



# Hepatocyte estrogen receptor alpha mediates estrogen action to promote reverse cholesterol transport during Western-type diet feeding

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## ABSTRACT

**Objective:** Hepatocyte deletion of estrogen receptor alpha (LKO-ER $\alpha$ ) worsens fatty liver, dyslipidemia, and insulin resistance in high-fat diet fed female mice. However, whether or not hepatocyte ER $\alpha$  regulates reverse cholesterol transport (RCT) in mice has not yet been reported.

**Methods and results:** Using LKO-ER $\alpha$  mice and wild-type (WT) littermates fed a Western-type diet, we found that deletion of hepatocyte ER $\alpha$  impaired *in vivo* RCT measured by the removal of <sup>3</sup>H-cholesterol from macrophages to the liver, and subsequently to feces, in female mice but not in male mice. Deletion of hepatocyte ER $\alpha$  decreased the capacity of isolated HDL to efflux cholesterol from macrophages and reduced the ability of isolated hepatocytes to accept cholesterol from HDL *ex vivo* in both sexes. However, only in female mice, LKO-ER $\alpha$  increased serum cholesterol levels and increased HDL particle sizes. Deletion of hepatocyte ER $\alpha$  increased adiposity and worsened insulin resistance to a greater degree in female than male mice. All of the changes lead to a 5.6-fold increase in the size of early atherosclerotic lesions in female LKO-ER $\alpha$  mice compared to WT controls.

**Conclusions:** Estrogen signaling through hepatocyte ER $\alpha$  plays an important role in RCT and is protective against lipid retention in the artery wall during early stages of atherosclerosis in female mice fed a Western-type diet.

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**Keywords** Estrogen; Insulin resistance; Cholesterol/metabolism; Sex differences; Reverse cholesterol transport

## 1. INTRODUCTION

Obese, premenopausal women are more insulin sensitive than body mass index-matched men [1,2], and have lower risk of coronary heart disease (CHD) with a less atherogenic plasma lipid profile [3,4]. Proatherogenic dyslipidemia with obesity is characterized by high levels of LDL-cholesterol and triglycerides and low levels of HDL-cholesterol. Insulin resistance promotes atherosclerosis by worsening dyslipidemia and other metabolic abnormalities [5]. Women have higher VLDL and LDL production rates but lower VLDL and LDL cholesterol levels than men due to enhanced plasma apoB-particle clearance in women that offsets VLDL and LDL production rates [6]. In addition, women have a greater apoA1 synthesis rate associated with higher HDL cholesterol levels [6,7].

One major atheroprotective mechanism of HDL is its ability to promote cholesterol efflux from foam cells and prevent lipid accumulation in the artery wall. To maintain efficient HDL-mediated cellular cholesterol efflux from foam cells, cholesterol and cholesteryl esters in HDL

particles are either removed by the liver through the scavenger receptor class B member I (SR-BI) pathway [8] or transferred *via* cholesteryl ester transfer protein (CETP) to apoB-particles in the blood and subsequently cleared through the LDL receptor (LDLR) or remnant receptor pathways [9]. The process of cholesterol efflux from foam cells to liver for removal from the body is referred to as reverse cholesterol transport (RCT). Impaired regulation of any step in RCT is correlated to CHD risk [9].

After menopause, CHD risk in women approaches that of men of the same age, suggesting that sex differences in CHD risk are in part influenced by estrogen signaling pathways [3,4]. The protective effects of estrogen signaling against insulin resistance are distributed across tissues including the central nervous system, macrophages/immune-system, adipose tissues, skeletal muscles, and the liver [2,6,10–12]. However, studies of the tissue-selective actions of estrogen on lipoprotein metabolism and its association to atherosclerosis using mouse models have been limited. Using apoE-null mice, endogenous ovarian hormones suppress atherosclerosis progression with Western-

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**Abbreviations:** ER $\alpha$ , estrogen receptor alpha; LKO-ER $\alpha$ , deletion of hepatocyte estrogen receptor alpha; CHD, coronary heart disease; SR-BI, scavenger receptor class B member I; PDZK1, PDZ domain containing 1; CETP, cholesteryl ester transfer protein; LDLR, LDL receptor; RCT, reverse cholesterol transport; TG, triglyceride; DG, diacylglycerol; WD, Western-type diet; WT, wild type; PLTP, phospholipid transfer protein; SAA1, serum amyloid A1; PON1, paraoxonase 1; FPLC, fast performance liquid chromatography

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type diet in sham-operated mice compared with ovariectomized mice, without changes in plasma cholesterol levels [13]. A similar finding was reported in another study using LDLR-null mice [14]. Although hormone replacement with exogenous estradiol reduced plasma lipids and atherosclerotic burden after ovariectomy, association between lesion area and plasma lipids was weak in studies using apoE- or LDLR-null mice [13,14]. These studies suggest that new mouse models are required to study estrogen signaling pathways in the regulation of lipoprotein metabolism and their potential contribution to the protective effects conferred by being female with regard to atherosclerosis development.

We previously reported that ovariectomy in female mice causes fatty liver by increasing lipogenesis and insulin resistance as determined by hyperinsulinemic-euglycemic clamp in high-fat diet fed mice [2]. Estradiol treatment corrected hepatic steatosis and insulin resistance during high-fat diet feeding in a manner that required hepatocyte ER $\alpha$  [2]. In this study, to investigate whether hepatic estrogen signaling regulates RCT and is protective against early stages of atherosclerosis, we fed female and male hepatocyte estrogen receptor  $\alpha$  knockout (LKO-ER $\alpha$ ) mice on C57BL/6J background a Western-type (WD) diet. Deletion of hepatocyte ER $\alpha$  increased insulin resistance, impaired HDL capacity for cholesterol transport from foam cells and hepatocyte HDL uptake *ex vivo* for both sexes. However, deletion of hepatocyte ER $\alpha$  decreased RCT *in vivo* accompanied with increases in early atherosclerotic lesion size at the aortic root sinus only in female mice. Thus, we show that hepatocyte ER $\alpha$  plays an important role in RCT to protect against lipid retention in the artery wall at early stages of atherosclerosis during WD feeding in female mice.

## 2. MATERIALS AND METHODS

### 2.1. *In vivo* reverse cholesterol transport (RCT) assay

LKO-ER $\alpha$  mice on a C57BL/6J background were made by breeding ER $\alpha$  flox/flox mice with expressing cre recombinase under the control of albumin promoter as described before [2,15]. A cohort of LKO-ER $\alpha$  mice and their wild type (WT) littermates (n = 8) were used for *in vivo* reverse cholesterol transport analysis, which was modified from the method of Tanigawa et al. [16]. To keep the body-weight similar between mice, 8-week old mice were fed a Western-type diet (WD, from Harlan, TD 88137) for a short-term (5 days) before the assay. 5 days of WD did not increase adiposity either make body composition different between LKO and WT mice in both sexes (Table 1). Peritoneal macrophages from WT female mice were collected 72 h after intraperitoneal injection of thioglycollate. Macrophages were first cultured in DMEM with 10% FBS for 2 h to allow for plate surface attachment and then cultured in DMEM with 0.2% BSA overnight. The next day, macrophages were loaded with 3  $\mu$ Ci/ml <sup>3</sup>H-cholesterol and 25  $\mu$ g/ml of acetylated LDL for 48 h. These labeled foam cells were washed twice, equilibrated in medium with 0.2% BSA for 6 h, centrifuged, and re-suspended in phenol red-free RPMI medium immediately before use. Then mice received intraperitoneal injections of the same amount of <sup>3</sup>H-cholesterol loaded foam cells (5  $\times$  10<sup>6</sup> cells/mouse and 1.1  $\times$  10<sup>6</sup> CPM/mouse) and were caged individually and fed WD ad libitum.

After 48 h, mice were euthanized and blood was collected by cardiac puncture, gallbladder was separated, and the liver was removed and frozen-flash for lipid extraction. Feces were collected continuously over 48 h prior to euthanizing.

Serum and liver lipids were extracted as described before [2]. The lipid layer was collected, evaporated, and re-suspended for scintillation counting. Gallbladders were lysed in 0.1 N NaOH for 4 h, and

**Table 1** — Sex dimorphism and genotype differences in physiology related to lipid metabolism.

	Female		Male	
	WT	LKO-ER $\alpha$	WT	LKO-ER $\alpha$
1st cohort_before WD				
Body weight (g)	18.2 $\pm$ 1.7	18.3 $\pm$ 1.6	24.2 $\pm$ 1.1 <sup>^</sup>	23.6 $\pm$ 2.3
Adiposity (%)	17.0 $\pm$ 1.7	16.3 $\pm$ 1.6	13.8 $\pm$ 1.8 <sup>^</sup>	13.2 $\pm$ 1.2
1st cohort_5-day WD				
Body weight (g)	18.1 $\pm$ 1.3	18.5 $\pm$ 1.2	24.3 $\pm$ 1.1 <sup>^</sup>	23.2 $\pm$ 1.4
Adiposity (%)	17.1 $\pm$ 2.8	16.9 $\pm$ 1.3	13.4 $\pm$ 2.0 <sup>^</sup>	12.4 $\pm$ 1.6
2nd cohort_7-month WD				
Body weight (g)	24.1 $\pm$ 2.2	26.3 $\pm$ 2.8	34.7 $\pm$ 3.9 <sup>^</sup>	34.6 $\pm$ 2.4
Adiposity (%)	19.3 $\pm$ 3.6	29.7 $\pm$ 2.1 <sup>**</sup>	23.6 $\pm$ 2.6 <sup>^</sup>	25.4 $\pm$ 2.5
Fasting insulin (ng/ml)	0.3 $\pm$ 0.1	0.7 $\pm$ 0.2 <sup>*</sup>	1.5 $\pm$ 0.7 <sup>^</sup>	2.1 $\pm$ 0.9 <sup>*</sup>
Fasting glucose (mg/dl)	142 $\pm$ 14	158 $\pm$ 20	169 $\pm$ 28 <sup>^</sup>	161 $\pm$ 25
Fasting cholesterol (mg/dl)	178 $\pm$ 11	214 $\pm$ 16 <sup>*</sup>	223 $\pm$ 7	222 $\pm$ 12
Fasting TGs (mg/dl)	70 $\pm$ 2	81 $\pm$ 7	78 $\pm$ 22	95 $\pm$ 31
Liver TGs ( $\mu$ g/mg)	79.5 $\pm$ 18.5	81.5 $\pm$ 11.7	103.9 $\pm$ 14.4	93.8 $\pm$ 22.9
Liver DG ( $\mu$ g/mg)	0.31 $\pm$ 0.06	0.36 $\pm$ 0.15	0.49 $\pm$ 0.23	0.47 $\pm$ 0.34
Liver cholesterol ( $\mu$ g/mg)	5.87 $\pm$ 1.06	4.39 $\pm$ 0.77 <sup>*</sup>	4.75 $\pm$ 1.16 <sup>^</sup>	4.54 $\pm$ 0.66
Liver phospholipids ( $\mu$ g/mg)	16.7 $\pm$ 5.7	12.6 $\pm$ 2.5 <sup>*</sup>	16.1 $\pm$ 4.3	13.8 $\pm$ 3.76 <sup>*</sup>

Data shown are mean  $\pm$  SD. Statistical analysis was performed by RM 2-Way ANOVA with multiple comparison test.  
<sup>\*</sup>P < 0.05; <sup>\*\*</sup>P < 0.01 in comparison to respective WT controls.  
<sup>^</sup>P < 0.05, WT male mice in comparison to WT female mice.

radioactivity was measured by scintillation counting. Feces of each mouse were soaked in 0.1 N NaOH overnight at 4  $^{\circ}$ C, then were homogenized the next day. One milliliter of the homogenized samples was subjected to scintillation counting. Protocols for all animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

### 2.2. Liver and serum lipid analysis

To further study the mechanism and early atherosclerosis, male and female LKO-ER $\alpha$  mice and their WT littermates on a C57BL/6J background (10  $\pm$  1 weeks, n  $\geq$  19 for each group) were fed a WD for 7 months. Then mice were sacrificed after a 5-hour fasting. Liver and serum were stored at  $-80^{\circ}$  C for future analysis.

Liver lipids were analyzed by the Lipid Core Laboratory of the Vanderbilt Mouse Metabolic Phenotyping Center. VLDL, LDL, and HDL were separated from serum using fast performance liquid chromatography (FPLC). Pooled serum from 2 or 3 mice were used for each run of FPLC. Cholesterol in serum and FPLC fractions were determined by enzymatic colorimetric assays (Cholesterol Reagent and Triglycerides GPO Reagent kits from *Infinity*).

### 2.3. Western blots

For liver proteins, frozen livers were lysed in T-PER tissue protein extraction reagent (*Thermo Scientific*) containing protease/phosphatase inhibitors (*Sigma*), and the protein concentrations were determined using BCA kit (*Thermo Scientific*). For serum proteins, 2  $\mu$ l of serum or 10  $\mu$ l of FPLC fractions (fractions of 25–30 for [Supplemental Figure 2](#)) were pooled for each sample and denatured in loading buffer (*Invitrogen*) containing reducing buffer (*Invitrogen*) and phospholipase and protease inhibitors (*Sigma*). Serum proteins were separated with gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibody (1:1000) at 4  $^{\circ}$ C overnight, and with 2nd antibody (1:10,000) at room temperature for 1 h. Rabbit anti-mouse apoB antibody was from *Lifespan Biosciences* (LS-C20729); rabbit monoclonal anti-LDL receptor antibody was from *abcam* (ab52818); rabbit anti-mouse apoA1 antibody was from

*Biodesign* (K23500R); rabbit anti-SR-BI antibody was from *Novus* (NB400-104); sheep polyclonal anti-PDZK1 antibody was from *R&D* (AF4997); rabbit anti-mouse PON1 was from *Sigma*; mouse anti-actin beta was from *Bio-Rad* (MCA5775GA); goat anti-SAA1 was from *R&D* (AF-2948); rabbit polyclonal anti-PLTP was from *Santa Cruz* (sc-32901); rabbit to mouse apoE antibody was from *abcam* (ab150032); rabbit polyclonal to adiponectin was from *abcam* (ab3455). IRDye 800CW goat anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-sheep IgG were from *LI-COR Biosciences*.

#### 2.4. *In vitro* cellular cholesterol efflux assay

The *in vitro* cellular cholesterol efflux assay was performed according to Asztalos et al. [17]. For preparation of HDL, 40  $\mu$ l of 20% PEG600 in 200 mM glycine buffer was added in 100  $\mu$ l of each serum sample, mixed, and incubated at room temperature for 20 min, then centrifuged at 10000 rpm for 30 min at 4 °C. For cells, peritoneal macrophages from female WT mice were collected 72 h after intraperitoneal injection of thioglycollate. Cholesterol labeled foam cells were prepared as above. These labeled foam cells were washed twice, equilibrated in medium with 0.2% BSA for 2 h, and a set of cells were collected for baseline <sup>3</sup>H-radioactivity. The remaining cells were treated with 1% of initial serum level from WT or LKO-ER $\alpha$  mice in DMEM with 0.2% BSA, cells treated with DMEM with 0.2% BSA were used as negative control and cells treated with apoA1 (50  $\mu$ g/ml, Meridian) as positive control. <sup>3</sup>H-radioactivities in media were determined using liquid scintillation counting and were used for cholesterol efflux calculation.

#### 2.5. *Ex vivo* hepatocyte HDL and LDL uptake assays

Primary hepatocytes from LKO-ER $\alpha$  and WT mice were isolated and cultured according to Foretz et al. [18]. Cells were plated in a 96-well plate at the density of  $4 \times 10^4$  cells/well. HDL-cholesterol uptake assay was performed using HDL Uptake Assay Kit (Fluorometric) (*abcam*, Cat#: ab204717), and LDL-cholesterol uptake assay was performed using LDL Uptake Assay Kit (Fluorometric) (*abcam*, Cat#: ab204716). Assays were performed according to the manufacturer's protocols. Briefly, primary hepatocytes were incubated in the assay media containing fluorescently-labeled HDL or fluorescently-labeled LDL for 3 h, then were washed, and the fluorescence was measured in a microplate reader at Ex/Em = 540/575 nm. Unlabeled-HDL or -LDL included in the kits is used for assay validation. The cell uptake was normalized by total protein amounts of each well.

#### 2.6. Atherosclerosis analysis

While sacrificing mice that were fed a WD for 7 months (see 2.2.), hearts and aortas were embedded in optimal cutting temperature (OCT) compound and snap-frozen for further analysis. Frozen sections of 10  $\mu$ m thickness were stained with oil-red-O, and serial 5  $\mu$ m cryosections of the aortic sinus area were used for immunostaining as previously described [19]. For immunostaining, sections were fixed in cold acetone for 10 min, then washed with PBS for 2 times, blocked in background buster (*Innovex*) at 37 °C for 1 h, then incubated with rat anti-mouse CD106 (*Millipore*) for identifying VCAM-1 at 4 °C for overnight. The next morning, sections were washed with PBS for 3 times, then incubated with Alexa Fluor 488 goat-anti rat IgG (*Invitrogen*) and at 37 °C for 1 h. Slides were washed, and cell nuclei were counterstained with Hoechst (*Vector Labs*, Burlingame CA). Images were captured using Olympus IX81 microscope. Quantification of images was performed as described before [19]. For quantification of VCAM-1 expression in the lesion, multi-channel images were split first and the density (green component only) of VCAM-1 was measured using Image J software. Blind analysis was performed for all images.

At least two sections from each mouse were analyzed and all the experimental mice ( $n \geq 19$ ) were used for analysis.

#### 2.7. Statistical analysis

All measurements passed D'Agostino & Pearson omnibus normality test ( $\alpha = 0.05$ ). For images, data are collected from two sections for each mouse, and mice that were fed a WD for 7 months were all included. Data shown are mean  $\pm$  SD. Statistical analysis was performed with Student *t* test or RM (repeated measures by both factors) 2-way ANOVA with Bonferroni's multiple comparison test.

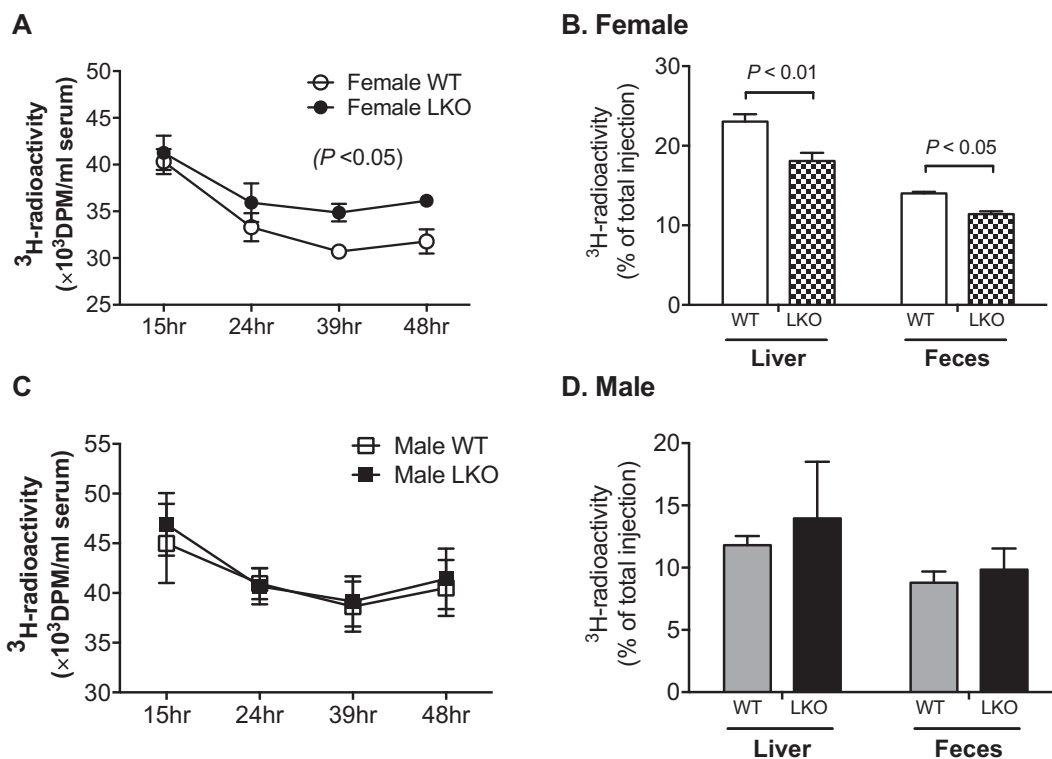
### 3. RESULTS

#### 3.1. Reverse cholesterol transport is impaired by the loss of hepatocyte ER $\alpha$ in female mice

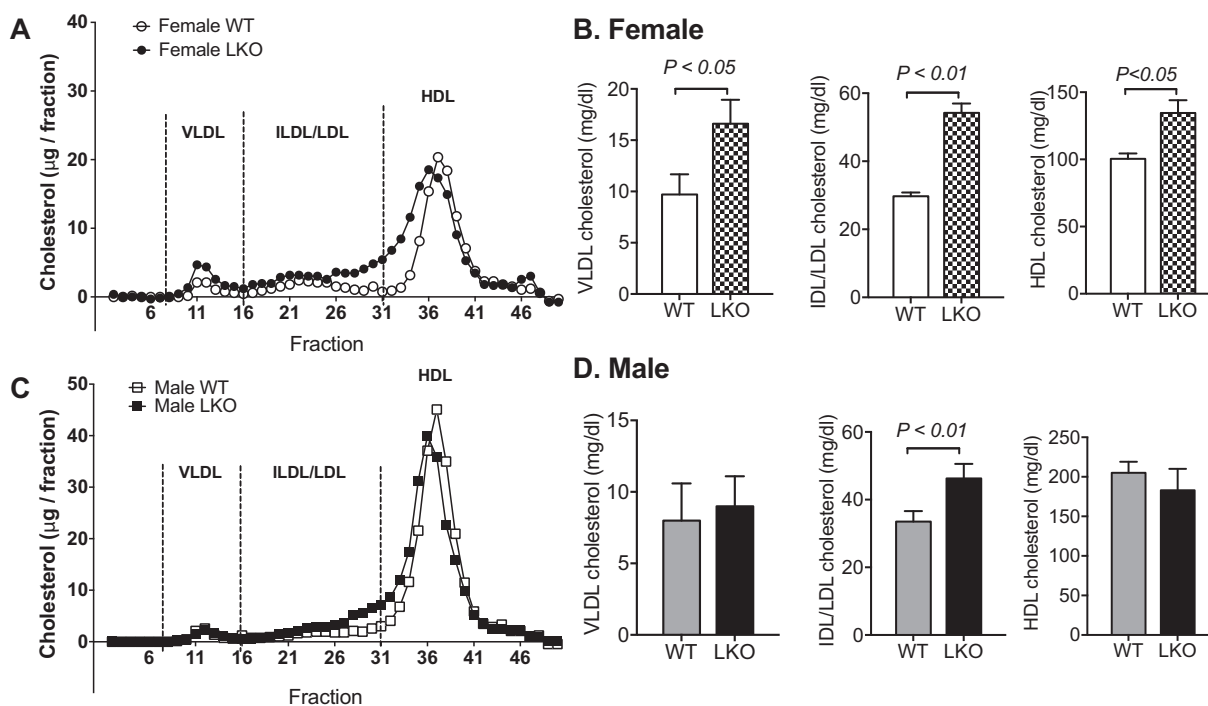
To determine whether hepatocyte ER $\alpha$  regulates whole-body cholesterol handling, we performed an *in vivo* reverse cholesterol transport study. LKO-ER $\alpha$  mice and their WT littermates were fed a WD for a short-term (5 days) and then were injected with <sup>3</sup>H-cholesterol loaded foam cells according to the methods of Tanigawa et al. [16]. In female WT mice, serum <sup>3</sup>H-radioactivity decreased by 18% from 15 h to 24 h (from  $40.3 \pm 4.8$  to  $33.3 \pm 5.4 \times 10^3$  dpm/ml, Figure 1A), further decreased by another 8% till 39 h, then leveled off. Serum <sup>3</sup>H-radioactivity decreased by 14% in female LKO-ER $\alpha$  mice (from  $41.3 \pm 5.8$  to  $35.9 \pm 6.6 \times 10^3$  dpm/ml, Figure 1A) and did not further decrease at later time points with a disappearance rate slower than their WT controls (Figure 1A). Deletion of hepatocyte ER $\alpha$  did not change the rate of serum <sup>3</sup>H-radioactivity disappearance in male mice (Figure 1C). Deletion of hepatocyte ER $\alpha$  reduced <sup>3</sup>H-cholesterol uptake by the liver by 22% in female mice ( $P < 0.01$ ) but did not alter <sup>3</sup>H-cholesterol liver uptake in male mice (Figure 1, B and D). Consistently, whole body <sup>3</sup>H-cholesterol removal into feces decreased by 18% in female LKO-ER $\alpha$  mice ( $P < 0.05$ ), but did not alter in male mice (Figure 1, B and D). There were no differences in body weight or adiposity between LKO-ER $\alpha$  mice and their WT littermates at this duration of feeding (Table 1). These results suggest that cholesterol clearance is decreased with the loss of hepatocyte ER $\alpha$  in female mice but not in male mice.

#### 3.2. Deletion of hepatocyte ER $\alpha$ caused the enlargement of HDL particles, an effect more pronounced in female mice

To better understand the mechanism for the impaired RCT seen in LKO-ER $\alpha$  mice, a cohort of mice were fed a WD for 7 months. Body fat composition increased 1.5-fold in female LKO-ER $\alpha$  mice compared to WT controls ( $19.3 \pm 3.6$  vs.  $29.7 \pm 2.1\%$ ,  $P < 0.01$ , Table 1) but did not change in male mice ( $23.6 \pm 2.6$  vs.  $25.4 \pm 2.5\%$ , Table 1). To understand why loss of hepatocyte ER $\alpha$  caused the increase in adiposity in female mice, we performed indirect calorimetry at the beginning of WD-feeding (Supplemental Figure 1). Energy expenditure was lower during light cycle than dark cycle in both sexes. Energy expenditure was lower in female LKO-ER $\alpha$  mice than their WT controls during dark cycle, and this difference was not seen in male mice (Supplemental Figure 1). Fasting insulin level increased 2.3-fold in female LKO-ER $\alpha$  mice ( $0.3 \pm 0.1$  vs.  $0.7 \pm 0.2$  ng/ml,  $P < 0.05$ ), and 1.4-fold in male LKO-ER $\alpha$  mice in comparison to their respective WT littermates ( $1.5 \pm 0.7$  vs.  $2.1 \pm 0.9$  ng/ml,  $P < 0.05$ , Table 1). Moreover, fasting cholesterol levels increased 1.2-fold in female LKO-ER $\alpha$  mice ( $178 \pm 11$  vs.  $214 \pm 16$  mg/dl,  $P < 0.05$ ) but did not change by the loss of hepatocyte ER $\alpha$  in male mice ( $223 \pm 7$  vs.  $222 \pm 12$  mg/dl, Table 1). The absence of hepatocyte ER $\alpha$  did not significantly alter serum TGs in female or male mice, although there was a trend for higher TGs in LKO-ER $\alpha$  mice (Table 1).



**Figure 1:** *In vivo* reverse cholesterol transport was impaired in LKO-ER $\alpha$  female mice. Mice were fed a WD for 5 days to keep body weight similar.  $^3\text{H}$ -cholesterol loaded foam cells were injected to mice, serum was collected at different time points. Mice were sacrificed 48 h later, liver and gallbladder were collected. Feces were also collected continuously for 48 h.  $^3\text{H}$ -radioactivities in serum in female (A) and male (C) mice.  $^3\text{H}$ -radioactivities in liver and gallbladder and in feces were determined in female (B) and male (D) mice. Data are expressed as mean  $\pm$  SD ( $n = 8$ ). Statistical analysis was performed with RM (repeated measures by both factors) 2-way ANOVA with Bonferroni's multiple comparisons tests. For Figure 1A,  $P < 0.05$  at 39 h time point and  $P < 0.01$  at 48 h time point.



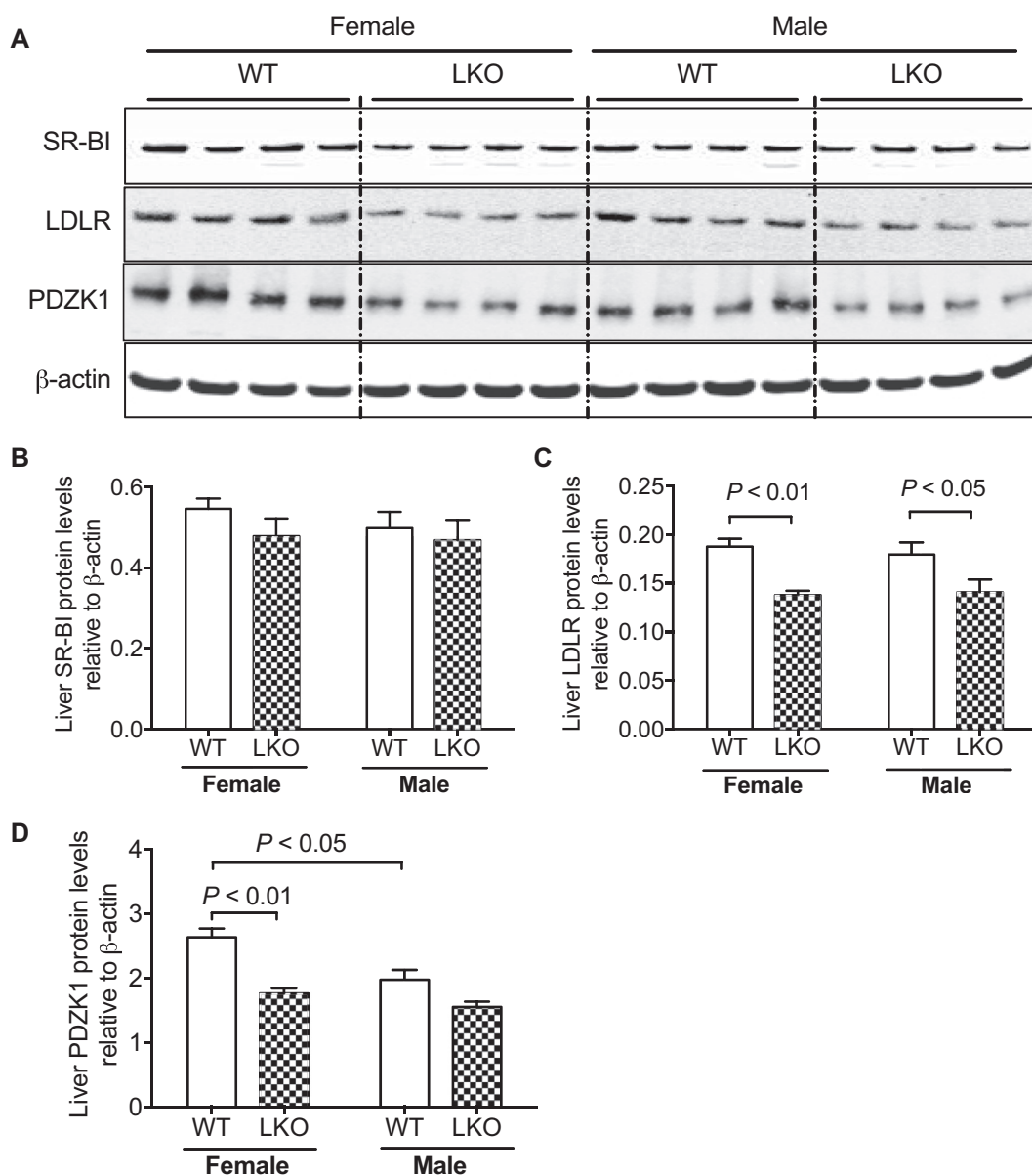
**Figure 2:** Cholesterol was distributed in enlarged HDL particles in LKO-ER $\alpha$  mice. Serum lipoproteins were separated using FPLC for female mice (A) and male mice (C). HDL-cholesterol, IDL/LDL-cholesterol and VLDL-cholesterol were determined (B and D). Student's  $t$ -test was used for statistical analysis for panels B and D.

Serum lipoproteins were separated by FPLC, and the cholesterol distribution in serum lipoprotein fractions was determined. Cholesterol was distributed in larger particles in female LKO-ER $\alpha$  mice as denoted by the shifted distribution curve toward the left in comparison to WT controls, and HDL and IDL/LDL cholesterol levels were higher in LKO-ER $\alpha$  mice (Figure 2, A and B). This shift in cholesterol distribution in serum lipoprotein fractions caused by the loss of hepatocyte ER $\alpha$  was less pronounced in male mice and only increased IDL/LDL-cholesterol levels in male LKO-ER $\alpha$  mice (Figure 2, C and D). To distinguish HDL from LDL particles, HPLC fractions of 25–30 from each run were pooled for immunoblotting of apoA1 (Supplemental Figure 2). ApoA1 was detected in the pooled fractions for LDL-sized particles from LKO-ER $\alpha$  mice in both sexes, demonstrating an enlargement of HDL particles in those mice (Supplemental Figure 2). The findings of enlarged

HDL particles and increased cholesterol levels in LDL fractions suggested that clearance of blood cholesterol is delayed in female LKO-ER $\alpha$  mice, which was less obvious in male LKO-ER $\alpha$  mice.

### 3.3. HDL-cholesterol clearance is impaired in the absence of hepatocyte ER $\alpha$ in female mice

After 7 months of WD feeding, liver cholesterol content was 20% lower in female LKO-ER $\alpha$  mice than in WT controls ( $5.87 \pm 1.06$  vs.  $4.39 \pm 0.77$   $\mu\text{g}/\text{mg}$ ,  $P < 0.05$ , Table 1), but deletion of hepatocyte ER $\alpha$  did not alter liver cholesterol content in male mice ( $4.75 \pm 1.16$  vs.  $4.54 \pm 0.66$   $\mu\text{g}/\text{mg}$ , Table 1). The absence of hepatocyte ER $\alpha$  did not change liver TGs or diacylglycerol (DG) content in either sex (Table 1). Liver phospholipids were reduced by the deletion of hepatocyte ER $\alpha$  in female and male mice (Table 1), and this reduction did



**Figure 3: Changes of liver receptor proteins for serum cholesterol clearance.** A. Immunoblots for liver SR-BI, LDLR, and PDZK1. B–D. Quantification of the blots for SR-BI, LDLR, and PDZK1, respectively. Results include all the animals that were fed a WD for 7 months. Data are expressed as mean  $\pm$  SD. Statistical analysis was performed with RM 2-way ANOVA with Bonferroni's multiple comparison test.

not change the percent contribution of each phospholipid species (Supplemental Figure 3).

We next compared protein levels of liver receptors for serum cholesterol uptake between WT and LKO-ER $\alpha$  mice. For the HDL receptor, SR-BI protein levels were similar between WT and LKO-ER $\alpha$  mice of both sexes (Figure 3, A and B). In contrast, LDLR protein levels were 22% lower in female LKO-ER $\alpha$  mice and 18% lower in male LKO-ER $\alpha$  mice than their respective WT controls (Figure 3, A and C). PDZK1 is an adaptor protein that plays an important role for HDL-cholesterol uptake mediated by SR-BI [20]. In ER $\alpha$ -expressing cells, PDZK1 expression is regulated by estrogen [21]. In the present study, deletion of hepatocyte ER $\alpha$  decreased PDZK1 protein levels by 33% in female mice ( $P < 0.01$ ) and by 22% that was not statistically significant in male mice (Figure 3, A and D).

To further understand the role of hepatic ER $\alpha$  in cholesterol uptake by the liver, primary hepatocytes were isolated from each group of mice and cultured to define hepatic HDL- and LDL-cholesterol uptake. As shown in Figure 4, deletion of hepatocyte ER $\alpha$  decreased HDL-cholesterol uptake by 15% in females ( $40.1 \pm 2.5$  vs.  $34.2 \pm 3.7$  ng/ $\mu$ g/hr,  $P < 0.05$ ), and by 16% in males ( $27.1 \pm 3.8$  vs.  $21.9 \pm 1.7$  ng/ $\mu$ g/hr,  $P < 0.05$ ). However, deletion of hepatocyte ER $\alpha$  did not alter LDL uptake in either female or male mice (Figure 4).

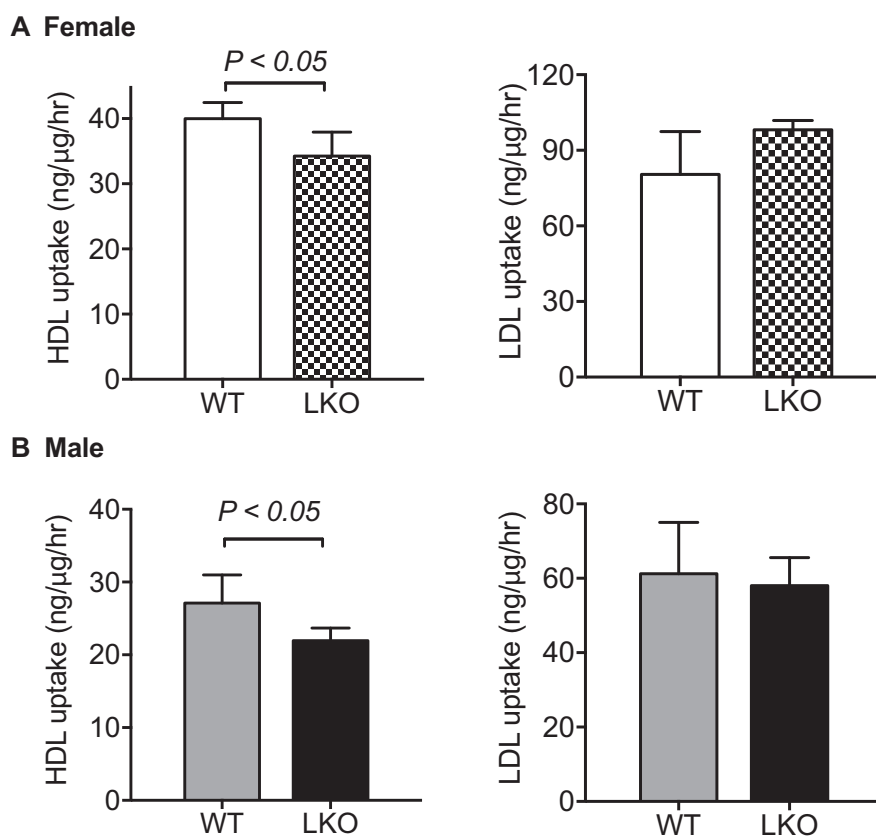
#### 3.4. HDL capacity for cellular cholesterol efflux is decreased by the loss of hepatocyte ER $\alpha$

Since HDL-cholesterol levels increased and HDL particle size was enlarged in LKO-ER $\alpha$  mice, we evaluated the HDL function with regard

to cholesterol efflux from foam cells. HDL was isolated from long-term WD fed mice and was incubated with foam cells loaded with  $^3$ H-cholesterol for 6 h. HDL capacity for cholesterol efflux was reduced by 20% by deletion of hepatocyte ER $\alpha$  in female mice ( $9.74 \pm 0.46\%$  vs.  $7.83 \pm 1.1\%$ ,  $P < 0.05$ ) and by 33% in male mice ( $9.65 \pm 1.2\%$  vs.  $6.47 \pm 0.98\%$ ,  $P < 0.01$ , Figure 5A).

We examined changes in serum apolipoproteins that play key roles in HDL cholesterol efflux capacity between groups. Deletion of hepatocyte ER $\alpha$  decreased serum apoE in both female and male mice (Figure 5, B and D). Levels for an acute inflammatory marker, serum amyloid A1 (SAA1), were significantly higher in male than in female mice. Deletion of hepatocyte ER $\alpha$  did not change SAA1 in female mice but decreased SAA1 in male mice (Figure 5, B and F). In addition, serum paraoxonase 1 (PON1) and adiponectin levels were higher in female mice but were not affected by the deletion of hepatocyte ER $\alpha$  (Figure 5, B, C and E). Both adiponectin and PON1 are protective against the development of insulin resistance [22,23]. The lower levels of PON1 and adiponectin in serum were consistent with the higher fasting insulin levels in male mice (Table 1). However, changes of serum PON1 and apoE were only partially related to protein amounts of PON1 and apoE in the liver (Supplemental Figure 4). Deletion of hepatocyte ER $\alpha$  significantly increased serum phospholipid transfer protein (PLTP) levels in both female and male mice (Figure 5, B and G). This observed change may be due to changes in liver phospholipids (Table 1).

Then we compared early stages of atherosclerosis to verify whether the impaired RCT seen in LKO-ER $\alpha$  mice would contribute to the lipid



**Figure 4: Hepatocyte uptake of HDL and LDL.** Hepatocytes were isolated from female (A) and male (B) LKO-ER $\alpha$  and WT mice. HDL and LDL uptake assay were performed after an overnight culturing. Data are from two independent experiments performed in duplicates ( $n = 4$  for each group). Data are expressed as mean  $\pm$  SD. Statistical analysis was performed with Student's  $t$ -test.

retention in the artery wall. Atherosclerotic lesion size at the aortic root sinus was determined after the oil-red-O staining. Atherosclerotic lesions developed in all LKO-ER $\alpha$  female mice, while the early lesion was not consistently detectable in WT controls (Figure 6A). The lesion size was increased 5.6-fold in female mice without hepatocyte ER $\alpha$  compared to WT controls ( $2981 \pm 1684$  vs.  $529 \pm 718 \times 10^3 \mu\text{m}^2$ ,  $P < 0.001$ , Figure 6, A and B). There was no increase in atherosclerotic lesions in male LKO-ER $\alpha$  mice compared to WT controls (data not shown). Expression of VCAM-1 over early foam cell lesions plays critical role for atherosclerosis progression by regulating the monocyte recruitment to artery walls [24]. As shown in Figure 6, VCAM-1 expression on the surface of the lesion area in female LKO-ER $\alpha$  mice was 1.44-fold of its expression in artery walls in female WT mice (Figure 6, C and D).

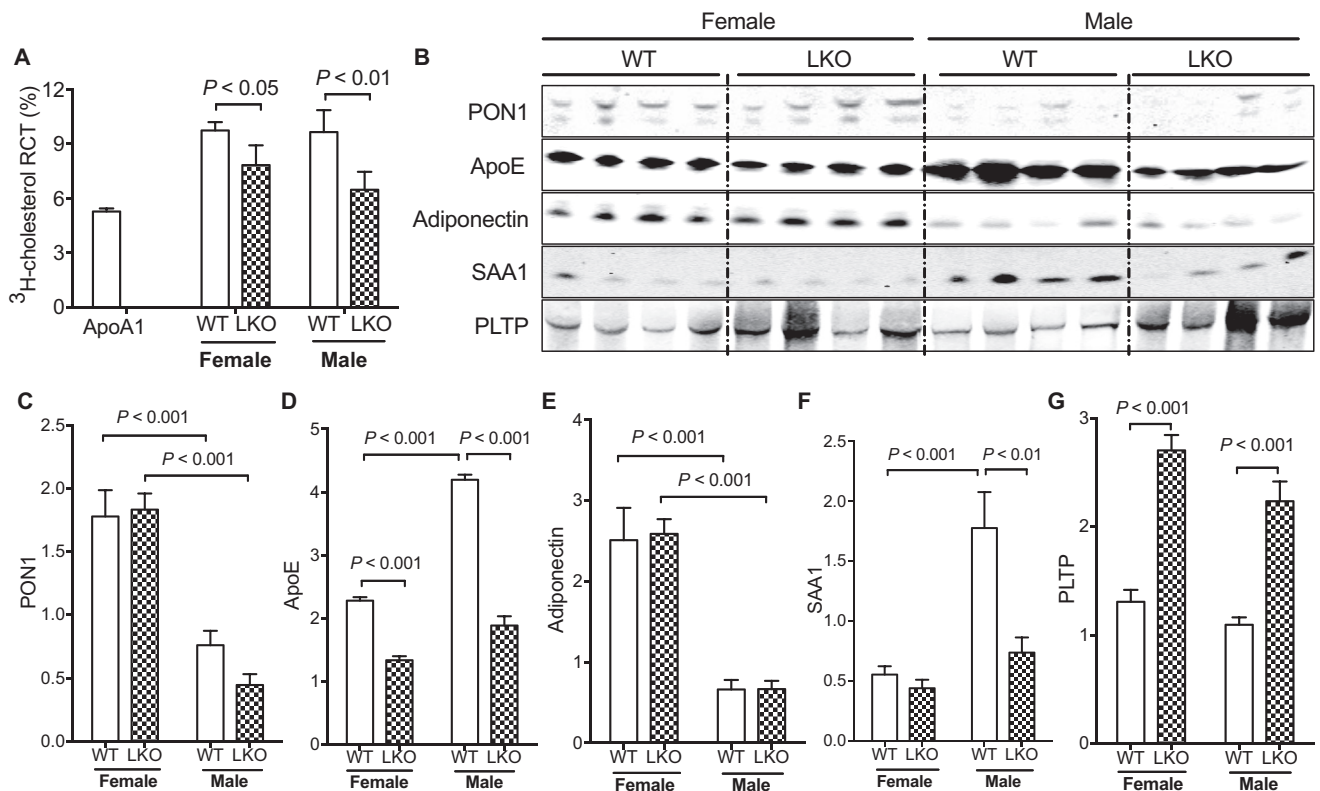
#### 4. DISCUSSION

In the present study, we show that hepatocyte ER $\alpha$  contributes to several key steps governing the development of atherosclerosis in female mice: cholesterol efflux from foam cells to HDL, HDL-delivery of cholesterol to hepatocytes, and removal of cholesterol from the body into feces, collectively termed reverse cholesterol transport. When hepatocyte ER $\alpha$  signaling was absent in female LKO-ER $\alpha$  mice, clearance of blood cholesterol through liver was decreased, leading to cholesterol accumulation in the circulation. Deletion of hepatocyte ER $\alpha$  increased early-stage atherosclerosis in female mice but not in male mice, indicating an important role of

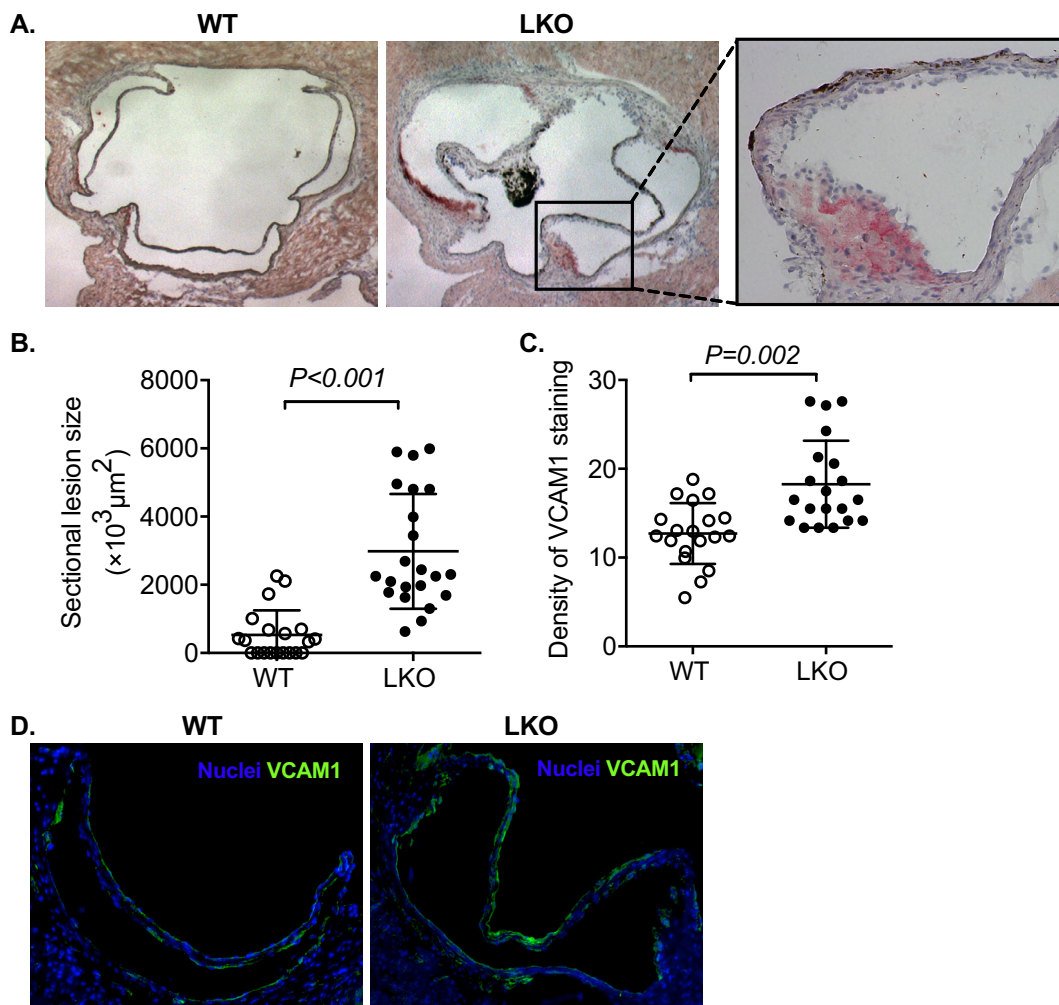
hepatocyte ER $\alpha$  for the protective effects of estrogen signaling with regard to atherosclerosis.

We show that the loss of hepatocyte ER $\alpha$  impairs HDL capacity for cholesterol efflux from foam cells. Although higher levels of HDL-cholesterol in plasma are associated with a lower risk of CHD, pharmacologic approaches to raise HDL-cholesterol have not reduced CHD risk [25–27]. Therefore, efforts to enhance HDL cholesterol efflux capacity or increase the whole-body reverse cholesterol transport, regardless of HDL level, may be of therapeutic interest. Indeed, cholesterol efflux capacity is inversely correlated with CHD independently of HDL-cholesterol level [28,29]. We previously reported that loss of ovarian hormones influences HDL protein composition with high-fat diet feeding [30]. Both PLTP and apoE play important roles in HDL capacity for cellular cholesterol efflux, and their activities are associated with CHD risk [31–33]. In the present study, HDL capacity for cholesterol efflux was impaired in both sexes mice by the loss of hepatocyte ER $\alpha$ , which was associated with exacerbated insulin resistance with WD, and changes of serum PLTP and apoE.

We report that deletion of hepatocyte ER $\alpha$  impairs blood cholesterol clearance and RCT, which leads to early stages of lipid retention in the artery wall. Humans carry the majority of blood cholesterol in LDL particles, thus delayed clearance of LDL cholesterol results in increased CHD risk [3]. Additionally, impairment of HDL-cholesterol clearance from the circulation increases CHD risk in humans with loss of function variant *SCARB1* (P376L). These individuals have a profound HDL-related phenotype with increases in large HDL particles [34]. Mice carry the majority of blood cholesterol in HDL particles and



**Figure 5: HDL capacity for cholesterol efflux was impaired in LKO-ER $\alpha$  mice.** A. HDL samples from long-term WD feeding cohort mice were used for the *in vitro* cholesterol efflux assays. Pure ApoA1 protein (50  $\mu\text{g}/\text{ml}$ ) was used as positive control. Cholesterol efflux was calculated as the percentage of  $^3\text{H}$ -radioactivity in media after 6 h of incubation to the  $^3\text{H}$ -radioactivity in cells before the treatment of HDL or ApoA1. B. Serum from the same cohort mice was used for immunoblotting. The quantification of the band density is shown in C-G. Data are expressed as mean  $\pm$  SD. Statistical analysis was performed with RM 2-way ANOVA with Bonferroni's multiple comparison test.



**Figure 6:** Deletion of hepatocyte ER $\alpha$  increased atherosclerosis in female mice after a long-term of Western-type diet. Female LKO-ER $\alpha$  mice and their wild-type (WT) littermates were fed a Western-type diet for 7 months. **A.** Cross-sectional area of proximal aortas was stained with oil-red-O, which was used for the quantification of atherosclerosis in **B.** **C–D.** VCAM-1 (green) positive staining is stronger in LKOER $\alpha$  mice than in WT controls and the quantification is shown in **C.** Nuclei were counterstained with Hoechst (blue). Data are collected from two sections for each mouse, and all experimental mice that were fed a WD for 7 months were included. Data are expressed as mean  $\pm$  SD.  $n \geq 19$ , statistical analysis was performed with Student's *t*-test.

are resistant to atherosclerosis. ApoE- or LDLR-null mouse models have increased LDL-cholesterol and been frequently used for atherosclerosis studies. However, apoE- and LDLR-null mice have impaired cholesterol clearance and are not ideal models for studying liver cholesterol uptake and RCT. Thus, clearance of blood cholesterol through HDL receptor, SR-BI, in the liver is important in mice, but this pathway is also applicable to humans. Impaired HDL-cholesterol uptake in SR-BI knockout mice with LDLR-null background increases plasma cholesterol and enlarges HDL particle size, leading to increased atherosclerotic burden [35–37]. In the present study, HDL-cholesterol uptake by the liver was impaired and led to early stages of atherosclerosis in female LKO-ER $\alpha$  mice in which hepatocyte estrogen signaling was reduced. Deletion of hepatocyte ER $\alpha$  decreased LDLR in both sexes mice, in line with our previous report that hepatocyte ER $\alpha$  signaling is involved in VLDL metabolism [2,15]. It has been reported that estrogen up-regulates mRNA expression of SR-BI, the HDL receptor, and promotes HDL-cholesterol uptake [38,39]. We did not see that liver SR-BI protein abundance was altered by the deletion of hepatocyte ER $\alpha$ . We did see that the loss of hepatocyte ER $\alpha$  down-

regulated the expression of PDZK1, the adaptor protein for SR-BI mediated HDL-cholesterol uptake. Mice without PDZK1 have increased plasma cholesterol, enlarged HDL particles, and increased atherosclerosis with WD [20,40,41], a similar phenotype to the female LKO-ER $\alpha$  mice in our study. PDZK1 is required for endocytosis of SR-BI from the basolateral membrane and traffics to the apical membrane to mediate rapid transport of cholesterol and cholesteryl ester from HDL into bile [42,43]. We cannot rule out that PDZK1 regulates the sub-cellular location of SR-BI in the present study, which might functionally reduce SR-BI action in LKO-ER $\alpha$  mice. Recently, SR-BI has been reported to be mainly expressed in sinusoidal endothelial cells in the liver and a transcytosis mechanism might be required for SR-BI function [44]. This study is consistent with our observation that SR-BI from liver tissues did not significantly decrease in LKO-ER $\alpha$  mice, in which ER $\alpha$  is deleted only in hepatocytes [2]. Future studies are required to clarify the underlying mechanism.

Our studies suggest that estrogen signaling through hepatocyte ER $\alpha$  protects against atherosclerosis by at least two means. First, hepatocyte ER $\alpha$  protects female mice from obesity and insulin resistance



after long-term WD. Obesity and insulin resistance may contribute to the development of atherosclerosis through multiple pathways [5]. HDL composition and binding to SR-BI for liver cholesterol uptake are impaired in obese mice [45]. In line with this, we observed that HDL capacity for cholesterol efflux was decreased in female LKO-ER $\alpha$  mice, which might be related to increased obesity and insulin resistance in these mice. Second, the absence of hepatocyte ER $\alpha$  decreases liver cholesterol uptake by downregulating its target gene PDZK1. In BMI-matched female LKO-ER $\alpha$  and WT mice after short-term WD, *in vivo* cholesterol efflux assays showed that liver cholesterol uptake was diminished in female LKO-ER $\alpha$  mice.

Results from *ex vivo* studies, i.e. cholesterol efflux capacity and hepatocyte HDL- and LDL-cholesterol uptake assays, showed the effects of the loss of hepatocyte ER $\alpha$  in both sexes. The diminished effects *in vivo* in male mice may be due to the difference of estrogen levels between male and female mice. We previously showed that blood estradiol levels are increased in male LKO-ER $\alpha$  mice but not in female LKO-ER $\alpha$  mice [2,15]. Estrogen's action in peripheral tissues such as macrophages/immune-system is also protective against atherosclerosis [12]. Furthermore, SAA1 is a contributor for atherosclerosis progression [46]. In current study, the loss of hepatic ER $\alpha$  decreased serum SAA1 only in male mice, which may have protected male LKO-ER $\alpha$  mice against atherosclerosis.

There may be discordance between the effects of naturally cycling estrogens and estrogen treatment approaches after menopause with regard to lipoprotein metabolism and atherosclerosis risk. Treatment of menopausal women with oral estrogens, which have a larger hepatic effect, increases HDL and lowers LDL. This effect is less potent with transdermal estradiol, which is delivered to the peripheral circulation and has less hepatic effects [47,48]. Our studies are limited to defining the effect of endogenous estrogen signaling through hepatocyte ER $\alpha$ , deficiency of which resulted in the accumulation of cholesterol in circulation, presumably because of impairment in HDL's ability to deliver cholesterol.

*In vivo*, the effects of estrogens to limit liver fat content are likely distributed among tissues, including estrogen signaling in adipose tissues, muscles, and the liver. In our previously published work, we showed that mice with ER $\alpha$  deletion from hepatocytes develop steatosis on high-fat diet [2]. Those mice were obese because they had undergone ovariectomy which leads to weight gain. By contrast, Hart-Unger et al. recently showed that female LKO-ER $\alpha$  mice with intact ovaries and without E2 treatment did not worsen fatty liver during high fat diet feeding compared to controls, but these mice were only minimally heavier than controls [49]. Comparison of these studies suggests the role of hepatocyte ER $\alpha$  to limit steatosis is more important in the setting of more severe obesity when fatty acid load to the liver would be higher [2,49]. Additionally, mice lacking ER $\alpha$  in skeletal muscle have severe metabolic dysfunction and impaired fatty acid utilization [50], which would be expected to worsen fatty liver by increasing fatty acid delivery to liver.

## 5. CONCLUSION

Hepatocyte ER $\alpha$  promotes HDL's ability for cholesterol efflux from foam cells by modulating molecular signaling within the liver in both female and male mice. Hepatocyte ER $\alpha$  promotes cholesterol uptake by the liver and whole-body reverse cholesterol transport, which process is especially important in females. Our results suggest that hepatocyte ER $\alpha$  is required for the actions of endogenous estrogens to protect against atherosclerosis. Given that hepatocyte ER $\alpha$  contributed to

steps of RCT in males as well, this pathway may be beneficially targeted to improve steps of reverse cholesterol transport in both males and females.

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## DISCLOSURES

All authors have nothing to disclose.

## CONFLICT OF INTEREST

None.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.molmet.2017.12.012>

## REFERENCES

- [1] Lundsgaard, A.M., Kiens, B., 2014. Gender differences in skeletal muscle substrate metabolism - molecular mechanisms and insulin sensitivity. *Frontiers in Endocrinology (Lausanne)* 5:195.
- [2] Zhu, L., Brown, W.C., Cai, Q., Krust, A., Chambon, P., McGuinness, O.P., et al., 2013. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. *Diabetes* 62:424–434.
- [3] Castelli, W.P., 1984. Epidemiology of coronary heart disease: the Framingham study. *The American Journal of Medicine* 76:4–12.
- [4] Wang, X., Magkos, F., Mittendorfer, B., 2011. Sex differences in lipid and lipoprotein metabolism: it's not just about sex hormones. *The Journal of Clinical Endocrinology and Metabolism* 96:885–893.
- [5] Semenkovich, C.F., 2006. Insulin resistance and atherosclerosis. *Journal of Clinical Investigation* 116:1813–1822.
- [6] Mittendorfer, B., 2005. Sexual dimorphism in human lipid metabolism. *Journal of Nutrition* 135:681–686.
- [7] Jensen, M.D., 1995. Gender differences in regional fatty acid metabolism before and after meal ingestion. *Journal of Clinical Investigation* 96:2297–2303.
- [8] Trigatti, B.L., Krieger, M., Rigotti, A., 2003. Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 23:1732–1738.
- [9] Rader, D.J., Tall, A.R., 2012. The not-so-simple HDL story: is it time to revise the HDL cholesterol hypothesis? *Nature Medicine* 18:1344–1346.
- [10] Mauvais-Jarvis, F., Clegg, D.J., Hevener, A.L., 2013. The role of estrogens in control of energy balance and glucose homeostasis. *Endocrine Reviews* 34:309–338.

- [11] Nielsen, S., Guo, Z., Albu, J.B., Klein, S., O'Brien, P.C., Jensen, M.D., 2003. Energy expenditure, sex, and endogenous fuel availability in humans. *Journal of Clinical Investigation* 111:981–988.
- [12] Ribas, V., Drew, B.G., Le, J.A., Soleymani, T., Daraei, P., Sitz, D., et al., 2011. Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *Proceedings of the National Academy of Sciences of the United States of America* 108:16457–16462.
- [13] Bourassa, P.A., Milos, P.M., Gaynor, B.J., Breslow, J.L., Aiello, R.J., 1996. Estrogen reduces atherosclerotic lesion development in apolipoprotein E-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 93:10022–10027.
- [14] Marsh, M.M., Walker, V.R., Curtiss, L.K., Banka, C.L., 1999. Protection against atherosclerosis by estrogen is independent of plasma cholesterol levels in LDL receptor-deficient mice. *The Journal of Lipid Research* 40:893–900.
- [15] Zhu, L., Martinez, M.N., Emfinger, C.H., Palmisano, B.T., Stafford, J.M., 2014. Estrogen signaling prevents diet-induced hepatic insulin resistance in male mice with obesity. *American Journal of Physiology - Endocrinology and Metabolism* 306:E1188–E1197.
- [16] Tanigawa, H., Billheimer, J.T., Tohyama, J., Zhang, Y., Rothblat, G., Rader, D.J., 2007. Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. *Circulation* 116:1267–1273.
- [17] Asztalos, B.F., de la Llera-Moya, M., Dallal, G.E., Horvath, K.V., Schaefer, E.J., Rothblat, G.H., 2005. Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux. *The Journal of Lipid Research* 46:2246–2253.
- [18] Foretz, M., Hebrard, S., Leclerc, J., Zarrinpashneh, E., Soty, M., Mithieux, G., et al., 2010. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *Journal of Clinical Investigation* 120:2355–2369.
- [19] Zhu, L., Giunzioni, I., Tavori, H., Covarrubias, R., Ding, L., Zhang, Y., et al., 2016. Loss of macrophage low-density lipoprotein receptor-related protein 1 confers resistance to the antiatherogenic effects of tumor necrosis factor- $\alpha$  inhibition. *Arteriosclerosis, Thrombosis, and Vascular Biology* 36:1483–1495.
- [20] Kocher, O., Krieger, M., 2009. Role of the adaptor protein PDZK1 in controlling the HDL receptor SR-BI. *Current Opinion in Lipidology* 20:236–241.
- [21] Ghosh, M.G., Thompson, D.A., Weigel, R.J., 2000. PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. *Cancer Research* 60:6367–6375.
- [22] Bednarska-Makaruk, M., Graban, A., Lipczynska-Lojkowska, W., Bochynska, A., Rodo, M., Krzywkowski, T., et al., 2013. Positive correlation of paraoxonase 1 (PON1) activity with serum insulin level and HOMA-IR in dementia. A possible advantageous role of PON1 in dementia development. *Journal of the Neurological Sciences* 324:172–175.
- [23] Lihn, A.S., Pedersen, S.B., Richelsen, B., 2005. Adiponectin: action, regulation and association to insulin sensitivity. *Obesity Reviews* 6:13–21.
- [24] Cybulsky, M.I., Iiyama, K., Li, H., Zhu, S., Chen, M., Iiyama, M., et al., 2001. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *Journal of Clinical Investigation* 107:1255–1262.
- [25] Schwartz, G.G., Olsson, A.G., Abt, M., Ballantyne, C.M., Barter, P.J., Brumm, J., et al., 2012. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *New England Journal of Medicine* 367:2089–2099.
- [26] Nissen, S.E., Tardif, J.C., Nicholls, S.J., Revkin, J.H., Shear, C.L., Duggan, W.T., et al., 2007. Effect of torcetrapib on the progression of coronary atherosclerosis. *New England Journal of Medicine* 356:1304–1316.
- [27] Barter, P.J., Caulfield, M., Eriksson, M., Grundy, S.M., Kastelein, J.J., Komajda, M., et al., 2007. Effects of torcetrapib in patients at high risk for coronary events. *New England Journal of Medicine* 357:2109–2122.
- [28] Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., et al., 2011. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *New England Journal of Medicine* 364:127–135.
- [29] Klancic, T., Woodward, L., Hofmann, S.M., Fisher, E.A., 2016. High density lipoprotein and metabolic disease: potential benefits of restoring its functional properties. *Molecular Metabolism* 5:321–327.
- [30] Martinez, M.N., Emfinger, C.H., Overton, M., Hill, S., Ramaswamy, T.S., Cappel, D.A., et al., 2012. Obesity and altered glucose metabolism impact HDL composition in CETP transgenic mice: a role for ovarian hormones. *The Journal of Lipid Research* 53:379–389.
- [31] Dove, D.E., Linton, M.F., Fazio, S., 2005. ApoE-mediated cholesterol efflux from macrophages: separation of autocrine and paracrine effects. *American Journal of Physiology - Cell Physiology* 288:C586–C592.
- [32] Schliitt, A., Bickel, C., Thumma, P., Blankenberg, S., Rupprecht, H.J., Meyer, J., et al., 2003. High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 23:1857–1862.
- [33] Yazdanyar, A., Yeang, C., Jiang, X.C., 2011. Role of phospholipid transfer protein in high-density lipoprotein-mediated reverse cholesterol transport. *Current Atherosclerosis Reports* 13:242–248.
- [34] Zanon, P., Khetarpal, S.A., Larach, D.B., Hancock-Cerutti, W.F., Millar, J.S., Cuchel, M., et al., Consortium, C. H. D. E., Consortium, C. A. E., Global Lipids Genetics, C, 2016. Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease. *Science* 351:1166–1171.
- [35] Braun, A., Trigatti, B.L., Post, M.J., Sato, K., Simons, M., Edelberg, J.M., et al., 2002. Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. *Circulation Research* 90:270–276.
- [36] Rader, D.J., Alexander, E.T., Weibel, G.L., Billheimer, J., Rothblat, G.H., 2009. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *The Journal of Lipid Research* 50(Suppl):S189–S194.
- [37] Rigotti, A., Trigatti, B.L., Penman, M., Rayburn, H., Herz, J., Krieger, M., 1997. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proceedings of the National Academy of Sciences of the United States of America* 94:12610–12615.
- [38] Fukata, Y., Yu, X., Imachi, H., Nishiuchi, T., Lyu, J., Seo, K., et al., 2014. 17 $\beta$ -Estradiol regulates scavenger receptor class BI gene expression via protein kinase C in vascular endothelial cells. *Endocrine* 46:644–650.
- [39] Lopez, D., McLean, M.P., 2006. Estrogen regulation of the scavenger receptor class B gene: anti-atherogenic or steroidogenic, is there a priority? *Molecular and Cellular Endocrinology* 247:22–33.
- [40] Ikemoto, M., Arai, H., Feng, D., Tanaka, K., Aoki, J., Dohmae, N., et al., 2000. Identification of a PDZ-domain-containing protein that interacts with the scavenger receptor class B type I. *Proceedings of the National Academy of Sciences of the United States of America* 97:6538–6543.
- [41] Kocher, O., Yesilaltay, A., Cirovic, C., Pal, R., Rigotti, A., Krieger, M., 2003. Targeted disruption of the PDZK1 gene in mice causes tissue-specific depletion of the high density lipoprotein receptor scavenger receptor class B type I and altered lipoprotein metabolism. *Journal of Biological Chemistry* 278:52820–52825.
- [42] Silver, D.L., 2004. SR-BI and protein-protein interactions in hepatic high density lipoprotein metabolism. *Reviews in Endocrine & Metabolic Disorders* 5:327–333.
- [43] Silver, D.L., Wang, N., Xiao, X., Tall, A.R., 2001. High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. *Journal of Biological Chemistry* 276:25287–25293.
- [44] Ganesan, L.P., Mates, J.M., Cheplowitz, A.M., Avila, C.L., Zimmerer, J.M., Yao, Z., et al., 2016. Scavenger receptor B1, the HDL receptor, is expressed abundantly in liver sinusoidal endothelial cells. *Scientific Reports* 6:20646.

- [45] Silver, D.L., Wang, N., Tall, A.R., 2000. Defective HDL particle uptake in ob/ob hepatocytes causes decreased recycling, degradation, and selective lipid uptake. *Journal of Clinical Investigation* 105:151–159.
- [46] Thompson, J.C., Jayne, C., Thompson, J., Wilson, P.G., Yoder, M.H., Webb, N., et al., 2015. A brief elevation of serum amyloid A is sufficient to increase atherosclerosis. *The Journal of Lipid Research* 56:286–293.
- [47] Mauvais-Jarvis, F., Manson, J.E., Stevenson, J.C., Fonseca, V.A., 2017. Menopausal hormone therapy and type 2 diabetes prevention: evidence, mechanisms, and clinical implications. *Endocrine Reviews* 38:173–188.
- [48] Salpeter, S.R., Walsh, J.M., Ormiston, T.M., Greyber, E., Buckley, N.S., Salpeter, E.E., 2006. Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes, Obesity and Metabolism* 8:538–554.
- [49] Hart-Unger, S., Arao, Y., Hamilton, K.J., Lierz, S.L., Malarkey, D.E., Hewitt, S.C., et al., 2017. Hormone signaling and fatty liver in females: analysis of estrogen receptor alpha mutant mice. *International Journal of Obesity (London)* 41:945–954.
- [50] Ribas, V., Drew, B.G., Zhou, Z., Phun, J., Kalajian, N.Y., Soleymani, T., et al., 2016. Skeletal muscle action of estrogen receptor alpha is critical for the maintenance of mitochondrial function and metabolic homeostasis in females. *Science Translational Medicine* 8:334ra54.