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**Author:** Beenakker, Jan Willem Maria  
**Title:** Unravelling the collagen network of the arterial wall  
**Date:** 2012-06-05
6 Collagen cross-linking in atherosclerotic lesions

Part of this chapter will be submitted as:

_The collagen cross-linking enzyme lysyl oxidase is associated with a stable phenotype of human atherosclerotic lesions_

6 Atherosclerotic plaques

6.1 Abstract

**Background**  Acute clinical complications of atherosclerosis such as myocardial infarction and ischemic stroke are usually caused by thrombus formation on the ruptured plaque surface. Collagen, the main structural protein of the fibrous cap, provides mechanical strength to the atherosclerotic plaque. The integrity of the fibrous cap therefore is likely to depend on collagen fiber cross-linking, a process controlled by the enzyme lysyl oxidase (LOX). We therefore investigate the LOX expression in human atherosclerosis and its effect on the stiffness of the fibrous cap.

**Methods and results**  We studied atherosclerotic plaques from patients undergoing carotid endarterectomy. LOX protein was detected in areas of the plaque rich in smooth muscle cells (SMC) and collagen; higher LOX mRNA and protein levels were associated with a more stable phenotype of the plaque. LOX mRNA levels in carotid plaques predicted future myocardial infarctions among operated patients. A positive correlation was observed between mRNA levels of LOX and osteoprotegerin (OPG), and negative correlations between LOX mRNA and markers of inflammation. The amount of the mature, LOX-mediated collagen cross-links in plaques correlated positively with serum level of OPG and with the stiffness of the fibrous cap.

**Conclusions**  LOX may contribute to the stabilization of atherosclerotic lesions and to the prevention of its lethal complications. Expression of LOX is negatively correlated with pro-inflammatory stimuli and positively correlated with OPG, suggesting that such mediators may control plaque LOX expression and hence plaque stability.

6.2 Introduction

Acute clinical complications of atherosclerosis such as myocardial infarction and ischemic stroke are caused by the thrombus formation on the plaque surface.\(^1,2\) The most frequent patho-anatomical substrate for sudden arterial thrombosis is a rupture of the fibrous cap that overlies the lipid core of the plaque.\(^1\) Fibrillar collagens types I and III, synthesized by vascular smooth muscle cells (SMC), are abundantly present in the fibrous cap and are an important factor for the biomechanical stability of the plaque.\(^3\) The strength of the fibrous cap depends not only on the amount of collagen, but also on cross-linking generated in the maturation of collagen fibers.\(^4\)
Lysyl oxidase (LOX) is an extracellular copper-dependent enzyme that catalyzes the formation of aldehydes from lysyl and hydroxylsyl residues within the C- and N-terminal telopeptides of collagen fibrils. This reaction leads to the formation of covalent intermolecular mature cross-links, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), which results in the insolubilization and stabilization of extracellular collagen.[5, 6] LOX gene deletion causes fetal death due to defects in the cardiovascular system including aortic aneurysms,[7] illustrating its importance for vascular pathophysiology.

Sites of atherosclerotic plaque rupture are characterized by the presence of activated macrophages, T cells, and mast cells that can influence collagen strength within the fibrous cap by producing pro-inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules.[1] Interferon IFN-γ produced by Th1 cells strongly inhibits the proliferation of vascular SMCs and the production of interstitial collagens and LOX by these cells.[8–10] Other proinflammatory cytokines such as tumor necrosis factor TNF-α induce expression of collagenolytic proteases by activated macrophages and mast cells in plaques.[1, 11]

Atherosclerotic lesions of mice with increased T-cell activation have smaller fibrous caps and display reduced amounts of mature, cross-linked collagen due to inhibition of LOX by cytokines of activated T cells.[12] Interestingly, two risk factors for atherosclerotic heart disease, hypercholesterolemia and hyperhomocysteinemia, can decrease LOX levels in cells.[10, 13] Anti-inflammatory mediators are thought to protect atherosclerotic plaques from rupture by expanding the population of vascular SMCs in the fibrous cap and by augmenting collagen production and LOX-dependent cross-linking of fibers. Transforming growth factor TGF-β, platelet-derived growth factor (PDGF) and statins all increase LOX expression and activity in vascular SMCs.[10] Treatment of Apoe-/- mice with osteoprotegerin (OPG), a decoy receptor for the receptor activator of nuclear factor κB ligand (RANKL), promotes vascular SMC accumulation, LOX-dependent collagen fiber formation and development of fibrous caps.[14]

We hypothesized that LOX-dependent collagen cross-linking plays an important role by stabilizing the atherosclerotic plaque and preventing fibrous cap rupture. Therefore, we investigated LOX expression and its cytokine regulation in human atherosclerosis and its effect on the stiffness of the fibrous cap.

### 6.3 Methods

**Human Specimen Collection** The studies were approved by the respective ethics committees for human studies. Human atherosclerotic plaque tissue was obtained from the Biobank of Karolinska Endarterectomies (BiKE).[15] and from the
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Biobank of St. Petersburg Investigation of Carotid Endarterectomies (SPICE) (table 6.4). Atherosclerotic plaques from carotid arteries were retrieved from patients during carotid endarterectomy, a surgical procedure in which material is removed from the inside of an artery, after providing informed consent. Pieces of carotid arteries were immediately frozen for RNA and protein extraction or embedded in TissueTek optical coherence tomography (OCT) compound (Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) and frozen for immunohistochemistry. 157 cases in BIKE were used for RNA analysis and 24 cases from SPICE for protein analysis.

**RNA analysis**  Total gene expression profiling was performed on 107 RNA samples from BIKE using Affymetrix Gene Array U133 Plus 2.0. All LOX analysis was based on the 215446_s_at probeset, which generated the highest signal values. Results from another probeset (204298_s_at) were similar, with a high correlation of 0.88. RNA from 157 human carotid endarterectomies from the same cohort was analyzed by real-time reverse-transcription polymerase chain reaction (RT-RT-PCR) as described previously.\(^\text{[16]}\) There was an overlap of 77 samples between the microarray and RT-RT-PCR cohort. Primers and probes for human lysyl oxidase and cyclophilin A, a house-keeping gene, were purchased as assays-on-demand (Applied Biosystems). Expression of LOX mRNA as assessed by RT-RT-PCR had a high correlation with the expression of the 215446_s_at probeset (Pearson correlation coefficient, \(r = 0.62\)).

The population of SMCs in the atherosclerotic lesion exhibits extensive phenotypic diversity and plasticity.\(^\text{[17]}\) SMCs in differentiated and dedifferentiated states differ morphologically and functionally, however it is difficult to clearly distinguish two populations based on existing SMC-markers. Therefore, to estimate the content of SMCs in atherosclerotic lesions we calculated the SMC-index that reflects the median of mRNA levels for all SMC-related genes as determined by gene expression array of total plaque mRNA (table 6.5).

**Immunohistochemistry and immunofluorescence**  To localize LOX in human atherosclerotic plaques, series of single immunohistochemical and double immunofluorescent stainings were performed. The primary antibodies that were used included mouse polyclonal antibody generated against a synthetic peptide of human LOX (anti-LOX antibody)\(^\text{[18]}\) (a generous gift from Professor Amato Giaccia, Department of Radiation Oncology, Stanford University, Stanford, CA); rabbit polyclonal antibodies to human von Willebrand factor (Dako), \(\alpha\)-smooth muscle cell-actin (\(\alpha\)sm-actin) (Abcam, Cambridge, UK) and collagen IV (Abcam); mouse monoclonal antibodies to human CD68 (Dako) and human CD163 (Dako); and a
mouse isotype control (Dako, Glostrup, Denmark). Immunohistochemical and fluorescent images were captured with a Leica DC480 color camera and with a Leica SP5x confocal microscope, respectively.

**Western blot analysis**  Active LOX enzyme in 4M urea extracts (5µg of protein per lane) from carotid plaques was visualized by Western blots using anti-LOX antibody\[^{18}\] diluted in 5% defatted dry milk/Tris-buffered saline/0.1% Tween 20. Optical density of the bands was measured using densitometry software, Fujifilm Image Gauge version 3.46.

**Collagen analysis**  Picrosirius red staining was used for the assessment of collagen fibers in lesions.\[^{19, 20}\] Briefly, 10µm formaldehyde-fixed sections of carotid plaques were stained for 1 hour in saturated picric acid containing 0.1% picrosirius red (Direct Red 80, Fluka, Buchs, Switzerland). The color of collagen fibers when identified using Picrosirius Red staining depends upon fiber thickness and packing of collagen fibers; as fiber thickness or alignment increases, the color under polarized light changes from green to yellow and red.\[^{21}\] All sections were analyzed under linear polarized light at magnification 200× in a Leica DRMB microscope and images were captured with a Leica DC480 color camera.

**Collagen cross-link analysis**  Analysis of mature collagen enzymatic cross-links, hydroxyllysylpyridinoline (HP) and lysylpyridinoline (LP)\[^{5}\] and non-enzymatic cross-links, pentosidine, was performed on ten slices (10µm) of frozen sections from individual carotid plaques by reverse-phase HPLC of amino acids and cross-links on a Micropak ODS-80TM column (150mm×4.6mm) (Varian, Palo Alto, CA) as described previously.\[^{22, 23}\] The quantities of the enzymatic mature cross-links (HP+LP) were expressed as the number of residues per collagen molecule, assuming 300 hydroxyprolyl (Hyp) residues per triple helix given that the prolyl hydroxylation level in collagen is stable.

**Enzyme-linked Immunosorbent Assay**  OPG, interleukin (IL)-6 and c-reactive protein (CRP) levels in serum and OPG in plaque tissue were measured with ELISA kits from R&D Systems (Minneapolis, MN). Neopterin was analyzed with an ELISA kit from BRAHMS (Hennigsdorf, Germany) and soluble RANKL with an Elisa from Peprotech (EC Ltd, London, UK) according to the manufacturer’s instructions.

**AFM nano-indentations**  The atomic force microscopy (AFM) nano-indentation experiments are performed as in ref.\[^{23}\]. In short, measurements were done using a Molecular Imaging Picoscan AFM (Agilent Technologies, Palo Alto,
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Figure 6.1:
(a) Histogram showing the distribution of measured Young’s moduli on the fibrous cap of an atherosclerotic plaque. For every location more than 1000 indentations have been performed on a grid of 34×34 points, reflecting the spread in stiffness of the 113μm×113μm probed area. The median of this distribution, 0.98×10²MPa for this measurement, is used as a representative measure for the stiffness of this location.
(b) Lox protein was detected in 4M urea extracts from human carotid plaques by Western blot using mouse anti-human lysyl oxidase antibodies. Representative photograph of a Western blot membrane shows two Lox-specific bands: inactive proenzyme (around 48–50kDa, single arrow) and active Lox enzyme (32kDa, double arrow).

CA) controlled with a custom scripting program written in LabVIEW (National Instruments, Austin, TX) and Visual Basic 6 (Microsoft, Redmond, WA). The nano-indentation was performed with a 2.0N/m cantilever (Olympus omcl type (Olympus, Hamburg, Gemany)) and was recorded with a National Instruments card at 100kS/s. MATLAB (The MathWorks, Natick, MA) was used to calculate the Young’s modulus for each indentation using the fiel method of Hassan et al.\textsuperscript{[24]} This method calculates the work needed for a certain deformation by integrating the force-distance curve. This measure for the stiffness is compared to a modeled curve, calculated using the Hertz model\textsuperscript{[25]} that would be expected for a conical indenter. The spring constant of the cantilever, necessary to relate the cantilever deflection with the applied force, is calibrated with the Sader method.\textsuperscript{[26]}

The assumption of the Hertz model of an isotropic, smooth substrate with a Young’s modulus independent of the applied force, is not met. Biological tissues tend to stiffen when they are deformed,\textsuperscript{[27, 28]} and the many different types of fibers make the sample far from isotropic. However, by keeping the loading rate approximately constant amongst different experiments and using the same force-setpoint, 100±16nN, the calculated values for the stiffness can still be used as a measure of the response of the tissue upon indentation. This “effective Young’s modulus” will reflect the local mechanical properties of the tissue under the set experimental conditions and can be used to compare indentations on different types of tissue.

By performing the indentations on a regular grid, a stiffness map of the tissue,
with a corresponding stiffness distribution, is made. At every location at least 1000 indentations on a grid of $34 \times 34$ points have been performed. For every indentation the effective Young's modulus was calculated. The median of all the stiffness measurements was used as a representative measure for the local stiffness, fig. 6.1a.

**Prediction of secondary events** For each of the bike patients the following two adverse event categories were defined: 1) death, and 2) myocardial infarction. All event types were taken from the Swedish Hospital Discharge Register and the Swedish Cause of Death Register. The retrieval of myocardial infarction incidence data from the Swedish Hospital Discharge Register and the Swedish Cause of Death Register is a reliable, validated alternative to the use of revised hospital discharge and death certificates. The follow-up time for patients free of myocardial infarction was $35 \pm 14$ months for the rt-rt-PCR cohort and $22 \pm 13$ months for the microarray cohort.

**Statistical Analysis** Spearman Rank Order Correlation was used to calculate the correlation coefficient and all correlations were adjusted for multiple comparisons by Bonferroni correction. The Mann-Whitney $U$ test was used for comparisons between 2 groups in in vitro experiments. Values were expressed as mean $\pm$ std. Values of $p < 0.05$ were considered significant. Analysis of follow up data was done using cox-regression as implemented in the survival package from the R programming language.

### 6.4 Results

**Expression of LOX in human atherosclerotic lesions**

We investigated LOX mRNA and protein in perioperatively obtained samples of human carotid plaques. LOX mRNA was highly expressed in human carotid plaques; with microarray data, LOX mRNA was detected among the top 10% of all genes. Using rt-rt-PCR, LOX mRNA was detected at a $ct$ value of 26–30. Using specific anti-LOX antibodies we were able to detect two forms of LOX: the proenzyme (approximately 48–50kDa) and the 32kDa mature enzyme in 4M urea extracts from plaque tissues (fig. 6.1b).

Immunostaining of human atherosclerotic lesions demonstrated prominent LOX expression in the fibrous cap and in the areas surrounding the necrotic core (fig. 6.2a). Expression of LOX was observed either intracellularly within $\alpha$SM-actin-positive $\alpha$SMCs (fig. 6.2b) or extracellularly, where it was localized in areas that
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contained thick mature collagen fibers, predominantly fibrillar collagens type I and III, and collagen type IV (fig. 6.3A–C,E). SMCs in the media of plaque-free vessel walls were generally LOX positive whereas the medial layer under the plaque contained predominantly LOX-negative SMCs (fig. 6.7). LOX protein was also detected in the immediate subendothelial space where it co-localized with collagen IV but not with von Willebrand factor-positive endothelial cells (fig. 6.3D–E). LOX protein co-localized with areas enriched with CD163+ macrophages (fig. 6.2A,E) and partially overlapped with CD68- positive areas (fig. 6.2A,F). However, expression of LOX varied within the lesions and between patients.

LOX is associated with a more stable phenotype of human plaques

Stable atherosclerotic plaque is characterized by an abundance of SMCs and mature fibrillar collagen type I and III in the fibrous cap. Previous studies in the lab of prof. Hansson in atherosclerosis-prone mice revealed that higher LOX mRNA and protein levels associate with better collagen fiber quality, i.e. with a high proportion of thick, cross-linked collagen fibers in lesions. To investigate whether LOX expression was associated with a more stable phenotype of human...
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Figure 6.3:
Double immunofluorescent staining of frozen sections of human carotid plaques with antibodies against LOX (A,C,E) αSM-actin (A), collagen type IV (C,E), and von Willebrand factor (D). LOX is stained red; cell type markers and collagen IV are green; co-localization of red and green results in yellow color. Nuclei are stained blue with DAPI. Original magnification 400× (A–C) and 630× (D,E). (B). Micrograph shows section of human plaque stained with Picosiris red dye for collagen fibers and visualized under polarized light. Original magnification 200×. Scale bars are 50µm. L, lumen.

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<th>Transcript</th>
<th>Correlation, rho</th>
<th>p-value</th>
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<td>0.2</td>
<td>0.02</td>
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<td>Procollagen α1(I)</td>
<td>0.51</td>
<td>&lt;0.001</td>
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<tr>
<td>Procollagen α1(III)</td>
<td>0.53</td>
<td>&lt;0.001</td>
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<td>Procollagen α1(IV)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase, α-subunit (P4HA)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase, β-subunit (P4HB)</td>
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<td>&lt;0.001</td>
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<td>Lysyl-hydroxylase-2 (PLOD2)</td>
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<td>&lt;0.001</td>
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Table 6.1:
Spearman rank correlation, rho, between mRNA levels of LOX and factors involved in collagen biosynthesis as analyzed by gene expression arrays of human carotid atherosclerotic lesions.

atherosclerotic lesions, we examined the correlation between the mRNA levels of LOX and mRNA for several markers of collagen biosynthesis in the human plaques. As shown in table 6.1, LOX mRNA correlated significantly with mRNA for three main procollagen types present in vascular tissue: procollagen type I, III and IV; it also correlated with mRNA for prolyl-4-hydroxylase and lysyl hydroxylase, enzymes that are important for cross-linking of collagen fibrils.[4]
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Figure 6.4:
Correlation between lysyl oxidase and collagen. Semiquantitative analysis of active LOX enzyme in human carotid plaques was performed by measuring optical density of the 32kDa-band. Amounts of collagen and enzymatically induced cross-links (hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP)) of mature collagen, and nonenzymatic pentosidine cross-links were determined in human carotid plaques by reverse-phase HPLC. Active LOX enzyme positively correlated with the percentage of collagen per mg of plaque tissue (left) and with the amount of mature enzymatic collagen cross-links per triple helix (TH) (pmol/pmol) (middle). It is negatively correlated with non-enzymatic cross-links, pentosidine (pmol/pmol) (right). Rho-Spearman rank correlation coefficient.

<table>
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<tr>
<th>Transcript</th>
<th>Correlation, rho</th>
<th>p-value</th>
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<td>OPG</td>
<td>0.6</td>
<td>&lt; 0.001</td>
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<td>CD74</td>
<td>-0.48</td>
<td>&lt; 0.01</td>
</tr>
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<td>HLA-DR</td>
<td>-0.44</td>
<td>&lt; 0.01</td>
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<td>Lipocalin-2</td>
<td>-0.318</td>
<td>&lt; 0.01</td>
</tr>
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<td>CD4</td>
<td>-0.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>RANKL</td>
<td>-0.26</td>
<td>NS</td>
</tr>
<tr>
<td>INF-γ</td>
<td>-0.1</td>
<td>NS</td>
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Table 6.2:
Spearman rank correlation, rho, between mRNA levels of LOX and markers of inflammation analyzed by gene expression arrays of human carotid plaques. NS indicates non significant results.

<table>
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<th>OPG in serum (ng/ml)</th>
<th>OPG in tissue (ng/mg)</th>
<th>SRANKL in serum (pg/ml)</th>
<th>IL6 in serum (pg/ml)</th>
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<tr>
<td>Collagen</td>
<td>0.26</td>
<td>0.43*</td>
<td>0.001</td>
<td>-0.1</td>
</tr>
<tr>
<td>(HP+LP)/TH</td>
<td>0.48*</td>
<td>0.27</td>
<td>0.05</td>
<td>-0.3</td>
</tr>
<tr>
<td>Pentosidine/TH</td>
<td>0.2</td>
<td>0.05</td>
<td>0.11</td>
<td>0.2</td>
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</table>

Table 6.3:
Spearman rank correlation, rho, between systemic markers of inflammation, percentage of collagen (% per mg tissue), and mature collagen cross-links (pmol/pmol) in human carotid plaques. *p < 0.05, TH-triple helix; mature insoluble collagen cross-links are hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).
Fibrogenic cells are the main source of LOX in tissues.\textsuperscript{[32]} Therefore, we assessed whether LOX mRNA levels were dependent on the content of collagen-producing SMCs in plaques. To overcome great phenotypic diversity and plasticity of SMCs in lesions\textsuperscript{[17]} we calculated a SMC index that reflected the median of mRNA levels for all SMC-related genes in vascular tissues (table 6.5). Although LOX mRNA correlated to the SMC index, the relationship was weak, suggesting that any changes of LOX expression in atherosclerotic plaque occur independently of SMC content (table 6.1 and fig. 6.8). We further evaluated whether LOX mRNA levels were associated with the amount of collagen in human atherosclerotic plaques. The amount of active LOX in plaques correlated positively with the amount of collagen and with the amount of mature collagen cross-links but negatively with non-enzymatic advanced glycation endproducts (AGE)-induced cross-links (pentosidine) (fig. 6.4).

**LOX levels correlate negatively to factors associated with a proinflammatory phenotype of human plaques**

To investigate if LOX was associated with vascular inflammation, we examined the correlation between the mRNA levels of LOX and several markers of inflammation in samples of carotid endarterectomies. As shown in table 6.2, LOX mRNA displayed significant positive correlation with OPG transcript levels and correlated negatively with mRNA levels for the MHC class II molecule HLA-DR and its invariant chain CD74, lipocalin-2 and the T-cell receptor associated protein CD4.

We further studied whether the quality of the collagen cap in human carotid plaques was associated with markers of systemic inflammation. The amount of mature collagen cross-links correlated positively with the systemic OPG level, but not with RANKL, IL-6, CRP or neopterin (table 6.3 and data not shown). Tissue OPG levels were associated with higher collagen content in carotid plaques (table 6.3).

**Local LOX levels correlate to local stiffness of the extra cellular matrix**

In a recent study by Hayenga et al., the atomic force microscope (AFM), has been used to show a stiffness difference between calcified and non-calcified regions of plaques in ApoE-/ mice.\textsuperscript{[33]} In this study, we used the AFM to locally measure the stiffness on different locations on the fibrous cap of human patients and to correlate this to the amount of LOX and collagen.

The AFM measurements, summarized in fig. 6.5, show a positive correlation between the LOX concentration and the stiffness of the tissue. On the whole plaque level, we measure a positive correlation between the LOX and collagen concentration, fig. 6.4. The absence of a correlation within the fibrous cap between the sample stiffness and collagen concentration, shows that a local increase in LOX,
which cross-links the collagen, is sufficient to locally increase the stiffness of the tissue.

Local LOX levels were not correlated to historically reported symptoms

The LOX expression level was not associated with any of the reported clinical symptoms, including amaurosis fugax, transient ischemic attack or stroke, registered before the operation. The level of LOX mRNA and active LOX protein were not affected by gender, age, body mass index, plasma cholesterol level, or pharmacological treatment with statins or angiotensin-converting enzyme inhibitors in two studied populations (data not shown).
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Figure 6.6:
Kaplan-Meyer plots for event-free survival from myocardial infarction in patients that had undergone carotid endarterectomy. The incidence of secondary myocardial infarctions in patients was determined using the Swedish Hospital Discharge Register and analyzed separately in patients with LOX mRNA expression above or below median in two biopsy cohorts: the microarray cohort (a) and the rt-RT-PCR cohort (b). Vertical lines indicate censored patients, i.e. the end of the follow-up period was reached without any event. The number of follow-up days after carotid endarterectomy is displayed on the x-axis.

LOX mRNA associated with better future outcome in patients with atherosclerosis

To investigate whether LOX mRNA levels were associated with the future outcome in carotid endarterectomy patients, we analyzed follow-up data for patients in the biopsy study. Data was analyzed from two subsamples of the biopsy cohort and stratified in tertiles by LOX expression. In the microarray subsample (69 patients), we found that higher LOX expression was associated with a significantly lowered incidence of myocardial infarction (cox regression coefficient of $-1.20 \pm 0.30$, $p = 0.024$; patients with a LOX mRNA expression above median have a 1.20 times lower change to suffer from a myocardial infarction compared to the below median group) (fig. 6.6a). The ability to predict myocardial infarction was validated in a larger cohort of 157 samples using rt-RT-PCR measurements. In this cohort, the cox-regression coefficient was $-1.46 \pm 0.745$, $p = 0.049$ (fig. 6.6b). Adjusting for age and gender effects gave similar results. No individual in the patient group with LOX expression above median was diagnosed with myocardial infarction within the follow-up period, while 12 individuals with LOX mRNA level below median suffered from myocardial infarction.

In total 12 deaths were registered in the biopsy cohort during follow-up period: three cases were related to cardiovascular pathology, one patient died of cancer, and the rest were unspecified. Lox transcript levels did not predict future death events.
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6.5 Discussion

The present study provides evidence for an important role of LOX in the stabilization of the human atherosclerotic plaque. Higher LOX mRNA and protein levels were associated positively with markers of atherosclerotic plaque stability, i.e. collagen synthesis and a high proportion of thick, cross-linked collagen fibers, and negatively with pro-inflammatory markers that characterize a vulnerable plaque. We detected LOX protein in regions of the fibrous cap that were rich in collagen and SMCs and in areas surrounding the necrotic core. Moreover, LOX levels were associated with increased synthesis and sufficient maturation of fibrillar collagens type I and III in human carotid lesions. Furthermore, locally the stiffness of the fibrous cap correlated positively with the amount of LOX protein.

The expression of LOX in plaques was associated with clinical events. Although LOX levels were not associated with symptoms of acute brain ischemia reported before the endarterectomy, LOX does seem to be protective in the long-term follow-up. Patients with the highest LOX mRNA had fewer secondary myocardial infarctions. By catalyzing collagen cross-linking and the formation of thick collagen fibers with high tensile strength, LOX may contribute importantly to the mechanical strength of the plaque and counteract the risk for plaque rupture. The lack of association between LOX levels and past symptoms may reflect the natural course of plaque progression. The time between the onset of a symptom and the operation varied from one day to three months, implying that plaques were captured at different phases of the healing process. Recent studies suggest that LOX is expressed by endothelial cells and that LOX expression can be inhibited by several known atherosclerotic risk factors such as TNF, low density lipoproteins and homocystein.\[10\] We could not detect LOX positive endothelial cells overlying human atherosclerotic plaques; however, LOX expression co-localized with basement membrane collagen IV and with SMCs in the immediate subendothelial space. Given that collagen IV is one of the substrates for LOX,\[5\] we assume that such subendothelial distribution of the enzyme helps to stabilize the endothelial layer by maintaining the integrity of the basement membranes. The amount of immunoreactive LOX protein in the subendothelial space varied substantially between different samples, possibly reflecting the degree of endothelial dysfunction.

In the last few years, novel biological functions of LOX have been reported, including the control of cell migration, adhesion and gene regulation.\[6\] It was shown that that LOX can modulate the chemotactic sensitivity of vascular SMCs and monocytes.\[34, 35\] We identified LOX-positive areas in the regions surrounding the necrotic core and in areas rich with α-sm-actin-positive SMCs and CD163+ macrophages. The haptoglobin receptor CD163 is a marker of “alternatively” acti-
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vated (M2) macrophages with profibrotic and anti-inflammatory potential.\cite{36, 37} Therefore, our findings may reflect a role for LOX in the recruitment of SMCs and M2 macrophages to the perinecrotic areas of plaques.

Impaired LOX-dependent collagen maturation has been suggested to play an important role in the pathogenesis of abdominal aortic aneurysms.\cite{7, 38} We observed that the medial layer under the atherosclerotic plaque predominantly contain LOX-negative SMCs. Considering the notion that atherosclerotic plaques grow outwards during progression,\cite{39} we speculate that the loss of LOX from medial SMCs promotes such an abluminal growth of the plaque.

The present findings in human atherosclerosis confirm and extend previous studies in mouse models, which demonstrated that excessive inflammation in atherosclerotic lesions leads to impaired fibrous cap formation by inhibiting LOX expression.\cite{12}

In the human atherosclerotic plaque, LOX mRNA expression correlated negatively to mRNA for several markers of immune activity including CD74, HLA-DR, lipocalin-2 and CD4. CD74 is the invariant chain associated intracellularly with HLA class II molecules and CD4 is the HLA class II co-receptor expressed on T cells. Both molecules are crucial for immune activation and increased in inflamed regions of atherosclerotic plaques.\cite{1, 40, 41} Their association with LOX confirms that local immune activity hampers plaque stability. Lipocalin-2, or neutrophil gelatinase-associated lipocalin (NGAL), is a glycoprotein found in neutrophil granules. It promotes MMP-9 activation and localizes in regions of atherosclerotic plaques with high proteolytic activity.\cite{42} Together, these data imply that inflammation and proteolysis, conditions known to destabilize plaques, are associated with reduced LOX expression. This, in turn, supports the hypothesis that plaque inflammation promotes rupture\cite{2} and suggests that reduction of LOX activity may contribute to the process.

Regions with higher LOX expression are stiffer compared to regions with lower LOX expression, confirming its stabilizing and strengthening effect on the collagen network. Further AFM studies could elucidate the effect of the various inflammatory systems on the collagen network and measure whether the spatial organization of the collagen has an influence on the fibrous cap strength.\cite{23}

OPG is a soluble member of the TNF receptor superfamily and acts as a decoy receptor by inhibiting RANKL ligation.\cite{43} Our recent findings provided evidence for atheroprotective and plaque stabilizing effects of OPG.\cite{14} They suggested that OPG may exert biological effects not only by inhibiting RANK ligation but also by directly affecting collagen synthesis and LOX-dependent collagen maturation within atherosclerotic lesions.\cite{14} Clinical studies show that RANK and its ligand are overexpressed in monocytes and T cells of patients with acute coronary syndromes.\cite{44} Our present data show a positive association between OPG levels
and markers of stability in human atherosclerotic plaques: OPG mRNA levels correlated positively to LOX mRNA expression and the SMC index. Furthermore, we observed significant positive correlations between serum levels of OPG and enzymatic cross-links of mature collagen in the plaques. In light of this, we speculate that OPG may promote plaque stabilization by increasing collagen synthesis and LOX-dependent collagen maturation.

The levels of LOX protein and mature collagen cross-links did not correlate with circulating levels of soluble RANKL or proinflammatory cytokines such as IL-6. In view of the negative correlations between mRNA for LOX and those for a range of proinflammatory mediators in the plaque, it will be important to investigate additional inflammatory molecules for correlation with plaque levels of LOX and collagen in order to identify biomarkers for plaque stability.

Taken together, our present findings support a role for LOX in the stabilization of atherosclerotic plaques. By promoting collagen cross-linking and the formation of thick collagen fibers with high tensile strength, LOX may reduce the risk for plaque rupture and the development of lethal complications of atherosclerosis. The negative correlation between LOX and markers of plaque inflammation and immunity suggest that inflammatory mediators may counteract stabilizing the effects of LOX, possibly by acting directly on LOX expression. Further studies are warranted to evaluate LOX and LOX associated molecules as biomarkers and therapy targets for plaque stability.

6.6 Supplementary methods

**RNA analysis** Total gene expression profiling was performed on 107 RNA samples from BiKE using Affymetrix Gene Array U133 Plus 2.0. Samples for microarray analysis were hybridized and scanned at the Karolinska Institute Affymetrix core facility; obtained cel-files were preprocessed and log2-transformed using rma-normalization.

Tissue samples and cells were lysed with RTL buffer (Qiagen, Valencia, CA). Total RNA was isolated with the Rneasy extraction kit (Qiagen) and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD). mRNA levels were assessed by rt-rt-PCR in a TaqMan universal polymerase chain reaction master mix (Applied Biosystems, Foster City, CA). Primers and probes for human lysyl oxidase and cyclophilin A were purchased as assays-on-demand (Applied Biosystems). Samples were analyzed in duplicates with an Abi Prism 7700 Sequence Detector (Applied Biosystems). Results were normalized to values for human cyclophilin A.
6.6 Supplementary methods

**Immunofluorescence** Acetone-fixed sections were incubated with 5% horse serum followed by a mouse monoclonal anti-human lysyl oxidase antibody\(^{[18]}\) (generous gift from Professor Amato Giaccia, Department of Radiation Oncology, Stanford University, Stanford, CA) or an isotype control (Dako, Glostrup, Denmark) and a polyclonal rabbit anti-human von Willebrand factor (Dako), rabbit polyclonal anti-human α-smooth muscle cell-actin (αsm-actin) (Abcam, Cambridge, UK) or rabbit polyclonal anti-human collagen IV (Abcam). Binding was detected with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) followed by a Texas Red streptavidin conjugate (Invitrogen, Carlsbad, CA) or a Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen). Lipid autofluorescence was blocked with 0.03% Sudan black B (Sigma Aldrich) in 70% alcohol. Nuclei were visualized with DAPI (Sigma Aldrich), and images were viewed in a Leica fluorescence microscope and captured with a confocal microscope Leica DMI.

**Immunohistochemistry** 10μm acetone-fixed sections of carotid plaques were stained by mouse monoclonal anti-human lysyl oxidase antibody\(^{[18]}\) or an isotype control (Dako); monoclonal mouse anti-human CD68 (Dako); monoclonal mouse anti-human CD163 (Dako). Antibody binding was detected using ImmPress reagent kit/Peroxidase and visualized with Vector NovoRed substrate kit (Vector laboratories).

**4M urea LOX extraction and western blot analysis** The active LOX protein is insoluble in neutral saline which likely reflects its tight association with its substrates in the extracellular space, but the enzyme can be rapidly solubilized by buffers supplemented with 4 to 6M urea.\(^{[32]}\) Therefore, multiple steps extraction was carried out at 0–4°C similar to the one described previously.\(^{[45]}\) Frozen samples of carotid plaques were cut into small pieces and homogenized for 2 min in 0.15M-NaCl/0.016M-potassium phosphate, pH 7.7, at a ratio of 4 ml of buffer/g of ground tissue. The homogenate was incubated in ice for 30 min and centrifuged at 15000 g for 10 min, and the pellet was extracted once more with the new buffer. The two buffered salt extracts were discarded. The resulting pellet obtained from the salt extractions was further extracted with 0.016M-potassium phosphate, and then with 4M-urea in this phosphate buffer. The urea-soluble material was decanted and kept and the pellet was extracted once more with 4M-urea/0.016M phosphate. The two urea extracts were pooled and protein concentration was determined by bicinchoninic acid (BCA) protein assays (Thermo Fisher Scientific, Rockford, USA) with bovine serum albumin as a standard.

4M urea extracts from carotid plaques (5μg of protein per lane) were sepa-
rated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and blotted to hybridization transfer membrane Hybond-P (Amersham, Little Chalfont, UK). Blots were blocked and incubated with primary antibodies (mouse monoclonal anti-human lysyl oxidase antibody\(^{18}\) and appropriate horseradish peroxidase-conjugated secondary antibodies diluted in 5% defatted dry milk/Tris-buffered saline/0.1% Tween 20). Immunoreactive proteins were visualized by the ECL Western blotting analysis system (Amersham). Optical density of the bands was measured using densitometry software Fujifilm Image Gauge version 3.46.

Figure 6.7:
Double immunofluorescent staining of frozen sections of human carotid plaques stained with antibodies against LOX (red) and \(\alpha\text{-sm}-\text{actin} \) (green); co-localization of red and green results in yellow; nuclei are stained blue with DAPI. LOX-positive \(\alpha\text{-smcs} \) dominate in plaque-free media (B), and LOX-negative \(\alpha\text{-smcs} \) are observed in the media under the plaque (B). Original magnification 400×

Figure 6.8:
Correlation between \(\alpha\text{-smcs} \) index and mRNA levels of LOX or OPG analyzed by gene expression arrays of human carotid plaques. Rho-Spearman rank correlation coefficient.
6.6 Supplementary methods

Values

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BIKEx</th>
<th>SPICE</th>
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<tbody>
<tr>
<td>Number of patines</td>
<td>107</td>
<td>106</td>
</tr>
<tr>
<td>Male/female</td>
<td>76.1% / 23.9%</td>
<td>79.2 % / 20.8%</td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>71.37 ± 8.61</td>
<td>62.6 ± 8.0</td>
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**Pharmacological treatment:**
- Antiplatelet agents: 91.3% (79.5% (n = 79))
- Statins: 78.2% (32.9% (n = 79))
- Angiotensin converting enzyme inhibitors: 25.4% (59.5% (n = 79))
- β-blockers: 46.4% (49.4% (n = 79))

Table 6.4: General characterization of patients from the biobanks BIKEx and SPICE.

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<tr>
<th>Gene name</th>
<th>Transcript acc. nr.</th>
<th>Probe set ID</th>
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<tr>
<td>kruppel-like factor 5 (intestinal)</td>
<td>NM_001730</td>
<td>209211_at</td>
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<tr>
<td>kruppel-like factor 5 (intestinal)</td>
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<td>1568574_x_at</td>
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<tr>
<td>osteopontin</td>
<td>NM_000582</td>
<td>209875_s_at</td>
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<tr>
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Table 6.5: smc-related genes that were analyzed by gene expression array of human carotid plaques and used to calculate smc index. Transcript accession numbers are taken out of the ncbi database; Probe set id corresponds to the Affymetrix Gene Array u133 Plus 2.0.
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### 6.7 Additional measurements on plaques of the abdominal aorta

The disease of atherosclerosis affects all vascular beds including coronary, carotid, aorta and peripheral arteries.\[^{[47]}\] Of these primary locations, surprisingly little is known about how atherosclerosis progresses in the aorta, the second largest manifestation of this disease.\[^{[48–52]}\] This lack of information is remarkable, especially considering the fact that the aorta is generally used in rodent studies of atherosclerotic disease. A recent cardiovascular MRI study showed, that during the progression of atherosclerosis, the vessel wall area significantly increased for both the carotid artery and abdominal aorta (table 6.6).\[^{[46]}\] The lumen area, however, did not change significantly for carotid arteries, but did significantly increase for the abdominal aorta. This difference in vascular remodeling could be the result of the difference in stiffness of the surrounding tissue, but could also have a more complicated origin. Furthermore, these measurements show a difference in plaque progression between different vascular beds.

In a large systematic histopathological study of 260 aortic samples, van Dijk et al. describe the development and progression of atherosclerosis in the abdominal aorta.\[^{[53]}\] The results of this study, summarized in fig. 6.9, show how the initially thick fibrous cap thins and eventually ruptures. The mechanism of cap weakening and thinning is currently a controversial issue. Macrophages are thought to thin the cap by secreting enzymes that degrade the extracellular matrix.\[^{[3, 54–57]}\] During the progression of the disease, the number of collagen producing smooth muscle cells declines, halting the production of collagen. Whether the primary cause of plaque thinning lies at the production or degradation side or both is a current issue of discussion.\[^{[54]}\] Another line of theories on cap weakening is based on the mechanical aspects of plaques, e.g., the blood exerts a pulsed pressure on the cap which could weaken the cap.\[^{[58]}\] A study by Bruke et al. provided evidence that silent plaque rupture could be a form of wound healing,\[^{[59]}\] implying

<table>
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<th>Δ/year in %</th>
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<td><strong>Carotid artery</strong></td>
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<tr>
<td>Lumen</td>
<td>31.18 ± 8.98</td>
<td>−1.23 ± 12.34</td>
</tr>
<tr>
<td>Wall</td>
<td>25 ± 9.61</td>
<td>8.67 ± 35.54</td>
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<table>
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<tr>
<th></th>
<th>Baseline (mm(^2))</th>
<th>Δ/year in %</th>
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</thead>
<tbody>
<tr>
<td><strong>Abdominal aorta</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>259.63 ± 85.89</td>
<td>4.97 ± 15.86</td>
</tr>
<tr>
<td>Wall</td>
<td>103.48 ± 33.72</td>
<td>13.24 ± 17.36</td>
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Table 6.6: Annual progression rate of atherosclerosis and change in lumen size per year for the carotid artery (\(n = 23\)) and abdominal aorta (\(n = 15\)).\[^{[46]}\] The wall area increased significantly for both the carotid artery and the abdominal aorta. The lumen area significantly increased for the abdominal aorta, but did not change significantly for the carotid arteries.
that plaque thinning and rupture could sometimes also be beneficial.

In the remaining of this chapter, we show some preliminary data on atherosclerotic plaques of the abdominal aorta. Although the results of these measurements still needs to be verified on a larger number of samples, they do demonstrate how the AFM, in combination with multi-photon microscopy, can become a valuable tool to study the progression of atherosclerosis.

The ability of the AFM to measure spatial variations of the stiffness within an atherosclerotic plaque, could yield important information on the weakening of the collagen cap. Finite element simulations on the rupture of carotid plaques showed that calculated maximum plaque stresses were found to be significantly higher in patients with acute symptoms than those in recently symptomatic patients. These models can however be improved significantly if the local physical properties of these plaques are known.

Our preliminary AFM measurements show, fig. 6.10a, a ten-fold difference in
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Figure 6.10:
AFM measurements on atherosclerotic plaques of the abdominal aorta.
(a) Stiffness measurements on the middle and the shoulder of the fibrous cap of the same plaque show a ten-fold difference in stiffness, suggesting the middle of cap is weaker than the shoulder.
(b) Stiffness measurements made with a ball tip at various distances from the necrotic core show how the tissue gets weaker near the necrotic core.

the stiffness between the middle of the plaque and the plaque shoulder. These measurements seem to show that the mechanism for plaque rupture in the abdominal aorta is different than in the coronary arteries, where they tend to rupture in shoulder regions of the plaque.\textsuperscript{[62, 63]} This difference could be the result of the difference in pressure and flow profiles between these arteries.

Multi-photon microscopy images of the collagen structure of a starting plaque, fig. 6.11a, show a thick barrier of multiple parallel collagen sheets, which preventing the contents of the necrotic core from entering the blood. Similar images of an older and thinner cap, fig. 6.11b, show how the collagen structure is disrupted. This damage is probably caused by the many collagen degrading enzymes of the necrotic core.\textsuperscript{[3, 54]} This hypothesis is strengthened by our AFM measurements which, by probing the stiffness as a function of the distance to the necrotic core, fig. 6.10b, showed a significant weakening of the tissue near the necrotic core.


**6.8 Acknowledgments**

We thank Drs Vladimir V. Shlomin, Michail L. Gordeev, Vera A. Ovchinnikova and Olga M. Moiseeva for the help during collection of carotid plaque; Ingrid Törnberg and Anneli Olsson for technical assistance. Janneke Ravensbergen, Annemieke v/d Zalm and Rogier van Dijk for their help with the plaques of the abdominal aorta.

**6.9 References**


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