Review Article
Modelling of atherosclerosis in genetically modified animals

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Abstract: Atherosclerosis is a lipid-driven, chronic inflammatory disease that leads to plaque formation at specific sites of the arterial tree. Being the common cause of many cardiovascular disorders, atherosclerosis makes a tremendous impact on morbidity and mortality rates of cardiovascular diseases (CVDs) in countries with higher income. Animal models of atherosclerosis are utilized as useful tools for studying the aetiology, pathogenesis and complications of atherosclerosis, thus, providing a valuable platform for the efficacy testing of different pharmacological therapies and validation of imaging techniques. To date, a large variety of models is available. Pathophysiological changes can be induced in animals by either an atherogenic diet or genetic manipulations. The discussion of advantages and disadvantages of some murine, rabbit and porcine genetic models currently available for the atherosclerosis research is the scope of the following review.

Keywords: Atherosclerosis, genetic animal models of human atherosclerosis, murine models of human atherosclerosis, rabbit models of human atherosclerosis, porcine models of human atherosclerosis

Introduction

In industrialized countries, atherosclerosis is the most significant contributor to CVDs that are accountable for over 50% of total mortality in these countries [1]. Atherosclerosis is the age-dependent disease, which can start at a young age and its prevalence and extent progress with years. Importantly, atherosclerotic lesion development can have a long asymptomatic phase [2, 3], and, in many cases, its first clinical manifestations emerge as lesions of the advanced stages that may cause the significant arterial occlusion with severe life-threatening consequences. In association with the presence of the atherosclerosis risk factors, many studies described the subclinical form of atherosclerosis in a large population of young adults [4-8]. Moreover, the high prevalence (up to 100%) of coronary atherosclerosis in asymptomatic teenagers and young adults was reported [9]. There are several different risk factors of atherosclerotic disease, including hyperlipidemia, hypertension, smoking, male gender, genetic disposition and diabetes mellitus. According to the current understanding, these conditions can cause damage to the vascular endothelium allowing lipid penetration into the vascular wall. Particularly, the increased plasma low-density lipoprotein (LDL) level is the most significant risk factor for the development of atherosclerosis and subsequent cardiovascular disease (CVD). Randomized clinical trials of lipid-lowering therapy revealed up to 30% decrease in main coronary events, thus, confirming the significance of hyperlipidemia as a major contributing risk factor of atherosclerosis [10]. Besides, hypercholesterolemia was reported to be the chief population attributable risk factor for coronary heart disease (CHD) [11]. Furthermore, the high content of blood cholesterol and LDL was found to predominantly contribute to the atherogenesis in both humans and animal experimental models [12, 13], hence, exposing hypercholesterolemia as an independent risk factor for the atherosclerotic disease. In addition, hypercholesterolemia is a monogenetic cause of familial hypercholester-
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Oleemia (FH), an autosomal dominant genetic disorder, in which premature atherosclerosis with subsequent CVD are inevitably developed [14].

It was established that development of the atherosclerotic lesion begins with the accumulation of the circulating modified LDL-cholesterol (LDL-C) in the subendothelial space of the arterial wall, therefore, the subendothelial lipid retention is the major process initiating the atherosclerotic plaque growth [15, 16]. Lipid accumulation is proportional to the plasma levels of circulating modified LDL-C and leads to the formation of foam cells. Further accumulation of foam cells in the arterial intimal cells results in the development of primary lesions and consequent fatty streaks, the early-stage lesions in the proatherogenic progression. Thus, LDL-C retention and its accumulation by foam cells in the arterial wall are the key processes leading to the development and progression of the atherosclerotic plaque. Moreover, the intracellular LDL-C retention is accompanied by the migration and accelerated proliferative activity of smooth muscle cells (SMCs), macrophages, lymphocytes, neutrophils and dendritic cells, and the increased synthesis of the components of the extracellular matrix by subendothelial cells [17, 18]. Additionally, in response to hyperlipidemia, both the innate and adaptive immune systems are intimately involved in the development of atherosclerotic plaque [19]. Atherosclerotic plaques develop primarily in the walls of large and medium-sized arteries causing thickening of the arterial wall that may lead to the significant narrowing of the arterial lumen and disturbed arterial vessel haemodynamics [20]. Also, for unknown reasons, the atherosclerotic plaques can rupture, often when they are small in size, and lead to the occlusive thrombosis resulting in myocardial infarction (MI) or stroke [21].

The high incidence of atherosclerosis encourages the investigation of its risk factors, causes, and pathomechanisms. In that regards, numerous animal models were explored. Thus, rabbits were the first species used to model atherosclerosis in the study dating back to the beginning of the 20th century that established the direct relationship between the high-cholesterol diet and atherosclerosis [22]. The application of the first models was limited due to the usage of high-fat diets with the noxious side effects. Since then, a number of species, such as mice, rats, guinea pigs, hamsters, birds, dogs, pigs and non-human primates were used for the modelling of human atherosclerosis. In general, animal modelling of atherosclerosis can be based on the accelerated plaque formation due to a cholesterol-rich/Western-type diet, manipulation of genes involved in the cholesterol metabolism, and the introduction of additional atherosclerosis risk factors. Genetically modified animal models were developed to produce spontaneous atherosclerotic lesions and nowadays are preferentially used to study the mechanisms of plaque formation and stability, as well as the development of therapeutic interventions and imaging techniques. None of the models is known to be ideal but each has its own advantages and disadvantages, in terms of precise mimicking of human atherosclerosis and the potential use for the translational research.

In this review, we will discuss the utility of different animal models of human atherosclerosis created by genetic engineering, noting their strengths and weaknesses that will help to increase the understanding of this chronic inflammatory disease and provide the robust foundation for the translational research.

Murine models of human atherosclerosis

Mice become the predominant species to study the experimental atherosclerosis for the following reasons: (i) rapid reproduction, (ii) large proportion of genes is in homology to humans and the ease of genetic manipulation, (iii) ability to monitor atherogenesis in a reasonable time frame, (iv) low cost, and (v) simple in-house maintenance [23, 24]. Despite, none of the animal models, including murine models, would replicate human atherosclerotic disease exactly, mice proven to be capable of developing lesion formation processes of close similarity. Like humans, mice spontaneously develop atherosclerotic lesions, in response to the cholesterol-rich diet [23]. Moreover, in humans, the formation of atherosclerotic plaques takes place at the specific arterial regions (vessel bifurcations), where the low and oscillatory endothelial shear stress occurs but varies considerably over a small distance [25, 26]. Mice develop atherosclerotic lesions in a similar manner [27].
However, several differences attributed to the developmental process of mouse atherosclerotic lesions should be considered, as they may limit the aptness of murine models to study human atherosclerosis. Thus, the manipulation of the atherogenic process and lipoprotein profile can be difficult in mice due to the substantial genetic differences in the lipid metabolism between mice and man. Cholesterol present in murine plasma is mostly carried out in the atheroprotective high-density lipoprotein (HDL) fraction [28], and atherogenic LDL and its precursors including very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) are rapidly cleared of the blood [29]. The most common lipoprotein pattern of humans suffering from coronary artery disease consists of elevated plasma levels of LDL-C and decreased HDL-C, with or without increases in plasma IDL and VLDL [30]. Moreover, mice and humans exhibit different HDL subclasses that can be indicative of the different levels of atheroprotection [31]. Additionally, mice do not express cholesteryl ester transfer protein (CETP), a plasma protein that attracted much attention as a possible target for atheroprotection in humans [23, 32]. Notably, CETR transfers cholesteryl ester from HDL to the apolipoprotein B (ApoB)-containing lipoproteins in exchange for triglycerides. Thus, the lipid profile observed in wild-type mice makes them more resistant to a dietary-induced elevation of LDL and development of atherosclerotic lesions, therefore, the metabolic murine models of human atherosclerosis were not in favour until the recent advances in gene-manipulating technologies. Besides, mice and humans differ in some other parameters that may influence atherogenesis. For example, the heart rate (over 300 bpm in mice, and 70-100 bpm in man), and atherosclerosis generation time (months in genetically modified mice and years in humans) [23].

Human and murine atherosclerosis differ in other features, including topography and morphology of lesions that also may limit the application of murine models. In humans, lesions develop more often in the coronary arteries, carotids and peripheral vessels, such as the iliac artery. Whereas, mice typically do not develop coronary atherosclerosis, except the most proximal regions. Their atherosclerotic lesions are more frequently observed in the aortic root, aortic arch and innominate artery [23]. There is the ingrowth of neo-vessels from vasa vasora into the base of lesions, which is a path for the inflammatory cell infiltration, and is important for the development of local inflammation and core necrosis in humans but such vessels are rarely present in murine lesions [23]. Additionally, taking into account that atherosclerosis development is commonly dependent on local inflammation, it is important to consider the differences in pathomechanisms of both adaptive and innate immune responses in mice and humans [33].

The aspects related to the development of advanced and complicated lesions are also dissimilar in mice models and humans [34] making difficult to study human advanced and complicated atherosclerosis, as well as plaque-stabilizing drugs. The most of murine models do not exhibit the development of unstable atherosclerotic plaque with overlying thrombosis that is the most often associated with clinically significant acute cardiovascular episodes in humans [35]. Also, the lesions acquiring in mice do not typically develop the thick fibrous cap that is frequently attributed to the chronic human atherosclerosis [23].

Another limitation associated with the utility of murine models is their size. The small size of mice can be limiting for some practical investigation procedures, such as visualization of coronary arteries, blood sample collection, and dissection of small arteries [36, 37]. Besides, the size constraints limit the stent intervention studies in mice. Stenting is proven to be a safe and effective approach for the management of acute cardiovascular events in humans, for example, ischemic stroke [38]. However, a successful model of murine in situ stenting demonstrating reduction in in-stent restenosis using interleukin-1 receptor-1 (IL-1R1)-deficient mice was described [39]. Taken together, to what extent mice can serve as accurate models of the human atherosclerotic disease is still the subject of discussion [40].

*Genetically-modified murine models of human atherosclerosis (Table 1)*

Regardless of the limitations, mice remain the favoured animals for the atherosclerosis modelling, since the simplicity of the genetic manipulation using gene knockout, tissue-specific
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### Table 1. Genetic murine models available for the atherosclerosis research

<table>
<thead>
<tr>
<th>Model type</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Utility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-/- mice</td>
<td>Spontaneously develop hyperlipidemia and extensive atherosclerosis of all stages morphologically identical to humans on the low-fat diet</td>
<td>The infrequency of crucial for humans plaque ruptures accompanied by mural thrombosis The low extent of coronary lesions</td>
<td>Suitable to study atherogenesis evaluating the mechanisms underlying atherosclerosis progression</td>
<td>[139-141]</td>
</tr>
<tr>
<td>LDLR-/- mice</td>
<td>Develop site-specific lesions in a time-dependent manner</td>
<td>The requirement for the long-term high-fat diet The low extent of coronary atherosclerosis</td>
<td>Can be used to facilitate lesion analysis at specific locations, particularly, to study the initiation of the fatty streak, inhibition of lesion progression and regression of advanced plaques</td>
<td>[142]</td>
</tr>
<tr>
<td>ApoE/LDLR double KO mice</td>
<td>Develop advanced atherosclerosis on the low-fat diet</td>
<td>Differences to humans in lipid profile</td>
<td>Suitable to study the anti-atherosclerotic effects of possible treatments, without the need of an atherogenic diet</td>
<td>[143-145]</td>
</tr>
<tr>
<td>ApoE*3-Leiden mice</td>
<td>Development of accelerated atherosclerosis and human-like lipoprotein profile</td>
<td>Lack of plaque rupture, thrombus formation, and/or haemorrhage</td>
<td>An accurate model for studying lipoprotein remnant metabolism and development of atherosclerotic plaques Suitable for anti-atherosclerotic drug development</td>
<td>[80, 145]</td>
</tr>
<tr>
<td>Tg ApoB100+/-/LDLR KO mice</td>
<td>Develop accelerated atherosclerosis on a chow diet and human-like lipoprotein profile</td>
<td>Absence of spontaneous plaque ruptures</td>
<td>Suitable to study atherosclerosis and tests of effects of new therapies</td>
<td>[78, 79]</td>
</tr>
<tr>
<td>LDLR/Apopoe-1 double KO mice</td>
<td>Develop severe cholesterolemia and spontaneous atherosclerosis diet which progressively worsens with age on a normal chow diet</td>
<td>Absence of spontaneous plaque ruptures</td>
<td>Superior model to study the progression of atheroma</td>
<td>[83]</td>
</tr>
<tr>
<td>PCSK9-rAAV mice</td>
<td>Develop severe and persistent hypercholesterolemia and advanced atherosclerosis, including calcification of plaque Induction of atherosclerosis in mice without germline genetic engineering, the rapid, easy and cost-effective approach</td>
<td>Absence of spontaneous plaque ruptures</td>
<td>Provide a flexible model of dyslipidemia and atherosclerosis</td>
<td>[93, 146, 147]</td>
</tr>
<tr>
<td>ApoE-/Fbn1C1039G+/- mice</td>
<td>Develop exacerbated atherosclerosis with plaque instability and spontaneous plaque ruptures</td>
<td>Required toxic Western-type diet</td>
<td>The perfect model for studying end-stage atherosclerosis investigating the role of major factors involved in plaque destabilization and rupture, as well as potential molecular targets for novel therapeutic interventions and the development of innovative molecular imaging strategies for vulnerable plaques</td>
<td>[104, 106, 107, 110]</td>
</tr>
</tbody>
</table>

Note: ApoE-/- - apolipoprotein E deficient mice; LDLR-/- - low-density lipoprotein receptor deficient mice; Tg ApoB100+/-/LDLR-/- KO mice - transgenic apolipoprotein B 100 positive/low-density lipoprotein receptor knock out mice; PCSK9-rAAV mice - proprotein convertase subtilisin/kexin type 9 recombinant adeno-associated virus mice; ApoE-/Fbn1C1039G+/- - apolipoprotein E-deficient Fibrillin-1 mutant mice.
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conditional gene expression, and gene knock-in techniques enables identification of genes contributing to the development of atherosclerosis and creating of transgenic models. Moreover, the relatively easy breeding of mice allowing the simultaneous manipulation of several genes in a single animal model represents an important advantage for the atherosclerosis research. The study showed that a combined interruption of genes influencing the development of the inflammatory component of the atherosclerotic process in apolipoprotein E deficient (ApoE-/-) hypercholesterolemic mice resulted in the inhibition of lesion development [41]. To date, most of the current genetic manipulations in mice applied for the atherosclerosis research rely on the disruption of normal lipoprotein regulation and metabolism generating non-HDL-based hypercholesterolemia. This can be the most readily achieved by the genetic knockout of ApoE or the LDL receptor (LDLR). Thus, ApoE-/- mice and low-density lipoprotein receptor-deficient mice (LDLR-/- mice) become the most commonly used genetic murine models of human atherosclerosis, which are going to be described in the next two subsections.

ApoE deficient mice

Apolipoprotein E (ApoE) is a lipoprotein that plays an important atheroprotective role in atherosclerosis development. Many of its anti-atherogenic effects that directly and indirectly involved in the lipoprotein metabolism were described [42]. ApoE is synthesized mainly in the liver, but it can be produced in some other tissues, such as brain, spleen, lung, kidney, arterial wall, and is present in high concentrations in the interstitial fluid [43, 44]. Except for LDL, ApoE is a structural component of all lipoprotein particles, as well as it is a high-affinity ligand for both LDLR and LDLR-related proteins. Its interaction with these receptors provides the transport of cholesterol and other lipids between various body cells. In particular, ApoE is a critical ligand for the effective hepatic clearance of plasma lipoproteins (diet-derived chylomicrons and liver-derived VLDL remnants) mediated by LDLR and LDLR-related proteins [45, 46]. It should be noted that ApoE functional roles within the LDL particles remain unclear.

ApoE gene deficiency in humans and animal models leads to the development of atherosclerosis, providing the evidence that the ApoE role in the mouse body is identical to that in humans. Various genetic mutations of functional LDLR pathway seen in patients with familial hypercholesterolemia (FH) lead to the impaired hepatic uptake and degradation of LDL, and, in turn, to a dramatic rise in plasma LDL, and an increased incidence of atherosclerosis and premature CVD [47]. Similarly, even on the standard chow diet, ApoE knockout (ApoE KO) mice develop a significant increase in plasma cholesterol level, with proatherogenic VLDL, as the most abundant circulating lipoprotein followed by the development of atherosclerosis [48].

ApoE KO mouse model was the first genetically modified murine model developed to study atherosclerosis [48]. The benefits of the use of this model are long-established. The early studies demonstrated that on the low-fat diet ApoE-/- mice rapidly develop atherosclerotic plaques, compared to wild-type mice, and, moreover, they can develop the morphologically identical lesions of all stages to human atherosclerosis [49, 50]. Since the ApoE-/- mice develop atherosclerotic lesion even on the low-fat feed, the highly toxic diet can be avoided [51]. Moreover, in the ApoE-/- mice, the progression from early to advanced lesion occurs in the similar fashion to that in humans: atherosclerotic lesions frequently develop at vascular branch points and grow rapidly into foam cells with fibrous plaques and necrotic lipid cores [52]. The spontaneous plaque development was identified in several vascular beds, predominantly in the aortic root, aortic arch and different branch points along the aorta in these mice [50]. The extensive atherosclerosis was seen in ApoE-/- mice at the age of 2-3 months [49]. Monocyte attachment to endothelial cells was detected from 6 weeks of age, foam cell lesions were developed after 8 weeks, and after 15 weeks, advanced lesions (fibrous plaques) were observed [50]. In 20 weeks, fibrous plaques containing SMCs, extracellular matrix, and an overlying fibrous cap were evident [50]. This time period can be accelerated in these mice by the feeding of the Western-type diet (0.15% cholesterol and 21% fat derived from milk fat), with the development of more advanced lesions. Accordingly, ApoE-/- mice fed with the Western-type diet developed extreme hypercholesterolemia accelerating the development and progression of atherosclerotic lesions comprising observable cholesterol crystals, necrotic core, and calcification [49]. The occurrence of the plaque rupture in these
mice remains to be a subject of debate for a past decade [53-56]. Some authors indicated that sporadic plaque ruptures may be observed, but they occur after a long period of time, and consequently, clinical events such as MI or ischemic stroke are almost never seen in these models [28]. Though, a plaque rupture may be stimulated in this model by the placement of perivascular collar or cuff [57]. The rarity of crucial for humans plaque rupture is a disadvantage of this model.

The major limitation to the use of this model is linked to the transfer of bone marrow from a mouse expressing ApoE into an ApoE-deficient recipient that significantly reduces plasma lipid levels and atherosclerosis [58]. Moreover, the extrapolation of data obtained in ApoE KO model to humans is difficult due to the differences in lipid metabolism, as most of the plasma cholesterol in these animals is in the form of VLDL but not LDL [59]. Besides, apart from the lipoprotein clearance, the presence of additional independent ApoE activities, such as anti-oxidative, anti-proliferative and anti-inflammatory that may lead to the development of atherosclerotic lesions irrespective of the plasma lipid level [44] can make an impact on the applicability of the results obtained in mice to humans. To date, considering the advantages and the limitations attributed to this model, it is widely used for studies of various pathways of atherosclerosis pathogenesis [60, 61] and drug discovery [62, 63].

**LDLR deficient mice**

LDLR-/- murine models were developed, in the attempt to overcome the limitations of ApoE-/- models, and they have revealed some advantages. The first advantage of LDLR-/- mouse model is based on the fact that LDLR does not have the variety of functions, therefore, the effects of its deficiency can be more straightforwardly attributed to the lipoprotein homeostasis. In this respect, the study showed that LDLR deficiency predominantly affecting lipoprotein uptake and clearance, resulted in a high prevalence of plasma LDL, as the main cholesterol-carrying lipoprotein in mice on the chow diet [64]. Noteworthy, the LDLR is a membrane receptor that mediates the endocytosis of cholesterol-rich LDL and maintains its plasma level. Besides, it facilitates the cellular uptake of ApoB- and E-containing lipoproteins.

Second, LDLR-/- mice have the ability to model human-like plaques commonly observed in human FH, including the lesions in aortic valves and the aortic root [65]. That is advantageous to study atherosclerosis accompanying FH, an autosomal dominant disorder caused by mutations in the LDLR gene [66]. Third, LDLR deficient mice were found to be useful for studying the relationship between diabetes and atherosclerosis, which are often co-exist, as compared to ApoE-/- mice, they are more susceptible to obesity and insulin resistance [67]. In addition, together with ApoE KO model, LDLR-/- mouse model may be useful to study mechanisms of the atherosclerosis regression.

Nevertheless, in case of resistance to an injury-induced neointimal formation, ApoE KO mice were found to be more useful, in comparison with LDLR-/- mice, for studying mechanisms of restenosis following angioplasty [68, 69]. Moreover, some reviewers indicated that, compared to ApoE deficient mice, when placed on a normal chow diet, LDLR-/- mice delay development of atherosclerosis, and higher cholesterol intake is required to accelerate the pathological process [70]. Variations in dietary cholesterol intake may lead to a problem of LDLR-/- mouse model standardization across different laboratories.

Interestingly, together with the transfer to a chow diet, reintroduction of ApoE and LDLR genes into ApoE-/- or LDLR-/- mice respectively, resulted in a sharp decrease in plasma cholesterol and a regression of established atherosclerosis [71]. The understanding of the atherosclerotic plaque regression is therapeutically relevant because the most cardiovascular disease patients will be treated after advanced plaques become established. Overall, the introduction of ApoE- and LDLR-deficient mouse models of atherosclerosis has transformed the understanding of the atherogenic process, and, hitherto, the mouse instantly became the most popular mammalian model of human atherosclerosis.

**ApoE/LDLR double knock out mice**

ApoE/LDLR double knock out (ApoE/LDLR-DKO) mice represent a model that develops both more severe hyperlipidemia and atherosclerosis than ApoE or LDLR single knockouts [72]. ApoE/LDLR-DKO mice demonstrated the
more pronounced progression of atherosclerosis even on a regular chow diet [73]. They displayed high levels of VLDL and LDL and the marked elevations in both ApoB-48, ApoB-100 fractions [74]. In that regards, this mouse model can be useful to study atherosclerosis and, consequently, the anti-atherosclerotic drugs, without the need of an atherogenic diet.

However, this double KO model does not closely reflect the lipid profiles of the disease in humans. This is partially due to the presence of an Apobec-1, RNA-specific cytidine deaminase enzyme in the mouse liver. According to the current understanding, this enzyme is a catalytic component of endosomes switching the ApoB-100-encoding mRNA to an mRNA coding for ApoB-48. Incorporation of ApoB-48 into VLDL inducts its rapid clearance by scavenger receptors, prior to conversion of VLDL into LDL particles. The study showed that even moderate Apobec-1 expression leads to aberrant hyper-editing [75]. Such editing results in the resistance of plasma LDL elevation in mice bringing out the limitation to the use of this model. It is possible to overcome the Apobec-1 activity by applying further gene manipulation strategies, such as transgene expression of human ApoB-100 or disruption of mouse Apobec-1 gene, therefore, creating other useful genetic murine models focused on the elevation of ApoB-100 levels that will be described below [76, 77]. The creation of the different gene knockout and transgenic mice that do not require dietary manipulations has enhanced the understanding of the mechanisms regulating plasma lipid levels.

Transgenic ApoB100+/+/LDLR-- knock out mice

Transgenic ApoB100+/+/LDLR-- knock out (TgApoB+/+/LDLR-- KO) mice are able to exhibit accelerated atherosclerosis on a chow diet, thus, they provide an excellent model for the atherosclerosis research. These mice showed atherogenic lipid profile of close resemblance to that in human atherosclerosis: dramatically elevated cholesterol and triglyceride plasma levels contained mainly in the IDL/LDL fraction, and significantly reduced HDL-C plasma levels [78]. It was reported that the lesion development does not require a high-fat, high-cholesterol diet (HFHC) intake in these mice, and they were able to develop complex and extensive atherosclerotic lesions involving approximately 15-20% of the aortic intimal surface (abdominal and terminal segments) [78]. This model was successfully used to study the anti-inflammatory effects of statins on the atherosclerotic plaque inflammation [79] and diet interventions on metabolic syndrome [80]. In addition, the hypercholesterolemia of TgApoB+/+/LDLR-- KO mice is associated with a clear locomotor deficit and impairment of the episodic-like memory, so these mice can also serve as a model for a cognitive and a psycho-motor decline [81].

LDLR/Apobec-1 double knock out mice

LDLR/Apobec-1 double knock out (LDLR/Apobec-1 DKO) mice are deficient in the ability to convert ApoB-100 to ApoB-48 in the liver, and, also in LDL clearance. They exhibit high levels of ApoB-100-LDL-C, closely reproducing the plasma lipid profiles of human Type II FH, the most frequent type of FH observed in humans [82]. The model is characterized by the development of severe spontaneous atherosclerosis on a normal chow diet and its thorough analysis of the plaque development and progression was described [83]. Thus, the lesions that spontaneously develop in LDLR/Apobec-1 DKO mice begin as fatty streaks (stage I, American Heart Association classification) in the proximal aortic regions, as early as 12 weeks, and progressively worsen with age. These lesions spread to distal regions and, by 72 weeks of age, can occupy over 60% of the entire arterial tree. These models can replicate histologically different stages of atheroma beginning with fatty streaks, progressing to a clear human stage IV atheroma and then to a thin cap fibrous atheroma. Cap rupture is unusual for this model but there is a piece of evidence for the cap erosion [83]. Accordingly, LDLR/Apobec-1 DKO is a potentially available important model to study the evolution of atheroma.

Apolipoprotein ApoE*3-Leiden mice

Apolipoprotein ApoE*3-Leiden mutation is associated with one of the genetic forms of hyperlipidemia [84]. Transgenic mice were generated using a genomic DNA construct containing the mutant ApoE and ApoC1 genes with all regulatory elements isolated from the APOE*3-Leiden proband [85]. The primary effect of the dominant ApoE*3-Leiden mutation is an
impaired clearance of triglyceride-rich lipoproteins (chylomicron- and VLDL-remnants) caused by a reduced affinity of the apolipoprotein ApoE*3Leiden for the LDLR3. The ApoE*3Leiden transgenic mice can develop diet-dependent hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. In these mice, when fed a mildly HFHC diet, early fatty-streak formation was observed in the aortic arch after 3 months, and late complex atherosclerotic lesions consisting of plaques with a necrotic core and a fibrous cap (stages IV and V) was registered after 3 to 6 months of feeding a severe HFHC diet [86]. Also, they showed dramatically elevated total plasma cholesterol and triglyceride levels attributed to an increase in VLDL/LDL particles [87].

One of the advantages to the use of this model is the presence of the functional ApoE, so the effects of hyperlipidemia can be studied independently of other ApoE activities. Another advantage is that the introduction of human CETP gene into ApoE*3-Leiden mice results in more human-like lipoprotein metabolism in ApoE*3Leiden. CETP mice [88]. These mice showed a human-like response to the clinically used lipid-modulating pharmacological interventions, such as statins, fibrate, ezetimibe, and niacin [89-91]. Based on numerous features in common with human atherosclerotic lesions and the similar diet-induced lipid metabolism, transgenic ApoE*3-Leiden mice were considered as one of the most accurate animal models of human atherosclerosis [92] posing a great value to study lipoprotein remnant metabolism and accelerated atherosclerosis. However, the disadvantage of this model is that ApoE3-Leiden mice lack plaque rupture, thrombus formation, and/or haemorrhage, the atherosclerotic events of profound importance for humans.

Proprotein convertase subtilisin/kexin type 9-recombinant adeno-associated virus mice

The construction of proprotein convertase subtilisin/kexin type 9-recombinant adeno-associated virus (PCSK9-rAAV) mouse model is a rapid, easy and cost-effective approach to study atherosclerosis [93]. Noteworthy, PCSK9, is a novel subtilase serine protease highly expressed in liver and intestine. It binds to hepatic LDLR on the cell surface promoting LDL lysosomal degradation that increases LDL plasma levels in humans and mice [94, 95]. The intracellular binding of PCSK9 was also described [96]. It was reported that PCSK9 gene regulates cholesterol homeostasis exclusively through the LDLR [97]. FH with severe hypercholesterolemia and early CVD can be associated with “gain-of-function” mutations in the PCSK9 gene [98]. In mice, AAV transduction with human or murine “gain-of-function” PCSK9 mutant gene resulted in doubling of serum cholesterol on the normal diet, compared to controls, and this effect was stable and preserved after 12 months post-transduction [28]. The Western-type diet exacerbated hyperlipidemia in these mice [28]. The lipoprotein profile of PCSK9<sup>pr</sup>-AAV mice fed with the Western-type diet revealed an equal distribution between VLDL and LDL particles [99].

Moreover, the atherosclerosis progression in PCSK9<sup>pr</sup> transgenic mice was observed in a dose-dependent manner of feeding [28]. Lesions resembling those of LDLR-/- mice occurred throughout the vasculature progressing to the fibro-atheromatous stage [100], and vascular calcification occurred within the time frame of 15-20 weeks [101]. In that regards, this model makes possible to conduct studies on vascular calcification of atherosclerotic plaques and therapeutic interventions by avoiding laborious and costly mouse colony generation allowing to model human atherosclerosis in animals with different genetic backgrounds. In addition, similarly to other described above murine models, the main limitation of this model is the absence of spontaneous plaque ruptures, the leading cause of acute events in humans.

Apolipoprotein E-deficient fibrillin-1 mutant (ApoE<sup>−/−</sup>-Fbn1C1039G+/−) mice

Fibrillin-1 (Fbn1) is a component of microfibrils associated with elastic fibres that provide structural support to a vessel wall [102]. Notably, mutations in the Fbn1 gene are the cause of the Marfan syndrome, a genetic disorder characterized by fragmentation of elastic fibres [103]. On a Western-type diet, ApoE deficient Fibrillin-1 mutant (ApoE<sup>−/−</sup>-Fbn1C1039G+/−) mice develop fragmentation of the elastic fibers and large atherosclerotic plaques with prominent features of plaque instability, including an enlarged necrotic core occupying about 30% of
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Figure 1. The utility of genetic rabbit models of human atherosclerosis. Note: WHHL - Watanabe Heritable Hyperlipidaemic; STH - St. Thomas’ Hospital; CETR - cholesteryl ester transfer protein; ApoB - apolipoprotein B; VLDL - very low-density lipoprotein.

Rabbit models of human atherosclerosis

Rabbits were the first animals to model atherosclerosis but, since the year 2000, there is a downward trend of using rabbit models, and that is probably due to the wide availability of murine models. However, rabbits have the same advantages as mice for modelling of human atherosclerosis, including ease of maintenance and availability, low economical cost, and are more appropriate for catheter-based procedures and non-invasive imaging. Moreover, rabbit models are considered to be the best models to study hyperlipidemia and, in turn, atherosclerosis because they possess several unique features of lipoprotein metabolism that are identical to humans and, there-

[108]. Spontaneous plaque ruptures were observed in this model [107, 109]. Several reviewers indicated that sudden death in ApoE-/Fbn1-C1039G+- mice fed with a Western-type diet can be observed in the time period between 16 and 23 weeks, with 50% mortality rate after 20 weeks [28]. Mice that died suddenly exhibited a significantly higher frequency of coronary stenosis, compared to survivors [107], suggesting that the plaque development in coronary artery plays an important role in cardiac death.

Taken together, ApoE-/Fbn1-C1039G+- mice can model human end-stage atherosclerosis, therefore, offer an opportunity to investigate the role of key factors involved in plaque destabilization and potential molecular targets for therapeutic interventions. Importantly, among all murine models, these mice are the best suited for spontaneous plaque rupture studies. They can be also used for the development of innovative molecular imaging strategies for vulnerable plaques [110].

total plaque area, strongly diminished collagen content (a thin fibrous cap with an important loss of collagen fibres), high level of inflammatory cytokines and matrix metalloproteinases, T-cell infiltration, SMC apoptosis, and numerous concealed caps, not only at the level of aortic valves but also in the brachiocephalic artery, and in different areas of the aorta [104-106]. Also, the elastin loss would result in fibrous cap exposure to increased biomechanical stress [106]. Moreover, similar to human pathology, elastin fragmentation in combination with a Western-type diet in these mice are the essentials for the development of atherosclerotic plaque neovascularization, intra-plaque haemorrhage, and plaque rupture resulting in MI, stroke and sudden death [104, 107]. These features are uncommon for murine atherosclerosis models but are known to favourably affect plaque progression and vulnerability in humans.
fore, advantageous for the atherosclerosis re-
search. The features of lipid metabolism attrib-
uted to rabbits are the following: (i) alike hu-
man, rabbits abundantly express plasma CETP,
an important regulator of the cholesterol meta-
bolism [111]; (ii) rabbit ApoB-containing lipo-
proteins are similar to those of humans in their
chemical composition and apoprotein content
(VLDL and LDL) [111]; (iii) ApoB mRNA editing
does not occur in a rabbit’s liver, hence, ApoB-
100-containing VLDL is produced and that is
similar to humans [111]; and (iv) finally, rabbits
are very susceptible to diet-induced atheroscle-
rosis [112]. In that regards, two strains, namely
Watanabe Heritable Hyperlipidaemic (WHHL)
and St. Thomas’ Hospital (STH) rabbits were
reported as relevant models for human hyper-
lipidemia and atherosclerosis (Figure 1) [113,
114]. Noteworthy, naturally defective in LDLR,
WHHL rabbits exhibit lipid metabolism pattern
identical to that seen in human FH [115]. The
recent advances in gene technology enabled
the generation of a variety of transgenic rab-
bits, therefore, providing a unique system to
study the properties important for human ath-
erosclerosis. One example, ApoB-100 trans-
genic rabbits were created, which are capable
to manifest combined hyperlipidemia with
reduced HDL-cholesterol concentrations allow-
ing to study of ApoB metabolism, therefore,
dietary manipulation and drug intervention
studies in these animals should be more rele-
vant to humans [116]. Other transgenic rabbit
models of human atherosclerosis highlighting
the effects of individual genes on lipoprotein
metabolism and atherosclerosis susceptibility
were also described [117].

Porcine models of human atherosclerosis
(Table 2)

Pigs, particularly minipigs, offer large-animal
models that have the human-like size and car-
diovascular anatomy of stronger genetic resem-
lance to humans. Having a large animal model
capable of developing human-like atheroscle-
sis is crucial for translational research.

In terms of atherosclerosis developmental pro-
cess, pigs have a number of important differ-
ces to humans, such as lack of CETP, the
requirement for high dietary cholesterol intake
typically combined with cholic acid, and lesion
progression is restricted to only foam cell for-
mation stage within a reasonable time frame
[12]. These differences caused some limita-
tions to the use of porcine models but genetic
engineering of minipigs helped to overcome the
disadvantages. Cloning by the somatic cell
nuclear transfer become the method of choice
for developing of both transgenic and gene-
edited animals. Most of the published genetic-
ally modified pig models were developed by
gene editing in porcine somatic cells followed
by the animal cloning [118-121].

It was found that atherosclerotic lesions de-
veloping in pigs exhibit overall morphology and
several specific pathological features that are
common for human lesions but not seen in
mice. Thus, lesions grow in the abdominal ao-
rita, iliofemoral arteries, and proximal segmen-
ts of the coronary arteries; initial foam cell
lesions evolve in an intima that also contains
connective tissue matrix and SMCs [12]. Pla-
que neovascularization, as well as calcification,
are also present [122]. Importantly, as in mu-
ine models, plaque ruptures are rare events in
pigs and that limits the suitability of porcine
models to study thrombotic complications [12].
Moreover, the development of spontaneous
atherosclerosis in pigs is rare, but pigs on HFHC
diet can develop advanced atherosclerotic
lesions similar in type and location to those
that occur in humans [123]. However, HFHC
diets are very expensive and require a long time
to manifest the disease, thus, making many
studies unaffordable.

Because of the closer phylogenetic relation-
ship [124], anatomy, physiology, body weight, lifes-
pan, and atherosclerosis pathology to humans
[125], the efficacy of drug testing in porcine
models may be more predictive than using murine models. Though, the absence of CETP
makes them unsuitable to study CETP inhibi-
tors or other lipid-altering interventions that
may require CETP for modelling the full effects
of lipids on atherosclerosis [126]. The predic-
tive value of porcine models for drug discovery
studies is currently unknown due to the scarcity
of the data. The close match of the porcine car-
diovascular system to humans, including pig
heart size, blood supply, coronary system func-
tion, the anatomy of the aorta [127], and the
relevant morphology of porcine coronary le-
sions allow validation of intravascular imaging
tools in vivo. Pigs offer an opportunity to per-
form examinations of atherosclerotic lesions in
conjunction with the evaluation of clinical imag-
ing end-points, hence, to decide which of the
end-points is most sensitive for the specific
### Table 2. Genetic porcine models available for the atherosclerosis research

<table>
<thead>
<tr>
<th>Model type</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Utility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg D374Y-PCSK9 minipigs</td>
<td>Develop moderate hypercholesterolemia and human-like atherosclerosis on the standard low-fat diet</td>
<td>The high-fat diet required to develop severe hypercholesterolemia and advanced atherosclerosis</td>
<td>Multiple expression of the mutant transgene may limit the utility for treatments designed to increase LDLR expression or reduce PCSK9 activity</td>
<td>[12, 122, 130-132]</td>
</tr>
<tr>
<td>LDLR/-/- minipigs</td>
<td>Develop advanced human-like atherosclerosis, including coronary lesions</td>
<td>The high-fat diet required</td>
<td>Suitable for coronary atherosclerosis research, development of plaque stabilization drugs and percutaneous diagnostic and interventional devices</td>
<td>[130, 133, 148]</td>
</tr>
<tr>
<td>ApoE/-/- minipigs</td>
<td>Develop moderate cholesterolemia and atherosclerosis on the low-fat diet</td>
<td>The high-fat diet required to develop prominent human-like dyslipidemia and progressive atherosclerosis</td>
<td>Suitable for translational studies of atherosclerosis</td>
<td>[136, 137]</td>
</tr>
<tr>
<td>ApoE /LDLR double KO minipigs</td>
<td>Develop atherosclerosis-related lipid metabolism</td>
<td>Validation of atherosclerosis phenotype is limited possibly due to the fact that in-frame mutations in both the ApoE and LDLR alleles may give rise to truncated ApoE and LDLR proteins with the partially retained function</td>
<td>Useful for studies determining the function of genes involved in the progression of human atherosclerosis</td>
<td>[138]</td>
</tr>
</tbody>
</table>

Note: Tg D374Y-PCSK9 minipigs - Transgenic D374Y-PCSK9 minipigs; LDLR - low-density lipoprotein receptor; LDL - low-density lipoprotein; LDLR /- - low-density lipoprotein receptor deficient; ApoE/- - apolipoprotein E deficient; ApoE/LDLR double KO minipigs - apolipoprotein E and low-density lipoprotein receptor double knockout minipigs.
pathology [128, 129]. In this respect, they are considered promising models bridging preclinical and human clinical studies. Thus, the pig is an important animal for the research of atherosclerosis and CVDs. Overall, limitations to porcine atherosclerotic models include the necessity for well-developed infrastructure to support animal maintenance, a long-time period to create and monitor lesions and substantial financial investment.

**D374Y-PCSK9 transgenic minipigs**

Yucatan or D374Y-PCSK9 transgenic minipig model was created by the liver-specific expression of the “gain-of-function” mutant D374Y in the PCSK9 gene that can cause a severe form of hypercholesterolemia and, ultimately, atherosclerosis [12]. They exhibited moderate hypercholesterolemia on standard feed and more severe hypercholesterolemia and human-like atherosclerosis were observed on HFHC diet [12]. Moreover, these animals have severely reduced hepatic LDLR levels, thus, augmented concentrations of LDL-C in combination with HFHC diet are sufficient conditions to induce atherosclerosis. The lesions were detected in the thoracic and abdominal aorta, the iliofemoral arteries, and the coronary arteries at 12 months of age [12]. At this age, Yucatan minipigs reach near-human weight making this model useful for testing different imaging techniques without modifications, such as clinical scanners and intravascular devices, in order to advance them into clinics.

Furthermore, the study reported that atherosclerotic lesions in these transgene minipigs recapitulated several histologic features of human atherosclerosis, such as the development of complex lesions with SMCs, extracellular matrix, and inflammatory infiltrate, in addition to macrophage-derived foam cells [122]. Atheromas demonstrated characteristics of advanced human plaques, including necrotic cores, fibrous tissue, calcification, plaque angiogenesis, and intraplaque haemorrhage [122]. However, with the mutant transgene being permanently overexpressed at nearly five hundred times over the normal level, these pigs may have limited utility for treatments designed to increase LDLR expression or reduce PCSK9 activity [130]. Potential benefits of this model include their human-like size, their well-described background, and the ability to induce progressive atherosclerotic lesion without the use of cholic acid, which has several unwanted side-effects. In addition, a few studies reported the suitability of this model for studies of the advanced coronary plaque formation mechanisms [131] and in vivo validation of coronary optical coherence tomography for plaque classification [132].

**LDLR deficient minipigs**

LDLR KO minipig model was generated using AAV-mediated delivery of gene targeting vector and somatic cell nuclear transfer in Yucatan minipigs with subsequent breeding of heterozygous [130]. Accordingly, in comparison to PCSK9-D374Y transgenic pigs, a more severe and more rapidly developing atherosclerosis occurs in this model, thus, decreasing the duration and costs of studies. Feeding HFHC diet, they develop advanced atherosclerotic lesions in the coronary arteries and abdominal aorta that closely resemble those in humans [130]. Thus, LDLR KO minipigs can be used for the development of human-like advanced coronary atherosclerosis, and, in turn, for translational research, particularly, for the development of plaque stabilization drugs and percutaneous diagnostic and interventional devices [133]. However, prolonged trials with the expensive HFHC diet makes this model cost-prohibitive for the broad application.

This model was reproduced using pig of different genetic background, such as domestic pigs that allowed to decrease the time of lesion development to four months on HFHC diet, and, moreover, to get more complicated human-like advanced plaques containing the necrotic core, hemorrhage, and calcification [133]. Besides, even more accelerated coronary plaque development can be achieved in these pigs with the use of coronary artery balloon injury. For instance, coronary plaques were detected eight weeks after the coronary artery injury [134]. Coronary angioplasty is an established procedure in domestic swine with normal cholesterol level but accelerated plaque development poorly resembles the natural course of human atherosclerosis, i.e. the lesions have abundant fibrotic tissue with little necrotic core formation [135]. This model can be useful for studies of specific atherosclerosis-related processes and imaging studies detecting a specifically localized plaque.
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**ApoE deficient minipigs**

In order to establish an improved large animal model of FH and atherosclerosis, ApoE/- mini-pigs were produced using the clustered regularly interspaced short palindromic repeat-associated protein 9 system (CRISPR/Cas9) to disrupt the ApoE gene in Bama miniature pigs [136]. The recent study demonstrated that similarly to PCSK9 transgenic pigs and LDLR KO pigs, ApoE/- pigs showed moderately increased plasma cholesterol levels on a low-fat diet resembling the human FH phenotype [136]. Moreover, when fed HFHC diet, these pigs showed severe hypercholesterolemia and developed progressive atherosclerotic lesions [136]. Likewise, another study showed that targeted gene KO of ApoE in Yucatan minipigs on the low-fat diet can cause remnant lipoproteinemia closely resembling the human familial dysbetalipoproteinemia, which on HFHC feed was significantly accentuated, and also, these minipigs displayed accelerated progressive atherosclerosis [137]. In that regards, ApoE/- minipigs could be beneficial for the elucidation of ApoE gene functions and translational studies of atherosclerosis because their serum lipid profile and atherosclerotic lesions can sufficiently recapitulate those in human disease.

**ApoE and LDLR double knockout minipigs**

ApoE/LDLR double knockout pigs were generated via CRISPR/Cas9 gene-editing approach targeting ApoE gene and LDLR simultaneously in Bama minipig embryonic fibroblasts, then they were used as nuclear donors to produce the double KO animals [138]. The gene-modified swine showed an abnormal lipid metabolism related to atherosclerosis from an early age. As compared to wild-type pig, at 2 months of age serum LDL-C was elevated by approximately 41%; there was also 57% elevation of serum total cholesterol and 120% elevation of serum triglycerides [138]. Atherosclerosis phenotype was not described in these pigs, possibly due to the fact that in-frame mutations in both the ApoE and LDLR alleles may give rise to truncated ApoE and LDLR proteins with the partially retained function [138]. Since ApoE and LDLR gene mutations play an important role in the atherosclerotic disease progression, this model can aid in determining the function of genes, thus, establish a valuable tool to study human atherosclerosis.

**Conclusion and comments for future**

Modelling atherosclerosis in genetically modified animals is currently mainstream in atherosclerotic research. The genetic animal models available to date can simulate all stages of plaque development providing an opportunity to study the pathogenesis of lesion formation, mechanisms of plaque vulnerability and regression and, therefore, evaluation of new therapeutics and imaging techniques. Each model differs in its potentials and possibilities of application. A good understanding of the similarities and differences between the animal model and human disease is important for the effective extrapolation of data for the translational application.

All described models of atherosclerosis rely on the hypercholesterinemia, as a major triggering factor, and this is one of the critical limitations because the real-life disease is usually multifactorial. In future studies, the inclusion of other risk factors of atherosclerosis may lead to better models of this complex disease with higher translational validity.

In addition, targeting LDL-lowering therapy to individuals at risk remains challenging, due to the lack of sensitive and specific diagnostic methods that can detect silent atherosclerosis prior to life-threatening clinical events. Further studies are required to solve this problem.

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**Disclosure of conflict of interest**

None.

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