Universiteit
Leiden
The Netherlands

Application of zebrafish and murine models in lipoprotein metabolism and atherosclerosis research

Verwilligen, R.A.F.

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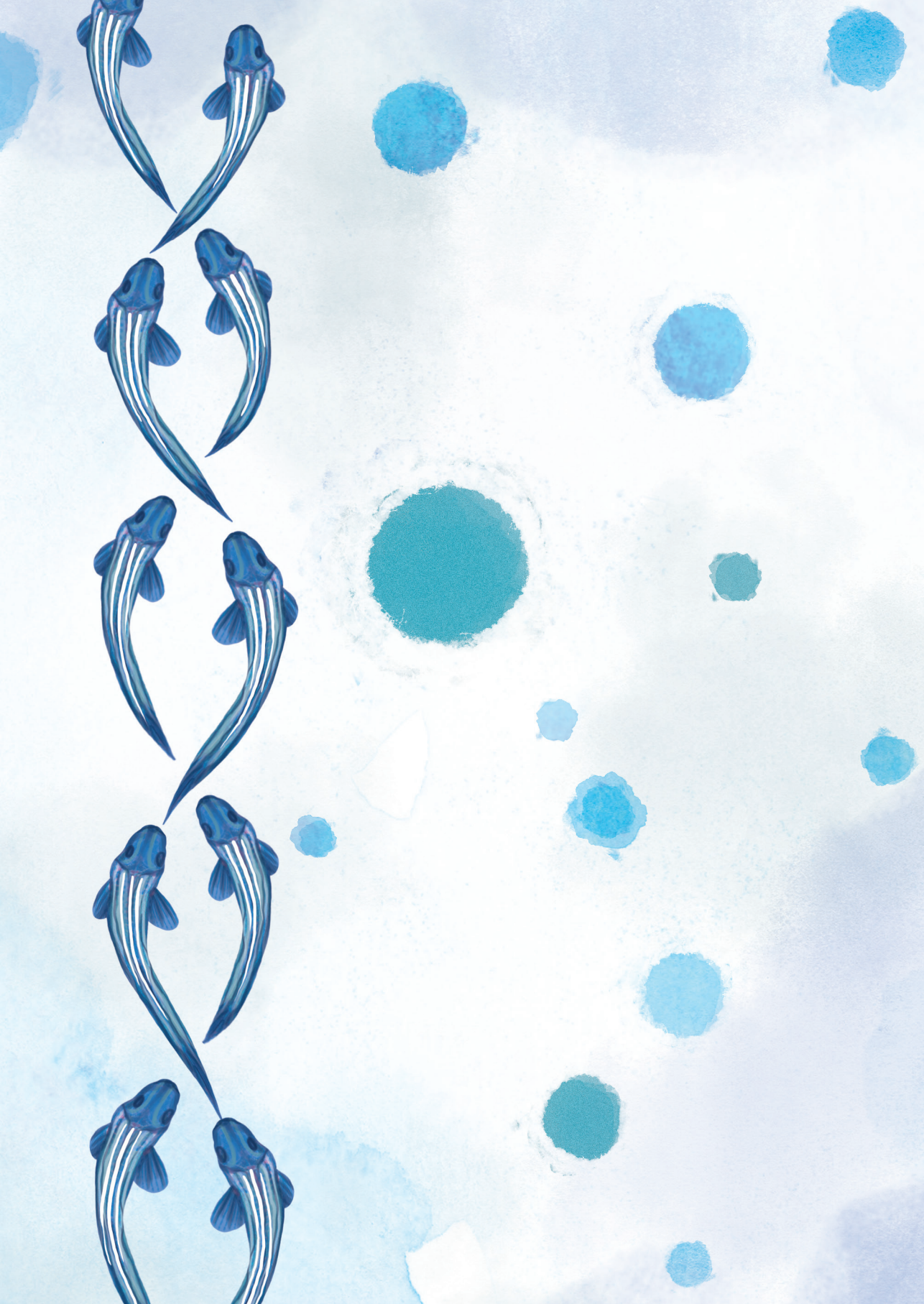

CHAPTER 1

GENERAL INTRODUCTION

1) Cholesterol and lipoprotein metabolism

For the last decade, it has been shown that balanced cholesterol levels and a functional cholesterol metabolism are of great importance for the prevention of different lipid-related diseases. Elevated cholesterol levels have been associated with an increased risk for cardiovascular disease. However, cholesterol and other types of lipids such as triglycerides, are crucial for multiple cellular and physiological processes throughout the body.

Cholesterol is known as the main sterol in mammals. The most prominent functions of cholesterol are to maintain cell membrane functionality and to serve as the precursor for steroid hormones synthesis such as cortisol and progesterone, bile acid synthesis and vitamin D synthesis[1,2]. The liver and intestine are two organs important in the control of cholesterol homeostasis. The intestine is mainly involved in the uptake of dietary as well biliary cholesterol, whereas the liver is more involved in the production of cholesterol and the regulation of systemic cholesterol metabolism[3,4]. Cholesterol from the liver can either be converted into bile acids or be packaged into apolipoprotein B-containing particles, which are secreted into circulation.

The packaging of cholesterol into lipoproteins is essential for its transport throughout the body as lipids are water insoluble and not able to circulate freely in the blood[5].

1.1) Lipoproteins

As mentioned above, lipoproteins play a key role in the uptake and transport of dietary lipids from the intestine and the transport of lipids produced in the liver to the periphery and vice versa[5].

Lipoproteins are complex vehicles that consist out of a hydrophobic core surrounded by a hydrophilic monolayered phospholipid shell. The hydrophobic core contains primarily neutral lipids, cholesteryl esters and triglycerides, whereas the hydrophilic shell mostly consists out of phospholipids with hydrophilic head groups, free cholesterol, and specific apolipoproteins.

There are different classes of lipoproteins based on their size, density, and apolipoprotein composition: chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL)[6]. Chylomicrons and VLDL are the largest particles and are primarily comprised of triglycerides, whereas the core of LDL, a remnant of VLDL, and HDL consists primarily out of cholesteryl esters (**Table 1**)[3]. Furthermore, lipoproteins are also classified according to their

apolipoproteins, that are acting as a ligand for lipoprotein receptors. The primary apolipoprotein of chylomicrons, VLDL and LDL is apolipoprotein B (ApoB), whereas the main apolipoprotein of HDL is apolipoprotein A1 (Apo A1)[7,8].

Table 1. Characteristics of human lipoproteins. Adapted from Wang *et al. (*2017) DOI: 10.5604/01.3001.0010.5495

Lipoprotein	Size (nm)	Density (g/mL)	Apolipoproteins	Core lipids
Chylomicrons	75-1200	<0.930	ApoA1, A-II, A-IV, ApoB48, ApoC, ApoE,	Triglycerides
Very-low-density lipoprotein	30-80	0.930-1.006	ApoB100, ApoC, ApoE	Triglycerides
Low-density lipoprotein	18-25	1.019-1.063	ApoB100	Cholesteryl esters
High-density lipoprotein	5-12	1.063-1.210	ApoA1, Apo A-II, ApoC, ApoE	Cholesteryl esters

1.2) Exogenous and endogenous lipid metabolism

The total body lipid levels are regulated by on the one hand the exogenous lipoprotein metabolism pathway and, on the other hand, the endogenous pathway (**Fig. 1**). In the exogenous pathway, dietary triglycerides and cholesterol are absorbed by the intestine and repackaged into chylomicrons molecules, that serve as a source of energy for peripheral tissues and organs. After repackaging, the nascent triglyceride-rich chylomicrons are secreted into the lymph whereafter they enter the blood circulation. To become mature chylomicrons, the nascent chylomicrons loose Apo A and require apolipoprotein C (ApoC) and apolipoprotein E (ApoE)[9,10]. In the circulation, the triglycerides inside the core of the chylomicrons are lipolyzed by lipoprotein lipase (LPL), which is primarily expressed on endothelial cells[11]. During this process, free fatty acids and glycerol are released and metabolized in muscle cells or brown and white adipose tissue either for generating energy or for storage[12]. Upon lipolysis of the triglycerides, the chylomicrons reduce in size and become cholesterol ester-rich chylomicron remnants, which are rapidly cleared from the circulation through the interaction of ApoE with the LDL receptor (LDLr), LDL receptor related protein (LRP), scavenger receptor class B type 1 (SCARB1), other remnant receptors and heparin sulphate proteoglycans[13–15].

When the supply of dietary cholesterol and triglyceride levels is not sufficient, lipids derived from the hepatic uptake of chylomicron remnants can be combined with de novo synthesized lipids and (re)enter the endogenous pathway as VLDL. In the endogenous pathway, the VLDL particles that contain ApoB100 as well as

newly synthesized ApoC and ApoE, circulate through the bloodstream as source for triglyceride-derived fatty acids, similarly as chylomicrons from the intestine. The triglycerides of VLDL are also hydrolyzed by LPL leading to the formation of intermediate-density lipoproteins (IDL)[16]. A part of these IDL particles is removed from the circulation through the interaction of ApoE with different receptors expressed on the liver including LDLr, LRP, SCARB1 and other remnant receptors. Further hydrolysis of the remaining IDL particles results in the formation of the smaller triglyceride-deprived LDL particles, which account for the majority of the transport of cholesterol in humans[17]. The main apolipoprotein on LDL is ApoB100. ApoB100 is involved in the clearance of LDL through its interaction with LDLr expressed on the liver, the predominant site of uptake. Besides the liver, organs and tissues in the periphery could also take up these cholesteryl ester-rich particles for regulation of their intracellular cholesterol metabolism. Together these processes form a continuous cycle thereby ensuring the continued supply of cholesterol and fatty acids to the tissues in the body.

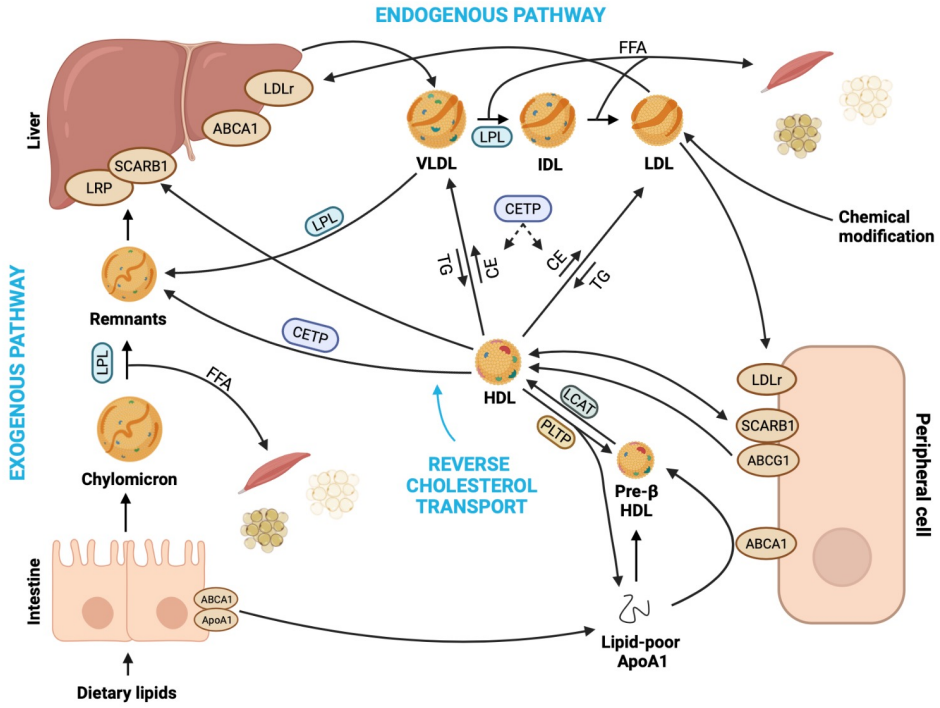

Figure 1. Schematic overview of pathways involved in human lipoprotein metabolism, including the exogenous, endogenous and reverse cholesterol transport pathways. Adapted from Kwan *et al.* (2007). DOI: 10.1681/ASN.2006091006

1.3) Reverse cholesterol transport and HDL metabolism

The reverse cholesterol transport pathway facilitates the transport of excessive cholesterol from peripheral tissues back to the liver, where it can be recycled or excreted into bile. The essential lipoprotein in this pathway is HDL. The main structural protein of HDL is ApoA1, which is produced by the liver and the intestine through the interaction of ApoA1 with the ATP-binding cassette transporter A1 (ABCA1)[18].

ABCA1 is also the primary cholesterol efflux transporter mediating the efflux of cholesterol from the periphery and liver to HDL, a process which leads to the formation of discoidal pre-β HDL particles. Further cholesterol enrichment via either passive diffusion or via active cholesterol transfer by ABCG1 and SCARB1, results in the formation of larger cholesterol-rich HDL particles[19].

For this to become mature HDL, the acquired cholesterol on the surface of HDL needs to be esterified by the enzyme lecithin-cholesterol acyltransferase (LCAT) to form cholesteryl esters. Subsequently, several lipases including phospholipid transfer protein (PLTP), are able to mediate the remodeling of the HDL particles. During this process, phospholipids as well as triglycerides are hydrolyzed and cholesteryl esters are transported to the core of the particle (add ref). In humans, the enzyme cholesterol ester transfer protein (CETP) facilitates the transport of HDL cholesteryl esters to triglyceride-rich lipoproteins such as VLDL and LDL[20]. This step links the reverse cholesterol transport pathway to the endogenous cholesterol pathway, in which cholesteryl esters are delivered to the hepatocytes for either elimination or biliary excretion or can be transported back to peripheral cells. Besides the transport of cholesteryl esters from HDL to VLDL/LDL via CETP, HDL cholesteryl esters can also directly be taken up through SCARB1, also known as the HDL receptor. Besides, when HDL is ApoE rich, HDL can be taken up via the LDLr, LRP or other remnant receptors.

1.4) Scavenger receptors in lipoprotein metabolism

The recruitment and accumulation of serum lipoproteins into the arterial wall, where they are susceptible to oxidation, induces a chronic pro-inflammatory cascade that initiates the pathogenesis of atherosclerosis. Atherosclerosis is considered a complex inflammatory disease related to high cholesterol levels resulting in vascular endothelial dysfunction and lipid accumulation in the arterial wall[21]. Recently, it has been demonstrated that oxidized phospholipids, part of oxidized LDL (oxLDL), are pro-inflammatory and therefore seen as the key component stimulating atherogenesis[22]. Modification of the LDL particles renders them susceptible for recognition by macrophage scavenger receptors, a diverse superfamily of membrane bound receptors that play an essential role in the removal of foreign and damaged molecules. In the

last several years, it has been shown that scavenger receptors are able to initiate pro-inflammatory signaling cascades influencing immune cell activation and as well as stimulate cellular lipid accumulation, linking this family of receptors to atherosclerosis development[23,24]. Brown and Goldstein *et al.* (1979) were the first to describe a key role for these receptors in the process of foam cell formation and early atherogenesis. They found that an increased cholesterol content of the cell does not inhibit the binding and internalization of (modified) lipoproteins via scavenger receptor pathways indicating that cells expressing these receptors would not be able to limit the uptake of the lipoproteins rendering them susceptible for foam cell formation[25].

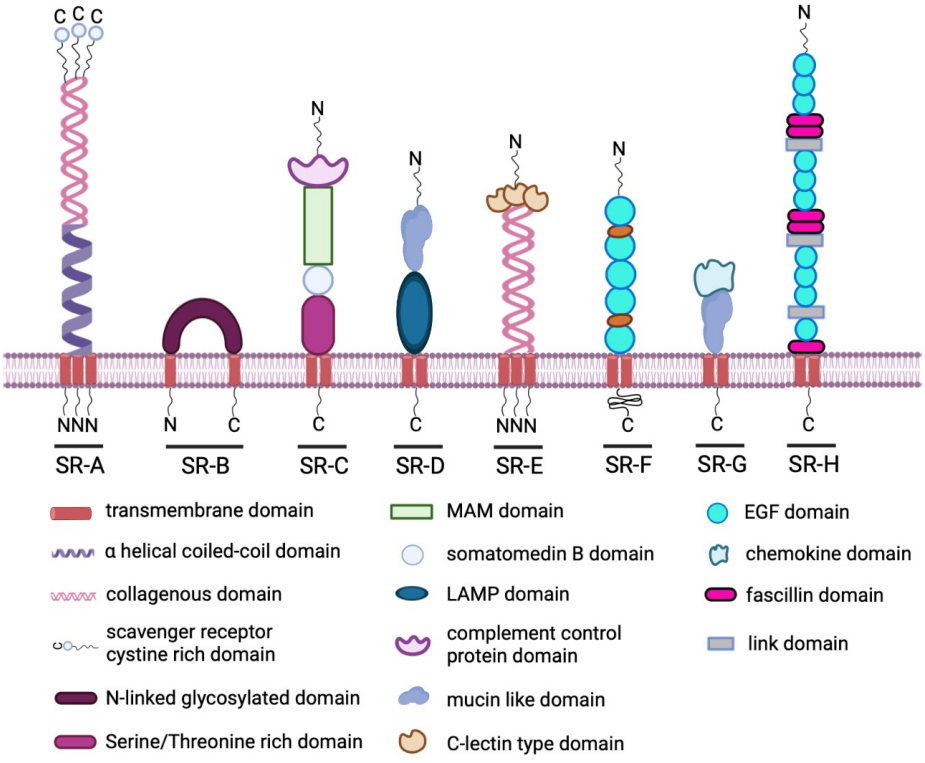

Figure 2. Schematic representation of classes of scavenger receptors. Adapted from: PrabhuDas *et al.* (2017). DOI: 10.4049/jimmunol.1700373

To date, there are 8 classes of functionally and structurally diverse scavenger receptors: class A, B, C, D, E, F, G, and H (**Fig. 2**). Most of these classes have been linked to the development of atherosclerosis, either by contributing to the uptake of modified lipoproteins resulting in foam cell formation, modulating the lipid efflux, regulating inflammatory mechanisms, or by actively participating

in the adhesion and migration of inflammatory cells, such as monocytes and macrophages[26]. In this thesis, the role of the class B receptor SCARB1 and class H receptors stabilin-1 and 2 in cholesterol metabolism and atherogenesis is extensively discussed.

2) Cardiovascular disease and atherosclerosis

Cardiovascular diseases (CVDs) are a major cause of morbidity and mortality[27]. CVDs include all the diseases that affect the heart and blood vessels such as myocardial infarction, coronary heart disease, angina pectoris, stroke, and peripheral artery disease. Major risk factors that increase the risk for developing CVD include high plasma cholesterol levels, e.g. due to high LDL levels, high triglyceride levels, a sedentary lifestyle, obesity, diabetes and hypertension[28]. Furthermore, people suffering from familial hypercholesterolemia, inflammatory or autoimmune diseases have an increased risk for the development of CVDs[29,30]. In 2019, 17.9 million people died of the consequences of CVD, representing 32% of all reported global deaths. In time, this cardiovascular mortality rate is expected to increase due to 1) aging and the increased prevalence of obesity in the general population[31] and 2) the fact that statins only lower the cardiovascular event rate by 20-40%[32]. A major problem is that current treatments for patients suffering from CVDs are only able to slow down the progression of atherosclerotic lesions. Therefore, there is still an urgent need for new therapeutic targets for the underlying pathology of CVD, atherosclerosis.

2.1) Early atherosclerosis

Atherogenesis is initiated by changes in the monolayer of arterial endothelial cells, which normally resist attachment of leukocytes. Upon irritative stimuli such as pro-inflammatory mediators or dyslipidemia, the expression of several adhesion molecules that capture leukocytes such as intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), and E-selectin, is increased on the surface of the endothelium[33]. Parallel changes in the permeability of the endothelial and in extracellular matrix composition promotes the transport of LDL particles into and retention inside the sub-endothelial space[34]. Within this sub-endothelial space, LDL particles are prone to oxidative modification leading to alterations in particle size and lipid composition[35]. OxLDL initiates inflammatory immune responses by activating the endothelium to upregulate adhesion molecules that capture leukocytes. Tissue macrophages derived from blood monocytes account for the majority of leukocytes inside early atherosclerotic lesions[36]. In the lesion, these macrophages take up this

oxLDL via scavenger receptors and become foam cells that are seen as the hallmark of atherosclerosis. Foam cells secrete inflammatory mediators such as tumor necrosis factor (TNF), interleukin 1 (IL-1), interleukin 6 (IL-6), and several chemokines including CCL5 and CCL2, that amplify the pro-inflammatory immune response[37].

2.2) Advanced atherosclerosis

As lesion formation progresses, many foam cells die by apoptosis due excess free cholesterol toxicity[38]. The ability of macrophages to phagocytose apoptotic cells, also known as efferocytosis, is diminished in the lesion due excessive cholesterol loading. As a result, cholesterol overloading of foam cells also stimulates secondary necrosis triggering the formation of a necrotic core with extracellular cholesterol deposition, typical for advanced atheroma. The formation of advanced atheroma's leads to a more pro-inflammatory environment and subsequently to the recruitment and accumulation of leukocytes including T cells. In the intima as well as in the adventitia, T cells are found to produce cytokines that are able to modulate immune responses causing either exacerbation or amelioration of atherosclerosis[39]. Besides T cells, vascular smooth muscle cells (VSMC) are able to migrate into the growing atherosclerotic lesion and produce collagen leading to fibrosis and the formation of a fibrous caps on top of the atherosclerotic lesion[40]. Fibrous lesions with small lipid pools are defined as stable lesions, whereas lesions with thin fibrous caps and large lipid pools are defined as vulnerable unstable lesions[41]. Stable lesions could be destabilized by the release of extracellular matrix degrading enzymes such as matrix metalloproteinases, which are produced by activated immune cells including macrophages[42]. Although atherosclerosis may remain clinically silent for many decades, gradual thinning of the fibrous cap or erosion of the endothelium overlying the atherosclerotic lesion could lead to disruption of the endothelial layers thereby stimulating the formation of a thrombus, which is defined as atherothrombosis[43]. Rapid occlusion of the arteries during atherothrombosis could lead to severe ischemic events such as myocardial infarctions and strokes.

2.3) Immune system and atherosclerosis

The interplay between lipids and immune cells is believed to be an important factor for the development of atherosclerosis[44] (**Fig. 3**). Whereas dyslipidemia initiates atherogenesis, it is the ongoing chronic inflammatory response, in which the innate as well as adaptive immune system play an essential role, that leads to the formation of advanced atherosclerotic lesions[45]. Multiple studies have shown that the immune cells observed in atherosclerosis have a diverse landscape and that macrophages and T cells are the most abundant cell-type present in the lesions[46–48].

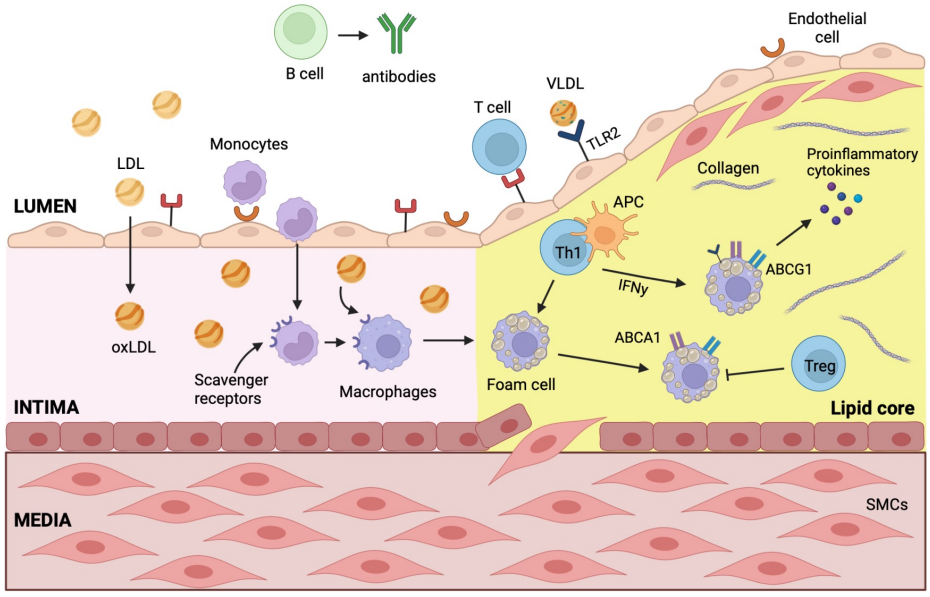

Figure 3. The development of atherosclerosis. Adapted from: Libby *et al.* (2011) DOI: 10.1038/nature10146.

2.3.1) Macrophages

During atherogenesis, macrophages play a pivotal role as they actively participate in (modified) lipoprotein uptake leading to their transformation into macrophage foam cells. Furthermore, these macrophages are also involved in the maintenance of the pro-inflammatory environment as well as in tissue repair and remodeling[49]. Due to the diverse functions of macrophages in atherogenesis, these cells have been proposed as an interesting therapeutic target for the development of novel therapeutic strategies to slow down or even regress the disease. Macrophages are derived from circulating monocytes which are recruited to the nascent atherosclerotic lesion upon environmental stimuli, such as the release of chemokines[50]. There, in the presence of macrophage colony-stimulating factors, the monocytes can differentiate into different subsets of macrophages[51].

The two major macrophage subsets on either end of the spectrum are the classically activated pro-inflammatory M1 macrophage subtype and the alternative anti-inflammatory M2 macrophage subtype. The polarization of macrophages into different subsets is a dynamic process and could be influenced by different micro-environmental factors. Endogenously formed toll-like receptor ligands, such as free fatty acids and oxidized lipoproteins, are likely to promote the polarization of macrophages into an M1 phenotype, whereas T-helper 2 cytokines in combination

of several immune complexes and bio-active lipids could stimulate the polarization into an M2 phenotype[52,53]. M1 macrophages are mainly located in the lipid core and plaque shoulder region. In contrast, M2 macrophages are predominantly located in the distal regions of the atherosclerotic lesion outside the lipid core and are associated with more stable and regressing lesions[54,55]. Although, M1 and M2 are the most predominant macrophage subsets present in atherosclerosis, recently, two new macrophage subsets have been described in the context of atherosclerosis, namely Mox and M4. The polarization into Mox macrophages is induced when macrophages are exposed to oxidized phospholipids such as present in oxLDL, and nuclear factor Erythroid 2-related factor 2 (NRF2) is activated[56,57]. Compared to M1 and M2 macrophages, Mox macrophages display reduced phagocytic activity and chemotaxis. Furthermore, activation of Mox macrophages leads to increased secretion of the pro-inflammatory cytokine IL-1β and release of reactive oxidative species, which in turn can stimulate atherogenesis[58]. M4 macrophages are also considered pro-inflammatory inflammatory as they release the pro-inflammatory cytokines IL-6 and TNFα and have been associated with instable lesions[59].

2.3.2) T cells

Recently, also T cells have come into the picture as an interesting therapeutic target for the intervention in atherosclerosis. T cells can either promote or inhibit immune responses, depending on their phenotype and thereby influence the pathogenesis of this chronic inflammatory disease. An efficient T cell response is generated when antigen presenting cells (APCs) such as macrophages and dendritic cells present cognate antigens, which they have engulfed from the micro-environment, to naïve T cells[39]. Upon antigen recognition via the interaction of APCs and T cells in secondary lymphoid organs, T cells undergo clonal expansion and differentiate into specific memory and effector T cells. After primary activation, the activated T cells migrate into nonlymphoid tissues where they undergo a second activation round by APCs present at sites of inflammation[60].

$CD4^+$ and $CD8^+$ T cells are the two major subtypes of T cells. Major histocompatibility complex (MHC) molecules are crucial for the recognition of antigenic peptides. $CD4^+$ and $CD8^+$ T cells express different MHC molecules; $CD4^+$ T cells express the MHCI molecule, while $CD8^+$ T cells express MHCII molecules. Both $CD4^+$ and $CD8^+$ T cells are found in human and murine atherosclerotic lesions, although the majority of T cells in the lesion are $CD4^+$ T cells instead of $CD8^+$ T cells[39,61]. Depending on the cytokines secreted in the micro-environment, T cells can differentiate into specific subsets including helper T cells (Ths), regulatory T cells (Tregs), and cytotoxic T cells (CTLs). There are different types of helper $CD4^+$ T cells, of which Th1, Th2, and Th17 cells

are the most common in the context of atherosclerosis. More recently also Th9 and Th22 subsets are discovered, but their role in atherosclerosis is still unknown. Each subset exerts different functions, influencing atherosclerosis in their own way. Th1 cells are considered to be pro-inflammatory, as it has been shown that Th1 deficiency reduces atherosclerotic lesion formation[62]. Tregs on the other hand are considered anti-inflammatory $CD4^+$ helper cells. Tregs regulate the immune response through the suppression of pro-inflammatory T cell subsets and therefore seen as an important player in atherosclerosis[63]. Whereas the role of Th1 cells and Tregs in atherosclerosis are extensively described, the precise role of Th2 and Th17 cells is controversial. Depending on the disease stage and the models used, the contribution of these cells to atherosclerosis susceptibility varies[64]. The same applies for cytotoxic $CD8^+$ T cells, which have shown to exert either pro-atherogenic effects as well as anti-inflammatory functions during atherosclerotic lesion development[65]. Although the role of T cells in atherosclerosis has been extensively studied, further studies are needed to elucidate the specific role of these individual subsets in the context of atherosclerosis before they can be targeted for the treatment of atherosclerosis.

3) Experimental animal models of atherosclerosis

Preclinical models of atherosclerosis have proven to be of great value for the identification of novel therapeutic targets against atherosclerosis. Although *in vitro* and *ex vivo* experimentation could give new insights in atherosclerosis-associated processes and therapy development, these models are yet not able to mimic the full complexity of the pathophysiology of atherosclerosis. As a result, animal models are essential for further elucidation of the onset and progression of the disease. Over the years, different mammalian models, such as non-human primates, swines, rabbits, rats and mice have been used to model the pathophysiology of atherosclerosis[66]. However, mice are the most commonly used animal model to date.

3.1) Mouse models of atherosclerosis

Wild-type (WT) mice do not develop spontaneous atherosclerosis in contrast to what is observed in humans. This could be explained by differences in plasma lipoprotein profiles due to the absence of CETP in mice. In mice, HDL is the main type of cholesterol circulating throughout the body, whereas in humans, it is LDL. Thanks to the wealth of genetic manipulations available, the mouse is the most widely used model for atherosclerosis. The most frequently used disease models are ApoE-, LDLr deficient or ApoE3-Leiden(-CETP) and all spontaneously develop hyperlipidemia and display increased susceptibility to atherosclerosis[67,68].

3.1.1) ApoE deficient mice

In 1992, Zhang *et al*. reported the first atherosclerosis mouse model with a deletion in the ApoE gene [67]. ApoE is a component of plasma lipoproteins and is involved in the recognition of amongst others the LDL receptor and LRP on hepatocytes. In line, ApoE deficient mice ($ApoE^{-/-}$) display impaired plasma lipoprotein clearance and have a disturbed triglyceride metabolism both contributing to the development of spontaneous atherosclerotic lesions on a regular chow diet[67,69,70]. Fatty streaks, mainly consisting of macrophage foam cells, can be observed as early as 8 weeks of age, whereas the first atherosclerotic lesions can be observed after 10 weeks[71]. Furthermore, there is a progressive rapid increase in atherosclerotic lesion size between 12 and 38 weeks of age[72]. Feeding $ApoE^{-/-}$ mice a Western-type diet (WTD), containing 0.15% cholesterol, 21% fat and 19.5% casein accelerates the development of atherosclerotic lesions in these animals, with foam cell accumulation already observed after 6 weeks of WTD challenge and the first lesions at 8 weeks[71]. Although the use of ApoE deficient mice has multiple advantages, also the disadvantages should be acknowledged. First of all, the lipid metabolism of $ApoE^{-/-}$ mice is not comparable to humans as the majority of the plasma cholesterol in $ApoE^{-/-}$ mice is in VLDL, while in humans it is primarily transported in the LDL fraction[73,74]. Moreover, besides its function in lipoprotein metabolism, ApoE also has other functions that can influence atherosclerosis, including anti-inflammatory, anti-proliferative, and anti-oxidative functions[75].

3.1.2) LDLr deficient mice

After the generation of the ApoE deficient mouse model, other models to study dyslipidemia and atherosclerosis and elucidate the factors and processes involved, were developed including the LDL receptor deficient mice ($LDLr^{-/-}$).

The $LDLr^{-/-}$ mouse model was developed to overcome the disadvantages associated with the use of $ApoE^{-/-}$ mice, as the LDLr is primarily involved in lipid metabolism and does not directly affect other atherosclerosis-influencing processes[76]. The LDLr is a surface receptor expressed primarily on liver cells that is able to clear lipoproteins from the circulation by binding ApoE and apoB100[69]. Feeding $LDLr^{-/-}$ mice a normal chow diet does not result in the development of atherosclerotic lesion[77]. To induce severe hypercholesterolemia and atherosclerosis, $LDLr^{-/-}$ mice need to be fed WTD to increase LDL and VLDL particles in the blood to above the threshold of 250 mg/dL required for induction of atherosclerosis in mice[78]. In humans, LDLr deficiency is associated with the development of familial hypercholesterolemia (FH). Human patients with FH have an increased risk for cardiovascular diseases and develop advanced atherosclerotic lesions at an early age. The $LDLr^{-/-}$ mouse model when

challenged with WTD displays some of the characteristics observed in FH patients, including the development of xanthomas as well as advanced atherosclerotic lesions. As a result, this model is very useful to study this disease and to develop new therapeutic strategies for FH.

Besides their usefulness in studying FH, LDLr$^{-/-}$ mice are valuable for research on the link between atherosclerosis and diabetes as these mice are more susceptible to develop insulin resistance and obesity compared to ApoE$^{-/-}$ mice[79–81].

3.1.3) ApoE3-Leiden/ ApoE3-Leiden-CETP mice

ApoE$^{-/-}$ and LDLr$^{-/-}$ mice are often used for atherosclerosis research, however these models do not fully resemble the human plasma cholesterol profile and and several anti-atherosclerotic drugs targeting the LDL/LDLr axis, including statins, cannot be properly tested in these models. Besides, the models lack CETP that is important for the exchange of lipids, such as cholesteryl esters and triglycerides, between VLDL, LDL and HDL. Two atherosclerosis mouse models that do have a more human-like lipoprotein metabolism, are the ApoE3-Leiden and, in particular, ApoE3-Leiden-CETP mice.

The ApoE3-Leiden transgenic mouse model expresses the human ApoE3-Leiden gene and exhibits a hyperlipidemic phenotype. Humans with mutations in the ApoE3 Leiden gene develop familial dysbetalipoproteinemia and are at increased risk for developing atherosclerosis at a young age[82]. The plasma cholesterol levels of ApoE3-Leiden mice are comparable to the levels observed in humans[83]. In line, ApoE3-Leiden mice also display an increased susceptibility to diet-induced atherosclerosis. However, the development of atherosclerotic lesions in these transgenic mice is relatively slow, as seen in humans[84]. After 18 weeks of WTD, the first signs of initial atherosclerosis development could be observed, while after 29 weeks these early fatty streaks developed into small atherosclerotic lesions. Moderate lesions could be observed after 31 weeks of WTD[85]. Because ApoE3-Leiden mice still express endogenous ApoE, these mice are less susceptible to atherosclerosis compared to ApoE$^{-/-}$ mice. ApoE3-Leiden mice, however, have proven very useful to test novel and existing lipid-lowering drugs, including statins. To further humanize this mouse model, ApoE3-Leiden mice have also been cross-bred with a transgenic mouse line expressing human CETP, to generate ApoE3-Leiden-CETP mice. The ApoE3-Leiden-CETP mice have a more human-like lipid metabolism compared to the ApoE3-Leiden mice as the expression of CETP shifts the cholesterol distribution form HDL to V(LDL). Moreover, the atherosclerotic lesions of these CETP-transgenic mice are 7.0 fold bigger compared to lesions of ApoE3-Leiden mice after x weeks WTD feeding[86]. A disadvantage of using ApoE3-

Leiden and ApoE3-Leiden-CETP mice for atherosclerosis research is that these mice develop atherosclerosis rather slowly. As a result, advanced atherosclerotic lesions are only visible after 31 weeks of WTD challenge, whereas in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice these are already visible after 8 weeks on WTD. As a result of these required long-term experiments, the associated costs for caretaking and housing of ApoE3-Leiden or ApoE3-Leiden-CETP mice are much higher as compared to experiments using ApoE$^{-/-}$ and LDLr$^{-/-}$ mice.

3.2.) Zebrafish models of atherosclerosis

For decades, mouse models have been used in medical research to study human disease. An important limitation of using mouse models to study atherosclerosis, is that it is only possible to do end-point measurements and imaging. A new upcoming inexpensive and powerful model to study human disease, including atherosclerosis, is the zebrafish (*Danio rerio*). The zebrafish and its translucent larvae are an upcoming model to study atherosclerosis as result of their lipid metabolism being comparable to humans and the possibility to perform real-time in vivo imaging. The development of new genetic approaches for the zebrafish, including transgenesis and mutagenesis, has also stimulated its widespread use as a model to study human disease[87–89].

3.2.1) Wild-type high cholesterol diet model

Stoletov *et al*. (2009) were the first to propose that WT zebrafish could be a suitable model to study temporal characteristics of early atherogenesis[90]. Feeding adult zebrafish for 8-12 weeks a 4% (w/w) high cholesterol diet (HCD) resulted in hypercholesterolemia characterized by plasma cholesterol levels reaching 800 mg/dL which is comparable to levels of LDLr$^{-/-}$ mice. This resulted in the development of fatty streaks in the dorsal aorta consisting of vascular lipid accumulation and cell infiltration, which appeared comparable to early lesions observed in mouse models and humans. No fatty streaks were found in the caudal vein of adult zebrafish. Besides using adult fish, Stoletov *et al*. have also shown that 5 days-old zebrafish could be used to study atherogenesis. Challenging 5 days-old transgenic *Fli1*:EGFP zebrafish larvae, in which the vasculature is labeled with a green fluorescent probe, with a 4% (w/w) HCD for 10 days resulted in vascular lipid accumulation in the caudal vein and as well as the dorsal aorta, but to a lesser extent. This is in contrast with our recent published study, in which we have reported that WT zebrafish larvae are not as susceptible to HCD-induced accumulation of macrophage foam cells in the caudal vein, as was suggested by Stoletov[91]. For this reason, further research into the use of WT zebrafish as a model for atherosclerosis is needed before this model can be widely incorporated as a model to study atherogenesis.

3.2.2) LDLr mutant

As previously mentioned, the LDLr$^{-/-}$ mouse is one of the most widely used mouse model to study atherosclerosis. In 2018, Liu *et al.* reported the generation of a new transgenic zebrafish model to study atherosclerosis: the *ldlr* mutant zebrafish[92]. The *ldlr* in zebrafish is duplicated resulting in the presence of two *ldlr* orthologs: *ldlra* and *ldlrb*. Due to the higher degree of conservation with the human LDL receptor, *ldlra* is defined as the *ldlr* receptor in zebrafish. Using CRISPR/Cas9 technology, 10 nucleotides of the *ldlra* were deleted leading to loss-of-function. Normal diet feeding of *ldlra* mutant zebrafish larvae resulted in activation of the *srebf2*-pathway, which is involved in cholesterol homeostasis and in the development of modest hypercholesterolemia. Moreover, feeding 4.5 days old *ldlra* mutant larvae with a HCD for only 5 days led to augmented hypercholesterolemia and the accumulation of lipids in the caudal vein, suggesting that this model could be useful to study processes involved in early atherogenesis and new therapeutic targets for hypercholesterolemia.

3.2.3) ApoC2 mutant

APOC2 is an important co-activating factor for LPL that is involved in the lipolysis of circulating triglyceride-rich lipoproteins, including chylomicrons and VLDL[93]. Deficiency of APOC2 leads to elevation of plasma triglyceride levels, which has shown to be a risk factor for atherosclerosis[94]. Using the transcription activator-like effector nuclease (TALEN) technique, Liu et al (2015) generated *apoc2* mutant zebrafish, which exhibited chylomicronemia and severe hypertriglyceridemia[95]. This closely resembles the characteristics of human patients suffering from APOC2 mutations[96]. Feeding 5 days-old *apoc2* zebrafish larvae a normal diet for 10 days resulted in the accumulation of lipids and macrophage foam cells in the vasculature, mimicking early human atherogenesis. Therefore, this mutant line could be very useful to study hypertriglyceridemia and its link to atherogenesis.

3.3.) Mouse versus zebrafish atherosclerosis models

Over the last years, it has become clear that animal models are of great importance for atherosclerosis research and that the focus on atherosclerosis animal models has shifted. Whereas processes involved in atherosclerosis are examined in different animal models including non-human primates, swine, rabbits and rats, the mouse is the most used atherosclerosis model at the moment[97-100]. However, recent advances have suggested that soon zebrafish could added to this array of animal models for studying dyslipidemia and atherosclerosis[101] (**Table 2-3**).

Compared to mouse models, zebrafish have multiple advantages comparing it to mice, making it an attractive model to study processes involved in the development of atherosclerosis (**Table 3**). Although the physiology of fish and humans differs in multiple ways, 70% of protein coding human genes are related to genes found in zebrafish. Moreover, 84% of genes known to be associated with human disease, including CETP, have a zebrafish counterpart[102]. Compared to mice, the zebrafish and its translucent larvae are inexpensive because of the low-cost housing and maintenance. Furthermore, this powerful model organism allows non-invasive cell tracking studies that can be used to study the pathology of atherosclerosis in real time. Using zebrafish for high-throughput screening would make the zebrafish a valuable model for the discovery of new pharmaceutical targets and drugs.

Table 2. Comparison of mouse and zebrafish models for atherosclerosis. Adapted from: Vedder *et al.* 2020.DOI:10.3389/fcvm.2020.00109

Comparison	**Zebrafish larvae**	**Zebrafish adults**	**Mice**
Maturation	3 months	3 months	2 months
Housing	Group (+-50) per big tank	Group (25-45) per big tank	5 per cage
Offspring	x	200-500 embryos	5-8 mice
Transparency	✓	x	x
Cholesterol metabolism			
Dominant lipoprotein	HDL	HDL	HDL
Source of energy	Lipids	Lipids	Carbohydrates
Cholesterol transport	HDL	HDL	HDL
CETP	✓	✓	x
Atherosclerosis			
Intima thickening	x	✓	✓
Lesions	x / ✓ Caudal vein	✓ Dorsal aorta	✓ aorta and carotids
Lesion classification	Type II Fatty streaks	Type II Fatty streaks	Type V to VI Advanced lesions
Oxygenated cholesteryl esters	In atherosclerotic lesions	In body liquids	In body liquids
Atherosclerosis models			
WT	x / ✓	✓	x
ApoE$^{-/-}$	x	x	✓
LDLr$^{-/-}$	✓	✓	✓
ApoC2$^{-/-}$	✓	✓	✓

Despite the advantages of zebrafish as model organism, it remains important to validate the mechanisms underlying zebrafish dyslipidemia and atherosclerosis before the zebrafish can become the new pioneering model for atherosclerosis research. Generation of ApoE$^{-/-}$ zebrafish would highly contribute to the use of zebrafish for studying atherogenesis. Combination of using zebrafish and mouse models for atherosclerosis could possibly give new insights and might be useful for studying mechanisms in depth. However, it is important that an appropriate animal model should always be chosen according your hypothesis.

Table 3. Advantages and limitations of using mouse and zebrafish for atherosclerosis research. + lowest levels of advantage; +++ highest level of advantage. Adapted from: Vasyutina *et al.* 2021. DOI:10.1016/j.metabol.2022.155138

Features	**Zebrafish**	**Mouse**
Breeding	**+++** - large offspring with identical genetic background - weekly (short period)	**++** - relatively fast reproduction and offspring homogeneity - 18-20 day pregnancy
Maintenance	**+++** - Automated housing systems - low costs	**+** - Multiple housing systems - higher costs
Genetic modification	**+++** - WT fish develop hyperlipidemia on HCD - genome editing is easy	**++** - Various atherosclerosis models worldwide available
Research options	**+++** - Embryo's and zebrafish larvae are optically transparent, allowing non-invasive live imaging - difficult to collect tissues due to small size	**++** - hard to perform live imaging - Classical histopathological, biochemical and other methods are widely used
human atherosclerosis	**+** - develop only type II initial lesions	**+++** - all types of lesions (initial to advanced)
Human lipid metabolism	**++** - Zebrafish have orthologs of important genes regulating the cholesterol metabolism - express CETP	**++** - main lipoprotein is HDL resulting in resistance for the development of atherosclerosis - CETP is absent

Thesis outline

With the research described in this thesis it is aimed to (1) validate the use of zebrafish in cholesterol metabolism and atherosclerosis research, (2) study the role of certain classes of scavenger receptors in lipoprotein uptake and cholesterol-based functions and (3) validated two immune-based potential targets for atherosclerosis with focusing cardiovascular side effects.

Establishing adequate atherosclerosis animal models is pivotal for studying the complex etiology and progression of the disease, as well as for screening of novel therapeutic targets. Over the years, different mammalian models, such as rats, rabbits, and mice, have been used to examine the pathophysiology of atherosclerosis. Although these rodent and rabbit models have provided scientists with the possibility to model different aspects of this disease, they all suffer from limitations, including the difficulty to execute high-throughput screening and to monitor atherogenesis over time. Recently, zebrafish have been proposed as a novel animal model for studying different aspects of atherosclerosis. In **chapter 2**, the use of zebrafish larvae as a model organism to study atherosclerosis is discussed. In our hands, wild-type zebrafish larvae seem not as susceptible to high cholesterol diet-induced accumulation of macrophage foam cells in the caudal vein, as previously suggested. Therefore, it is important to clearly define atherosclerosis when studying a new animal experimental model, as the application of different definitions can affect the interpretation of the results. Although zebrafish appear less prone to atherosclerosis development compared to mammalian models, **chapter 3** highlights the use of zebrafish as a model to study lipoprotein metabolism and the importance of stabilin-1 and 2 in apoB-lipoprotein scavenging including the uptake of oxLDL and even LDL. Stabilin-1 and 2 are class H scavenger receptors, which are evolutionarily ancient membrane bound receptors. During the emergence of the first primitive multicellular organisms, different family members with a varied structure arose that function as scavenger receptors. One ancient receptor, belonging to the class B sub-family of scavenger receptors is scavenger receptor class B1 (SCARB1).

SCARB1 was identified as the high-density lipoprotein (HDL) receptor, which facilitates the uptake of cholesteryl esters from HDL and is involved in the reverse cholesterol transport pathway. Comparative studies of vertebrate SCARB1 have shown that SCARB1 in general is well conserved between different species, except in zebrafish. In **chapter 4**, we provide evidence that the involvement of SCARB1 in plasma cholesterol metabolism and steroidogenesis arose as novel functions during mammalian evolution, as this seems absent in *scarb1*$^{-/-}$ zebrafish. This data could have consequences for the

usefulness of non-mammalian model in diseases in which cholesterol metabolism plays an important role, such as in atherosclerosis. It has become apparent that besides cholesterol, also the immune system is involved in the development of atherosclerosis. Transcriptional activator protein arginine methyltransferase 5 (PRMT5), a catalyzer of posttranslational protein methylation, regulates inflammatory macrophage activation as well as T cell differentiation, which are both important processes in atherosclerosis. Therefore, PRMT5 might be an interesting target for the modulation of atherosclerosis. In **chapter 5**, the role of PRMT5 in atherosclerosis was examined. Although PRMT5 is involved in multiple inflammatory processes, inhibition of PRMT5 did not affect atherosclerosis development or lesion composition. It, however, did induce hepatic lipid accumulation. Since PRMT5 inhibition is a potential target for cancer treatment, our study would caution long term administration as this possible side effect can lead to fatty liver disease. Another immune target that has been described as a key regulator of the immune system and is primarily expressed on macrophages and T cells is Interleukin-4 induced gene 1 (IL4I1). Recently, IL4i1 has shown to be promising target for treatment of cancer and autoimmune diseases, such as multiple sclerosis. Its role in atherosclerosis, however, is still unknown. Single-cell sequencing data from human atherosclerotic plaques has shown that IL4I1 is highly expressed in macrophages, suggesting that it might play a key role in atherosclerosis. Therefore, in **chapter 6** the immune effects of IL4i1 in the context of atherosclerosis development were examined. Inhibition of IL4i1, using the inhibitor CB-668, stimulated a pro-inflammatory immune environment without affecting initial atherosclerotic lesion development. Thus, although IL4i1 is anti-inflammatory, no protective effects of IL4i1 inhibition were found in the context of atherosclerosis development. Also, no negative effects were found, indicating that in contrast to PRMT5 inhibition no cardiometabolic effects need to be taken into account when IL4i1 inhibition is applied in the context of cancer and other autoimmune diseases.

Finally, in **chapter 7**, the results described in this thesis are summarized and discussed also describing future perspectives.

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