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# Low Levels of Nogo-B in Human Carotid Atherosclerotic Plaques Are Associated With an Atheromatous Phenotype, Restenosis, and Stenosis Severity

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**Objective**—Reticulon-4/Nogo (Nogo-B) protects mouse arteries from lumen loss by reducing smooth muscle cell (SMC) migration and intimal thickening. Our goal was to determine plaque and circulating levels of Nogo-B in atherosclerotic and control subjects. Therefore, we studied the relationships between local Nogo-B, plaque characteristics, and clinical data in patients undergoing carotid endarterectomy.

**Methods and Results**—Western blot analysis showed that endarterectomy specimens from the femoral (n=19) and carotid arteries (n=145) contained significantly less Nogo-B than nonatherosclerotic mammary arteries (n=8;  $P<0.003$ ) and aortas (n=15;  $P=0.03$ ). Immunohistochemistry revealed that in atherosclerotic lesions, Nogo-B was expressed by macrophage/foam cells, SMC rich, and neo-vascularized areas. Atheromatous plaques (>40% fat content) showed a significant reduction in Nogo-B expression ( $P=0.002$ ). Nogo-B expression levels were significantly lower in patients with more than 90% of carotid stenosis ( $P=0.04$ ) or restenotic lesions after prior carotid intervention (duplex;  $P=0.01$ ). In contrast, plasmatic levels of Nogo-B (soluble Nogo-B) did not differ between atherosclerotic subjects (n=68) and risk-factor matched controls (n=63;  $P=0.5$ ).

**Conclusion**—Our findings suggest that local reduction of Nogo-B in atherosclerotic tissue might contribute to plaque formation and/or instability triggering luminal narrowing. In contrast, plasma Nogo-B levels are not associated with clinically manifested atherosclerotic disease. (*Arterioscler Thromb Vasc Biol.* 2007;27:1354-1360.)

**Key Words:** atherosclerosis ■ restenosis ■ vascular biology ■ Nogo-B

Arterial lumen loss is determined by geometric arterial remodeling,<sup>1</sup> intimal thickening,<sup>2</sup> and/or atherosclerotic plaque formation.<sup>3,4</sup> Carotid stenosis is a typical manifestation of atherosclerotic disease affecting about 9 to 12% of patients.<sup>5</sup> Atherosclerotic plaques are caused by an abnormal cellular proliferation and migration, lipid deposition, and extracellular matrix accumulation.<sup>6</sup> Rupture prone plaques are associated with a high inflammatory component, large necrotic/apoptotic lipid core accompanied by elevated proteolytic activity.<sup>7,8</sup> On rupture of the fibrous cap, the lipid core serves as a substrate for thrombus formation triggering arterial occlusion and subsequently acute coronary syndromes, cerebrovascular events, and cardiac death. Our current knowledge of mechanisms underlying plaque growth, destabilization, and rupture remains incomplete. Therefore, identification of novel players in plaque formation and

progression will contribute to a better understanding of atherosclerotic syndrome.

Recent findings revealed that a member of the reticulon family of proteins, reticulon 4 B, also known as Nogo-B, protects mouse arteries from lumen loss after arterial injury.<sup>9</sup> Acevedo et al showed that human endothelial cells (ECs), smooth muscle cells (SMCs), and healthy murine arteries contain high levels of Nogo-B, and Nogo-B expression rapidly decreases after arterial injury. Consistently, Nogo A/B-deficient mice exhibited an accelerated neointima formation on femoral artery injury.<sup>9</sup> Functional studies revealed that the N terminus region of Nogo-B promotes EC adhesion and migration and blocks platelet derived growth factor-induced migration in SMCs, suggesting that Nogo-B serves as positive regulator of EC functions and as a negative regulator of SMC migration.<sup>9</sup>

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The presence or potential roles of Nogo-B have not been described yet in normal human blood vessels or during atherosclerosis. Intimal hyperplasia and advanced atherosclerosis share common features like the presence of a local inflammatory response, increased protease activity, endothelial dysfunction, and smooth muscle cell migration. Considering the aforementioned report on the protective role of Nogo-B in neointima formation in mice, we hypothesized that Nogo-B expression may be decreased in another highly prevalent arterial obstructive disease, eg, human atherosclerotic tissue. In addition, we investigated whether Nogo-B expression would be mainly lowered in plaques with an unstable plaque phenotype.

Our findings demonstrate a strong reduction of Nogo-B levels in human carotid atherosclerotic plaques compared with nonatherosclerotic arteries while circulating levels of Nogo-B did not differ between atherosclerotic subjects and controls. This attenuation of Nogo-B expression was most evident in plaques with an atheromatous phenotype. Low Nogo-B in carotid plaques was associated with a high degree of carotid stenosis in patients undergoing carotid endarterectomy. Our findings support the hypothesis that local down-regulation of Nogo-B contributes to plaque formation and/or instability accelerating luminal narrowing.

## Methods

### Clinical Studies

Subjects analyzed in this study were included in 2 ongoing clinical studies called ATHERO-EXPRESS<sup>10</sup> and SMART.<sup>11</sup> Demographic and risk factors data of the populations studied are depicted in the supplemental Tables I and II, available online at <http://atvb.ahajournals.org>

### Athero-Express

Differential Atherosclerotic plaque Expression of mRNA and proteins in relation to cardiovascular events and patient characteristics.

This study was approved by the ethical committees of the participating hospitals in accordance with institutional guidelines. Patients filled out questionnaires covering (among others) history of cardiovascular disease, cardiovascular risk factors, physical activity, family history of vascular disease, and medication.

The degree of luminal stenosis was based on duplex ultrasonography before intervention. Duplex criteria for stenosis were a combination of peak systolic velocity greater than 125 cm/s and a gamma (the ratio between peak systolic velocities in the stenotic area and end diastolic velocity in distal common carotid artery) greater than 12.

Human mammary arteries (n=8) were surgically obtained from patients scheduled for coronary bypass surgery. Human coronary arteries (n=6) were obtained from elderly subjects at autopsy who did not die of any cardiovascular causes, although obtained arterial segments showed different degree of silent atherosclerosis. Aortic specimens from the proximal anterior part of the ascending thoracic aorta (n=15) were collected from cardiac transplantation recipients and cadaveric organ donors. Femoral (n=19) and carotid atherosclerotic plaques (n=145) were obtained by endarterectomy. Carotid endarterectomy (CEA) was performed under general anesthesia by an open noneversion technique. Atherosclerotic plaques were dissected at the bifurcation into the internal and external carotid arteries. Immediately after dissection the atherosclerotic plaque was transported to the laboratory. The atherosclerotic segment was dissected in parts of 0.5 cm. The culprit lesion was fixated in 4% formalin solution and embedded in paraffin for plaque characterization. Adjacent segments were immediately frozen in liquid nitrogen (LN<sub>2</sub>) and subsequently processed for protein isolation.

### Characterization of Atherosclerotic Lesions

CEA specimens were stained and semiquantitatively scored microscopically by 2 independent observers as reported earlier.<sup>10</sup>

### Protein Isolation

Proteins were isolated from atherosclerotic segments adjacent to the paraffin embedded segment that was used for immunohistochemistry. Total proteins were extracted from samples using Tri-Pure Isolation Reagent (Roche) according to the manufacturer's protocol. Total protein concentration was determined using the Bio-Rad DC protein assay.

### Determination of Matrix Metalloproteinase Activity

In atherosclerotic plaques, matrix metalloproteinase (MMP)-2, MMP-8, and MMP-9 activities were measured using Biotrack activity assays RPN 2617, RPN 2635, and RPN 2634, respectively (Amersham Biosciences).

### Immunohistochemistry

Serial cross-sections (5  $\mu$ m) from human mammary arteries, human coronary arteries, and human carotid endarterectomies samples were deparaffinized and rehydrated. After blocking with 10% (v/v) normal rabbit serum, sections were incubated overnight at 4°C with goat anti-human Nogo-B (N-18; Santa Cruz Biotechnology Inc; 2  $\mu$ g/mL) diluted in PBS containing 1% BSA. After washing, sections were incubated with biotinylated rabbit anti-goat (DAKO; 1.6  $\mu$ g/mL). Sections were incubated with horseradish peroxidase (HRP) labeled streptavidin (Vector; 1  $\mu$ g/mL). Color was developed using diaminobenzidine as substrate for 10 minutes. Counterstaining was performed using Mayer hematoxylin. Nonimmune negative controls were obtained avoiding the primary antibody. ECs were identified by staining with antibody against CD31, macrophages with an antibody against CD68, and SMCs with an antibody against alpha actin.

### SDS-PAGE and Western Blotting

Equal amount of total protein (10  $\mu$ g) extracted from different samples were boiled in  $\beta$ -mercaptoethanol-containing buffer for 5 minutes, separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell Dassel, Germany). Protein loading was further confirmed using Ponceau Red S staining. The membranes were blocked overnight and incubated with goat anti-human Nogo-B (N-18; 0.2  $\mu$ g/mL) for 1 hour at RT in PBS containing 5% nonfat dry milk /0.1% Tween-20. After several washing steps, membranes were incubated rabbit HRP-anti-goat IgG (Sigma; 1:80000). Signal was detected by enhanced chemiluminescence. Thereafter, membranes were reprobed with mouse anti-human beta actin ( $\beta$ -actin) antibodies (Sigma; 1  $\mu$ g/mL), and Rabbit anti-mouse antibody (DAKO) was used as secondary antibody. Cell lysates from neuroblastoma SH-Y5Y and extracts from pooled human mammary arteries (n=6) were used as positive controls for Nogo-B in all blots. In every gel, expression levels of Nogo-B and  $\beta$ -actin in pooled mammary arteries (n=6) were considered as 100. Nogo-B and  $\beta$ -actin expression levels in atherosclerotic and nonatherosclerotic tissues were standardized and calculated as percentages relative to standard positive control.

### SMART (Second Manifestations of ARterial Disease)

The SMART study is an ongoing, single-center, prospective cohort study in which patients are referred to the University Medical Center Utrecht for the first time because of atherosclerotic vascular disease or treatment of atherosclerotic risk factors.<sup>11</sup>

More than 4000 patients have been enrolled. All patients underwent duplex scanning of the carotid arteries, abdominal ultrasound to detect aneurysm formation, ECG examination, as well as brachial-ankle index measurements. For detailed information regarding clinical investigations we refer to previously published reports.<sup>11</sup> Control patients from the SMART study were frequency-matched for sex, age, and 1 of 3 major risk factors (hypertension, diabetes mellitus, and hyperlipidemia). These patients revealed neither clinically evident nor silent peripheral atherosclerosis. The SMART cohort was

used to answer the research question whether Nogo-B could serve as a plasma biomarker for the presence of atherosclerotic disease.

### **Determination of the Intima-Media Thickness**

In all patients included in the SMART study intima-media thickness (IMT) was measured in the left and right common carotid arteries as a measure for the extent of atherosclerosis in SMART population. Arteries were examined in anterolateral, posterolateral, and medio-lateral direction using an ATL Ultramark 9 machine (Advanced Technology Laboratories) equipped with a 10-MHz linear array transducer. The intima-media surface of the selected area was calculated online using built-in software of the ultrasound system. The mean IMT of the 6 measurements in each patient was calculated. Investigators who performed the ELISAs were blinded for the outcome of IMT measurements.

### **Amino Nogo-B ELISA and Detection of Nogo-B in Plasma**

A secreted form of amino Nogo-B (amino acids 1 to 200) was engineered by inserting a myc epitope and a polyhistidine sequence at the 3' region of the human Nogo-B cDNA (nucleotides 1 to 600) followed by a stop codon, and this fragment was cloned in front of a secretory signal for alkaline phosphatase. For production of soluble amino Nogo-B, CHO cells were transiently transfected and conditioned media collected for 72 hours. Secreted Nogo-B was purified to homogeneity using a nickel affinity matrix. Two antibodies that recognize the amino Nogo-B were used to develop a sandwich ELISA, N18 (goat, anti-human Nogo Santa Cruz Biotech, sc-11027; recognizing epitopes 1 to 18) and 1761A (rabbit, anti-human Nogo anti-serum; Imgenex, IMG-5346A; recognizing epitope 14 to 30). N-18 was coated on a 96-well microtiter plate as the capture Ab (0.2  $\mu$ g/mL) overnight, followed by washing with Tris-buffered saline (TBS). Nonspecific binding was blocked by the addition of casein blocker (BioRad 161 to 0782) in TBS followed by washing in TBS tween. Standard curves were generated with purified amino Nogo-B (1 to 100 nmol/L dilutions) followed by incubation for 1 hour at room temperature. The detecting Ab (1761, 1:5000 dilution) was then incubated for 1 hour at room temperature, followed by the addition of goat  $\alpha$ -rabbit-alkaline phosphate (AP) conjugating Ab (1:5000 dilution). AP activity was assessed using p-nitrophenyl phosphate (Sigma S0942-100TAB) as a substrate. Standard curves were linear in the range of these concentrations with a correlation coefficient of  $r^2=0.98$  to  $0.99$ . For detection of Nogo-B in human plasma, duplicate samples (10 to 20  $\mu$ L) were incubated as indicated for amino-Nogo-B standard and processed accordingly.

### **Data Analysis and Statistics**

Data analysis was performed using SPSS 11.5 (SPSS Inc). Comparison of Nogo-B expression levels between different arteries and patient groups was done by Mann-Whitney *U* test. The Mann-Whitney *U* test was also used to test the association between Nogo-B measurements and semiquantitatively measured plaque characteristics. Values of local and soluble Nogo-B levels are presented as mean  $\pm$  SEM. Probability values  $<0.05$  were considered statistically significant.

## **Results**

### **Identification of Nogo-B in Human Atherosclerotic Lesions**

We first studied the presence of Nogo-B in atherosclerotic tissues harvested from different vascular beds and nonatherosclerotic arteries. Nogo-B expression levels were determined by semiquantitative Western blotting in nonatherosclerotic human mammary arteries ( $n=8$ ) and aortas ( $n=15$ ), atherosclerotic human coronary arteries (CA,  $n=6$ ), and endarterectomy specimens from the femoral (FEA,  $n=19$ ) and carotid arteries (CEA,  $n=145$ ; Figure 1). The Nogo-A/B antibody, N-18, detected 2 bands with relative molecular weights

between 55 KD and 50 kDa indicating the existence of 2 variants of Nogo-B (Nogo-B1 and B2; Figure 1A) but did not detect a higher molecular weight form of the Nogo A isoform (220 KDa), consistent with previous work in murine blood vessels.<sup>9</sup> Western blotting extracts from atherosclerotic lesions showed significant lower levels of both variants of Nogo-B compared with nonatherosclerotic mammary arteries and aortas (CEA= $16.45 \pm 1.12\%$ , FEA= $11.1 \pm 4.35\%$ , and CA= $25 \pm 5.77\%$  of standard positive control,  $P<0.05$ , Figure 1A and 1B).

To rule out that the relationships between Nogo-B, plaque characteristics, and clinical data presented in this manuscript are not simply reflecting changes in the total plaque cellularity, we also determined  $\beta$ -actin expression levels by Western blotting (Figure 1A). The expression patterns of Nogo-B and  $\beta$ -actin were totally distinct and did not show any statistically significant association within the carotid plaques ( $R=0.13$ ;  $P=0.10$ ; Figure 1C).

As shown by immunohistochemistry (Figure 2), in nonatherosclerotic arteries Nogo-B was distributed throughout the vessel wall with a strong signal in the endothelial layer and the smooth muscle cells within the media (Figure 2A, 2C, and 2D). In carotid plaques ( $n=15$ ), Nogo-B was expressed by smooth muscle cells (Figure 2I and 2J) and endothelial cells from the vasa vasorum (Figure 2K and 2L). Nogo-B also was evident in macrophage/foam cells rich areas (Figure 2G and 2H).

### **Nogo-B Expression Levels and Plaque Characteristics**

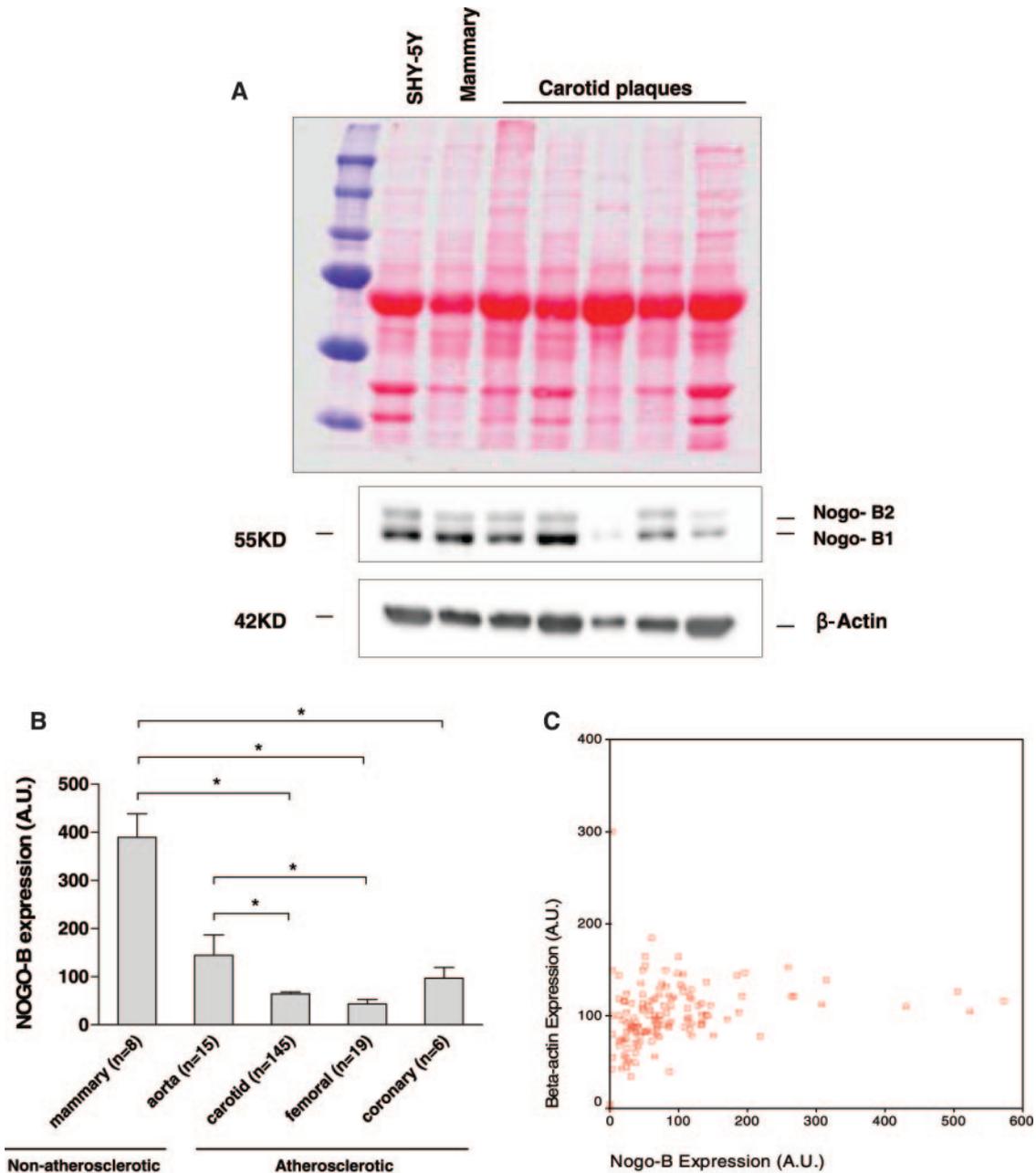
Next, we looked into the relationship between Nogo-B levels and different plaque characteristics. Carotid plaques containing more than 40% of fat (atheromatous phenotype) showed significantly lower Nogo-B levels in comparison to plaques with no or minor fat content ( $P=0.002$ ; Table 1). No relation was found between Nogo-B expression levels and other plaque characteristics such as number of macrophages and/or smooth muscle cells, amount of collagen or calcifications (Table 1).

### **Nogo-B Expression Levels, Proteolytic Activity, and Inflammation**

Because atherosclerotic plaque instability is related to inflammation and elevated MMP activity, we further investigated the relationship between Nogo-B expression levels, local MMP activity, and proinflammatory cytokine levels. A positive relation was found between Nogo-B and MMP-2 activity in carotid plaques ( $P=0.01$ ; Table 2). On the contrary, no relation was detected between Nogo-B and MMP-8 or MMP-9 activity (Table 2). Levels of either IL-6 or IL-8 did not show any relationship with Nogo-B (not shown).

### **Nogo-B Expression Levels and Clinical Data**

We also analyzed the relationship between Nogo-B, classical risk factors, and different clinical symptoms associated with atherosclerosis. Nogo-B expression levels were not related to any of the classical risk factors of atherosclerosis (Table 3). However, Nogo-B levels were significantly lower in those patients that revealed a high percentage carotid artery stenosis ( $>90\%$ ) and patients with restenosis after prior CEA (Table 3).

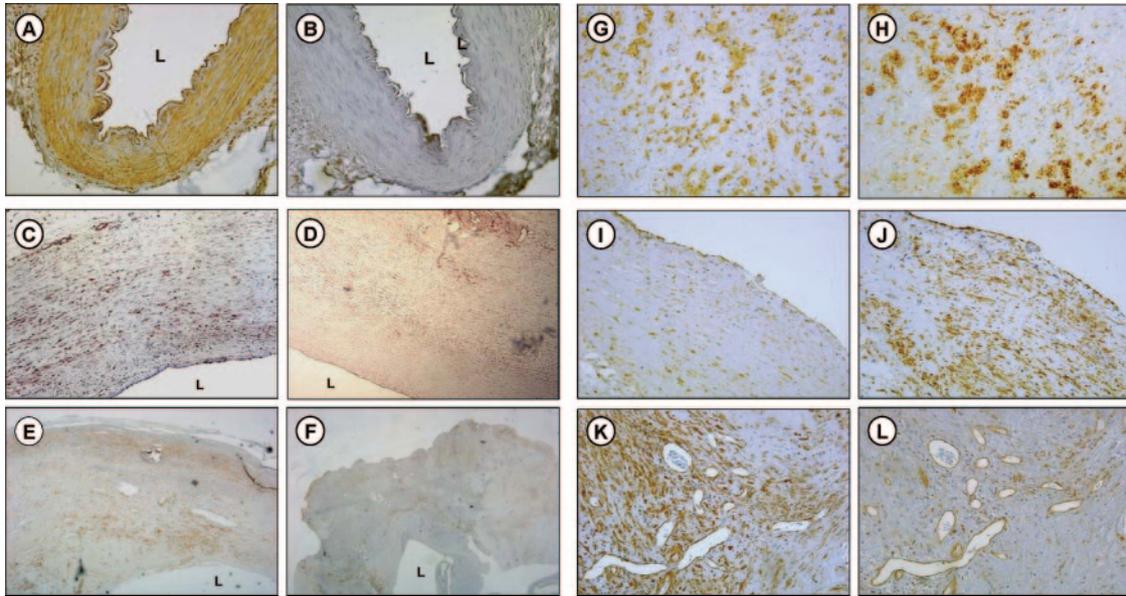


**Figure 1.** Identification of Nogo-B in different human vascular beds. A, Representative nitrocellulose membrane stained with Ponceau Red S showing protein loading (top). Representative Western blots for Nogo-B and  $\beta$ -Actin expression in human mammary and human carotid endarterectomy samples (bottom). Total lysates from the human neuroblastoma cell line SHY-5Y were used as positive control for Nogo A and B antibody. B, Densitometric analysis of Nogo-B expression levels in nonatherosclerotic mammary arteries (n=8), aortas (n=15), and atherosclerotic specimens of carotid endarterectomies (n=145), femoral endarterectomies (n=19), and coronary arteries (n=6). \* $P$ <0.001 mammary vs carotid; \* $P$ <0.001 mammary vs femoral; \* $P$ <0.003 mammary vs coronary; \* $P$ =0.03 aorta vs carotid; \* $P$ =0.007 aorta vs coronary. AU indicates Arbitrary Units. C, Relationship between  $\beta$ -actin (Arbitrary Units) and Nogo-B levels (Arbitrary Units) in Carotid endarterectomies;  $R=0.13$ ,  $P=0.10$ .

### Identification of Nogo-B in Plasma of Atherosclerotic and Control Subjects

To examine whether Nogo-B can be released into plasma and whether Nogo-B levels in the plasma are also affected during atherosclerosis, we determined circulating Nogo-B levels (soluble Nogo-B) in subjects that came to the hospital with clear manifestation of atherosclerotic disease (n=68) and risk-factor matched controls (n=63) included in the SMART study by ELISA (Figure 3A and 3B). The ELISA was

developed to detect the amino terminus of human Nogo A and B, using a sandwich technique with antibodies that recognize amino acids 1 to 18 and 14 to 30. Using this assay, Nogo-B levels did not differ between atherosclerotic patients and controls ( $46.64 \pm 10.65$  versus  $73.35 \pm 23.48$  nmol/L, respectively,  $P=0.5$ ) despite trends toward a reduction in patients with atherosclerosis. We also evaluated the relationship between soluble Nogo-B levels and intima-media thickness (IMT) which represents the severity of atherosclerotic



**Figure 2.** Immunodetection of Nogo-B in human tissue. Paraffin-embedded sections (5  $\mu$ m) from human mammary artery (A), human carotid (C), human aorta (D), and human carotid plaques (n=15; E, F, G, I, and K) were incubated with goat anti-human Nogo-A/B antibody (N-18). Adjacent sections were stained with anti-CD-68 (Macrophages, H) anti-alpha actin (SMCs, J), and anti-CD-31 (ECs, L) antibodies. Nonimmune control is shown in B. Brown staining indicates protein abundance. L indicates lumen.

disease. No correlation was found between soluble Nogo-B and IMT ( $P=0.62$ , Figure 3C).

**Discussion**

In the central nervous system, reticulon-4/Nogo-B has been described as a strong myelin-associated inhibitor which blocks axonal regeneration after damage.<sup>12,13</sup> In the arterial wall, it was demonstrated that Nogo-B inhibits intimal growth

and subsequent luminal narrowing in mice.<sup>9</sup> The presence and expression pattern of Nogo-B in human atherosclerotic lesions has not been established previously.

In the present study, we identified Nogo-B in human atherosclerotic plaques. Two variants of Nogo-B (Nogo-B1 and B2) are present in both nonatherosclerotic and atherosclerotic tissues. However, Nogo-B expression levels are significantly lower in atherosclerotic lesions in comparison to healthy mammary arteries. The mechanisms that influence local Nogo-B levels in atherosclerotic plaques are presently unknown. Pressure but not cellular stretch has been associated with an upregulation of Nogo-B levels in nonvascular cells.<sup>14</sup> It remains unknown whether initiation and/or progression of atherosclerosis with the presence of proatherogenic stimuli might trigger signaling cascades that reduce the

**TABLE 1. Relationship Between Nogo-B Expression (Arbitrary Units) and Carotid Plaque Characteristics (Semi-Quantitative). Note That Carotid Endarterectomy Samples Containing Larger Amount of Fat (>40%) Are Strongly Associated to Low Nogo-B Expression Levels**

	Nogo-B		P Value
	Median	Interquartile Range	
<b>Fat</b>			
<40%	65	30–106	0.002
>40%	37	20–63	
<b>Macrophages</b>			
No/Minor	56	24–90	0.86
Moderate/Heavy	50	26–94	
<b>Smooth muscle cells</b>			
No/Minor	41	22–80	0.15
Moderate/Heavy	63	25–103	
<b>Collagen</b>			
No/Minor	63	22–101	0.99
Moderate/Heavy	51	25–89	
<b>Calcifications</b>			
No/Minor	47	23–88	0.67
Moderate/Heavy	61	25–94	

**TABLE 2. Relationship Between Nogo-B Expression (Arbitrary Units) and MMP Activity (mmol/mL) in Carotid Plaques. Note That Carotid Endarterectomies Containing Low Nogo-B Levels Show Low MMP-2 Activity**

	Nogo-B		
	Median	Interquartile Range	P Value
<b>MMP-2 activity</b>			
<median	40	20–67	0.01
>median	71	32–115	
<b>MMP-8 activity</b>			
<median	49	23–100	0.77
>median	61	24–94	
<b>MMP-9 activity</b>			
<median	58	25–106	0.36
>median	48	22–86	

**TABLE 3. Relationship Between Nogo-B Expression Levels (Arbitrary Units) and Clinical Symptoms. Note That Carotid Endarterectomies From Subjects With High Stenosis Degree and Restenosis After Prior CEA Show Significantly Reduced Levels of Nogo-B**

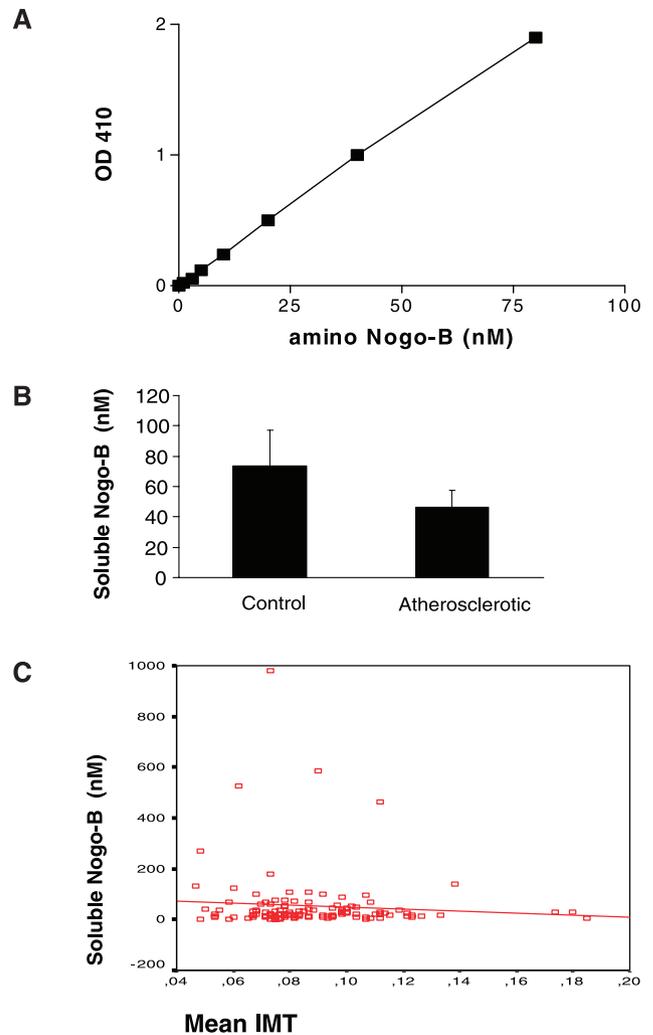
	Nogo-B		P Value
	Median	Interquartile Range	
Carotid degree of stenosis by duplex			
<90%	68	37–109	0.04
>90%	46	19–86	
Restenosis or <i>de novo</i> stenosis			
De novo	61	26–103	0.01
Restenosis	13	0–31	
History of stroke			
No	49	21–100	0.38
Yes	61	25–100	
History of myocardial infarction			
No	63	22–106	0.59
Yes	39	29–84	
History of angina pectoris			
No	65	33–104	0.13
Yes	39	19–97	

expression and/or promote the degradation of Nogo-B in response to injury.

As shown by immunohistochemistry, in healthy mammary arteries and aortas Nogo-B is distributed through the vessel wall with a strong signal in the endothelial layer and the smooth muscle cells within the media. This observation is in agreement with the previous reports which have identified expression of Nogo-B in cultured human endothelial cells and smooth muscle cells as well as mouse fibroblasts.<sup>9,15</sup> In carotid plaques, Nogo-B is present in smooth muscle cells and endothelial cells from the vasa vasorum. Interestingly, Nogo-B also seems to be concentrated in macrophage/foam cells rich areas. This has not been reported in human tissues before. Recently, Rosseau and coworkers did report the expression of Nogo-B in RAW264 macrophages, although its role remains to be established.<sup>16</sup>

Another finding is that Nogo-B levels in carotid plaques are inversely related with the presence of large lipid pools. It is thought that in advanced atherosclerotic lesions, apoptosis of macrophages/foam cells contribute to lipid-rich necrotic cores formation.<sup>17</sup> In this context, it has been suggested that Nogo-B might promote apoptosis in cancer cells.<sup>18–21</sup> Despite the overall reduction in Nogo-B levels in plaques, a local accumulation of Nogo-B in macrophages might contribute to cell death facilitating lipid accumulation. However, it should be considered that other members of the Nogo family have demonstrated an antiapoptotic effect.<sup>22</sup> Therefore, the involvement of proteins from Nogo family in apoptotic process is still controversial and requires further investigation.<sup>23</sup>

We have searched for associations between expression levels of Nogo-B and well-established factors related to plaque vulnerability such as MMP activity and proinflamma-



**Figure 3.** Identification of soluble Nogo-B in human plasma. A, Representative standard curve for amino Nogo-B ELISA. Standard curves were linear in the range between 0 and 100 nmol/L with a correlation coefficient of  $r^2=0.98$  to  $0.99$ . For detection of Nogo-B in human plasma, duplicate samples (10 to 20  $\mu$ L) were incubated as indicated for amino-Nogo-B standard and processed accordingly. OD 410 indicates optical density. B, Levels of soluble Nogo-B (nmol/L) in atherosclerotic patients ( $n=68$ ) and risk-factor matched controls ( $n=63$ ) from the SMART population.  $P=0.5$ . C, Relationship between intima-medial thickness (IMT) and soluble Nogo-B levels in the SMART population;  $r^2=0.43$ ,  $P=0.62$ .

tory cytokines levels and have observed a positive relationship between Nogo-B expression levels and MMP-2 activity. Recently, we have reported that high levels of MMP-2 are associated with a smooth muscle cell-rich stable plaque phenotype.<sup>24</sup> Thus, this observation suggests that high Nogo-B levels may contribute to plaque stabilization. We also found that Nogo-B levels are not influenced by previous use of medication to treat cardiovascular diseases like statins (not shown). The correlation between plaque Nogo-B levels, patient's clinical history, and clinical complications revealed data supporting the idea that local downregulation of Nogo-B might accelerate luminal narrowing in atherosclerotic and restenotic disease. Indeed, we found that Nogo-B levels were inversely associated with high preoperative degree of carotid stenosis and restenosis after prior carotid endarterectomy.

These observations are in line with animal experimental data of Acevedo et al, who demonstrated that Nogo-B impairs neointima formation following arterial ligation in mice.<sup>9</sup>

We also investigated whether circulating levels of Nogo-B (soluble Nogo-B) can serve as a surrogate marker of human atherosclerosis. Despite a trend to reduction, no differences were found in plasma of atherosclerotic when compared with control patients (Figure 3). A potential explanation that soluble Nogo-B was not decreased in plasma of atherosclerotic patients may be that plasma levels reflect the total amount of Nogo-B in the blood. Possibly, in atherosclerotic patients, nonaffected arteries may also continuously release Nogo-B into the circulation and this might mask the differences between the groups.

In summary, here we report for the first time that low levels of Nogo-B in carotid plaques are strongly associated with an atheromatous (vulnerable) phenotype and stenotic lesions in subjects receiving operative treatment. These data suggest that endogenous levels of Nogo-B may be considered atheroprotective, consistent with the preclinical data documenting enhanced neointima in Nogo-B deficient mice.<sup>9</sup> Therefore, elucidation of new ways to upregulate vascular Nogo-B levels and/or prevent its loss during atherogenesis may represent a novel target to prevent acceleration and destabilization of atherosclerotic plaques.

### Limitations of the Study

This is the first observational study of Nogo expression in human arteries and as such, we cannot make inferences regarding causality. Although our data suggests that the reduction of Nogo-B expression could play a role in plaque progression and plaque destabilization, it could also merely be a consequence of atherosclerosis. We feel, however, that a causal role of lowered Nogo B expression in plaque progression is supported by previously published studies in mice<sup>9</sup> and more recently in rabbits.<sup>25</sup>

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### Disclosures

None.

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