

RESEARCH ARTICLE

# A Western-Fed Diet Increases Plasma HDL and LDL-Cholesterol Levels in ApoD<sup>-/-</sup> Mice

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## Abstract

**Objective:** Plasma apolipoprotein (apo)D, a ubiquitously expressed protein that binds small hydrophobic ligands, is found mainly on HDL particles. According to studies of human genetics and lipid disorders, plasma apoD levels positively correlate with HDL-cholesterol and apoA1 levels. Thus, we tested the hypothesis that apoD was a regulator of HDL metabolism.

**Methods & Results:** We compared the plasma lipid and lipoprotein profiles of wild-type (WT) C57BL/6 mice with apoD<sup>-/-</sup> mice on a C57BL/6 background after receiving a high fat-high cholesterol diet for 12 weeks. ApoD<sup>-/-</sup> mice had higher HDL-cholesterol levels ( $61 \pm 13$ -apoD<sup>-/-</sup> vs.  $52 \pm 10$ -WT-males;  $37 \pm 11$ -apoD<sup>-/-</sup> vs.  $22 \pm 2$  WT-female) than WT mice with sex-specific changes in total plasma levels of cholesterol and other lipids. Compared to WT, the HDL of apoD<sup>-/-</sup> mice showed an increase in large, lipid-rich HDL particles and according to size various quantities and sizes of LDL particles. Plasma levels of lecithin:cholesterol acyltransferase in the control and apoD<sup>-/-</sup> mice were not different, however, plasma phospholipid transfer protein activity was modestly elevated (+10%) only in male apoD<sup>-/-</sup> mice. An *in vivo* HDL metabolism experiment with isolated Western-fed apoD<sup>-/-</sup> HDL particles showed that female apoD<sup>-/-</sup> mice had a 36% decrease in the fractional catabolic rate of HDL cholesteryl ester. Hepatic SR-BI and LDLR protein levels were significantly decreased; accordingly, LDL-cholesterol and apoB levels were increased in female mice.

**Conclusion:** In the context of a high fat-high cholesterol diet, apoD deficiency in female mice is associated with increases in both plasma HDL and LDL-cholesterol levels, reflecting changes in expression of SR-BI and LDL receptors, which may impact diet-induced atherosclerosis.



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## Introduction

Cardiovascular disease (CVD) is the leading cause of death in Western countries. Plasma low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein (HDL)-C levels correlate positively and negatively with CVD risk respectively [1, 2]. Although statins reduce LDL-C and with it the incidence of CVD, new therapeutic options are needed to raise plasma HDL-C in ways that are atheroprotective. CETP-inhibitors raise circulating plasma HDL-C levels, but to date none have prevented CVD [3]. Thus, the mechanisms by which HDL-C levels are increased are likely to be more relevant to atheroprotection than the actual HDL-C-raising itself [4]. The surface of HDL particles is approximately 85% protein and the HDL proteome is heterogeneous [5]. HDL-proteins occur as specific clusters in HDL subclasses, each exerting distinct biological functions—including regulation of cholesterol efflux, and anti-inflammatory, anti-oxidative, anti-thrombotic, and vasodilatory activities [6, 7]. Consequently, investigating the biological effects of HDL-associated proteins on the function of this lipoprotein is a key to understanding how HDL reduces CVD risk.

Apolipoprotein D (ApoD) is a 29-kDa glycoprotein associated mainly with plasma HDL and to a lesser extent with LDL and very low-density lipoproteins (VLDL) [8, 9]. The impact of apoD on lipid metabolism was partially clarified by recent studies revealing that apoD regulates triglyceride metabolism. Hepatic over-expression of mouse apoD reduced plasma triglyceride levels by increasing lipoprotein lipase (LPL) activity and the catabolism of triglyceride-rich particles [10]. Moreover, ApoD deficiency in mice is associated with reduced adipose tissue-LPL levels and hypertriglyceridemia [11]. Although these observations indicate that apoD regulates triglyceride metabolism, its role in the regulation of HDL, its major carrier, is unknown.

Human apoD levels are reduced in hypocholesterolemic diseases that result from deficiencies in proteins that modulate HDL-C levels, including Tangier disease and familial lecithin-cholesterol acyltransferase (LCAT) deficiency. Both diseases are associated with lower apolipoproteins levels, an effect that was most profound for apoD [12]. In a study of single nucleotide polymorphisms in the human apoD gene of African Blacks, the Phe36Val SNP was associated with increased HDL<sub>3</sub>-C and apoAI concentrations in females [13]. Another study showed a strong positive correlation between apoD and apoAI levels, and HDL lipid content (cholesterol and phospholipids) in healthy male subjects [14]. The association between HDL and apoD may be linked to the biological activities of HDL-associated remodeling enzymes, such as LCAT, phospholipid transfer protein (PLTP), and paroxonases (PON). ApoD complexes with LCAT, and the presence of apoD in proteoliposome particles composed of either apoAI or CI stimulates the esterification activity of LCAT [15]. PLTP, which transfers phospholipids among lipoproteins and modulates HDL particle size, forms a complex composed of six apolipoproteins including apoD [16]. A human proteomics study revealed that apoD is preferentially found in the smaller, denser HDL<sub>3</sub> subpopulation and is modestly positively correlated with the levels of

PON1 and 3, proteins localized to small HDL<sub>3</sub> [6]. The functional relationships between apoD and HDL-associated enzymes, PON and PLTP, are unknown. Collectively, however, these data suggest that apoD may regulate both HDL-C levels and its functionality, although no studies have determined the mechanism by which apoD influences plasma HDL-cholesterol. Since apoD is often up-regulated under stress conditions or disease states, mice were fed an atherogenic Western diet [8, 17, 18]. We tested the hypothesis that apoD affects circulating plasma HDL-cholesterol levels by altering the lipid and protein content of HDL and the catalytic activities of associated enzymes.

## Materials and Methods

### Mice

In-house 6–8 week old-aged matched male and female C57BL-6 and apoD<sup>-/-</sup> mice were fed a Western Diet (Harlan-TD88137) for 12 weeks. Mice had ad-libitum access to water. Animals were weighed and bled monthly after an overnight fast. At time of sacrifice, mice were fasted overnight and anesthetized with a ketamine/xylazine cocktail. Plasma and serum were collected via the retro-orbital plexus. Plasma, sera, and liver were stored at  $-80^{\circ}\text{C}$  until further use. The City College of New York Institutional Animal Care and Use Committee approved all procedures.

### Fast Protein Liquid Chromatography (FPLC)-size exclusion analysis of pooled plasma samples

Pooled plasma ( $\sim 150\ \mu\text{l}$ ) from each genotype ( $n=4-6$ ) was injected onto Superose HR6 10/300 GL FPLC column (GE Healthcare). Lipoproteins were eluted with 24 mL of elution buffer [0.15 M NaCl, 1 mM EDTA, 0.2% w/v of sodium azide in 1X phosphate saline buffer (PBS)] and 0.5 mL fractions were collected at a flow rate of 0.5 mL/min.

### Plasma and Serum lipid analyses

Total cholesterol, free cholesterol, triglycerides, non-esterified fatty acids, and phospholipids levels were measured in plasma or sera and FPLC samples according to manufacturer instructions from Wako Chemicals or Infinity Triglycerides. The total cholesterol levels in FPLC fractions were measured with 2X Cholesterol E buffer. Plasma HDL-cholesterol measurements were determined after precipitation of apoB-containing lipoproteins. Briefly, plasma samples were mixed with equal volumes of dextran sulfate (20 g/L) and magnesium chloride (1 M) solution pH 7.0 and incubated for 10 minutes at room temperature followed by centrifugation for 30 minutes at  $4^{\circ}\text{C}$ . The supernatant was measured for cholesterol with the Cholesterol E kit (Wako).

## SDS-PAGE and Western Blotting

FPLC HDL fractions (25  $\mu$ L), plasma (1  $\mu$ L), or liver extract (25–50  $\mu$ g) were reduced with  $\beta$ -mercaptoethanol/Laemmli buffer, separated on 4–20% mini Protean TGX gels (Bio-Rad), transferred to a PVDF membrane, and probed with primary antibodies for mouse apoAI (Abcam-ab20453), mouse apoD (Santa Cruz-sc34763), SR-BI (Novus Biologicals-NB400-104), LDLR (BioVision-3839-100), and  $\beta$ -actin (Thermo Scientific-MA-91399). Proteins were detected with ECL reagent (Promega) and the pixel intensities of bands were quantified by the Image J program (NIH). For total protein analysis, 10–20  $\mu$ g of total protein was loaded on 4–20% mini TGX gel and gel stained with SimplyBlue SafeStain (Invitrogen) to detect apolipoproteins' levels in pooled FPLC fractions.

## Plasma activities of LCAT and PLTP

Plasma LCAT and PLTP activities were measured with the Roar Biomedicals Kits per vendor instructions.

## Ion mobility of plasma lipoproteins

The lipoprotein concentrations and particle size from pooled plasma (100  $\mu$ L) were determined by gas-phase differential electrophoretic mobility method as previously described [19].

## Liver lipids measurement and relative mRNA expression

Livers (50 mg) from C57BL/6 and apoD<sup>-/-</sup> mice were homogenized in PBS with a bullet blender for 5 minutes at 4°C with zirconium oxide beads (ZrOB05 and ZrOB10-Next Advance). The homogenate was collected and diluted after centrifugation at 10,000 rpm and 4°C. An aliquot of homogenate was solubilized with an equal volume of 0.25% or 1% deoxycholate to measure cholesterol and triglyceride levels, respectively. Samples were incubated for 5 minutes at 37°C. Infinity Cholesterol or Triglycerides liquid stable reagents (ThermoScientific) were added to samples and glycerol & cholesterol standards for 5–15 minutes, and absorbance read at 500 nm. Hepatic lipid levels were normalized to total protein (BCA assay).

Total RNA was isolated from liver (50 mg) with Trizol Reagent (Invitrogen) after homogenization with ZrOB05 beads (Next Advance). cDNA was synthesized with 1  $\mu$ g RNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories). The relative mRNA expression was determined by real-time PCR using Taqman Fast Advanced Mastermix (Invitrogen) with Taqman Gene Expression Assay and calculated by the comparative  $C_T$  method,  $\Delta\Delta C_T$ . The following expression assays were used: apoAI (Apoa1- mm00437568\_g1), apoE (ApoE-Mm01307193\_g1), ATP-binding cassette transporter (Abca1- mm00442646\_m1), LDL-receptor (Ldlr, mm00440169\_m1), liver X receptor- $\alpha$  (Lxr $\alpha$ , mm00443451\_m1), scavenger receptor B1 (Scarb1, mm00450234\_m1), and sterol regulatory element binding

transcription factor 2 (Srebf2, mm01306292\_m1), and beta-2-microglobulin (b2m-mm00437762\_m1).

### HDL turnover studies

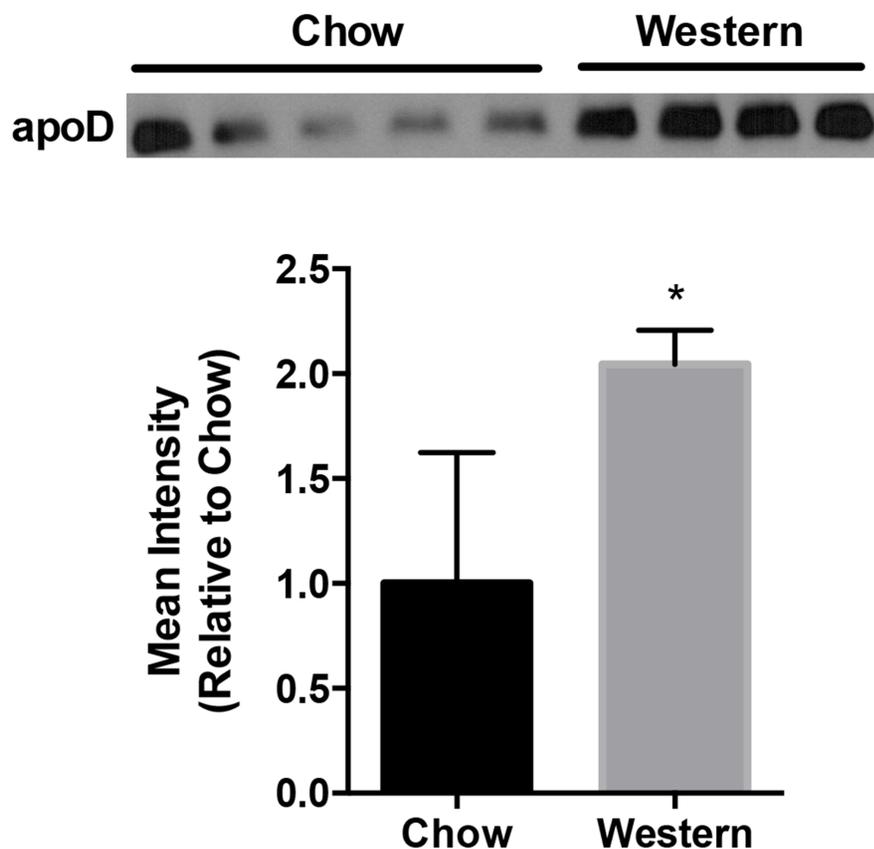
apoD<sup>-/-</sup> HDL was isolated from pooled mouse plasma after 12 weeks on a Western diet by sequential ultracentrifugation (density,  $1.063 < d < 1.21$ ). HDL was labeled with <sup>3</sup>H-1,2,3-cholesteryl oleate (Perkin Elmer) as previously described [20] and 250,000 to 1 million counts per minute of <sup>3</sup>H-CE-HDL was injected into the retro-orbital sinus of Western-fed mice under isofluroane anesthesia. Blood was collected at 5 minutes and 1, 5, 10, 24, and 48 hours, and liver and feces at 48 hours. Plasma decay curves were generated by subtracting the initial 5 min. injection point from each time point. The fractional catabolism was fitted to a biexponential curve using the WinSAAM modeling program, and FCR (per hour) was calculated from the normalized area under the fitted curve [20].

### Statistical analysis

Data are reported as mean  $\pm$  SD and statistical significance was analyzed by a student's t-test using the GraphPad 6.0 software;  $p < 0.05$  was considered significant.

## Results

We investigated the role of apoD on HDL metabolism in mice on a high fat/cholesterol diet. Given that plasma expression of apoD increased 2-fold on a Western diet (WD) compared to chow diet in WT mice (Fig. 1), we analyzed plasma lipids in male and female C57BL/6 and apoD<sup>-/-</sup> mice after 12 weeks on WD diet. Following a WD, plasma HDL-C was significantly higher in both male ( $61 \pm 13$ -apoD<sup>-/-</sup> vs.  $52 \pm 10$ -WT,  $p < 0.001$ ) and female apoD<sup>-/-</sup> mice ( $37 \pm 11$ -apoD<sup>-/-</sup> vs.  $22 \pm 2$ ,  $p < 0.001$ ) compared to WT mice (Table 1). This observation was not seen on a chow diet (data not shown). However, the commonality in HDL-cholesterol levels diverged for other lipid parameters between the sexes in apoD<sup>-/-</sup> mice (Table 1). In female apoD<sup>-/-</sup> mice, a significant increase in total cholesterol ( $90 \pm 10$ -WT vs.  $140 \pm 14$ - apoD<sup>-/-</sup>,  $p < 0.05$ ) was observed due to changes in both HDL and non-HDL cholesterol levels (LDL, VLDL). Both plasma esterified cholesterol (CE) and free cholesterol (FC) were increased with the greatest change in CE (+42%). Finally, there were substantial decreases in both circulating triglyceride and NEFAs in female apoD<sup>-/-</sup> compared to control mice. In contrast, in male apoD<sup>-/-</sup> mice there were no significant differences in other plasma lipid parameters except for a decrease ( $0.6 \pm 0.1$ -apoD<sup>-/-</sup> vs.  $0.7 \pm 0.1$ -WT,  $p < 0.05$ ) in non-esterified fatty acids (NEFAs). Thus, a WD dramatically impacted plasma lipids in female apoD<sup>-/-</sup> mice compared to males. In the context of these clear differences in HDL-C lipids in apoD<sup>-/-</sup> mice on an atherogenic diet, we conducted more



**Fig. 1. Up-regulation of plasma apoD levels in Western-fed WT mice.** Western blot and semi-quantitative analyses of apoD levels in WT mice fed a 12-week chow and Western diets. Data is representative of both sexes, n=4–5 per group, mean ± SD, \*p<0.05.

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detailed analyses of the effects of apoD deficiency on HDL content, size, and proteins.

**Table 1. Lipid parameters in male and female WT and apoD<sup>-/-</sup> mice after 12 weeks on a Western diet.**

| Lipid Parameters  | WT Female (n=7–9) | apoD <sup>-/-</sup> Female (n=7–9) | WT Male (n=9–12) | apoD <sup>-/-</sup> Male (n=9–12) |
|-------------------|-------------------|------------------------------------|------------------|-----------------------------------|
| Total cholesterol | 90 ± 10           | 140 ± 14*                          | 174 ± 30         | 178 ± 34                          |
| HDL -C            | 22 ± 2            | 37 ± 11**                          | 52 ± 10          | 61 ± 13**                         |
| Non-HDL-C         | 65 ± 13           | 103 ± 17**                         | 123 ± 35         | 116 ± 28                          |
| FC                | 34 ± 5            | 44 ± 9*                            | 39 ± 6           | 42 ± 7                            |
| CE                | 54 ± 7            | 92 ± 13****                        | 135 ± 28         | 136 ± 30                          |
| Triglycerides     | 60 ± 10           | 43 ± 7**                           | 81 ± 15          | 84 ± 9                            |
| Phospholipids     | 132 ± 17          | 145 ± 18                           | 170 ± 30         | 160 ± 11                          |
| NEFA (mEq/L)      | 0.6 ± 0.1         | 0.4 ± 0.1*                         | 0.7 ± 0.1        | 0.6 ± 0.1*                        |
| Body weight (g)   | 26 ± 3            | 27 ± 3                             | 30 ± 5           | 34 ± 5                            |

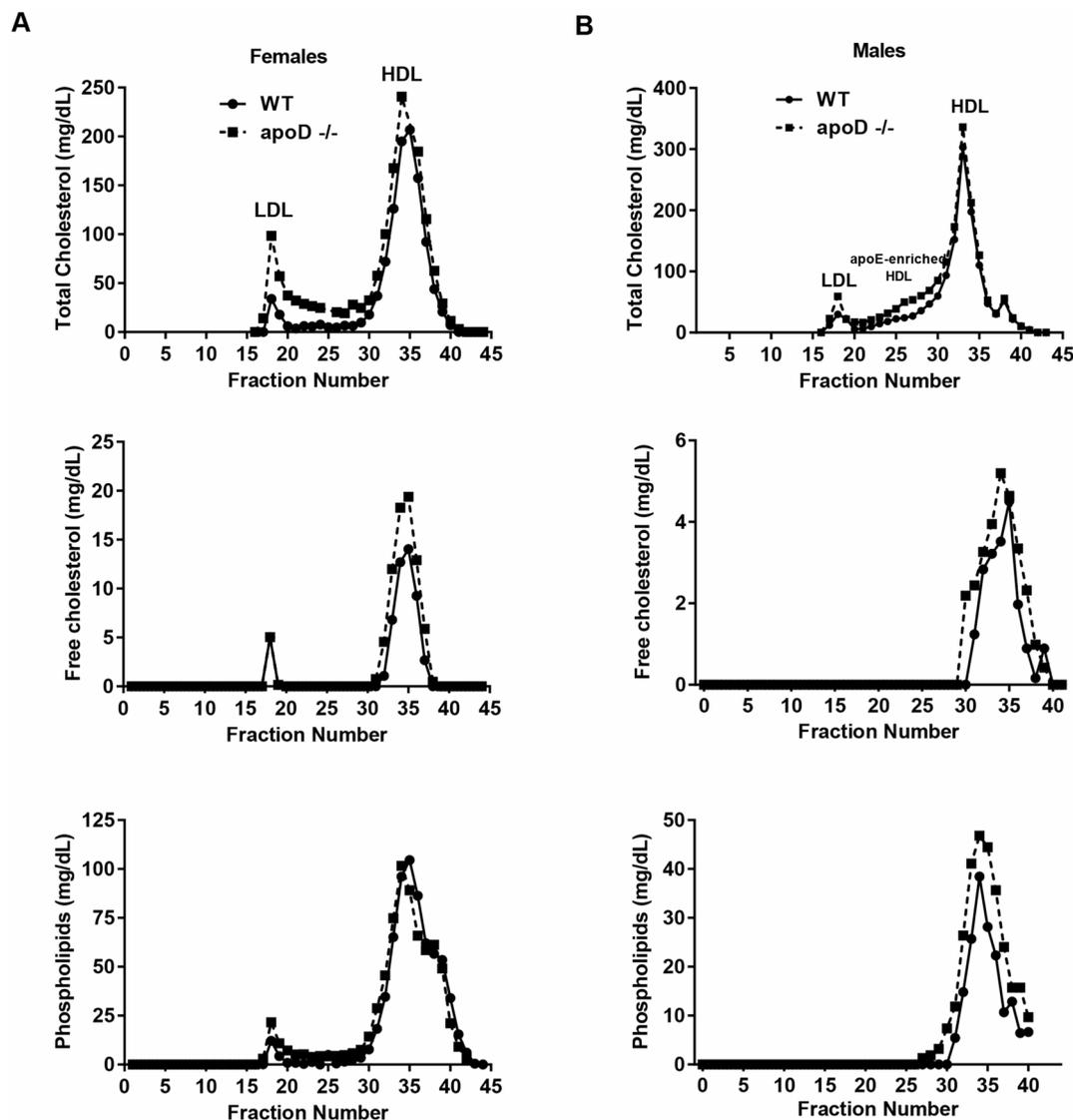
Non-HDL-C and cholesteryl esters (CE) are calculated values. Values represent the mean ± SD, \*p<0.05, \*\*p<0.001, and \*\*\*\*p<0.0001. NEFA, n=4–12. FC= free cholesterol.

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The lipid and apolipoprotein compositions of HDL were determined in fractions isolated by size-exclusion chromatography (SEC). For female mice, apoD deficiency increased total cholesterol in both LDL and HDL fractions as well as FC in HDL particles (Fig. 2A). Semiquantitative apoAI levels from SEC-HDL fractions (31–37) were similar for both female WT and apoD<sup>-/-</sup> mice (Fig. 3A). Also, plasma apoAI levels were unchanged (Fig. 3B). The effect of apoD deficiency on lipoprotein properties in male mice was different from that of females. Size exclusion of pooled plasma, showed that compared to male WT mice, male apoD<sup>-/-</sup> mice had increased apoE-enriched HDL, which was larger as assessed by total cholesterol levels (Fig. 2B). The HDL fractions (30–40) were slightly higher in total cholesterol, free cholesterol, and phospholipids in male apoD<sup>-/-</sup> mice compared to WT (Fig. 2B). Levels of apoAI in the larger lipid-rich HDL fractions (fractions 32–40) were lower in male apoD<sup>-/-</sup> vs. WT mice (Fig. 3A). Moreover, SEC fractions 24–28 from apoD<sup>-/-</sup> mice were more apoE-rich (lane 2) but had reduced apoAI levels (lane 4) than the corresponding fractions from WT mice (lanes 1&3) (Fig. 3C). Analysis of circulating plasma levels of apoAI were not different in male apoD<sup>-/-</sup> compared to WT mice (Fig. 3B). These data suggest that apoD influences both the heterogeneity in protein and lipid content of circulating HDL-C levels with a shift towards larger, lipid rich HDL in a sex-dependent manner.

We next quantified particle sizes and lipoprotein subpopulation concentrations by ion mobility. For both sexes, there was an increase in the concentration of HDL particles of all sizes in apoD<sup>-/-</sup> versus control mice, with the majority of particles in smaller denser HDL<sub>3/2a</sub>. The greatest increase in concentration of HDL was, however, in larger HDL<sub>2b</sub> particles in apoD<sup>-/-</sup> mice (Table 2). The amount of the smaller particles, HDL<sub>3</sub> and HDL<sub>2a</sub> (76.5–105 Å), were increased by 2% in female apoD<sup>-/-</sup> mice, whereas, only a 23% increase was seen in male apoD<sup>-/-</sup> mice compared to WT mice. The larger HDL<sub>2b</sub> particles (105–145 Å) were increased 28% in female mice and 69% in male apoD<sup>-/-</sup> mice. The greater increase of large HDL particles in males correlates well with the increase in apoE-HDL particles by size exclusion column analysis. Interestingly, there was also an increase in LDL particles with sex-dependent changes in the quantity and particle sizes in apoD<sup>-/-</sup> mice (Table 2). LDL particle sizes and concentrations were elevated ~2-fold for male apoD<sup>-/-</sup> mice; however, in females there was only a 15% increase in total LDL particles, with the most profound change in very small atherogenic LDL particles (LDL<sub>4a-b</sub>) compared to WT mice. Overall, most of the circulating lipoproteins were HDL particles in apoD<sup>-/-</sup> mice, which correlate with the observed changes in circulating plasma and by size exclusion chromatography analyses.

To explain the increases in both plasma HDL-C and HDL particle size in apoD<sup>-/-</sup> mice on a Western diet, the activities of HDL-modifying proteins such as LCAT and PLTP were measured. LCAT activity was unchanged in both sexes of apoD<sup>-/-</sup> mice compared to control mice (Fig. 4A). In contrast, there was a significant but small (+10%) increase in PLTP activity in male apoD<sup>-/-</sup> mice,

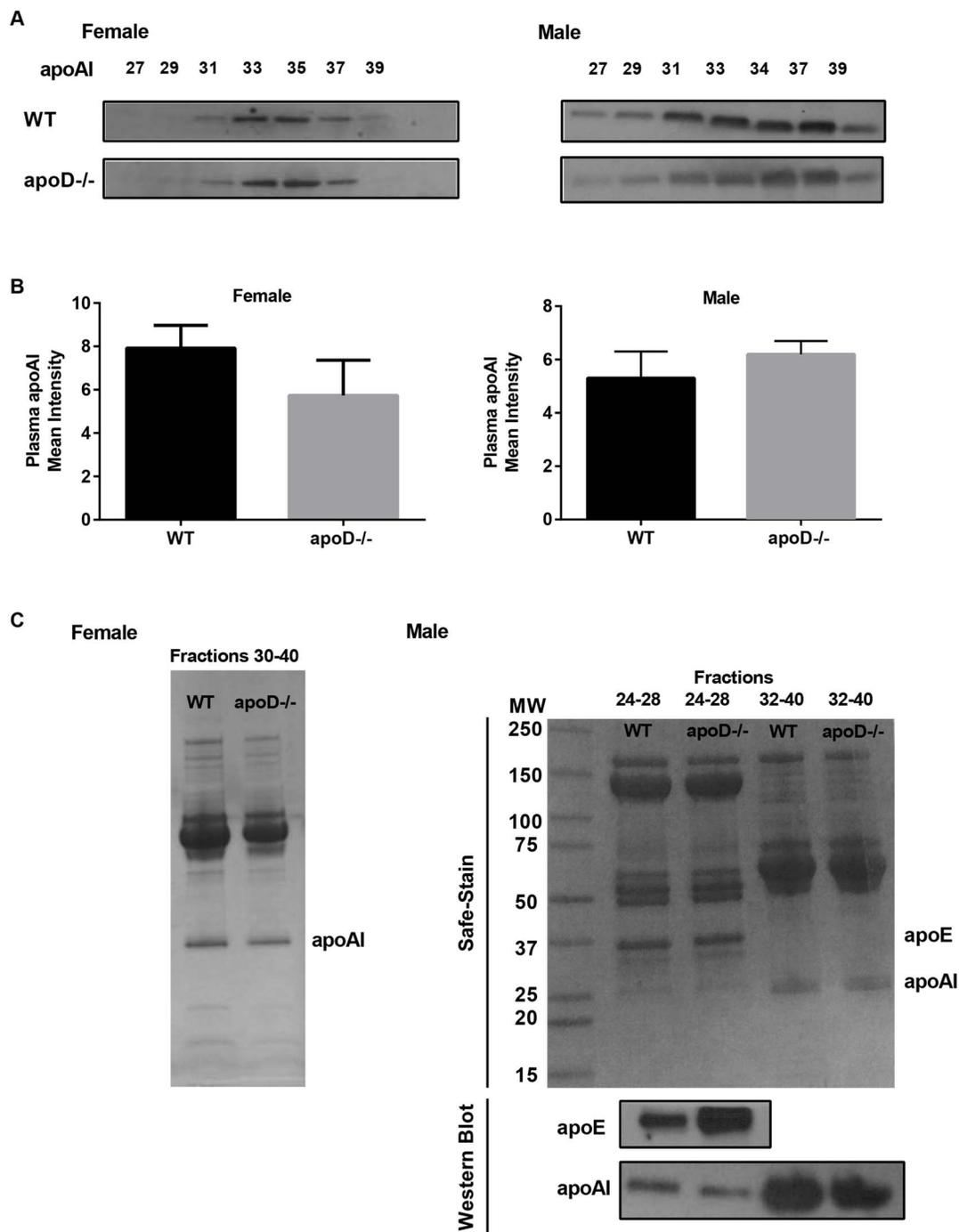


**Fig. 2. ApoE-HDL enrichment and lipids content changes in HDL particles.** Size exclusion chromatography lipid profiles (total cholesterol, free cholesterol, phospholipids) of pooled plasma from A) female and B) male WT and apoD<sup>-/-</sup> mice after 12 weeks on a Western diet.

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with no differences in females (Fig. 4B). The difference in PLTP activity may partially explain the changes in HDL particle sizes in male apoD<sup>-/-</sup> mice.

Hepatic lipid levels and gene and protein expression were assessed to uncover a possible metabolic relationship between apoD and HDL-cholesterol levels. There were no differences found in hepatic cholesterol and triglyceride levels between control and apoD<sup>-/-</sup> mice after the 12-week atherogenic diet (Fig. 5A–B). Moreover, we considered the differential expression of genes involved in HDL biogenesis and remodeling. We found no differences in the relative expression of cholesterol transcription factor genes, sterol-regulatory element binding protein-2 (SREBF-2) and liver X receptor  $\alpha$  (LXR $\alpha$ ) in male mice, but in female apoD<sup>-/-</sup>



**Fig. 3. No change in plasma apoAI, but changes in apolipoproteins content in HDL-size exclusion chromatography fractions.** A) Western blot analysis of apoAI levels from the SEC-HDL fractions. B) Semi-quantitative analysis of plasma apoAI levels. C) SimpleStain and Western blotting for apolipoprotein levels of pooled SEC-HDL fractions (10–20  $\mu$ g).

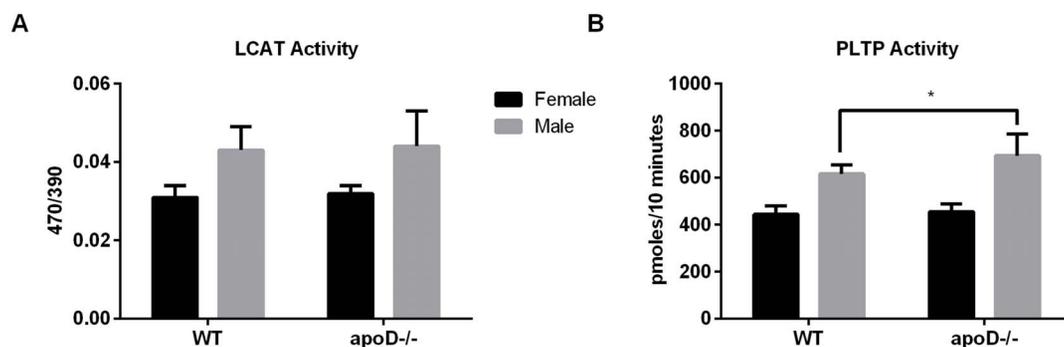
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**Table 2.** Lipoprotein particle size and concentrations (nmol/L) analyzed by IM methodology from pooled plasma of WT and apoD<sup>-/-</sup> mice fed a Western diet for 12 weeks.

| Lipoprotein size fractions | Female |                     | Male  |                     |
|----------------------------|--------|---------------------|-------|---------------------|
|                            | WT     | apoD <sup>-/-</sup> | WT    | apoD <sup>-/-</sup> |
| <b>Total HDL</b>           | 11241  | 12488               | 4668  | 6416                |
| Large 2b                   | 3832   | 4897                | 1474  | 2493                |
| Small 2a+b                 | 7409   | 7591                | 3194  | 3923                |
| <b>Total LDL</b>           | 37.21  | 43.32               | 39.68 | 84.12               |
| Large 1                    | 6.91   | 5.37                | 6.57  | 12.83               |
| Medium 2a                  | 3.12   | 3.56                | 4.25  | 9.57                |
| Small 3a                   | 6.03   | 5.81                | 6.02  | 14.21               |
| Small 3b                   | 3.05   | 4.04                | 3.89  | 9.00                |
| Very small 4a              | 4.83   | 6.99                | 7.24  | 16.78               |
| Very small 4b              | 8.99   | 13.48               | 11.71 | 21.73               |
| <b>Total VLDL</b>          | 7.01   | 6.56                | 20.32 | 20.92               |
| Large                      | 0.54   | 0.66                | 7.89  | 6.62                |
| Medium                     | 2.53   | 2.67                | 7.88  | 8.21                |
| Small                      | 3.94   | 3.23                | 4.55  | 6.09                |

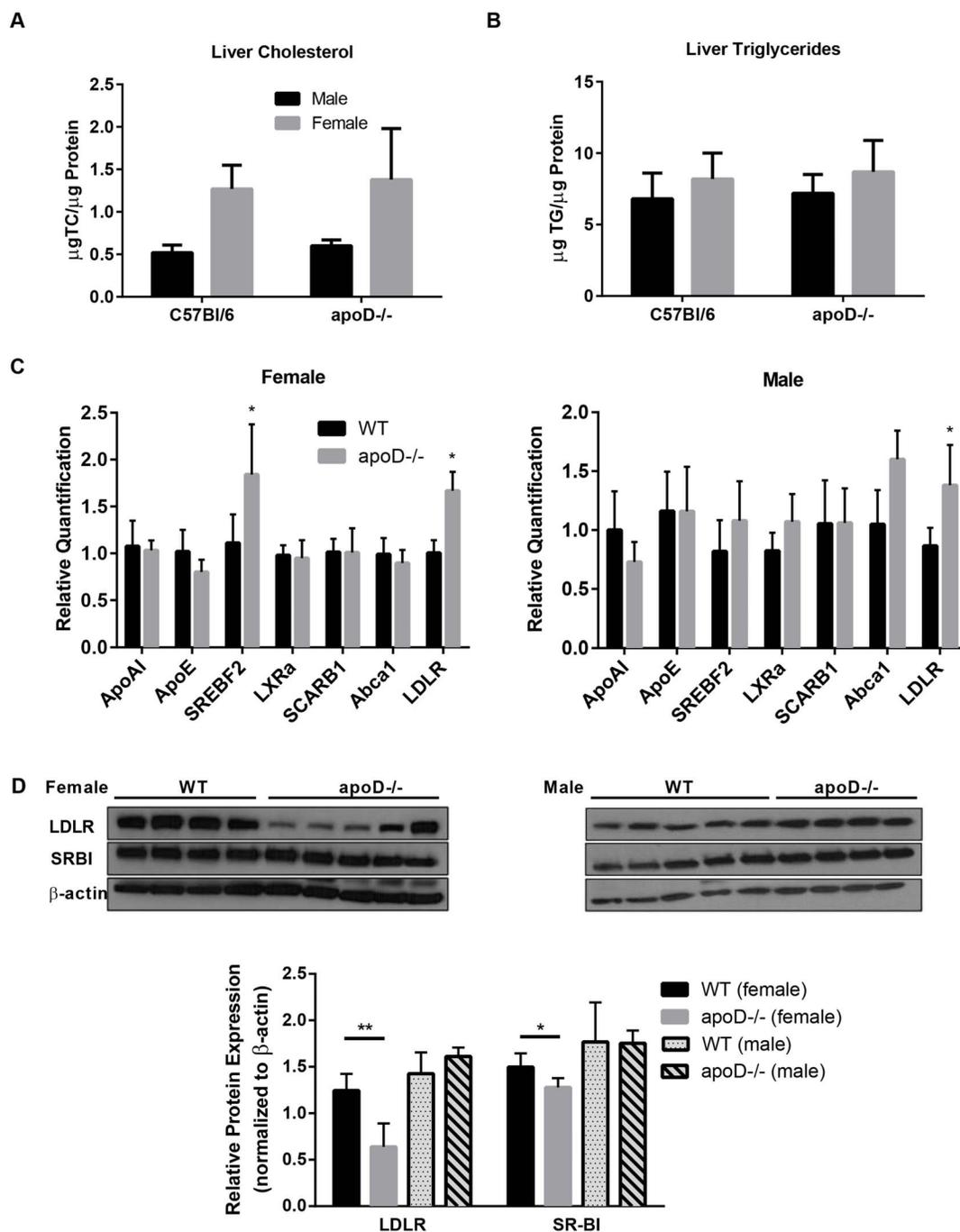
doi:10.1371/journal.pone.0115744.t002

mice there was a 1.5-fold increase in SREBF-2 (Fig. 5C). Furthermore, similar expression levels of apoAI and apoE were found in all mice (Fig. 5C). Finally, genes involved in hepatic HDL catabolism or synthesis, i.e., SCARB1, LDLR, and ABCA1, were analyzed; only a ~1.5- difference in LDLR expression was observed for both sexes in apoD<sup>-/-</sup> mice (Fig. 4C). Analysis of liver protein expression of both LDLR and SR-BI receptors showed the opposite effect than the mRNA expression in female apoD<sup>-/-</sup> mice (Fig. 5D). Both plasma membrane receptors were significantly down-regulated between 15–54% (SR-BI: 1.3 ± 0.1-apoD<sup>-/-</sup> vs. 1.5 ± 0.2-WT, p<0.05; and LDLR: 0.6 ± 0.3-apoD<sup>-/-</sup> vs. 1.3 ± 0.2-WT, p<0.005) in female apoD<sup>-/-</sup> mice. There were no changes in the protein expression of both these receptors in male mice (SR-BI: 1.8 ± 0.1-apoD<sup>-/-</sup> vs.



**Fig. 4. Increased PLTP activity.** A) Plasma LCAT and B) PLTP activities from female and male WT and apoD<sup>-/-</sup> mice on a Western diet for 12 weeks. n=7–14, values are mean ± SD, \*p<0.05.

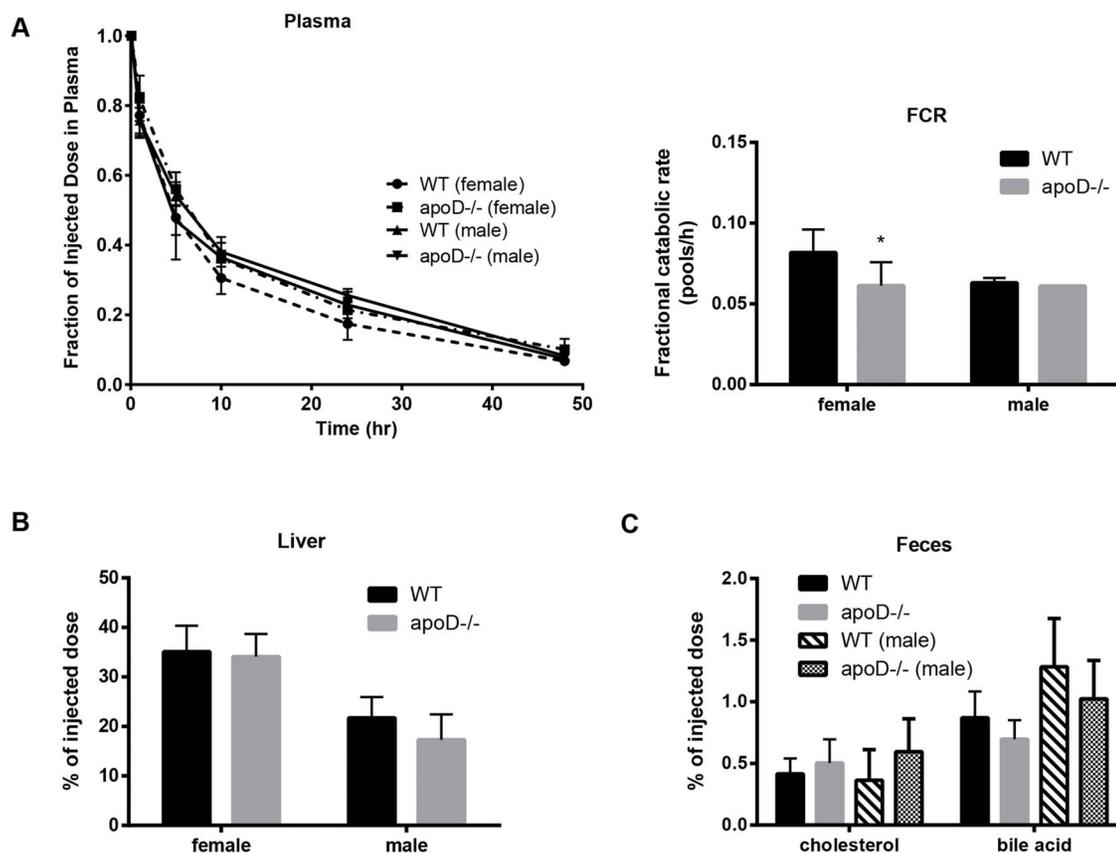
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**Fig. 5. Decrease in the relative protein expression of SRBI and LDLR in female apoD<sup>-/-</sup> mice but opposite pattern for mRNA expression.** Liver levels of A) Cholesterol and B) Triglyceride C) Relative expression of genes and D) Protein expression levels of LDLR and SRBI after 12 weeks on a Western diet. n=5–7 per group, mean ± SD, \*p<0.05, \*\*p<0.01.

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1.8 ± 0.2-WT; and LDLR: 1.6 ± 0.1-apoD<sup>-/-</sup> vs. 1.4 ± 0.1-WT) (Fig. 5D). These findings indicate that the increase in plasma LDL-C and HDL-C in female apoD<sup>-/-</sup> mice could be attributed to a decrease in hepatic receptor protein



**Fig. 6. A decrease in <sup>3</sup>H-CE HDL fractional catabolic rate in female apoD<sup>-/-</sup> mice only.** Mice were fed a Western diet for 12 weeks and <sup>3</sup>H-cholesteryl oleate-labeled apoD<sup>-/-</sup>-HDL was injected. A) Changes in plasma <sup>3</sup>H-CE-HDL and calculated fractional catabolic rate (FCR), B) <sup>3</sup>H-cholesterol (% of injected dose) in the liver, C) <sup>3</sup>H-sterol and bile acids (% of injected dose) in feces. The data represent n=4–6 per group, mean ± SD, \* indicates statistical significant, p<0.05.

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expression, and that other HDL-associated proteins may have led to the accumulation of apoE-enriched HDL in the plasma of male apoD<sup>-/-</sup> mice.

To determine the metabolic basis for an increase in plasma HDL levels, we investigated the catabolism of apoD<sup>-/-</sup> <sup>3</sup>H-CE-HDL in mice. No substantial differences in HDL turnover or sterol tissue levels were observed in male mice (Fig. 6). In contrast, there was a 36% decrease in the HDL-CE FCR in female apoD<sup>-/-</sup> mice in comparison to WT mice (Fig. 6A, 0.082 ± 0.014 vs. 0.061 ± 0.014 pools/h, p<0.05). However, there were no differences observed in hepatic <sup>3</sup>H-cholesterol counts or fecal extractions of <sup>3</sup>H-cholesterol or bile acids in both sexes (Fig. 6B–C). Thus, down-regulation of the HDL receptor or the protein content of apoD<sup>-/-</sup>-HDL particles may explain the differences in HDL catabolism and the increase in plasma HDL-levels in female apoD<sup>-/-</sup> mice.

## Discussion

This study investigated apoD as a candidate regulator of plasma HDL-C metabolism. ApoD occurs predominantly on HDL and several studies suggest that plasma apoD levels are associated with those of HDL or apoAI levels [12, 13, 14]. This is the first study to provide evidence that apoD deficiency modulates plasma HDL-C and LDL-C levels. We observed a profound increase in circulating plasma HDL-cholesterol levels and particle size in the apoD<sup>-/-</sup> mice on an atherogenic diet. These observations were confirmed by size-exclusion chromatography, as well as lipoprotein-lipid and -protein analyses. PLTP but not LCAT activity was modestly changed by apoD deficiency in male apoD<sup>-/-</sup> mice only. Despite being <5% of HDL-protein in WT mice, apoD deficiency altered the distribution of the major HDL protein and HDL particle size, which were associated with higher circulating plasma HDL-C levels [21]. More importantly, in female apoD<sup>-/-</sup> mice there was substantial decrease in plasma <sup>3</sup>H-CE-HDL metabolism, which coincided with a decrease in the expression of the hepatic HDL receptor, SR-BI. These data suggest that reduction of plasma apoD levels plays a role in plasma cholesterol homeostasis.

The reduction in SR-BI protein levels may underlie the reduced FCR for HDL cholesteryl esters and thus cause elevated HDL-C levels in female vs. male apoD<sup>-/-</sup> mice. SR-BI is the key regulator of HDL-C plasma in the liver, and it mediates the selective uptake of CE from circulating HDL, leading to a reduction in HDL particle size [22]. Hepatic SR-BI deficient or SR-BI knockout mice have significantly elevated plasma HDL-C levels while overexpression of SR-BI has the opposite effect [23–27]. Furthermore, the plasma HDL phenotype in female apoD<sup>-/-</sup> mice resembles that of SR-BI<sup>-/-</sup> mice with increased plasma HDL-cholesterol and particle size with no changes in plasma apoAI levels [23]. ApoD may be directly involved in regulation of SR-BI levels as SR-BI<sup>-/-</sup>: apoE<sup>-/-</sup> double knockout mice, a mouse model of coronary heart disease (CHD), had significantly higher apoD mRNA expression levels [28]. Our data show that apoD deficiency produces modest, but significant decreases in hepatic SR-BI levels in female apoD<sup>-/-</sup> mice, which could be one mechanism for a lower HDL-CE turnover rate and higher HDL plasma levels.

ApoD deficiency also modifies plasma cholesterol homeostasis via the regulation of LDL-cholesterol. In humans, apoD occurs mainly on HDL; only 5% of apoD occurs in human LDL and VLDL particles [29]. Quantitative trait loci analysis suggested that chromosome 3, specifically the apoD locus, may influence the plasma concentration of small, dense LDL-cholesterol [30]. A possible mechanism for the observed changes in LDLR expression and plasma LDL-C levels is post-transcriptional regulation since the mRNA levels of the LDLR gene were increased, but protein expression was decreased. The LDLR is regulated on a post-translational level, particularly by proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of the low-density lipoprotein receptor (IDOL). PCSK9 affects LDLR stability and trafficking by promoting its degradation by lysosomes, whereas IDOL ubiquitinates LDLR for proteasomal

degradation [31–33]. Additional studies are needed to investigate hepatic Srebps, PCSK9, or IDOL pathways to explain the increase in LDL-C levels.

The mechanistic regulation of plasma HDL-C in male apoD<sup>-/-</sup> mice is unclear. Male apoD<sup>-/-</sup> mice showed no difference in CE-HDL catabolism, despite an increase in PLTP activity and apoE-enriched HDL particles. The increased PLTP activity in the male mice could be involved in the appearance of a minor population of spherical apoE-enriched HDL particles. Future studies will be required to determine the mechanism for changes in HDL-C levels in male apoD<sup>-/-</sup> mice.

## Conclusions

This study demonstrated that apoD may directly or indirectly modulate the levels, size, and apolipoprotein content of HDL in a sex-dependent manner. Thus, our new findings suggest that in female mice the absence of apoD may promote a pro-atherogenic phenotype via its regulation of circulating HDL-C concentrations, reflecting SR-BI deficiency, and the increase in small-dense LDL-C levels and particles. These data may support the notion that expression of apoD is protective under different stress conditions and especially in different animal models of CHD such as ischemia/reperfusion-induced myocardial infarction or diet-induced atherosclerosis [25].

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## Author Contributions

Conceived and designed the experiments: KA. Performed the experiments: EMAA SN US BR LL SMMT KA MDK. Analyzed the data: EMAA US BR SMMT KA. Wrote the paper: KA.

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