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The important role of apolipoprotein A-II in ezetimibe driven reduction of high cholesterol diet-induced atherosclerosis

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HIGHLIGHTS

- Ezetimibe alleviates high cholesterol diet-induced zebrafish pro-atherosclerotic lesions by increasing Apo A-II expression.
- Ezetimibe increases Apo A-II expression through HNF4 and PPARα transcriptional factors in HepG2 cells.
- Increased Apo A-II is beneficial to the recovery of macrophages cholesterol efflux, which could reduce the risk of atherosclerosis.

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ABSTRACT

Background and aims: It has been well established that ezetimibe blocks cholesterol absorption to prevent the negative effects of a high-fat diet in atherosclerosis. However, the exact mechanism is unknown. Here we use a transgenic zebrafish, which expresses different fluorescent proteins on either endothelial cells or granulocytes and macrophages, to explore the specific mechanism of ezetimibe and its role in reducing atherosclerosis-related hypercholesteremia.

Methods: Zebrafish larvae were exposed to a control diet, high cholesterol diet (HCD) or a HCD with ezetimibe treatment. Both the control diet and high cholesterol diet were mixed with red or green fluorophore labeled cholesteryl ester to trace lipid distribution. Isobaric tags were used for relative and absolute quantification to examine protein expression profiles of zebrafish larvae in the different treatment groups. To knock down Apo A-II and investigate the role of Apo A-II in the anti-atherosclerotic function of ezetimibe, we used morpholinos to target zebrafish *Apoa2* mRNA. To confirm ezetimibe regulatory role on Apo A-II expression, siRNA against *HNF4*, *PPARα*, and *SREBP1* were transfected into HepG2 cells.

Results: The results show that ezetimibe increased the expression of Apo A-II but failed to reduce vascular lipid accumulation and macrophage recruitment induced by the HCD diet when Apo A-II was knocked down. Finally, we found that ezetimibe increased the expression of Apo A-II through HNF4 and PPARα transcriptional factors.

Conclusions: Our data indicates that ezetimibe may not only prevents atherosclerosis by inhibiting cholesterol absorption in the intestine, but also by increasing the expression of Apo A-II in hepatocytes, thereby enhancing reverse cholesterol transport and removing excess cholesterol from the periphery.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette sub-family G member 1; ASCVD, atherosclerotic cardiovascular disease; EZE, ezetimibe; HCD, high cholesterol diet; HDL, high density lipoprotein; iTRAQ, isobaric tags for relative and absolute quantification; MIF, mean fluorescent intensity; MO, morpholino oligonucleotides

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1. Introduction

Atherosclerosis is a chronic vascular inflammatory disease, which is mainly induced by hyperlipidemia, so that nowadays most clinical therapies generally target lipid metabolism to slow disease progression. For instance, statins block endogenous synthesis of cholesterol, and ezetimibe blocks the absorption of lipids from small intestines [1–4]. Besides the consumption of lipids, as fuel for the body metabolic processes and the use of cholesterol as an integral structure of the cell membrane, our body uses an important pathway to exclude excess peripheral lipids and balance lipid metabolism, namely the reverse cholesterol transport pathway (RCT) [5].

The RCT pathway consists of cholesterol transport proteins like ABCA1 on the peripheral cells membrane, and HDL and HDL receptors on hepatocyte cell membranes. RCT utilizes HDL receptor to uptake HDL lipids to synthesize bile and secrete lipid within the bile from the liver to the small intestine [6]. HDL consists of apolipoproteins, such as Apo A-I, A-II, Apo D and Apo E, etc [7]. Hyperlipidemia may impair RCT by reducing either ABCA1 expression on peripheral cells, so that excess lipids cannot be transported to HDL or apolipoproteins and speed-limited enzymes expression for bile synthesis in hepatocytes, so that liver cells cannot receive and process lipids for excretion [8]. An impaired RCT pathway is a key cause of hyperlipidemia [9].

Ezetimibe is believed to target the Niemann-Pick C1-Like 1 (NPC1L1) sterol receptor, a cellular protein expressed on enterocytes and in the liver, which serves to tightly regulate dietary and biliary cholesterol absorption [4]. Using ezetimibe as a control for the study of a Chinese herb function on regulation of lipid metabolism, we were surprised to find that it regulates Apo A-II expression as well. The expression of Apo A-II in hepatocytes is regulated by a series of transcription factors, as apo A-II gene has a complex array of proximal, middle, and distal regulatory elements, designated from A to N, situated in the apo A-II promoter region. These elements can be separated into eight proximal and middle (AIIA to AIIH) and six distal (AIII to AIIN) elements [10]. It has been shown that orphan nuclear receptors, such as hepatic nuclear factor 4 (HNF-4), v-Erb related receptor 2 (EAR-2), EAR-3, and apolipoprotein regulatory element 1 (ARP-1), interact with this J site of apo A-II promoter [11,12]. HNF-4 induces, whereas EAR-2, EAR-3, and ARP-1 reduce, apo A-II gene transcription on binding to their elements [12]. The apo A-II promoter is also trans-activated by retinoid X receptor (RXR) and peroxisome proliferator-activated receptor a (PPARα), strongly suggesting that the heterodimer RXR/PPAR could be a positive physiological regulator of the apo A-II transcriptional rate [13]. Several studies have shown that sterol regulatory element binding protein 1 (SREBP-1) recognizes regulatory elements AIIB, AIICD, AIIDE, AIIK and AIIHI [13]. Thus, we explored the effects of HNF-4, PPARα, SREBP-1 siRNA to see whether any of their targets are involved in ezetimibe up-regulation of Apo AII expression and the ensuing lipid efflux from macrophages to HDL.

2. Materials and methods

2.1. Zebrafish

All zebrafish lines are gifts from Professor Yiyue Zhang and Professor Wenqing Zhang, Department of Developmental Biology, Southern Medical University. *Tg (fli1: eGFP)* is a transgenic animal with eGFP-fused transcription factor ERGB, which expresses only in endothelial cells. *Tg (flk1: mCherry)* contains a mCherry-fused vascular endothelial growth factor receptor 2 (VEGFR-2), which also expresses only in endothelial cells. *Tg (lyz: DsRed)* fish, is transfected with DsRed-fused lysozyme-3-protein, which expresses in granulocytes only. *Tg (coronina1a: eGFP)* is a transgenic fish with eGFP fused coronin 1A expressed in myeloid cells.

All maintenance and procedures of animals were in accordance with the requirements of the Ethical Committee of Southern Medical University. A high-cholesterol food (HCD) was prepared by dissolving cholesterol (BioSharp) in diethyl ether to achieve 4% (w/w) cholesterol in the food after ether evaporation. One group of HCD-fed larvae was exposed to 40 μg/ml ezetimibe added into the fish tank water during the feeding period. For the purposes of studying vascular lipid accumulation in larvae, both control and HCD food were supplemented with 10 μg/g of fluorophore labeled cholesteryl ester, either in red (cholesteryl BODIPY® FL C12, Molecular Probes) or green fluorescent (cholesteryl BODIPY® 542/563 cholesteryl ester, Molecular Probes) color.

2.2. In vivo confocal microscopy

For *in vivo* confocal microscopy, zebrafish larvae were anesthetized with 0.02% tricaine and mounted in 0.5% low melting agarose and observed with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Images of zebrafish larvae were captured every 200 nm, vascular lipid, neutrophils and myeloid cells accumulation, blood vessel thickness and permeability of the endothelial cell layer and macrophages lipid colocalization were documented and analyzed using the Olympus Fluoview software.

2.3. iTRAQ analysis

Zebrafish larvae (50 larvae per group) were euthanized by prolonged exposure to tricaine and non-digested food was removed from the animals' abdomens. Animals were pooled, homogenized in dissolution buffer (0.1% SDS in 500 mM triethylammonium bicarbonate, pH 8.0) and sonicated with a sonicator (Thermo Scientific, Rockford, IL). The proteins were collected by centrifugation and measured the concentration by the Bradford assay (Thermo Scientific, Rockford, IL). 200 mg of proteins from each sample was reduced with 5 mM tris-(2-carboxyethyl) phosphine at 60 °C for 1 h, alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) at room temperature for 10 min and digested with trypsin 1:20 (E/S, w/w) at 37 °C for 18 h. Subsequently, each tryptic digest was labeled for 1 h with one of the eight iTRAQ reagents according to the manufacturer's protocol (AB Sciex, Foster City, CA). Labeled peptides were separated with SCX column and subjected to LC-MS/MS analysis with LTQ Orbitrap Velos (Thermo Fisher, USA). The MS/MS raw data were blasted against the Ensembl Danio Rerio database for peptide identification and quantification using Mascot 2.3.02. Proteins with FDR ≤ 0.01 were qualified for further quantification data analysis. Fold change cutoff ratio (FC) of ≥ 1.2 or ≤ 0.833 was set as the threshold to identify differently expressed proteins. A minimum of one unique peptide was required to identify and relatively quantify a protein.

2.4. Microinjection

Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools (Philomath). These MOs were designed complementary to the end of exon 2 and the beginning of intron 2 in *apoa2* gene with the sequences: apoa2-MO1-S : AGAACTATTTTTTACCTTGGAGTGCRC; RC: GCACTCCAAGGTAAAAAAT-

AGTTCT. MO1-S and control MO were prepared in RNase-free ultrapure water (5 ng/nl), and each embryo was injected 1 nl MO solution, totally 5 ng MO per embryo.

2.5. siRNA and siRNA transfection

To knockdown the specific transcriptional factor, siRNAs were constructed. *HNF4* siRNA forward: 5′-CAUGUACUCCUGCAGAUU UTT-3′, reverse: 5′-AAAUCUGCAGGAGUACAUGTT-3’; *PPARα* siRNA forward: 5′-GCCCGUUAUCUGAAGAGUUTT-3′, reverse: 5′-AACUCUUC AGAUAACGGGCTT-3’; *SREBP*1 forward: 5′-GGAGGCUUCUCUACAGG AATT-3′, and reverse: 5′-UUCCUGUAGAGAAGCCUCCTT-3’. The siRNAs were delivered with Lipofectamine re-agent (Invitrogen) in

OptiMEM (Gibco) at a concentration of 1 μg/ml.

2.6. Cell culture

HepG2 cells (human hepatocellular carcinoma cell line) and THP-1 (human acute monocytic leukemia cell line) monocytes were maintained in RPMI 1640 medium containing 10% (v/v) FBS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). For cholesterol efflux assay, THP-1 cells were induced with phorbol-12-myristate-13-acetate (PMA) at a final concentration 0.1 μg/ml for 48–72 h before experiment.

2.7. Cholesterol efflux

The cholesterol efflux assay was conducted using Cholesterol Efflux Assay Kit (Sigma, St. Louis, MO, USA). Cells were seeded in 1×10^5 cells/well in a 96 well plate and washed with serum free RPMI 1640 medium after adherence. 100 μl fluorescence-labeled cholesterol reaction mix buffer was added to each well and cells were incubated for 16 h under standard tissue culture conditions. The mix buffer was removed and the cells were washed gently with incomplete RPMI 1640 medium. Fresh medium that was diluted with cholesterol acceptor Apo A-II was added to the cells and incubated for 5 h under 37 °C. The supernatants were transferred to a new 96 well plate and the fluorescence intensity of media (Fm) was measured with a microplate reader (Fm, λex = 482/λem = 515 nm). The cells were lysed in the plate with 100 μl per well with cell lysis buffer (Catalog Number MAK192E, Sigma). The cell lysates were transferred to the supernatant medium and the fluorescence intensity of cell lysates was measured (Fc, λex = 482/λem = 515 nm). The rate of cholesterol efflux is expressed as a proportion of cholesterol movement from cells to the acceptor proteins relative to cholesterol absorbed by the cells. The following formula is used: % *Choleserol Efflux* = (*F*m/(*F*c + *F*m)) × 100%.

2.8. Statistics

Data in graphs are presented as means ± SE. Differences among experimental groups were evaluated by one-way ANOVA. $p < 0.05$ was considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. Ezetimibe reduces HCD-induced vascular lipid accumulation in zebrafish larvae

Transgenic zebrafish larvae is a suitable hyperlipidemia model, whose body is transparent during the first 30 dpf and *Fli1: eGFP* line expresses GFP specifically in endothelial cells, which allows a dynamic observation of the processes of vascular lipid accumulation. In this study, *Fli1: eGFP* larvae were fed a normal diet or a high cholesterol diet (HCD) containing 4% cholesterol with or without ezetimibe treatment, a well-known cholesterol absorption inhibitor, from 5 days post fertilization (dpf) when fish begin free feeding. Both the normal diet and high cholesterol diet were mixed with red fluorescent cholesteryl ester. In Fig. 1A, more red fluorescent lipid accumulation in caudal veins was observed in HCD-fed larvae compared with normal diet larvae. Except for the majority of the lipid deposits located in the caudal vein, some deposits were also found in the dorsal aorta and at sites of blood vessel bifurcation of HCD fed larvae as well (Fig. 1A). These results are consistent with Yury Miller publication and may be due to the direct connection between large arteries and veins at this development stage in zebrafish [14].

3.2. Ezetimibe reduces HCD-induced vascular myeloid cell accumulation in zebrafish larvae

The recruitment of macrophages to blood vessel and engulfment of lipid by macrophages is an early event in the pathogenesis of atherosclerosis in human. Feeding HCD to *Coroninala: eGFP/Fli1: mCherry larvae*, whose macrophages expressed eGFP, and endothelial cells expressed mCherry only (Supplementary Fig. 2), resulted in the recruitment of green fluorescent macrophages cells to the caudal vein and dorsal aorta (Fig. 1C). We also fed *Coroninala: mCherry larvae* with green fluorescent fluorophore labeled cholesterol to investigate the relationship between vascular lipid accumulation and macrophage adhesion. It was observed that more red fluorescence labeled macrophages and green fluorescence labeled lipid colocalized in caudal veins than control and ezetimibe groups (Fig. 1E). Ezetimibe reduced HCD induced macrophages recruitment and adhesion efficiently. The co-localization between lipid and macrophages in ezetimibe group was also weaker than in the HCD group (Fig. 1E).

3.3. Ezetimibe alleviates endothelial layer disorganization and permeability in HCD-fed larvae

An apparent thickness of the endothelial cell (EC) layer at sites of turbulent flow and formation of large vacuole-like EC boundaries indicates loss of EC alignment, which may be followed by infiltration of macrophages. In HCD-fed larvae, we observed irregularity in the endothelial layer morphology and apparent thickness of the EC layer in caudal blood vessels (Fig. 2C), and possible leakage of lipid substances from vessels. Remarkably, ezetimibe treatment attenuated HCD-induced EC disorganization and lipid leakage in the peripheral vasculature and relieved the thickness of the EC layer in the caudal vein (Supplementary Fig. 4A).

Recent evidence suggests that neutrophils play an important role in the initiation phase of atherosclerosis [1]. Feeding HCD to *lyz: DsRed2* larvae, whose monocyte/macrophages and granulocytes express DsRed2, resulted in expansion of red fluorescent myeloid cells beside the caudal vein, suggesting inflammation and vascular barrier dysfunction in the vascular wall (Fig. 2A). Feeding HCD to *lyz:DsRed2/coroninala : eGFP* larvae, we found expansion of neutrophils and macrophages outside the caudal vessels (Fig. 2B), while ezetimibe reduced the numbers of neutrophils and macrophages outside the vessel effectively.

3.4. HCD reduces apo A-II protein expression in zebrafish, while ezetimibe resumes apo A-II expression

We utilized the isobaric tags for relative and absolute quantitation (iTRAQ) method to compare the protein profiles between any 2 groups among zebrafish larvae fed a normal diet, HCD only or HCD plus EZE (Table 1). A total of 2765 peptides were identified after mass spectrometry analysis of iTRAQ labeled samples and they matched to 908 proteins (Table 2). The property and functions of identified proteins were classified with the Gene Ontology (GO) and Cluster of Orthologous Groups of proteins (COG) analysis (Supplementary Figs. 3 and 4) (lipid transport and metabolism related candidates were screened out (Supplementary Table 1). The abundance of those proteins was compared between any two groups, only the variation of protein abundance is more than 1.2 times and p value < 0.05, were accepted as changed protein. At last, we found a total of 288 proteins were up- or down-regulated significantly in these 3 group comparisons (Table 3; Supplementary Fig. 3C). Twelve candidates in the 288 proteins were found to change significantly in all 3 group comparisons after the intersectional clustering of differentially expressed proteins (Fig. 3A). Apolipoprotein A-II (Apo A-II) is the only one member of the apolipoprotein family (Table 4). Other members of HDL related apolipoproteins, such as Apo A-I, Apo A-IV and Apo-C1, were also changed in some

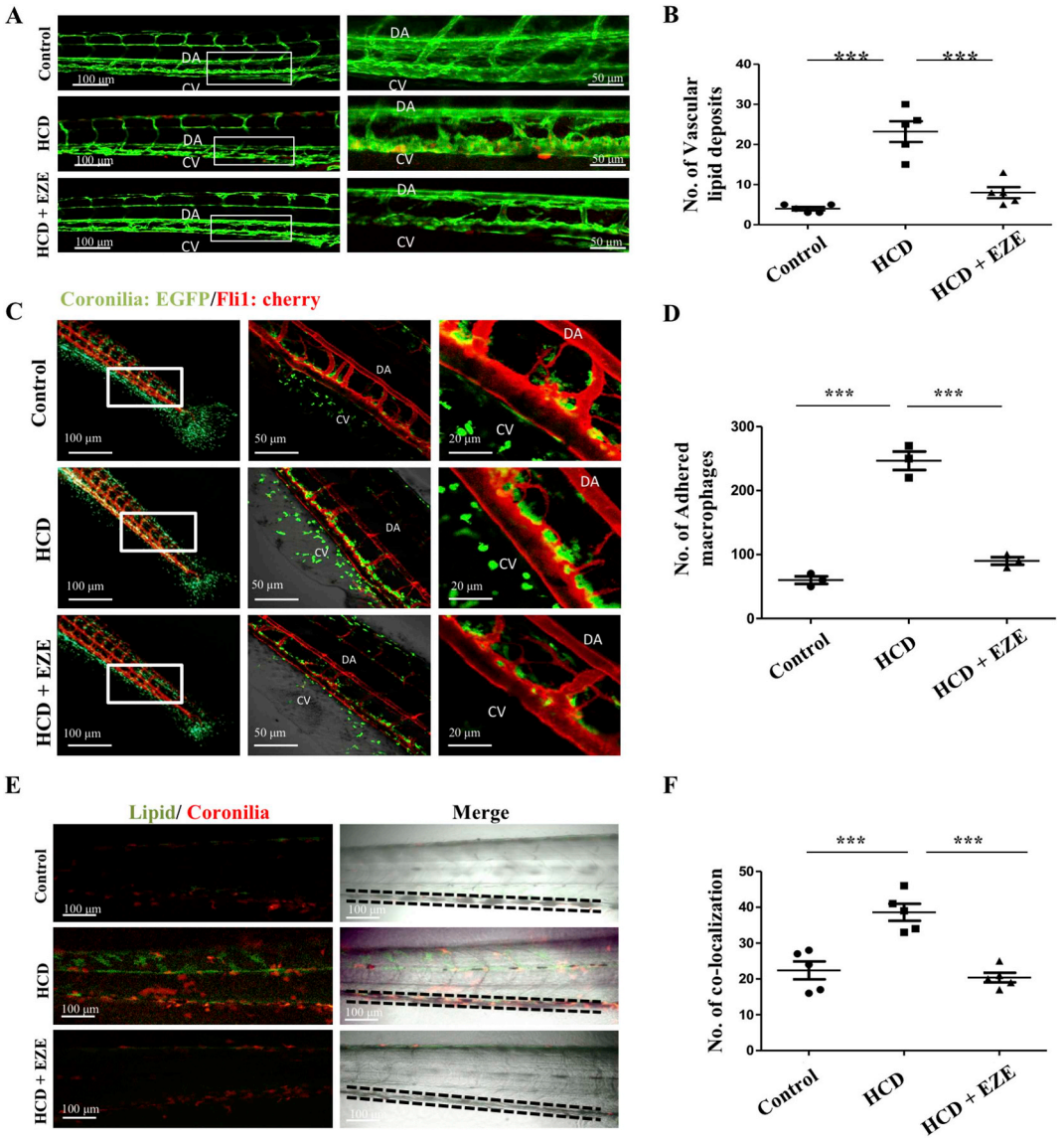


Fig. 1. Ezetimibe reduces HCD-induced vascular lipid accumulation in zebrafish.
Tg (fli1: eGFP), Tg (coroninala : eGFP) and Tg (coroninala : eGFP/fli1: mCherry) larvae were fed normal diet or HCD (4% cholesterol) supplemented with or without ezetimibe for 10 days from 5 dpf. (A) To track lipid accumulation, red fluorophore-labeled cholesteryl ester (10 μg/g) was mixed with the food. Images of live larvae show accumulation of red fluorescent lipid in the caudal vein and dorsal aorta. (B) Statistical analysis of red fluorescence, indicating lipid accumulation (n = 5). (C and D) Five-day-old *coroninala : eGFP/fli1: eGFP larvae* were fed control, HCD or HCD with ezetimibe for 10 days, and green fluorescent macrophages accumulated along the caudal vein were imaged and statistically analyzed. (E) To track lipid accumulation, HCD was labeled with green fluorophore-labeled cholesteryl ester. Images of caudal vasculature in live larvae showed lipid deposits (green) and macrophages (red). Both lipid deposits and macrophages colocalized in bright field backgrounds. (F) The co-localization of red fluorescent macrophages and green fluorophore-labeled cholesteryl ester accumulated within 50 μm of the caudal vein (delineated by dotted lines) was counted and statistically analyzed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

specific comparisons, but Apo A-II was the only one changed in every comparison (Table 5). We verified this finding by immunoblotting and quantitative PCR (Fig. 3B–F), which are consistent with iTRAQ results.

3.5. Ezetimibe increases Apo A-II expression through HNF4 and PPARα

To verify ezetimibe apo A-II-induction function, we treated human HepG2 cells with different doses of ezetimibe. The results in Fig. 4A and B showed that ezetimibe increased Apo A-II mRNA and protein level in a dose-dependent manner. We also investigate the influence of ezetimibe on Apo A-II expression in the human intestine epithelial cell line FH. The results in Fig. 4D showed that ezetimibe has no significant influence on Apo A-II expression in FH cells. Cholesterol overloaded cells expressed less Apo A-II protein in a dose dependent manner (Supplementary Fig. 5), while ezetimibe treatment reversed this phenomenon. According to references [10], the transcription of apoa2 genes is regulated by several transcriptional activators like HNF4, PPARα and SREBP1. We used siRNA to block the expression of those transcriptional activators in HepG2 cells and stimulated cells with ezetimibe. The results showed that both *HNF4* siRNA and *PPARα* siRNA reduced the expression of Apo A-II effectively (Fig. 4F).

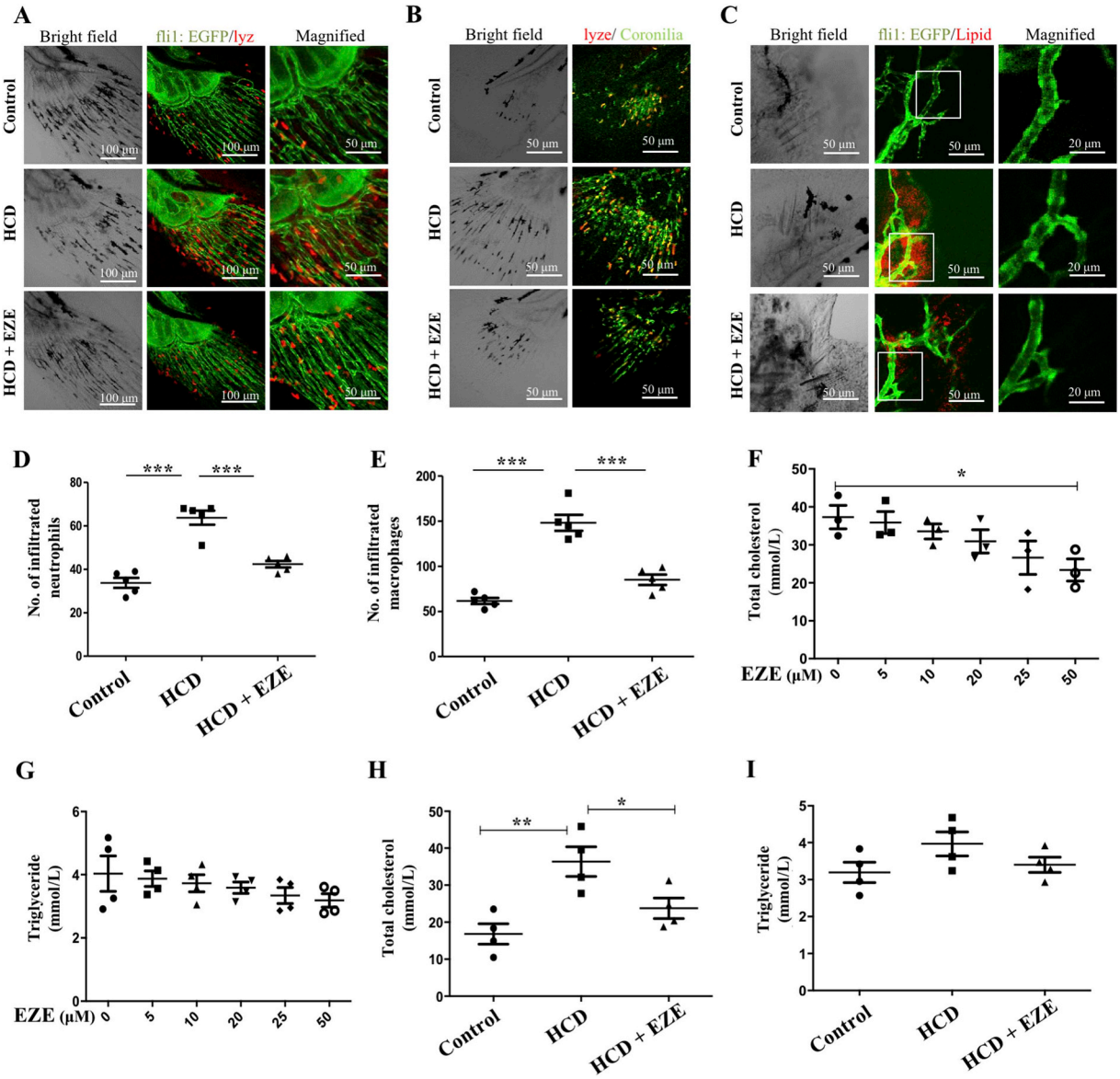


Fig. 2. Ezetimibe reduces endothelial layer disorganization and neutrophils recruitment in HCD-fed larvae.
Tg (fli1: eGFP), Tg (fli1: eGFP/lyz: DsRed2) or Tg (coroninala : eGFP/lyz: DsRed2) larvae were fed normal diet, HCD (red fluorescent cholesteryl ester analog labeled) supplemented with or without ezetimibe for 10 days from 5 dpf. (A) Images of caudal fin vasculature in live *Tg (fli1: eGFP/lyz: DsRed2) larvae* showed endothelial cells (green) and infiltrated neutrophils (red). (B) Images of caudal fin vasculature in *Tg (coroninala: eGFP/lyz: DsRed2)* showed macrophages (green) and neutrophils (red). (C) Images of the details of caudal fin vasculature in *Tg (fli1: eGFP) larvae* showed lipid (red) and endothelial cells morphology (green). (D) The infiltrated red fluorescent cells in Fig. 2C were counted and statistically analyzed (n = 5). (E) Infiltrated macrophages (green fluorescent cells) were counted and statistically analyzed (n = 5). (F–I) Total cholesterol (TC) and total triglycerides (TG) of 3 month old zebrafish larvae were tested (n = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Concentration, volume and density of loading samples.

Groups	Concentration (μg/μl)	Volume (μl)	Total protein (μg)
Control	1.25	400	500
HCD	1.50	400	600
HCD + EZE	0.97	400	388

Table 2
Number of spectra, peptides and proteins identified by LCM/MS.

Total spectra	78793
Spectra	9458
Unique spectra	5437
Peptide	2765
Unique peptide	2059
Protein	908

Table 3
Number of changed proteins in each group.

Groups	Up-regulated	Down-regulated	Total
Control-*vs*-HCD	30	44	74
Control-*vs*-HCD + EZE	77	75	152
HCD-*vs*-HCD + EZE	23	39	62

3.6. Ezetimibe reverses cholesterol-overload-induced decline of cholesterol efflux

Defective cholesterol efflux is a major cause of foam cell formation [15]. To investigate the role of Apo A-II in the cholesterol efflux in macrophages, we blocked Apo A-II expression with *HNF4* siRNA in HepG2 cells and collected the medium to treat cholesterol overloaded THP-1 cells (Fig. 4H and I). It was shown that ezetimibe increases Apo A-II level in the medium of human HepG2 cells compared with the control group. *HNF4* siRNA reduced Apo A-II concentration significantly compared with control and ezetimibe group. Addition of ezetimibe to *HNF4* siRNA pretreated HepG2 cells did not increase Apo A-II expression in the cell medium (Fig. 4H). In Fig. 4I, we found that Äpo A-II rich medium could increase the cholesterol efflux from THP-1 cells significantly compared with Apo A-II poor medium.

3.7. Knockdown of Apo A-II impairs ezetimibe protective effects

To investigate the role of apo A-II in ezetimibe protective function, we used antisense morpholino oligonucleotides complementary to the translational initiation site of zebrafish *apoa1* and *apoa2* mRNA to knockdown their expression in zebrafish embryos. We fed 5 dpf larvae with HCD or HCD plus ezetimibe for 3 additional days after injecting apo A-II MO to zebrafish embryos due to the short chronergy of morpholino oligonucleotides [16]. It was found that 3 days of HCD was enough to induce vascular lipid accumulation (Fig. 5A). As we observed in Fig. 5, knockdown of apo A-II aggravated vascular lipid accumulation, but when ezetimibe treatment was included, these effects still could not be inhibited (Fig. 5A).

4. Discussion

Ezetimibe is recommended as second line therapy for subjects intolerant to statins or unable to achieve target LDL cholesterol levels on statins alone by several major medical practice groups, including the American College of Cardiology [4]. Although the application of the first line therapy, statins has reduced around one third of atherosclerosis-related events, statin many side effects have been a major limitation for many patients [17,18]. A new therapy, involving PCSK9 inhibition targeting the reverse cholesterol transport pathway, has since been developed [19]. Our data suggests ezetimibe functions on lowering plasma lipid levels, which may also involve RCT, and is Apo A-II dependent. Many of PPARα and LXR agonists have been discussed in relation to their regulation of RCT protein expression, since the PPARα/LXR complex targets RCT pathway-associated genes, including genes encoding membrane lipid transporters, such as ATP-binding cassette subfamily A type 1 (*ABCA1*), *ABCG1*, *ABCG5* and *ABCG8* [20,21]; genes for apolipoproteins (Apos), such as *apo A-I*, *apo A-II*, *apo E* [22]; and genes for lipid transfer proteins and cholesterol metabolizing enzymes, such as cholesterol 7-α-hydroxylase (*CYP7A1*), a rate-limiting enzyme for bile synthesis, and HDL receptor gene for scavenger

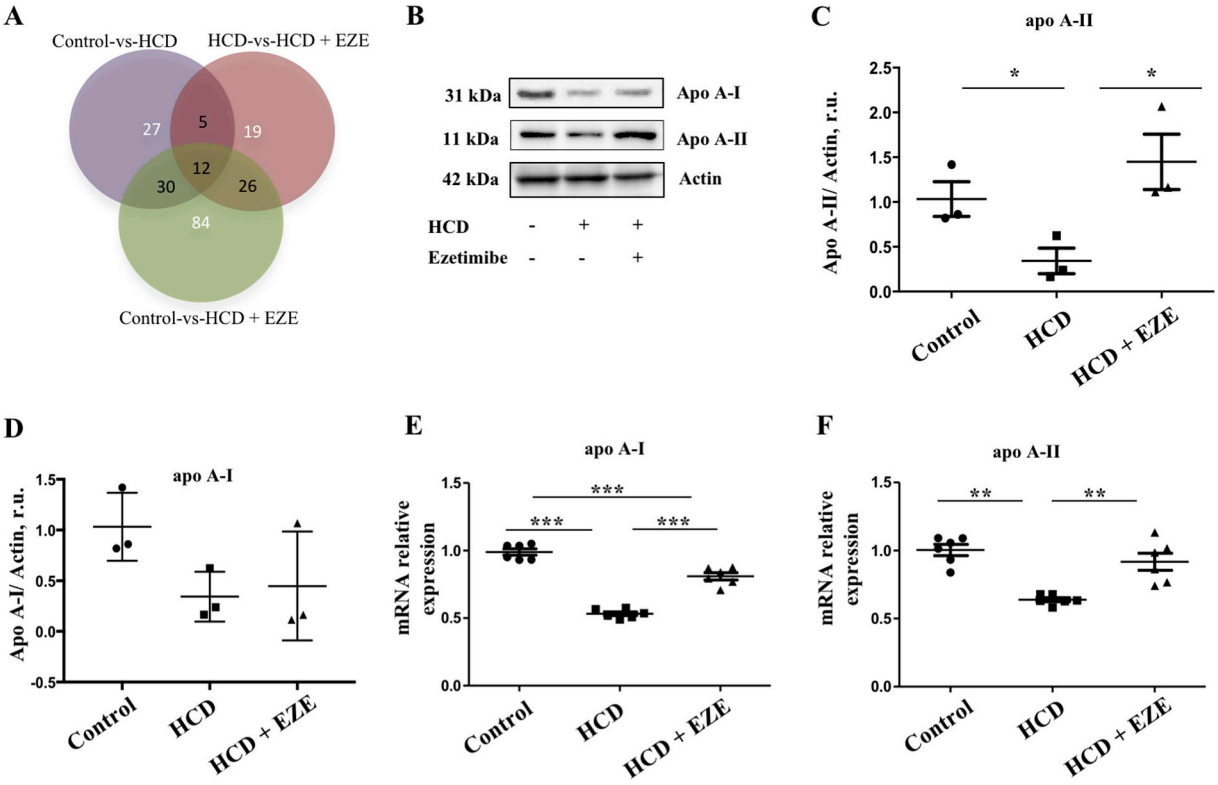


Fig. 3. iTRAQ analysis of differential protein profiles in control diet, HCD and HCD, with ezetimibe fed zebrafish.
Fifty larvae in each experimental group as mentioned in Fig. 1 were homogenized and subject to isobaric tags for relative and absolute quantitation (iTRAQ), to compare the differences in protein expression profile. (A) Venn diagram showing overlap between protein identification in three iTRAQ experiments. (B) Expression of Apo-AII in different groups of zebrafish was assayed with Western blot. (C and D) The intensity of the bands of targeted protein was calculated, then normalized to the internal control and statistically analyzed (n = 3), r.u., relative intensity. (E and F) The relative mRNA expression of apo A-I and -II in normal diet, HCD and HCD with ezetimibe fed larvae was determined by quantitative PCR.

Table 4
12 candidates were screened and exhibited after the intersectional clustering of differentially expressed protein.

Accession Number	Protein	Con *vs.* HCD	Control *vs.* HCD + EZE	HCD *vs.* HCD + EZE
ENSDARG00000005162	Tropomyosin 3	0.808	0.638	0.812
ENSDARG00000011201	Ribosomal protein, large P2,-like	0.825	0.636	0.711
ENSDARG00000045676	Calumenin-A	0.8	0.649	0.799
ENSDARG00000057867	LIM and SH3 protein 1	0.811	0.617	0.748
ENSDARG00000013752	Troponin I type 2a	0.452	0.741	1.475
ENSDARG00000005513	Nascent polypeptide-associated complex subunit alpha	0.707	0.548	0.761
ENSDARG00000032405	Zgc:85975	0.791	0.65	0.809
ENSDARG00000025850	40S ribosomal protein S21	0.74	0.624	0.828
ensdarg00000042502	Histone H2A	1.422	1.743	1.204
ENSDARG00000074242	SERPINE1 mRNA-binding protein 1a	0.8	0.58	0.67
ENSDARG00000015866	Apolipoprotein A-II	1.463	0.706	0.476
ENSDARG00000007275	Si:ch211-251b21.1	1.509	1.948	1.254

The MS/MS raw data were blasted against the Ensembl Danio Rerio database for peptide identification and quantification using Mascot 2.3.02. The abundance of those proteins was compared between any two groups, only the variation of protein abundance more than 1.2 times and p value < 0.05 were accepted as changed protein. 12 candidates were found to change obviously in all 3 group comparisons after the intersectional clustering of differentially expressed proteins. The accession number, protein name, fold change of protein abundance in different comparisons were exhibited as above.

Table 5
The hyperlipidemia and HDL related proteins were presented.

Accession number	Protein	Con *vs.* HCD	Sig	Con *vs.* HCD + EZE	Sig	HCD *vs.* HCD + EZE	Sig
ENSDARP00000112199	Apolipoprotein A-Ib	1.319	*	1.25	*	0.962	
ENSDARP00000058963	Apolipoprotein A-II	1.463	*	0.706	*	0.476	*
ENSDARP00000118222	Apolipoprotein C-I	0.972	*	0.424	*	0.428	*
ENSDARP00000025613	Apolipoprotein A-I	0.848	*	0.53	*	0.604	*
ENSDARP00000111399	Apolipoprotein A-IV b	0.963		0.531	*	0.54	*
ENSDARP00000064375	Superoxide dismutase [Cu-Zn]	0.702	*	0.597	*	0.933	
ENSDARP00000092919	COX17 cytochrome *c* oxidase assembly homolog	0.828	*	0.9		1.008	
ENSDARP00000055160	Cytochrome *c* oxidase subunit	0.765	*	0.677	*	0.869	
ENSDARP00000090311	Cytochrome *c* oxidase subunit Vb 2	0.745	*	0.819		1.079	

The fold change of abundance of other members of HDL related apolipoproteins, such as Apo A-I, Apo A-IV and Apo-C1 were also exhibited as above. Some hyperlipidemia related proteins like cytochrome C, superoxide dismutase were also screened out. The fold change cutoff ratio (FC) of ≥ 1.2 or ≤ 0.833 and p value < 0.05 were set as the threshold to identify differently expressed proteins.

receptor BI [23,24]. Our results show that ezetimibe could increase apo A-II expression in both zebrafish and human hepG2 cells. It has been reported that the transcription of *apoa2* gene is regulated by several transcriptional factors like hepatic nuclear factor 4 (HNF-4), v-Erb related receptor 2 (EAR-2), EAR-3, apolipoprotein regulatory element 1 (ARP-1) and also by retinoid X receptor (RXR) and peroxisome proliferator-activated receptor a (PPARα). Although we found ezetimibe regulates Apo A-II expression through HNF4 and PPAR-α pathway, its underlying molecular mechanism still needs to be explored further. At least, we have shown that ezetimibe can lower plasma lipid levels not only by inhibition of cholesterol absorption from the small intestine, but also by activation of the reverse cholesterol transport pathway. A combination of statin and ezetimibe treatment in clinic, may not only block cholesterol synthesis and uptake, but also activate the RCT pathway, which can remove excess cholesterol from periphery, and a synergistic effect reducing hyperlipoidemia and halting atherosclerosis progression could be expected.

Apolipoprotein (apo-) A-II is the second most abundant protein of the high density lipoprotein (HDL) family [25,26]. HDLs have an atheroprotective effect due to their anti-oxidative, anti-inflammatory effects, and ability to carry out reverse cholesterol transport [8]. Transgenic mice expressing hapo A-I or hapo A-II and mice deficient in endogenous apo A-I or apo A-II (apo A-I-KO or apo A-II-KO, respectively) were studied for the components contribution of HDL and their protective effects against atherosclerosis [27]. Mouse HDL are a homogeneous population of large particles; upon expression of hapo A-I, a smaller HDL population appeared, resembling human HDL, and plasma HDL levels increased. With a moderate expression of hapo A-II, smaller-sized HDL also appeared, and their proportion increased with hapo A-II expression levels, whereas HDL levels did not increase [27]. In keeping with the importance of apo A-I for HDL synthesis, apo A-I-KO mice displayed drastically decreased plasma HDL, but surprisingly, did not develop aortic lesions when fed an atherogenic diet. Unexpectedly, apo A-II-KO mice had even lower plasma HDL than apo A-I-KO mice, and developed atherosclerosis. It appears that hapo A-I and hapo A-II are important for size heterogeneity of human HDL but both apo A-I and apo A-II are important for HDL formation and/or structural stability. However, apolipoprotein A-II might be a key regulatory factor of HDL against atherosclerosis [25,26]. Our data shows that ezetimibe stimulates hepatocyte expression of apo A-II, and knockdown of apo A-II expression, by interfering with its translation in Zebrafish, attenuates ezetimibe prevention of vascular lipid accumulation and macrophages adhesion in the plaque area in zebrafish animal model. Thus, ezetimibe-induced apolipoprotein A-II expression is crucial for the prevention of high cholesterol diet-induced atherosclerosis.

In summary, we found that ezetimibe prevents atherosclerosis not only by its inhibition of cholesterol absorption from the small intestine, but also by activation of reverse cholesterol transportation, since its activation of hepatocytes expression of apo A-II and its atheroprotective effect were Apo A-II dependent. We propose that a combination of statin and ezetimibe treatment in the clinic may have a better therapeutic effect.

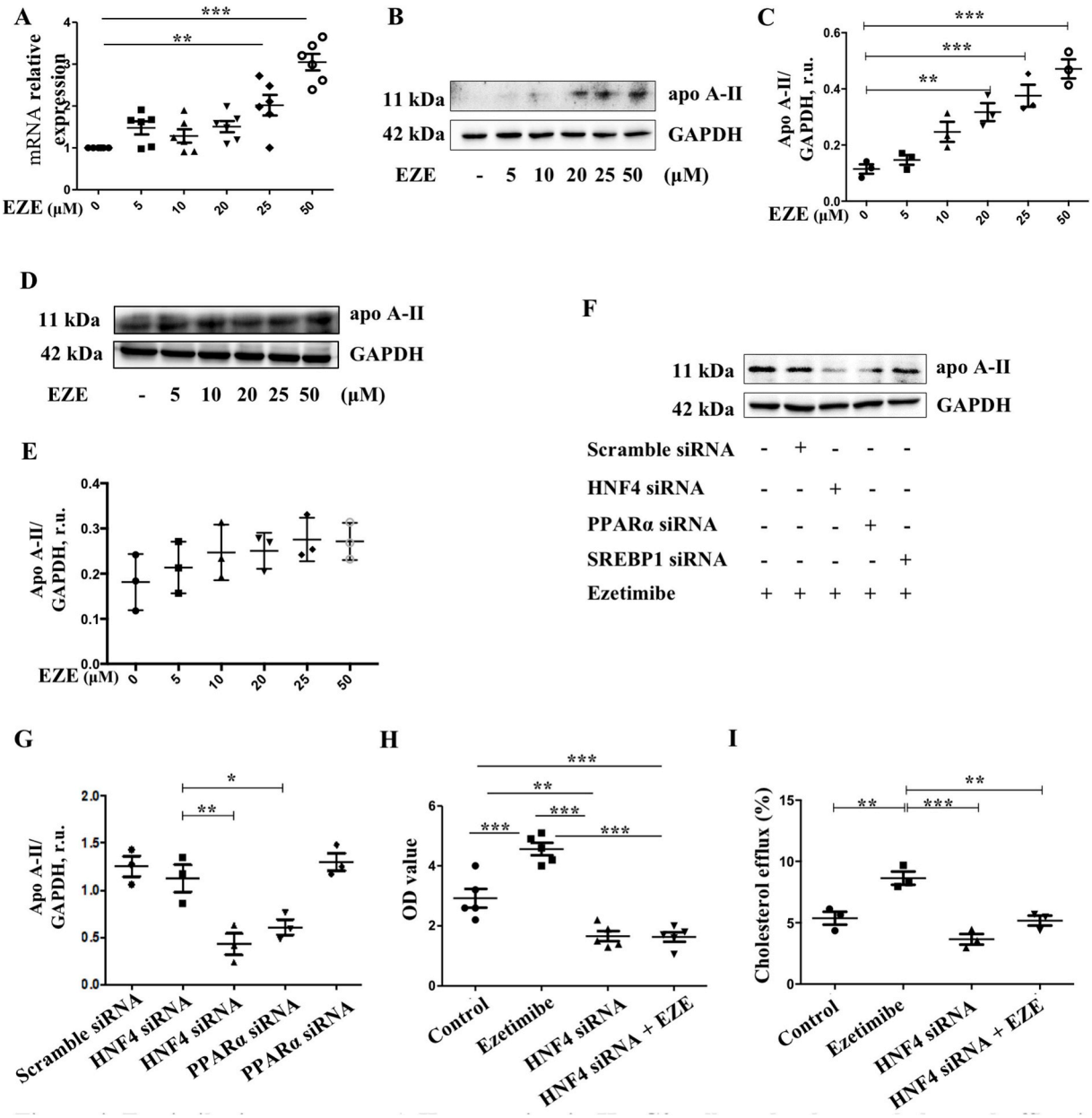
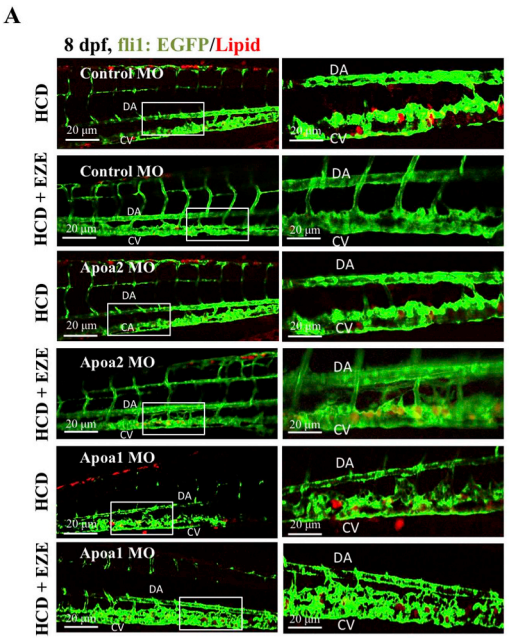


Fig. 4. Ezetimibe increases apo A-II expression in HepG2 cells and enhances cholesterol efflux in human primary macrophages cells.
(A) Human HepG2 cells were treated with different doses of ezetimibe for 48 h. After stimulation, the mRNA of cells was extracted and reverse transcribed to cDNA. The cDNA was subject to quantitative PCR to determine the transcriptional levels of apo A-II. (B) Human HepG2 cells were treated with different doses of ezetimibe for 48 h. After stimulation, proteins were collected from cell lysates and subject to Western blot to determine the expression level of Apo A-II. (C) Statistical analysis of Western blot results from panel B, r.u., relative intensity. (D) Human intestinal epithelial cell line FH was exposed to different doses of cholesterol for 48 h and Apo A-II expression was determined by Western blot. (E) Statistical analysis of Western blot results from panel D, r.u., relative intensity. (F) HepG2 cells were pretreated with *HNF4, PPARα* and *SREBP1* siRNA and exposed to 40 μg/ml ezetimibe for 48 h. Expression of Apo A-II in HepG2 cells of different groups was detected by Western blot. (G) Statistical analysis of Western blot results from panel F, r.u., relative intensity. (H) HepG2 cells were grouped into control, ezetimibe, HNF4 + PPARα siRNA and HNF4 + PPARα siRNA + ezetimibe. The medium was collected and the concentration of Apo A-II determined by ELISA. (I) Stimulate differentiated THP-1 cells with collected medium from panel F, cholesterol efflux from macrophages was calculated and statistically analyzed.



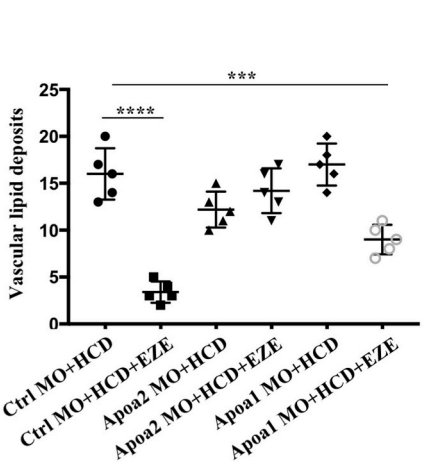


Fig. 5. Knockdown of Apo A-II attenuates ezetimibe prevention of lipid accumulation in zebrafish.
Tg (Fli1: eGFP) or Tg (coroninala: eGFP/fli1: mCherry) embryos were injected with morpholino oligonucleotides (MO) complementary to the translational initiation site of zebrafish *apoa1* and *apoa2* mRNA to knockdown their expression in zebrafish embryos. The MO injected larvae embryos were then divided into groups of control MO larvae fed HCD diet, control MO larvae fed HCD with ezetimibe, apoa2 MO larvae fed HCD and apoa2 MO larvae fed HCD with ezetimibe for 3 days from 5dpf. All HCD were mixed with red fluorophore-labeled cholesteryl ester. (A) Images of caudal vasculature in live *Tg (Fli1: eGFP) larvae* show vascular lipid accumulation (red) and endothelial cells (green). (B) The vascular lipid accumulation from panel (A) was statistically analyzed (n = 5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Wei Wu and Ming Zhao conceived and designed the studies, wrote the manuscript and completed all analyses. Yi Yan and Fei He contribute equally and performed the experiments and data analysis. Zhonghao Li, Ruoting Xu, Ting Li, Jinyu Su and Xianyan Liu contributed to the acquisition of data. All authors approve the final version of the submitted manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.11.016.

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