Unravelling the collagen network of the arterial wall

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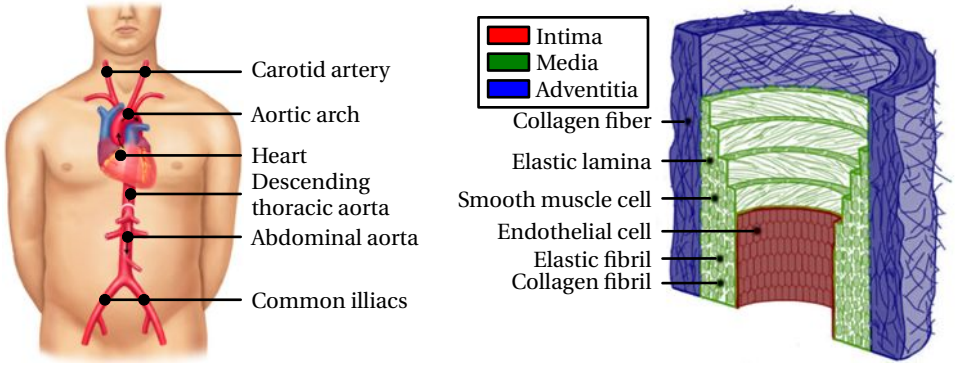
2 Background



(a) Cardiovascular system (b) Aortic wall

Figure 2.1: Anatomy of the human cardiovascular system (a) and the aortic wall (b).[1]

2.1 The aorta

The aorta, the largest artery in the human body, originates from the left ventricle of the heart and extends down to the abdomen where it branches into two smaller arteries, the common iliacs. It is now well recognized that, beyond serving a conduit function, the aorta undertakes through its mechanical properties important roles in regulating left ventricular performance and the arterial function of the entire cardiovascular system.[2–4]

The mechanical properties of the aorta allow it to serve as an elastic reservoir.[5] The aorta expands with blood each time the heart contracts (systole) and then recoils elastically to continue the supply of blood to the small peripheral vessels while the heart is refilling (diastole). This phenomenon is often described by the windkessel model[6–8], but this model has been also criticized widely for being too simple and not useful to describe various diseased states.[4, 9, 10]

2.1.1 Anatomy of the aorta

The human aortic wall consists of three distinct layers, depicted in fig. 2.1b, which, together, give rise to its mechanical properties. The innermost layer, the tunica intima, consists of a thin layer of endothelial cells and is supported by the internal elastic lamina. These endothelial cells are in direct contact with the blood flow. The intima is particularly important in the disease of atherosclerosis (chapter 6).

The middle layer, the tunica media, is mainly composed of smooth muscle cells and elastin. The elastin is arranged into lamellae, between which collagen fibers,

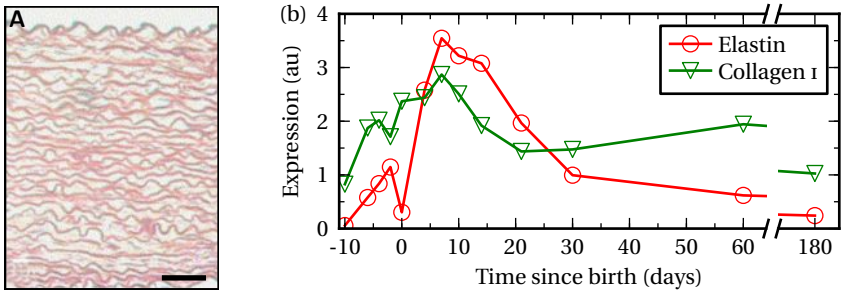


Figure 2.2:
(A) Circumferential section of the aortic wall showing the wavy elastin fibers (Verhoeff's elastica, scale bar is approximate 100 µm).[14]
(b) Collagen and elastin expression in the developing mouse aorta, showing a decrease in the elastin expression a few weeks after birth. Collagen, however, remains expressed during the whole lifespan.[15]

proteoglycans and smooth muscle cells are found. Thin elastic fibers connect the lamellae into a three-dimensional continuous network.[11, 12] Interestingly, the number of lamellae does not change after birth.[5]

The outermost layer of the vessel wall, the adventitia, starts next to the external elastic lamina. This layer consists of a collagen-rich extra cellular matrix and helps to prevent vascular rupture at extremely high pressures.[13] The adventitia of the aorta also contains the vasa vasorum, a network of small blood vessels that provide oxygen and nourishment to the cells in the vessel wall.

2.1.2 The extra cellular matrix of the aorta

The mechanical characteristics of blood vessels are determined by both active and passive components. The extra cellular matrix (ECM), a network composed mainly of collagen, elastin and proteoglycans, is responsible for the passive part, whereas smooth muscle cells are responsible for the active part. The macromolecules of the ECM are synthesized by three vascular cell types: intimal endothelial cells, medial smooth muscle cells and adventitial fibroblasts.

Elastin

In tissues where elastic recoil is necessary, elastic fibers consisting mainly of elastin are present in the extra cellular matrix. In the aorta, elastin constitutes as much as 40% of the total dry weight.[5, 16] Elastin is synthesized by the cells in the vessel wall as a soluble precursor molecule tropoelastin and is cross-linked by lysyl oxidase.[17] The elastic fibers of the aorta are composed of an elastin core

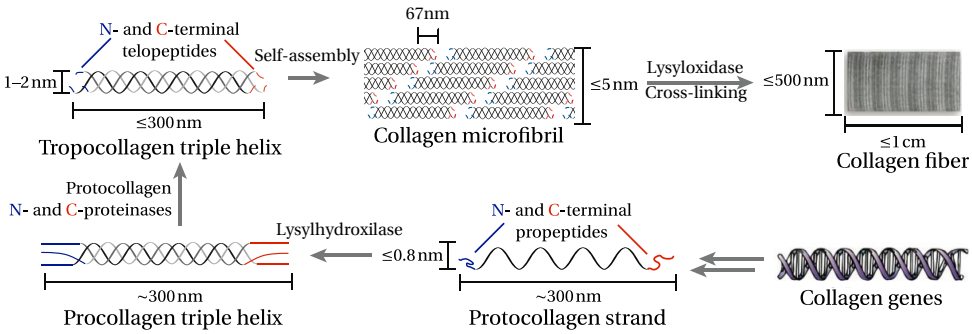


Figure 2.3: The assembly of collagen fibers.[23]

and are surrounded by a mantle of fibrillin-rich microfibrils.[18] Elastin contains a high number of cross-links per monomer, 15–20 compared to 1–4 cross-links per collagen monomer,[5] which is important for its recoil properties.

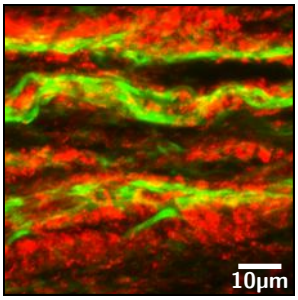
When the elastic lamellae in the aortic wall are unpressurised, they appear wavy in longitudinal and transverse sections, as is shown in fig. 2.2A. With increasing pressure and distention, there is a progressive straightening of the lamellae and the inter-lamellar distance decreases. At 10 kPa (75 mm Hg), the low end of the physiological pressure range, the lamellae are straight and give the appearance of regular concentric cylinders.[1]

The high degree of cross-linking is also responsible for the fibres longevity. Shapiro et al. showed, using ^{14}C turnover and aspartic acid racemization, that the elastin fibre is a metabolically stable unit over the human lifespan.[19] Other studies suggest that less than 1% of the total body elastin pool turns over in a year.[20]

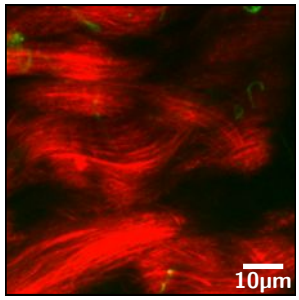
In most tissues elastin expression occurs over a narrow window of development, starting in mid gestation and ending in the postnatal period,[21, 22] see fig. 2.2b. This explains the incomplete repair of elastin during adult life which is a key element in many diseases. The extensive loss of medial elastin, for example, is traditionally considered the hallmark of aneurysm formation, although it is now acknowledged that aneurysmal growth is also related to an impaired collagen homeostasis (see chapter 4).

Collagen

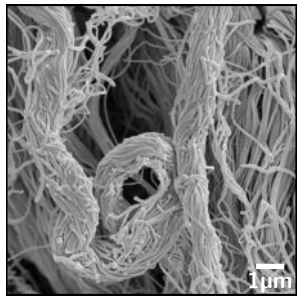
Collagen is the main constituent of the extra cellular matrix of animal connective tissues, compromising one-third of the total proteins in humans. In contrast to elastin, collagen fibers are rather stiff polymers.[24, 25] Currently twenty-eight different types of collagens have been identified.[26, 27] Collagen types I and III



(a) Media



(b) Adventitia



(c) SEM

Figure 2.4:
(a), (b) Multi-photon images of the porcine aortic wall showing the elastin (green) and collagen (red) fibers. In the media (a) the collagen fibers appear to be located around the elastin fibers, while they form a intertwined network in the adventita (b).
(c) SEM image of collagen fibers in the adventita of the human arterial wall shows a highly organized network (5.0 kV, 7000×).

are the major collagens in blood vessels, representing 60% and 30% of vascular collagens respectively.[28–30]

In vivo, individual collagen triple helices, known as tropocollagen, assemble in a complex, hierarchical manner that ultimately leads to the macroscopic fibers and networks observed in tissue, bone, and the vascular wall (fig. 2.3). This process is very dependent on the specific amino acids and a minor mutation can therefore already cause serious diseases such as the Ehlers-Danlos syndrome.[31, 32]

Collagen deposition in the medial layer is best characterized by small, interdispersed collagen fibrils that run mainly parallel to the main axis of the smooth muscle cells as well as to the streaks of elastin protruding from the elastic lamellae (fig. 2.4a).[12] Adventitial collagen, on the other hand, is arranged in a loose knitting of highly organized ribbon-like collagen bands that brace the medial and intimal layers of the vessel wall, fig. 2.4b. These different architectures appear optimal for realizing the different functionalities for the aortic medial and adventitial layers, elastic recoil and resilience, respectively.[33, 34] At physiological pressure, less than 10% of the collagen fibers are engaged, whereas at higher pressures, the vessel becomes progressively less distensible as more collagen fibers are being stretched.[35]

Proteoglycans

Proteoglycans are a group of diverse macromolecules that contain core proteins to which one or more glycosaminoglycans are covalently attached. The proteo-

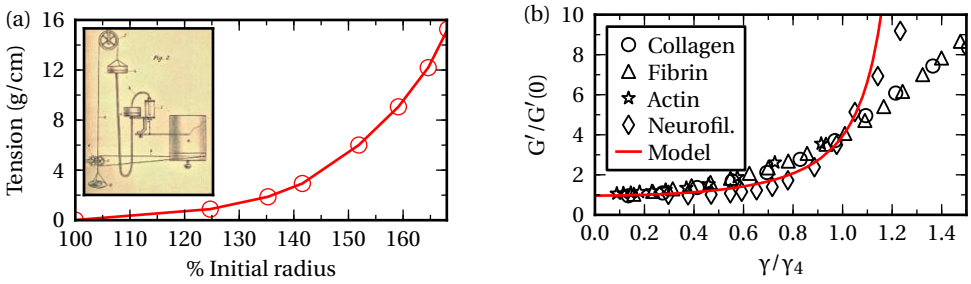


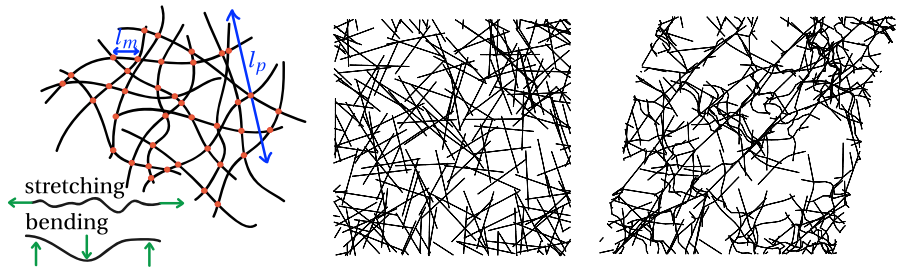
Figure 2.5:
(a) The mechanical response of the human iliac artery to inflation, showing its non-linear response to stress.[48]
(a inset) Schematic representation of the setup with which Charles Roy studied the elasticity of the aorta.[49] A detailed explanation of the setup can be found in ref. [1].
(b) Scaled modulus-strain curves for various biopolymer networks compared to model proposed by Storm et al. The shear moduli of the networks of collagen, fibrin, F-actin and neurofilaments are measured in a strain-controlled rheometer.[50]

glycans found in greatest abundance in the vessel wall can be categorized into two classes: large proteoglycans, that form an interconnected polymeric network by interactions with hyaluron, and small leucine-rich proteoglycans.[5] Electron microscopy and immunofluorescence studies strongly suggest the association of proteoglycans with specific regions of the banding pattern of collagen fibrils and also indicate that proteoglycans can form bridges between fibrils.[36–40]

Although proteoglycans constitute a minor component of vascular tissue (2% to 5% by dry weight),[41] they have an important influence on the mechanical properties of the tissue. Proteoglycans have a net negative charge under physiological conditions and produce a swelling stress which depends on the ionic strength of their environment.[42, 43] The inhomogenous distribution of proteoglycans across the vessel wall, showing a higher concentration in the intimal and medial layers than in the adventitia,[44, 45] gives rise to a residual stress in the aortic wall.[46, 47]

2.2 The physics of networks

One of the remarkable physical properties of many different types of biological tissues is its non-linear behavior under stress. The harder the tissue is strained, the stiffer it becomes. This strain-stiffening behavior allows for small deformations of tissues like the skin,[51] aorta[1] and blood clots,[52] but prevents large deformations that could threaten tissue integrity. The earliest quantitative study of vascular elasticity appears to be the work of Charles Roy (1881).[49] He con-



(a) Model (b) No strain (c) Large strain

Figure 2.6:
The model proposed by Onck et al., describes the network as fibers with a long persistence length, l_p, compared to the mesh size of the network, l_m, e.g. a network of collagen fibers. This renders the effect of entropic stiffening minimal compared to the penalty of bending, resulting in non-affine deformations of the network during strain. Images adopted from ref. [58, 59].

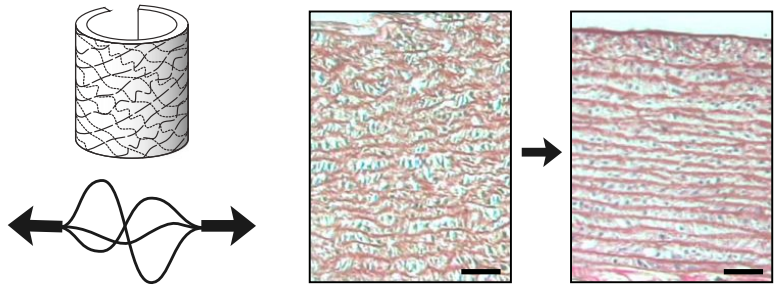
structed an ingenious gravity-driven apparatus, shown in the inset of fig. 2.5a, that performed in vitro inflation of blood vessel segments, measured instantaneous pressure and volume change, and plotted the resulting P – V curves to a rotating smoked-drum kymograph.

In recent years many different experimental and theoretical studies have shed a first light on the physical principles that determine the mechanical properties of tissues. Most experimental studies comprise of an in vitro system in which one or two different isolated components of the extra cellular matrix are combined to make a gel and reveal a rich interplay between fibers, linkers and cells.[53–55] Rheological measurements on, for example, reconstituted, collagen showed that these gels exhibit a similar strain-stiffening behavior as has been observed in vivo.[56, 57]

2.2.1 Theoretical models

Experimental observations such as the ones described in the previous section are the input of many different theoretical studies, which make an effort to explain the observed mechanical response. In 2005, C. Storm et al. proposed a model in which the force-response of networks is dominated by entropy.[50] In this model all the fibers react individually to the applied deformation. Because there are many curled-up configurations of a fiber and there is only one perfectly straight configuration, stretching a flexible filament reduces its conformational entropy and thus produces an opposing force.[50, 60–62] The collective non-linear behavior of this tissue model is due to the sum of nonlinear response of all the individual fibers.

The model proposed by Onck et al. argues that the entropic contributions to



(a) Model (b) Input from histology

Figure 2.7:
The model proposed (a) by Fonk et al. describes the vessel wall as an elastic medium in which fibers of different lengths are embedded. As the vessel wall is stretched (b), the fibers are sequentially recruited which causes the stiffness of the tissue to increase. Many of the different parameters of this model are obtained from histological experiments. Scale bars are approximate 100µm. Images are adopted from ref. [14, 69].

the stiffness of the tissue are of minor importance compared to the nonaffine network rearrangements that occur during strain, fig. 2.6.[59] This model shows a strain-stiffening behavior can be the result of a transition from a bending-dominated response at small strains to a stretching-dominated response at higher strains.[58, 59, 63–65]

The long thermal persistence length of collagen fibers, ~1cm, compared to a typical cross-link spacing of ~2µm, renders the entropic stiffening effects in collagen gels to be minimal.[53] However, for other biopolymers such as actin, where the persistence length of the filaments is in the order of the distance between cross-links, entropic stiffening of the individual fibers is more likely to be an important component. Other recent studies have shown that the specific details of the individual cross-links also play an important role in the mechanics of the whole system.[53, 66–68] A system composed of randomly oriented rods connected by flexible cross-links, for example, already represents nonlinear network behavior.[66]

A theoretical model of the aortic wall

The models mentioned above describe the behavior of polymer gels composed of filaments and cross-links. These models predict the response measured in different in vitro systems, but these models still lack the complexity of real tissues in which not only many different components are present, but in which also the specific spatial organization of the different fibers greatly contributes to the mechanical response.[70, 71]

Another class of models, specifically developed for the vessel wall, describes the elastin fibers as an elastic reservoir in which collagen fibers of varying length are embedded.[14, 69, 71–77] As the stress increases, the collagen fibers are sequentially recruited causing an increasing stiffness of the tissue, as is illustrated in fig. 2.7. This model nicely fits the measured strain-stiffening of the arterial wall and has been used to study the effect of elastin degradation[74] and aging[73] on vessel mechanics. This mean-field approach, however, lacks the details of the interactions between the different components at the fiber level as is described by the earlier models and is therefor unable to describe the ECM remodeling occurring in many diseases.

2.3 The approach of this thesis

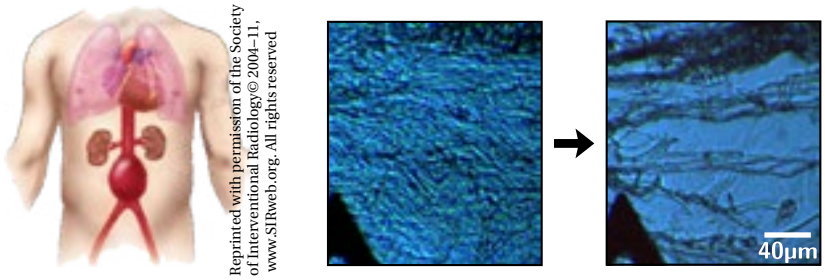
In this thesis we present our study of how the different components of the ECM determine the mechanical properties of the aortic wall and how they are related to different diseases. By this study we also hope to contribute to the process which is currently made to get a better understanding of the physics of tissues at the fiber level. We do this by locally probing the mechanical properties with the Atomic Force Microscope, while different optical techniques will be used to link these findings to the network structure of the tissue. The different techniques used in the thesis are described in chapter 3.

Aneurysms of the abdominal aorta

Aneurysms are localized dilatations of the vessel wall that are caused by a segmental weakening of the vessel wall, fig. 2.8a.[78, 79] Although aneurysms generally are without clinical symptoms, larger aneurysms may rupture, and bleeding from a ruptured aneurysm is responsible for more than 15,000 annual deaths in the United States alone.[80]

Aneurysm formation relates to defects in the extra cellular matrix resulting in attenuation and ultimate failure of the vessel wall.[81] Remarkably, although numerous studies have looked for putative quantitative changes in aortic collagen, results reported to date are controversial.[82–84] With the exception of rare mutations in the collagen III gene, such as the vascular type of Ehlers-Danlos syndrome, no clear association between impaired collagen homeostasis and aneurysm growth and/or rupture has been identified.

In this thesis the aortic wall of an healthy individual is compared to an aneurysm of the abdominal aorta (AAA) and to the aortic wall of a patient with the Marfan syndrome. The Marfan syndrome is a systemic disorder caused by mutations in the ECM protein fibrillin-1.[85–88] Fibrillin-1 microfibrils are thought



(a) AAA (b) Tissue digestions

Figure 2.8:
(a) An aneurysm of the abdominal aorta (AAA) is a localized dilatation of the arterial wall.[91]
(b) The effect of collagenase treatment on the adventitia of the arterial wall. In the lower left corner of the image the AFM cantilever, used for the mechanical probing, is visible.

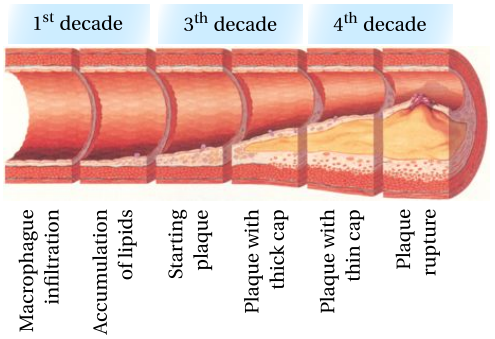
to confer important biomechanical properties in connecting, anchoring and maintaining tissues and organs.[86] The clinical manifestations of Marfan are varied in range and involve many organs.[89] The majority of anomalies are found in the cardiovascular, respiratory, ocular and skeletal system. Aortic dilatation is the most common cause of morbidity and mortality under Marfan patients.[90]

In search of the collagen defect(s) underlying aneurysm formation, we applied an integrated approach of biochemical analyses, multiple imaging modalities, and mechanical analysis to identify the putative collagen defect in AAA and in Marfan syndrome. Results of this evaluation, chapter 4, show that advanced stages of aneurysmal disease are characterized by distinct defects in the network structure of adventitial collagen rather than by purely biochemical defects.

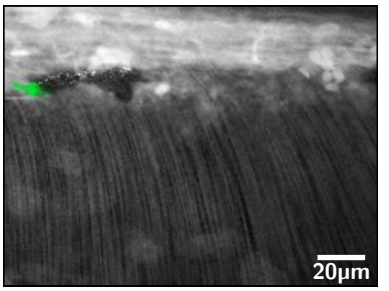
Proteolytic alterations of the arterial wall

The behaviour of cells is largely influenced by their surroundings.[92–94] While the biochemical interaction between cells and their environment has been widely studied in different systems, more recently different studies have shown the importance of the physical interaction of the cells with their surroundings, for example its mechanical properties. Fibroblastic cells, for example, spread less on a soft substrate[95] and have been observed to migrate toward stiffer substrates.[96]

Cells are not only able to respond to mechanical cues in their local environment, they are also able to change the mechanical properties of the same environment. The highly remodeled vessel wall of aneurysms, are, for example, linked with a highly elevated number of neutrophils.[97–99] One of the questions is whether the structural rearrangements of the aorta cause the inflammation or are a result of the inflammation.



(a) Atherosclerosis



(b) Migrating neutrophil

Figure 2.9:
(a) The timeline of atherosclerosis starts with endothelial dysfunction which causes an inflammatory response due to the infiltration of lipids into the vessel wall. The onset of the disease already starts at the first decade, while its clinical manifestations start from the fourth decade.[100]
(b) Migrating neutrophils (green) alter the collagen network (white) in a zebrafish cancer model.

In chapter 5, we examine the effects of enzymatic digestion of the extra cellular matrix of the aortic wall, e.g. due to the contents of neutrophils, in order to better understand how cells change the mechanical properties of their environment. By starting with real tissue and selectively removing different elements, we are able to measure the contribution of the different constituents of the ECM to the mechanical properties of the whole tissue.

We do not only show that enzymatic treatment of the aortic wall greatly reduces its stiffness, but also that a simple treatment with the contents of neutrophils is able to mimic the in chapter 4 observed change in mechanical response from a healthy aorta to an aneurysm.

Atherosclerotic plaques

Atherosclerosis is a leading cause of death in the western world and is responsible for coronary heart disease, the majority of strokes and limb ischemia.[101] In atherosclerosis, cholesterol and components of the immune system accumulate in the vessel wall. Although luminal narrowing by such an atherosclerotic plaque contributes to some of the clinical manifestations, it is the rupture of such a plaque, followed by the formation of a blood clot, that causes the most acute and serious clinical manifestations of this disease.[102, 103]

Atherosclerosis typically starts in early adolescence and is usually found in most major arteries. Figure 2.9a schematically show the progression of the disease. The main cause of atherosclerosis is still a topic of many studies, but

the common thought is that the process is initiated by the infiltration of LDL cholesterol in the vessel wall due to endothelial dysfunction.[104] The presence of these lipids in the vessel wall leads to an inflammatory response, triggering macrophages to enter the vessel wall. The macrophages absorb the lipids, but are not able to process them, and will eventually rupture. This results in a greater amount of inflammatory signals, triggering the recruitment of more macrophages, continuing the cycle.

The contents of the ruptured macrophages are highly thrombogenic and contain many ECM degrading enzymes.[101, 105] The blood is protected from this so called necrotic core by a thick layer of collagen, which is secreted by smooth muscle cells. During the progression of the disease, the collagen cap gets thinner by the loss of the collagen producing smooth muscle cells on the one hand, and the ECM degrading enzymes inside the core on the other. A plaque with such a thin collagen cap can eventually rupture, bringing the highly thrombogenic core into contact with the blood. This triggers the formation of a thrombus (blood clot), which can block the blood supply and give rise to many lethal complications.[101, 103, 105, 106]

The combination of an altered ECM synthesis and degradation in the collagen cap leads to a remodeling of the collagen network, which affects the mechanical stability of the plaque.[105] Lysyl oxidase (LOX) is an extracellular enzyme that catalyzes the cross-linking of collagen fibrils, which results in the stabilization of extracellular collagen.[107, 108] In chapter 6 we show that higher LOX MRNA and protein levels are associated with a more stable phenotype of atherosclerotic plaques. This suggests that by promoting collagen cross-linking and the formation of thick collagen fibers with high tensile strength or an increased resistance to degradation by enzymes in the core, LOX may reduce the risk for plaque rupture and the development of lethal complications of atherosclerosis.

Migrating neutrophils in a zebrafish cancer model

The interactions between malignant tumor cells and their micro environment have a central role on tumor progression.[109, 110] The zebrafish, Danio rerio, has become an import animal model for cancer and immune research over the last decade.[111, 112] Many molecular and cellular components that operate during tumorigenesis are conserved between zebrafish and mammals.[113] The transparency of zebrafish, in combination with the availability of various tissue specific fluorescent reporter transgenic lines, allow for high resolution analysis of the tumor progression and the interactions between the tumor cells and the host microenvironment in vivo. Several tumor transplantation assays with human and mammalian cells have been developed to study different aspects of tumor

malignancies in embryo and adult zebrafish, such as tumor cell invasion.[114–117]

We have adopted the currently available multi-photon imaging techniques to be used on zebrafish. In chapter 7 we also present the results of a novel xenograft model that allows the visualization of all tumor development hallmarks. This new model elucidates how the transmigration of neutrophils remodels the collagen matrix, fig. 2.9b, and facilitates the invasion of tumor cells.

2.4 References

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