Galectin-3 and Lp(a) plasma concentrations and advanced carotid atherosclerotic plaques: correlation with plaque presence and features

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ABSTRACT

Introduction: atherosclerosis is one of the leading causes of death and morbidity worldwide. It consists in the development of plaques in the intima media layers of arteries due to lipid accumulation and oxidation, causing massive inflammation. We aim to better understand the role of Galectin-3 (Gal-3) and Lipoprotein(a) [Lp(a)] as possible peripheral markers of plaque presence.
Methods: Gal-3 and Lp(a) were measured in plasma samples from 99 patients undergoing carotid endarterectomy and 78 healthy controls, by immunometric assays. Plaques were classified histologically, according to the American Heart Association (AHA) guidelines as type Va (fibroatheroma), Vb (mainly calcific) and VI (complicated lesion).
Results: Gal-3 and Lp(a) plasma levels are higher in patients compared to controls [19.8 ng/mL (SD 5.8) vs 14.0 ng/mL (3.6)], p<0.0001 and 8.4 mg/dL (IQR 4.0-25.1) vs 4.7 mg/dL (2.4-12.7), p=0.0003, respectively). Analysis of ROC curves confirmed the discriminating power of these markers obtaining an area under the curve of 0.806 (p<0.0001) for Gal-3 and 0.657 (p=0.0001) for Lp(a). At multivariate logistic regression, Gal-3 and Lp(a) plasma levels were associated with plaque presence independently of each other as well as of age, sex, LDL-C levels and previous myocardial infarction with an odds ratio of 1.22 (95%CI 1.08-1.38, p=0.002) and 1.05 (1.00-1.09, p=0.048) respectively. No differences of Gal-3 and Lp(a) plasma levels were observed among the plaque types.
Conclusion: our data showed that Gal-3 and Lp(a) are reliable markers of advanced atherosclerotic plaques. The absence of differences among the different lesion types suggests that the increase of Gal-3 and Lp(a) is independent of the specific plaque features.

INTRODUCTION

Atherosclerosis is a progressive chronic inflammatory process of the arteries underlying several clinical conditions according to the artery involved. Carotid atherosclerosis represents an important risk factor for cerebrovascular ischemia leading to ischemic stroke, a major cause of morbidity and mortality in the developed countries (1, 2). The pathologic process leading to atherosclerosis is complex. It is commonly characterized by an altered cellular permeability of the arterial walls and the focal sub-endothelial accumulation of LDL cholesterol (LDL-C), developing atherosclerotic plaques characterized by inflammation and oxidation (3, 4). The presence of oxidized LDL-C particles in the sub-endothelial space of the interstitial arteries is described as the crucial event for the formation of foam cells and induction of inflammatory response (5). Studies on the lipid component present in atherosclerotic lesions showed that another class of lipoproteins, the

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lipoprotein (a) [Lp(a)] may be involved in the formation and progression of atherosclerotic plaques. Lp(a) is a modified LDL particle with the apolipoprotein (a) [Apo(a)], covalently attached to apolipoprotein B-100 of LDL particle by a single thioester bond. The physiological functions of Lp(a) are still unknown. Similarly to other lipoproteins, Lp(a) is also susceptible to oxidative modifications, leading to extensive formation of pro-inflammatory and pro-atherogenic oxidized phospholipids, oxysterols lipid-protein adducts in Lp(a) particles (6). Moreover, Apo(a) has several kringle domains rich in disulfide bridges similar to many coagulation and fibrinolysis proteins. In particular, a strong homology between Apo(a) and plasminogen has been demonstrated and different studies suggest that Apo(a) is able to inhibit, by a competitive inhibition mechanism, the binding of plasminogen to its receptor, thereby interfering with fibrinolysis and inducing a prothrombotic status (7). It may be inferred that Lp(a) is able both to amplify oxidative stress and inflammation-related atherogenesis and to stimulate prothrombotic mechanisms leading to acute events such as stroke or acute myocardial infarction (AMI). Lp(a) plasma levels show a significant inter-individual variability as they are inversely related to the Apo(a) size that is genetically determined (8). There are several forms of Apo(a) based on the repetition number of the kringle IV type 2 domain. A low repetition number (i.e. a small Apo(a) molecule) is associated with high Lp(a) circulating concentrations (9).

Despite its well-known atherogenic role, Lp(a) could be considered a re-emerging risk factor for cardiovascular disease since the previous methodologies were unable to measure all the Lp(a) length forms. Recently, available methods based on antibodies recognizing the fixed region of the protein, have improved the accuracy of the assays, being able to measure the Lp(a) containing Apo(a) with different lengths (10).

Inflammation is the main process involved in the development of atherosclerotic plaque and macrophages play a pivotal role through the synthesis and secretion of pro-inflammatory mediators including cytokines and matrix metalloproteinase (11). Several molecules involved in pathobiology of atherogenesis as well as some genetic conditions have been investigated over the years attempting to identify their role as markers or predictors of cardiovascular diseases (12-15). Galectin-3 (Gal-3) is a β-galactoside-binding lectin belonging to the Galectin family secreted by macrophages (16). Once secreted, Gal-3 acts in an autocrine way activating macrophages and other inflammatory cells and promoting the migration of monocytes into vascular walls. It is a multifunctional, pleiotropic protein involved in several biological processes among which macrophage chemotaxis, phagocytosis, oxidative stress, cell proliferation, and deposition of type-1 collagen in the extracellular matrix (ECM) (17). It is ubiquitously expressed and, although it is predominantly located in the cytoplasm, it has also been detected in the nucleus, on the cell surface and in the circulation (16). Circulating Gal-3 levels significantly increase under several pathological conditions such as AMI, heart failure, acute coronary syndrome, cancer and renal diseases (18-21). Upregulation of Gal-3 has been described both in rodent models of atherosclerotic disease and human atherosclerotic lesions (22). The majority of animal studies have consistently indicated the correlation between Gal-3 plaque levels and atherosclerosis development (23, 24). In vitro studies suggested that Gal-3 could promote the migration of monocytes into vascular walls (22). Other studies reported that Gal-3 is correlated with matrix metalloproteinases which play an important role in plaque destabilization (25) and modulates vascular calcification (26). However, few clinical studies have been performed to assess the role of Gal-3 as a circulating marker of atherosclerotic plaque presence and features.

The aim of this study was to investigate the association of Lp(a) and Gal-3 plasma levels with the presence of carotid atherosclerotic plaque and identify possible differences between the different plaque types in a population of patients undergoing carotid endarterectomy.

METHODS

Study population

We enrolled 99 consecutive patients undergoing carotid endarterectomy for stenosis ≥70% or stenosis ranging from 50% to 70% associated to clinical symptoms according to the American Heart Association (AHA) guidelines. Patients were enrolled at the Department of Public Health (Vascular Surgery Unit) of the University of Naples Federico II. Physiological, pathological and anamnestic data were collected for each patient. The gathered data included age, body mass index (BMI) calculated dividing the weight (kg) by the height squared meters, and cardiovascular risk factors including arterial hypertension, diabetes mellitus, dyslipidemia, obesity, smoking and previous AMI. Patients were considered hypertensive if they had systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or were receiving anti-hypertension therapy. Diabetes mellitus was defined as non-fasting glucose >10.5 mmol/L, fasting plasma glucose >6.9 mmol/L and/or glycosylated hemoglobin > 47.5 mmol/L, treatment for diabetes mellitus, or self-reported physician diagnosis of diabetes mellitus. Dyslipidemia was defined according to the American Association of Clinical Endocrinologists and the American College of Endocrinology Guidelines (27) or if patients were under treatment with statins or fibrates. Patients were defined as obese if their BMI was ≥30 kg/m^2. Smokers were classified as current smokers or ex-smokers (>100 cigarettes in their lifetime but not in the last 28 days). Symptomatic patients were defined as those having a history of ischemic stroke, transient

ischemic attack or amaurosis fugax.

We recruited, also, 78 healthy blood donors as controls from a previous study (28).

The study was performed according to the current version of the Helsinki Declaration and informed consent was obtained for each patient.

Biochemical analysis

Lipid parameters including total cholesterol, HDL-cholesterol and triglycerides, as well as Lp(a) and Gal-3 were measured on plasma of patients and controls. Since high Gal-3 levels could be due to cardiac fibrosis, we firstly verified that this condition was not present in our population. Then, to rule out any recent cardiac damage, we also measured brain natriuretic peptide (BNP) and high sensitivity troponin I (hsTnI), which were both found within the reference ranges. In addition, to exclude the presence of renal failure as an additional cause of increased Gal-3 levels, we verified that the levels of urea and creatinine were within the reference intervals. Patients' blood samples were collected in a tube with EDTA before undergoing carotid endarterectomy. Plasma was separated by centrifugation at 3 000 rpm for 10 minutes and subsequently frozen in aliquots at -80°C until testing. Plasma from control subjects was obtained as previously described (28).

Lipid parameters were measured by enzymatic colorimetric method on the ACHITECT i2000R System (Abbott Laboratories, Wiesbaden, Germany). LDL-C levels were calculated by the Friedewald formula. Gal-3, BNP and hsTnI plasma levels were measured by chemiluminescent microparticle immunoassay on the ARCHITECT i1000R System (Abbott). Lp(a) plasma levels were measured by particle enhanced immunonephelometry on the BN ProSpec System (Siemens). All measurements were performed on the same plasma sample.

Histopathological analysis of carotid plaques

Surgically removed carotid plaques were collected from patients as previously described (29). Each endarterectomy specimen was immediately frozen at -80°C and stored at the biobank of CEINGE S.C.a r.l. Biotecnologie Avanzate.

Carotid plaques were histologically stained with hematoxylin–eosin according to the AHA guidelines (30, 31). Briefly, plaques were classified as type IV (atheroma), Va (fibroatheroma), Vb (mainly calcific) and VI (complicated lesion).

Histopathological analysis was performed at the Department of Advanced Biomedical Sciences of the University of Naples Federico II by an experienced pathologist at a two-headed microscope together with a second pathologist.

Statistical Analysis

The normality of variables distribution was evaluated using Kolmogorov-Smirnov test. Parametric variables are reported as mean (standard deviation, SD); non parametric variables are reported as median and interquartile range (IQR). T-test, Mann-Whitney, ANOVA and Kruskal-Wallis were used to compare data between groups. Fisher exact test was used to evaluate frequency differences of sex and AMI between patients and controls. For multivariate logistic regression, the Odds Ratio (OR) and the related 95% Confidence Interval (95%CI) were reported. Sex was codified as 0(male) or 1(female), whereas AMI was codified as 0(no AMI) or 1(AMI).

Data were analyzed using Predictive Analytics SoftWare 18.0 (SPSS Inc.). The Receiver Operating Characteristic (ROC) curve analysis was performed by MedCalc version 11.5.1. Area Under the Curve (AUC) values were reported with the 95%CI and the significance level was calculated against the null hypothesis AUC = 0.5 using the DeLong method. The optimal threshold values for Lp(a) and Gal-3 were determined by the farthest point from the bisector of the ROC curve. The Hanley & McNeil test was used to compare the AUCs of Gal-3 and Lp(a). A p value <0.05 was considered significant.

RESULTS

This study included a total of 177 subjects, 99 patients with stenosis of the carotid artery undergoing carotid endarterectomy and 78 healthy controls. Demographic, clinical and biochemical characteristics of patients and controls are shown in Table 1.

The Gal-3 and Lp(a) plasma values were significantly higher in patients than in controls, whereas the LDL-C levels were lower in patients compared to controls as showed in Table 1.

The discriminating power of Gal-3 and Lp(a) was assessed by ROC curves analysis, showing that both Gal-3 and Lp(a) plasma concentrations identify the presence of carotid atherosclerosis with high accuracy (Figure 1). The best thresholds to distinguish between patients and controls and their respective sensitivity and specificity are indicated in Figure 1.

Plasma Gal-3 is a better parameter than Lp(a) to distinguish atherosclerotic patients from controls (p= 0.003 at the comparison of ROC curves).

We observed that the values of Gal-3 and Lp(a) did not statistically differ between patients with a previous AMI (n= 17) and patients without AMI (n=82). The Gal-3 plasma concentrations were 20.3 ng/mL (SD 7.3) versus 19.7 ng/mL (5.5), p=0.701; the Lp(a) plasma concentrations were 12.3 mg/dL (IQR 4.1-28.1) versus 8.1 mg/dL (3.9-24.6), p=0.985.

To evaluate the association of Gal-3 and Lp(a) with the presence of atherosclerotic plaques independently of age, sex, LDL-C and AMI, we performed a multivariate logistic regression analysis. Although no differences of gender frequency were observed we decided to include this parameter in the multivariate model in order to verify

Table 1
Demographic, clinical and biochemical characteristics of patients and controls.

	Patients (n=99)	Controls (n=78)	p value
Age, years	71 (63-76)	61 (60-63)	<0.0005
Gender, n male	70	56	1.000
BMI, kg/m^2	26.9 (4.2)	n.a.	
Arterial hypertension, n	83	n.a.	
Diabetes, n	40	n.a.	
Dyslipidaemia, n	73	n.a.	
Obesity, n	21	n.a.	
Smoking, n	42	n.a.	
Acute myocardial infarction, n	17	0	<0.0001
High sensitivity Troponin I, pg/mL	3.3 (2.5-4.8)	2.8 (2.2-3.9)	0.057
Brain natriuretic peptide, pg/mL	10.0 (10.0-19.7)	10.0 (10.0-18.1)	0.954
Glucose, mmol/L	5.22 (4.55-6.1)	5.11 (4.72-5.49)	0.384
Total cholesterol, mmol/L	3.96 (1.03)	5.15 (0.68)	<0.0005
HDL-cholesterol, mmol/L	0.99 (0.28)	1.37 (0.30)	<0.0005
LDL-cholesterol, mmol/L	2.35 (0.82)	3.23 (0.61)	<0.0005
Triglycerides, mmol/L	1.22 (0.90-1.60)	1.12 (0.89-1.42)	0.186
Galectin-3, ng/mL	19.8 (5.8)	14.0 (3.6)	<0.0005
Lipoprotein(a), mg/dL	8.4 (4.0-25.1)	4.7 (2.4-12.7)	<0.001
Symptomatic, n	35		
Type of plaque,			
IV	2		
Va	11		
Vb	43		
VI	43		

Values are reported as mean (standard deviation); non parametric variables are reported as median (interquartile range); n.a. information not available

if the found associations were independent from this potential confounding factor. Among the lipid parameters differing between patients and controls, we included in the multivariate model only the LDL-C because a correlation between LDL-C and total cholesterol or HDL-C was observed. The Gal-3 and Lp(a) plasma levels remained significantly associated with plaque presence as shown in Table 2. We cannot include other clinical data (such as BMI, hypertension, diabetes, obesity, smoking) in the regression model because these information are lacking for the controls. However, among patients no association was found between Gal-3 or Lp(a) levels and the presence of hypertension, diabetes, obesity or smoking.

Comparing Gal-3 and Lp(a) levels among the different plaque types, no differences were found. In fact, Gal-3 levels (ng/mL) were 13.9 and 23.1 in the 2 type IV plaques, 20.2 (3.2) in type Va, 20.4 (6.1) in type Vb and 19.3 (6.0) in type VI (p=0.810 at ANOVA), whereas Lp(a) levels (mg/dL) were 3.9 and 27.8 in the 2 type IV plaques, 12.3 (2.5-39.9) in type Va, 8.1 (4.0-22.5) in type Vb and 8.4 (5.2-24.5) in type VI (p=0.953 at Kruskal-Wallis). Moreover, no differences of Gal-3 and Lp(a) levels were found between complicated (type VI – Gal-3

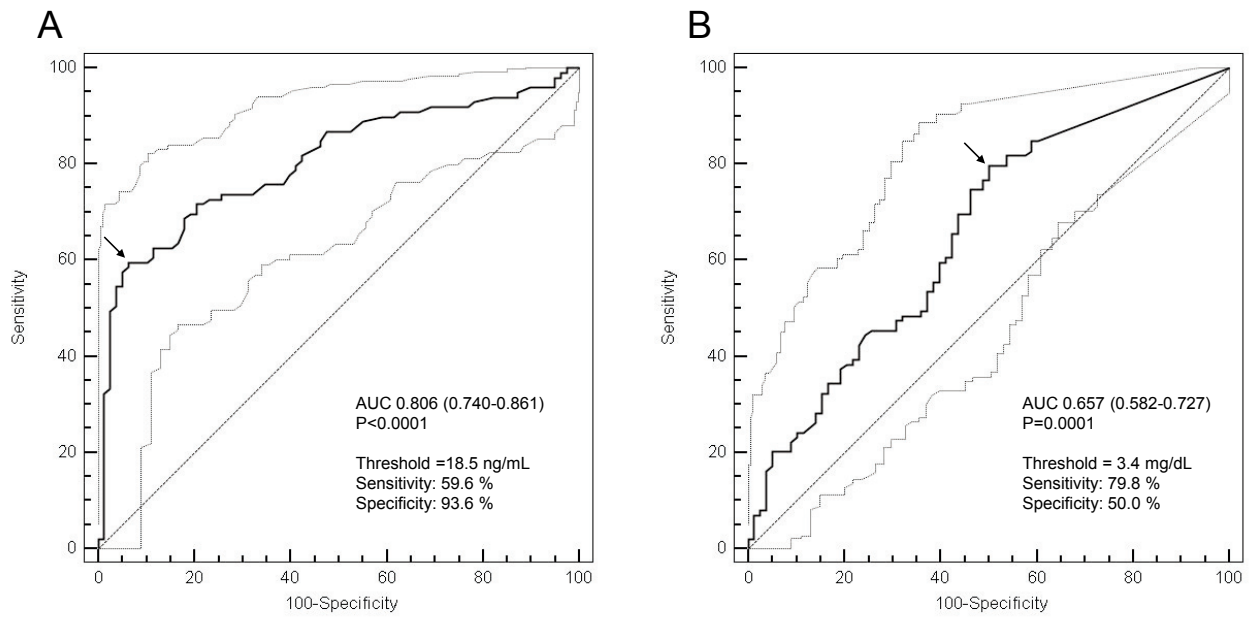

Figure 1
ROC curves of Galectin-3 and Lipoprotein(a)
Panel A: ROC curve of Galectin-3; Panel B: ROC curve of Lipoprotein (a). The ROC curve is indicated with a bold line; the dotted lines indicate the 95% confidence interval (CI) and the bisector. The best threshold to separate patients and controls is indicated with an arrow. AUC: area under the curve

Table 2
Odds ratio obtained at multivariate logistic regression using the presence of atherosclerotic plaque as dependent variable.

Predictive variables	Significance	OR	95% CI for OR
Age	0.014	1.097	1.019-1.181
Gender	0.725	0.813	0.258-2.567
Lipoprotein(a)	0.048	1.045	1.000-1.091
Galectin-3	0.002	1.217	1.076-1.377
LDL-C	p<0.0005	0.945	0.922-0.968
AMI	0.998	0.000	

OR, Odds ratio; CI, Confidence Interval; LDL-C, LDL-cholesterol.

19.3 (6.0) and Lp(a) 8.4 (5.2-24.5)) and uncomplicated plaques (type IV, Va and Vb - Gal-3 20.3 (5.6), p=0.379 and Lp(a) 8.4 (3.8-26.5), p=0.972) or between symptomatic [Gal-3 18.6 (5.3) and Lp(a) 7.6 (3.3-24.5)]and asymptomatic patients (Gal-3 20.5 (6.0), p=0.115 and Lp(a) 8.5 (4.5-26.1), p=750.

DISCUSSION

In this study we analyzed the association of Gal-3 and Lp(a) plasma values with the presence of advanced carotid atherosclerotic plaque. We found that patients with carotid plaque had higher levels of Gal-3 and Lp(a) than healthy controls.

Increased levels of Lp(a) are a well-known marker of cardiovascular events (32); as different results were obtained using different methods on the same sample, standardized methods were deemed necessary to measure all the Lp(a) length forms (33). The currently available methods allow to bypass the measurement differences among the length forms giving rise to a more accurate analysis of the association of Lp(a) levels to

cardiovascular diseases and acute events. Using a standardized method, we verified that Lp(a) levels alone are able to distinguish between patients with advanced plaque from healthy subjects.

We also observed that patients showed lower LDL-C levels than healthy controls; this could be due to the fact that patients were under statin therapy that influences LDL-C values but has a minor effect on Lp(a). As to Gal-3, there are several evidences demonstrating the association of its circulating levels with cardiovascular disease: Madrigal-Matute et al. found significantly higher Gal-3 levels in patients with carotid atherosclerosis in comparison with controls (34) and Ozturk et al. showed that circulating Gal-3 is a predictor of coronary atherosclerosis in patients with diabetes mellitus type 2 (35). Recently, increased Gal-3 circulating concentrations were found to be associated with high intima media thickness in patients with initial atherosclerosis (36) as well as with unfavorable outcomes after ischemic stroke (37). Our results are in agreement with the above mentioned findings. Since increased Gal-3 levels are associated with heart failure severity, cardiac fibrosis and renal dysfunction (21), we firstly verified that in our population no differences were present between patients with or without a previous AMI. Furthermore, to rule out any recent cardiac event or renal disease, we analyzed BNP, hsTnI, urea and creatinine in patient plasma. We concluded that the increased levels of Gal-3 cannot be attributed to these other pathological conditions.

The relationship between circulating Gal-3 and plaque characteristics has not been thoroughly investigated and only the correlation of high Gal-3 levels with calcified plaques (calcium score evaluated by imaging) was found (35). In our study, no differences of Gal-3 levels were observed among the different plaque types histologically assessed. The difference between our results and the latter study could be attributed to the different method used to evaluate plaque features. In addition, no differences were observed between complicated and uncomplicated plaques, suggesting that the increase of Gal-3 could be a marker of the various atherosclerotic lesion types.

We also observed that the association of Gal-3 and Lp(a) with plaque presence was independent of each other and of age, sex, LDL-C and previous AMI. In particular, the observed ORs indicate that increased levels of Gal-3 represent a risk factor more relevant than Lp(a) levels. Additional support to this datum was provided by the comparison of Gal-3 and Lp(a) ROC curves showing that Gal-3 is significantly more powerful in the discrimination between atherosclerotic patients and controls.

However, a limitation to the study is that we were unable to evaluate the role of several factors (BMI, hypertension, diabetes, obesity and smoking) on the association of Gal-3 and Lp(a) levels with plaque presence because this information about controls were lacking.

In conclusion, our findings suggest that high plasma concentrations of both Gal-3 and Lp(a) are reliable biomarkers of advanced atherosclerotic plaque, independently of the specific plaque type.

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CONFLICTS OF INTEREST

None

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