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

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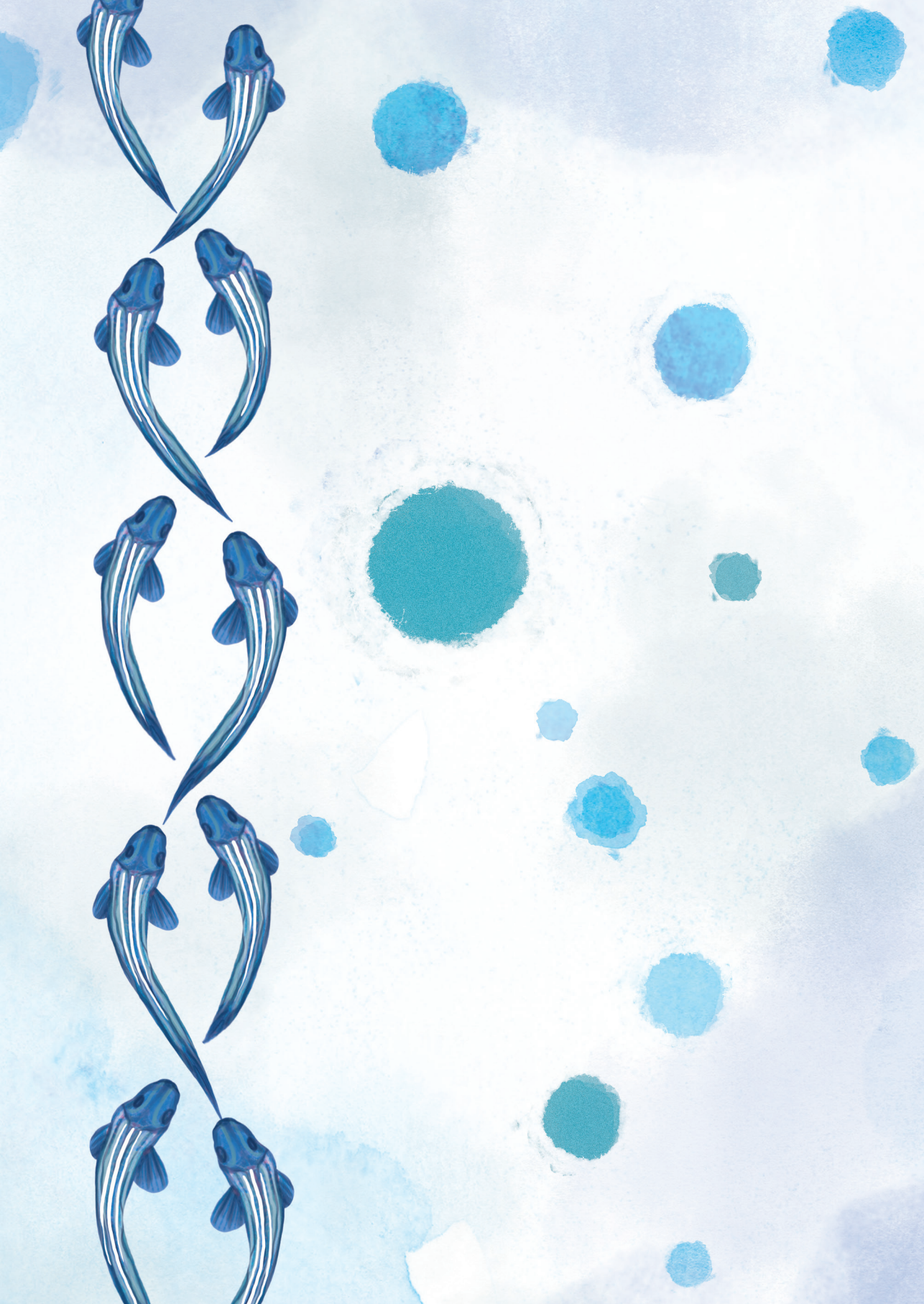

CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives

Background

Cardiovascular diseases are still a major concern for the global health[1]. The main underlying pathology of this disease is atherosclerosis which is characterized by the accumulation of lipids and immune cells in the arterial wall leading to a chronic local inflammation and lesion formation. Several health risk factors including smoking and physical activity contribute to the development of atherosclerosis. However, dyslipidemia is described as the main risk factor. Current therapies focus on lowering plasma cholesterol levels, as 1 mmol/L LDL reduction already can result in 20% reduction in future cardiovascular events[2,3]. However, inter-patient variability of lipid-lowering treatments such as statins or protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors could lead to a low response rate (i.e. hypo- and hyper-responders) and therefore to a reduced therapy effectiveness[4–6]. Furthermore, these lipid-lowering treatments only reduce the risk of atherosclerosis and are not able to reverse the burden that is already present. Therefore, new therapeutic targets that are able induce atherosclerosis regression need to be identified. For the identification of novel therapeutic targets for atherosclerosis, pre-clinical research using cell lines and animal models is of great importance. Each model has its own advantages and disadvantages. Selecting the right model, to study either certain aspects concerning the pathogenesis of atherosclerosis or to validate new potential drug targets, is essential for the outcome and validity of the study.

In this thesis, we 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.

Zebrafish as model organism for cholesterol metabolism and atherosclerosis research

Zebrafish are widely used as an animal model for biomedical research. Low maintenance costs, its translucent larvae, a high level of genetic similarity to humans, and the application of CRISPR genome editing make this small tropical fish an interesting animal model to study different types of diseases, including development and cardiovascular lipid-driven diseases. The development of the cardiovascular system is highly conserved between species and, while in the early days zebrafish were primarily used to study developmental issues, it is now an upcoming model to study molecular mechanisms underlying the regulation of vascular diseases including atherosclerosis. Until now, mice are the most commonly used model to study

atherosclerosis. Stoletov *et al*, however, opened new opportunities for using zebrafish in cardiovascular research[7]. In contrast to wild-type mice which are resistant to the development of atherosclerosis, in wild-type zebrafish atherosclerosis can be induced. More in detail, Stoletov *et al.* showed that feeding adult zebrafish with a 4% w/w high cholesterol diet could replicate processes involved in early atherogenesis, including vascular lipid accumulation and lipoprotein oxidation. Although Stoletov *et al.* and several others demonstrated early atherogenesis in wild-type zebrafish larvae, we have shown that wild-type zebrafish are not as prone to develop early atherogenesis as was previously suggested (*chapter 2*). The underlying reasons for the contradictory results are still unknown. Multiple factors are involved in atherogenesis and even small differences in for example zebrafish husbandry could affect the microbiome and immunity (add ref) and therefore the susceptibility of zebrafish to develop vascular lipid deposition.

In our study, we defined early atherosclerosis in zebrafish as the accumulation of lipid-laden macrophages, particularly because in mammals macrophages make up the majority of myeloid cells present in early atherosclerotic lesions. We used a macrophage-specific transgenic zebrafish line, whereas Stoletov *et al.* did not examine the uptake of lipids by macrophages and only registered the general vascular lipid accumulation. Notably, other studies using broader myeloid-lineage specific transgenic zebrafish lines did find co-localization of myeloid cells and lipid, indicating that other myeloid cells, such as neutrophils, could drive early atherogenesis in zebrafish[8–10]. Therefore, it is important to note that the definition of early atherogenesis applied impacts the conclusions drawn from the studies.

Another important factor is that early atherogenesis in zebrafish is mainly reported in the caudal vein and not in the aorta, where in mammals atherosclerotic lesions are located. This aspect, in combination with the absence of macrophage co-localization, raises the question how translatable the findings on atherosclerosis in wild-type zebrafish to humans are and hence how useful research performed in zebrafish is for increasing the understanding of this disease?

Because of these questions, more research is needed to fully understand the pathophysiology of atherosclerosis in zebrafish and to determine how translatable this upcoming model is. For lipoprotein and cholesterol metabolism-based research, the zebrafish seems to function as a proper model. Most of the genes implicated in lipid metabolism are highly conserved[11,12]. Apolipoprotein E has two zebrafish orthologs: apoea and apoeb, but the identity to its human ortholog is only 27.5% [13,14]. In addition, human apoB has three zebrafish orthologs: apoBa, apoBb1 and apoBb2

with an identity of 51.6%, 42.5%, and 27.5%, respectively. Mainly apoBa and apoBb1 share syntenic gene regions with human apoB and not the apoBb2 variant[15]. However, there are also some differences regarding certain metabolic pathways that should be taken into account. For example, the lipoprotein composition differs in zebrafish as they only produce apoB100 containing chylomicrons instead of apoB100 and apoB48 containing chylomicrons, possibly altering the hepatic clearance of this class of lipoproteins[15,16].

An important family of receptors that recognize epitopes with a negative charge such as apolipoproteins on lipoproteins are scavenger receptors. Because of this function scavenger receptors are essential for lipoprotein clearance are scavenger receptors. These receptors have shown to be involved in the development and progression of atherosclerosis by initiating pro-inflammatory signaling in immune cells as well as accumulation of modified lipoproteins, such as oxLDL. There are different classes of scavenger receptors and each scavenger receptor class has its own function and can take up different kinds of ligands, depending on its structure and properties. In this thesis, we described the role of 2 classes of scavenger receptors using genetic modified zebrafish deficient for either the class H scavenger receptors STAB1 and STAB2 (*chapter 3*) or the class B scavenger receptor SCARB1 (*chapter 4*).

The role of the class H receptors STAB1 and STAB2 in cholesterol metabolism and early atherogenesis *in vivo* was largely unknown. *In vitro studies* using transfected HEK cells and freshly isolated liver sinusoidal endothelial cells suggested that STAB1 and STAB2 play an essential role in lipoprotein uptake, including the uptake of oxLDL[17]. In line with this research, in *chapter 3* it is described that in zebrafish STAB1 and STAB2 influence the cellular uptake of apoB-containing lipoproteins *in vivo*. Total-body deficiency of these two receptors led to an enhanced clearance of these lipoproteins by macrophages of the caudal vein thereby stimulating to their transformation to lipid-laden macrophages but not leading to early atherosclerosis. Important to note is that these receptors are expressed on endothelial cells as well as macrophages. Nahon *et al.* have already shown that hematopoietic STAB1 deficiency does not alter the atherosclerosis susceptibility of LDLr$^{-/-}$ mice, suggesting that the expression of this receptor on endothelial cells has a protective role in atherosclerosis[18]. Besides the role of STAB1 in atherosclerosis, Kayashima *et al.* has shown that STAB2/ApoE double knockout mice are protected against atherosclerosis indicating that STAB2 is a modifier gene of atherosclerosis[19]. Interestingly, a recent study has shown that genetic deficiency and antibody-mediated inhibition of STAB1 as well as STAB2 protect against the progression of atherosclerosis in ApoE knockout and LDLr knockout mice[20].

The interplay between endothelial cells and macrophages in the removal of oxidatively modified lipoproteins from the circulation can possibly affect the susceptibility to atherosclerosis. The same accounts for liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs) in the liver. Zebrafish do not have these cells, but they do have cells that are homolog to mammalian LSECs, named SECs. The caudal vein of zebrafish embryo's is lined with this special type of endothelial cells, that are known to clear particles such as (oxidized) lipoproteins from the blood circulation. Our studies described in chapter 3 have shown that zebrafish are a good model to study the function of SECs making use of the transparency of zebrafish larvae. However, to address the individual contribution of stabilin 1 and 2 to LSEC function, lipoprotein metabolism, and foam cell formation, it would be of interest to examine these processes in endothelial-specific and/or macrophage-specific *stab1* and *stab2* knockout zebrafish.

Another scavenger receptor which is known for its role in mammalian HDL metabolism is SCARB1. This multi-ligand scavenger receptor plays an important role in the mammalian reverse cholesterol transport. Deficiency of SCARB1 impairs the delivery of HDL cholesteryl esters to the liver, leading to increased HDL cholesterol levels in the circulation and an increased atherosclerosis susceptibility. Although SCARB1 is highly conserved between different species, the extracellular loop which is important for cholesterol handling, is not conserved in zebrafish. As a result, HDL binding and selective uptake of cholesteryl esters by SCARB1 might be dysfunctional or limited. In chapter 4, it was examined whether the cholesterol transport function of SCARB1 arose early or later during mammalian evolution. Hereto, we generated zebrafish lines deficient for *scarb1*. From studies in other species than zebrafish, it is known that SCARB1 deficiency not only influences HDL cholesterol metabolism, but also carotenoid uptake and pigment development, steroid production, and fertility[21–26]. Whereas the hypothesis was that *scarb1* deficiency in zebrafish would lead to higher cholesterol levels and impaired steroidogenesis, we surprisingly showed that *scarb1* does not play a key role in the zebrafish HDL cholesterol metabolism or cortisol production, suggesting that other (scavenger) receptors might play a prominent role or can take over the role in these processes in zebrafish. As evidenced in Chapter 3, it is likely not stabilin 1 and stabilin 2 which were found not to be involved in HDL metabolism. It would be of interest to examine the role of the LDLr receptor in the uptake of HDL in zebrafish as currently the function of this receptor in HDL metabolism and cholesteryl ester uptake in zebrafish is largely unknown. Ldlra$^{-/-}$ zebrafish do have higher serum cholesterol levels leading to vascular lipid deposition when fed with a 4% high cholesterol diet[27]. Although a disbalance in cholesterol metabolism has been associated with reduced fertility in humans and mice[23,28–30], *ldlra*$^{-/-}$ fish do not have

fertility issues. *Scarb1*$^{-/-}$ zebrafish, however, did display fertility problems, despite that their cholesterol levels were the same compared to wild-type zebrafish. This data suggested that the major decrease in fertility and reduced survival of the offspring is independent of the plasma cholesterol or lipoprotein uptake. Whereas in mammals the reduced reproduction rate is mainly driven by reduced female fertility, our study has shown that male as well as female *scarb1*$^{-/-}$ zebrafish suffer from fertility issues. Because in mammals it is suggested that vitamin A as well as E could play a role fertility, it would be of interest to investigate whether these factors could influence the reduced fertility observed in *scarb1*$^{-/-}$ zebrafish.

Overall, the zebrafish has proven to be a valuable animal model for research into the pathology of different diseases including cancer[31], epilepsy[32], autism[33], diabetes mellitus type 2[34], nervous systems disease[35], amyotrophic lateral sclerosis[36], osteoporosis[37], and bacterial infection[38]. Although zebrafish have also been proposed as an upcoming and relevant model for atherosclerosis research[39], this thesis has provided contradictory findings that limit its value for studying vascular lipid deposition. Zebrafish, however, did emerge as a useful model to examine several processes underlying the pathogenesis of atherosclerosis i.e. lipoprotein uptake by macrophages and immune cell migration.

Identification of new immune-based targets for atherosclerosis

Several clinical studies have shown that modulation of the immune system could positively affect cardiovascular outcomes[40–42]. As it has become apparent that besides cholesterol, the immune system is involved in atherosclerosis, two possibly new therapeutic immune targets were identified and validated in this thesis: PRMT5 (*chapter 5*) and IL4I1 (*chapter 6*).

PRMT5

PRMT5 is a member of the PRMT family of epigenetic modifiers. Considering its already described functions in inflammation and metabolism, *in chapter 5* the role of PRMT5 in the development of atherosclerotic lesions was evaluated. *In vitro* experiments in cultured macrophages and an *in vivo* study in atherosclerosis-susceptible LDLr$^{-/-}$ mice were performed in which PRMT5 functioning was blocked using the specific PRMT5 inhibitor GSK3326595. As evidenced by a change in M1 and M2 marker gene expression pattern and augmented M1/m2 ratio, GSK3326595 dependent inhibition of PRMT5 was able to alter the macrophage phenotype. Important to mention is that these effects were observed only upon IFNg stimulation and not when other stimuli were added. This could suggest that PRMT5 is associated with IFNg signaling, which is a known factor in immune regulation and in atherosclerosis[43,44]. Furthermore, Fan

et al. (2016) have suggested that in response to IFNg, PRMT5 is translocated from the cytoplasm into the nucleus of macrophages where it can bind to the promotor of the MHCII complex and activate its transcription. In accordance, our *in vitro* study showed that PRMT5 inhibition by GSK3326595 significantly downregulates the relative expression level of MHCII after IFNg stimulation. Although GSK3326595 treatment did prime peritoneal macrophages to a pro-inflammatory M1 phenotype in response to exposure of IFNg, chronic *in vivo* treatment with a low dose of GSK3326595 did not impact the inflammatory state

(T cell subsets and activation state) or the susceptibility to atherosclerosis. However, it might still be of interest to investigate if a higher dose of GSK3326595 would be able to modulate atherosclerosis susceptibility by regulation of the inflammatory state. Low-dose GSK3326595 treatment did activate genes involved in hepatic fatty acid acquisition, including SREBF1, FASN, and CD36 and significantly increased hepatic triglyceride levels. Our data suggests that PRMT5 inhibition by GSK3326595 is not a valuable therapeutic approach to modulate atherosclerosis susceptibility. However, GSK3326595 is currently being tested as anti-cancer drug therapy in both preclinical studies in mice and in clinical phase I and II studies. Because our data showed that low-dose GSK3326595 treatment is able to induce hepatic triglyceride accumulation, it is important to consider that long-term pharmacological inhibition of PRMT5 to treat cancer may cause severe side effects in liver, i.e. induce non-alcoholic fatty liver disease. Clearly, further research is needed to examine these liver-specific side effects and we recommend that this is taken into account in the ongoing trials.

IL4I1

IL4I1 is a secreted enzyme which catabolizes phenylalanine, thereby producing phenylpyruvate, ammonia, and hydrogen peroxide[45]. In addition, IL4I1 has been shown to regulate the immune response and, in particular inhibit T and B cell functions, by oxidizing phenylalanine[45]. As a result of its immune regulatory activities, IL4I1 has emerged as a promising therapeutic target in cancer and multiple sclerosis[46,47]. According to single cell sequencing data from human atherosclerosis plaques, IL4I1 is significantly upregulated in macrophages and colocalizes with TREM2^{hi} macrophages. Furthermore, it promotes M2 macrophage polarization which can affect T cell functions[48]. Since previous research have demonstrated that IL4I1 is involved in the regulation of innate and adaptive immune responses, IL4I1 may affect the development and progression of atherosclerosis. In our study described in chapter 6 we showed that inhibition of IL4I1 using CB-668 did not lead to enhanced (early) atherosclerosis development, whereas it did stimulate a pro-inflammatory immune environment. However, it is important to note that different immune cells play distinct roles in

the variety of stages of atherosclerosis. Macrophages are more important in the early developmental stages of atherosclerosis and T cells play a more prominent role during atherosclerosis progression in murine models. Because IL4I1 is expressed on macrophages as well as T cells, it would be of interest to examine whether inhibition of IL4I1 does affect advanced stages of atherosclerotic lesion development as in this stage T cells are more involved. Currently CB-668 is tested as new therapeutic strategy for cancer patients. A side effect of anti-cancer treatments often is the development of cardiovascular diseases[49]. Our study highlights that in this setting the usage of CB-668 does not lead to an increased risk of early atherosclerosis development. This might suggest that it would be safe for treatment of cancer patients without any evident atherosclerosis, although we cannot exclude any long-term effects of the treatment. Moreover, it still would be important to test if the usage of CB-668 in cancer patients suffering from atherosclerosis would still be beneficial.

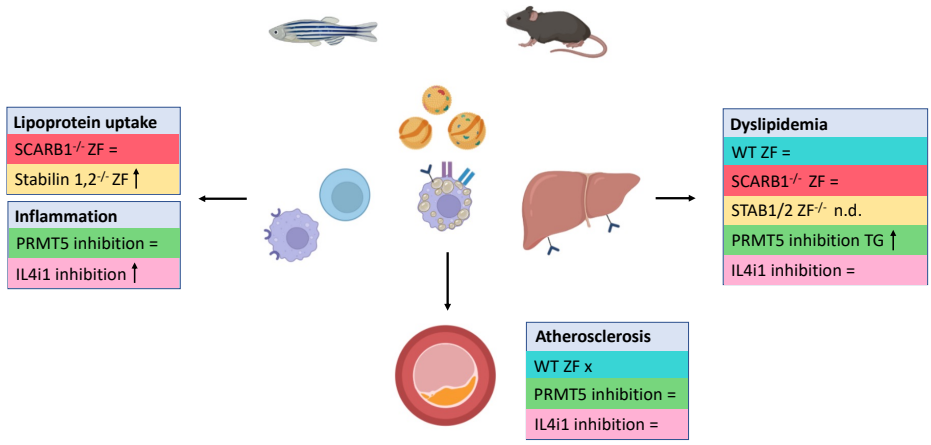

Figure 1. Schematic overview of the targets examined in this thesis and their effects in aspects involved in atherosclerosis. n.d.= not determined.

Future Perspectives

In the last decade a lot of research has focused on the pathophysiology of atherosclerosis thereby reducing the gap in knowledge and increasing the possibility of finding potential novel targets for the treatment of atherosclerosis. For designing atherosclerosis research, it is of great importance to choose the right animal model as multiple animal models exist and each model has their own limitations and advantages. Recently, the zebrafish has been proposed as a potential animal model to examine early atherosclerosis. The studies described in this thesis show that wild-type zebrafish are not the best model to study early atherogenesis, i.e.

accumulation of cholesterol-laden macrophages. Genetically-modified zebrafish, including the *ldlra*$^{-/-}$ zebrafish might be more suitable for this. Whereas there are a few studies that have shown the possibility of inducing atherosclerosis, our study is so far the only study that tried to visualize the co-localization of macrophages during a 10-day high cholesterol diet challenge. Therefore, it is still of great importance that atherosclerosis susceptibility of *ldlra*$^{-/-}$ zebrafish will be validated in multiple labs and that the cellular composition of these plaques is investigated. To achieve this, clearly standardized protocols are needed and manuscripts should provide all important details that might influence the outcome of the experiments. This includes for example: sort of diet, fluorescent label used, zebrafish strain, developmental stage etc. A helpful guideline that ensures that the study is reported in enough detail are the Animals in Research: Reporting *In vivo* Experiments (ARRIVE) guidelines. Reporting animal research in adherence with these guidelines helps readers and reviewers to evaluate the study set-up and when desirable give the possibility to reproduce the methods or findings.

This thesis has proven that zebrafish could be useful for studying certain aspects of the lipoprotein metabolism related to atherosclerosis development and therefore useful for translational research into an important risk factor underlying the pathophysiology of atherosclerosis. The use of zebrafish has evident advantages; it is a very time and cost-efficient model allowing systematic image- and genetic interventions and even target-driven drug discovery[10]. For future research it is of great importance that, before acknowledging (genetically-modified) zebrafish as an established model organism, this specific model is fully characterized and the aspects of atherosclerosis are clearly defined so that the reliability and value of the research findings and conclusions based on these outcomes can be properly assessed. Although zebrafish are becoming more popular for atherosclerosis research, mice models are still the golden standard to validate new potential targets. From ethical point of view, it would be eventually ideal if the mechanisms underlying atherosclerosis and potential novel drug targets could be studied using *in vitro* models. At the moment, several *in vitro* models including two and three-dimensional cell models, have been developed as potential platforms to examine certain aspects of atherosclerosis and to validate new potential therapeutic targets[50]. The advantages of using these *in vitro* models for drug screening is that the efficacy and toxicity of a certain drug can already be examined in a shorter period of time and hence more cost effectively compared to testing in animal models. Although in the coming years *in vitro* models, particularly the three-dimensional models such as a vessel-on-a-chip, show potential for examining atherosclerosis mechanisms and therefore will gain more popularity, the established *in vitro* models are so far still not capable to

fully model the pathophysiology of human atherosclerosis and the interaction with the complex immune systems and regulation of metabolism. The future will show what models are the best for examining the mechanisms behind atherosclerosis and eventually help to discover novel potential lipid and/or immune-based therapeutic targets that can be used for the treatment of atherosclerosis.

Conclusion

With the studies presented in this thesis we a) examined whether zebrafish could be used as animal model for cholesterol metabolism and atherosclerosis research, b) studied the function of scavenger receptors SCARB1 and stabilin 1 and 2 in cholesterol metabolism, and c) investigated the potential of 2 novel immune-based therapeutic targets, PRMT5 and IL4I1, in atherosclerosis (**Fig. 1**). The results (1) highlight the importance of a proper definition for early atherosclerosis in zebrafish as this could impact the outcome of the studies (2) underline the importance (and difficulty) of choosing the right animal model for your research as each model has its advantages and limitations, (3) indicate that the function of certain scavenger receptors in lipoprotein metabolism differs between mammals and vertebrates such as zebrafish, and (4) suggest that the identification and validation of new therapeutic targets against atherosclerosis is complex and that future therapeutic strategies to treat atherosclerosis will need to include the combination of lipid lowering- and immune blocking agents.

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