± 12 | 44 ± 12 | 0.017 |
| LDL (mg/dl) | 97 ± 34 | 103 ± 44 | 0.313 |
| Triglycerides (mg/dl) | 140 ± 95 | 144 ± 95 | 0.776 |
| FPG (mg/dl) | 136 ± 48 | 134 ± 47 | 0.797 |
| hsCRP (mg/L) | 3.44 ± 1.01 | 1.58 ± 0.55 | <0.001 |
| WBC (cells/μL) | 7601 ± 2023 | 7470 ± 1689 | 0.670 |
| RBP-4 (mg/L) | 51.44 ± 16.23 | 38.39 ± 8.85 | <0.001 |
| Omentin-1 (ng/ml) | 490.41 ± 172 | 603.20 ± 202.43 | <0.001 |
| TPA (mm$^2$) | 0.93 ± 0.37 | 0.50 ± 0.24 | <0.001 |
| GSM score | 44.36 ± 15.40 | 63.63 ± 22.75 | <0.001 |

Data are means ± SD. n, number of patients; CAD, Coronary artery disease; PAD, peripheral arterial disease; BMI, Body-mass index; BP, blood pressure; TChol, total cholesterol; FPG, Fasting Plasma Glucose; hsCRP, high sensitivity C-reactive protein; WBC, White Blood Cells; RBP4, retinol binding protein-4; TPA, total plaque area; GSM score, Gray Scale Median score.

### 3.4. Correlations

We evaluated the relation of TPA and GSM with various clinical and biochemical features. In univariate analysis, TPA correlated positively with RBP4 ($r = 0.211$, $p = 0.011$), systolic blood pressure ($r = 0.264$, $p = 0.047$) and hsCRP ($r = 0.241$, $p = 0.020$). After entering a multiple regression analysis model, adjusted for traditional cardiovascular risk factors, such as age, sex, diabetes, hypertension, dyslipidemia and BMI, RBP4 ($p = 0.025$) and hsCRP ($p = 0.004$) remained independent determinants of TPA (Table 4a). There was also a significant positive association of GSM score with hsCRP ($r = 0.177$, $p = 0.022$) and omentin-1 ($r = 0.169$, $p = 0.041$). Among the latter variables only hsCRP (beta = 0.185, $p = 0.036$) was independently associated with GSM score in multivariate analysis (Table 4b).

By simple logistic regression analysis adjusted for age, sex, diabetes, dyslipidemia and hypertension, the presence of ipsilateral stroke was independently associated with the GSM score within the HGCS [Exp(B) = 1.189 (CI: 1.080–1.395), $p = 0.033$], but not with adipokines levels.

**Table 3**
Comparison of variables between symptomatic and asymptomatic patients within group of high-grade carotid stenosis undergoing carotid revascularization.

| Variables | Symptomatic patients ($N = 73$) | Asymptomatic patients ($N = 152$) | $p$ |
|---|---|---|---|
| Men/Women | 49/24 | 112/40 | 0.298 |
| Age (years) | 66 ± 10 | 69 ± 9 | 0.701 |
| Smokers ($n$) | 40 (54.8%) | 75 (49.3%) | 0.272 |
| Diabetes ($n$) | 33 (45.21%) | 53 (34.87%) | 0.065 |
| Hypertension ($n$) | 58 (79.45%) | 132 (86.84%) | 0.207 |
| Dyslipidemia ($n$) | 68 (93.15%) | 147 (96.08%) | 0.776 |
| CAD ($n$) | 22 (30.14%) | 55 (35.95%) | 0.395 |
| BMI (kg/m$^2$) | 26.83 ± 3.88 | 27.20 ± 3.61 | 0.876 |
| WHR | 0.927 ± 0.098 | 0.958 ± 0.85 | 0.412 |
| Fat-mass (%) | 30.60 ± 8.46 | 33.35 ± 7.82 | 0.080 |
| Systolic BP (mmHg) | 135 ± 20 | 135 ± 17 | 0.959 |
| Diastolic BP (mmHg) | 76 ± 12 | 79 ± 10 | 0.116 |
| Total cholesterol (mg/dl) | 177 ± 41 | 171 ± 47 | 0.841 |
| HDL (mg/dl) | 48 ± 14 | 47 ± 12 | 0.181 |
| LDL (mg/dl) | 101 ± 37 | 95 ± 37 | 0.679 |
| Triglycerides (mg/dl) | 140 ± 81 | 140 ± 101 | 0.970 |
| FPG (mg/dl) | 110 ± 44 | 104 ± 50 | 0.411 |
| hsCRP (mg/L) | 5.51 ± 1.12 | 2.45 ± 0.76 | <0.001 |
| WBC (cells/μL) | 7643 ± 2085 | 7580 ± 1920 | 0.772 |
| RBP-4 (mg/L) | 50.61 ± 10.62 | 51.84 ± 17.96 | 0.852 |
| Omentin-1 (ng/ml) | 458.02 ± 170.05 | 505.95 ± 187.26 | 0.004 |
| TPA (mm$^2$) | 0.96 ± 0.39 | 0.92 ± 0.37 | 0.398 |
| GSM score | 27.24 ± 12.87 | 52.58 ± 24.43 | <0.001 |

Data are means ± SD. CAD, Coronary artery disease; BMI, Body-mass index; BP, blood pressure; FPG, Fasting Plasma Glucose; hsCRP, high sensitivity C-reactive protein; WBC, White Blood Cells; TPA, total plaque area; GSM score, Gray-Scale Median score.

**Table 4a**
Independent associations of total plaque area with variables using linear standard multiple regression analysis ($R^2 = 0.201$, $p = 0.005$).

| Variables | Total plaque area | | |
|---|---|---|---|
| | beta | $p$ | 95% CI |
| Age | 0.510 | 0.220 | 0.107–1.051 |
| Sex | 0.138 | 0.202 | 0.054–0.321 |
| Diabetes | 0.194 | 0.095 | 0.059–0.323 |
| Hypertension | 0.014 | 0.934 | −0.261–0.289 |
| Dyslipidemia | 0.138 | 0.331 | 0.010–0.697 |
| BMI | 0.221 | 0.198 | 0.110–0.356 |
| RBP4 | 0.242 | 0.021 | 0.193–0.312 |
| hsCRP | 0.289 | 0.006 | 0.219–0.425 |

CI, confidence intervals; hsCRP, high-sensitivity C-reactive protein.

## 4. Discussion

To our knowledge this is the first study evaluating the relationship of novel adipokines, RBP-4 and omentin-1, with ultrasonographically assessed carotid TPA and density. Our cross-sectional study showed significantly higher RBP4 and lower omentin-1 circulating levels in patients with carotid atherosclerosis compared to individuals without atherosclerotic manifestations. Moreover, serum RBP-4 levels were positively related to plaque area scoring (TPA), independent of traditional cardiovascular risk factors or symptoms. On the other hand, low omentin-1 serum levels were associated with echolucent plaques and symptoms only in univariate analysis.

Our results support a potential atherogenic role of RBP-4. Elevated RBP4 concentrations were found in patients with established carotid atherosclerosis. Moreover, they were independently related to greater carotid TPA, an emerging and quantified measure

**Table 4b**
Independent associations of Gray-Scale Median (GSM) score with variables using linear standard multiple regression analysis ($R^2 = 0.221$, $p = 0.029$).

| Variables | Gray-scale median score | | |
|---|---|---|---|
| | beta | $p$ | 95% CI |
| Age | 0.034 | 0.591 | −0.498 to −0.598 |
| Sex | 0.165 | 0.067 | 0.021–0.542 |
| Diabetes | 0.107 | 0.227 | −0.371–0.401 |
| Hypertension | 0.177 | 0.080 | 0.017–0.393 |
| Dyslipidemia | 0.098 | 0.274 | −0.155–0.357 |
| BMI | 0.145 | 0.104 | 0.099–0.192 |
| Omentin-1 | 0.106 | 0.449 | 0.032–0.319 |
| hsCRP | 0.185 | 0.036 | 0.162–0.201 |

CI, confidence intervals; hsCRP, high-sensitivity C-reactive protein; BMI, body-mass index.

of atherosclerosis burden and a valid prognostic factor of cardiovascular events [23]. Most recently Dessein et al. (2014) documented the relationship of RBP4 with carotid plaque presence among patients with rheumatoid arthritis [24]. This is the first study demonstrating a positive linear relationship of RBP4 with the magnitude of carotid atherosclerotic lesions. It has been widely considered that central obesity, insulin resistance, dyslipidemia, and inflammation are risk factors for carotid atherosclerosis [25]. Most, but not all, recent clinical and experimental studies have reported the involvement of RBP4 in the latter pro-atherogenic complex mechanisms [13,26–28]. In contrast, other studies have disputed the mediating role of RBP4 in insulin resistance [29] or its contribution to atherosclerotic plaque formation [30].Therefore; it remains unclear whether increased RBP4 reflects a causative factor or a bystander of atherosclerotic cardiovascular disease.

Although our study was not designed to investigate the underlying molecular mechanisms, it implicated the involvement of RBP4 in carotid atherosclerosis progression. Most importantly, the coexistence of CAD or male gender only merely attenuated the impact of established carotid atherosclerosis on RBP4 levels. On the other hand, the absence of interaction between circulating RBP4 and plaque-related symptomatology limits its role in stable atherosclerotic lesions. This has been previously confirmed in subpopulations with coronary [31] or carotid atherosclerosis [32]. Perhaps, RBP4 is involved in advanced atherosclerosis; however, the underlying mechanisms regulating carotid plaque vulnerability are not related to RBP4.

Two previous studies have reported the inverse relationship between circulating omentin-1 and early carotid atherosclerosis [16,33]. Recently, Yoo et al. demonstrated low circulating omentin-1 was proved as an independent determinant of carotid plaque existence among type 2 diabetic patients [34]. Our study extended previous scientific knowledge by demonstrating suppressed serum omentin-1 concentrations in patients with established carotid atherosclerosis compared to non-atherosclerotic controls. Moreover, the carotid-related symptomatology was associated with further reduction in omentin-1. That effect seemed superior to the degree of carotid stenosis, since asymptomatic HGCS showed only a non-significant trend for lower omentin-1 compared to asymptomatic LGCS. Perhaps, the omentin-1-associated regulatory mechanisms of plaque rupture superimpose those of plaque development.

Experimental studies have suggested several atheroprotective and anti-inflammatory mechanisms of omentin-1 [35–37]. Despite limited clinical evidence implicating the relationship between omentin-1 and acute coronary syndromes, the underlying mechanisms remain elusive [38,39]. In the present study, omentin-1 inversely correlated with GSM score, however that relationship was faded out after adjustment for established cardiovascular risk factors. Since we drew blood samples at least after 15 days from the last stroke/TIA, it is unclear whether omentin-1 assay serves as biomarker of the acute phase of plaque destabilization. It may simply indicate the extent of cerebrovascular damage and concomitant carotid atherosclerosis. However, the circulating biomarker profile of subjects with remote symptoms of carotid stenosis may remain at least partially different from that of never-symptomatic subjects. Future studies enrolling patients with acute ischemic stroke/TIA and advanced carotid atherosclerosis will shed more light on this issue.

Despite the strengths of our study, we are well aware of some limitations. Notwithstanding the large sample, the cross-sectional design of our study prevented from clarifying the role of RBP4 and omentin-1 as bystanders or causative agents in carotid atherosclerosis progression. On the other hand, the assessment of circulating biomarkers does not necessarily reflect the release of the biomarker from the index lesion, since a sufficiently high release is required to affect its systemic level. All symptomatic patients entered our study at sub-acute or chronic phase after event (>15 days). Thus, our study might have underestimated the differences in inflammatory markers between symptomatic and other groups, regarding the excessive inflammatory reaction at acute phase. In order to gain further insight into the prognostic role of novel adipokines, future cohort studies should prospectively assess their levels in combination with long-term clinical outcomes. Finally, it would be interesting to assess adipokines levels with intermediate carotid stenosis.

In conclusion, circulating RBP4 concentrations were higher in patients with established carotid atherosclerosis than controls and paralleled the degree of carotid stenosis. Most importantly RBP4 was independently associated with TPA. Inversely, low omentin-1 levels were positively associated with either carotid-related symptomatology or low GSM score, but not after multivariate analysis. Further studies will clarify the pathogenetic role of these adipokines at acute phase of cerebrovascular events and will evaluate their contribution to stroke-patients prognosis. Finally, a widespread usage of carotid plaque echogenicity index (GSM score) and adipokines may assist the monitoring of carotid plaque vulnerability along time or after intensive pharmaceutical therapy (e.g. statins).

## Conflict of interest

All authors declare no conflict of interest.

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