Targeting glycolysis in endothelial cells to prevent intraplaque neovascularization and atherogenesis in mice

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Chapter 3

Animal models of atherosclerosis

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Abstract

An ideal animal model of atherosclerosis resembles human anatomy and pathophysiology and has the potential to be used in medical and pharmaceutical research to obtain results that can be extrapolated to human medicine. Moreover, it must be easy to acquire, can be maintained at a reasonable cost, is easy to handle and shares the topography of the lesions with humans. In general, animal models of atherosclerosis are based on accelerated plaque formation due to a cholesterol-rich/Western-type diet, manipulation of genes involved in the cholesterol metabolism, and the introduction of additional risk factors for atherosclerosis. Mouse and rabbit models have been mostly used, followed by pigs and non-human primates. Each of these models has its advantages and limitations. The mouse has become the predominant species to study experimental atherosclerosis because of its rapid reproduction, ease of genetic manipulation and its ability to monitor atherogenesis in a reasonable time frame. Both Apolipoprotein E deficient ($ApoE^{-/-}$) and LDL-receptor ($LDLr^{-/-}$) knockout mice have been frequently used, but also ApoE/LDLr double-knockout, ApoE3-Leiden and PCSK9-AAV mice are valuable tools in atherosclerosis research. However, a great challenge was the development of a model in which intraplaque microvessels, haemorrhages, spontaneous atherosclerotic plaque ruptures, myocardial infarction and sudden death occur consistently. These features are present in $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice, which can be used as a validated model in pre-clinical studies to evaluate novel plaque-stabilizing drugs.

Keywords: animal models, atherosclerosis, plaque rupture, ApoE, LDL receptor, PCSK9

Introduction

Atherosclerosis is a progressive inflammatory disease characterized by accumulation of lipids in the arterial vessel wall, which starts early in life. Disease progression leads to build-up of atherosclerotic plaques that cause narrowing of the arterial lumen. Atherosclerotic plaques often remain stable for years, but can rapidly become unstable, rupture and trigger thrombus formation. Accordingly, in addition to restriction of the vessel lumen, the presence of atherosclerotic plaques is linked to an increased risk of acute cardiovascular events such as myocardial infarction (MI) and stroke. The use of animal models of atherosclerosis is an essential tool to improve the understanding of the molecular mechanisms behind atherosclerotic plaque formation and progression, as well as the occurrence of plaque rupture and its associated cardiovascular events. Moreover, animal models allow to assess novel pharmacological treatments that can prevent or slow down the onset of atherosclerosis. In general, animal models for atherosclerosis are based on accelerated plaque formation due to: (1) a cholesterol-rich/Western-type diet, (2) manipulation of genes involved in the cholesterol metabolism, and (3) the introduction of additional risk factors for atherosclerosis, such as diabetes.

In this review, we will discuss the animal models that have contributed to the understanding of atherosclerosis and its clinical consequences, and that allow significant improvement in treatment.

Numerous studies have shown that high plasma levels of low-density lipoprotein (LDL) represent one of the most prominent risk factors of atherosclerosis. Indeed, LDL tends to accumulate in the sub-endothelial space of the arterial wall and progressively undergoes oxidative modifications to form oxidized LDL (oxLDL). This induces an inflammatory response characterized by overexpression of chemotactic proteins such as monocyte chemoattractant protein-1 (MCP-1), and adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin) by endothelial cells. [1, 2] Adhesion molecules promote the infiltration of blood-carried monocytes into the inflamed arterial wall. After differentiation into macrophages, these cells engulf oxLDL, transform into foam cells and contribute to plaque development by secreting multiple mediators of the inflammatory process in the vessel wall. [3] The inflammatory response also promotes recruitment of circulating

monocytes and T-cells that stimulate the migration of vascular smooth muscle cells (SMCs) from the tunica media into the sub-endothelial space where they exhibit abnormally high proliferation and secrete extracellular matrix proteins that also contribute to atheroma growth.[1] Advanced human plaques are characterized by a large necrotic core, many lipid laden and activated macrophages, few SMCs, intraplaque neovascularisation and haemorrhages, and a thin fibrous cap that separates the plaque from the blood stream. Rupture of the fibrous cap of such high-risk vulnerable plaques leads to luminal thrombosis, arterial occlusion or embolism in distant vascular beds, resulting in MI, stroke or sudden death. [4] In humans, atherosclerotic plaques can usually be found in the aorta, coronary arteries and in the carotid and cerebral arteries. [5]

In 1908, Ignatowski investigated for the first time plaque formation in the aortic wall of rabbits that were fed a cholesterol-rich diet. [6] Since then many other animal species such as mice, birds, pigs and non-human primates have been used as an experimental model of atherosclerosis. [1, 7]

An ideal animal model resembles human anatomy and pathophysiology, and has the potential to be used in medical and pharmaceutical research to obtain results that can be extrapolated to human medicine. Moreover, it is important that animals used as models are easy to acquire, can be maintained at a reasonable cost, are easy to handle and have well-defined genetic characteristics. A valuable animal model for atherosclerosis research not only shares the crucial aspects of the disease process with humans but also the topography of the lesions. In addition, the animals preferably develop lesions in a spontaneous manner after consumption of a diet similar as in humans. [8] Although several animals develop atherosclerotic plaques after a cholesterol-rich diet, the topography of the lesions is not always similar as compared to humans. Furthermore, it is important to note that in the majority of atherosclerosis models, animals do not spontaneously develop the complications seen in humans such as plaque rupture, MI, stroke and sudden death.

Mouse models of atherosclerosis

Over the past decades, the mouse has become the predominant species to study experimental atherosclerosis because of its rapid reproduction, ease of genetic

manipulation and its ability to monitor atherogenesis in a reasonable time frame.[8-11] However, mice are relatively resistant to the development of atherosclerosis due to their significantly different lipid profile as compared to humans. Therefore, genetic manipulation of their lipid metabolism is mandatory.[8, 12] In mice, most of the cholesterol is transported in high density lipoprotein (HDL) like particles. Accordingly, mice contain only low concentrations of the atherogenic LDL and very low density lipoprotein (VLDL). Mice deficient in the receptor clearing these LDL particles ($LDLr^{-/-}$ mice) develop significantly higher plasma levels of cholesterol. Apolipoprotein E (ApoE) is a glycoprotein synthesized mainly in the liver and the brain and functions as a ligand for receptors that clear chylomicrons and VLDL remnants.[12] Deficiency in this glycoprotein ($ApoE^{-/-}$) leads to increased plasma levels of total cholesterol, mostly in the VLDL and chylomicron fractions,[13] which are quadrupled by a high-fat or Western-type diet.[14] Both mouse models have extensively been used to study the mechanisms underlying the initiation and progression of atherosclerosis. Atherosclerotic lesions in mice develop in regions of the vasculature subjected to low and/or oscillatory shear stress. [9, 10] Predilection sites in the mouse are the aortic root, lesser curvature of the aortic arch and branch points of the brachiocephalic, left carotid and subclavian arteries. However, on a high-cholesterol diet, $ApoE^{-/-}$ mice develop plaques more rapidly and with a more advanced phenotype as compared to $LDLr^{-/-}$ mice [15], making the $ApoE^{-/-}$ model widely used in experimental atherosclerosis studies.

The Apolipoprotein (Apo) E3-Leiden mutation is associated with a genetic form of hyperlipidaemia. Therefore, ApoE3-Leiden transgenic mice can also be used as a model for atherosclerosis, but in comparison with $ApoE^{-/-}$ and $LDLr^{-/-}$ mice, they show rather low levels of total plasma cholesterol and triglycerides when fed a normal diet. Nevertheless, these mice are highly responsive to fat-, sugar-, and cholesterol-containing diets resulting in strongly elevated lipoprotein profiles.[16] Regardless of lesion development, varying from fatty streaks to mild, moderate, and severe plaques, ApoE3-Leiden mice lack the critical events such as plaque rupture, thrombus formation, and/or haemorrhage, which are of major importance in human atherosclerosis. [17, 18]

High plasma levels of lipoprotein (a) [Lp(a)], which is a complex of LDL and a large glycoprotein called Apolipoprotein (a) [Apo(a)], is an independent risk factor for the

development of atherosclerosis in humans.[19, 20] Virtually all species other than primates lack Apo(a), hampering the use of convenient animal models to study its role in atherosclerotic plaque development. Therefore, transgenic mice that express human Apo(a) are used. When fed a Western-type diet, these mice show the presence of macrophage-like cells in combination with the development of fatty-streak lesions at the base of the aorta. [21] In humans, plasma Apo(a) is almost entirely covalently bound to LDL, whereas in mice, Apo(a) circulates as non-lipoprotein associated Apo(a). [21] Therefore, Apo(a) transgenic mice can be used to identify the role of Apo(a) in atherogenesis, independent of human LDL.
The most commonly used mouse models of atherosclerosis are described in detail below and in Figure 1.

Apolipoprotein E deficient (ApoE$^{-/-}$) mice

ApoE is a glycoprotein with a molecular size of approximately 34 kDa. It is synthesized mainly in the liver and brain, and is a structural component of all lipoprotein particles except low-density lipoproteins. It serves as a ligand for cell-surface lipoprotein receptors whose function is to clear chylomicrons and VLDL remnants. It is also synthesized by monocytes and macrophages. [22] Other functions include cholesterol homeostasis, local redistribution of cholesterol within tissues, immunoregulation and dietary absorption and biliary excretion of cholesterol. [23, 24]

In 1992, the first line of ApoE$^{-/-}$ mice was developed almost contemporaneously in two laboratories. [13, 14] The deletion of the ApoE gene was done in mouse embryonic stem cells by homologous recombination. ApoE$^{-/-}$ mice were healthy, had a similar body weight as wild-type mice, and were born at the expected frequency.[14, 25] However, their lipoprotein profile disclosed significant differences with the wild-type mates. The ability of ApoE$^{-/-}$ mice to clear plasma lipoproteins is severely impaired resulting in plasma cholesterol levels of 400-600 mg/dl when fed a normal diet, whereas wild-type mice have levels of 75-110 mg/dl. [26, 27] This drastic change is due to an increase in VLDL-sized particles. The development of significant hypercholesterolaemia, even when fed a normal diet, suggests that in the absence of an environmental stimulus, deficiency of ApoE is sufficient to cause massive changes in lipoprotein metabolism. Furthermore, the lack of ApoE boosts the

sensitivity to dietary fat and cholesterol. After several weeks of feeding a Western-type diet (consisting of 21% fat and 0.15% cholesterol, which is similar to the everyday diet of Western countries), plasma cholesterol levels double in wild-type mice, whereas in ApoE-deficient mice a fourfold increase in total plasma cholesterol is observed. [14] Extensive atherosclerosis is seen in mice on both types of diet by 2 to 3 months of age. [28] On the other hand, heterozygous ApoE-deficient mice do not show an increase in plasma cholesterol levels even when fed a Western-type diet, presuming that a 50% decrease in ApoE is not sufficient to increase plasma lipids. Of note, plasma cholesterol levels in mice are not affected by age or sex of the animal. [26]

The entire spectrum of atherosclerotic lesions is present in ApoE$^{-/-}$ mice. [25] Monocyte attachment to endothelial cells is noticed from 6 weeks of age, and after 8 weeks foam cell lesion development is detectable. After 15 to 20 weeks, intermediate lesions are present containing mostly SMCs as well as fibrous plaques consisting of SMCs, extracellular matrix and a necrotic core covered with a fibrous cap. [26] In more advanced lesions, fibro-fatty nodules are a nidus for calcification and plaques become more calcified with time. [29] When fed a Western-type diet, the time course for lesion formation is tremendously accelerated. [25] Compared to mice fed a low-fat diet, lesions are 3-4 times larger within the same period of time. This response implies a diet-dependent mechanism, i.e. increased fat leads to increased plasma cholesterol, which in turn leads to increased atherosclerosis, which resembles the diet-dependency of atherosclerotic heart disease observed in humans. [14]

ApoE$^{-/-}$ mice tend to develop atherosclerotic plaques at vascular branch points, with predilection for the aortic root, the lesser curvature of the aortic arch, the principal branches of the aorta as well as the pulmonary and carotid arteries. [26] Sequential events of plaque formation in ApoE$^{-/-}$ mice are considerably similar to those in well-established larger animal models of atherosclerosis and in humans. [26] Although this mouse model is used by many research groups, it has some limitations. For instance, ApoE is a multifunctional protein that has an impact on inflammation, oxidation, reverse cholesterol transport by macrophages, and smooth muscle proliferation and migration. These functions might affect atherosclerotic plaque development in ApoE$^{-/-}$ mice, independent of plasma lipid levels. [30] Furthermore, not

LDL, which is characteristic of human atherosclerosis, but VLDL is the most abundant lipoprotein in ApoE$^{-/-}$ mice. [14] However, the major limitation of the 'classical' mouse models of atherosclerosis is the rarity of plaque rupture and thrombosis, [31, 32] whereas these events are fairly common in humans and can lead to MI and stroke. [25] It has been suggested that this might be due to the tiny diameter of the mouse vessels; as the vessel diameter decreases, the surface tension increases exponentially, impeding the likelihood of plaque rupture. [25] However, also other explanations have to be taken into account as discussed below in the section 'mouse models of atherosclerotic plaque rupture'.

A method for the induction of accelerated atherogenesis and plaque rupture is the placement of a perivascular collar or cuff, mainly in ApoE$^{-/-}$ mice. In their study, Sasaki et al., claim that cuff placement around the left carotid artery results in an animal model of plaque rupture. By using the ligation technique to induce neo-intimal hyperplasia, they observed lipid- and collagen-rich lesions accompanied with intraplaque haemorrhage and plaque rupture. Furthermore, a decrease in collagen content, and formation of fibrinogen-positive thrombi were detected, analogous to plaque rupture in humans. [33] Along with this observation, perivascular carotid collar placement also reproduces the induction of rapid and site-controlled atherosclerosis [34], while maintaining the structural integrity of the endothelium. Formed plaques are located primarily in the area proximal to the collar. The advantages of this model over the conventional animal models of mechanically induced atherosclerosis include the closer resemblance to human plaque morphology and endothelial expression pattern. [34]

LDL receptor-deficient (LDLr$^{-/-}$) mice

The LDL receptor is a membrane receptor with a molecular weight of 160 kDa, which mediates the endocytosis of cholesterol-rich LDL and thus maintains the plasma level of LDL. It also facilitates the cellular uptake of apolipoprotein B- and E-containing lipoproteins. LDL receptor deficiency along with mutations in the gene encoding for the LDL receptor count for the phenotypic events described in familial hypercholesterolaemia. [35, 36] Mice with a targeted inactivation of the LDL receptor were created in 1993. [37, 38] Compared with wild-type, LDLr$^{-/-}$ mice display modestly

elevated plasma cholesterol levels and develop no or only mild atherosclerosis when fed a normal diet. [38] In terms of lipoprotein particles, the increase is higher among IDL and LDL sized particles, whereas HDL and triglycerides remain unaffected. [37, 38] It is worth to note that this is different from ApoE$^{-/-}$ mice, in which cholesterol is primarily accumulated in large lipoprotein particles such as chylomicron remnants, VLDL and IDL particles (*vide supra*).[14, 39] The response to high-fat/high cholesterol Western-type diets shows a remarkable change in lipoprotein profile of these mice with a high probability for atherosclerotic lesion development.

The plaques that develop in LDLr$^{-/-}$ mice are generally the same as those seen in ApoE$^{-/-}$ mice. [40] A Western-type diet induces larger and more advanced lesions with a collagen-rich fibrous cap, a necrotic core containing cholesterol clefts and cellular enrichment adjacent to the lumen. [41] The plaque development occurs in a time-dependent manner, initially in the proximal aorta, and spreading toward the distal aorta. Similar to humans, the locations where the blood flow is disturbed are more prone to atherosclerotic lesions. [40] By making LDLr$^{-/-}$ and ApoE$^{-/-}$ mice homozygous for the ApoB-100 allele, total plasma cholesterol levels of approximately 300 mg/dl were obtained on a normal diet. LDLr$^{-/-}$ ApoB$^{100/100}$ mice developed more atherosclerotic lesions than the ApoE$^{-/-}$ ApoB$^{100/100}$ mice, even with a normal diet. [42, 43]

The LDLr$^{-/-}$ mouse model has some advantages in comparison with ApoE$^{-/-}$ mice. Firstly, plasma cholesterol is mostly carried by LDL particles, which generates a more human-like lipid profile. Secondly, the absence of the LDL receptor does not have an impact on inflammation as compared to ApoE deficiency. Thus, atherosclerotic plaque development in this mouse model is based on elevated plasma lipid levels and not caused by other functions linked to the LDL receptor. [8] Thirdly, the LDLr$^{-/-}$ mouse model shares the characteristics observed in human familial hypercholesterolaemia, which is caused by the absence of functional LDL receptors. [44, 45]

ApoE/LDL receptor double-knockout mice

Introduced shortly after ApoE$^{-/-}$ and LDLr$^{-/-}$ mice, ApoE/LDL receptor double knock out mice represent a model that develops more severe hyperlipidaemia and

atherosclerosis than the former ones. [46] It is an animal model with spontaneous atherosclerotic plaque development and it has been reported that even on regular chow diet, the progression of atherosclerosis is usually more marked in ApoE/LDL receptor double knock out mice than in mice deficient for ApoE alone. [47] There is no significant difference in the lipoprotein profile of the double knockouts compared to ApoE$^{-/-}$ mice, they both have high levels of VLDL and LDL [48], except the marked elevations in B48 and B100 apolipoproteins. [49] This mouse model is considered suitable to study the anti-atherosclerotic effects of possible treatments, without the need of an atherogenic diet. [25]

ApoE3-Leiden mice

Although ApoE$^{-/-}$ mice and LDLr$^{-/-}$ mice are the two most frequently used mouse models for atherosclerosis, also ApoE3-Leiden mice are utilized in many studies. Apolipoprotein (Apo)E3-Leiden is associated with a genetic form of hyperlipidaemia and is particularly expressed in a Dutch family. Transgenic mice have been generated using a genomic 27-kilobase DNA construct (containing the ApoE gene, ApoC1 gene and all regulatory elements) isolated from the APOE3-Leiden proband, to study the effect of the ApoE3-Leiden mutation *in vivo.* [17, 50]

Remarkably, although these mice are less susceptible for atherosclerosis than the ApoE deficient mice, they also show dramatically elevated total plasma cholesterol and triglyceride levels when fed a Western-type diet. This is mainly attributed to an increase in VLDL/LDL particles, which demonstrates that ApoE3-Leiden mice have a human-like lipoprotein profile. [51] Another advantage is that ApoE3-Leiden mice have the ability to synthesize functional ApoE. This offers the possibility to study the effect of elevated plasma lipid levels without disturbing inflammatory processes, which is an important limitation of the 'classical' ApoE$^{-/-}$ mouse model. [52]

The ApoE3-Leiden mice develop atherosclerotic lesions in the aorta and large vessels when fed a Western-type diet. Lesions are also observed in the proximal coronary arteries, the aortic root, the aortic arch and its main branch points, the thoracic aorta, the abdominal aorta, the renal artery branch points, the abdominal aorta bifurcation, and the iliac artery bifurcations. It is interesting to note that this strain develops early foam cell lesions on normal chow diet. However, more complex

and advanced lesions are observed after 1, 3 and 6 months of Western-type diet feeding. [17]

ApoE3-Leiden mice are used as a model to elucidate factors involved in the metabolism of ApoE and the aetiology of familial dyslipidaemia in particular. Furthermore, ApoE3-Leiden mice are utilized to study complications of venous bypass grafting, a clinical procedure that bypasses an atherosclerotic obstruction in an artery. Similar to humans, the grafted vein in this model undergoes remodelling, which is a consequence of exposure to higher blood pressure and shear stress but also vessel injury due to surgery. This process results in the formation of intimal hyperplasia and accelerated atherosclerosis, which may lead to obstruction of the graft. [17, 53, 54]

Because similarities were found between lesions in vein grafts and native atherosclerosis, a murine model of vein graft disease has been established. [55] In this model, the thoracic caval vein of a donor mouse is grafted in the carotid artery of a receiver mouse. This procedure has been used in mice that are susceptible to atherosclerosis (ApoE$^{-/-}$ or ApoE3-Leiden mice) so that vein grafts with accelerated atherosclerosis could be studied. [56-58] It has been show that vein grafts in these transgenic mice are morphologically similar to rupture-prone plaques in humans. The lesions in this model have the typical characteristics of late stage atherosclerosis, including the presence of foam cells, a large necrotic core, intraplaque neovascularization, calcification and cholesterol clefts. [59]

PCSK9-AAV mice

Besides the abovementioned models, a new line of mouse model without germline genetic engineering is emerging in the research field of atherosclerosis. The so called pro-protein convertase subtilisin/kexin type 9 (PCSK9) - adeno associated virus (AAV) mice were described independently by two research groups in 2014 as a rapid, versatile and cost-effective animal model for atherosclerosis. [60, 61] PCSK9, a newly identified human subtilase, is a serine protease with plasma concentrations of ≈100 to 200 ng/mL and it is highly expressed in the liver. [62, 63] Several studies have shown that PCSK9 reduces hepatic uptake of LDL by increasing the endosomal and lysosomal degradation of LDL receptors. [64] In brief, after protein

maturation and secretion, circulating PCSK9 binds the LDL receptors on the cell surface and is subsequently co-internalized together with the receptor. This distracts the normal recycling process of the receptor to the plasma membrane and promotes degradation in the lysosome. [63]

Recombinant AAV vectors support long-term transgene expression in many animal models [65-67] and humans. [68] Following single intravenous injection with human D374Y [61] or murine D377Y [60] gain-of-function mutant PCSK9, mice were stably expressing $PCSK9^{DY}$ mRNA in the liver. AAV viral infection does not elicit any adverse effects in the animals and no signs of liver damage or immunologic response were observed following infection. At 30 days after injection, total serum cholesterol in $PCSK9^{DY}$-AAV transgenes was doubled compared to control mice. These differences remained the same even after 1 year post-infection, confirming a chronic effect of a single AAV injection. [61] Western-type diet exacerbated hyperlipidaemia in $PCSK9^{DY}$-AAV mice, leading to plasma cholesterol levels of up to 1165 mg/dl, while chow diet fed mice barely reached 316 mg/dl. The lipoprotein profile of Western-type diet fed $PCSK9^{DY}$-AAV mice showed an equal distribution between VLDL and LDL particles. [61] $PCSK9^{DY}$ transgenic mice develop atherosclerosis in a dose-dependent manner. Hyperlipidaemia provokes the build-up of lesions throughout the vasculature resembling those of $LDLr^{-/-}$ mice, which is exacerbated by HFD feeding. [60, 61] Aortic root lesions show advanced plaque development with foam cells, smooth muscle cells, macrophage infiltration and fibrous tissue, but importantly, lesions progress to the fibro-atheromatous stage. [60, 61] and within the time frame of 15-20 weeks, vascular calcification occurs. [69] When Roche-Molina et al. combined $PCSK9^{DY}$ expression and ApoE deficiency, they revealed an expected synergistic effect: the lesions doubled in size with no significant differences in lipoprotein profile as compared to single mutants on the same diet. [61]

Overall, the induction of hyperlipidaemia and atherosclerosis in animals with different genetic backgrounds, the robust stability after single administration of mutant human PCSK9 and the fact that there are no major biosafety concerns in using AAVs as vectors, makes the PCSK9-AAV model a valuable tool in atherosclerosis research.[61]

Mouse models of atherosclerotic plaque rupture

Despite major advances in cardio- and cerebrovascular research, plaque rupture remains the leading cause of acute events. [70] Therefore, the need for the development of plaque-stabilizing therapies is high. Several research groups have tried to develop suitable models of plaque rupture for the last 15 years but in these models rupture occurs only sporadically, after a long period of time, or depends on mechanical injury .[11, 71, 72] Moreover, the reproducibility is low and events as seen in humans are rarely observed.

As discussed earlier, atherosclerotic plaques in mice develop in specific sites such as the aortic root, the lesser curvature of the aortic arch and the branch points of the brachiocephalic, the left carotid and the subclavian arteries. However, mice show only minor plaque development in the coronary and carotid arteries, which are the main sites of atherosclerotic plaque development in humans. [8-10] To induce plaque rupture in mice, several approaches based on surgical (such as arterial ligation or the positioning of a cuff around an artery) or genetic manipulation have been proposed (Table 1). Although these models have been useful in understanding the concepts of plaque rupture, none of them exhibit the full combination of the characteristics seen in human vulnerable/ruptured plaques. Moreover, plaque rupture with a superimposed occlusive thrombus, the most common complication of human atherosclerosis, is rarely observed. [73] Consequently, clinical events such as MI or ischemic stroke are almost never seen in these models. [9, 70] Furthermore, most of these models do not show 'spontaneous' plaque ruptures. When spontaneous ruptures are observed, they only occur sporadically and after a long period of time. However, recently a model of consistent, spontaneous atherosclerotic plaque ruptures in mice has been described, as discussed below.

Apolipoprotein E-deficient Fibrillin-1 mutant ($ApoE^{-/-}Fbn1^{C1039G+/-}$) mice

The extracellular matrix is a complex network of predominantly elastin and collagen, which is essential to provide structural, adhesive and biochemical signalling support to the vessel wall. In elastic arteries, elastin is the most abundant protein. The elastic fibres comprise the elastin core, which is surrounded by a mantle of fibrillin-rich

microfibrils. [74]The elastic-fibre-associated microfibrils have as the main structural component fibrillin-1, a large glycoprotein of about 350 kDa, whose major role is in binding and sequestering growth factors, such as transforming growth factor-β (TGF-β), as well as providing the scaffold for the deposition and the cross-linking of elastin. [75, 76]

Recently, we reported the effect of an impaired elastin structure of the vessel wall on the progression of atherosclerosis by cross-breeding ApoE$^{-/-}$ mice with mice containing a heterozygous mutation (C1039G$^{+/-}$) in the fibrillin-1 (Fbn1) gene. [76] Mutations in the Fbn1 gene lead to the Marfan syndrome, a genetic disorder characterized by fragmentation of elastic fibres. [77]This results in increased arterial stiffening, elevated pulse pressure and progressive aortic dilatation. [76, 78, 79] Moreover, the mutation leads to the development of highly unstable plaques in ApoE$^{-/-}$ mice, resulting in spontaneous plaque rupture with end-points including MI and sudden death. [76, 80] Importantly, these events do not – or only very occasionally – occur in ApoE$^{-/-}$ mice on a Western-type diet or in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice fed a normal diet. [80, 81] These findings underscore the importance of elastin fragmentation in combination with a Western-type diet as prerequisites for atherosclerotic plaque rupture in mice.

ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice have significantly larger plaques with a highly unstable phenotype, characterized by a large necrotic core (occupying about 30% of total plaque area), and a strongly diminished collagen content. Accelerated atherogenesis in these mice is likely the result of enhanced vascular inflammation, leading to increased monocyte attraction, oxidation and accumulation of lipids. [82] Inducible nitric oxide synthase (iNOS), a marker for activated macrophages and inflammation, is significantly more expressed in plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on either Western-type diet or normal diet as compared to ApoE$^{-/-}$ mice on Western-type diet. Accordingly, inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are highly increased. In addition, a higher infiltration of T-cells and their activation marker interferon-γ (IFN-γ) is present, the latter playing an important role in collagen turn-over by inhibiting SMCs to synthesise collagen, required to repair and maintain fibrous cap integrity. [83, 84] Moreover, matrix metalloproteinase (MMP)-2, -9, -12 and -13 expression or activity

is increased in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice. MMP-2 and MMP-9 are implicated in both atherosclerosis and angiogenesis. [85] For example, ApoE$^{-/-}$ mice lacking MMP-2 develop smaller and more stable plaques, whereas macrophages overexpressing active MMP-9 promote neovascularisation, intraplaque haemorrhage [86, 87] and features of plaque rupture in ApoE$^{-/-}$ mice. [87] In the latter case, those features were attributed to elastin degradation, underscoring its role in plaque destabilisation and rupture. MMP-12 and MMP-13 additionally contribute to elastin and (type I) collagen degradation, respectively. Taken together, in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet enhanced collagen/extracellular matrix breakdown together with decreased synthesis and repair are likely responsible for weakening of the fibrous cap and rendering it more rupture-prone. [84, 85]

Extensive neovascularisation and intraplaque haemorrhages consistently occur in the brachiocephalic and common carotid arteries of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. These features are rarely seen in murine atherosclerosis models but are known to highly affect plaque progression and vulnerability in humans. [88] In ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet, intraplaque neo-vessels, likely arising from adventitial vasa vasorum, clearly sprout out of the media. [89, 90] Neo-vessels are not only present at the base of the plaque but are also frequently observed in its centre, similar to human pathology. [88, 90] Angiogenesis requires extracellular matrix degradation by proteases, including MMPs, to enable endothelial cell migration into the surrounding tissue. [85] In addition, degradation of the extracellular matrix induces release of sequestered angiogenic factors such as vascular endothelial growth factor (VEGF) and TGF-β [85, 86], also observed in ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice on Western-type diet. The extent of neovascularisation in ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice correlates with the degree of elastin fragmentation in the vessel wall. However, degradation of the extracellular matrix alone is not sufficient to induce neovascularisation in atherosclerotic plaques, because microvessels are not present in plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a normal diet. This observation indicates that an additional factor is needed to trigger plaque neovascularisation. Hypoxia, a well-known angiogenesis trigger [91], is strongly increased in plaques of brachiocephalic and carotid arteries in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. By contrast, hypoxia in the ascending aorta is minor, which likely explains the

absence of neo-vessels at that site. Thus, the highly permeable arterial wall, due to degradation of the extracellular matrix, combined with intraplaque hypoxia seems required for neo-vessel formation in atherosclerotic plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. Importantly, those neo-vessels are highly leaky. Moreover, the presence of intraplaque erythrocytes near neo-vessels at the base of the plaque points to intraplaque haemorrhages, substantiating ruptured neo-vessels as source of intraplaque bleeding. [86, 88, 92] Erythrocytes are important sources of free cholesterol, thereby increasing necrotic core size. Hence, neovascularisation, besides supplying plaques with leukocytes and lipoproteins, can promote focal plaque expansion when microvessels rupture or become thrombotic. [88, 91, 92] Taken together, these observations in this mouse model are in line with current concepts of human vulnerable plaques.

In addition to enhanced plaque vulnerability, plaque rupture is consistently present in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet, but only very rarely in ApoE$^{-/-}$ mice on a Western-type diet. Moreover, fibrin-rich mural thrombi are present in brachiocephalic, carotid and coronary arteries and ascending aortas. Both intrinsic (i.e. a highly unstable plaque phenotype) and extrinsic factors (i.e. forces acting on the plaque) are elementary for plaque rupture. [93] In general, rupture occurs when the mechanical stress applied on the fibrous cap exceeds its tensile strength. The latter is mainly determined by the collagen content of the plaque, which is significantly decreased in plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. [84, 93] Elevated pulse pressure (as a consequence of arterial stiffening) [76] leads to repetitive plaque deformation, increasing the tensile stress on the cap. [79, 94] When applied chronically, this can lead to plaque fatigue, making it prone to rupture. [93, 94] Moreover, due to the progressive aortic dilatation and outward remodelling (as a result of the large plaques), the collagen and elastin fibres of the cap are stretched and become more rigid, increasing the susceptibility to mechanical stress. Aortic dilatation is highly pronounced in the ascending aorta of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet, suggesting that this mechanism is responsible for rupture of unstable plaques at this site. In brachiocephalic and carotid arteries, intraplaque neovascularisation and haemorrhage are frequently present, further increasing plaque size and vulnerability to rupture.

In addition, sudden death is observed in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet, mainly between 16 and 23 weeks, with 50% mortality after 20 weeks. Moreover, ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet that died suddenly show a significantly higher frequency of coronary stenosis compared to survivors, suggesting that the presence of coronary artery plaque plays an important role in cardiac death. The majority of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet show infarcted areas, which compromise cardiac function even more. Although it is not known whether the increased infarcted area is the result of plaque rupture or due to pronounced plaque formation and coronary artery stenosis, these findings are remarkable because coronary artery plaque and spontaneous MIs almost never develop in ApoE$^{-/-}$ mice on a Western-type diet. Also in humans, differences in fibrillin-1 genotype have shown to greatly affect plaque progression and severity of coronary artery disease, underscoring the pathophysiological relevance of fibrillin-1 mutations in cardiovascular disease. [79]

Thus, elastin fragmentation in combination with a Western-type diet leads to plaque destabilisation and rupture in ApoE$^{-/-}$ mice. ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice show many features of human end-stage atherosclerosis, such as an enlarged necrotic core, a thin fibrous cap with an important loss of collagen fibres, outward remodelling and the presence of intraplaque microvessels and haemorrhage, resulting in plaque rupture, MI and sudden death. Therefore, ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet offer the opportunity to investigate the role of key factors involved in plaque destabilisation, including intraplaque neovascularisation, which will provide more insight into the mechanisms of plaque disruption and potential targets for therapeutic interventions. [80, 95-97]

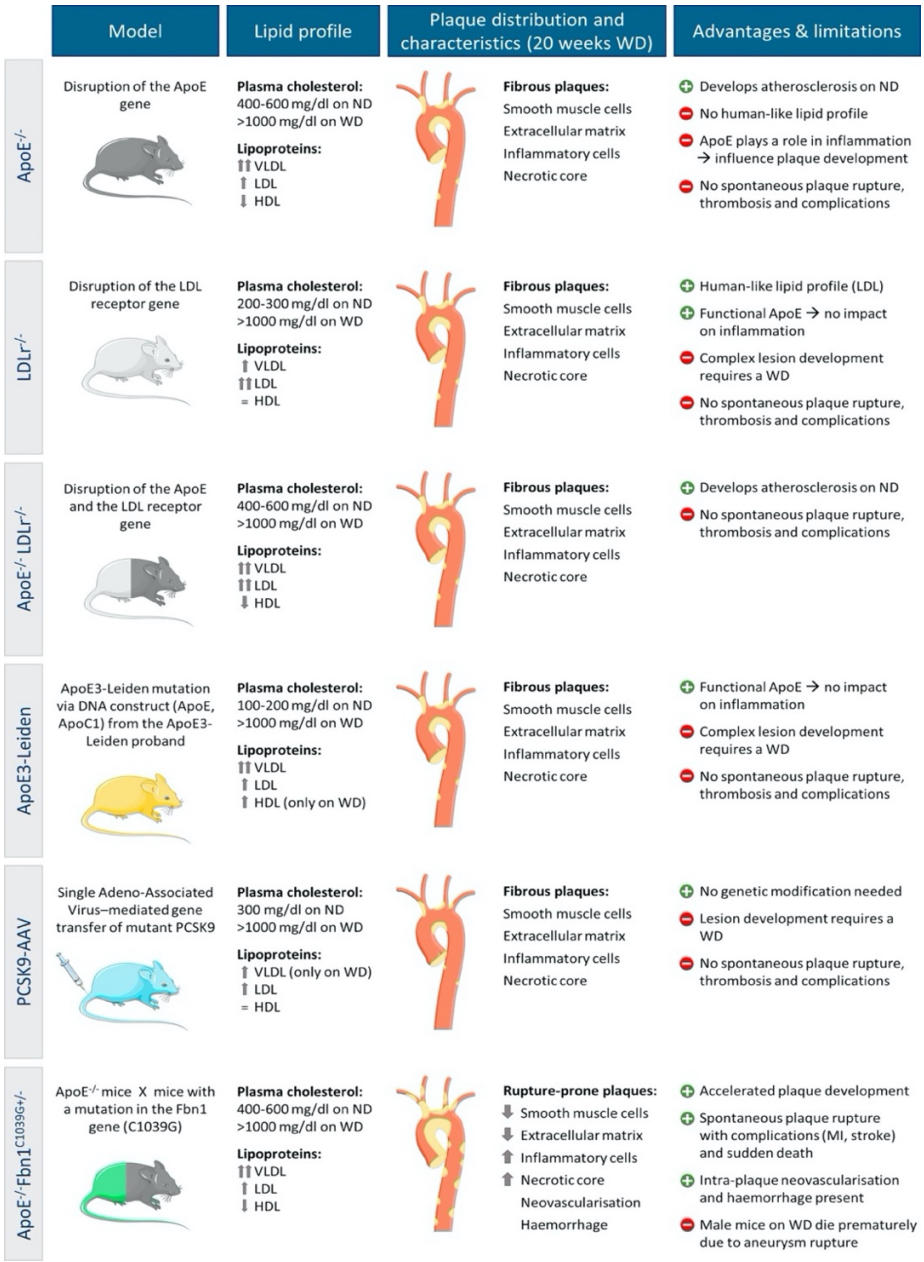

Figure 1. Overview of current mouse models of atherosclerosis. This figure describes the different models with their total plasma cholesterol levels on normal (ND) and Western-type diet (WD), lipoprotein profile, plaque characteristics, advantages and limitations. The distribution of the plaques in the thoracic aorta and the complexity is shown for mice fed a WD for 20 weeks. The composition of the most complex lesions at that time point is displayed (usually found in the aortic root or brachiocephalic artery).

Rabbits

The rabbit has been one of the most frequently used animals in atherosclerosis research because of their easy handling and relatively inexpensive maintenance. [8] However, there has been a reduced trend of using this animal model since 2000, probably due to the availability of ApoE and LDL receptor knock-out mice. [98] Multiple approaches and models have been used to study atherosclerosis and its complication in rabbits, including genetically hypercholesterolaemic rabbits such as Watanabe heritable hyperlipidaemic rabbits (WHHL) [99], New Zeeland White rabbits fed a cholesterol-rich diet [100], and very recently ApoE$^{-/-}$ rabbits. [101]

Rabbits have a lipoprotein metabolism that is similar to humans (except for their hepatic lipase deficiency) and show significant differences with mice. Unlike mice, in which HDL is the predominant plasma lipoprotein, rabbits transport significant amounts of cholesterol via ApoB-containing particles (VLDL and LDL).[102] Consequently, rabbits have been useful to point out the role of elevated plasma cholesterol as a critical factor in the initiation of atherosclerosis. Limitations of rabbit models include a highly abnormal diet required for the development of hypercholesterolaemia, massive inflammation and hepatic toxicity due to the long term high cholesterol feeding.[1]

Watanabe heritable hyperlipidaemic (WHHL) rabbits

WHHL rabbits are a mutant strain that shows spontaneous hypercholesterolaemia and atherosclerosis due to a defect in the LDL receptor.[99] Homozygous WHHL rabbits fed a normal diet are hypercholesterolaemic from birth with LDL as the predominant lipoprotein. They exhibit various types of atherosclerotic lesions ranging from early fatty streaks to advanced lesions in the aorta, coronary arteries and cerebral artery.[100, 103] These rabbits also show an increased risk of MI. The WHHL rabbit was one of the first rabbit models in which the effect of statins to suppress plaque destabilization and to reduce thrombogenicity was investigated. High-fructose and high fat–diet fed WHHL rabbits develop early insulin resistance and glucose tolerance and show aortic lesions with a lipid core and calcifications.

This model has allowed researchers to investigate the effect of insulin resistance on atherosclerosis lesion formation.[104]

New Zealand White (NZW) rabbits

NZW rabbits are commonly used to study atherosclerosis. NZW rabbits that are fed a normal diet have low plasma cholesterol levels (mostly < 50 mg/dl) and consequently do not develop spontaneous atherosclerosis. However, supplementing the diet with 0.3-0.5% cholesterol increases the plasma cholesterol level up to 1000 mg/dl. The plaque composition is determined by the level of dietary cholesterol and the duration of cholesterol feeding. One protocol uses adult rabbits that are fed a cholesterol-rich diet (1.0-1.5% cholesterol) for a short period of time (about 8 weeks). By using such a diet, rabbits develop severe hypercholesterolaemia with plasma cholesterol levels between 1500 and 3000 mg/dl, which are never seen in humans, resulting in atherosclerotic plaques primarily composed of macrophage-derived foam cells. For this reason, the most used protocol lasts 20 to 26 weeks and consists of a diet containing 0.3% cholesterol. On average, cholesterol levels rise to about 800 mg/dl. This protocol develops atherosclerotic plaques in the aortic arch and thoracic aorta, rather than in the abdominal aorta (less pronounced plaque formation) [100, 102], whereas in humans, plaques are commonly found in the abdominal aorta. Coronary atherosclerosis is also observed in cholesterol-fed rabbits but is usually restricted to the left coronary arterial trunks. Depending on the length of the cholesterol feeding, also plaque calcification occurs. However, there is no evidence for spontaneous plaque rupture.

Apolipoprotein E knockout (ApoE$^{-/-}$) rabbits

Recently, ApoE$^{-/-}$ rabbits have been reported as a model to study the relationship between atherosclerosis and human hyperlipidaemia.[101] ApoE$^{-/-}$ rabbits can be generated using genome editing enzymes such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs) or RNA-guided CRISPR-associated protein 9 (Cas9) endonucleases. Because the rabbit lipoprotein profile is similar to humans [105], the ApoE$^{-/-}$ rabbit represents an attractive alternative to the

ApoE$^{-/-}$ mouse. Even on a normal diet, ApoE$^{-/-}$ rabbits show mild hyperlipidaemia with plasma total cholesterol levels around 200 mg/dl. However, when fed a cholesterol-rich diet (0.3% cholesterol and 3% soybean oil for 2 weeks) their plasma total cholesterol levels increase to about 1000 mg/dl (vs. about 170 mg/dl in cholesterol-fed wild-type rabbits). ApoE$^{-/-}$ rabbits develop more pronounced aortic atherosclerosis than wild-type rabbits when fed with a cholesterol diet for 10 weeks.[101] Because both ApoE and the LDL receptor play an important role in mediating cholesterol metabolism, ApoE$^{-/-}$ rabbits together with LDL receptor-deficient WHHL rabbits may be valuable models for the study of human hyperlipidaemia: ApoE$^{-/-}$ rabbits show elevation of remnant lipoproteins, whereas WHHL rabbits have high levels of LDL accompanied by low HDL.[101]

Large animal models (pigs and non-human primates)

Although small animal models have provided insight into the mechanisms that drive atherosclerosis, additional strategies are required to translate these findings into improved prevention and treatment of symptomatic atherosclerosis in humans. Efficient large animal models of atherosclerosis may be useful to deal with these challenges. Indeed, the translation of the knowledge obtained from studies in mice to the development of drugs for human atherosclerosis can benefit from a bridging tool such as porcine models of atherosclerosis. Not only the effects of pharmacological treatments on atherosclerosis can be studied in such models but also clinical imaging end-points can be evaluated as guiding tool for subsequent phase II clinical trials.[106] Gene-editing tools for large animals have made it possible to create gene-modified minipigs that develop atherosclerosis with many similarities to humans in terms of predilection for lesion sites and histopathology. For instance, minipigs with liver-specific expression of human D374Y-PCSK9 show severe hypercholesterolaemia and development of progressive atherosclerotic lesions.[107] Together with existing porcine models of atherosclerosis that are based on spontaneous mutations or severe diabetes, such models may provide new approaches for translational research in atherosclerosis [106]. Non-human primates show hypercholesterolaemia when fed a high fat/high cholesterol diet and develop coronary fibro-fatty atherosclerotic plaques, similar to humans.[8] Yet, working with

monkeys is expensive, highly regulated, and requires very specialized laboratory animal science skills. Therefore, these models are not frequently used. A few years ago, however, also knockout non-human primates have been created, which may reinforce the interest in large animal models with accelerated atherosclerosis.[106, 108]

In conclusion, many efforts have been made to develop animal models that resemble human atherosclerosis as good as possible. However, each of the current animal models has its advantages and limitations, as summarized in Table 2. A great challenge was the development of an animal model of spontaneous (i.e. without mechanical interventions) plaque rupture with human-like endpoints such as MI, stroke and sudden death. These features are present in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice, making it a promising model to evaluate potential plaque stabilizing therapies.

Table 1. Mouse models of atherosclerotic plaque rupture

Strain	mechanism	Duration (weeks)	Plaque disruption	Luminal thrombus	Intraplaque neo-vessels	IPH	Outward remodelling	'Human-like' complications	Comments	Ref.
ApoE$^{-/-}$	'ageing'	60	12%	3%	N.D.	N.D.	N.D.	Coronary thrombosis	Long term experiment Low rate of plaque rupture and thrombosis	109
ApoE$^{-/-}$	Mixed C57BL/6-129SvJ background	30-65	52%	73%	N.D.	N.D.	N.D.	MI ('some cases') sudden death	Long term experiment mixed background	110
ApoE$^{-/-}$	Collar; Ad-p53 in SMC + phenylephrine	15-17	40%	5%	N.D.	35%	N.D.	N.D.	Plaque rupture not spontaneous , complicated manipulation	111
ApoE$^{-/-}$	Active MMP-9 overexpression in Mφ	41	40%	Fibrin deposition (100%)	N.D.	90%	N.D.	Sudden death (20%)	Long term experiment, complicated manipulation	87
ApoE$^{-/-}$	Cuff (+ ligation)	13-14	29-63%	17-42%	N.D.	31-47%	N.D.	N.D.	Plaque rupture not spontaneous	33
ApoE$^{-/-}$; TM$^{Pro/Pro}$	Collar, genetic hypercoagulability	16-17	+	+	N.D.	+	+	N.D.	Plaque rupture not spontaneous	112
ApoE$^{-/-}$	Partial ligation carotid + renal arteries	16	+	50%	N.D.	80%	N.D.	N.D.	Plaque rupture not spontaneous	113
ApoE$^{-/-}$	uPA overexpression in Mφ	43-48	78%	Fibrin deposition (67%)	N.D.	61%	N.D.	N.D.	Long term experiment, complicated manipulation	114
ApoE$^{-/-}$	Tandem stenosis	14-22	32%	+	+	50%	+	N.D.	Plaque rupture not spontaneous	72
ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$	Elastin fragmentation	20-35	50-70%	carotid	+	90%	+	MI, stroke, sudden death	Spontaneous plaque rupture	80

IPH, intraplaque haemorrhage; Ref., reference; +, present; MI, myocardial infarction N.D., not determined; Ad, adenovirus; Mφ, macrophages; uPA, Urokinase-type plasminogen active

Table 2. Most important advantages and limitations of commonly used models of atherosclerosis

	Advantages	Limitations
Mice	• Relatively cheap • Efficient • Easy crossbreeding • Low cost for pharmacological intervention studies	• Differences with human plaques: – Less coronary plaque formation – No intraplaque neovascularisation and haemorrhage – Rare plaque rupture and thrombosis However, these limitations are overcome in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice.
Rabbits	• Allows translation research such as catheterisation of the aorta (coronary arteries are too small) • Relatively cheap • Easy to breed and handle	• Differences in the localisation of the plaques as compared to humans: – Plaques are most pronounced in the aortic arch and descending thoracic aorta (in contrast to the abdominal aorta in humans)
Pigs	• Similarities with human plaques: – Localisation: coronary arteries, abdominal aorta, ileo-femoral – Neovascularisation towards the plaque	• Plaque development mostly ends in the foam cell stage (this can be overcome with the selection of natural mutants, mechanical damage, introduction of other risk factors, genetic engineering with minipigs) • Thrombosis due to plaque rupture is rare (in contrast to humans) • Relatively expensive and more difficult to handle
Non-human primates	• Very similar plaque formation as compared to humans (both micro- and macroscopic) • Plaque formation in the coronary arteries	• Non-human primates are expensive and highly regulated • Specialized training is needed

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