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Immune mechanisms behind plaque vulnerability: experimental and clinical studies

Olga Ovchinnikova

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From the Experimental Cardiovascular Research Unit,
Department of Medicine and Center for Molecular Medicine,
Karolinska University Hospital,
Karolinska Institutet, Stockholm, Sweden

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To my family and friends

and to all others who made me who I am

Beroende av andra människor, inte friheten från dem, är vad som gör oss till människor: beroende av och tacksamheten mot andra människor.

Göran Rosenberg

(***Eng***: Dependence on other people, not freedom from them, is what makes us human: dependence on and gratitude towards others)

(***Rus***: Зависимость от других людей, а не свобода от них - вот то, что делает нас людьми: зависимость и благодарность к людям)

ABSTRACT

Physical disruption of atherosclerotic plaques causes many acute thrombotic complications such as myocardial infarction and stroke. The resistance of the atherosclerotic plaque to disruption depends in part on the integrity of its fibrous cap, which prevents contact between the highly thrombogenic lipid core and the circulating blood. The fibrillar collagens types I and III synthesized by smooth muscle cells (SMCs) largely determine the tensile strength of the cap.

Sites of plaque rupture display signs of active inflammation that can impair plaque stability. Macrophages and mast cells release a set of collagen-degrading enzymes. Additional possible mechanisms include inhibited expression of procollagen genes and induction of death or reduced renewal of the collagen-producing SMC population, both phenomena promoted by T cell-derived interferon-γ (IFNγ). However, little attention has been given to the post-translational modification of collagen fibers in the fibrous caps. It is known that efficient extracellular cross-linking of collagen catalyzed by the enzyme lysyl oxidase (LOX) confers biomechanical properties and proteolytic resistance of the mature collagen fiber. Thus, failure of collagen maturation may lead to a defective extracellular matrix in the fibrous cap.

Using atherosclerosis-prone mice and samples of human carotid endarterectomies, we investigated whether pro- and anti-inflammatory mediators can affect the LOX-dependent collagen maturation in atherosclerotic lesions, thus leading to plaque weakening.

To study the effect of T cell-driven inflammation, we used genetically modified mice with hypercholesterolemia and disrupted TGFβ signaling in T cells (*Apoe-/-* x CD4dnTβRII). These mice developed larger atherosclerotic lesions with augmented levels of IFNγ, increased numbers of activated macrophages and, importantly, impaired maturation of collagen fibers, consistent with a vulnerable phenotype (Paper I). Analysis of mRNA and protein content showed a significant decrease of LOX in aortae of *Apoe-/-* x CD4dnTβRII mice. T cell-driven inflammation in these mice provoked a limited selective increase in the expression of proteinases that degrade the extracellular matrix, but no increase in collagen fragmentation was detected. Therefore, we concluded that exaggerated T cell-driven inflammation limits the extracellular maturation of collagen in the atherosclerotic plaque.

The stability of atherosclerotic lesions was investigated in *Apoe-/-* mice after treatment with osteoprotegerin (OPG), a cytokine of the TNFR superfamily and a circulating decoy receptor for the receptor activator of nuclear factor κB ligand (RANKL) (Paper II). Treatment with OPG facilitated accumulation of SMCs and increased formation of mature collagen fibers within the lesions of *Apoe-/-* mice. Aortic mRNA level of LOX was also upregulated in treated animals. In cell culture studies, OPG promoted proliferation of rat aortic SMCs. Therefore, we suggested that osteoprotegerin may be a possible mediator of lesion stabilization.

We further investigated if a similar pattern as that obtained in the animal experiments could also be found in the human disease (Paper III). We were able to detect LOX protein in SMC- and collagen-rich areas of human carotid lesions. A higher LOX mRNA and protein were associated with a more stable phenotype of the plaques. Examination of gene expression in plaques revealed a positive correlation between mRNA expression of LOX and mRNA for OPG, and a negative correlation between LOX mRNA and markers of inflammation. This data suggests that LOX may contribute to the stabilization of human atherosclerotic lesions and that its expression is controlled by inflammation.

In paper IV we reported that mRNA and protein content of 5-lipoxygenase activating protein (FLAP) were highly upregulated in aortae of *Apoe-/-* x CD4dnTβRII mice compared with *Apoe-/-* littermates. FLAP immunoreactive protein co-localized with CD68+ macrophages. Augmented *ex vivo* formation of leukotriene B4 in aortae of transgenic mice further supported functional significance of the increased level of FLAP. Treatment with the FLAP-inhibitor MK-886 not only decreased the number of CD3+ cells in lesions and IFNγ mRNA levels in aortae of *Apoe-/-* x CD4dnTβRII mice, but, most importantly, significantly reduced atherosclerotic lesion size. Although FLAP inhibition did not have any significant effect on collagen synthesis, it can be considered as a possible therapeutic tool to stabilize the plaque by reducing the degree of local inflammation.

In summary, the findings of this thesis identify extracellular maturation of collagen, catalyzed by LOX, as important in maintaining the stability of the fibrous cap in the atherosclerotic lesion. The process of collagen maturation is regulated by pro- and anti-inflammatory mediators within the plaque, and it may serve as a target for development of new diagnostic and therapeutic tools.

CONTENTS

1 LIST OF PUBLICATIONS

This thesis is based on the following original studies which will be referred to by their Roman numerals.

I. **Ovchinnikova O**, Robertson AK, Wågsäter D, Folco EJ, Hyry M, Myllyharju J, Eriksson P, Libby P, Hansson GK. T cell activation leads to reduced collagen maturation in atherosclerotic plaques of Apoe(-/-) mice. Am J Pathol. 2009 Feb;174(2):693-700.

II. **Ovchinnikova O**, Gylfe A, Bailey L, Nordström A, Rudling M, Jung C, Bergström S, Waldenström A, Hansson GK, Nordström P. Osteoprotegerin Promotes Fibrous Cap Formation in Atherosclerotic Lesions of ApoE-Deficient Mice. Arterioscler Thromb Vasc Biol. 2009 Oct;29(10):1478-80b

III. **Ovchinnikova O**, Folkersen L, Lindeman JHN, Ueland T, Aukrust P, Hedin U, Gavrisheva NA, Shlyakhto EV, Olofsson P, Hansson GK. The collagen cross-linking enzyme lysyl oxidase is associated with a stable phenotype of human atherosclerotic lesions. *Manuscript 2010*

IV. Bäck M, Sultan A, **Ovchinnikova O**, Hansson GK. 5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation. Circ Res. 2007 Apr 13;100(7):946-9

Note: In paper II, the two first authors contributed equally and the two last authors share senior authorship.

2 LIST OF ABBREVIATIONS

Apoe-/- mice	Apolipoprotein E knockout mice
AGE	Advanced glycation endproducts
αSM-actin	α-actin of smooth muscle cells
BLT receptor	Receptor for leukotriene B4
CysLT	Receptor for cysteinyl leukotrienes
DDR	Discoidin domain receptor
EC №	Enzyme Commission number
FLAP	5-lipoxygenase activating protein
Hsp47	Heat shock protein 47
HP	Hydroxylysylpyridinoline
Hyl	Hydroxylysyl residues
IFNγ	Interferon-γ
IL	Interleukin
LDL	Low density lipoproteins
Ldlr-/- mice	Low density lipoproteins receptor knockout mice
LH	Lysyl hydroxylase
5-LO	5-lipoxygenase
LOX	Lysyl oxidase
LP	Lysylpyridinoline
LT	Leukotriene
Lys	Lysyl residues
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
OPG	Osteoprotegerin
P4H	Prolyl-4-hydroxylase
PDGF	Platelet-derived growth factor
PDI	Protein disulfide isomerase
PLOD	Procollagen-lysine 2-oxoglutarate 5-dioxygenase (LH)
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RT-PCR	Reverse-transcription polymerase chain reaction
SMCs	Smooth muscle cells
TGFβ	Transforming growth factor-β
TIMP	Tissue inhibitor of MMPs
TNFR-SF	Tumor necrosis factor receptor superfamily
TNF-SF	Tumor necrosis factor superfamily

Ambition:
The world makes way for those who know
where they are going

3 INTRODUCTION

Cardiovascular diseases and specifically atherosclerosis are among the main causes of death globally [1, 2]. Atherosclerosis is responsible for ischemic heart disease, ischemic stroke and critical limb ischemia. An atherosclerotic plaque causes progressive luminal narrowing and results in stable clinical manifestations of atherosclerosis. On the other hand, thrombus formation on the plaque surface leads to acute and lethal clinical manifestations of atherosclerosis such as unstable angina, myocardial infarction and stroke. The incidence of atherosclerotic complications increases globally forcing scientists to look for new strategies for prediction, prevention, and treatment [3].

An atherosclerotic lesion (atheromata) is an eccentric focal thickening of the intima, the innermost layer of the artery [1]. It results from a complex interaction between blood elements, disturbed flow and vessel wall abnormalities. Consecutive pathological processes in the intima lead to plaque growth initiation, progression and complications. Such processes include (a) lipid retention and accumulation in the subendothelial space; (b) inflammation with increased endothelial permeability, endothelial activation and immune cell recruitment; (c) growth with smooth muscle cell (SMCs) proliferation, migration and matrix synthesis; (d) degeneration and necrosis with debris accumulation; (e) calcification; and (f) thrombosis with platelet recruitment and fibrin formation [1].

Mature atherosclerotic plaques (types IV and Va according to the classification of American Heart Association [4]) typically consist of two main components: a soft lipid core and a fibrous cap which separates the core from the lumen [5]. The stability of the atherosclerotic plaque and its resistance to disruption depend in part on the integrity of the fibrous cap that is composed of SMCs and a collagen-rich extracellular matrix (Figure 1). An inverse relationship has been demonstrated between cap thickness and peak circumferential stress in the plaque [6]. The extracellular matrix content, and particularly fibrillar collagens types I and III synthesized by SMCs, usually determine the stability and strength of tissues, including arteries. Collagen can tolerate much greater tensile stress than elastin [7]. Therefore, collagen-poor fibrous caps are more fragile.

The most frequent pathoanatomical substrate for sudden arterial thrombosis is a physical disruption of the fibrous cap and exposure of the highly thrombogenic lipid core to factors of blood coagulation [5, 8]. Pathologists have identified a number of characteristics of atherosclerotic plaques that caused fatal thrombi. These unstable plaques, contain large lipid cores and thin fibrous caps (<65μm), and are infiltrated with activated immune cells such as macrophages and T cells [1, 9]. Inflammatory cells can influence the function of SMCs and collagen strength within the fibrous cap by producing pro-inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules [1]. Loss of SMCs and altered collagen metabolism can lead to thinning of the collagen-rich fibrous cap and hence to its destruction [9].

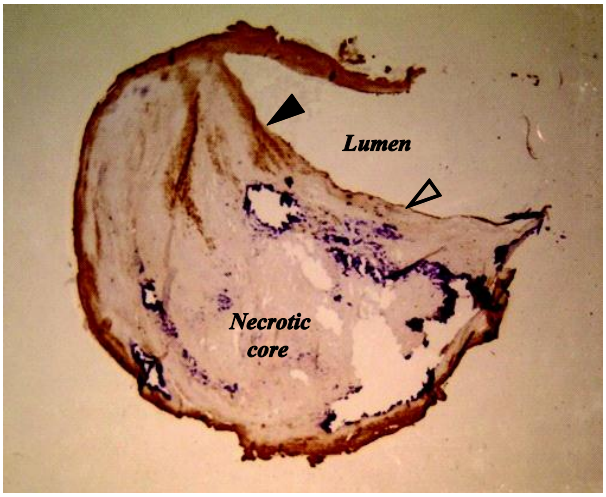

Figure 1. Human carotid plaque stained for α-actin positive SMCs. The fibrous cap is denoted by head arrows: filled arrow head – SMC-rich region; empty arrow head – SMC-poor region of the cap. Original magnification x10.

Ample scientific literature of the past two decades strongly suggests that a disbalance between collagen synthesis and degradation is the main cause of collagen loss from the fibrous cap [1, 9]. Based on numerous *in vitro* and *in vivo* studies, it is now believed that collagen gene expression can be hampered by cytokines that are produced in

activated T cells [10, 11], whereas mature collagen fibers in the fibrous cap can be degraded by macrophage-derived proteolytic enzymes [7, 9, 12].

Little attention has been paid to changes in the maturation of collagen in determining plaque stability. The tensile strength of collagen and its resistance to proteolytic enzymes largely depend on the efficiency of intramolecular and intermolecular cross-linking generated in a complex multistep process of collagen maturation [13]. However, it is still unclear if fragile plaques have a low concentration of mature collagen cross-links.

Therefore, we focused our investigations on the mechanisms involved in collagen maturation within the atherosclerotic lesion and on the effect of pro- and anti-inflammatory stimuli on the efficiency of collagen cross-linking in the fibrous cap.

4 COLLAGENS IN ATHEROSCLEROSIS

Members of the collagen family are the most abundant proteins in the extracellular matrix and are the major structural elements of all connective tissues. Collagens contribute to stability and maintain structural integrity of tissues and organs including the vasculature.

4.1 COLLAGEN TYPES AND THEIR BASIC STRUCTURE

28 genetically distinct collagen types have been described so far. They are divided into several groups based on their structure and supramolecular organization (Table 1) [13, 14]. Fibril-forming (fibrillar) collagens are the most abundant and wide-spread family. Their torsional stability and tensile strength provide mechanical stability of tissues.

All members of the collagen family have one characteristic feature in common: they are composed of three α-chains organized in a right-handed triple helix (Figure 2). The triple helix can be formed by three identical chains (homotrimers) as in collagens type II, III, VII, and X, or by two or more different chains (heterotrimers) as in collagens type I, IV, V, VI, IX, and XI. A structural basis for the triple helical assembly is a presence of glycine, the smallest amino acid, in every third position of the polypeptide chain – $(Gly\text{-}X\text{-}Y)_n$. The α-chains assemble so that all glycyl residues are positioned in the center of the triple helix (Figure 2). This configuration allows a close packing along the central axis of the triple helix [13]. In the triplet, X is often a proline, and Y is frequently a hydroxyproline (Figure 2).

In the intracellular space the procollagen monomer is flanked by N- and C-propeptides that have important functions in procollagen processing. The C-propeptide plays a fundamental role in the initiation of triple helix formation, and the N-propeptide is thought to be involved in the regulation of the primary fibril diameter (Figure 2).

The processed collagens consist of a central triple helical region (collagenous domain) and two non-helical regions called telopeptides at the N- and C-terminal. Triple helical regions form domains of 300 nm in length (about 1000 amino acids) as in fibril-forming collagen, or contain much shorter domains alternating with non-triple helical interruptions like in other collagen types [13, 14].

Table 1. The various collagen types and respective major collagen families (modified after [13]).

Type	Molecular composition	Genes (genomic localization)	Tissue distribution
Fibril-forming (fibrillar) collagens			
I	$[\alpha1(I)]_2\ \alpha2(I)$	COL1A1 (17q21.31-q22) COL1A2 (7q22.1)	Bone, dermis, vessel wall, tendon, ligaments, cornea
II	$[\alpha1(II)]_3$	COL2A1 (12q13.11-q13.2)	Cartilage, vitreous body, nucleus pulposus
III	$[\alpha1(III)]_3$	COL3A1 (2q31)	Skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen, etc)
V	$\alpha1(V),\alpha2(V),\ \alpha3(V)$	COL5A1 (9q34.2-q34.3) COL5A2(2q31) COL5A3 (19p13.2)	Lungs, cornea, bone, fetal membranes; together with collagen type I
XI	$\alpha1(XI),\alpha2(XI),\ \alpha3(XI)$	COL11A1(1p21) COL11A2 (6p21.3) COL11A3 = COL2A1	Cartilage, vitreous body
Basement membrane collagens			
IV	$[\alpha1(IV)]_2\ \alpha2(IV);\ \alpha1\text{-}\alpha6$	COL4A1 (13q34) COL4A2 (13q34) COL4A3 (2q36-q37) COL4A4 (2q36-q37) COL4A5 (Xq22.3) COL4A6 (Xq22.3)	Basement membranes
Microfibrillar collagens			
VI	$\alpha1(VI),\alpha2(VI),\ \alpha3(VI)$	COL6A1 (21q22.3) COL6A2 (21q22.3) COL6A3 (2q37)	Widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils			
VII	$[\alpha1(VII)]_3$	COL7A1 (3p21.3)	Skin, dermal-epidermal junctions; oral mucosa, cervix
Hexagonal network-forming collagens			
VIII	$[\alpha1(VIII)]_2\ \alpha2(VIII)$	COL8A1 (3q12-q13.1) COL8A2 (1p34.3-p32.3)	Vessel wall, Descemet's membrane
X	$[\alpha1(X)]_3$	COL10A1 (6q21-q22.3)	Hypertrophic cartilage
FACIT collagens			
IX	$\alpha1(IX),\alpha2(IX),\ \alpha3(IX)$	COL9A1 (6q13) COL9A2 (1p33-p32.2)	Cartilage, vitreous humor, cornea
XII	$[\alpha1(XII)]_3$	COL12A1 (6q12-q13)	Perichondrium, ligaments, tendon
XIV	$[\alpha1(XIV)]_3$	COL14A1 (8q23)	Dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$[\alpha1(XIX)]_3$	COL19A1 (6q12-q14)	Human rhabdomyosarcoma
XX	$[\alpha1(XX)]_3$	COL20A1 (20q13.33)	Corneal epithelium, embryonic skin, sterna cartilage, tendon
XXI	$[\alpha1(XXI)]_3$	COL21A1 (6q12.3-11.2)	Vessel wall
Transmembrane collagens			
XIII	$[\alpha1(XIII)]_3$	COL13A1 (10q22)	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$[\alpha1(XVII)]_3$	COL17A1 (10q24.3)	Dermal – epidermal junctions
Multiplexins			
XV	$[\alpha1(XV)]_3$	COL15A1 (9q21-q22)	Fibroblasts, SMCs, kidney, pancreas
XVI	$[\alpha1(XVI)]_3$	COL16A1 (1p34)	Fibroblasts, amnion, keratinocytes
XVIII	$[\alpha1(XVIII)]_3$	COL18A1 (21q22.3)	Lungs, liver

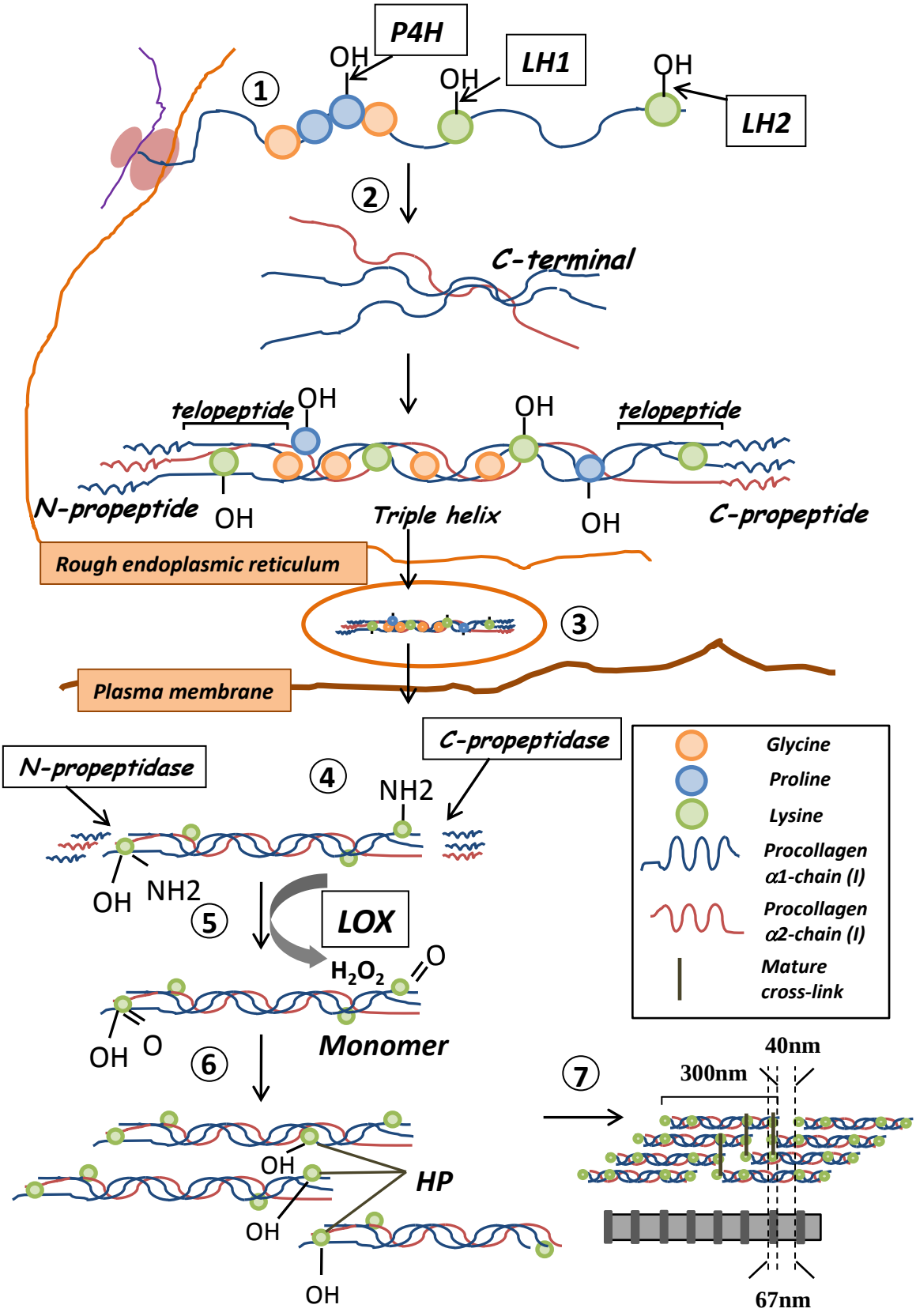

Figure 2. Schematic presentation of collagen type I structure and synthesis. (1), collagen translation in the rough endoplasmic reticulum and hydroxylation of prolyl and lysyl residues with prolyl-4-hydroxylase (P4H) and lysyl hydroxylase (LH); (2), triple helix assembly from C-terminal; (3), transport in vesicles and secretion; (4), cleavage of C- and N-propeptides; (5), oxidation of lysyl and hydroxylysyl residues in telopeptides; (6), Amadori rearrangement of immature links and formation of mature non-reducible cross-links, HP – hydroxylysylpyridinoline (as an example); (7), organization of collagen fibrils in the characteristic quarter-staggered manner and the scheme of "banded fibrils" appearance of collagen on electron micrographs.

Collagen type IV with a flexible triple helix makes a three-dimensional structure to form basement membranes. Collagenous domains of collagen type VI yield the ultrastructural appearance of beads on a string through formation of dimers and tetramers [13, 14].

The short non-helical telopeptides of the processed collagen monomers are involved in the covalent cross-linking of fibrils in the extracellular space. They also help linking collagen to other molecular structures of the surrounding matrix [13, 14]. In the extracellular space, collagen fibrils are aligned and assembled in the generally accepted quarter-stagger model [15]. On electron micrographs, fibrils appear as an alternating light and dark pattern, which gives them a name "banded fibrils" (Figure 2) [14].

4.2 COLLAGEN LOCALIZATION IN ATHEROSCLEROTIC LESIONS

Collagen is the major extracellular component of atherosclerotic plaques. Collagen type I and III together represent approximately 60% of the total protein content and at least 90% of the total collagen content of the plaque [16-18]. Other collagens frequently detected in the atherosclerotic lesion are collagen type IV, V, VI and VIII [17, 19].

The localization of collagens in atherosclerotic plaques varies at different stages of the disease [17]. Collagens type I and III are diffusely co-distributed in the thickened intima at all stages during plaque progression [16, 17, 20]. Their mRNA expression is significantly increased in atherosclerotic plaques compared with underlying media or intima of the normal artery both in humans and in animals [21-24]. In the advanced and complicated lesions, collagens type I and III are mostly present in the fibrous cap but not in the lipid core [16, 17, 20, 24]. The distribution of these two collagens in the fibrous cap varies resulting in collagen deficient regions and collagen-rich regions [20, 24].

Collagen type IV is present in the subendothelium of the normal intima where it forms the endothelial basement membrane. Collagen type IV occurs also in the basal lamina of the SMCs [17, 18], and its deposits can be observed in calcified tissues and in small new vessels of advanced lesions [17].

Collagens type V and VI are not detected in the intima of normal vessels or in early lesions, whereas their content increases with lesion progression. Both collagen types appear together with collagens type I and III in later stages of lesion development [17]. Collagen type V is located in the subendothelium and associated with SMC surface; collagen type VI is found between collagen type I fibers in the media and in the subendothelium [18].

Collagen VIII is widely distributed in the vessel wall and can be secreted by monocytes and macrophages as well as by SMCs [25, 26]. It has been detected in all three layers of the normal vessel wall and depositions of collagen type VIII have been observed in atherosclerotic lesions at early stages of development. In advanced lesions, it is deposited together with fibrillar collagens in the fibrous cap, the plaque shoulders and the plaque base [19].

Sparse or no collagen can be detected in the lipid core of advanced atheromatas [17].

4.3 COLLAGEN FUNCTIONS IN ATHEROSCLEROSIS

The role of collagen in atherogenesis is rather diverse. At some stages of the disease collagen can be considered an enemy, and at some stages – a friend. At later stages of atherogenesis collagen provides the tensile strength of the intima and guards against fibrous cap rupture [9, 27]. However, collagen can also promote plaque growth by stimulating cell migration and providing permissive matrix for lipid retention [18, 27].

4.3.1 Collagen functions in plaque progression

SMCs in the atherosclerotic plaque can migrate, divide and synthesize extracellular matrix in response to mechanical or chemical (cytokines and growth factors) influences. These SMCs have the synthetic phenotype and are regarded as poorly differentiated in contrast to the contractile phenotype of SMCs in the media [28, 29]. SMCs cultured from atherosclerotic lesions secrete more collagen compared with the cells isolated from regions of normal arteries [30, 31]. Newly synthesized collagen contributes to the thickening of the vessel wall and, together with the cellular

components, is responsible for the occlusion of the vessel lumen caused by the growing atherosclerotic plaque [18].

Apart from the space-filling role, collagen provides an anchorage for cells and exerts an important influence on their phenotype and behavior through receptor-mediated contacts. The principal collagen binding receptors that are expressed on SMCs include integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_3$) and the discoidin domain receptor (DDR1 and DDR2) tyrosine kinases [27]. *In vitro* experiments have shown that integrins bind both native and heat-denatured collagen [27, 32]. DDRs are expressed in atherosclerotic plaques and can be activated only by collagen in its native, triple-helical form [33, 34]. Collagens may also modulate cell function indirectly by acting as binding sites for other matrix components such as thrombospondin, von Willebrand factor, and fibronectin [18].

Collagens are believed to be critical regulators of SMC phenotype alterations, proliferation and migration in the atherosclerotic plaque via integrin-mediated pathways [18, 27, 35, 36]. The polymerization state of collagen is an important determinant of this aspect. SMCs proliferate and move faster when plated on monomeric collagen compared with collagen fibers [18, 27]. The monomeric collagen induces the expression of many genes that are important in regulation of cells spreading, whereas polymerized collagen type I acts as a suppressive agent [27, 37]. Newly synthesized or degraded collagen is also an important matrix for cell migration. Treatment of cultured cells with inhibitors of collagen synthesis or collagenolysis attenuates SMC migration and invasion [38-40].

The same observations have been made for collagen type IV. Soluble collagen IV added to the media can stimulate migration of cultured SMCs [27] whereas a mature collagen type IV net likely serves to maintain SMC quiescence by forming basement membranes around individual cells [29].

The collagen-binding receptor DDR1 can also control the SMC response to injury. *Ddr-/-* SMCs exhibit reduced proliferation, migration and proteolytic activity in response to collagen type I *in vitro* [41], and overexpression of DDRs rescues these deficits [33, 42].

In addition to its influence on SMCs, collagen is capable of regulating phenotype and function of inflammatory cells present in the atherosclerotic plaques via activation of integrins, DDRs and class A scavenger receptors. Signals initiated by collagen-receptor interactions regulate all aspects of macrophage biology including differentiation, migration, production of inflammatory cytokines and elaboration of matrix degrading enzymes [43, 44]. Apolipoprotein E knockout (*Apoe-/-*) mice deficient also in the genes for integrin α1 and low density lipoproteins receptor knockout (*Ldlr-/-)* mice deficient also in DDR1 developed smaller plaques with a reduced content of macrophages and T cells [45, 46].

Finally, collagens may be important for binding and retention of native and oxidized low density lipoproteins (LDL) in the atherosclerotic plaque [47].

4.3.2 Collagen functions in advanced plaques

Major complications of atherosclerosis are caused by the fissuring or rupture of the fibrous cap. In advanced atherosclerotic lesions the distribution of collagen within the intima is not uniform. Generally speaking, fibrous caps are more collagen-rich than inner parts of the lesions [17]. Such fibrous cap localization of collagen in atheromata is of key clinical importance. Collagens fulfill a mechanical function providing tensile strength and playing an important role in maintaining plaque stability. Collagen type I modulates tensile strength of the vascular tissue, while collagen type III accounts for its elasticity [18].

The protective role of collagens within the fibrous cap is unquestionable as long as the integrity of the atheromata surface is preserved. With rupture of the fibrous cap, circulating platelets come in contact with collagen fibers in the plaque. Collagen then interacts with platelet integrins, which may initiate coagulation and thrombus formation [18].

Intimal calcification is a feature of advanced atherosclerotic lesions [48]. Mineral crystals can be deposited along collagen fibers. Furthermore, an exposure of aortic SMCs to collagen type I increases their mineralization and calcium incorporation [27, 49].

4.3.3 Functions of other collagens in atherosclerosis

Collagen type IV constitutes the general scaffold for the basement membrane and is involved in the process of cell adhesion [19, 50]. Its distribution around SMCs in atherosclerotic lesions suggests a relation to the morphological changes of SMCs from contractile to synthetic phenotype. The degree of collagen type IV expression can be an indicator of the prevailing SMC phenotype, because with thickening of the basement membrane SMCs decrease their proliferative capacity [17, 40]. Localization of collagen type IV around calcified tissue and neovessels suggests a possible involvement in the processes of calcification and neovascularization [17].

It is believed that collagen type V can interact with collagen type I to regulate fibril diameter [51, 52]. Collagen type VI forms microfibrils and exhibits unique adhesive properties compared to other extracellular matrix components and cells. It is involved in the adhesion and activation of platelets and SMCs and can bind to various collagen types, heparin and von Willebrand factor [18, 19, 53, 54].

Collagen type VIII stabilizes the vascular wall. It plays a role in uniting other components of the extracellular matrix, and can contribute to the elasticity of the vessel wall through interaction with the elastic system. Cellular and extracellular distribution of collagen type VIII in advanced atherosclerotic lesions may imply that it has a role in vascular repair and plaque stabilization [19].

5 COLLAGEN METABOLISM IN ATHEROSCLEROSIS

As mentioned earlier, the overall stability and flexibility of connective tissue depends on a basic framework of collagen fibers. Therefore, the strength and stability of collagen fibers are tightly regulated by the complex multistep processes of collagen biosynthesis and catabolism.

The formation of mature fibrillar collagen involves many steps beyond gene transcription. Nascent procollagen polypeptides undergo a series of posttranslational modifications which include intracellular and extracellular steps (Figure 2). Intracellular processes of hydroxylation, glycosylation and self-association into triple helical structures are followed by extracellular cleavage of the N- and C-propeptides, cross-linking of side chains and self-aggregation into multimeric collagen fibers [13, 55]. This multistep process requires three collagen hydroxylases, two collagen glycosyltransferases, two specific proteinases that cleave off the N- and C-propeptides and one specific oxidase that initiates cross-linking (Figure 2) [55]. Failure at any step of collagen biosynthesis results in fragile fibers that cannot withstand mechanical forces to the necessary extent and are more susceptible to proteolytic degradation.

Additional mechanisms behind collagen fragility include an increase in extracellular non-enzymatic glycation- or oxidation-induced collagen cross-linking that occurs with ageing or in patients with diabetes. Non-enzymatic collagen cross-linking leads to formation of stiff and brittle collagen fibers [56].

Mature collagen fibers can be efficiently degraded by at least two types of proteases: matrix metalloproteinases (MMPs) and cysteine proteases [9, 40, 57, 58].

This chapter will give an overview of the main steps of collagen metabolism with emphasis on the process of extracellular collagen cross-linking and the enzyme catalyzing this important extracellular step of collagen biosynthesis, **lysyl oxidase** (LOX). LOX-dependent collagen maturation in the atherosclerotic plaque has been the main focus of the work presented in the papers of this thesis.

The biochemistry of proteolytic enzymes and their involvement in the process of atherosclerotic plaque destabilization have been a target of numerous investigations [9, 40, 57, 58]. Therefore, only a brief overview of this process will be presented.

5.1 TRANSCRIPTION AND TRANSLATION

All α-chains of various collagens are encoded by separate genes with a unique localization in the genome. Most collagen genes display a complex exon-intron pattern, ranging from 3 to 117 exons. The mRNA of α-chain of fibrillar collagens can be encoded by more than 50 exons [13, 14].

Different mRNA species can be generated by multiple transcription initiation sites, alternative splicing of exons or a combination of both. In addition to splicing, the pre-mRNA undergoes capping at the 5' end and polyadenylation at the 3' end [13]. Ribosome-bound mRNA is translated into prepro-collagen chain that is transported to the lumen of the rough endoplasmic reticulum for further modifications [13].

5.2 INTRACELLULAR POSTTRANSLATIONAL MODIFICATION

After removal of the signal peptide by a signal peptidase, prolyl and lysyl residues in the procollagen chains undergo hydroxylation by enzymes such as prolyl-3-hydroxylase, prolyl-4-hydroxylase and lysyl hydroxylase (Figure 2). Such hydroxylation of residues in the primary procollagen sequence is a crucial step in the organization of the triple-helical structures. It also permits formation of intermolecular cross-links, which gives collagen fibers enormous tensile strength [59].

Conversion of proline to hydroxyproline is catalyzed by collagen **prolyl-4-hydroxylase** (P4H, Enzyme Commission number (EC №) 1.14.11.2), an $\alpha_2\beta_2$ tetramer located within the lumen of the endoplasmic reticulum [60]. P4H has at least three isoenzymes in human. Each isoenzyme has a distinct α-subunit that is bound to a protein disulfide isomerase (PDI). PDI serves as β-subunit for all three enzymes. P4H requires a set of co-factors that include Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate [61]. In the fibril-forming collagens, approximately 50% of the prolyl residues contain a hydroxyl group. In conditions of suboptimal activity of P4H, the underhydroxylated procollagen chains misfold and are either secreted from the cells at a slow rate or targeted for intracellular degradation [62, 63]. Reduction in the content of 4-hydroxyproline in secreted procollagen chain reduces the stability of fibers at physiological conditions [13, 64]. Moreover, the degree of proline hydroxylation affects collagen resistance to proteolytic

attacks [65, 66] and ability to recognize and bind other components of extracellular matrix [67].

PDI has several functions such as (i) to catalyze the formation of intrachain and interchain disulfide bonds; (ii) to serve as the β-subunit in collagen P4H; (iii) and to act as a chaperone that binds nascent procollagen chains and prevents their aggregation [55].

The conversion of lysyl residues (Lys) to hydroxylysyl residues (Hyl) is catalyzed by enzyme **lysyl hydroxylase (LH)** (procollagen-lysine 2-oxoglutarate 5-dioxygenase, *PLOD*, EC № 1.14.11.4) in the endoplasmic reticulum. The enzyme has at least three isoenzymes (LH1, 2 and 3) [13, 55]. Hydroxylation of Lys can occur in the telopeptide regions in procollagen chains where it is catalyzed by LH2 ("telopeptide lysyl hydroxylase", TLH), and in the triple helical part of the procollagen chain catalyzed by LH1 ("helical lysyl hydroxylase", HLH) [56, 68]. Hydroxylation of Lys in the telopeptide region is a crucial step for the future enzymatic cross-link formation after procollagen secretion.

Mutations in the human *PLOD1* gene for LH1 results in Ehlers-Danlos syndrome characterized by generalized fragility of connective tissues [55]. Mice lacking the *Plod1* gene develop aortic rupture due to abnormal morphology of collagen fibrils [69]. Mutations in the *PLOD2* gene for LH2 that are identified in the patients with Bruck syndrome result in underhydroxylation of Lys in telopeptide regions, formation of aberrant cross-links and, thus, in bone fragility, scoliosis and osteoporosis [55]. On the other hand, the excessive hydroxylation of Lys in telopeptide regions by LH2 can result in adverse effects leading to excessive fibrogenesis [56, 68].

Hyl also represent sites for the attachment of sugar moieties by glucosyl transferases. These hydroxylated and glycosylated chains then self-assemble into helical trimers starting from the alignment of the C-terminal domains [13].

The efficient folding of the procollagen chains also depends on the presence of further enzymes: PPI (peptidyl-prolyl *cis-trans*-isomerase) and the collagen specific chaperone heat shock protein 47 (Hsp47) [13, 55].

Hsp47 is a heat shock-inducible glycoprotein that is expressed selectively within the endoplasmic reticulum of cells that synthesize and secrete procollagen type I and III

[70]. A transient physical association between Hsp47 and procollagen within the endoplasmic reticulum serves to stabilize procollagen. It prevents premature aggregation of monomers into oligomeric forms and modulates their transfer to the Golgi apparatus before export from the cell [71]. Homozygous knockout of this gene in mice is lethal at the embryonic stage, indicating that this protein is essential for normal development [72].

5.2.1 Intracellular modification of collagen in atherosclerotic lesions

Information about the role of intracellular collagen modifying enzymes in atherogenesis is rather limited. The majority of studies have been done on animal models of atherosclerosis and showed that expression of enzymes accompanies the increase in collagen accumulation during early progression of the disease.

SMCs from cholesterol-fed rabbits have increased mRNA level for the α-subunit of P4H [73]. P4H enzymatic activity is increased in atherosclerotic lesions of various avian and animal models of atherosclerosis after exposure to common risk factors such as sympathetic nerve system activation and hyperlipidemia [74-76]. Expression of Hsp47 mRNA is induced in rat carotid arteries after balloon injury [77]. Hector et al demonstrated increased LH1 activity, as determined by higher concentration of Hyl, in human atherosclerotic plaques compared with underlying media [78]. All these observations of increased collagen synthesis support the notion that SMCs acquire synthetic phenotype at early stages of atherogenesis.

On the other hand, it has been known for decades that the absence of vitamin C, a required co-factor for P4H, impairs formation of stable collagen and leads to fragility of blood vessels [79]. *Apoe-/-* mice that are unable to synthesize ascorbic acid have decreased collagen content in the lesions. This impairs the biomechanical strength of the plaques and makes them potentially vulnerable to rupture [80]. An increased amount of Hsp47 protein is detected in the SMC-rich fibrous cap of advanced lesions of *Apoe-/-* mice [81]. Moreover, studies on human atherosclerotic plaques demonstrated localization of α(III)-subunit of P4H and Hsp47 in the fibrous cap of advanced human carotid atherosclerotic lesions [82, 83]. Therefore, this limited data

suggests that efficient intracellular modification of collagen may determine plaque stability.

5.3 SECRETION

After processing and correct assembly into triple helices, procollagen is packed into secretory vesicles within the Golgi compartment and transported into the extracellular space. After secretion of procollagen, C- and N-propeptides are cleaved off by two specific proteases, the procollagen C-proteinase and the procollagen N-proteinase, respectively (Figure 2) [13].

5.4 EXTRACELLULAR PROCESSING AND MODIFICATION

Stabilization of newly formed and secreted collagen fibrils is achieved by the formation of covalent cross-links between neighboring collagen fibrils. Collagen cross-links can be divided into two types: enzymatic cross-links (LH- and LOX- mediated) and non-enzymatic cross-links (advanced glycation endproducts (AGE) induced cross-links).

5.4.1 Enzymatic collagen cross-linking. Lysyl oxidase

The tensile properties of collagen fibers result from intermolecular cross-links that connect the nonhelical ends of two collagen monomers (telopeptides) with the triple helical part of the third adjacent monomer according to the quarter-stagger model (Figure 2) [15]. Each monomer of the fibril-forming collagens has four cross-linking sites: one in each telopeptide and two in the triple helical region, close to its N- and C-terminal ends (Figure 2) [84]. The formation of collagen cross-links is a joint effort of two enzymes: the intracellular LH that was described earlier and the extracellular enzyme LOX. Excessive formation of enzymatic cross-links does not occur in physiological conditions due to tight control of the expression of LOX [56].

The extracellular copper-dependent enzyme LOX initiates the formation of stable non-reducible collagen cross-links by oxidizing specific Lys and Hyl in the telopeptide regions of collagen into aldehyde to give allysine and hydroxyallysine, respectively [56, 84, 85].

The stoichiometry of the reaction catalyzed by LOX is:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2 \quad [85]$$

This LOX-catalyzed step is important in the initiation of cross-link formation. Further aggregation of collagen fibrils into fibers occurs spontaneously.

Cross-links that result from the hydroxyallysine pathway are more stable and predominate in connective tissues that bear large mechanical loads [84]. The hydroxyallysine in the telopeptide of one collagen monomer reacts with either a Lys or a Hyl in the triple helix on a neighboring monomer to give difunctional cross-links (ketoimine bonds). These immature cross-links further undergo Amadori rearrangement and then bind another Lys or Hyl in the telopeptide of third collagen monomer to form trifunctional non-reducible cross-links. These final mature Hyl-derived cross-links are hydroxylysylpyridinoline (HP), derived from three Hyl, and lysylpyridinoline (LP), derived from one Lys and two Hyl (Figure 2) [56, 68, 84]. HP cross-links are the most abundant and present in virtually all mature tissues including blood vessels [84, 86].

5.4.1.1 The LOX enzyme

LOX (protein-6-oxidase, EC № 1.4.3.13) is a copper amine oxidase that catalyzes the formation of covalent cross-links within collagen and elastin fibers [87]. It plays a central role in the repair of connective tissues all over the body including the cardiovascular system [84]. Decreased LOX activity is associated with disorganization of connective tissues as seen in copper transport disorders, such as cutis laxa and Menkes syndrome [87]. Lathyrism, a condition caused by toxic effects of *Lathyrus odoratus* seeds (sweet peas) that contain the irreversible LOX-inhibitor beta-aminopropionitrile (BAPN), is characterized by abnormal collagen cross-linking due to inactivation of LOX [88]. Increased LOX activity is observed in several fibrotic conditions such as Alzheimer's disease, proliferative retinopathy and heart failure [87, 89-91].

LOX requires pyridoxal phosphate (vitamin B6) and tyrosyl-lysine quinone as essential co-factors [85]. Vitamin B6 deficiency in rats leads to a 25% decrease in enzymatic collagen cross-link formation in bones [92].

LOX is expressed and secreted by vascular SMCs and other fibrogenic cells [87]. LOX protein is localized in the extracellular compartment of several tissues such as skin, aorta, heart, lung, liver and cartilage [85, 93, 94]. The catalytically active enzyme has also been documented within nuclei of vascular SMCs and fibroblasts [95], and, once secreted and proteolytically processed, mature LOX can translocate back to the nuclei of vascular SMCs [96].

LOX is secreted from cells in the form of a catalytically inactive 50 kDa proenzyme (proLOX) which is proteolytically cleaved to yield the 32 kDa active enzyme (Figure 3). This predominantly occurs in the extracellular space, although intracellular proteolytic processing cannot be excluded [97]. The propeptide is required for efficient secretion of proLOX, and for optimal activation of the LOX enzyme in the extracellular space [98]. Interestingly, the conversion of proLOX is catalyzed by the same enzyme that cleaves the C-terminal domain of the procollagen chain - the procollagen C-proteinase (bone morphogenetic protein-1) [99, 100]. This represents a highly integrated mechanism for the formation of cross-linked collagen fibers. Other extracellular proteases can also cleave proLOX at the correct physiological site but with lower efficiency [100].

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 6 M urea [85, 97].

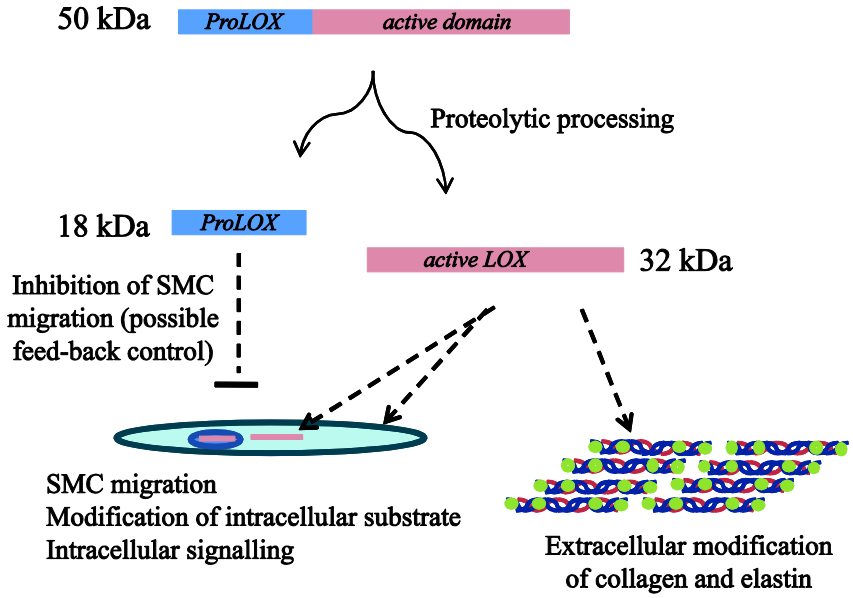

Figure 3. LOX structure and function. Modified from [101].

Thorough studies of the biochemistry of LOX have revealed that the substrate specificity of LOX is not restricted to elastin and collagen. Purified LOX oxidizes a number of basic, globular proteins with pI values > 8, such as histones and basic fibroblast growth factor, but does not oxidize neutral or acidic proteins [102-104]. There is evidence suggesting that exposure of cells to LOX increases intracellular endproducts of the reaction catalyzed by LOX, such as H_2O_2 [105]. This data suggests that additional LOX substrates are either intrinsic membrane proteins or proteins that are tightly bound to the cell surface. Moreover, the presence of LOX in the cytosolic and nuclear compartments may suggest that LOX can control cellular homeostasis [95, 96]. Therefore, LOX might play a critical role in other biological processes beyond the oxidation of structural proteins and stabilization of the extracellular matrix. LOX has been implicated in regulation of tissue development, cell proliferation, intracellular signal responses, and cell migration. It can also act as either an antagonist or a protagonist of malignant processes [106].

In vitro studies have suggested that LOX can be a potent chemoattractant. Purified active LOX is able to induce strong chemotactic responses in human monocytes and vascular SMCs [105, 107]. These responses are mediated by H_2O_2, the product of amine oxidation by LOX, which is markedly elevated in vascular SMCs upon exposure

to LOX. H_2O_2 induces changes in the content and architecture of cytoskeletal components and adhesive cell proteins, which results in directed cell migration [105]. Lucero et al [108] suggested that the chemotactic response of vascular SMCs to LOX can also be mediated via oxidation of cell surface proteins such as platelet-derived growth factor (PDGF) receptor-β to induce PDGF-induced chemotaxis.

Another line of evidence suggests that LOX may affect the cell phenotype and play an important role in suppressing oncogenic cellular transformation [106, 109, 110]. LOX-dependent cross-linking of matrix surrounding ductal breast carcinoma may represent a defense mechanism against invasion [111]. In contrast, it has been observed that LOX mRNA is upregulated in invasive breast cancer and that active LOX facilitates invasion by several lines of malignant cells via H_2O_2 production [106]. However, the detailed mechanisms involved in pro- and antioncogenic cellular responses to LOX are beyond the scope of the present work.

The importance of translocation of active LOX into the nuclei and nuclear distribution of the enzyme is still unclear. However, given that histones 1 and 2 can be LOX substrates *in vitro*, it has been suggested that LOX activity within nuclei can be linked to the regulation of nuclear chromatin condensation and changes of the availability of promoter regions to transcriptional factors [106]. In line with this hypothesis, it has been shown that LOX can regulate mRNA expression of human collagen type III gene and elastin [112-114].

It has to be noted that the 18 kDa propeptide (LOX-PP) that is enzymatically cleaved of the secreted proLOX has distinct functions in addition to preventing premature activation of LOX (Figure 3). LOX-PP has a unique structure and no sequence similarities to any other LOX-like proteins [93]. LOX-PP epitopes accumulate in injured arteries [115]. Synthetic LOX-PP can inhibit proliferation and TNF stimulated MMP-9 synthesis in cultured vascular SMCs [115].

5.4.1.2 The LOX family

In addition to LOX, at least four genetically distinct LOX-like (LOXL) proteins have been described: LOXL1, LOXL2, LOXL3 and LOXL4 [93]. All five proteins have related but different functions and carry a certain degree of homology. This makes them

one family that in turn is subdivided in two subfamilies (Table 2). The first LOX subfamily includes LOX and LOXL1 that bear the highest homology. They are secreted as proproteins that are proteolytically cleaved to release active enzymes. The members of the other subfamily (LOXL2, 3 and 4) contain four scavenger receptor cysteine-rich (SRCR) domains in the regions following the signal peptide, and as a result exist in stable, distinctly folded protein structures [106]. SRCR domains are known to mediate protein-protein interactions in cell adhesion and cell signaling [93].

The pattern of expression of different members of the LOX family is partially overlapping (Table 2). However, LOX is responsible for 80% of lysyl oxidase activity in aortic SMCs, indicating that LOX is the main isoenzyme in these cells [116]. LOX, LOXL1, LOXL2 and LOXL3 proteins are all expressed in the cardiovascular system. LOXL2 is highly expressed in the fetal heart, and LOXL3 expression is restricted to the adult aorta [117].

All members of the LOX family may have different substrate specificity [93]. It has been suggested that LOX has the highest substrate specificity to collagen type I [106], whereas LOXL1 is essential for elastic fiber homeostasis [118]. Mice lacking LOXL1 do not deposit normal elastic fibers and develop multiple organ disorders including vascular abnormalities with concomitant tropoelastin accumulation [118]. Mutations in the human gene for LOXL1 are associated with systemic elastic microfibrillopathy [119].

Expression of LOXL2 has been observed in senescent fibroblasts and is associated with premature ageing [120]. The protein has also been found to be upregulated in several conditions characterized by liver fibrosis [101]. Like LOX itself, all four members of the LOX family have been implicated in the malignant transformation of various cell lines [101].

Table 2. Characteristics of the different members of the LOX family. Adapted from [93, 121].

Family member	Human chromosome	Highest mRNA levels	Similarity to LOX, %
LOX	5q23	Aorta, heart, lung, kidney, skin, placenta	100
LOXL1	15q24	Heart, placenta, skeletal muscle, lung, pancreas	85
LOXL2	8p21	Fetal heart, prostate, uterus, placenta	58
LOXL3	2p13	Brain, heart, uterus, aorta	65
LOXL4	10q24	Placenta, lung, kidney, testis, pancreas, ovary	62

5.4.2 Non-enzymatic collagen cross-linking

AGE-related injury can promote the development of many age- and diabetes-related disorders, including atherosclerosis. It involves activation of growth factors and initiation of inflammatory reactions as well as enhancement of vascular stiffening, angiogenesis, and extracellular matrix accumulation [122].

Collagen in the blood vessel wall has a relatively long biological half-life and with time undergoes significant non-enzymatic glycosylation (glycation). In contrast to the beneficial effects of the enzymatic collagen cross-links, non-enzymatic AGE cross-links, such as pentosidine and glucosepane, deteriorate the biological and mechanical properties of tissues. The formation of AGE cross-links promotes fibrosis and decreases connective tissue flexibility, making them partly responsible for many fibrotic complications including atherosclerosis, especially in diabetic patients [122, 123]. Collagen glycation may impair collagen's functional interaction with the cellular components within the vessel wall by modifying its binding to integrins [50].

The chemistry of formation of AGE cross-links in collagen fibers somewhat resembles the mechanism of LOX-dependent cross-linking. However, it involves the formation of the aldehydes from glucose, ketose, or other metabolic intermediates. These aldehydes react with Lys or Hyl in collagen monomer to form a glycosyl-Lys via Schiff base formation. Such non-enzymatic cross-linking involves Lys or Hyl in helical domains of collagen monomer, but not in telopeptides. This distinguishes non-enzymatic collagen

cross-linking from enzymatic. Modified residues are stabilized by spontaneous Amadori rearrangement and further undergo reaction with Lys or arginine in adjacent collagen monomer to form irreversible inter-helical AGE cross-links [56, 124]. Such changes are accelerated in various diseases with a strong metabolic component, such as diabetes mellitus and end-stage renal disease [124].

5.4.3 Extracellular cross-linking of collagen in atherosclerotic lesions (Papers I and III)

In the vascular wall, the expression of LOX is restricted to fibrogenic SMCs. However cultured endothelial cells can also synthesize LOX [125]. The role of LOX in cardiovascular development and diseases has been studied mainly using animal models and cultured cells, and has mainly been focusing on the role of LOX in the development of aortic aneurysm [126, 127]. Inactivation of the *Lox* gene in mice results in fetal death due to a defective cardiovascular system including large aortic aneurysms caused by collagen and elastin abnormalities [128]. Animals on copper-deficient diets often die from aortic ruptures [129].

In humans, disorders in copper metabolism as seen in Menkes disease have been associated with a decreased activity of LOX and increased risk for development of myocardial infarction and abdominal aneurysm [130]. A clinical case of spontaneous coronary artery dissection due to a dramatic decrease in LOX levels and increased extracellular matrix disorganization has been reported recently [131].

Very little attention has been given to the role of extracellular collagen maturation in determining the stability of atherosclerotic plaques. Earlier studies found increased LOX enzyme activity in the aortic lesions of atherosclerosis-prone rabbits and a significant increase in LOX mRNA expression in rat models of vascular restenosis. These observations could reflect accumulation of SMCs during lesion formation [132-134].

It is widely accepted that thinning and rupture of the fibrous cap of atherosclerotic lesions leads to thrombotic events that are more dangerous than artery occlusion [135]. It has been shown that systemic hypercholesterolemia downregulates LOX mRNA

expression in a porcine model of diet-induced atherosclerosis [136], and LOX and procollagen mRNA expression is reduced in the arterial wall of diabetic rats compared with controls [137].

The lack of studies characterizing vascular LOX expression and function in atherogenesis stimulated us to address this question in murine and human atherosclerosis (**Papers I and III)**.

In **paper I**, we investigated how T cell-driven inflammation affects plaque morphology in *Apoe-/-* mice. We observed the reduction in amount of mature collagen in atherosclerotic plaques under hyperinflammatory conditions. Since we did not detect any differences in procollagen synthesis in these mice, we thought it would be relevant to study LOX. We detected LOX mRNA and active protein in aortae of hypercholesterolemic mice and found that enzyme expression was decreased in lesions with severe local inflammation and therefore, presumably, a more vulnerable phenotype (Figure 4A).

In **paper III,** we investigated the role of LOX in human atherosclerosis and were able for the first time to localize LOX protein in human lesions. Predominant expression of LOX was observed in regions rich in collagen and SMCs in the fibrous cap and surrounding the necrotic core (Figure 4B). We, therefore, suggested that LOX may contribute to fibrous cap strengthening and necrotic core incapsulation given that LOX can induce SMCs migration [105, 108].

Higher LOX mRNA and protein expression in human lesions was associated with a more stable phenotype of the plaque, because (1) LOX mRNA correlated significantly with mRNAs for procollagens, P4H and LH; and (2) the amount of active LOX protein positively correlated with the percentage of total collagen and with increased amounts of the mature enzymatic collagen cross-links (HP and LP) in atheromatas (Figure 4D).

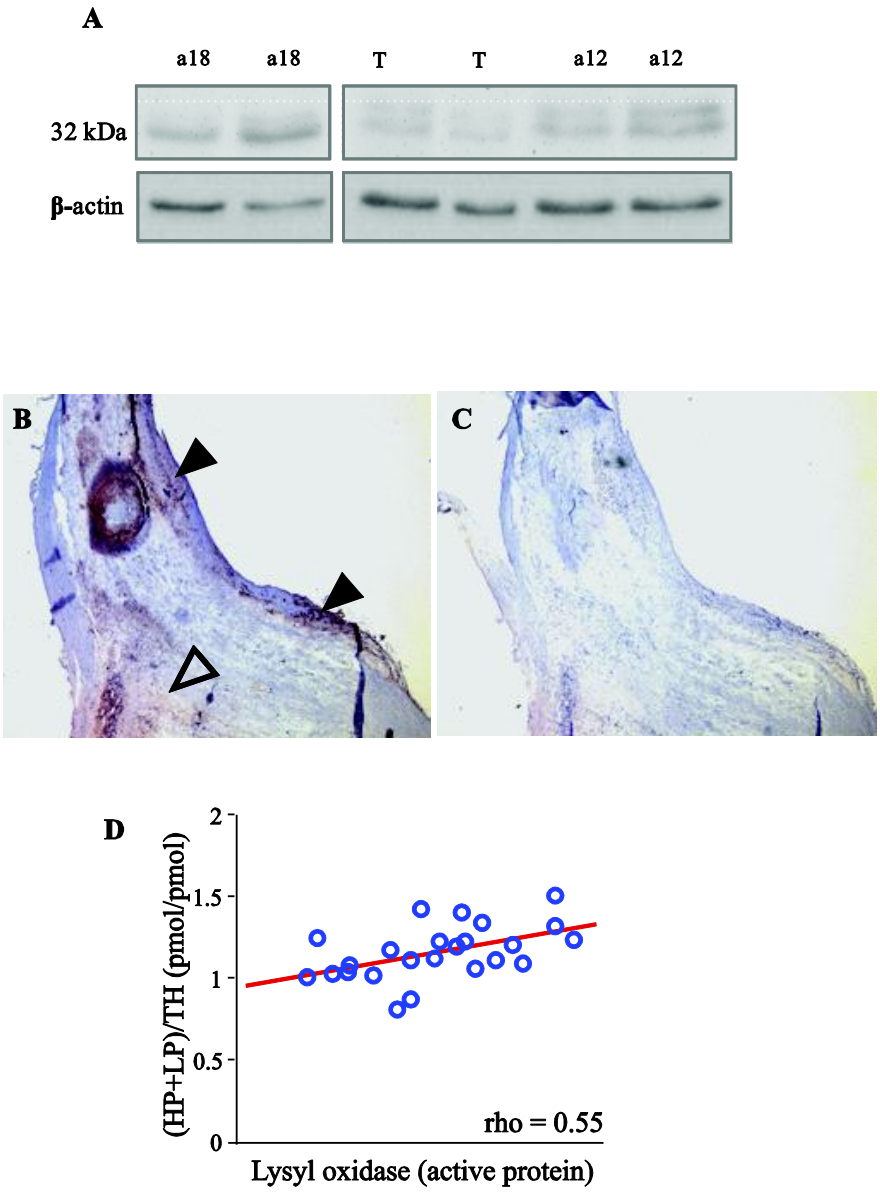

Figure 4. (A) Active LOX protein was detected by western blotting in aortae of 12 week old (a12) and 18 week old (a18) Apoe-/- mice, and in mice with hyperinflamed plaques (T). (B) LOX protein was detected in human carotid lesions in the fibrous caps (filled arrow heads) and around necrotic areas (empty arrow head). Magnification x25. The round intensively stained structure is a section artifact. (C) Human carotid lesion stained with mouse immunoglobulins unspecific to LOX. (D) A positive correlation between active LOX and collagen cross-links in human atherosclerotic lesions as determined by the semi-quantitative western blot analysis of LOX and chromatographic analysis of collagen cross-links, respectively. HP-hydroxylysylpyridinolines, LP- lysylpyridinolines, TH-triple helix.

Although recent *in vitro* studies suggest that LOX is expressed by endothelial cells and its downregulation is associated with endothelial dysfunction [125], we could not detect LOX positive endothelial cells overlying human atherosclerotic plaques. Instead, LOX

protein was localized in the immediate subendothelial space where it was co-localized with basement membrane collagen IV and with SMCs. Given that collagen IV is one of the substrates for LOX, we assumed that such subendothelial distribution of the enzyme helps to stabilize the endothelial layer by maintaining the integrity of the basement membranes.

Decreased LOX-catalyzed collagen maturation in the media has been suggested as one of the mechanisms behind the development of abdominal aortic aneurysm [126, 128]. In **paper III,** we observed that LOX protein is lost from SMCs in the media below the atherosclerotic plaque. This may explain why atherosclerotic plaques grow outwards during their progression.

Our data shows that LOX controls collagen maturation that is required for the structural integrity of the fibrous cap. Moreover, recent data further suggests anti-inflammatory properties of LOX, namely an ability to inhibit MCP-1 secretion from SMCs, thus preventing monocyte infiltration into vascular tissue [138]. Altogether, data suggests that increased LOX can be potentially beneficial to clinical outcomes of atherosclerosis.

5.5 COLLAGEN DEGRADATION

Mature collagen within the atherosclerotic plaque can be degraded by several families of matrix-degrading proteases. The most abundant proteases in the plaque are MMPs and cysteine proteases released by activated macrophages and mast cells [139-141]. Several members of these enzyme families have been found in vulnerable regions of atherosclerotic plaques and associated with clinical manifestations of acute vascular events and plaque progression [142-146].

5.5.1 MMPs

The family of zinc- and calcium-dependent MMPs consists of more than 20 structurally related proteases [40]. MMPs can be divided into five groups according to their substrate specificity: interstitial collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, -11 and -7), membrane type MMPs (MMP-14, -15,

-16 and -17) and others, including the metalloelastase MMP-12 [40]. Except for the membrane-type MMPs that are integrated in the plasma membrane, all MMPs are secreted into the extracellular space. Macrophages represent the major source of MMPs, but SMCs, endothelial cells, T cells and mast cells can also secrete a subset of this protease family [40].

Almost half the members of the MMP family are present in the arterial wall [9, 140]. The interstitial collagenases, MMP-1, -8, -13, and -14, can efficiently degrade intact fibrillar collagens types I and III into 3/4 and 1/4 fragments which then become targets for other MMPs. The gelatinases, MMP-2 and -9, degrade mainly gelatin (denatured collagen) as well as nonfibrillar collagens, including type IV, and elastin. The stromelysins, MMP-3, -7, and -10, have a rather wide specificity [140]. Together MMPs can degrade practically all components of extracellular matrix found in plaques.

The activity of MMPs is regulated at multiple levels including regulation of transcription, secretion, proteolytic activation of inactive proforms and inhibition by specific tissue inhibitors of MMPs (TIMP-1, -2, -3 and -4). All MMPs are effectively inhibited by forming 1:1 complexes with TIMPs [140].

The role of MMPs in the process of plaque destabilization is rather controversial. On one hand, there is indirect evidence suggesting that macrophage derived MMPs in atheromata weaken the fibrous cap and promote disruption [9]. On the other hand, another line of evidence points to a plaque stabilizing role of certain MMPs due to their capacity to regulate SMC migration and proliferation; this should favor fibrous cap formation and repair after vascular injury [140]. Although functional genetic polymorphisms of some MMPs lead to increased prevalence of cardiovascular morbidity, the overall picture is still not consistent [12].

The collagenases MMP-1, -13, and -8 have been detected in vulnerable human atherosclerotic plaques and colocalized with cleaved collagen fragments in situ [143, 146]. Numerous subsequent studies have localized MMP-2, -3,-7, -9, -11 and -12 to unstable and highly inflamed regions of human lesions, and correlated their high serum level with thrombotic complications [12, 40, 139].

However, it is very difficult to differentiate whether the level of MMPs is a cause or an effect of plaque destabilization. Increased local expression of proteases can simply reflect greater macrophage infiltration into the lesions [40]. The ability of MMPs to facilitate SMC migration to sites of injury can imply a beneficial role of MMPs in wound healing [29].

Several animal studies support a plaque destabilizing potential of MMPs. *Apoe-/-* mice carrying a "knock-in" of a collagenase-resistant form of procollagen accumulates more collagen in their lesions than those with wild-type collagen [147]. Compound *Apoe-/-* x *Mmp13-/-* knockout mice show increased accumulation and maturation of collagen compared with *Apoe-/-* x *Mmp-13+/+* counterparts [148]. MMP-14, a membrane-associated MMP, also appears to contribute to collagenolysis and plaque destabilization in experimental atherosclerosis [149, 150].

In contrast, *Apoe-/-* mice also deficient in MMP-2, -3, and -9 develop SMC-poor lesions in brachiocephalic arteries. Plaques in these mice show signs of possible ruptures as defined by a greater number of buried fibrous caps [149, 152]. Macrophage-selective overexpression of human MMP-1 in *Apoe-/-* mice leads to decreased lesion size [151]. Therefore, these MMPs were assumed to promote plaque stability. However, MMP-3 and -9 have harmful effects at atherosclerotic prone sites in aorta [153, 154]. Moreover, MMP-9 has differential effects on plaque progression at various stages of atherogenesis in mice. Overexpression of MMP-9 in advanced lesions leads to development of plaque with vulnerable morphology, whereas overexpression of MMP-9 in early or intermediate lesions does not appear harmful [155].

5.5.2 Cathepsins

In addition to MMPs, certain cathepsins may contribute to plaque destabilization [57]. Cathepsins have potent elastolytic and collagenolytic activities [57]. Furthermore, cathepsin S has been implicated in the promoting of immune responses. The enzyme helps efficient antigen presentation by processing invariant chain of major histocompatibility complex (MHC) class II [156]. Finally, there is evidence that cathepsins proteolytically modify LDL and enhance the extracellular accumulation of LDL-derived lipid droplets within the arterial intima during atherogenesis [157].

The SMC-derived cathepsins S, K, L and F have been detected in human atheromata and localized to breaks in the elastic laminae [145, 157, 158]. Hyperlipidemic mice deficient for cathepsin S develop smaller lesions with better preserved elastin and decreased inflammation but reduced number of SMCs and collagen content [159]. Thus, it is possible that the cathepsins are important in promoting plaque destabilization primarily due to their capacity to initiate and maintain inflammation in the plaques and degrade elastin, whereas their effect on collagen metabolism within the fibrous cap is still questionable.

In **paper I**, we investigated whether the degree of proteolytic degradation of collagen was increased in murine lesions with severe local inflammation and poor collagen quality. We analyzed several members of MMP and cathepsin families on the levels of mRNA, protein and proteolytic activity. We observed that only mRNA encoding MMP-13 and cathepsin S were increased under severe T cell-driven inflammation. mRNA for MMP-2, -3, -8, -12 and 14, TIMP-1, cathepsin K and L, and neutrophil elastase remained unchanged in mice with hyperinflammatory lesions when compared to controls (Figure 5A). The activity of proteolytic enzymes analyzed by gel zymography and the degree of collagen fragmentation did not change in mice with activated T cells. Instead we observed the reduction in LOX mRNA and protein as described earlier. Our study showed that T cell-driven inflammation mainly acts on LOX-dependent collagen maturation rather than on its degradation. Therefore, we suggest that the local environment within the atherosclerotic plaque may play a key role in a critical plaque weakening by providing more available collagen substrates to certain subsets of proteolytic enzymes.

5.6 CONCLUDING REMARKS: WHICH CAME FIRST, THE CHICKEN OR THE EGG?

Collagens are not inert structures but are dynamic and multifaceted frameworks existing in atherosclerotic plaques. Collagen fibers accumulate at the onset of atherosclerosis, but eventually stabilize the fibrous cap preventing plaque rupture [160]. Clinically, patients are diagnosed with atherosclerosis at advanced stages of disease when lesions are established. Therapeutically, stabilization of the fibrous cap and thus prevention of plaque rupture are more relevant.

It is believed that the weakening of the fibrous cap is caused by an imbalance between decreased collagen synthesis and increased collagen degradation [135]. However, broad-range synthetic inhibitors of MMPs are not used in preventive cardiological practice [40]. Moreover, animal models clearly suggest that specific proteases promote plaque stabilization, and the time course of atherogenesis is critical for the activation of some proteases [140, 155]. Mature, insoluble collagen is resistant to the majority of proteolytic enzymes and has specific cleavage sites only for collagenases [65]. Therefore, increased degradation of collagen fibers in the fibrous cap is not solely the causative factor in plaque rupture.

We suggest that defective maturation of secreted collagen fibers contributes to the pathogenesis of plaque weakening. Previous studies demonstrate an abnormal biochemical composition and atypical organization of collagen in the fibrous cap [161]. These collagens are susceptible to fracture under stress. In addition, insufficient maturation of collagen fibers exposes additional MMP-cleavage sites which make collagen more susceptible to proteolytic degradation [65, 66].

Collagen cross-linking catalyzed by LOX modulates the biomechanical properties of collagen [106]. Here, we demonstrate that LOX is active in murine and human atherosclerotic plaques, and its presence is associated with a stable plaque phenotype (**Papers I and III**). Hence, defective cross-linking because of low LOX activity could promote disorganization of the extracellular matrix in the fibrous cap. LOX inhibition shifts the balance of collagen to the immature soluble forms. These forms are prone to degradation by a broader spectrum of MMPs [66, 101, 106]. Therefore, the observed increase in collagen degradation in unstable atherosclerotic plaques may be a result of collagen fibers “vulnerability” upon LOX deficiency.

Immune mechanisms that regulate LOX-dependent collagen cross-linking in atherosclerotic plaques were evaluated and are presented in the chapter 6 of the thesis. Further investigation into these mechanisms would be helpful when developing new tools for diagnosis and prevention of the vascular complications of atherosclerosis.

6 REGULATION OF ATHEROSCLEROTIC PLAQUE STABILITY

Sites of atherosclerotic plaque rupture are characterized by the presence of activated macrophages, T cells, and mast cells. These cells can affect the biosynthetic function of SMCs and collagen strength within the fibrous cap by producing pro-inflammatory cytokines and proteases [1]. SMCs can also interact with immune cells through direct cell-to-cell surface contacts, as seen with CD40L-CD40 ligation [162, 163].

Cytokine networks within the atherosclerotic plaque are complex [164]. We focused on exploring cytokine regulation collagen biosynthesis in the fibrous cap.

Collagen fiber formation may be controlled at several levels: (1) transcription and mRNA stability; (2) translation; (3) posttranslational modification; (4) intracellular and extracellular cross-linking and fiber deposition; and (5) degradation. Coordinated regulation of collagen synthesis and modification is critical in maintaining the integrity of the fibrous cap.

Experiments in isolated cell culture systems have shown that many cytokines are capable of modulating collagen biosynthesis [93, 165, 166]. However, the complexity of the atherosclerotic plaque does not allow us to draw any firm conclusions based entirely on *in vitro* data. Out of the many cytokines present in the atheromata and those that control collagen biosynthesis, only some can influence the composition of the extracellular matrix in atherosclerotic lesions in animals [164]. Genetic and therapeutic manipulations in hyperlipidemic mice show that transforming growth factor (TGF)β [167] and osteoprotegerin (**Paper II and III**) are the most potent pro-fibrotic and plaque stabilizing agents, whereas interferon (IFN)γ [168, 169], interleukin (IL)-18 [170-172] and IL-6 [173] promote plaque destabilization.

Other factors - such as hypoxia, hyperlipidemia, and mechanical forces - can influence collagen production and maturation in atherosclerotic lesions [125, 160].

6.1 IMMUNOLOGICAL FACTORS AFFECTING PLAQUE STABILITY

Activated T cells are present in atherosclerotic lesions at all stages of plaque development [10, 174]. CD4+ T cells and IFNγ, the cytokine produced in Th1 subset, exist in human atherosclerotic lesions and are associated with disease complications such as acute coronary syndrome [10, 175]. Regions of human atherosclerotic plaques that have ruptured are infiltrated with T cells [176]. *Apoe*-knockout mice that lack CD4+ cells (*Apoe-/-* x scid/scid mice) develop less atherosclerosis than their immunocompetent littermates and transfer of CD4+ T cells reverses this effect [177]. Uncontrolled T cell activation due to lack of TGFβ suppressive signaling leads to rampant inflammation and accelerated atherosclerosis in a hypercholesterolemic animal [178].

Th1 cells secrete ***IFNγ***, which exerts its proatherogenic role by 1) stimulating expression of adhesive molecules in endothelial cells and promoting recruitment of T cells and macrophages to the plaques; 2) enhancing the efficiency of antigen-presentation; and (3) increasing the production of pro-inflammatory cytokines and proteolytic enzymes in macrophages [179]. Exogenous treatment to *Apoe-/-* mice with IFNγ increases atherosclerosis [180]. In contrast, targeted gene deletions of IFNγ or its receptor lead to reduced atherosclerosis in the same animal model [168, 181].

Apart from its proinflammatory properties, IFNγ can promote plaque destabilization by thinning the fibrous cap. The cytokine is a powerful inhibitor of SMC proliferation [182-184], and is capable of inhibiting basal as well as IL-1, PDGF, or TGFβ-stimulated procollagen gene expression in SMCs [11]. Animal models have supported the antifibrotic effects of IFNγ. *Apoe* and IFNγ receptor double knockout mice show a reduction in atherosclerotic lesion size, but a marked increase in lesion collagen content [168]. Moreover, IFNγ can inhibit intimal thickening in the rat carotid balloon injury model [185, 186].

In human atherosclerotic plaques, expression of procollagen type I is negatively associated with the proximity of T cells [24]. Active T cells from patients with acute coronary syndrome can induce vascular SMC apoptosis via direct contact [187].

Apart from inhibiting procollagen transcription, IFNγ affects enzymes important for posttranslational collagen maturation. Recombinant IFNγ attenuates P4H and LOX

gene expression and activity in cultured fibrogenic cells including vascular SMCs [188-190].

In **paper I,** we addressed the question of what mechanisms are involved in the T cell-mediated plaque vulnerability. We used the *Apoe-/-* mice, which express a dominant negative TGFβ receptor II under control of the CD4 promoter (*Apoe-/-* x CD4dnTβRII) [178]. In these mice, T cells produce a non-functional TGFβ receptor II, do not respond to anti-inflammatory TGFβ signaling and are markedly activated. This leads to increased production of proinflammatory cytokines, including 100-fold increase in INFγ mRNA in aortae.

Apoe-/- x CD4dnTβRII mice develop advanced atherosclerotic lesions earlier than their *Apoe-/-* littermates [178] and, by the age of 15 weeks, they show signs of myocardial infarction (unpublished data), which suggests that lesions in these mice are more vulnerable. Indeed, histological analysis of lesions in the aortic roots revealed massive infiltration of macrophages and activated T cells and reduced collagen mass [178].

The lesions of *Apoe-/-* x CD4dnTβRII mice in **paper I** had increased amount of thin, immature fibers and decreased amount of thick mature fibers compared to lesion-size matched *Apoe-/-* controls. We, therefore, concluded that the collagen quality was impaired in the hyperinflamed lesions of these mice (Figure 5A). Surprisingly, the level of α-chain of procollagen I and III mRNA was not changed, and we could not detect any increase in collagen fragmentation in *Apoe-/-* x CD4dnTβRII mice. Instead, we observed that levels of LOX mRNA and protein were significantly reduced in aortae of *Apoe-/-* x CD4dnTβRII mice (Figure 5B). We also evaluated other factors involved in collagen maturation, namely P4H, LH and Hsp47. The effect of aggravated inflammation on expression of these molecules was not consistent. We hypothesize that the failure of extracellular LOX-dependent collagen maturation is a novel mechanism by which activated T cells may hamper plaque stability.

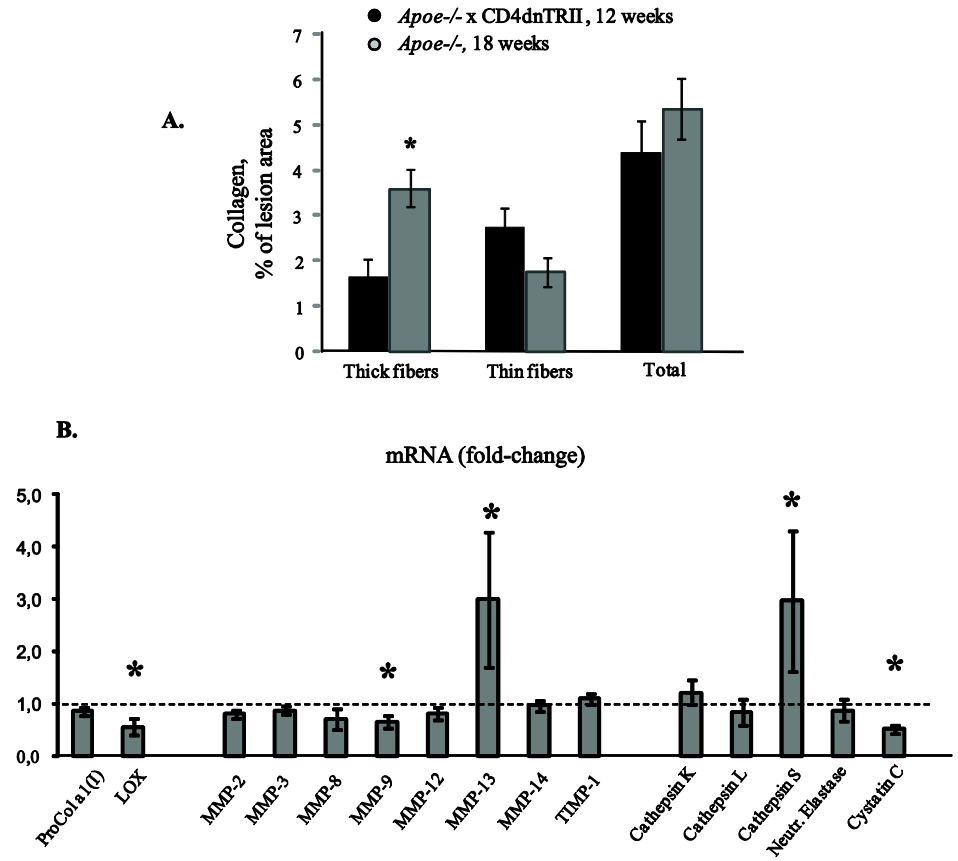

*Figure 5. (A) Collagen composition in lesions of Apoe-/- x CD4dnTβRII mice and Apoe-/- controls. The graph shows the percentage of total collagen as well as areas of thick or thin fibers divided by lesion area. Morphometric analysis of micrographs stained with Picrosirius Red. (B) Aortic expression of mRNA for procollagen α1(I), LOX and collagen degrading enzymes in Apoe-/- x CD4dnTβRII mice. Results are presented as fold-change compared to Apoe-/- mice (dotted line). *P<0.05 versus control.*

Proinflammatory cytokines, such as IL-1β and TNF, and T cells via direct contact can activate macrophages to produce MMPs and cathepsins [40, 57, 139]. This suggests that inflammation can affect collagen metabolism by decreasing production of stable collagen fibers and increasing their degradation. In **paper I**, we observed that the effect of T cell-driven inflammation on collagen degradation was modest. Measurement of the gene expression profiles of proteolytic enzymes revealed that the hyperinflamed aortae overexpressed only MMP-13 and cathepsin S (Figure 5B). Cathepsin S protein expression was consistently increased. However, no increase in collagen fragmentation in aortae of mice with activated T cells was observed. Our results do not negate a

potentially important role for MMPs in plaque destabilization, rather, they supports the idea that T cells affect principally collagen maturation.

In **paper III** we investigated whether T cell-driven inflammation can promote plaque vulnerability by reducing LOX-mediated collagen stabilization in human atherosclerosis. Gene expression patterns in human carotid plaques show that LOX mRNA expression correlates negatively to mRNA levels of several pro-inflammatory markers such as CD74, HLA-DR, lipocalin-2 and CD4 (Table 3). 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 increased in inflamed regions of atherosclerotic plaques [1, 191]. 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 [192]. Together, this data suggests that inflammation and proteolysis, conditions known to destabilize plaques, are associated with reduced LOX expression.

Table 3. Correlation between mRNA levels of LOX and proinflammatory markers analyzed by gene expression arrays of human carotid plaques.

Transcript	Spearman rank correlation, rho	p-value
CD74	- 0.48	< 0.01
HLA-DR	- 0.44	< 0.01
Lipocalin-2	- 0.31	< 0.01
CD4	- 0.3	< 0.01
RANKL	- 0.26	NS
INF-γ	- 0.1	NS

NS indicates non-significant results

Other factors can also facilitate fibrous cap weakening by decreasing collagen synthesis and cross-linking. Basic fibroblast growth factor inhibits collagen production and Hsp47 expression [82, 193, 194], and endothelial cell-derived nitric oxide reduces collagen production by cultured SMCs [195, 196].

Insulin-like growth factor-I inhibits mRNA for procollagens α1(I) and α1(III) and P4H activity in rats [197]. Administration of IL-18, a proinflammatory cytokine which induces IFNγ enhances atherogenesis and decreases plaque stability in *Apoe-/-* mice [172, 198]. IL-18 deficiency, in contrast, attenuates atherosclerosis progression and promotes plaque stability by increasing collagen deposition [170, 171].

TNF and IL1β inhibit LOX expression and activity in cultured endothelial cells [199], SMCs [127] and in the vascular wall in rats [199]. In *Apoe-/-* mice, IL-6 deficiency reduces LOX mRNA levels and collagen content in atherosclerotic plaques [173]. Atherogenic concentrations of LDL and homocysteine, well-known risk factors for atherosclerosis, reduce LOX expression and activity in endothelial cells [136, 200].

Other condition related to collagen disorders is ascorbate deficiency, which hampers proline hydroxylation, causing scurvy, a disease characterized by reduced connective tissue strength and increased fragility of blood vessels [79]. Chronic vitamin C deficiency compromises collagen deposition in atherosclerotic lesions in *Apoe-/-* mice [80].

In vivo evidence presented here supports findings from other studies and shows that LOX-dependent stabilization of collagen fibers can be hampered by proatherogenic stimuli in the atherosclerotic plaques. This can lead to plaque weakening and thrombotic events.

6.2 MEDIATORS WHICH SUPPORT PLAQUE STABILITY

TGFβ is an anti-inflammatory, immunosuppressive cytokine [201], and is a powerful stimulator of collagen synthesis in vascular SMCs [11, 202, 203]. The promoter of the procollagen α1(I) gene bears a TGFβ activation element which suggests that TGFβ can influence collagen synthesis directly [204]. Neutralization of TGFβ in hyperlipidemic mice leads to formation of large atherosclerotic lesions with signs of active inflammation and decreased collagen content, supporting a critical role of TGFβ in matrix production and plaque stability [167, 205]. Patients with advanced atherosclerosis have markedly decreased serum levels of active TGFβ [206]. Stimulation of fibroblasts and vascular SMCs with TGFβ results in a significant increase in P4H, LH and LOX production and activity, implying that this growth factor can influence multiple steps of collagen biosynthesis [93, 188, 190, 207-210]. Consequently, absence of TGFβ signaling to vascular SMCs reduces mRNA expression and activity of LOX in mouse aortae, which leads to aneurysm formation [211].

In **papers II and III,** we show that ***osteoprotegerin (OPG)***, a member of the tumor necrosis factor receptor superfamily, promotes atherosclerotic plaque stability in mice and humans by affecting SMC accumulation and LOX-dependent collagen maturation. A detailed description of the role of OPG in atherosclerosis progression and complications is given in the section 6.3 below.

Among other mediators, PDGF enhances SMC migration, collagen production and LOX expression in vascular SMCs [108, 160, 212]. Similarly, granulocyte macrophage colony-stimulating factor can increase collagen content in atherosclerotic lesions and positively regulate LOX expression in vascular SMCs [213-215].

Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (statins) are believed to prevent plaque rupture through their lipid-lowering and pleotropic effects [216, 217]. Statins increase synthesis of procollagen type I in *Apoe-/-* mice [218], and abrogate the reduction of vascular LOX expression triggered by high levels of LDL *in vivo* and *in vitro* [219]. This data suggests that one of the pleotropic effects

of statin might be stabilization of the fibrous cap through an increase of enzymatic collagen maturation.

Mechanical strain can stimulate collagen synthesis in cultured SMCs or *ex vivo* artery preparations which links local hemodynamic forces to plaque collagen production [160]. Furthermore, genes of procollagen, P4H and LOX are transcriptional targets of hypoxia-inducible factor 1α (HIF1α), which suggests that hypoxia is a strong regulator of fibrosis [220-222].

6.3 THE RANK-RANKL-OPG AXIS IN PLAQUE STABILITY

Receptor activator of nuclear factor-κB (RANK, TNFRSF11A), its ligand (RANKL, TNFSF11) and the decoy receptor for RANKL - osteoprotegerin (OPG, TNFRSF11B) - belong to proteins of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF) and Tumor Necrosis Factor Superfamily (TNFSF), and are associated with atherosclerosis development and plaque destabilization [223-225].

OPG is a soluble decoy receptor for RANKL and prevents RANKL interaction with its transmembrane receptor – RANK [223]. OPG is a basic secretory glycoprotein with 7 distinct structural domains. It can function as a 60 kDa monomer that has two apoptosis-mediating death domains and a heparin-binding region at the carboxyl terminal [225]. The formation of OPG homodimers is required for its binding to RANKL [226].

The RANK-RANKL-OPG axis was initially recognized as a potent regulator of bone metabolism [227]. RANK is expressed on the surface of osteoclast precursors such as monocytes, macrophages, and dendritic cells. RANKL is expressed by osteoblasts, stromal cells and T cells. The RANK-RANKL interaction leads to osteoclast formation, fusion, differentiation, activation, and survival, leading to enhanced bone resorption [223, 227]. OPG, which also is produced from osteoblasts, serves to hamper this interaction and prevent bone loss. Mice lacking a functional *Opg* gene develop severe osteoporosis [228] and OPG administration effectively prevents bone resorption [229]. *Opg-/-* mice suffer from a profound calcification in the large arteries and partial aortic dissection [228], which suggests that OPG might be important in vascular pathology.

Nevertheless, OPG's impact in cardiovascular disease development remains unclear [224, 225, 230, 231]. It has been shown that OPG is present in the arterial wall in significant amounts [232]. Vascular SMCs and endothelial cells can produce OPG along with monocytes, T and B cells [233], and its release can be enhanced by stimulation with TNF or IL-1β [225]. OPG is expressed in healthy vessels as well as in atherosclerotic plaques [234]. Patients with neurological symptoms or acute coronary syndrome have elevated OPG expression compared to non-symptomatic cases [235, 236]. Increased levels of OPG have been associated with increased risk for coronary artery disease, stroke and cardiovascular mortality not only in patients but also in the general population [224, 225, 230, 237, 238]. Epidemiological studies show a positive relationship between serum levels of OPG and prevalence and severity of coronary artery disease, cerebrovascular disease and peripheral vascular disease. Therefore, OPG was considered to be an independent risk factor for cardiovascular morbidity. However, it did not predict recurrent myocardial infarction in patients and failed to predict ischemic stroke in a healthy population [239, 240].

Experimental evidence conflicts with these clinical findings. Mice lacking *Opg* develop significantly larger atherosclerotic lesions compared to *Opg*+/+ x *Apoe-/-* mice [241]. Administration of OPG to *Ldlr-/-* mice reduces calcification in the lesions, although it does not affect lesion size or the degree of inflammation [242]. In line with results from animal models, studies of functional genetic polymorphisms in humans show no association between polymorphisms in the promoter region of OPG gene with aortic calcification or coronary artery disease [243, 244]. This data further suggests that increased plasma OPG is perhaps a marker but not a mediator of adverse cardiovascular prognosis.

In **paper II,** we investigated the effect of OPG administration on atherosclerosis development in *Apoe-/-* mice. Although OPG did not reduce plaque size and hamper inflammation in treated animals, it contributed to fibrous cap formation and lesion stabilization by promoting SMC accumulation and increasing collagen content. The increase in collagen content in lesions corresponded with elevated expression of LOX mRNA. This suggests that OPG is able to promote plaque stabilization by enhancing collagen cross-linking and strength (Figure 6). We showed also that administration of

recombinant murine OPG to cultured rat aortic SMCs significantly increased their proliferation. RANKL together with OPG did not affect the growth response. We suggest, therefore, that OPG impacts SMC growth and collagen matrix formation independently of RANKL.

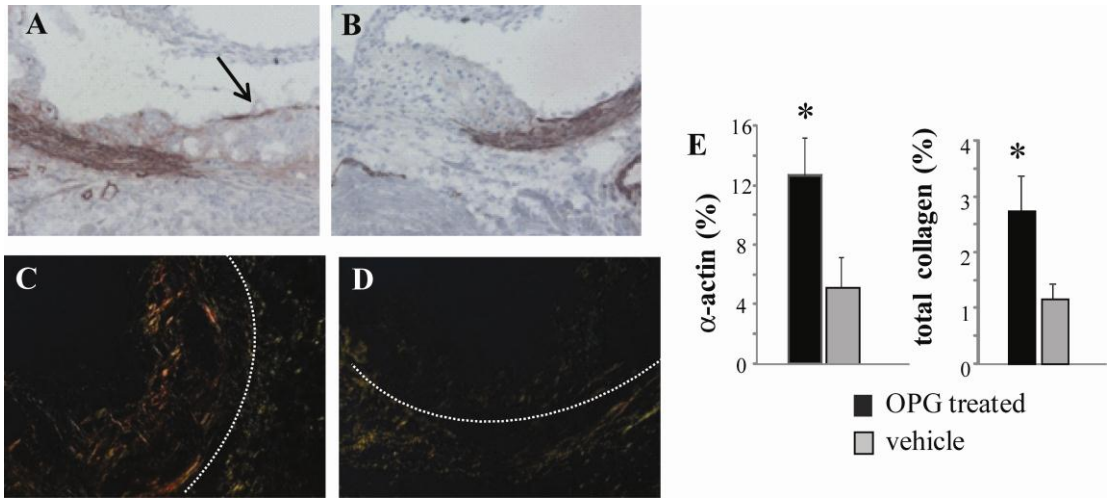

*Figure 6. (A-E) Micrographs of immunohistochemical staining with an antibody against SMC marker αSM-actin (A-B), and micrographs of sections stained with picrosirius red showing the collagen fiber composition (C-D) in atherosclerotic lesions of OPG- (A,C) and vehicle-treated mice (B,D). Note the cap-like structure in the lesion after OPG treatment (single arrow). The dashed line separates the intima from the media. (E) Area occupied by αSM-actin+ SMCs or fibrous collagen expressed as % of total cross-section lesion area. *) p<0.05 vs. vehicle control.*

Following the plaque stabilizing role of OPG described in paper II, in **paper III** we focused on OPG role in human carotid plaques. Global gene array analysis in human carotid plaques showed that OPG mRNA expression correlated both positively to LOX mRNA expression (Figure 7A) and to the amount of SMCs in lesions. A significant positive correlation existed between serum levels of OPG and mature enzymatic collagen cross-links in the plaques (Figure 7B). In line with this, OPG treatment of cultured human aortic SMCs led to increased levels of procollagen type I gene. Therefore, we found further support for the notion that OPG may promote plaque stabilization by increasing collagen synthesis and LOX-dependent collagen maturation.

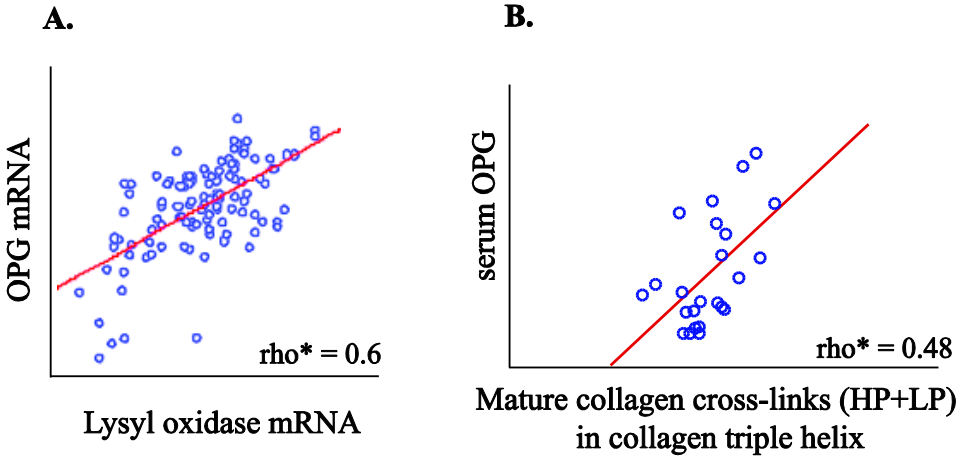

Figure 7. A significant positive correlation exists between mRNA expression of OPG and LOX according to gene array analysis of human carotid plaques (A); Serum levels of OPG correlate to the amount of mature enzymatic collagen cross-links in human carotid plaque samples (B).

Epidemiological studies describing the potentially harmful role of OPG in atherosclerosis conflict with the related experimental evidence to date. A plausible explanation for this discrepancy may be that OPG exerts its protective action locally in the vasculature. OPG concentration in human arteries is at least 100 times higher than in plasma (our own unpublished data and [230]). Thus, high levels of OPG in serum can reflect on-going injuries to the vessel wall and possible "leakage" of OPG from the atherosclerotic plaque. Supporting this hypothesis, OPG in supernatants from endarterectomy explants from symptomatic patients is elevated [235]. Therefore, new experimental strategies to maintain OPG within atherosclerotic plaque where it may facilitate fibrous cap stability are warranted.

6.4 LEUKOTRIENES IN PLAQUE STABILITY

Leukotrienes are lipid inflammatory and constrictive mediators derived from the 5-lipoxygenase pathway of arachidonic acid metabolism [245]. Metabolites of the 5-lipoxygenase pathway have strong proinflammatory activities in the cardiovascular system and thus may have an important role in atherogenesis.

Enzyme 5-lipoxygenase (5-LO) and 5-LO activating protein (FLAP) initiate the metabolism of arachidonic acid from membrane phospholipids and lead to formation on an unstable precursor leukotriene (LT)A4. FLAP has no enzymatic activity, but stabilizes 5-LO and improves its interaction with the substrate [246]. LTA4 can be further metabolized on the same cells or can be transferred to the neighboring cells such as leucocytes, SMCs, endothelial cells or platelets [245, 247]. Further LT synthesis follows two distinct pathways: either hydrolyzation into LTB4, or conjugation with glutathione to form the cysteinyl-LT: LTC4, LTD4 and LTE4 (known as slow reacting substances of anaphylaxis) (Figure 8)[247].

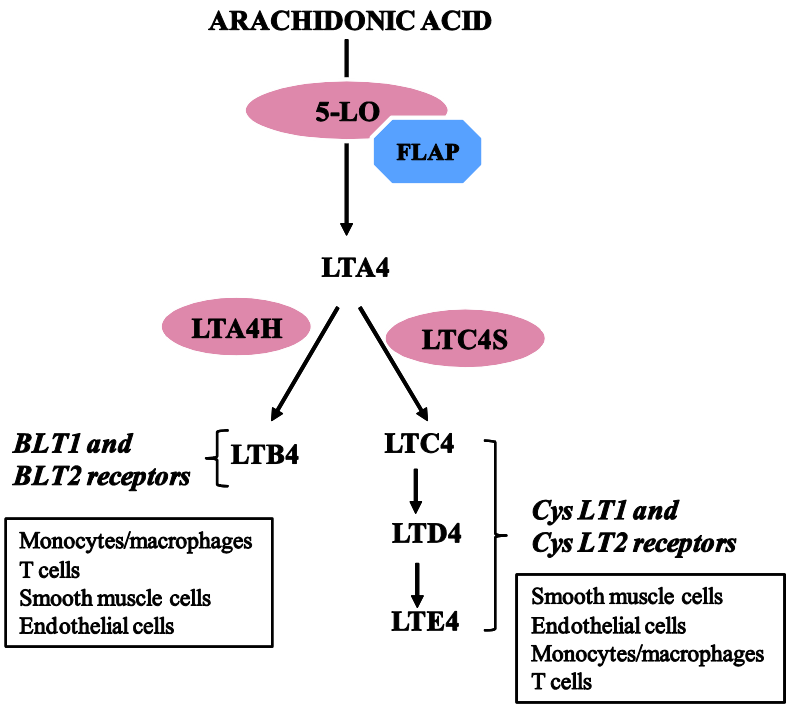

Figure 8. The scheme of the metabolism of arachidonic acid through 5-lipoxygenase (5-LO) pathway. FLAP – 5-LO activating protein, LTA4H – LTA4 hydrolase, LTC4S – LTC4 synthase, BLT and CysLT – receptors for BLT4 and cysteinyl-LTs, respectively. Adapted from [247].

LTs act in an autocrine/paracrine manner: LTB4 exerts its action via activation of G-protein-coupled cell surface receptors, BLT1 and BLT2. Cysteinyl-LTs activate receptors CysLT1 and CysLT2 [247].

The 5-LO pathway is involved in atherosclerosis progression. Genetic studies on mice identified loci on chromosome 6 that are associated with reduced 5-LO expression and confer resistance to atherosclerosis in C57BL/6J and in *Ldlr-/-* mice [248-250]. 5-LO was found to be expressed in atherosclerotic lesions of *Apoe-/-* and *Ldlr-/-* mice colocalizing with macrophage-rich areas [250]. Furthermore, 5-LO, FLAP, LTA4 hydrolase and BLT1 are significantly increased in human atherosclerotic lesions [251-253]. Variations of 5-LO, FLAP and LTA4 hydrolase genes show association with cardiovascular disease and atherosclerotic complications such as myocardial infarction and stroke [254-257]. Compound *Apoe-/-* x *Blt1-/-* deficient mice develop smaller atherosclerotic lesions with decreased numbers of SMCs, macrophages and T cells [258, 259]. Administration of FLAP inhibitors to hyperlipidemic mice protects them from atherosclerosis [260]. Dual inhibition of cyclooxygenase and 5-LO not only reduces lesion size and degree of local inflammation in *Ldlr-/-* mice, but also stabilizes the plaque by increasing SMC number and collagen content, and reduces proteolytic activity in lesions [261]. However, there are studies that argue with the involvement of 5-LO pathway in the pathogenesis of atherosclerosis [262].

The role of LTB4 in atherosclerosis is defined by its ability to recruit inflammatory cells and to maintain immune activation within the atherosclerotic lesion [247]. LTB4 exerts its action by stimulating BLT1 and BLT2 receptors on monocytes and T-cells, by upregulating chemokine production and by inducing nuclear factor (NF)-κB pathway [252, 258, 263]. LTB4 is thought to promote plaque vulnerability by increasing the activity of MMPs [263, 264]. In line with this, in diabetic patients 5-LO expression and LTB4 production are associated with increased levels of MMPs [265]. The role of LTB4 in collagen biosynthesis is unclear.

In **paper IV,** we investigated the role of FLAP in atherosclerosis. *Apoe*-/- mouse model possessing T cells lacking TGFβ suppression (*Apoe-/-* x CD4dnTβRII mice) was used [178]. These mice provide the model with predominantly activated T cell-driven inflammation. In the aortae of these mice, FLAP gene expression, assessed by

microarray analysis, was significantly upregulated compared to *Apoe-/-* controls. A 2 to 3- fold increase of FLAP mRNA in aortae, analyzed by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR), and a significant elevation of FLAP protein in lesions of *Apoe-/-* x CD4dnTβRII mice were also observed. In lesions, FLAP co-localized with CD68+ macrophages but not with T cells. LTB4, a product of FLAP-mediated reaction, was significantly elevated after *ex vivo* stimulation of aortae of *Apoe-/-* x CD4dnTβRII mice. As these mice have strongly activated T cells, it can be suggested that T cells increase FLAP expression and LTB4 production in macrophages in lesions. Supporting a proatherogenic role for FLAP, therapeutical inhibition of FLAP by administration of FLAP-inhibitor MK-886 significantly reduced atherosclerotic burden in *Apoe-/-* x CD4dnTβRII mice. Moreover, it decreased local inflammation defined by reduced number of T cells in the lesions and mRNA level for IFNγ in aortae of these mice.

Therefore, the study suggests that the FLAP/LT signaling has an important role in adaptive immunological circuits in vascular inflammation. Activated by T cells macrophages increase their production of LTB4 via FLAP-mediated pathway. LTB4, in turn, recruits more T cells into lesions and maintain their activation by enhancing NFκB pathway [252].

LOX expression and collagen maturation was impaired in mice with defective TGFβ signaling **(paper I)**. Administration of a FLAP inhibitor failed to restore LOX and procollagen type I expression (unpublished data) (Figure 9), suggesting that metabolites of the 5-LO pathway do not influence collagen stability in atherosclerotic plaques. Instead, these molecules exert their action primarily by attracting more inflammatory cells into lesions and by promoting secretion and activation of collagen degrading enzymes.

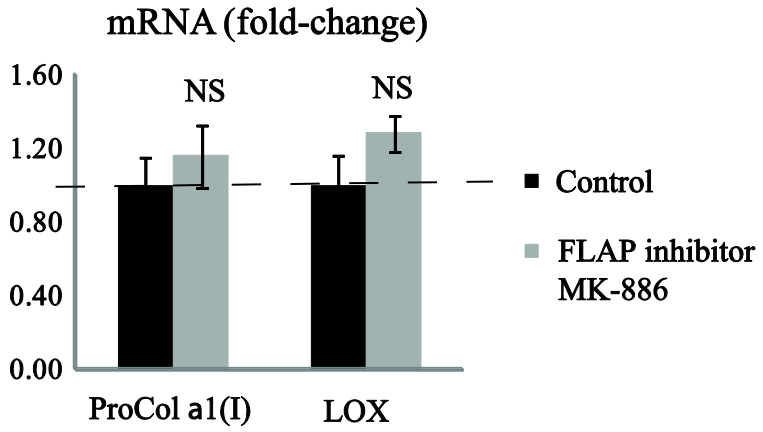

Figure 9. Aortic mRNA expression of procollagen type I and LOX in Apoe-/- x CD4dnTβRII mice treated with either a FLAP-inhibitor (MK-886) or tylose as a control. Results are expressed as fold-change mRNA expression compared with tylose-treated animals. NS denotes non-significant results.

7 METHODOLOGICAL CONSIDERATIONS

Our current understanding of atherosclerosis is based on molecular and biochemical evidence collected from animal models, cultured cells, human atherosclerotic lesions and from clinical as well as epidemiologic studies [1]. This thesis work encompasses all of these approaches to unravel the mechanisms causing plaque vulnerability.

7.1 MOUSE MODELS

Experiments in animals are needed to dissect the individual pathogenic steps and determine causality [1]. Under normal conditions laboratory mice do not develop atherosclerosis. Targeted deletion of the genes involved in lipid metabolism leads to severe hypercholesterolemia and spontaneous atherosclerosis [1]. Mice that lack apolipoprotein E (*Apoe-/-*) is an atherosclerosis-prone model used to study atherosclerosis [266]. Without a high-fat diet, these mice develop advanced atherosclerotic lesions already at the age of 18-weeks. We used these mice to study the effects of OPG injections in atherosclerosis development in **paper II**.

The *Apoe-/-* mouse is also a widely used model for further genetic manipulations. By mating these mice with knockout mice lacking immunoregulatory genes, it is possible to clarify the role of immunologic and inflammatory mechanisms in atherosclerosis [266]. A mouse strain that has a dominant negative TGF-β receptor under the CD4 promoter exists and is useful because these mice have T cells which produce non-functional TGFβ receptor and do not respond to anti-inflammatory TGFβ signaling [267]. This leads to an accelerated activation of T cells and increased production of INFγ. These mice were crossed with the *Apoe* knockout mouse strain to specifically address the role of TGFβ in atherosclerosis (*Apoe-/-* x CD4dnTβRII mice) [178]. Lesions of *Apoe-/-* x CD4dnTβRII mice display increased macrophage infiltration and MHC class II expression on cells which reflects elevated local INFγ production. Thus, we can investigate the effects of unsuppressed T cell-driven inflammation on plaque progression. We used *Apoe-/-* x CD4dnTβRII mice in **papers I and IV**.

7.2 HUMAN BIOBANKS (BIKE, SPICE)

Experimental evidence derived from non-human animal models must be validated by testing in humans or on human biological samples. Therefore, we analyzed human atherosclerotic plaques from two biobanks of carotid endarterectomies generated in Karolinska University Hospital, Stockholm, Sweden, and Almazov Federal Heart, Blood and Endocrinology Center and City Hospital #2, St. Petersburg, Russia. Upon informed consent, atherosclerotic plaques from carotid arteries were retrieved from patients during carotid endarterectomies. This biological material was stored frozen with the intent to be used for future research projects which would carry out molecular and biochemical strategies. Patient data and biographies were also collected.

The Biobank of Karolinska Carotid Endarterectomies (BiKE) was established in 2003 in collaboration between the Department of Vascular Surgery and the Cardiovascular Research laboratories at the Center for Molecular Medicine at Karolinska University Hospital [253, 268-270]. BiKE currently contains more than 450 atherosclerotic lesions. The Biobank of St. Petersburg Investigation of Carotid Endarterectomies (SPICE) was started in 2005 as a part of the collaboration between Karolinska Institutet and Almazov Federal Heart, Blood and Endocrinology Center (Table 4). SPICE contains more than 100 patients. Its clinical database is currently under development.

Table 4. General characterization of patients from biobanks BiKE and SPICE.

Parameters	**Values**	
	BiKE	**SPICE**
Number of patients	107	144
Male / Female	76.1% / 23.9	79% / 21%
Age, years (mean ± SD)	71.37 ± 8.61	62 ± 8.1
Pharmacological treatment:		
Antiplatelet agents	32.6%	80%
Statins	21%	38%
Angiotensin converting enzyme inhibitors	25.4%	58%
β-blockers	46.4%	53%

RNA from 144 human carotid endarterectomies from BiKE was analyzed by real-time RT-PCR **(paper III)**. Total gene expression profiling was performed on 69 RNA samples from the same cohort, using Affymetrix Gene Array U133 Plus 2.0. The samples were hybridized and scanned at the Karolinska Institute Affymetrix core facility; obtained cel-files were pre-processed and log2-transformed using RMA-normalization. Frozen sections, protein extracts, and serum from 24 human carotid endarterectomies from SPICE were also included.

A limitation in this study is the lack of an ideal mouse model or method to investigate plaque vulnerability. Human lesion samples are healed after the rupture and markers and mediators in these events are captured after the incident. Collagen composition is an important factor that protects the plaque against rupture. Therefore, we aimed to assess inflammatory mediators in collagen dynamics by using animal models and validated these mechanisms using human biological samples. Overlaying these two approaches can model how plaque stability is regulated in humans.

7.3 COLLAGEN ANALYSIS

Collagen content in tissues can be analyzed by histological techniques and biochemical methods. Apart from immunostaining against individual collagen types, histological staining using different dyes can be used. Traditional staining of fibrous tissues such as van Gieson and the various forms of trichrome are limiting. They do not stain thin collagen fibers [271, 272]. Picrosirius red binds specifically to collagen and stains all different collagen fibers including thin ones. The method can be powered by microscopic examination of stained tissues under polarized light, which allows the generation of high-contrast images of the collagenous skeleton in tissues including atherosclerotic plaques. A fundamental physical property of collagen fibers, its natural birefringence, favors the use of Picrosirius red method over other staining techniques. Due to its anisotropic properties, collagen fibers appear bright when viewed with polarized light. Picrosirius red dye also has an anisotropic molecular organization and when bound to collagen in an ordered manner, it enhances collagen birefringence [272]. The intensity of birefringence depends on thickness and orientation of fibers. Well

organized, thick and tightly packed collagen fibers retard the linear polarized light passing though the tissue. Such fibers appear much brighter and have bright orange-red colors (Figure 10). Thin and poorly packed fibers appear less bright and have a green color [273]. Finally, the method also helps to avoid identification of non-collagenous proteins such as proteoglycans and fibronectin [271]. We used the method in **papers I, II, III**.

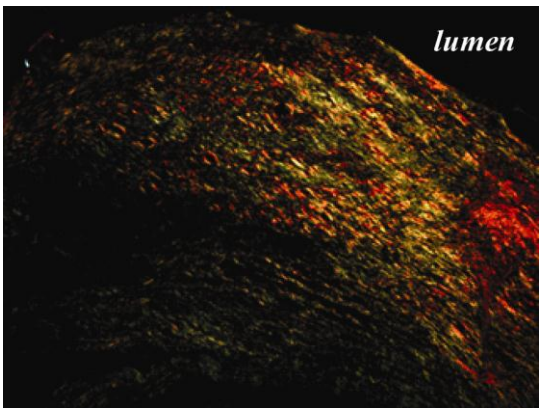

Figure 10. Micrograph presents a sample of human carotid plaque stained with Picrosirius red and visualized under polarized light. Magnification x100.

A limitation to the Picrosirius red method is that it does not allow distinguishing, non-reducible, enzymatic cross-links from non-enzymatic cross-links. This makes it unsuitable for analyzing collagen quality in complex, highly inflamed tissues, such as human atherosclerotic plaques. Therefore, in **paper III** we complemented the histological examination of collagen with biochemical analysis of different mature collagen cross-links. A high-performance liquid chromatographic assay of tissue extracts allows differentiated analysis of enzymatic cross-links such as hydroxylysylpyridinoline (HP) and lysyl-pyridinoline (LP), and non-enzymatic cross-links such as pentosidine. The cross-links are detected due to their native fluorescence. The quantities of each cross-link can be then expressed as the number of residues per collagen chain, assuming 300 hydroxyproline residues per triple helix given that the prolyl hydroxylation level in collagen is stable [274].

8 Quality matters: CONCLUDING REMARKS

The existing paradigm states that activated inflammatory cells in the plaques cause fibrous cap weakening by shifting the balance toward increased proteolytic degradation of collagen fibers. This thesis presents a new understanding to this concept. The strength of the fibrous cap does not only depend on the amount of collagen, but also on the efficiency of fiber cross-linking generated in complex multistep process of collagen maturation Proinflammatory mediators inhibit posttranslational maturation of collagen which leads to collagen fragility and reduce its resistance to proteolytic attacks and hemodynamic strain.

In **paper I,** T cell-driven inflammation reduces expression of LOX, the enzyme essential for collagen cross-linking. This results in a disorganization of collagen fibers in atherosclerotic lesions of *Apoe-/-* x CD4dnTβRII mice. In **paper II,** OPG treatment to *Apoe* knockout mice stabilized atherosclerotic lesions by promoting SMC accumulation, LOX-dependent collagen cross-linking and development of fibrous caps. In **paper III,** using human carotid atherosclerotic plaques, LOX protein was localized in the fibrous cap and perinecrotic regions. These areas contained both collagen and SMCs. A higher LOX mRNA and protein were associated with a more stable phenotype of the plaques. Examination of gene expression in plaques revealed a positive correlation between LOX and OPG and a negative correlation between LOX and markers of inflammation. In **paper IV,** activated Th1 cells in atherosclerotic plaques of *Apoe-/-* x CD4dnTβRII mice increased production of a lipid mediator, LTB4, in macrophages by enhancing 5-LO-mediated pathway. Secretion of LTB4 by macrophages could influence T cells by recruiting more T cells into lesions and activating NFκB pathway. Pharmaceutical FLAP inhibition provided a possible therapeutic tool to stabilize the plaque by reducing local inflammation. However, it did not have any significant effect on collagen synthesis.

A summary of the findings presented in this thesis is in Figure 11. Mechanisms that influence the stability of the atherosclerotic plaque are depicted here. The stability of the atherosclerotic plaque depends on the mechanical properties of collagen fibers in the fibrous cap. LOX catalyzes the extracellular cross-linking of fibrillar collagens and mediates their thermal and mechanical stability. Sufficient extracellular maturation of

collagen by LOX is important to maintain the stability of the collagen-rich fibrous cap. Catalyzing the formation of cross-links in the basement membrane collagen IV, LOX supports the integrity of the endothelial layer. Finally, LOX stabilizes the plaque by inducing the migration of SMCs to the fibrous cap and to perinecrotic regions of the plaques (**Paper III**).

Collagen fiber stability depends on the balance between proinflammatory and anti-inflammatory stimuli in the atherosclerotic plaque. Activated Th1 cells produce IFNγ and promote the development of a highly inflammatory microenvironment in the fibrous cap by activating adjacent cells. This leads to decreased secretion of procollagen and reduced production of LOX (**Paper I and III**). Insufficient cross-linking of collagen makes it "vulnerable". Weak fibers cannot resist hemodymanic strain and are more prone to proteolytic degradation. Collagen-degrading proteases have better access to cleavage sites of loose-packed collagen fibers and degrade them more efficiently. All these lead to fibrous cap weakening and eventually to plaque rupture.

Activated by Th1 cells, macrophages produce proinflammatory mediators, including LTB4 that sustains the proinflammatory cascade within the atherosclerotic plaque (**Paper IV**). LTB4 is capable of inducing MMPs production in macrophages in an autocrine/paracrine manner.

The impact of proinflammatory mediators is balanced by anti-inflammatory, profibrotic agents, such as OPG, that is produced by SMCs and endothelial cells in the lesion. OPG can increase proliferation and induce collagen synthesis in SMCs (**Paper II and III**). OPG can also induce production of LOX, which promotes stabilization of newly secreted collagen fibers.

In summary, this thesis presents novel evidence showing that LOX-dependent collagen cross-linking has an important role in the stabilization of the atherosclerotic plaques. Cross-linked collagen fibers have higher tensile strength, which reduces the risk for the fibrous cap rupture and thrombotic events. The process of collagen maturation is regulated by pro- and anti-inflammatory mediators within the atherosclerotic plaque. The evidence presented here, for the first time, offers a new understanding to the impact of immunological mediators in development of myocardial infarction and stroke, and provides potential targets for plaque stabilization therapy.

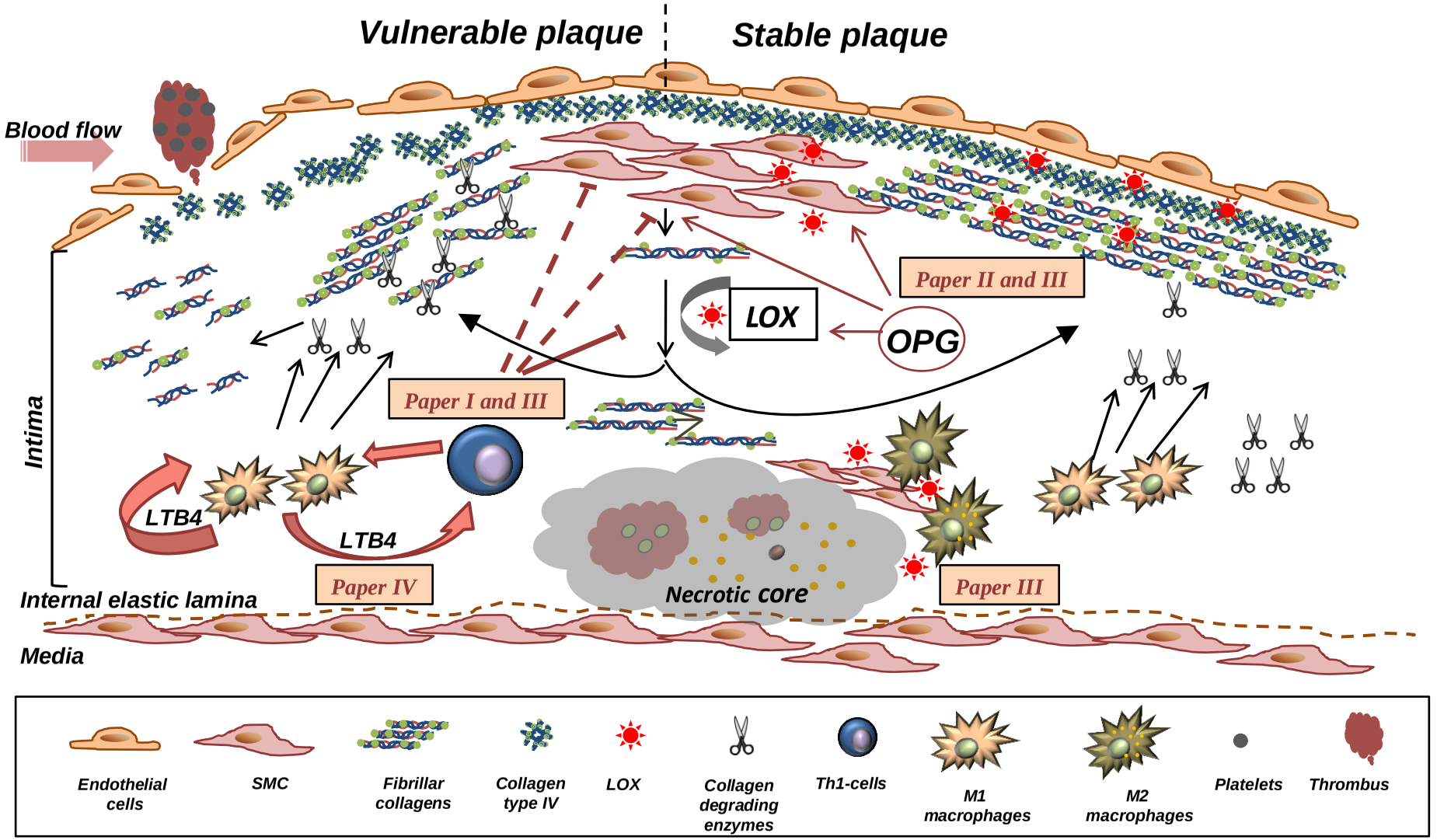

Figure 11. Concluding remarks. Explanations are in the text.

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