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 for 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 ≤ 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
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. 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 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.

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. 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 2500g 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 qScript 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% H2O2, 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 χ2 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.
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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).

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 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/m²†      | 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, 10⁹/L† | 7.6 (2.5)   | 5.6 (1.2)      | <0.001  |
| Platelets, 10⁹/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  |

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/m²†      | 26.2 (3.8)      |
| Current smoking, % (n) | 40.4 (59)    | 40.4 (59)      |
| Hypertension, % (n) | 65.1 (95)     | 65.1 (95)      |
| Diabetes mellitus, % (n) | 22.6 (33)     | 22.6 (33)      |
| Degree of stenosis, %* | 80 (50–99)  | 80 (50–99)     |
| Echolucent plaque, % (n) | 55.5 (81)    | 55.5 (81)      |
| Aspirin treatment, % (n) | 80.7 (131)   | 80.7 (131)     |
| Statin treatment, % (n) | 92.5 (135)   | 92.5 (135)     |
| CRP, mg/L*       | 8.8 (36.8)      |
| Leukocyte count, 10⁹/L* | 7.8 (2.1)   | 7.8 (2.1)      |
| Platelets, 10⁹/L* | 279 (78)       |
| Total cholesterol, mmol/L* | 4.2 (1.1)  | 4.2 (1.1)      |
| LDL cholesterol, mmol/L* | 2.4 (0.8)  | 2.4 (0.8)      |
| HDL cholesterol, mmol/L* | 1.3 (0.5)  | 1.3 (0.5)      |
| Triglycerides, mmol/L* | 1.5 (1.0)   | 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.

Data were analyzed using Student’s t test, Mann–Whitney U test, or χ² tests and numbers are presented as percentage (numbers) or *median (min–max) 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 macro-phage 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 $\alpha$-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
have reported increased serum levels of sLOX-1 in patients with acute ischemic stroke.\textsuperscript{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,\textsuperscript{6} and sLOX-1 levels have been used in a formula to predict ischemic stroke during follow-up.\textsuperscript{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 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.
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 and appears to be significantly higher in acute coronary syndrome than in stable CAD. sLOX-1 is also associated with coronary in-stent restenosis in patients with stable CAD as well as with increased risk of periprocedural myocardial infarction in stable CAD patients undergoing elective percutaneous coronary intervention. 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 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 ß-ACTIN). SMC indicates smooth muscle cells.
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, neurons, platelets, cardiomyocytes, as well as in renal tissue and lung tissue 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. 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. In addition, different animal models have confirmed upregulation of OLR1 expression in the vasculature during inflammatory and oxidative conditions. 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. Here we extend these finding by showing increased OLR1 mRNA expression in 8 human plaques as compared with 2 controls by the use of qPCR. 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 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 onset.

**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.
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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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 |
|-----------------|-----|----|-----|-------|-------|------|-------|------|------|
| Spearman’s 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

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.

|          | 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 | 0.324 | 0.162 |
| N         | 126  | 142 | 64  | 71    | 74    | 77   | 127   | 138 | 143  |
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.