ORIGINAL RESEARCH

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Increased Levels of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 in Ischemic Stroke and Transient Ischemic Attack

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***Background*—**Soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) has been shown to be increased in patients with acute ischemic stroke. Here, we evaluated plasma sLOX-1 levels and vascular carotid plaque LOX-1 (ie, *OLR1*) gene expression in patients with ischemic stroke and transient ischemic attack (TIA) with particular focus on their relation to time since symptom onset.

***Methods and Results*—**Plasma sLOX-1 (n=232) and carotid plaque *OLR1* gene expression (n=146) were evaluated in patients who were referred to evaluation for carotid endarterectomy, as well as in healthy control plasma (n=81). Patients were categorized according to presence of acute ischemic stroke or transient ischemic attack (n=35) ≤7 days, >7 days ≤3 months (n=90), >3 months (n=40), or no reported symptoms before study inclusion (n=67). Our major findings were the following: (1) Patients with carotid atherosclerosis had increased plasma sLOX-1 levels as compared with controls. (2) Plaque *OLR1* mRNA levels were increased in carotid plaques (n=146) compared with nonatherosclerotic vessels (ie, common iliac arteries of organ donors, n=10). (3) There were no differences in sLOX plasma levels or *OLR1* gene expression when analyzed according to the time since relevant cerebral ischemic symptoms. (4) Also patients with severe carotid atherosclerosis without any previous ischemic events had raised sLOX-1 levels. (5) Immunostaining showed colocalization between LOX-1 and macrophages within the carotid plaques. (6) Also patients with acute stroke (within 7 days) caused by atrial fibrillation (n=22) had comparable raised sLOX-1 levels.

***Conclusions*—**sLOX-1 levels are elevated in patients with ischemic stroke and transient ischemic attack independent of cause and time since the ischemic event. **(*J Am Heart Assoc*. 2018;7:e006479. DOI: 10.1161/JAHA.117.006479.)**

Key Words: cerebrovascular disease/stroke • inflammation • ischemic stroke

The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a transmembrane endocytosis receptor of oxidized low-density lipoprotein involved in the development of atherosclerotic disease. LOX-1 is regarded as the main oxidized low-density lipoprotein scavenger receptor of endothelial cells, and is also expressed on the cell surface of other cells involved in the pathogenesis of atherosclerosis, such as monocytes/macrophages and smooth muscle cells (SMC).[1] Although the basal expression of LOX-1 is low, it is induced by oxidative and inflammatory stimuli.[1] Membrane-bound LOX-1 can be proteolytically cleaved into a soluble form. This is induced by inflammatory stimuli such as tumor necrosis factor, interleukin-8, and interleukin-18, and can be detected in the circulation as soluble LOX-1 (sLOX-1).[2,3]

Although the biological functions of sLOX-1 are elusive, there are several reports of increased sLOX-1 levels in patients with coronary artery disease (CAD), and sLOX-1 appears to have potential as a prognostic biomarker among patients with acute coronary syndrome and/or stable CAD.[4] In contrast, data on sLOX-1 level in patients with carotid atherosclerosis and ischemic stroke are scarce.[5,6] Moreover, although the regulation of LOX-1 gene expression (ie, *OLR1*) is comprehensively studied in atherosclerosis-related in vitro and animal studies, to the best of our knowledge, there is only one relatively small study that has measured *OLR1* gene expression in human plaques (n=8) and control arteries (n=2).[7]

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Accompanying Tables S1 through S3 and Figures S1, S2 are available at http://jaha.ahajournals.org/content/7/2/e006479/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- Soluble LOX-1 (sLOX-1) levels in plasma were increased in patients with carotid atherosclerosis independent of the time since the ischemic event and even in patients without any ischemic event.
- Patients with an acute ischemic stroke caused by atrial fibrillation had raised sLOX-1 levels comparable to those with an acute ischemic cerebral event related to carotid atherosclerosis.
- Patients with carotid atherosclerosis showed increased plaque expression of LOX-1 (mRNA levels of *OLR1*) with no relation to time since the ischemic event.

What Are the Clinical Implications?

- Systemic and plaque expression of LOX-1 could be a marker of plaque atherosclerosis rather than the acute ischemic event.
- The regulation of LOX-1 in atrial fibrillation should merit further investigation.

The purpose of the present study was to examine LOX-1 levels in human carotid atherosclerosis by analyzing both sLOX-1 and the corresponding carotid plaque *OLR1* mRNA expression in a large population of patients with acute ischemic stroke or transient ischemic attack (TIA), with particular focus on their relation to time since symptom onset.

Methods

Because of ethical restrictions from the Regional Committee for Medical and Research Ethics in South-East Norway, the data from the individual patients will unfortunately not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Ethics

The protocols were approved by the Regional Committee for Medical and Research Ethics in South-East Norway, reference ID S-09276c, 2009/5237, and 2014/2078. All participants gave signed informed consent and the study protocols were in agreement with the principles of the Declaration of Helsinki.

Study Population

Patients who were referred to carotid endarterectomy were enrolled in the Oslo Cohort study between 2005 and 2014 at the Department of Neurology, Oslo University Hospital Rikshospitalet.[8] All patients (n=232) had moderate (50–69%) or severe (≥70%) stenosis in the internal carotid artery. Carotid plaques (n=146) were collected during carotid endarterectomy (CEA). The patients were classified according to the presence of preoperative symptoms of ischemic stroke or TIA ipsilateral to the stenotic carotid artery ≤7 days, >7 days ≤3 months, >3 months before study inclusion, or no reported symptoms. The patients with no relevant symptoms had carotid stenosis that was coincidentally detected during clinical examination of patients with coronary or peripheral artery disease. The indication for CEA was based on current guidelines[9] that allow evaluation of patient-specific factors, such as age, sex, and comorbidities as well as recent studies suggesting that CEA within 30 days after stroke is associated with a decreased risk of restenosis.[10]

Blood sampling was performed 48 hours before the operative procedure, and was accessible from 232 patients. In 81 patients, both blood samples and carotid plaques obtained at the same time point were available. The carotid stenosis and plaque echolucency were classified by precerebral color triplex ultrasound and computed tomography angiography according to consensus criteria.[11,12] Exclusion criteria were concomitant infections, connective tissue disease, heart failure, malignancies, as well as kidney and liver disease.

The collection of human samples is an ongoing study at the Oslo University Hospital and unfortunately, all parameters are not available for all patients because of reasons like too little plasma volume and lack of routine clinical chemistry analyses. There were no dropouts.

For comparison of plaque analyses, arterial tissue samples were obtained from nonatherosclerotic common iliac arteries of organ donors with no history of cardiovascular disease, deceased from sudden death and approved for organ transplantation (n=10). For comparison of plasma analyses, blood samples were obtained from age-matched control subjects (n=81). These controls were recruited from the same area of Norway as the patients, and were healthy individuals as evaluated by clinical examination, disease history, and normal levels of C-reactive protein (CRP).

For comparison we also included a control group of patients with acute ischemic stroke (<7 days since onset of symptoms) caused by atrial fibrillation (n=22, Table S1).

Blood and Tissue Sampling Protocol

Atherosclerotic carotid plaques collected during carotid endarterectomy were rapidly frozen in liquid nitrogen and stored at −80°C until further analyses. The control arteries obtained from organ donors were processed and stored the same way as plaques. The organ donors were adequately circulated during the whole operating procedure. Peripheral

venous blood was collected from control subjects and patients and drawn into pyrogen-free tubes with ethylenediaminetetraacetic acid as anticoagulant. The tubes were promptly immersed in melting ice and centrifuged within 30 minutes at 2500*g* for 20 minutes to obtain platelet-poor plasma. All samples were stored at −80°C until analysis and thawed <3 times (ie, all samples were analyzed at the same time). We cannot exclude the possibility of degradation attributable to storage time that varied between the samples. On the other hand, by analyzing all samples at the same time and including representative samples from all subgroups in each enzyme-linked immunosorbent assay (ELISA) plate, we have minimized the influence of intra- and intervariation of the ELISA on our results.

Measurements of sLOX-1

sLOX-1 levels were measured in undiluted plasma by ELISA (Uscn Life Science Inc., Wuhan, China) according to the protocol of the manufacturer. The inter- and intracoefficients of variation were <10%.

RNA Isolation, cDNA Synthesis, and Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated from whole homogenized atherosclerotic plaques and control arteries with the use of RNase-free conditions and the RNeasy spin columns, as described by the company (Qiagen, Hilden, Germany). Isolated RNA was treated with DNase (Qiagen) and stored at −80°C until further analysis. RNA concentrations and purity based on the 260/280 and the 260/230 ratio were assessed by spectrophotometer absorbance (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE). Equal amounts of RNA were loaded into the cDNA synthesis and synthesis of cDNA was performed using the q-Script cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantification of mRNA was performed using Perfecta SYBR Green Fastmix ROX (Quanta Bioscience) or TaqMan assays (Applied Biosystems, Foster City, CA) and the 7900HT Fast Real-Time PCR System (Applied Biosystems) with the accompanying software SDS 2.4. As small sample volumes (mean 3.4; min 0.5; max 13.8 μL) were used in the analyses of human samples, operator-dependent variability in pipetting was avoided as robotics performed all steps involving cDNA synthesis and reverse transcription quantitative polymerase chain reactions. All primer sequences can be provided upon request. For each transcript, reverse transcription quantitative polymerase chain reaction was conducted in duplicates. Target transcript levels were quantified by the comparative Ct method using the mean of 2 nonregulated reference genes as endogenous control (ie, *GAPDH* and β-*ACTIN*).

Immunohistochemistry

Formalin-fixed cryosections (10 μm) of atherosclerotic carotid plaques were exposed to high-temperature antigen retrieval in citrate buffer (pH 6). After blocking in 0.5% bovine serum albumin in PBS, samples were incubated with primary antibodies overnight at 4°C. Primary antibodies used were the following: mouse anti-LOX-1 (MAB1798, R&D Systems, Minneapolis, MN), rabbit anti-CD31 (ab76533, Abcam, Cambridge, MA), rabbit anti-α smooth muscle actin (ab124964, Abcam) and mouse anti-CD68 (m0876, Agilent Technologies, Santa Clara, CA). For immunohistochemistry, the slides were treated with 0.3% H_2O_2, followed by a 2-hour incubation with peroxidase-conjugated secondary antibodies (Impress-Vector, Vector Laboratories, Burlingame, CA), rinsed, and developed with chromogen for immunoperoxidase staining (DAB Plus, Vector Laboratories) for 3 minutes. The sections were counterstained with hematoxylin. For immunofluorescence, the sections were washed and counterstained with Alexa Fluor 568- and 488-conjugated IgG, respectively (Thermo Fisher Scientific, Waltham, MA). Nuclei were stained with diamidino-2-phenylindole (ProLong Gold antifade reagent; Thermo Fisher Scientific). Oil-red-O staining was performed to analyze lipid content. Images were obtained on a Nikon Eclipse E400 microscope with ×100 and ×400 magnification.

Miscellaneous

Standard blood chemistry and lipid parameters were evaluated in plasma using in-house routine laboratory methods. Levels of CRP were determined using a particle-enhanced, high-sensitive immunoturbidimetric assay (hsCRP, Tina-Quant CRP Gen.3; Roche Diagnostica, Basel, Switzerland) with a minimal detectable concentration of 0.6 mg/L.

Statistical Analysis

Statistical analyses were performed using Student *t* test or Mann–Whitney *U* test as appropriate. When comparing more than 2 groups, the Kruskal–Wallis test was performed a priori. If significant, Mann–Whitney *U* test was used for post hoc testing between each group. Categorical data were analyzed by χ^2 tests. Correlation analysis was calculated using Spearman's rank correlation coefficient. Correction for potential confounders was done by linear regression. Correction for multiple testing with the Benjamini-Hochberg false discovery rate procedure was performed using R, version 3.1.2 and statistical analyses were conducted using Prism version 6.0 (GraphPad software, La Jolla, CA) and SPSS for Windows statistical software (version 22, SPSS Inc, Chicago, IL). $P<0.05$ was considered statistically significant. Data are presented as mean±SEM unless otherwise stated.

Results

Study Population

Plasma samples were obtained from patients within 2 days before planned CEA (n=232; of these, 38 did not undergo operation because of severe stroke judged to have irreversible pathology, increased preoperative risk (eg, comorbidity and multiple stenoses and unwillingness to be operated on) as well as from healthy control subjects (n=81) (Table 1). The patients were classified according to presence of preoperative symptoms of ischemic stroke or TIA ≤7 days (n=35), >7 days ≤3 months (n=90), >3 months (n=40) before study inclusion, or no reported symptoms (n=67). The patients had lower levels of total cholesterol and LDL cholesterol as compared with the controls, probably because of the wide use of statins in the patient group. A major proportion of the patients were smokers and had hypertension or diabetes mellitus. Age has a major influence on plaque stability,[10] and importantly, the controls were age-matched as compared with the patients. However, the controls had a lower fraction of males and, as expected, had lower CRP and leukocyte values (Table 1).

Table 1. Plasma Analysis: Baseline Variables in Patients With Carotid Atherosclerosis and Controls

	Patients (N=232)	Controls (N=81)	*P* Value
Age, y†	67 (8.9)	66 (5.6)	0.1
Male sex, % (n)	65.1 (151)	42.0 (34)	<0.001
BMI, kg/m^{2}†	26.2 (4.4)	...	...
Current smoking, % (n)	46.1 (107)	...	...
Hypertension, % (n)	67.7 (149)	0	...
Diabetes mellitus, % (n)	17.2 (40)	0	...
Aspirin treatment, % (n)	82.9 (184)	0	...
Statin treatment, % (n)	86.8 (191)	0	...
Degree of stenosis, %*	80 (50–100)	...	...
Echolucent plaque, % (n)	52.2 (121)	...	...
CRP, mg/L†	5.8 (9.5)	2.0 (3.0)	<0.001
Leukocyte count, 10^{9}/L†	7.6 (2.5)	5.6 (1.2)	<0.001
Platelets, 10^{9}/L†	282 (76.7)	...	...
Total cholesterol, mmol/L†	4.3 (0.9)	5.9 (1.0)	<0.001
LDL cholesterol, mmol/L†	2.4 (0.8)	3.7 (0.8)	<0.001
HDL cholesterol, mmol/L†	1.3 (0.4)	1.8 (0.5)	<0.001
Triglycerides, mmol/L†	1.5 (0.7)	1.2 (0.7)	0.004
HbA1c, %†	6.0 (1.2)	5.5 (0.8)	<0.001

BMI indicates body mass index; CRP, C-reactive protein; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Data were analyzed using Student *t* test, Mann–Whitney *U* test, or χ^2 tests and numbers are presented as percentage (numbers) or *median (min–max) or †mean (SD).

Characteristics of the patients who donated carotid atherosclerotic plaques (n=146) are presented in Table 2. The common iliac arteries used as controls were obtained from deceased organ donors (n=10) with no history of cardiovascular disease (age: 44.4±14.7 years [mean and SD], 62% males).

Increased sLOX-1 Levels in Plasma in Patients With Carotid Atherosclerosis

In general, plasma levels of sLOX-1 were markedly increased in patients with carotid atherosclerosis (n=232) as compared with healthy controls (n=81) (0.67 ng/mL versus 0.99 ng/mL *P*<0.001). However, there were no significant differences in sLOX-1 levels between the different subgroups of patients (ie, acute ischemic stroke or TIA within 7 days, >7 days ≤3 months, >3 months before study inclusion, or no reported symptoms) (Figure 1A). Moreover, 38 patients did not undergo surgery (CEA) and there was no difference in sLOX-1 between these patients and those who underwent CEA (*P*=0.61). Furthermore, 104 of the patients had ischemic stroke and 61 had TIA, but we could not find any differences in sLOX-1 levels between these 2 groups of patients (1.00 [0.11–2.63] ng/mL versus 0.95 [0.23–7.31] ng/mL, respectively, *P*=0.80). Thus, it

Table 2. Baseline Variables in Patients and Controls Carotid Samples

	Patients (N=146)
Age, y*	69 (7.9)
Male sex, % (n)	66.4 (97)
BMI, kg/m^{2}*	26.2 (3.8)
Current smoking, % (n)	40.4 (59)
Hypertension, % (n)	65.1 (95)
Diabetes mellitus, % (n)	22.6 (33)
Degree of stenosis, %*	80 (50–99)
Echolucent plaque, % (n)	55.5 (81)
Aspirin treatment, % (n)	80.7 (131)
Statin treatment, % (n)	92.5 (135)
CRP, mg/L*	8.8 (36.8)
Leukocyte count, 10^{9}/L*	7.8 (2.1)
Platelets, 10^{9}/L*	279 (78)
Total cholesterol, mmol/L*	4.2 (1.1)
LDL cholesterol, mmol/L*	2.4 (0.8)
HDL cholesterol, mmol/L*	1.3 (0.5)
Triglycerides, mmol/L*	1.5 (1.0)
HbA1c, %*	6.2 (1.5)

BMI indicates body mass index; CRP, C-reactive protein; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Numbers given as percent (numbers) or *mean (SD).

seems that the increase of sLOX-1 may be related to the chronic atherosclerotic process rather than to time since the acute ischemic event or severity of the event.

The control group had a lower proportion of males and lower levels of CRP and leukocyte counts, but importantly, the difference in sLOX-1 between patients and controls was also significant after adjusting for sex, CRP, and total leukocyte counts as well as age, representing potential confounders (linear regression, $P<0.004$).

Increased sLOX-1 Levels in Patients With Acute Ischemic Events Caused by Atrial Fibrillation

For comparison, we analyzed plasma levels of sLOX-1 in a group of patients with very recent (<7 days) cardioembolic ischemic stroke or TIA caused by atrial fibrillation (n=22). As shown in Figure S1, these patients also had increased sLOX-1 levels as compared with healthy controls, but with no difference in sLOX-1 level when compared with patients with very recent acute ischemic event (<7 days) caused by carotid atherosclerosis. Six of these patients used statins and 12 used antiplatelet drugs, but importantly, the use of these medications did not significantly influence sLOX-1 levels: sLOX-1 with (n=6) and without (n=16) statins: 1.30 (0.20–1.54) ng/mL versus 1.11 (0.43–1.69) ng/mL, respectively, $P=0.73$ and sLOX with (n=12) and without (n=10) antiplatelet drugs: 1.22 (0.76–1.60) ng/mL versus 1.10 (0.20–1.69) ng/mL, respectively, $P=1.0$.

Increased Plaque Expression of *OLR1* in Plaques of Patients With Carotid Atherosclerosis

Examination of *OLR1* mRNA levels in carotid plaques (n=146) and control (n=10) arteries showed increased *OLR1* gene expression in carotid plaques (Figure 1B). In a similar pattern to our findings for sLOX-1, there were no differences in plaque *OLR1* levels between patients with acute ischemic stroke or TIA within 7 days (n=21) before study inclusion and the other subgroups of patients (ie, an ischemic event >7 days and <3 months [n=76], >3 months before study inclusion [n=21] or with no events [n=28]) (Figure 1B). The presence of LOX-1 within the plaques was also analyzed at protein level (ELISAs from plaque lysates), showing the same pattern as for the mRNA data with no difference between recent symptoms (within 2 months) and no reported symptoms differences in relation to time since the acute event (Figure S2).

No Association Between sLOX-1 in Plasma and *OLR1* Gene Expression in Carotid Atherosclerosis

To evaluate the association between sLOX-1 in the circulation and *OLR1* gene expression in carotid plaques, these parameters were evaluated in a subgroup of patients in which both parameters were obtainable (n=81). There was, however, no significant correlation between carotid plaque *OLR1* mRNA expression and plasma sLOX-1 levels (Figure 1C).

Correlations of sLOX-1 and *OLR1* Gene Expression With Clinical Characteristics of the Patient Group

Tables S2 and S3 present correlations of sLOX-1 and *OLR1* mRNA expression with body mass index, plasma lipids, and inflammatory markers. In general, few significant correlations were revealed. Thus, except for a positive correlation of sLOX-1 with triglycerides ($r=0.25$, $P<0.023$) and body mass index ($r=0.36$, $P=0.009$), no other significant correlations were found between plasma sLOX-1 and the clinical parameters described in Table S1. Moreover, there were no correlations between plaque *OLR1* gene expression and any of the clinical parameters (Table S2).

Correlation of *OLR1* Gene Expression With Cellular Markers in Carotid Plaques

The cellular distribution of membrane-bound LOX-1 has been shown to include cells relevant to atherosclerosis, such as macrophages, endothelial cells, and SMC. Thus, we examined the correlation of *OLR1* with relevant cellular markers within carotid plaques. Plaque *OLR1* gene transcript levels showed a significant positive correlation with mRNA levels of the macrophage markers *CD68*, *CD14*, and *CD163* (Figure 2A through 2C) as well as with the endothelial marker *CD31* (Figure 2D). In contrast, plaque *OLR1* mRNA levels showed a negative correlation with the SMC marker α-ACTIN (*ACTA2*) (Figure 2E).

LOX-1 Is Localized to Macrophages and Endothelial Cells in Carotid Plaques

To further relate LOX-1 expression to macrophages, endothelial cells, and SMC within the lesion, we performed immunostaining of carotid plaques (Figure 3). These analyses showed LOX-1 to be present throughout the atherosclerotic lesions (Figure 3C) with a particular strong colocalization to lipid-loaded (Figure 3A) and macrophage-rich areas (Figures 3B and 4A). LOX-1 staining was also seen in the proximity of endothelial cells and SMC, but the immunostaining was weaker and the colocalization was not as evident (Figure 4B and 4C).

Discussion

Serum sLOX-1 has recently been suggested as a biomarker in acute ischemic cerebrovascular disease, as 2 different studies

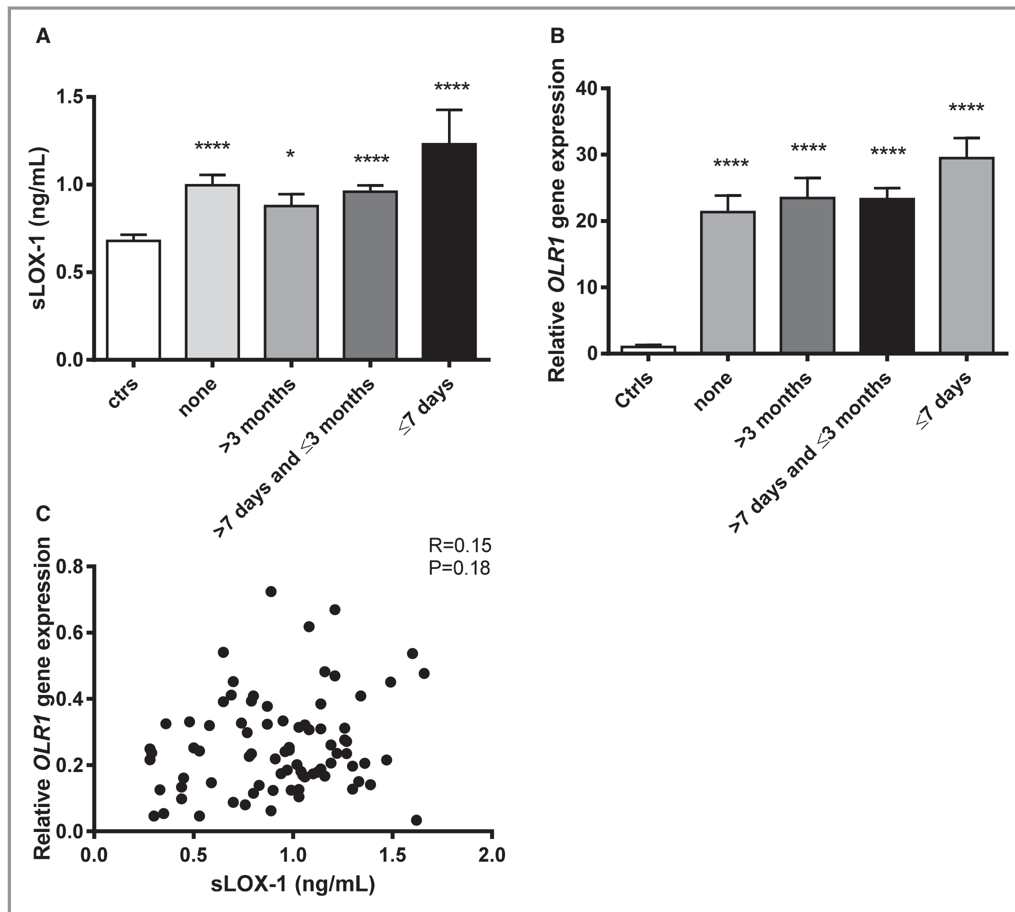

Figure 1. Plasma sLOX-1 and plaque *OLR1* mRNA expression in patients with carotid atherosclerosis. A, *Soluble (s)LOX-1* is increased in plasma of patients with carotid atherosclerosis (n=232) as compared with healthy controls (n=81) ($P<0.001$) with no differences between subgroups of patients with carotid atherosclerosis (ie, ischemic stroke or TIA <7 days [n=35], >7 days and ≤3 months [n=90], >3 months [n=40] before study inclusion or no reported symptoms [n=67]). B, *OLR1* gene expression is increased in carotid plaques (n=146) as compared with control arteries (n=10). There are no differences in *OLR1* gene expression between subgroups of patients with carotid atherosclerosis (ie, ischemic stroke or TIA <7 days [n=21], >7 days and ≤3 months [n=76], >3 months [n=21] before study inclusion, or no reported symptoms [None; n=28]). C, No correlation between plasma sLOX-1 and plaque *OLR1* gene expression in patients with carotid atherosclerosis (Spearman's r=0.15, P=0.18, n=81). Data are presented as mean and SEM. Analyses were performed using Kruskal–Wallis test, Mann–Whitney *U* test, and Spearman's rank correlation. *$P<0.05$, and ****$P<0.0001$ vs controls (Mann–Whitney). ctrs indicates controls; sLOX-1, soluble lectin-like oxidized low-density lipoprotein receptor 1; TIA, transient ischemic attack.

have reported increased serum levels of sLOX-1 in patients with acute ischemic stroke.[5,6] In both studies, blood samples were collected closely after stroke onset (ie, within 24 hours and 3 days, respectively). Herein we confirm these findings by showing increased sLOX-1 levels in patients with acute ischemic stroke or TIA within 7 days. However, our findings also show that increased levels of sLOX-1 in patients with carotid atherosclerosis are not restricted to patients with acute ischemic stroke or TIA, but are also observed in patients with cerebral ischemic events regardless of time since symptom onset, and even in patients with carotid atherosclerosis without any previous ischemic events. Consequently, we suggest that increased sLOX-1 levels are not only merely related to acute ischemic events, but could also potentially be a feature of the chronic atherosclerotic process within the carotid plaques.

The serum sLOX-1 level have been reported to predict functional outcome in patients with large artery ischemic stroke,[6] and sLOX-1 levels have been used in a formula to predict ischemic stroke during follow-up.[13] In general, however, the clinical use of sLOX-1 in ischemic stroke patients is elusive. In contrast, the role of sLOX-1 as a biomarker in

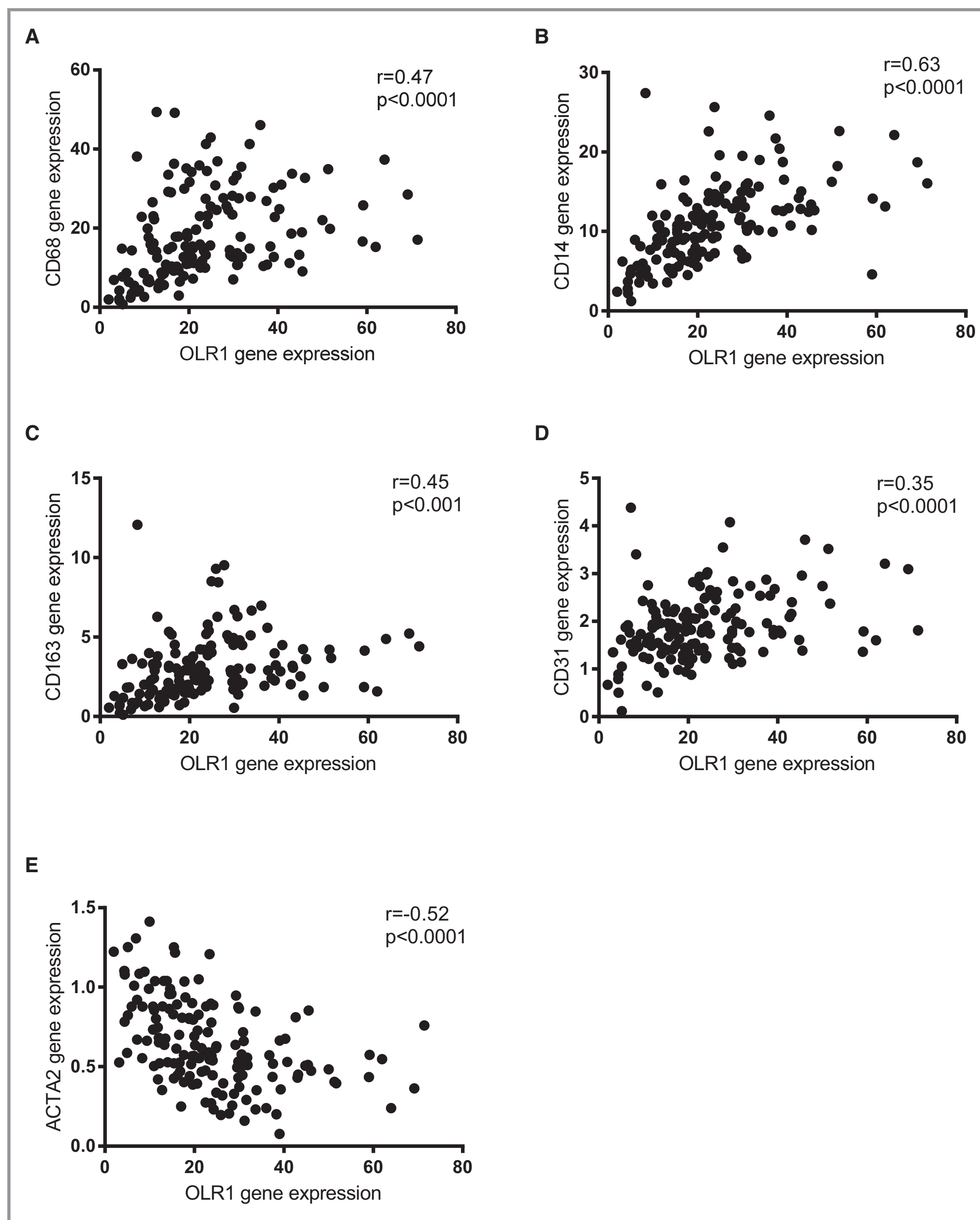

Figure 2. Correlations of plaque *OLR1* gene expression with mRNA levels of cell markers in carotid plaques (n=146). There is a positive correlation of *OLR1* gene expression and the macrophage markers (A) *CD68*, (B) *CD14*, and (C) *CD163*. There is also a positive correlation with the endothelial marker *CD31* (D) but a negative correlation with the SMC marker *ACTA2* (E). The mRNA levels are normalized to the mean of 2 reference genes (*GAPDH* and β-*ACTIN*). SMC indicates smooth muscle cells.

ischemic heart disease is thoroughly evaluated. Indeed, in CAD, several studies imply that sLOX-1 has potential as a sensitive biomarker for acute coronary syndrome[14–18] and appears to be significantly higher in acute coronary syndrome than in stable CAD.[14,18] sLOX-1 is also associated with coronary in-stent restenosis in patients with stable CAD[19] as well as with increased risk of periprocedural myocardial infarction in stable CAD patients undergoing elective percutaneous coronary intervention.[20] The present study expands our knowledge on sLOX-1, demonstrating that increased sLOX-1 is not only restricted to acute coronary events but is also seen in acute ischemic stroke or TIA. However, elevated sLOX-1 levels also seem to be a feature of carotid atherosclerosis, at least partly independently of previous ischemic events, time since symptom onset, or undergoing CEA or not. Thus, it is possible that LOX-1 level

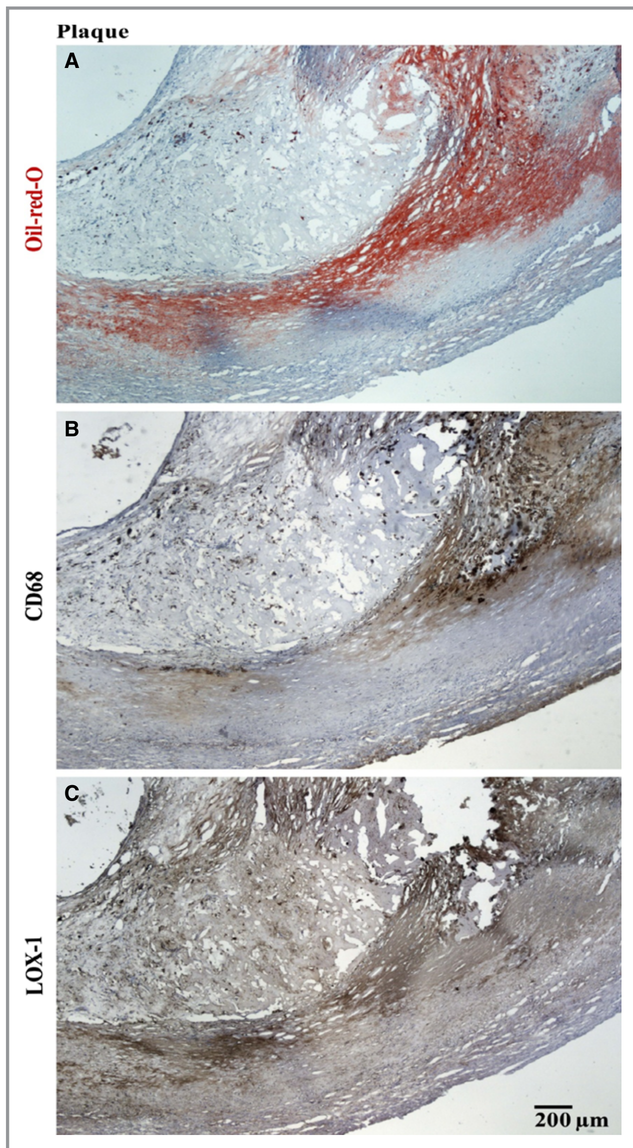

Figure 3. Histological staining of representative carotid plaque. Immunohistochemical staining of carotid plaque for lipid accumulation (A) Oil-red-O staining and (B) $CD68^+$ cell (ie, macrophages), and (C) LOX-1 show lipid accumulation, macrophage infiltration, and LOX-1 distribution throughout the carotid plaque. LOX-1 indicates lectin-like oxidized low-density lipoprotein receptor 1.

may be related to the chronic atherosclerotic process and in a lesser degree to the pathways that are activated during an acute ischemic event. We also found raised sLOX-1 levels in patients with acute ischemic stroke caused by atrial fibrillation compared with controls, with similar levels as in those with acute ischemic events caused by carotid atherosclerosis, suggesting that the pathogenesis of the acute ischemic event only in a minor degree influences LOX-1 levels. However, to elucidate the regulation of sLOX-1 in stroke related to atrial fibrillation, more studies are needed. Such studies should include a larger number of patients, as well as data from patients with atrial fibrillation without ischemic stroke and ideally patients with atrial fibrillation without carotid atherosclerosis.

There are several possible sources of sLOX-1 in the circulation. Although it is tempting to assume that the main shedding of sLOX-1 originates from the vasculature, studies on human and/or animal cell lines have shown expression of LOX-1 in several nonvascular cells, including adipocytes,[21] neurons,[22] platelets,[23] cardiomyocytes,[24] as well as in renal[25,26] and lung tissue[27] during pathological conditions. Hence, although we hypothesize that sLOX-1 is a marker for carotid atherosclerosis, we cannot rule out the possibility that increased sLOX-1 in the circulation may have a nonvascular cause. However, although we found no significant correlation between plaque *OLR1* mRNA levels and sLOX-1 in plasma, lack of statistical correlations do not rule out any causal relationship. Indeed, the regulation of membrane-bound LOX-1 and its release into the circulation is modified by numerous stimuli, such as cytokines, modified lipoproteins, statins, and metalloproteinases.[1,3,28–30] Therefore, it is not unlikely that there is a nonlinear relationship between plaque mRNA levels of *OLR1* and the amounts of sLOX-1 that are shed from the atherosclerotic lesions into the circulation.

Numerous in vitro studies have shown that oxidative as well as inflammatory stimuli increase *OLR1* gene expression in macrophages, endothelial cells, and probably also in SMC.[31–34] In addition, different animal models have confirmed upregulation of *OLR1* expression in the vasculature during inflammatory and oxidative conditions.[30] However, the exploratory foundation of *OLR1* expression in the human vasculature in vivo is rather scarce. Previously, Kataoka and coworkers showed increased *OLR1* gene expression in 8 human plaques as compared with 2 controls by the use of qPCR.[7] Here we extend these finding by showing increased *OLR1* mRNA expression in a much larger cohort of patients with carotid atherosclerosis (n=146), using qPCR. In addition, immunohistochemistry examination confirmed LOX-1 to be present throughout the human atherosclerotic plaque, with particularly strong colocalization to plaque macrophages. Moreover, similar to our data on sLOX-1, we could not detect any transcriptional differences when separating patients with regard to time since onset of symptoms. Thus, our findings support some previous studies,[7,35] suggesting that LOX-1 may be involved in the chronic atherosclerotic process and not only to plaque destabilization with development of subsequent ischemic events.

The present study has some limitations, such as lack of longitudinal data and lack of data on the association between sLOX-1 levels and forthcoming clinical events. Further, the lack of exact data on time between symptom

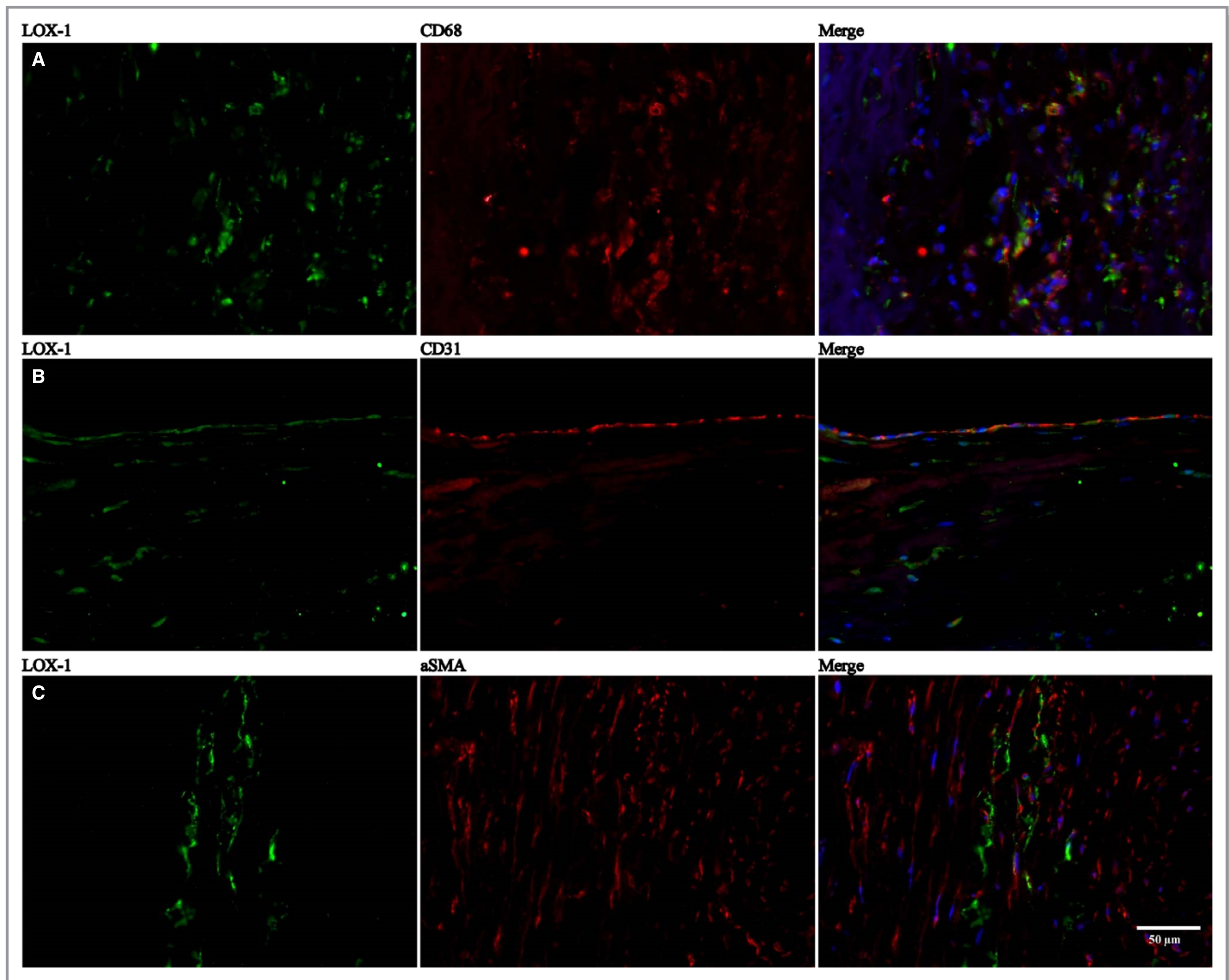

Figure 4. Fluorescence immunohistochemical staining of carotid plaques. Fluorescence immunohistochemical staining demonstrates colocalization between LOX-1 (green) and (A) CD68 (macrophages, red), (B) CD31 (endothelial cells, red), and (C) aSMA (red). LOX-1 indicates lectin-like oxidized low-density lipoprotein receptor 1; aSMA, alpha smooth muscle cells.

onset and blood sampling for each individual patient as well as the use of iliac arteries as nonatherosclerotic controls, a low number of patients in some subgroups of patients such as those with atrial fibrillation, and the imbalanced number of patients and healthy controls represent limitations of the study. Also, our data are purely descriptive and associations do not necessarily imply any causal relationship. However, our findings are based on examination of a rather large cohort of patients with carotid atherosclerosis and include investigation of LOX-1 both systemically and within the atherosclerotic lesion. To further elucidate the clinical use of sLOX-1, future studies should investigate whether sLOX-1 could be a prognostic marker in patients with established carotid atherosclerosis. Forthcoming studies should also examine whether sLOX-1 levels could predict cerebrovascular events or development of carotid atherosclerosis in apparently healthy individuals.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. Acute stroke due to atrial fibrillation (AF)

	Patients **N=22**
Age, (years)*	74.4 (12.7)
Male sex, %(n)	50 (11)
Hypertension, % (n)	54.5 (12)
Diabetes mellitus, % (n)	18.2 (4)
Anti-coagulation, % (n)	54.5 (12)
Aspirin treatment, % (n)	22.7 (5)
Statin treatment, % (n)	27.3 (6)

Data were analyzed using Student`s *t*-test, Mann-Whitney *U* test or chi-square tests and numbers are presented as percentage (numbers) or *mean (SD). BMI; body mass index.

Table S2. Correlations of plasma sLOX-1 with plasma lipids and inflammatory markers

	BMI	HT	TG	LDL-C	HDL-C	Chol	HbA1C	CRP	Leuk
Spearmans' r LOX-1	0.36	0.14	0.25	0.17	-0.08	0.02	0.02	0.01	0.02
value	0.009	0.120	0.023	0.842	0.727	0.842	0.842	0.842	0.842
N	171	220	154	157	165	169	205	209	226

Correlations were calculated by the Spearman's rank correlation test and are presented by Spearman's r and FDR-corrected p values (Benjamini-Hochberg). BMI; body mass index, HT; hypertension, TG; triglycerides, LDL-C; LDL Cholesterol; HDL-C, HDL cholesterol, Chol; total cholesterol, CRP; C-reactive protein, Leuk; plasma leukocyte count.

Table S3. Correlations of plaque OLR1 expression with plasma lipids and inflammatory markers

	BMI	HT	TG	LDL-C	HDL-C	Chol	HbA1C	CRP	Leuk
Spearman's r *OLR1*	0.073	0.03	0.26	0.06	0.225	0.03	0.019	0.13	0.18
P value	0.754	0.870	0.162	0.858	0.162	0.870	0.870	0.324	0.162
N	126	142	64	71	74	77	127	138	143

Correlations were calculated by the Spearman's rank correlation test and are presented by Spearman's r and FDR-corrected p values (Benjamini-Hochberg). BMI; body mass index, HT; hypertension, TG; triglycerides, LDL-C; LDL Cholesterol; HDL-C, HDL cholesterol, Chol; total cholesterol, CRP; C-reactive protein, Leuk; plasma leukocyte count.

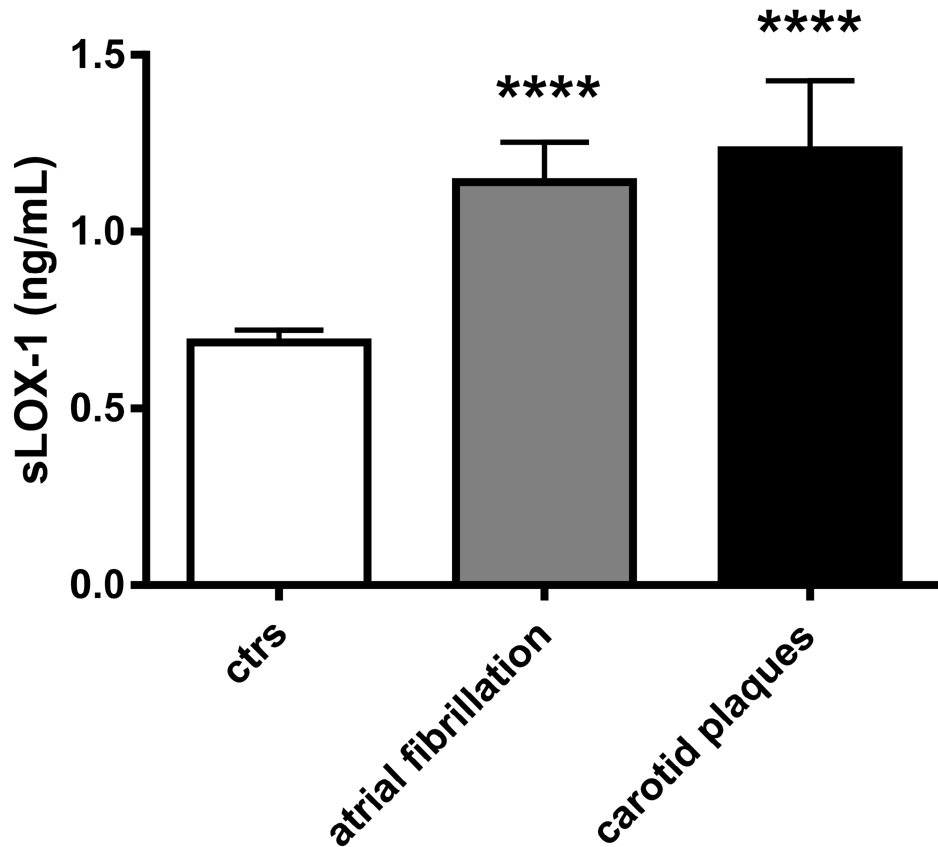

Figure S1. Increased sLOX-1 in acute ischemic event. To investigate sLOX-1 as a specific marker for carotid atherosclerosis stroke, we compared to a patient group with ischemic stroke with reported atrial fibrillation. There was no difference between the two patient groups, and both groups were increased compared to controls.

Ctrs; controls, ****=$p<0.0001$.

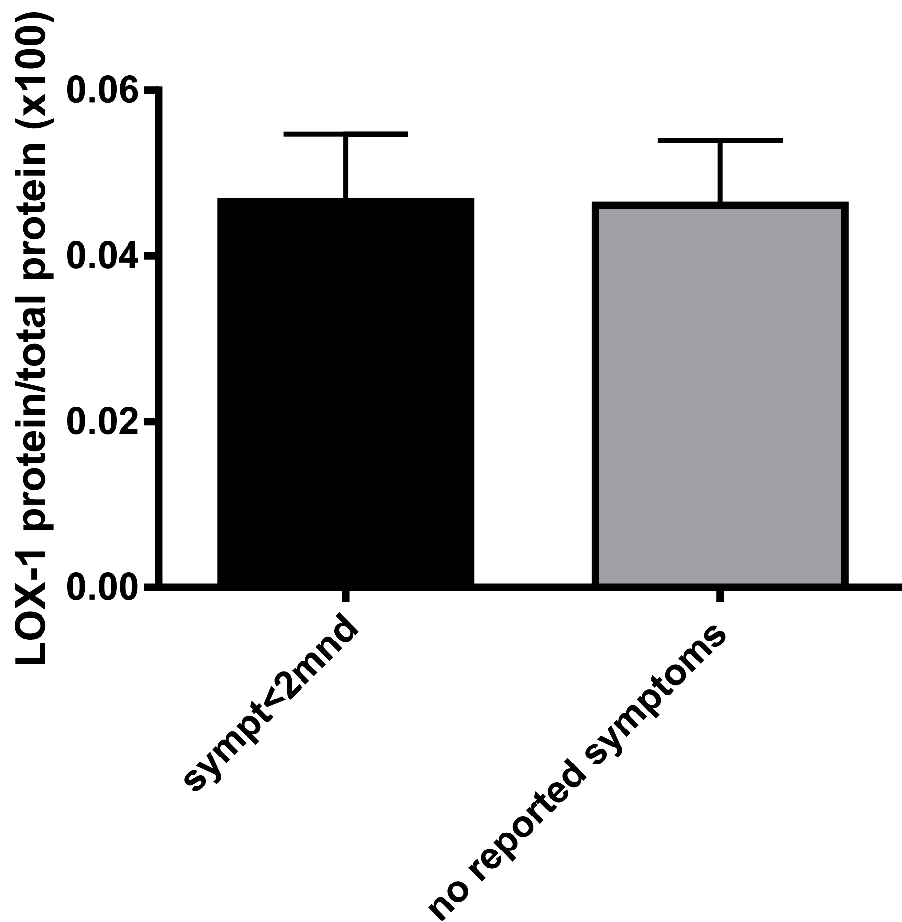

Figure S2 LOX-1 protein levels in carotid atherosclerotic plaque. To verify the LOX-1 protein presence in the atherosclerotic plaque, protein level in plaque lysat was measured with the use LOX-1 ELISA kit. Protein level was detectable but there was no difference between plaques with reported events <2 months and no reported events.