Galectin-3, a Biomarker Linking Oxidative Stress and Inflammation With the Clinical Outcomes of Patients With Atherothrombosis

Julio Madrigal-Matute, PhD; Jes Sandal Lindholt, MD, PhD; Carlos Ernesto Fernandez-Garcia, BSc; Alberto Benito-Martin, PhD; Elena Burillo, PhD; Guillermo Zalba, PhD; Oscar Beloqui, PhD; Patricia Llamas-Granda, BSc; Alberto Ortiz, MD, PhD; Jesus Egido, MD, PhD; Luis Miguel Blanco-Colio, PhD; Jose Luis Martin-Ventura, PhD

Background—Galectin-3 (Gal-3) participates in different mechanisms involved in atherothrombosis, such as inflammation, proliferation, or macrophage chemotaxis. Thus, there have been committed intensive efforts to elucidate the function of Gal-3 in cardiovascular (CV) diseases. The role of Gal-3 as a circulating biomarker has been demonstrated in patients with heart failure, but its importance as a biomarker in atherothrombosis is still unknown.

Methods and Results—Because Gal-3 is involved in monocyte-to-macrophage transition, we used fresh isolated monocytes and the in vitro model of macrophage differentiation of THP-1 cells stimulated with phorbol myristate acetate (PMA). Gal-3 release is increased by PMA in human monocytes and macrophages, a process involving exosomes and regulated by reactive oxygen species/NADPH oxidase activity. In asymptomatic subjects (n=199), Gal-3 plasma levels are correlated with NADPH oxidase activity in peripheral blood mononuclear cells (r=0.476; P<0.001) and carotid intima-media thickness (r=0.438; P<0.001), a surrogate marker of atherosclerosis. Accordingly, Gal-3 plasma concentrations are increased in patients with carotid atherosclerosis (n=158), compared to control subjects (n=115; 14.3 [10.7 to 16.9] vs. 10.4 [8.6 to 12.5] ng/mL; P<0.001). Finally, on a 5-year follow-up study in patients with peripheral artery disease, Gal-3 concentrations are significantly and independently associated with an increased risk for CV mortality (hazard ratio=2.24, 95% confidence interval: 1.06 to 4.73, P<0.05).

Conclusions—Gal-3 extracellular levels could reflect key underlying mechanisms involved in atherosclerosis etiology, development, and plaque rupture, such as inflammation, infiltration of circulating cells and oxidative stress. Moreover, circulating Gal-3 concentrations are associated with clinical outcomes in patients with atherothrombosis. (J Am Heart Assoc. 2014;00: e000785 doi: 10.1161/JAHA.114.000785)

Key Words: atherothrombosis • biomarkers • inflammation • mortality • oxidative stress

Atherothrombosis remains as the main cause of death in the Western and developing countries. The underlying pathological process is arterial wall thickening resulting from the formation of atherosclerotic plaque, which is frequently complicated by thrombus, thereby giving rise to the possibility of acute coronary syndrome or stroke.

Galectins are a family of highly conserved mammalian lectins that regulate inflammation.1 Moreover, galectin-3 (Gal-3) is involved in proliferation, macrophage chemotaxis, phagocytosis, neutrophil extravasation, oxidative stress, apoptosis, and angiogenesis.2 These mechanisms are involved in cardiovascular (CV) diseases (CVDs), among them atherosclerosis and heart failure (HF). On one hand, Gal-3 has been involved in the pathogenesis of experimental atherosclerosis.3–7 The beneficial effect of decreasing Gal-3 by either gene inactivation or therapeutic modulation to reduce lesion size in apolipoprotein E–deficient mice has been mainly attributed to the proinflammatory properties of Gal-3.3,5,8 More recently, it has been also suggested that Gal-3 could be involved in

From the Vascular Research Lab, IIS, Fundación Jiménez Díaz, Autónoma University, IRSIN, Madrid, Spain (J.M.-M., C.E.F.-G., A.B.-M., E.B., P.L.-G., A.O., J.E., L.M.B.-C., J.L.M.-V.); Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY (J.M.-M.); Vascular Research Unit, Viborg Hospital, Viborg, Denmark (J.S.L.); Division of Cardiovascular Sciences, CIMA University of Navarra, Pamplona, Spain (G.Z.); Department of Biochemistry and Genetics (G.Z.) and University Clinic (O.B.), University of Navarra, Pamplona, Spain; Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain (J.F.-G.).

*Dr Madrigal-Matute, Dr Lindholt, and Dr Fernandez-Garcia contributed equally to this work.

Correspondence to: Jose Luis Martin-Ventura, PhD, Vascular Research Lab, IIS, Fundación Jiménez Díaz, Autónoma University, Av Reyes Católicos 2, 28040 Madrid, Spain. E-mail: jlmartin@ffjd.es

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vascular smooth muscle cell (VSMC) osteogenic differentiation. On the other hand, Gal-3 may play a function in the pathophysiology of HF through promotion of myocardial fibrosis and inflammation, two related processes involved in myocardial remodeling. Gal-3 is required for inflammatory and fibrotic responses to aldosterone in VSMC in vitro and in vivo, suggesting a key role for Gal-3 in vascular fibrosis. It has been recently demonstrated that inhibition of Gal-3 function by genetic disruption or pharmacological intervention halts the progression of cardiac remodeling, attenuates myocardial fibrogenesis, and preserves left ventricular function.

Gal-3 does not contain a classical signal sequence to be secreted. However, the finding of extracellular Gal-3 suggests the existence of a yet undefined secretory pathway. In this respect, Gal-3 has been found to be associated with exosomes. Exosomes are membranous vesicles released by cells into extracellular fluids, thereby mediating intercellular communication in physiological and pathological processes. The potential role of Gal-3 as a circulating biomarker of CVDs has been mainly tested in HF patients. Interestingly, Gal-3 levels predict 4-year mortality in patients with HF, independent of echocardiographic markers of disease severity.

Our hypothesis was that Gal-3 could be a potential biomarker of atherothrombosis. For that purpose, this study was designed to (1) address potential sources and regulatory mechanisms involved in extracellular Gal-3 levels in atherosclerosis, (2) analyze the systemic Gal-3 concentrations in subclinical (asymptomatic subjects with known carotid intima-media thickness [IMT; STUDY 1]) and clinical (patients with carotid atherosclerosis [STUDY 2] and peripheral arterial disease [PAD; STUDY 3]) atherosclerosis, and (3) evaluate the potential prognostic value of systemic Gal-3 determination in PAD patients with a 5-year follow-up. Description of the different cohorts analyzed is shown in Figure 1.

### Methods

#### Cell Culture

Human THP-1 monocytic cell line was purchased from ATCC (CRL-1593; Manassas, VA) and cultured in RPMI 1640 (BioWhittaker Verriers, Belgium) supplemented with 10% decomplemented (heat-deactivated) FBS, 2 mmol/L of L-glutamine, and antibiotics. For experiments, cells were preincubated with 0% FBS during 24 hours. Phorbol myristate acetate (PMA) and the NADPH oxidase inhibitor, apocynin, were from Sigma-Aldrich (St. Louis, MO).

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy volunteers by Ficoll density gradient centrifugation, and monocytes were further isolated by automated magnetic-activated cell separation using CD14 MicroBeads (Miltenyi Biotec, Cambridge, MA). Monocytes were maintained in suspension culture for 16 hours in RPMI supplemented with 10% FBS and then in 0.5% FBS during stimulation.

### Patients

**Asymptomatic subjects (STUDY 1)**

A group of 199 asymptomatic subjects in whom global risk assessment was performed in the course of a general health checkup by the Internal Medicine Department (University Clinic of Navarra, Navarra, Spain) were studied (Table 1). Subjects were free from clinically apparent atherosclerotic disease based on: (1) absence of history of coronary disease, stroke, or PAD and (2) normal electrocardiogram and chest X-ray results. Coronary heart disease was defined by: (1) self-reported myocardial infarction (MI), angina, or use of nitroglycerin and (2) self-reported history of coronary angioplasty or coronary artery bypass surgery. Cerebrovascular disease (CBD) was defined as self-reported stroke, transient ischemic attack (TIA), or carotid endarterectomy. Symptoms of intermittent claudication were queried in a questionnaire, together with the physician interview. In all subjects, absence of history of coronary disease, stroke, or PAD was recorded.

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**Figure 1.** Patients analyzed in this study. Gal-3 indicates galectin-3; IMT, intima-media thickness.
Additional exclusion criteria were the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of nonsteroidal anti-inflammatory drugs in the 2 weeks before entering the study. In all subjects, carotid ultrasonography was performed to determine IMT. Subjects were examined by the same 2 sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. The between-observer intraclass correlation coefficient was 0.76 \( (P<0.001) \), and the between-subject repeatability was 0.82 \( (P<0.001) \). The corresponding coefficients of variance were 5% and 10%, respectively.

Patients were also excluded if they had advanced carotid atherosclerosis according to IMT measurements \( (>1.7 \text{ mm}) \). Atheroma plaques were defined as echogenic structures encroaching on the vessel lumen with a distinct area of 50% greater than the intimal plus media thickness of neighboring sites.

The presence of cardiovascular risk (CVR) factors, such as diabetes mellitus (DM), arterial hypertension (AHTN), dyslipidemia, obesity, and smoking habits were assessed. Blood pressure was measured twice on the right-upper arm with a random-zero mercury sphygmomanometer in patients in the sitting position. Patients were considered as hypertensives if they had systolic blood pressure (SBP) \( >139 \text{ mm Hg and/or diastolic blood pressure (DBP) } >89 \text{ mm Hg or were receiving antihypertensive drugs. Subjects with a positive history of DM or with fasting glucose levels } >125 \text{ mg/dL were considered as diabetics. Smoking was defined as current smokers or nonsmokers, with a smoker defined by the history of smoking } \geq 10 \text{ cigarettes per day } >1 \text{ year). Subjects with a body mass index } \geq 30 \text{ kg/m}^2 \text{ were classified as obese. Dyslipidemia was diagnosed as the presence of at least 1 of the following characteristics: total cholesterol } >200 \text{ mg/dL; low-density lipoprotein (LDL)-cholesterol } >130 \text{ mg/dL, and high-density lipoprotein (HDL)-cholesterol } <35 \text{ mg/dL. According to institutional guidelines, subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accord with the Declaration of Helsinki, and the Ethical Committee of the University Clinic of Navarra approved the protocol.}

**Table 1.** Baseline Clinical Characteristics of the Asymptomatic Population (STUDY 1)

| Total Population | \( n=199 \) |
|------------------|-------------|
| Age, y           | 56 (49 to 62) |
| Gender (male/female), % | 84/16 |
| BMI, kg/m²       | 28 (26.1 to 31.2) |
| SBP, mm Hg       | 125 (120 to 140) |
| DBP, mm Hg       | 80 (80 to 85) |
| Arterial hypertension, % | 51 |
| Diabetes mellitus, % | 13 |
| Obesity, %       | 35 |
| Dyslipidemia, %  | 59 |
| Smoking, %       | 22 |
| Glucose, mg/dL   | 95 (88 to 106) |
| Total cholesterol, mg/dL | 207 (186 to 236) |
| HDL-cholesterol, mg/dL | 51 (42 to 62) |
| LDL-cholesterol, mg/dL | 130 (111 to 158) |
| Triglycerides, mg/dL | 96 (72 to 136) |
| Serum creatinine, mg/dL | 0.90 (0.80 to 1.00) |
| Glomerular filtration rate (mL/min per 1.73 m²) | 87.1 (77.7 to 95.9) |
| Galectin, mg/dL   | 7.3 (5.75 to 9.75) |
| Superoxide production, RLU/s | 20.5 (12.3 to 29.0) |
| Mean carotid IMT, mm | 0.65 (0.60 to 0.75) |
| hs-CRP, mg/L     | 1.69 (0.82 to 3.03) |
| Medication       |             |
| Antihypertensives, % | 33 |
| Oral hypoglycemics, % | 9 |
| Statins, %       | 16 |
| Continuous variables are expressed as median (IQR) values, and categorical variables as percentages values. BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; LDL, low-density lipoprotein; RLU/s, relative light units/second; SBP, systolic blood pressure. |

**Table 2.** Clinical Characteristics of Patients With Carotid Atherosclerosis and Controls (Free of Carotid Atherosclerosis) (STUDY 2)

|                      | Controls (\( n=115 \)) | Atherosclerosis (\( n=158 \)) |
|----------------------|------------------------|-------------------------------|
| Gender (male/female, %) | 100/0                  | 82/18                         |
| Age, median (IQR), y  | 65 (64.9 to 65.1)      | 70 (62 to 76)                 |
| Dyslipemia, %         | 64                     | 29                            |
| Current smoking, %    | 50                     | 34                            |
| Diabetes, %           | 38                     | 15                            |
| AHTN, %               | 48                     | 82                            |

Additional exclusion criteria were the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of nonsteroidal anti-inflammatory drugs in the 2 weeks before entering the study. In all subjects, carotid ultrasonography was performed to determine IMT. Subjects were examined by the same 2 sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. The between-observer intraclass correlation coefficient was 0.76 \( (P<0.001) \), and the between-subject repeatability was 0.82 \( (P<0.001) \). The corresponding coefficients of variance were 5% and 10%, respectively.
under treatment (supervised diet, hypoglycemic oral medication, and insulin) or a basal glycemia >120 mg/dL and/or glycosylated hemoglobin >6.5%. We defined hypertension (HTN) as SBP >140 mm Hg and/or DBP) >90 mm Hg measured during the examination (after the participant had been sitting for at least 30 minutes) or if the participant was already taking hypotensive medication. Dyslipidemia was diagnosed as the presence of at least 1 of the following characteristics: total cholesterol >200 mg/dL; LDL-cholesterol >130 mg/dL, or triglycerides >200 mg/dL; or the participant was already taking statins or fibrates medication. Smoking was defined as current smokers or nonsmokers (including ex-smokers, those who stopped smoking at least 6 months before the inclusion in the study), with a smoker defined by the history of smoking (≥10 cigarettes per day >1 year).

The ethical committee on human research at IIS, Fundación Jiménez Díaz, Autónoma University (Madrid, Spain) approved the study, which was performed in accord with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

One hundred fifteen controls with no stenosis in the carotid artery were recruited from a screening program for both carotid atherosclerosis and abdominal aortic aneurysm between 65-year-old men. The absence of vascular disease was confirmed with a physical examination and an ultrasound scan.

**PAD patients (STUDY 3)**

Three hundred nine patients with intermittent claudication or critical lower-extremity ischemia were enrolled prospectively from a clinical trial. Sixty-seven (22%) died within the first 5 years. PAD was diagnosed during clinical examination. The lowest ankle-brachial index (ABI) of the 2 legs was used in the analysis. Acute lower-limb ischemia cases were excluded. Patients were followed for up to 5 years. The median follow-up period was 4.31±1.52 years. Deaths from all and CV causes were identified in the nation-wide Danish National CPR registry. A thorough medical history was recorded in all patients, including details of previous MI, a history of angina pectoris, AHTN, previous CBD, smoking status, DM, and ankle and brachial SBPs. The information came from medical records or directly from patients. Patients were considered as smokers if actively smoking or having discontinued smoking within 2 years. Diabetes was defined by history of DM or the use of oral antidiabetic drugs and/or insulin. HTN was defined by any history of HTN with use of antihypertensive drugs for that purpose. A history of previous MI, a history of angina pectoris and CBD combined with stroke, TIA, or carotid reconstruction reported in the registry of hospital discharge diagnoses based upon the World Health Organization codes International Classification of Diseases (ICD)-8 (before 1994) and ICD-10 (from 1994). The study was approved by the research ethics committee for North Jutland, Viborg, and Aarhus counties.

**Determination of Superoxide Production**

NADPH oxidase-dependent superoxide production was measured using 5 μmol/L of lucigenin (Sigma-Aldrich) in 400 000 PBMCs isolated from blood samples with Lymphoprep or in 10⁶ THP-1 under basal conditions and in response to 3.2 μmol/L of PMA (Sigma-Aldrich) during 30 minutes (THP-1) or 1 hour (PBMCs). Luminescence measurements (1 second) were recorded every 15 to 30 seconds along an interval of 1 hour in a plate reader luminometer (Luminoskan Ascent; Thermo Labsystems, Milford, MA). The value of the area under the curve (AUC) was used to quantify chemiluminescence. A buffer blank was subtracted from each reading. We have previously reported that lucigenin measurements closely correlated with an independent measurement of superoxide production using superoxide dismutase-inhibitable ferricytochrome c reduction.

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction**

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was used for reverse transcription with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction (PCR) was performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems), according to the manufacturer’s protocol, using the ΔΔCt method as previously described. Quanti- fication of human Gal-3 mRNA levels was performed by amplification of cDNA using SYBR® Premix Ex TaqTM (Takara Bio Inc., Otsu, Japan). mRNA levels of Gal-3 were normalized to 18S mRNA content. Sequences of primers were as follows: Gal3: 5’- TITGCCCTGGGGAGTGTGCCT -3’ (sense) and 5’- TGGGCTTCACCGTGCCCAGAA -3’ (antisense); 18S: 5’- CCGTCGTAGTCCGACCATAA -3’ (sense) and 5’- CAGCTTGCAACACTCTCCC -3’ (antisense). Expression levels are given as ratio to housekeeping gene 18S, and data are expressed as fold versus basal values.

**Exosome Isolation and Characterization**

Exosome-like vesicles were isolated from conditioned media of CD14+ monocytes or THP-1 (1×10⁶ cells) and human plasma (1 mL), as previously described. Plasma was obtained from 10 patients with carotid atherosclerosis and from 10 controls randomly selected from the above-described population (STUDY 2). Briefly, cell supernatants
were centrifugated at increasing speeds in order to discard cells (300 g, 10 minutes), dead cells (3000 g, 10 minutes), and cell debris (17 000 g, 30 minutes). Two ultracentrifugations (100 000 g, 70 minutes) were performed to pellet the small vesicles that correspond to exosomes and to discard contaminating proteins. Exosomes were resuspended in lysis buffer for SDS-PAGE analysis or paraformaldehyde (PFA) for electron microscopy (EM). Exosomes from cell-culture supernatant of CD14+ monocytes and THP-1 cells and from plasma were characterized by nanoparticle tracking analysis21 (NanoSight with the collaboration of Mittelbrunn M and Baixauli F from the National Center of Cardiovascular Research, Madrid; Figure 2). We further confirmed the presence of exosomes in cell-culture supernatant by the detection of the positive TSG101 exosome marker (Abcam, Cambridge, MA) by Western blot (Figure 3A). To purify exosomes from plasma, the speed and duration of centrifugation steps were increased, as previously described.20 For standard EM, exosomes were resuspended in PFA and embedded on Formvar carbon-coated EM grids (PELCO; Ted Pella, Inc., Redding, CA). Examination of samples under the electron microscope at 80 kV disclosed 50- to 90-nm cup-shaped membrane vesicles consistent with exosome-like vesicles (Figure 3B). Smaller 10- to 20-nm lipid particles were also observed within the same preparations (not shown).

**ELISA**

Soluble concentrations of Gal-3 were quantified in exosomes, cell-conditioned media, and plasma by a commercially

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Figure 2. Exosome characterization. Representative NTA of exosomes isolated from (A) CD14+ monocytes, (B) THP-1 cells, and (C) plasma. NTA indicates nanoparticle tracking analysis.

Figure 3. Exosome characterization. A, Serial centrifugations were performed in order to isolate exosomes from THP-1 conditioned media. Last lane represents the second 100 000g centrifugation step to discard contaminating proteins and is labeled by 100 000g (-=negative). The pellet obtained in each step was resuspended in lysis buffer to analyze the expression of typical exosome marker TSG101. Representative Western blot is shown. B, Electronic microscopy shows exosomes (arrows) isolated from human plasma. Scale bar is shown (0.1 μm). TSG101 indicates tumor susceptibility gene 101.
available kit (eBioscience, Inc., San Diego, CA). As displayed in the manufacturer’s instructions, expected Gal-3 values in plasma ranged between 4.67 and 10.30 ng/mL and in serum between 0.62 and 6.25 ng/mL. The intra- and interassay variability were 6.4% and 11.4%, respectively. High-sensitivity C-reactive protein (hs-CRP) was determined by a commercially available kit (RAP002; BioVendor, Modrice, Czech Republic). The intra- and interassay variability were 5.4% and 6.1%, respectively.

Statistical Analysis

Statistics were performed using SPSS software (12.0; SPSS, Inc., Chicago, IL). Probability plots and one-sample Kolmogorov-Smirnov tests were used to check for normal distributions of data.

In vitro experiments were performed at least 3 times. Results are expressed as mean±SEM and were analyzed by the Student t test (2-tailed, significant differences at P<0.05).

In the asymptomatic population (STUDY 1), continuous variables are expressed as median (interquartile range [IQR]) values. Categorical variables were reported as percentages. Univariate association was assessed by Pearson’s correlation test. Multivariable linear regression analysis was conducted with carotid IMT or Gal-3 as dependent variables, including in the model the traditional risk factors and those variables that were significant in the univariate analysis.

Gal-3 levels in carotid atherosclerotic patients (STUDY 2) are expressed as median (IQR) and were analyzed by Mann-Whitney’s test. Logistic regression analysis adjusted by risk factors was conducted with atherosclerosis presence as the dependent variable. Receiver operating characteristic (ROC) curve analysis was performed to discriminate carotid sclerosis patients from control subjects. For the analysis of the ROC curve, the null hypothesis was that the test had a performance similar to the diagonal line (the area under the curve was 0.5). If the lowest 95% confidence limit for the AUC was more than 0.5, a significant predictive test was said to be present.

For patients with peripheral arterial disease (STUDY 3), continuous variables are presented as median (IQR); categorical variables are presented as percentages. Hereafter, subjects were divided by median into 2 groups. Associations with a P value below 0.1 between the variables and GAL3 levels or death were considered to be potential confounders and adjusted for in survival analyses. Cox’s proportional hazard regression analysis with adjustments for age, gender, smoking status, DM, ABI, HTN, previous acute myocardial infarction (AMI), previous ischemic cerebral event, present angina pectoris, and HTN were performed to evaluate an association between Gal-3 and CV mortality. Ninety-five percent confidence intervals (CIs) were calculated for each comparison.

Results

Gal-3 Is Expressed in Human Monocytes and Released by Exosomes Under Oxidative Stress

We analyzed the effect of PMA, a known inducer of NADPH activity, in Gal-3 expression and release by human CD14+ monocytes isolated from healthy volunteers. PMA induced NADPH oxidase-dependent superoxide production at 30 minutes (not shown). PMA increased mRNA expression of Gal-3 at 24 hours (Figure 4A). Moreover, Gal-3 extracellular levels were increased in both whole conditioned media and in exosomes isolated from conditioned media of PMA-stimulated monocytes at 24 hours (Figure 4B and 4C). Pretreatment with apocynin (an NADPH/ reactive oxygen species [ROS] inhibitor) reversed PMA-induced Gal-3 mRNA expression and Gal-3 release in monocytes (Figure 4). We further confirmed the increase in Gal-3 mRNA expression and secretion in the in vitro model of macrophage differentiation of THP-1 cells stimulated with PMA for 24 hours (Figure 5).

Gal-3 Plasma Levels Are Correlated With the Etiology of Atherosclerosis in Asymptomatic Human Subjects

We analyzed Gal-3 plasma levels in a test population of 199 asymptomatic subjects in which phagocytic NADPH oxidase-dependent superoxide production in PBMCs and carotid IMT had been measured (Table 1). Gal-3 was positively correlated with phagocytic NADPH oxidase-dependent superoxide production (r=0.476; P<0.001; Figure 6A), which remained highly significant after controlling for age and sex (r=0.450; P<0.001; Table 3). In a multivariable analysis, the association between Gal-3 levels and phagocytic NADPH oxidase-dependent superoxide production remained statistically significant after adjusting for some potential factors that might be regulating Gal-3 levels (Table 4). Interestingly, a positive correlation between Gal-3 and carotid IMT was observed (r=0.438; P<0.001; Figure 6B), which remained highly significant after controlling for age and sex (r=0.376; P<0.001; Table 3). No correlation between Gal-3 and other clinical parameters was observed (Table 3). In a multivariable analysis, the association between plasma Gal-3 levels and carotid IMT remained statistically significant after adjusting for some potentially confounding CV risk (CVR) factors (Table 5). Interestingly, increased Gal-3 levels were observed in asymptomatic subjects with carotid
atherosclerotic plaques, compared to those without plaques (6.70 [5.36 to 8.17] vs. 8.05 [7.05 to 12.1] ng/mL, P < 0.001).

Gal-3 Plasma Levels Are Increased in Carotid Atherosclerotic Patients

We analyzed Gal-3 plasma levels in a group of 158 patients with carotid atherosclerosis and 115 controls (Table 2). Gal-3 levels in carotid atherosclerotic patients were higher than in control subjects (14.3 [10.7 to 16.9] vs. 10.4 [8.6 to 12.5] ng/mL; Figure 7A). In contrast, no significant differences were observed for hs-CRP (2.3 [1.1 to 5.2] vs. 1.7 [0.9 to 3.5]). Logistic regression analysis showed that the significant association between increased Gal-3 levels and atherosclerosis persisted after adjustment for risk factors (Table 6). ROC curve analyses showed that Gal-3 levels were predictors of carotid atherosclerosis presence (AUC [95% CI]= 0.7 [0.64 to 0.76]; P < 0.001; Figure 7B). Furthermore, Gal-3 concentration in exosomes isolated from plasma from 10 patients with carotid atherosclerosis was higher than in exosomes isolated from 10 controls (3.2 [2.4 to 8.2] vs. 1 [0.6 to 4.6] ng/mL; P < 0.05; Figure 7C).

Circulating Levels of Gal-3 in PAD Patients Are Associated With CV Mortality Risk

Finally, we analyzed Gal-3 concentrations in PAD patients (n=309) with a 5-year follow-up. In Table 7, the proportions of potential clinical demographic and treatment confounders are displayed. No differences in Gal-3 levels among the different subgroups analyzed were observed (not shown). Gal-3 concentrations above the median were significantly associated with an 80% increase on total mortality risk, compared to Gal-3 below the median, which increased to 97% after adjustment for the above-mentioned potential confounders (not shown). Interestingly, Gal-3 levels above the median were significantly associated with a 75% increase on CV mortality risk, compared to Gal-3 below the median, which increased to 124% after adjustment for the above-mentioned potential
confounders (adjusted hazard ratio [HR] = 2.24; 95% CI; 1.06 to 4.73; \( P < 0.05 \); Figure 8). In this multivariable analysis, male gender, age, and current angina pectoris increased independently overall mortality risk during the next 5 years (Table 8).

**Discussion**

The main results of the present study are the following: (1) Gal-3 release is increased by PMA in human monocytes

![Figure 5.](image1)  

**Figure 5.** Gal-3 expression and release by THP-1 monocytes. A, Gal-3 mRNA quantification by real-time PCR in THP-1 treated with PMA (3.2 μmol/L, 3 to 24 hours) in the absence or presence of apocynin (3 mmol/L, 30 minutes of preincubation). Gal-3 level quantification by ELISA in (B) conditioned media and (C) exosomes isolated of conditioned media from different experimental conditions (24 hours). Values shown are mean±SEM of 3 independent experiments. *\( P < 0.05 \) versus basal; #\( P < 0.05 \) versus PMA. Apo 3 indicates apocynin (3 mmol/L); B, basal; Gal-3, galectin-3; PCR, polymerase chain reaction; PMA, phorbol myristate acetate.

![Figure 6.](image2)  

**Figure 6.** Gal-3 plasma levels in asymptomatic subjects (STUDY 1). Positive correlation of Gal-3 with (A) phagocytic NADPH oxidase-dependent superoxide production and (B) carotid IMT in asymptomatic subjects (n=199). Gal-3 indicates galectin-3; IMT, intima-media thickness; RLU/s, relative light units/second.
and macrophages, a process involving exosomes and regulated by ROS/NADPH oxidase activity; (2) Gal-3 plasma levels are correlated with NADPH oxidase activity and carotid IMT in asymptomatic subjects; (3) Gal-3 plasma levels are increased in patient with carotid atherosclerosis and PAD, compared to controls; and (4) Gal-3 concentrations are significantly and independently associated with increased CV mortality risk in patients with PAD.

### Table 3. Correlation Coefficients of Mean Carotid IMT and Galectin With Clinical and Laboratory Parameters in the Asymptomatic Population (Adjusted by Age and gender) (STUDY 1, n=199)

| Variable                      | Carotid IMT | Galectin |
|-------------------------------|-------------|----------|
| BMI, kg/m²                    | 0.097       | 0.022    |
| SBP, mm Hg                    | 0.188       | 0.011    |
| DBP, mm Hg                    | 0.129       | 0.036    |
| Glucose, mg/dL                | 0.245       | 0.130    |
| Total cholesterol, mg/dL      | 0.032       | 0.103    |
| HDL-cholesterol, mg/dL        | -0.125      | -0.011   |
| LDL-cholesterol, mg/dL        | 0.054       | 0.032    |
| Triglycerides, mg/dL          | 0.113       | 0.147    |
| hs-CRP, mg/L                  | 0.072       | 0.103    |
| Serum creatinine, mg/dL       | -0.034      | 0.059    |
| Glomerular filtration rate    | -0.018      | -0.088   |
| Galectin, mg/dL               | 0.376       | <0.001   |
| Carotid IMT, mm               | —           | 0.376    |
| Superoxide production, RLU/s  | 0.630       | <0.001   |

### Table 4. Multiple Linear Regression Analysis With Galectin Levels as Dependent Variable (STUDY 1, n=199)

| Independent Variables | β     | P Value | Partial R² (%) |
|-----------------------|-------|---------|----------------|
| Superoxide production, RLU/s | 0.163 | <0.001 | 21.3           |
| Age                   | 0.043 | 0.044   | 3.1            |
| Gender, male/female   | -0.276 | 0.693 | 0.0            |
| Body mass index, kg/m²| 0.087 | 0.193   | 0.2            |
| Hypertension, n/y     | -1.085 | 0.077  | 1.2            |
| Smoking, n/y          | -0.663 | 0.578  | 0.1            |
| Glucose, mg/dL        | -0.008 | 0.472  | 0.2            |

Adjusted for age, gender, body mass index, hypertension, smoking, and glucose. R² for the total population was 26.1%. B indicates regression coefficient; R², partial correlation coefficient after adjustment; RLU/s, relative light units/second.

### Table 5. Multiple Linear Regression Analysis With Carotid IMT as Dependent Variable (STUDY 1, n=199)

| Independent Variables | β     | P Value | Partial R² (%) |
|-----------------------|-------|---------|----------------|
| Galectin, ng/mL       | 0.013 | <0.001 | 19.0           |
| Age                   | 0.002 | 0.020   | 7.0            |
| Gender, male/female   | 0.035 | 0.104   | 1.2            |
| Body mass index, kg/m²| 0.001 | 0.836   | 0.9            |
| Hypertension, n/y     | 0.063 | 0.001   | 4.0            |
| Smoking, n/y          | 0.036 | 0.294   | 0.3            |
| Total cholesterol, mg/dL | 0.001 | 0.278   | 0.4            |
| Glucose, mg/dL        | 0.001 | 0.036   | 1.8            |

Adjusted for age, gender, body mass index, hypertension, smoking, total cholesterol, and glucose. R² for the total population was 34.6%. IMT indicates intima-media thickness; R², partial correlation coefficient after adjustment; β, regression coefficient.
Gal-3 is Released by Exosome-Like Vesicles

Gal-3 functions depend on its cellular localization. Intracellularly, Gal-3 can participate in signaling pathways and modulate different processes, such as cell migration and apoptosis. Extracellularly, Gal-3 is involved in cell adhesion, cytokine production, and chemotaxis, as well as modulation of receptor functions. The mechanisms involved in Gal-3 secretion are not completely established, but it has been proposed to involve exosomes. Interestingly, proteomic studies have identified Gal-3 in exosomes derived from dendritic and cancer cells. In the present study, we have shown that Gal-3 is released by activated monocytes, at least in part, associated with exosomes mediated by ROS, although other non-exosome secretion mechanisms could also be involved. Moreover, increased Gal-3 levels were found in exosomes isolated from plasma of patients with carotid atherosclerosis, compared to control subjects. This could suggest increased release by activated monocytes in vivo (in agreement with our in vitro studies). However, we could not discard the contribution of other cell types, neither an increased passive/active release by dysfunctional/normal endothelium, as previously shown for microparticles.

Gal-3, IMT, and Atherosclerosis

Different studies have highlighted the use of IMT as a surrogate marker of CVR prediction, although it has been recently suggested that evidence of atherosclerosis (plaque) is more strongly and independently associated with CV events. In the present study, we have shown that Gal-3 plasma levels correlate with IMT in asymptomatic subjects.

Table 6. Logistic Regression Analysis With Atherosclerosis as Dependent Variable (STUDY 2, Controls [n=115] and Atherosclerosis [n=158])

|          | B     | SE    | Sig  | Exp (B) |
|----------|-------|-------|------|---------|
| Constant | 12.271| 18.375| 0.504| 213 405.4|
| Sex      | -8.777| 18.226| 0.630| 0.000   |
| Age      | -0.089| 0.029 | 0.002| 0.915   |
| Current smoking | -0.219| 0.332 | 0.509| 0.803   |
| Hypertension | 1.137| 0.345 | 0.001| 3.119   |
| Diabetes | 0.731 | 0.401 | 0.068| 2.078   |
| Dyslipidemia | 0.942| 0.342 | 0.006| 2.565   |
| Galectin | -0.115| 0.032 | 0.000| 0.892   |

Figure 7. Gal-3 plasma levels in patients with carotid atherosclerosis (STUDY 2). A, Gal-3 concentrations in plasma of patients with carotid atherosclerosis (n=158) or controls (n=115) (P<0.001, respectively). B, ROC curve to discriminate carotid sclerosis patients (n=158) from control subjects (n=115). C, Galectin-3 concentration of patients with carotid atherosclerosis and controls (n=10; P<0.05). Galectin-3 indicates galectin-3; ROC, receiver operating characteristic.
after adjusting for classical risk factors. Moreover, we have shown that Gal-3 plasma levels are increased in patients with carotid atherosclerosis and PAD, compared to control subjects. In the same line, Gal-3 plasma levels are increased in HF patients. Pathological biomarkers are not specific of a disease, but rather reflect a biological activity associated with pathology. These results suggest that Gal-3 circulating levels are increased in CVDs, probably reflecting the underlying activation of common pathological mechanisms (macrophage activation and oxidative stress).

Table 7. Clinical Characteristics in PAD Population (STUDY 3, n=309)

| Independent Variable | %         |
|----------------------|-----------|
| Gender (male/female) | 60.5/39.5 |
| Smoking              | 60.5      |
| Diabetes mellitus    | 14.2      |
| Hypertension         | 49.5      |
| Previous ischemic cerebral event | 9.7 | |
| Previous acute myocardial infarction | 13.3 | |
| Previous or present angina pectoris | 23.0 | |
| Chronical obstructive pulmonary disease | 9.9 | |
| Hyperlipidaemia      | 37.7      |
| Use of low-dose aspirin | 57.8   |
| Use of warfarin      | 5.0       |
| Use of beta-blocker  | 18.8      |
| Use of ACE inhibitor | 18.1      |
| Use of calcium blocker | 21.6   |
| Use of statin        | 19.9      |
| Chronical critical ischemia | 50.9 |
| Age, y               | 67.1 (62.1 to 72.1) |
| Systolic blood pressure, mm Hg | 144 (130 to 160) |
| Diastolic blood pressure, mm Hg | 78 (70 to 85) |
| Pulse pressure, mm Hg | 65 (55 to 80) |
| Height, m            | 1.70 (1.65 to 1.75) |
| Weight, kg           | 71.5 (60.0 to 80.0) |
| Body mass index, kg/m² | 24.5 (22.0 to 27.5) |
| Max. walking distance, m | 100 (11.5 to 200) |
| Lowest ankle brachial blood pressure index | 0.78 (0.55 to 1.00) |
| Total cholesterol    | 5.20 (4.40 to 6.10) |
| hs-CRP               | 11.0 (3.27 to 39.7) |
| S-Galectin 3         | 4.75 (2.47 to 14.7) |

ACE indicates angiotensin-converting enzyme; hs-CRP, high-sensitivity C-reactive protein; PAD, peripheral arterial disease.

Figure 8. Gal-3 serum levels predict CV mortality in PAD patients (STUDY 3). Kaplan-Meier’s curves for CV mortality according to levels above or below median of serum Gal-3 in patients with symptomatic PAD. CV indicates cardiovascular; Gal-3, galectin-3; PAD, peripheral arterial disease.

Gal-3, PAD, and Mortality

Previous studies have suggested the predictive value of Gal-3 determination in patients with acute HF. To address the potential association of Gal-3 concentrations with clinical outcome in atherothrombosis, we investigated the usefulness of serum Gal-3 for predicting death in a population of symptomatic PAD patients. This is the first study demonstrating the effect of circulating Gal-3 on the outcome of patients with lower-extremity atherosclerosis. We observed that Gal-3 levels were significantly and independently associated with an increased risk for total and CV mortality in patients with PAD. Patients with Gal-3 concentrations above the median had 2-fold higher risk of death within 5 years, compared to the levels below the median, independently of CRP. This observation is consistent with recent survival data from other patient populations. Gal-3 serum levels independently predicted an adverse prognosis in patients with chronic HF and found the same independent 2-fold risk by having levels belonging to the concentrations above the median. It has also been associated with the outcomes in cancer and in the population in general. However, in the general population, the increased risk by augmented levels by the SD was only 9%. In that study, women had higher levels, as well as a stronger Gal-3 association with CVR factors. Such trends

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could not be observed in our cohort. This may be a result of the fact that women in the present study were already ill and thus looked more like the male PAD population. On the whole, these results suggest that circulating Gal-3 concentrations are associated with the clinical outcomes in patients with CVDs.

**Strengths and Limitations**

Apart from the robust prospective cohort study design used in the present study, some other strengths and potential limitations should be considered. The strengths are the consecutive enrollment of all PAD patients being observed at a primary referral single vascular surgical center at a district hospital. This ensures that patients are nonselective patients referred for vascular surgical evaluation serving a well-defined population. On the other hand, this is a descriptive, hypothesis-generating work and it is not truly population based, because many asymptomatic persons in the population were not enrolled. Moreover, although we adjusted for several well-known confounders in CVR factor research, residual confounding may have persisted, especially because of the lack of possibility to adjust for HF and coexisting cancers.

**Conclusions**

Gal-3 has unique features for being a good pathological biomarker of atherothrombosis (eg increased expression in activated macrophages/foam cells and atherosclerotic plaques and increased release to the blood in subclinical/clinical atherosclerosis). Circulating Gal-3 concentrations are associated with the clinical outcomes in patients with CVDs. However, future studies are needed to assess the clinical utility of Gal-3 determination for CVR prediction.

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**Disclosures**

None.
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