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# Stabilin 1 and 2 are important regulators for cellular uptake of apolipoprotein B-containing lipoproteins in zebrafish

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ABSTRACT

*Background and aims:* Scavenger receptors form a superfamily of membrane-bound receptors that bind and internalize different types of ligands, including pro-atherogenic oxidized low-density lipoproteins (oxLDLs). *In vitro* studies have indicated a role for the liver sinusoidal endothelial cell receptors stabilin 1 (*stab1*) and 2 (*stab2*) in oxLDL clearance. In this study, we evaluated the potential role of *stab1* and *stab2* in lipoprotein uptake in zebrafish, an upcoming model for studying cholesterol metabolism and atherosclerosis.

*Methods:* Lipoproteins were injected in the duct of Cuvier of wild-type (ABTL) or *stab1* and *stab2* mutant (*stab1*$^{-/-}$*stab2*$^{-/-}$) zebrafish larvae at 3 days post-fertilization. To examine the effect of stabilin deficiency on lipoprotein and cholesterol metabolism, zebrafish larvae were challenged with a high cholesterol diet (HCD; 4% w/w) for 10 days.

*Results:* Lipoprotein injections showed impaired uptake of both LDL and oxLDL into the vessel wall of caudal veins of *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish, which was paralleled by redistribution to tissue macrophages. Total body cholesterol levels did not differ between HCD-fed *stab1*$^{-/-}$*stab2*$^{-/-}$ and ABTL zebrafish. However, *stab1*$^{-/-}$*stab2*$^{-/-}$ larvae exhibited 1.4-fold higher mRNA expression levels of *ldlra* involved in (modified) LDL uptake, whereas the expression levels of scavenger receptors *scarb1* and *cd36* were significantly decreased.

*Conclusions:* We have shown that stabilins 1 and 2 have an important scavenging function for apolipoprotein B-containing lipoproteins in zebrafish and that combined deficiency of these two proteins strongly upregulates the clearance of lipoproteins by macrophages within the caudal vein. Our current study highlights the use of zebrafish as model to study lipoprotein metabolism and liver sinusoidal endothelial cell function.

## 1. Introduction

Atherosclerosis is a complex disease in which the recruitment and accumulation of serum lipoproteins in the arterial wall, where they are subjected to oxidation, induce a chronic pro-inflammatory cascade leading to endothelial dysfunction and plaque development.

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 [1]. 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. The main advantages of using zebrafish are its conserved lipid metabolism, its optical transparency, and the possibility of non-invasive cell tracking. Several studies have shown that wild-type zebrafish larvae are susceptible to high cholesterol diet (HCD) induced atherosclerosis [2,3]. The exact mechanisms underlying the pro-atherogenic effects observed in wild-type zebrafish larvae, however, are yet unknown. This is particularly relevant as our recent study showed that wild-type zebrafish are rather protected against the accumulation of macrophage foam cells and early atherosclerosis [4].

In line with commonly used mammal atherosclerosis models, feeding zebrafish larvae a HCD induces profound lipoprotein oxidation,

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important for the onset of atherosclerosis [5]. Thus, the elimination process of modified and oxidized lipoproteins might, at least partly, determine atherosclerosis susceptibility of zebrafish larvae. It is important to note that, whereas atherosclerosis in mammals develops in arteries, studies on atherosclerosis in zebrafish larvae focus on lipid deposition in the caudal vein. The caudal vein of zebrafish embryo's is lined with sinusoidal endothelial cells (SECs), a special type of endothelial cells [6]. Evolutionary, SECs are the zebrafish homolog of liver sinusoidal endothelial cells (LSECs) in mammals. LSECs form a barrier between blood in liver sinusoids and the hepatocytes, and together with the liver resident macrophages called Kupffer cells, constitute a dual scavenging system for the clearance of blood-borne macro molecules [7].

In atherosclerosis, scavenger receptor-mediated uptake of oxidatively modified lipoproteins by endothelial cells and macrophages in the sub-endothelial space of the arterial wall stimulates the progression of disease [8,9]. The interplay between endothelial cells and macrophages in the removal of oxidatively modified lipoproteins from the circulation, similarly as for LSECs and Kupffer cells in the liver, can likely influence atherosclerosis susceptibility. The majority of the research on effects of oxLDL in atherosclerosis focused on the role of the endothelial lectin-like oxidized low density lipoprotein receptor 1 (*lox-1*), a class E scavenger receptor, while for macrophages *sr-a* (class A) and *cd36* (class B) are considered the primary oxLDL receptors. SECs in the caudal vein of zebrafish express several types of scavenger receptors, including class B (SR-B) and class H (SR-H) receptors [10]. Particularly, the class H scavenger receptors stabilin-1 (*stab1*) and stabilin-*2* (*stab2*) facilitate nanoparticle uptake in zebrafish larvae [6]. Interestingly, *in vitro* studies showed that endocytosis of mildly oxLDL by LSECs is also mediated by *stab1* and *stab2* [11]. In this study, the potential role of scavenger receptors *stab1* and *2* in lipoprotein metabolism in zebrafish is examined.

## 2. Materials and methods

### 2.1. Zebrafish maintenance

All zebrafish lines (ABTL, *stab1*$^{-/-}$, *stab2*$^{-/-}$*and stab1*$^{-/-}$*stab2*$^{-/-}$ *mpeg1*:rfp) were supplied by the in-house fish facility of the Gorlaeus Laboratories, Leiden University [6,12]. All experiments were performed using these individual lines. Adult zebrafish were maintained on a 14-h-light/10-h dark cycle at 28 °C and fed Artemia (AM) and a mixture of GEMMA Micro 300 (200–500 μm, Skretting) and GEMMA Diamond M-0.5 (0.5 mm Skretting) (PM) daily. Fertilization was performed by natural spawning at the beginning of the light cycle by setting up single crosses of the transgenic zebrafish lines. Eggs were raised at 28.5 °C in egg water (60 μg/mL Instant Ocean sea salts). Zebrafish larvae were fed GEMMA micro 75 (50–100 μm, Skretting) twice daily, starting from 5 days post fertilization (dpf). For high cholesterol diet (HCD) feeding, 4% w/w cholesterol (Sigma, Cat 8503) was added to the diet by dissolving the cholesterol in diethyl ether and mixing this with the diet, allowing sufficient time for diethyl ether evaporation afterwards. For the purpose of studying vascular lipid accumulation in larvae, both the control diet and HCD were supplemented with a fluorescent cholesteryl ester analog (cholesteryl BODIPY FLC$_{12}$, Invitrogen, Cat. C-3927MP), a label that was previously shown to be incorporated into lipids and stored in lipid droplets [13,14]. All experimental protocols were approved by the Animal Welfare Body of Leiden University and were performed in compliance with the project license AVD1060020185964, 1-01 issued by the Central Authority for Scientific Procedures on Animals (CCD), the Dutch government guidelines, and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

### 2.2. Zebrafish intravenous injections

Three dpf zebrafish embryos (from different clutches (n = 6)) were anesthetized in 0.01% tricaine and embedded in 0.4% agarose (Serva, Cat. 11382.02) containing tricaine before injection. 1 or 2 nL volumes were calibrated and injected into the duct of Cuvier using a Femtojet 5247 micro-injector (Eppendorf). The following concentrations were injected: human Bodipy™ FL-LDL (Thermofisher, L3483, 0.5 mg/mL), human DiO-oxLDL (0.5 mg/mL) (mildly to heavily oxidized; 24 h with 10 μM copper sulfate solution) (Kalen Biomedical, Cat. 770282-9) and human DiO-HDL (0.5 mg/mL) (Kalen Biomedical, Cat. 770380-9). The lipoproteins were characterized before injection and the size and charge were measured by dynamic light scattering (DLS) on a Zetasizer Nano (Malvern Instruments, Malvern, UK) before injection (Supplemental Table 1). N is the number of total individual fish used for the experiment per group.

### 2.3. Zebrafish imaging and quantification

For *in vivo* confocal microscopy, anesthetized larvae were laterally aligned in 0.4% low melting agarose containing tricaine in 40 mm glass bottom dishes (WilCo Wells, cat. GWST-5040). Stereo images were captured with a Leica DMI4000B fluorescent microscope. Confocal Z-stacks were captured on a Leica SPE confocal microscope, using a 10× air objective or a 40× water-immersion objective. For whole-embryo views, 3–5 overlapping z-stacks were captured to cover the complete embryo. The gain, offset and laser intensity settings were identical between stacks and sessions. Images were processed and quantified using the Fiji distribution of ImageJ. To quantify co-localization of macrophages with LDL, oxLDL and HDL, the co-localization events were blinded and manually counted in the vascular segments' posterior to the cloaca.

### 2.4. Quantitative RTqPCR

Total RNA was isolated from 3 dpf or 15 dpf zebrafish larvae (HCD study) (n = 7, pool of 5 larvae) by the acid guanidinium thiocyanate-phenol chloroform extraction method according to Chomczynski and Sacchi [15]. cDNA was synthesized from 1 μg of total RNA using Maxima H Minus Reverse Transcriptase (200 U/μL, ThermoFisher, Cat. EP0741). mRNA levels were quantitively determined using SYBR Green Technology (Eurogentec) on the QuantStudio 6 Flex (Applied Biosystems). *Rpl37, rplp0, β-actin 1, rps18, and tuba1b* (averaged) were used as reference genes. The relative gene expression of *hmgcra*, *ldlra, srebf2, scarb1*, *cd36*, *marco*, *abca1,* and *abcg1* were determined (Table 1). The relative gene expression was calculated by subtracting the threshold cycle number (Ct) of the genes of interest from the reference Ct values and raising 2 to the power of this difference. The dCt values of multiple replicates were averaged for further analysis. To exclude variations in the relative gene expression of reference genes, the average Ct values of the reference genes were used.

**Table 1**
RTqPCR primer sequences.

| | Forward primer | Reverse primer |
|---|---|---|
| *rpl37* | 5′-taagagacgcagcaccacaggaac-3′ | 5′- tggaaacacgttatgaggagctggac-3′ |
| *rplp0* | 5′-agattcctccttgggtgcctcctc-3′ | 5′-cagactacacattcccctggctgag-3′ |
| *β-actin 1* | 5′-gtgctgttttcccctccattgttgg-3′ | 5′-aggagtctttctgtcccatgccaac-3′ |
| *rps18* | 5′-atacagccaggtccttgctaatggtc-3′ | 5′-gaccgcgacgaccagttgttttg-3′ |
| *tuba1b* | 5′-tgctcgtggtcactacactattggc-3′ | 5′-ttggacttcttgccgtagtcgacag-3′ |
| *hmgcra* | 5′-gggagagctgtcactcatgtctgc-3′ | 5′-gcatgttcctggagcttcttgcaag-3′ |
| *ldlra* | 5′-caaactccacacagaaacaccagctg-3′ | 5′-cgggttgtcaaagtggatgctgttg-3′ |
| *srebf2* | 5′-gtgtcagtcgcacaacaacacctg-3′ | 5′-tctcagagtcaggagaaggtcacagac-3′ |
| *scarb1* | 5′-aatggggctttttcactggttgtgc-3′ | 5′-acatgcagtacatcttcatcgctctcg-3′ |
| *cd36* | 5′-aggcttcaggtcaacatgatgtacgg-3′ | 5′-ttttggccgtctcatcgtccagaac-3′ |
| *marco* | 5′-cgataatttggatgcgctggtggtg-3′ | 5′-tcggttccagtgcatctcaactcatc-3 |
| *abca1a* | 5′-tcggatggccatcatggtgaacg-3′ | 5′-atgaactccatcaccggctccag-3′ |
| *abcg1* | 5′-ttgtgggtcctgttacagccatcc-3′ | 5′-ccagcccgtagatggatagaattaccc-3′ |

### 2.5. Lipid extraction

Zebrafish larvae pools (n = 6, pools of 5 larvae 15 dpf, HCD study) were homogenized in 200 μL homogenization buffer (20 mM Tris, 1 mM EDTA, pH = 8.0) and sonicated using the Branson SFX250 sonifier (Emerson, Ferguson, Missouri, U.S.) for 4 s with a 1/4th inch tapered micro-tip and an output of 3 W. The tip of the sonifier remained immersed in the solution to prevent foam formation. After this, 180 μL of the homogenate was transferred to a 2 mL tube for extraction of lipids. Protein content in the homogenates was determined using a BCA assay. A volume of 750 μL of a chloroform and methanol mixture (1:2) was added to each of the samples, after which the samples were vortexed for 30 s. The samples were incubated for 10 min at room temperature, 250 μL of chloroform was added, and the samples were vortexed as mentioned above. Following this, 250 μL of the homogenization buffer was added and the samples were vortexed for another 30 s and incubated for 5 min at 25 °C. After the incubation, the samples were centrifuged at 3000 rpm for 5 min. The bottom organic phase was transferred to a 1.5 mL Eppendorf tube and the samples were left in the fume hood overnight to allow chloroform evaporation. The thin film of lipids that remained in the tube was then dissolved in 200 μL 2% Triton X-100/Milli-Q and sonicated for 15 s.

### 2.6. Cholesterol assay

The amount of free and total cholesterol extracted from the homogenates of zebrafish larvae pools (n = 6, pools of 5 larvae 15 dpf, HCD study) was determined using an enzymatic colorimetric assay (Roche Diagnostics). Precipath (Roche Diagnostics, Cat. 11285874) was used as an internal standard. The concentrations of free and total cholesterol in the homogenates were determined by enzymatic colorimetric assays with 0.025 U/mL cholesterol oxidase (Sigma, Cat. 228250), 0.065 U/mL cholesterol peroxidase (Sigma, Cat. P8375), and 15 μg/mL cholesterol esterase (Sigma, Cat. 228180) in reaction buffer (1.0 mol/L KPi buffer, pH = 7.7 containing 0.01 mol/L phenol, 1 mmol/L 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). Absorbance was measured at 490 nm.

### 2.7. Statistical analyses

All graphs are expressed as means ± SEM. Statistical analysis were performed in Graphpad Prism 9. Statistical differences between the experimental groups were evaluated using either a Student's t-test or a two-way ANOVA (corrected for multiple testing using the Bejamini-Hochberg procedure). Values of $p < 0.05$ were considered statistically significant.

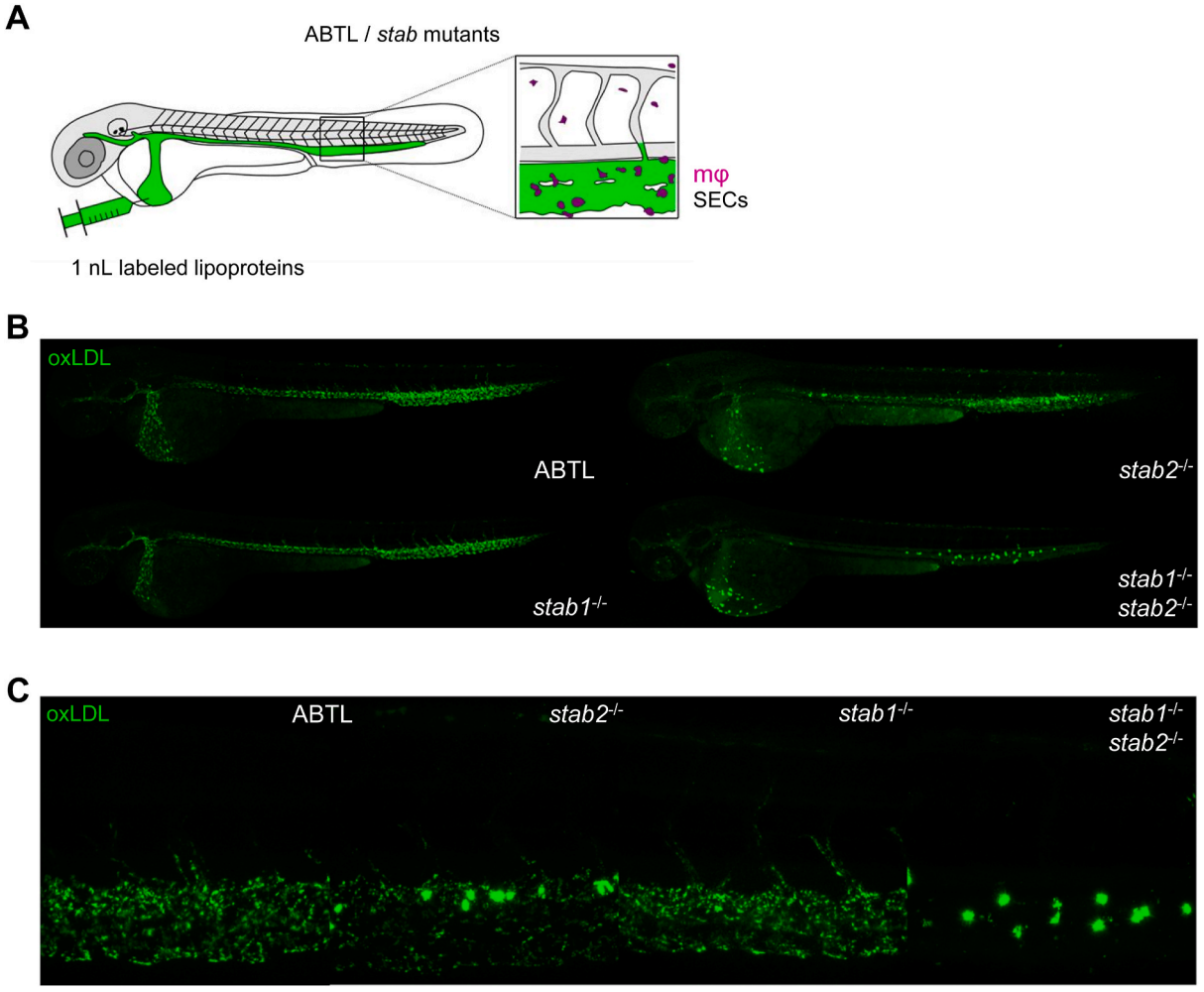


**Fig. 1.** Absence of scavenger receptors *stab*1 and/or *stab*2 reduces oxLDL uptake by endothelial cells.
(A) Schematics of experimental set-up; injection of fluorescent labeled lipoproteins in 3 dpf ABTL or stabilin knockout zebrafish larvae. (B) Photomicrographs showing an overview of 3 dpf ABTL, *stab*1$^{-/-}$, stab2$^{-/-}$, and *stab*1$^{-/-}$stab2$^{-/-}$ zebrafish larvae after injection of oxLDL (0.5 mg/mL; green). (C) Uptake of oxLDL (0.5 mg/mL; green) in the caudal vein of 3dpf ABTL, *stab*1$^{-/-}$, stab2$^{-/-}$ and *stab*1$^{-/-}$stab2$^{-/-}$ zebrafish larvae. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 3. Results

### 3.1. Impaired scavenging function of SECs in absence of the scavenger receptors stab1 and stab2 stimulates the uptake of apolipoprotein B (apoB)-containing lipoproteins by caudal vein macrophages

Scavenger receptors are known to play a role in lipoprotein removal during early atherosclerosis. We examined the importance of stabilin 1 and 2 for the uptake of lipoproteins by the vessel wall. Injection of fluorescent DiO-oxLDL in the duct of Cuvier of 3 dpf wild-type (ABTL), stabilin 1 mutants (*stab1*$^{-/-}$), stabilin 2 mutants (*stab2*$^{-/-}$), or stabilin 1 and 2 double mutants (*stab1*$^{-/-}$*stab2*$^{-/-}$) demonstrated decreased vessel wall uptake of oxLDL (green) in the stabilin double mutants compared to ABTL zebrafish larvae (Fig. 1A–B). Looking more closely, in the *stab1*$^{-/-}$ mutants, the uptake of oxLDL in the vessel wall is slightly decreased compared to ABTL, whereas the uptake of oxLDL in *stab2*$^{-/-}$ mutants is visibly diminished and completely absent in *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants (Fig. 1C). Notably, the decreased vessel wall uptake in the *stab2*$^{-/-}$ and *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish larvae was paralleled by large accumulation of oxLDL in dot-like structures (Fig. 1C). These enlarged

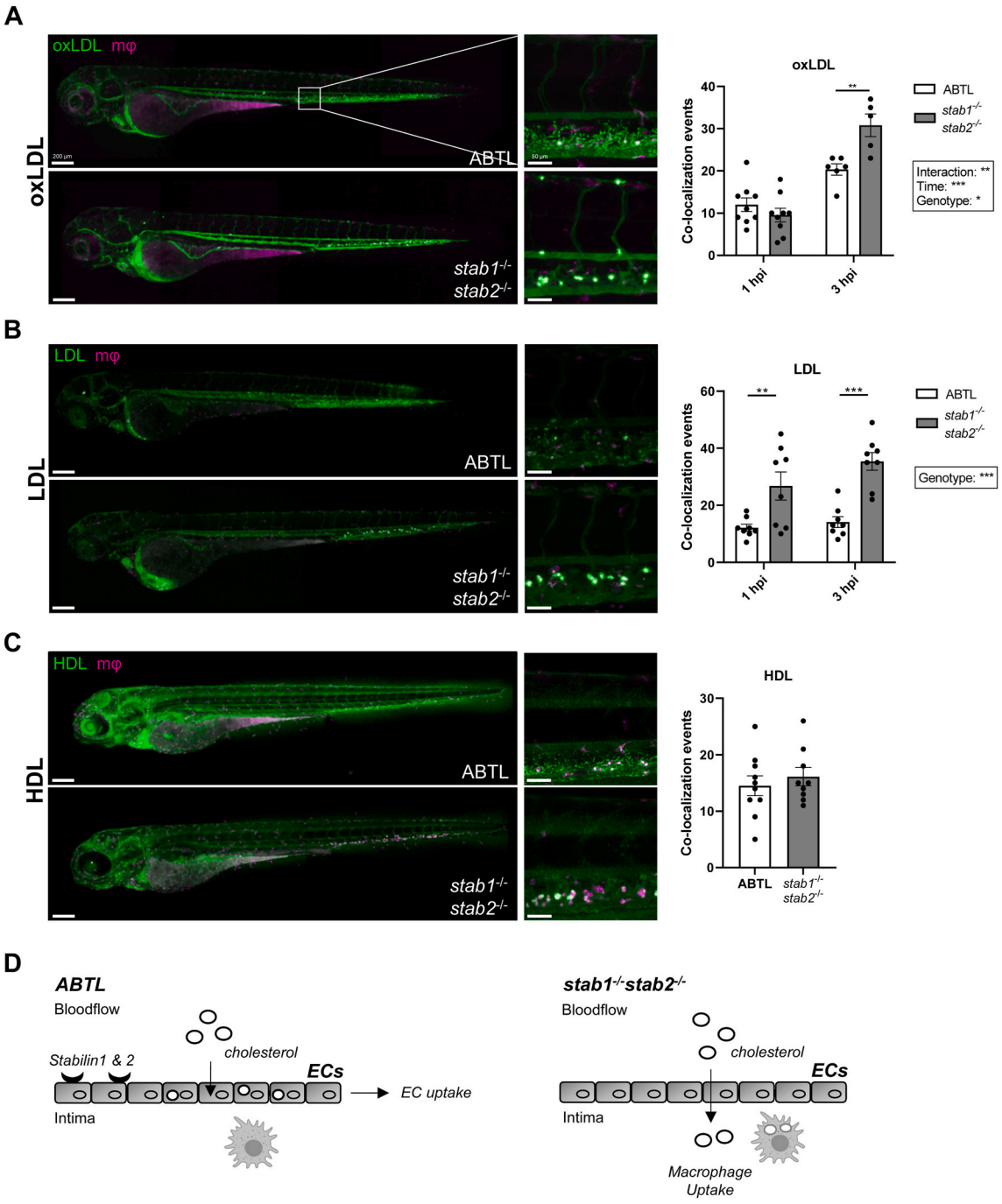


**Fig. 2.** Absence of scavenger receptors *stab*1 and *stab*2 reduces ApoB containing lipoprotein clearance by endothelial cells.
(A–C) Uptake of lipoproteins by endothelial cells and macrophages in 3dpf ABTL and *stab*1$^{-/-}$stab2$^{-/-}$ *mpeg1*:rfp transgenic zebrafish at 3 h post injection (hpi) ((ox) LDL) or 24 hpi (HDL) and the quantification of co-localization events between lipoproteins (green) and macrophages (purple) after 1 hpi (n = 9), 3 hpi (n = 6) or 24 hpi (n = 10). (D) Overview of possible mechanisms regarding cellular cholesterol uptake in ABTL and *stab*1$^{-/-}$stab2$^{-/-}$. Means ± SEM; $p < 0.01$**; $p < 0.001$***. Magnification: 10× and 40×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

green dots are likely macrophages that have taken up the oxLDL, because macrophages express multiple other scavenger receptors on their surface that are able to engulf lipoproteins. To further show the specific contribution of macrophages as professional scavenging cells to the clearance of the lipoproteins in *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants, wild-type and knockout zebrafish lines with transgenic expression of *mpeg1*:rfp were generated, enabling visualization of macrophage/lipoprotein co-localization. Injection of DiO-oxLDL in *mpeg1*:rfp ABTL larvae showed that, in the presence of functional stabilin scavenger receptors, oxLDL was mainly taken up by smaller vessel wall cells, probably endothelial cells, instead of macrophages. Combined deletion of *stab1* and *stab2* did not influence the number of co-localization events of the ox-LDL label and *mpeg1*:rfp macrophages at 1 h post injection (ABTL 13 vs *stab1*$^{-/-}$*stab2*$^{-/-}$ 9 co-localization events). However, at 3 h a prominent 1.5-fold increase ($p = 0.001$) was observed in absence of *stab1* and *stab2*. As evident from the fluorescent photomicrographs not only more macrophages accumulated oxLDL, but also the amount of oxLDL associated with the macrophages was dramatically increased (Fig. 2A) (sFig. 1). These results were independent of the macrophage numbers as the amount of total body macrophages did not differ between ABTL (n = 43) and *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish larvae (n = 38) (ABTL 60 vs *stab1*$^{-/-}$*stab2*$^{-/-}$ 59 macrophages; $p > 0.05$; data not shown). Surprisingly, *stab1* and *stab2* also influenced the clearance of native LDL. At 1 and 3 h post injection of Bodipy™ FL-LDL a 2.2-fold (1 h; $p = 0.003$) and 2.5-fold (3 h; $p < 0.0001$) increase in co-localization events with macrophages was observed in the stabilin mutants (Fig. 2B) (sFig. 2). Notably, the effects of stabilin deficiency on the macrophage association of native LDL were higher and evident earlier after injection than the effects seen with oxLDL. *In vitro* studies have already demonstrated that stabilin receptors have a high affinity for the binding of oxLDL, however the affinity of stabilins for native LDL and a role in removal from the circulation has not been described.

To examine whether stabilins are also involved in the association of non-oxidized, apolipoprotein A1-containing lipoproteins, human DiO-HDL was injected into 3 dpf zebrafish larvae. One hour after injection, HDL was still observed in the circulation and not taken up by endothelial cells or macrophages (sFig. 4). Therefore, we quantified the co-localization events 24 h post injection. In contrast to the apoB-containing lipoproteins LDL and oxLDL, stabilin deficiency did not affect the uptake of HDL by macrophages (ABTL 15 vs *stab1*$^{-/-}$*stab2*$^{-/-}$ 16 co-localization events) (Fig. 2C) (sFig. 3). The observed effects thus appear specific for apoB-containing lipoproteins. It is important to note that different fluorophores (DiO and Bodipy) used for the labeling of lipoproteins might possibly interfere with the uptake of these lipoproteins by macrophages. However, the striking effect of stabilin deficiency on DiO-oxLDL in the context of unchanged DiO-HDL uptake suggests that fluorescent labelling of lipoproteins is not the driving force in the effect of total body stabilin deficiency on lipoprotein uptake.

Together, these data suggest that 1) stabilins play a role in the clearance of apoB-containing (oxidized) lipoproteins and 2) that macrophages, probably via uptake by other scavenger receptors, compensate for the loss of endothelial *stab1* and *stab2* by scavenging more (ox) LDL (Fig. 2D).

### 3.2. Stab1 and 2 deficiency does not result in major compensatory changes in the expression levels of scavenger receptors in 3 dpf larvae

To examine whether combined *stab1* and *2* deficiency induces changes in the expression pattern of genes involved in scavenging and lipoprotein uptake, as these might compensate for the loss of *stab1* and *2*, mRNA of 3 dpf ABTL and *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish larvae was isolated for further analysis (n = 7, pool of 5 fish). A significant 1.9-fold increase in macrophage receptor with collagenous structure (*marco*) was observed ($p = 0.04$), whereas no difference in the relative expression of scavenger receptors class B type 1 (*scarb1*) or cluster of differentiation 36 (*cd36*) was detected (Fig. 3A). In addition, the expression levels of HMG-CoA reductase (*hmgcra*) and sterol regulatory element binding transcription factor 2 (*srebf2*), both important for cholesterol synthesis, were not significantly changed between ABTL and stabilin mutants (Fig. 3B). The mRNA expression levels of LDL receptor (*ldlra*), important for uptake of (un)modified apoB-containing lipoproteins, were also unchanged in *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants (Fig. 3B). This suggests that the increased macrophage uptake of LDL and oxLDL in *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants was not due to an increase in the LDL receptor but rather related to the increased expression of *marco*. The ATP-binding cassette transporter sub-family A member 1 (*abca1a*) and ATP-binding cassette sub-family G member 1 (*abcg1*), both cholesterol efflux transporters, were not affected by stabilin deficiency (Fig. 3C). Thus, *stab1* and *stab2*

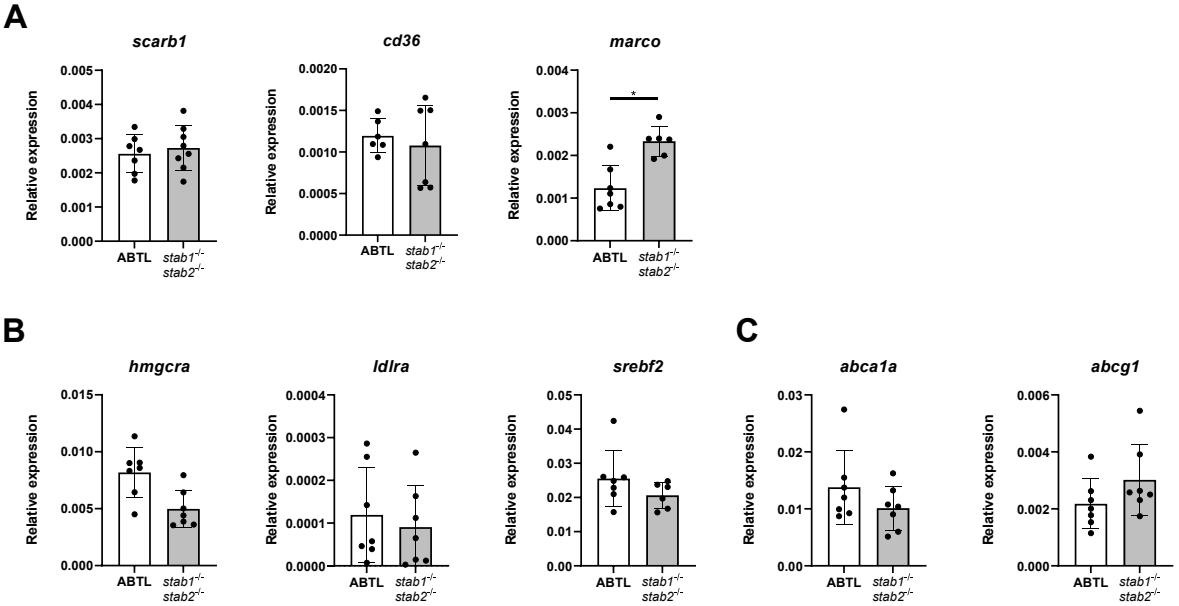


**Fig. 3.** Stabilin 1 and 2 deficiency does not affect genes involved in cholesterol synthesis and uptake in 3 days post fertilization old zebrafish larvae.
The effect of *stab1* and *2* deficiency on the relative expression of genes (A) involved in scavenging (B) cholesterol metabolism (C) and cholesterol efflux. Means ± SEM (n = 7, pool of 5); $p < 0.05$*.

deficiency is not associated with major compensatory regulation of key genes involved in cholesterol metabolism at a development stage prior to the initiation of feeding.

### 3.3. Stab1 and 2 deficient zebrafish display compensatory upregulation of the LDL receptor involved in cholesterol acquisition under high cholesterol diet feeding conditions

Previously, we have reported that ABTL zebrafish are protected against HCD-induced atherosclerosis, as we did not find vascular lipid deposition after 10 days of HCD feeding [4].

This was probably due the absence of an effect on total body cholesterol levels as result of compensatory downregulation of the cholesterol acquisition genes HMG-CoA reductase and the LDLreceptor [4]. Interestingly, in response to a HCD, the relative gene expression of *stab2* was also decreased (5.4-fold lower as compared to a low cholesterol diet, $p = 0.007$), while the relative expression of *stab1* remained unaltered (Fig. 4A). This decrease implies that *stab2* might play a more prominent role in the metabolism of cholesterol in zebrafish than *stab1*.

To further challenge the system and investigate the effects of stabilin deficiency on the total cholesterol levels and cholesterol homeostasis under high cholesterol conditions, ABTL *mpeg1*:rfp and *stab1*$^{-/-}$*stab2*$^{-/-}$ *mpeg1*:rfp zebrafish larvae were fed HCD supplemented with 10 μg/g Bodipy FLC12 cholesteryl ester for 10 days (Fig. S5A). As expected, no signs of vascular lipid deposition in the caudal vein were observed in 15 dpf old ABTL zebrafish larvae. However, despite the observed increase in uptake of native and oxidized LDL by macrophages in de caudal vein, *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish also did not develop vascular lipid deposition in response to HCD feeding (Fig. S5C). In accordance, the total body cholesterol content was unchanged (23.0 ± 1.8 μg/mg for ABTL *mpeg1*:rfp, as compared to 23.2 ± 1.2 μg/mg for *stab1*$^{-/-}$*stab2*$^{-/-}$ *mpeg1*:rfp zebrafish) (Fig. 4B). To investigate possible compensatory changes in response to the HCD feeding, the effects of *stab1* and *2* deficiency on the expression of genes important in whole body cholesterol acquisition and homeostasis, were determined (n = 7, pool of 5 fish). The mRNA expression levels of *ldlra,* involved in lipoprotein uptake, were 1.4-fold

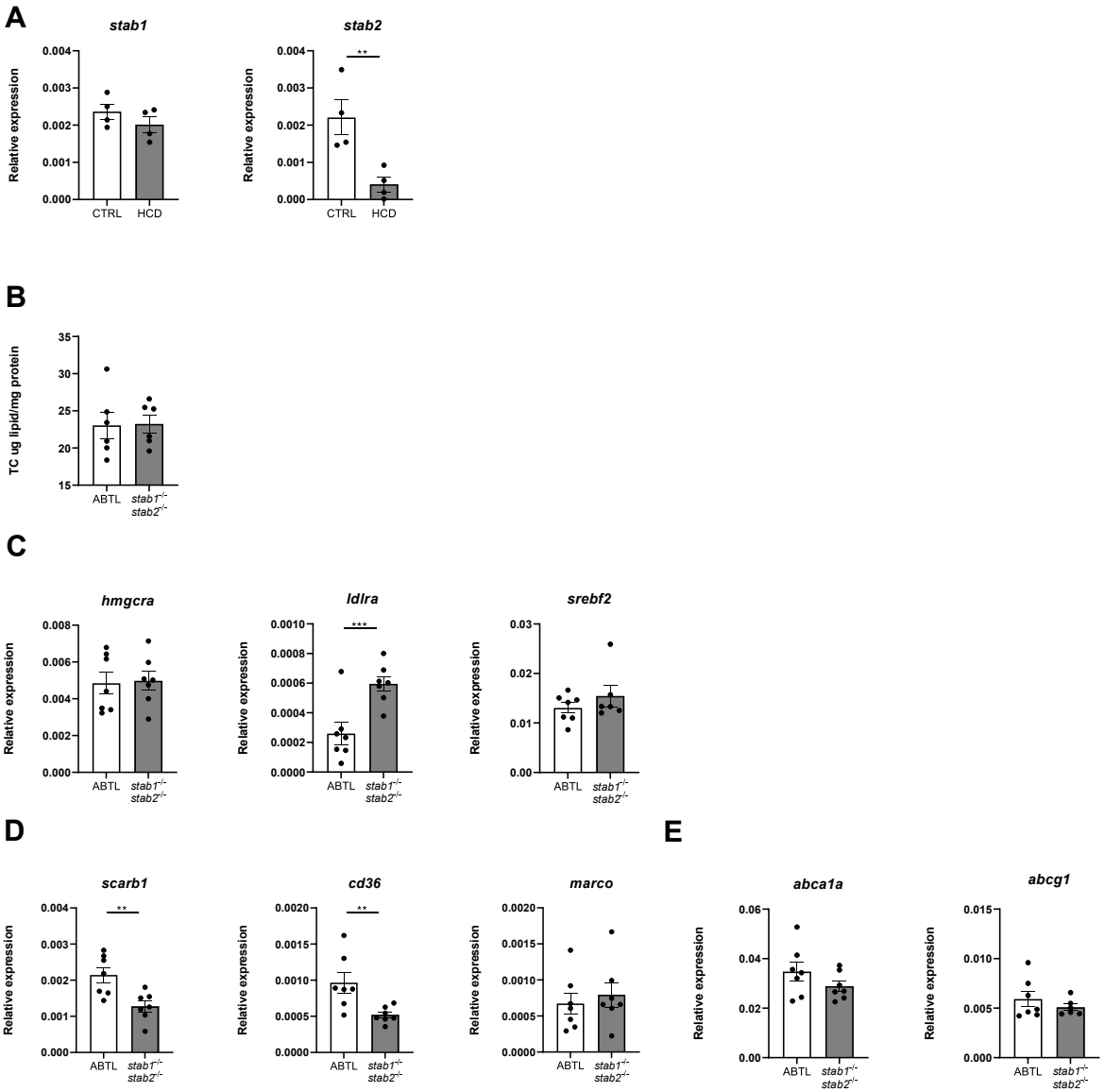


**Fig. 4.** Stabilins are an important player in zebrafish total-body cholesterol homeostasis after challenged with a 10 day high cholesterol diet (HCD).
(A) Relative expression of *stab1* and *2* in ABTL zebrafish (15 dpf old) after 10 day normal (CTRL) or either 10 day high cholesterol diet (HCD). (B) Whole body total cholesterol levels in 15dpf ABTL and *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish fed with a HCD for 10 days. (C) The effect of *stab1* and *2* deficiency after 10 days of HCD on relative expression of genes involved in cholesterol acquisition (D) scavenging and (E) cholesterol efflux. Means ± SEM (n = 7; pool of 5 fish); $p < 0.01$**; $p < 0.001$***.

higher ($p < 0.001$). Notably, *hmgcra* as well as cholesterol synthesis-related transcription factor *srebf2* showed no differences in stabilin double mutants as compared to ABTL controls challenged with (Fig. 4C). No difference was found in the relative gene expression of the scavenger receptors *marco,* whereas surprisingly *scarb1* (1.1-fold decrease, $p = 0.008$) and *cd36* (0.9-fold decrease, $p = 0.005$) were significantly downregulated (Fig. 4D). The expression levels of *abca1*a and *abcg1* were not changed in the *stab1*$^{-/-}$*stab2*$^{-/-}$ group (Fig. 4E). Together, these data might suggest that the observed increase in LDL receptor expression compensates for the loss of stabilin 1- and 2-mediated cellular cholesterol uptake.

## 4. Discussion

In this study, we examined the role of stabilins in the clearance of (modified) lipoproteins and early atherogenesis using a zebrafish model. Considering its many advantages and its highly conserved lipid metabolism, the zebrafish has been proposed as an upcoming alternative animal model to study the processes involved in atherogenesis, including lipoprotein uptake, endothelial function, and macrophage foam cell formation which is the hallmark of early atherogenesis.

Here, we described a key role for stabilins in the clearance of apoB-containing lipoproteins and highlighted the protective effects of a functional dual scavenging system, comprised of SECs and macrophages, in zebrafish larvae. We showed that deficiency of *stab1* and *stab2* led to redirected clearance of (ox)LDL in the caudal vein from endothelial cells to macrophages. While the loss of *stab1* and *stab2* did not result in vascular lipid deposition in the caudal vein of zebrafish larvae, we did observe whole body compensatory changes after 10 days of HCD, underlining the importance of stabilins in cholesterol homeostasis in zebrafish larvae under these conditions.

To date, this is the first *in vivo* study showing a role for stabilins in lipoprotein metabolism. In 2010, Li et al. were the first to suggest a role for *stab1* and *stab2* in the LSEC-mediated endocytosis of oxLDL in transfected HEK cells and freshly isolated rat LSECs in culture [11]. They showed that *stab1*-transfected HEK cells actively took up both mildly oxLDL ($oxLDL_{3h}$) and heavily oxLDL ($oxLDL_{24h}$), while *stab2*-transfected HEK cells preferred heavily oxLDL. In line with *Li* et al. (2010), we found that stabilins also play a role in apoB-containing lipoprotein uptake *in vivo*. Our study demonstrated that fluorescently-labeled mildly to heavily oxidized LDL (LDL incubated with 10 μM copper sulfate solution for 24 h, labeled with carbocyanine dye) is actively taken up via stabilins expressed by cells on the inner vascular lining.

In *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants, deficiency of total body *stab1* and *2* led to an impaired clearance of oxLDL and was paralleled by a redistribution of oxLDL to macrophages in the caudal vein. Considering that we used *stab1* and 2 double knockout mutants, this cannot be mediated by *stab1*, which is highly expressed on macrophages in wildtype larvae. Thus the findings suggest that other macrophage scavenger receptors play an important role in the uptake of oxLDL in absence of stab1. To our surprise, stabilins also facilitated the uptake of native LDL in zebrafish larvae, as evidenced by increased uptake by macrophages in the stabilin mutants. LDL has not been described as a stabilin ligand before. Li et al. previously showed that *stab1* or *stab2*-transfected HEK cells take up LDL and that in *stab2*-transfected HEK cells, the uptake of LDL was even in the same range as the uptake of mildly oxLDL [11]. This was, however, attributed to the role of the LDL receptor in these cells. Close inspection of the figures of the manuscript of Li et al., however, shows that uptake of LDL by *stab1*-or *stab2*-transfected HEK cells is slightly higher as compared to the uptake by non-transfected cells. Notably, a recent study, examining the effects of apoB lipoproteins during embryogenesis and pathological conditions in zebrafish, has shown that apoB-containing lipoproteins in zebrafish could regulate angiogenesis and activate antiangiogenic mechanisms in endothelial cells [16,17]. Uptake of native LDL by endothelial cells thus not only is related to their scavenging function, but actually has an important physiological role in zebrafish.

Recently, it has been shown that particle size is an important parameter for cellular uptake of nanoparticles [18]. In SECS, the uptake and elimination of nanoparticles are mainly mediated by the *stab2* receptor [6]. A more recent study has added to these findings that *stab1*, the *stab2* homolog, is required for endothelial clearance of small nanoparticles (<50 nm) and cooperates with *stab2* in the removal of larger nanoparticles [12]. These results suggest partial redundancy between these two scavenger receptors and highlight particle size as a critical parameter for receptor specificity. Besides, other biological factors and small differences in structural domains of *stab1* and *stab2*, which are highly similar, could influence whether the fluorescent lipoproteins are taken up by *stab1* or/and *stab2*. The diameter of fluorescently labeled LDL was 25 ± 1.1 nm whereas the diameter of fluorescent mildly to heavily oxLDL was 35 ± 0.98 nm. Injection of oxLDL in *stab1*$^{-/-}$, *stab2*$^{-/-}$, and *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish larvae demonstrated that these receptors cooperate when taking up oxLDL from the bloodstream confirming a role for both these stabilin receptors in lipoprotein uptake and therefore suggesting a possible role in atherogenesis. Interestingly, stabilin deficiency did not influence the uptake of the smaller lipoprotein HDL (±12 nm). It has been shown that stabilins are able to take up particles up to sizes smaller than 10 nm[12]. Therefore, the differences in recognition of HDL as compared to oxLDL and native LDL can likely not be explained by the size of HDL, but are rather the consequence of other structural differences between HDL and the apoB-containing lipoproteins.

Important to note is that the LDL receptor binding domain in zebrafish apoE is highly conserved. It was shown that apolipoprotein E (apoE) as well as A-I (apoA-I) genes are present in zebrafish and that the deduced amino acid sequences of zebrafish apoE and apoA-I have an identity of 27.5% and 25.6% respectively to the human orthologs [19,20]. Furthermore, 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 [21]. To our knowledge no competition studies have been performed using the LDL receptor of zebrafish origin and human and zebrafish apoE-containing lipoproteins. It therefore cannot be excluded that binding of the injected human lipoproteins to zebrafish lipoprotein receptors and/or subsequent intracellular processing differs from endogenous zebrafish lipoproteins [19,21].

To date, there are no studies published regarding atherosclerosis development in stabilin 1 and 2 double knockout mutants. This is mainly because the lifetime of *stab1*$^{-/-}$*stab2*$^{-/-}$ mice is reduced due to the development of several abnormalities, including liver fibrosis and nephropathy [22]. More recent, Kim et al. has demonstrated that pregnant *stab1*$^{-/-}$*stab2*$^{-/-}$ female mice suffer from defects in placental development and maintenance, indicating that *stab1* and *stab2* are involved in reproduction and placentation in mice [23]. Because zebrafish do not have a placenta, these defects were not observed *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish, that are viable and reproduce normally, making zebrafish a good model organism to study the role of stabilins in lipoprotein uptake and early atherogenesis. However, despite the observed increased co-localization of macrophages with (ox)LDL, resembling foam cell formation, *stab1*$^{-/-}$*stab2*$^{-/-}$ mutant zebrafish did not develop atherosclerotic lesions in response to the HCD feeding. For future research, it would be interesting to investigate whether *stab1*$^{-/-}$*stab2*$^{-/-}$ mutants generated in a *ldlra*$^{-/-}$ background would develop more vascular lipid deposition compared to single *ldlra*$^{-/-}$ zebrafish larvae, as it has been published that even 5 day HCD feeding of *ldlra*$^{-/-}$ mutants results in accumulation of vascular lipid deposits [24].

Macrophages are not only the main cell type involved in early-stage atherosclerosis, but together with LSECs (in zebrafish SECs) they also form a potent and functional scavenging system. LSECs are responsible for the elimination of bloodborne antigens and molecules, including pro-atherogenic oxLDL [25]. Inefficient clearance of pro-atherogenic

substances by LSECs due to chronic inflammation could subsequently lead to more oxidative stress and damaged liver sinusoids, in turn increasing the risk of atherosclerosis [26]. Importantly, ageing is a well-known risk factor for dysfunctional LSECs and can cause substantial morphological as well as functional changes including endothelial thickening [27–29]. Besides, ageing reduces the endocytic capacity of LSECs, which is especially important for the oxLDL clearance by stabilins [30]. This impaired LSEC function could stimulate the onset of age-related atherosclerosis and therefore could be a promising therapeutic target. Therefore, it would be interesting to examine whether we would observe atherosclerosis in different age stages of zebrafish including, young-adult and especially aged zebrafish.

In conclusion, our study has demonstrated that stabilins have an important apoB-lipoprotein scavenging function in zebrafish and that deficiency of these class H scavenger receptors strongly upregulates the clearance of lipoproteins by macrophages within the caudal vein. Although zebrafish appear less prone to atherosclerosis development compared to mammalian models, this study does highlight the use of zebrafish as a model to study lipoprotein metabolism and LSEC function. In this context, for future studies it would be of interest to generate endothelial and/or macrophage-specific *stab1* and, particularly, *stab2* knockout zebrafish, allowing verification of the individual contribution of these specific cells to lipoprotein metabolism.

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## CRediT authorship contribution statement

**Robin A.F. Verwilligen:** Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Lindsay Mulder:** Validation, Investigation, Formal analysis, Data curation. **Frans J. Rodenburg:** Data curation, Formal analysis. **Amy Van Dijke:** Investigation, Data curation, Formal analysis. **Menno Hoekstra:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration. **Jeroen Bussmann:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration. **Miranda Van Eck:** Supervision, Writing – review & editing, Funding acquisition, Project administration.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. dmSupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2022.02.018.

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