Hepatic scavenger receptor class B type 1 knockdown reduces atherosclerosis and enhances the antiatherosclerotic effect of brown fat activation in APOE*3-Leiden.CETP mice

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Hepatic Scavenger Receptor Class B Type 1 Knockdown Reduces Atherosclerosis and Enhances the Antiatherosclerotic Effect of Brown Fat Activation in APOE*3-Leiden.CETP Mice

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OBJECTIVE: Brown fat activation attenuates atherosclerosis development by accelerating triglyceride-rich lipoprotein turnover and/or stimulation of reverse cholesterol transport via the SRB1 (scavenger receptor class B type 1). The aim of this study was to investigate the specific role of hepatic SRB1 in the atheroprotective properties of brown fat activation.

APPROACH AND RESULTS: *APOE*3-Leiden.CETP* mice, a well-established model of human-like lipoprotein metabolism and atherosclerosis, were treated with vehicle or adenoassociated virus serotype 8-short hairpin RNA, which decreased hepatic SRB1 protein levels by 40% to 55%. After 2 weeks, mice without or with hepatic SRB1 knockdown were treated with vehicle or the β3-adrenergic receptor agonist CL316243 to activate brown fat for 4 weeks to determine HDL (high-density lipoprotein) catabolism and for 9 weeks to evaluate atherosclerosis. Surprisingly, hepatic SRB1 knockdown additively improved the beneficial effects of β3-adrenergic receptor agonism on atherosclerosis development. In fact, hepatic SRB1 knockdown per se not only increased HDL-cholesterol levels but also reduced plasma triglyceride and non-HDL-cholesterol levels, thus explaining the reduction in atherosclerosis development. Mechanistic studies indicated that this is due to increased lipolytic processing and hepatic uptake of VLDL (very low density lipoprotein) by facilitating VLDL-surface transfer to HDL.

CONCLUSIONS: Hepatic SRB1 knockdown in a mouse model with an intact ApoE (apolipoprotein E)-LDLR (low density lipoprotein receptor) clearance pathway, relevant to human lipoprotein metabolism, reduced atherosclerosis and improved the beneficial effect of brown fat activation on atherosclerosis development, explained by pleiotropic effects of hepatic SRB1 knockdown on lipolytic processing and hepatic uptake of VLDL. Brown fat activation could thus be an effective strategy to treat cardiovascular disease also in subjects with impaired SRB1 function.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ brown adipose tissue ■ cardiovascular diseases ■ lipoprotein ■ triglyceride

Atherosclerosis remains the most common cause of cardiovascular diseases. A prominent risk factor for atherosclerosis is dyslipidemia, that is, high levels of plasma low density lipoprotein-cholesterol and triglycerides, and low levels of high density lipoprotein-cholesterol (HDL-C). Remarkable efforts have been put into developing lipid-lowering medications such as statins and PCSK9 (proprotein convertase subtilisin-like kexin type 9) inhibitors. However, only 30% of all cardiovascular events can be prevented by such treatment strategies,[1–3] illustrating the need for new therapeutic strategies.

Brown fat is an emerging target to combat cardiometabolic diseases.[4–6] We previously showed that brown fat activation by cold exposure and β3-adrenergic receptor (β3-AR) agonism induces LPL (lipoprotein lipase)-mediated lipolysis of TRLs (triglyceride-rich lipoproteins),

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Nonstandard Abbreviations and Acronyms

β3-AR	β3-adrenergic receptor
ApoE	apolipoprotein E
AAV8	adenoassociated virus serotype 8
CETP	cholesteryl ester transfer protein
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein-cholesterol
HL	hepatic lipase
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
Pcsk9	proprotein convertase subtilisin-like kexin type 9
shRNA	short hairpin RNA
SRB1	scavenger receptor class B type 1
TC	total cholesterol
TRL	triglyceride-rich lipoprotein
VLDL	very low-density lipoprotein

Highlights

- Hepatic SRB1 (scavenger receptor class B type 1) knockdown in a humanized mouse model with an intact ApoE (apolipoprotein E)-LDLR (low density lipoprotein receptor) clearance pathway not only increases plasma HDL (high-density lipoprotein)-cholesterol but also reduces plasma triglyceride and non-HDL-cholesterol, thus resulting in the reduction in atherosclerosis development.
- Hepatic SRB1 knockdown does not impair but additively improves the beneficial effects of brown fat activation on cholesterol metabolism and atherosclerosis development.
- Brown fat activation could also be an effective strategy to treat cardiovascular disease in subjects with impaired SRB1 function.

thereby reducing plasma triglycerides. The generated cholesterol-enriched TRL remnants are subsequently taken up by the liver, thus reducing circulating (V) low-density lipoprotein-cholesterol levels and ameliorating atherosclerosis in *APOE*3-Leiden.CETP* (*E3L.CETP*) mice.[7] Nevertheless, the cholesterol-lowering effects explained only ≈30% of atheroprotective effects of brown fat activation.

Interestingly, β3-AR agonism also increases HDL-C in both *E3L.CETP* mice[8] and in humans.[9] A human study further shows that brown fat activation by short-term cooling increases small HDL particles with increased cholesterol efflux capacity in vitro.[10] The increase in HDL may well contribute to the antiatherogenic effect of brown fat activation, especially since β3-AR agonism promotes reverse cholesterol transport as dependent on the hepatic SRB1 (scavenger receptor class B type 1) in mice.[8]

SRB1 is highly expressed by hepatocytes and facilitates the selective uptake of HDL-derived cholesteryl esters.[11] SRB1-deficiency in both ApoE (apolipoprotein E)-knockout and LDLR (low density lipoprotein receptor)-knockout mice aggravates atherosclerosis development.[12,13] These combined data suggested a main antiatherogenic role of SRB1 by mediating a crucial step in reverse cholesterol transport. However, it should be noted that ablation of SRB1 in both ApoE$^{-/-}$ and LDLR$^{-/-}$ mice assessed the role of this HDL receptor in absence of physiological catabolism of (V)LDL by the ApoE-LDLR clearance pathway.

Since hepatic SRB1-mediated cholesterol clearance may thus contribute to the atheroprotective effects of brown fat activation, the aim of this study was to evaluate whether knocking down hepatic SRB1 attenuates the therapeutic effectiveness of brown fat activation on atherosclerosis development. To this end, *E3L.CETP* mice, a well-established model of human-like lipoprotein metabolism with an intact ApoE-LDLR pathway for (V) LDL clearance, received a short hairpin RNA (shRNA) targeting SRB1, as delivered by adenoassociated virus serotype 8 (AAV8) that has been widely used to transduce hepatocytes.[14,15] The effects of knocking down SRB1 on the beneficial effects of β3-AR agonism on lipoprotein metabolism and atherosclerosis development were evaluated.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and its Data Supplement. Additional detailed materials and methods are included in the Data Supplement.

Animals and Treatments

Hemizygous *APOE*3-Leiden* (*E3L*) mice were crossbred with homozygous human CETP (cholesteryl ester transfer protein) transgenic mice to generate heterozygous *E3L.CETP* mice.[16] Please see the Major Resources Table in the Data Supplement. Mice were housed in standard conditions at room temperature (22 °C) with 40±5% relative humidity and a 12-h light/dark (7 AM lights on; 7 PM lights off) cycle. Water and standard laboratory diet (801203, Special Diets Services, United Kingdom) were available ad libitum, unless indicated.

In a first experiment, 9- to 12-week-old female mice were fed a Western-type diet (Altromin, Germany) containing 15% cacao butter, 1% corn oil, and 0.15% (wt/wt) cholesterol. After a run-in period of 3 weeks, mice were randomized into 2 groups based on plasma lipid levels and body weight and received an injection via the tail vein with an AAV8 vector loaded with shRNA targeting SRB1 (Vector Biolabs, 5×10^{11} genome copies/mouse) or saline as control. Two weeks after injection, mice in each group were again randomized based on plasma lipid levels and body weight into 2 subgroups and additionally treated with the β3-AR agonist CL316243

(Tocris Bioscience Bristol, United Kingdom; 20 μg·mouse^{-1}) or vehicle (saline) 3× per week by subcutaneous injections between 13:00 and 15:00 hours for additional 9 weeks to evaluate atherosclerosis development. This resulted in the following 4 treatment groups (n=11 mice per group): (1) vehicle (ctrl), (2) CL316243 (β), (3) shSRB1 treatment (shSRB1), (4) shSRB1 treatment+CL316243 (shSRB1+β). Body weight and plasma lipid levels were measured at the indicated time points throughout the intervention period. Blood samples for LPL activity assay (see below) were collected after 8 weeks of vehicle or CL316243 treatment. After 9 weeks of treatment, plasma ApoA1 and CETP levels were measured as described below. VLDL (very low density lipoprotein) clearance was assessed (see below) and mice were euthanized by CO_2 suffocation and perfused with ice-cold saline via the heart. Organs were isolated for Western blotting and other analysis.

To rule out an effect of the AAV vector itself on plasma lipids, in a second experiment, 9- to 12-week-old female mice were again fed the Western-type diet. After a run-in period of 3 weeks, mice were randomized into 3 groups based on plasma lipid levels and body weight and received an injection into the tail vein with an AAV8 vector loaded with shRNA targeting SRB1 (shSRB1, Vector Biolabs, 5×10^{11} genome copies/mouse) or with a scrambled sequence (scrambled, Vector Biolabs, 5×10^{11} genome copies/mouse), or saline (ctrl). Body weight was determined every 2 weeks, and plasma lipids were measured after 2 and 6 weeks of treatment.

The set-up of the third experiment was similar to that of the first experiment, with the exception that mice were treated with CL316243 or vehicle for 4 instead of 9 weeks. At the end of treatment, in vivo plasma decay and hepatic uptake of HDL-cholesteryl oleyl ether was measured as described below.

Our study adhered to the guidelines as described in the ATVB Council Statement for considering "sex difference" as a biological variable.[17] We used female mice because only female *E3L.CETP* mice develop Western-type diet-induced dyslipidemia and atherosclerosis.[7,18–20] Our study also adhered to the guidelines for experimental atherosclerosis studies described in the AHA Statement.[21] The animal experiment was approved by the Animal Ethical Committee of Leiden University Medical Center, Leiden, The Netherlands (AVD1160020173305, PE. 18.034.019, PE. 18. 034. 049). All animal procedures performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Statistical Analysis

Interaction between treatment and genotype and difference between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test without testing normality and variance. Difference between 2 groups in Figure IV in the Data Supplement was determined using 1-way ANOVA with the *LSD* post hoc test if *F* achieved statistical significance ($P<0.05$) and significant variance inhomogeneity was not observed. The square root (SQRT) of the lesion area was transformed, and univariate regression of analyses was performed to test for significant correlations between atherosclerotic lesion area and plasma non-HDL-C/HDL-C/triglyceride exposure. Multiple regression analysis was performed to predict the contribution of plasma non-HDL-C/HDL-C/triglyceride exposure to the atherosclerotic lesion area. Probability values <0.05 were considered statistically significant. All statistical analyses were performed with the GraphPad Prism 8.0.1 for Windows except for univariate and multiple regression analyses, which were performed with SPSS 25 for Windows.

RESULTS

Hepatic SRB1 Knockdown Increases HDL-C and Decreases Non-HDL-C and Additively Improves β3-AR Agonism-Induced Reduction in Plasma Non-HDL-C and Increase in HDL-C Levels

In a first experiment, to investigate the role of hepatic SRB1 in the beneficial effects of brown fat activation on cholesterol metabolism and atherosclerosis development, *E3L.CETP* mice were injected with shRNA-loaded AAV8 to downregulate liver-specific expression of SRB1.[15] Hepatic SRB1 was downregulated by ≈40% to 55% at both mRNA and protein level as compared with control mice, as assessed after 11 weeks of injection upon termination (Table; Figure IA and IB in the Data Supplement). shSRB1-AAV8 did not reduce SRB1 protein levels in both adrenal glands and gonadal white adipose tissues (Figure IE and IF in the Data Supplement), while it increased SRB1 protein levels in descending thoracic aortas (+48%, Figure IG in the Data Supplement). SRB1 knockdown had no effect on the hepatic expression of other genes related to HDL metabolism, including *Apoa1, Abca1, Abcg1,* and *Lcat* (Table).

Two weeks after shSRB1-AAV8 injection, both control and SRB1 knockdown mice were treated with vehicle or the β3-AR agonist CL316243 to activate brown fat. β3-AR agonism prevented body weight gain and decreased gonadal white adipose tissue and intrascapular brown adipose tissue) mass in control mice, which is in line with our previous observations,[7] confirming the beneficial effects of β3-AR agonism on adiposity, and which was also observed in hepatic SRB1 knockdown mice (Figure IIA through IIC in the Data Supplement). Hepatic SRB1 knockdown per se had no effects on adiposity (Figure IIA through IIC in the Data Supplement).

We next explored the effects of hepatic SRB1 knockdown on the plasma lipid-modulating effects of brown fat activation. First, we showed that SRB1 knockdown in control mice increased plasma ApoA1 (+31%, shSRB1 versus ctrl, Figure IIIA in the Data Supplement) and HDL-C levels (Figure 1A and 1G), as well as HDL-C exposure (+31%, shSRB1 versus ctrl, Figure 1B), confirming the role of hepatic SRB1 in HDL metabolism. β3-AR agonism in control mice increased plasma HDL-C levels (Figure 1A and 1G)

Table. Hepatic Gene Expression

	Gene	ctrl	β	shSRB1	shSRB1+β
HDL formation and processing	*Apoa1*	1.00±0.09	0.96±0.12	1.24±0.08	1.07±0.10
	Abca1	1.00±0.05	0.89±0.04	1.03±0.03	0.98±0.04
	Abcg1	1.00±0.06	0.91±0.06	0.98±0.04	0.72±0.02*†
	CETP	1.00±0.06	0.87±0.11	1.01±0.05	0.67±0.09‡
	Lcat	1.00±0.08	0.86±0.04	0.92±0.04	0.93±0.03
VLDL production and secretion	*Apob*	1.00±0.05	0.91±0.05	0.97±0.02	0.98±0.05
	Mttp	1.00±0.07	1.10±0.08	1.01±0.03	1.26±0.07‡
	Srebp1c	1.00±0.05	1.01±0.08	1.10±0.07	1.24±0.08
Lipolytic processing	*Lpl*	1.00±0.10	1.12±0.12	0.94±0.07	0.92±0.06
	Hl	1.00±0.06	0.88±0.04	0.86±0.05	0.89±0.06
	Angptl4	1.00±0.08	1.17±0.11	1.05±0.06	1.39±0.19
	Apoc2	1.00±0.04	1.18±0.08§	1.09±0.05	1.39±0.07‡
Hepatic uptake of cholesterol	*Ldlr*	1.00±0.07	0.75±0.05‖	1.09±0.05	0.79±0.06*
	Lrp1	1.00±0.08	0.71±0.05¶	0.70±0.05¶	0.54±0.04†
	Srb1	1.00±0.07	0.99±0.07	0.49±0.05¶	0.59±0.06#
	Pcsk9	1.00±0.21	0.59±0.13§	1.02±0.19	0.39±0.07†‖

Values are expressed as mean fold change±SEM. n=10/11 mice per group. β indicates β3-AR agonist CL316243; and shSRB1, ShRNA targeting scavenger receptor class B type 1. §P<0.05, ‖P<0.01, ¶P<0.001 vs ctrl, †P<0.01, #P<0.001 vs β, ‡P<0.01, *P<0.001 vs shSRB1.

and HDL-C exposure (+36%, β versus ctrl, Figure 1B) without effects on plasma ApoA1 levels (Figure IIIA in the Data Supplement). Two-way ANOVA analysis revealed that there was no interaction effect between genotype and treatment on HDL-C exposure (2-way ANOVA, P=0.840 for genotype×treatment). Thus, in hepatic SRB1 knockdown mice, β3-AR agonism still increased HDL-C levels (Figure 1A and 1G) and HDL-C exposure (+21%, shSRB1+β versus β; +26%, shSRB1+β versus shSRB1; Figure 1B).

As expected, β3-AR agonism reduced plasma total cholesterol (TC) levels (Figure 1C), resulting in reduced TC exposure (−18%, β versus ctrl, Figure 1D). Interestingly, hepatic SRB1 knockdown per se also decreased plasma TC levels (Figure 1C) and TC exposure (−15%, shSRB1 versus ctrl, Figure 1D) as compared with vehicle-treated control mice, and in SRB1 knockdown condition, β3-AR agonism still reduced plasma TC levels (Figure 1C) and TC exposure (−14%, shSRB1+β versus shSRB1, Figure 1D). The reduced TC by β3-AR agonism was attributed to non-HDL-C in both control mice (−21%, β versus ctrl) and SRB1 knockdown mice (−19%, shSRB1+β versus shSRB1; Figure 1E through 1G). In addition, SRB1 knockdown additively improved non-HDL-C-lowering effects of β3-AR agonism (−15%, shSRB1+β versus β; −19%, shSRB1+β versus shSRB1; Figure 1E through 1G). Taken together, both brown fat activation and hepatic SRB1 knockdown in *E3L.CETP* mice improve dyslipidemia by reducing plasma non-HDL-C levels and increasing HDL-C levels, without significant interaction between brown fat activation and hepatic SRB1 knockdown.

Hepatic SRB1 Knockdown Decreases Plasma Triglycerides and Additively Improves β3-AR Agonism-Induced Reduction in Plasma Triglyceride Levels

β3-AR agonism reduced plasma triglyceride levels (Figure 2A) and triglyceride exposure (−34%, β versus ctrl; Figure 2B). Notably, hepatic SRB1 knockdown per se also decreased plasma triglyceride levels (Figure 2A) and triglyceride exposure (−29%, shSRB1 versus ctrl; Figure 2B). Under condition of hepatic SRB1 knockdown, β3-AR agonism still reduced plasma triglyceride levels (Figure 2A) and triglyceride exposure (−23%; Figure 2B), although there was a marginal interaction effect of hepatic SRB1 knockdown on triglyceride-lowering effects of β3-AR agonism (2-way ANOVA, P=0.036 for genotype×treatment). Triglyceride distribution over lipoproteins revealed that the reduced triglyceride was mainly confined to VLDL (Figure 2C). In control mice, β3-AR agonism did not affect the hepatic expression of genes related to VLDL production (ie, *Apob*, *Srebp1c*, and *Mttp*). In SRB1 knockdown mice, β3-AR agonism did not affect the expression of *Apob* and *Srebp1c*, while increasing *Mttp* expression (+25%, shSRB1+β versus shSRB1).

To exclude an effect of the AAV8 vector per se on plasma lipids, in a second experiment, we treated an additional group of mice with an AAV8 loaded with a scrambled shRNA sequence (scrambled). Indeed, after 6 weeks of treatment, scrambled-AAV8 did not induce any significant effects on body and liver weight or plasma lipid levels, while we again observed that shSRB1-AAV8 increased plasma

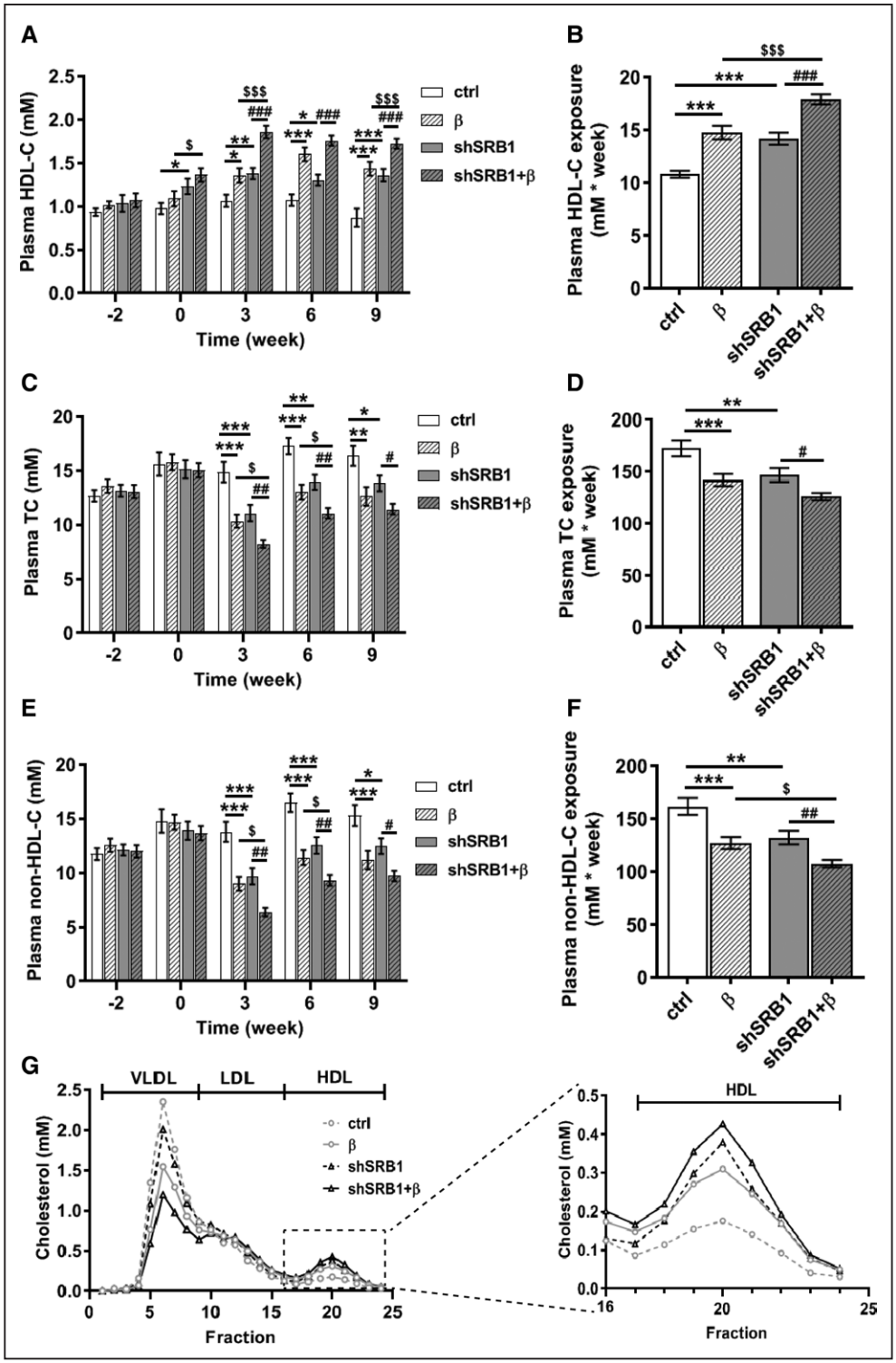

Figure 1. Hepatic SRB1 (scavenger receptor class B type 1) knockdown increases HDL (high-density lipoprotein)-C and decreases non-HDL-C and additively improves β3-adrenergic receptor (AR) agonism-induced reduction in plasma non-HDL-C and increase in HDL-C levels.

E3L.CETP mice fed a Western-type diet and pretreated with vehicle (ctrl) or an adenoassociated virus serotype 8 loaded with an shRNA targeting SRB1 (scavenger receptor class B type 1; shSRB1) at wk 2 were treated with vehicle or the β3-AR agonist CL316 243 (β) from wk 0 for 9 wks. Plasma samples were collected at indicated time points to determine plasma levels of (**A**) HDL cholesterol (-C), (**C**) total cholesterol (TC), and (**E**) non-HDL-C. **B**, Plasma HDL-C exposure, (**D**) plasma TC exposure, and (**F**) plasma non-HDL-C exposure were calculated accordingly (n=10/11 mice per group). **G**, Plasma samples obtained at 9 wks were pooled per group to determine cholesterol distribution over lipoproteins. Values are mean±SEM. Differences between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs ctrl; $*P*<0.05, $$$*P*<0.001 vs β; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs shSRB1.

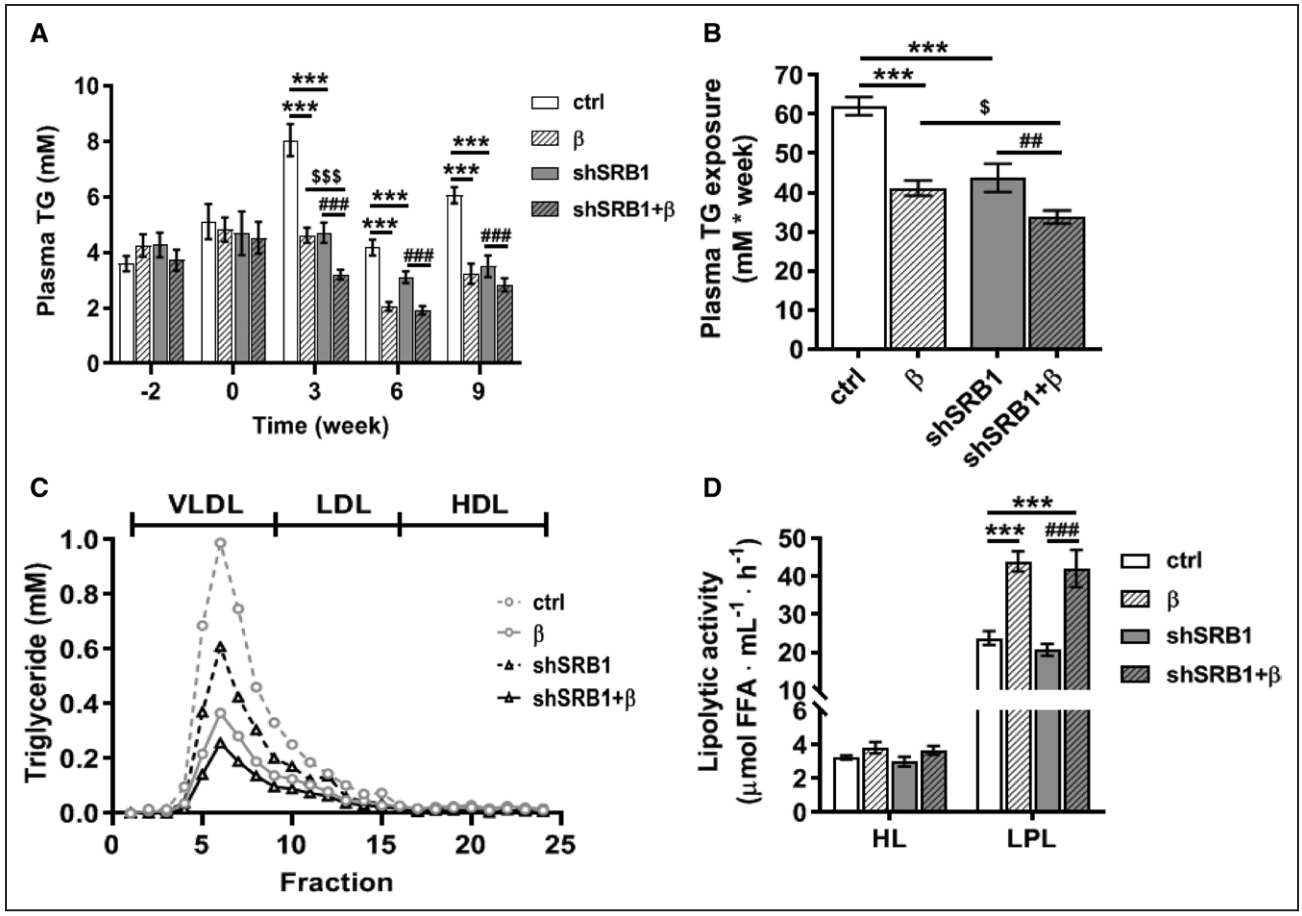

Figure 2. Hepatic SRB1 (scavenger receptor class B type 1) knockdown decreases plasma triglycerides (TGs) and additively improves β3-adrenergic receptor (AR) agonism-induced reduction in plasma TG levels.
E3L.CETP mice fed a Western-type diet and pretreated with vehicle (ctrl) or an adenoassociated virus serotype 8 loaded with an shRNA targeting SRB1 (shSRB1) at wk-2 were treated with vehicle or the β3-AR agonist CL316 243 (β) from wk 0 for 9 wks. Plasma samples were collected at indicated time points to determine (**A**) plasma TG levels and (**B**) plasma TG exposure (n=10/11 mice per group). **C**, Plasma samples at 9 wks were pooled per group to determine TG distribution over lipoproteins. **D**, After 8 wks of vehicle or CL316 243 treatment, mice were intravenously injected with heparin. Before and 10 min after injection plasma was collected. Endogenous HL (hepatic lipase) and LPL (lipoprotein lipase) activity was determined using a substrate mixture containing glycerol tri[^{3}H]oleate-labeled VLDL (very low density lipoprotein)-like particles in the presence or absence of 1 M NaCl, which inhibits LPL activity (n=10/11 mice per group). Values are mean±SEM. Differences between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test. ***P<0.001 vs ctrl; \$$P$<0.05, \$\$\$$P$<0.001 vs β; ##$P$<0.01, ###$P$<0.001 vs shSRB1. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.

HDL-C levels (+30%, shSRB1 versus ctrl; +50%, shSRB1 versus scrambled) and reduced plasma levels of TC (−20%, shSRB1 versus ctrl; −25%, shSRB1 versus scrambled), non-HDL-C (−25%, shSRB1 versus ctrl; −31%, shSRB1 versus scrambled), and triglyceride (−40%, shSRB1 versus ctrl; −40%, shSRB1 versus scrambled; Figure IVC through IVF in the Data Supplement).

β3-AR agonism did not influence hepatic gene expression of hepatic lipase (*Hl*), lipoprotein lipase (*Lpl*), and *Angptl4* but increased *Apoc2* expression in both control (+19%, β versus ctrl) and SRB1 knockdown mice (+27%; shSRB1+β versus shSRB1; Table), which may imply that β3-AR agonism increases endogenous LPL activity. Indeed, postheparin plasma was collected after 8 weeks of vehicle or CL316243 treatment to show that β3-AR agonism increased total endogenous LPL activity in both control mice (+84%, β versus ctrl) and shSRB1 knockdown mice (+76%, shSRB1+β versus ctrl), without effects on endogenous HL (hepatic lipase) activity (Figure 2D). SRB1 knockdown per se did not influence HL or LPL activity (Figure 2D). Together, these data show that brown fat activation in *E3L.CETP* mice reduces plasma triglyceride related to enhanced endogenous LPL activity, both of which were not influenced by hepatic SRB1 knockdown.

Hepatic SRB1 Knockdown on Top of β3-AR Agonism Increases VLDL Surface Phospholipid Transfer and VLDL Clearance, Without Affecting Kinetics of HDL Cholesteryl Ether

Next, we investigated the effect of SRB1 knockdown in combination with β3-AR agonism on plasma VLDL

catabolism by injection of VLDL-mimicking particles[22] that were double-labeled with [^{3}H]dipalmitoylphosphatidylcholine (ie, surface marker) and [^{14}C]cholesteryl oleate (ie, core marker). β3-AR agonism accelerated [^{3}H]dipalmitoylphosphatidylcholine clearance from the circulation in control and SRB1 knockdown mice (Figure 3A). β3-AR agonism increased total activity of [^{3}H]dipalmitoylphosphatidylcholine transferred to HDL in SRB1 knockdown mice as an indication of increased surface phospholipid transfer to HDL, while no effect was observed in control mice (Figure 3B). In addition, β3-AR agonism increased the ratio of [^{3}H]dipalmitoylphosphatidylcholine in HDL over non-HDL in both control and SRB1 knockdown *E3L.CETP* mice (Figure 3C).

Although β3-AR agonism and hepatic SRB1 knockdown downregulated hepatic LDL receptor (*Ldlr*) and LDLR related protein 1 (*Lrp1*) expression (Table), combination treatment increased LDLR protein levels (Figure IA, IC, and ID in the Data Supplement). β3-AR agonism increased [^{14}C]cholesteryl oleate clearance from the circulation (Figure 3D) and increased uptake of [^{14}C]cholesteryl oleate by the liver in both control mice (+26%, β versus ctrl) and SRB1 knockdown mice (+28%, shSRB1+β versus shSRB1; Figure 3E). SRB1 knockdown combined with β3-AR agonism further increased plasma clearance (Figure 3D) and tended to further increase hepatic uptake of [^{14}C]cholesteryl oleate (*P*=0.078, +18%, shSRB1+β versus β; Figure 3E).

As it is possible that HDL-cholesteryl esters could be transferred to (V)LDL in the presence of CETP and subsequently taken up by the liver to contribute the cholesterol-lowering effects of hepatic SRB1 knockdown, we determined the effects of the various treatments on hepatic *CETP* expression and plasma CETP levels. While the combination treatment decreased hepatic *CETP* expression, hepatic SRB1 knockdown, β3-AR agonism, and the combination did not influence plasma CETP levels (Figure IIIB in the Data Supplement) or plasma decay and hepatic uptake of HDL-cholesteryl oleyl ether (Figure VA and VB in the Data Supplement).

Collectively, these data suggest that hepatic SRB1 knockdown increases plasma HDL as avid acceptor of VLDL-derived surface remnants, which accelerates LPL-mediated VLDL lipolysis and subsequent hepatic uptake of VLDL core remnants.

Hepatic SRB1 Knockdown Additively Improves β3-AR Agonism-Induced Attenuation in Atherosclerosis Development

After 9 weeks of hepatic SRB1 knockdown combined with β3-AR agonism, atherosclerosis development was evaluated. The aortic roots of the hearts were isolated and stained to evaluate atherosclerotic lesion area and severity. β3-AR agonism markedly reduced atherosclerotic lesion area through the aortic root (Figure 4A and 4B), resulting in lower mean atherosclerotic lesion area (−38%, β versus ctrl; Figure 4C). Hepatic SRB1 knockdown per se also clearly reduced atherosclerotic lesion area (−42%, shSRB1 versus ctrl; Figure 4A through 4C) and lesion severity (Figure 4A and 4D) as compared with vehicle-treated control mice. SRB1 knockdown did not abrogate β3-AR agonism induced attenuation in atherosclerotic lesion area (2-way ANOVA, *P*=0.402 for genotype×treatment). Rather, β3-AR agonism in SRB1 knockdown mice further reduced lesion area (−43%, shSRB1+ β versus β; −39%, shSRB1+ β versus shSRB1; Figure 4A through 4C). We further analyzed association between plasma lipid exposure and atherosclerosis development. Non-HDL-C exposure (R^2=0.315, *P*<0.0001; Figure 4E), HDL-C exposure (R^2=0.190, *P*=0.004; Figure 4F), and triglyceride exposure (R^2=0.202, *P*=0.003; Figure 4G) were all associated with the square root (SQRT) of atherosclerotic lesion area, indicating all these factors contributed to the reduced atherosclerosis development.

Finally, we characterized atherosclerotic lesion composition by analyzing the relative plaque content of smooth muscle cells, collagen, and macrophages. β3-AR agonism and SRB1 knockdown per se, as well as their combination had no significant effect on lesion composition including smooth muscle cells, collagen, and macrophages (Figure 5A through 5D, Figure VI in the Data Supplement) and did not affect the ratio of stable markers (ie, smooth muscle cell and collagen area) versus the unstable marker (ie, macrophage area; Figure 5E). β3-AR agonism under the condition of SRB1 knockdown increased the ratio of smooth muscle cell and collagen area versus macrophage area (+28%, shSRB1+β versus β; Figure 5E).

DISCUSSION

Previously, we demonstrated that brown fat activation by β3-AR agonism reduced atherosclerosis development, which could be explained by both LDLR-dependent hepatic clearance of TRL remnants[7] and increased SRB1-mediated reverse cholesterol transport.[8] While previous studies have demonstrated the crucial role of the ApoE-LDLR pathway in the antiatherogenic effects of brown fat activation,[7,23] the specific role of hepatic SRB1 in the antiatherogenic effect of brown fat activation was still obscure. By using the *E3L.CETP* mouse model, we now demonstrated that hepatic SRB1 knockdown in the presence of an intact ApoE-LDLR clearance pathway for TRL remnants did not attenuate the antiatherogenic effects of β3-AR activation. Rather, hepatic SRB1 knockdown per se appeared to improve hyperlipidemia and consequently reduce atherosclerosis development.

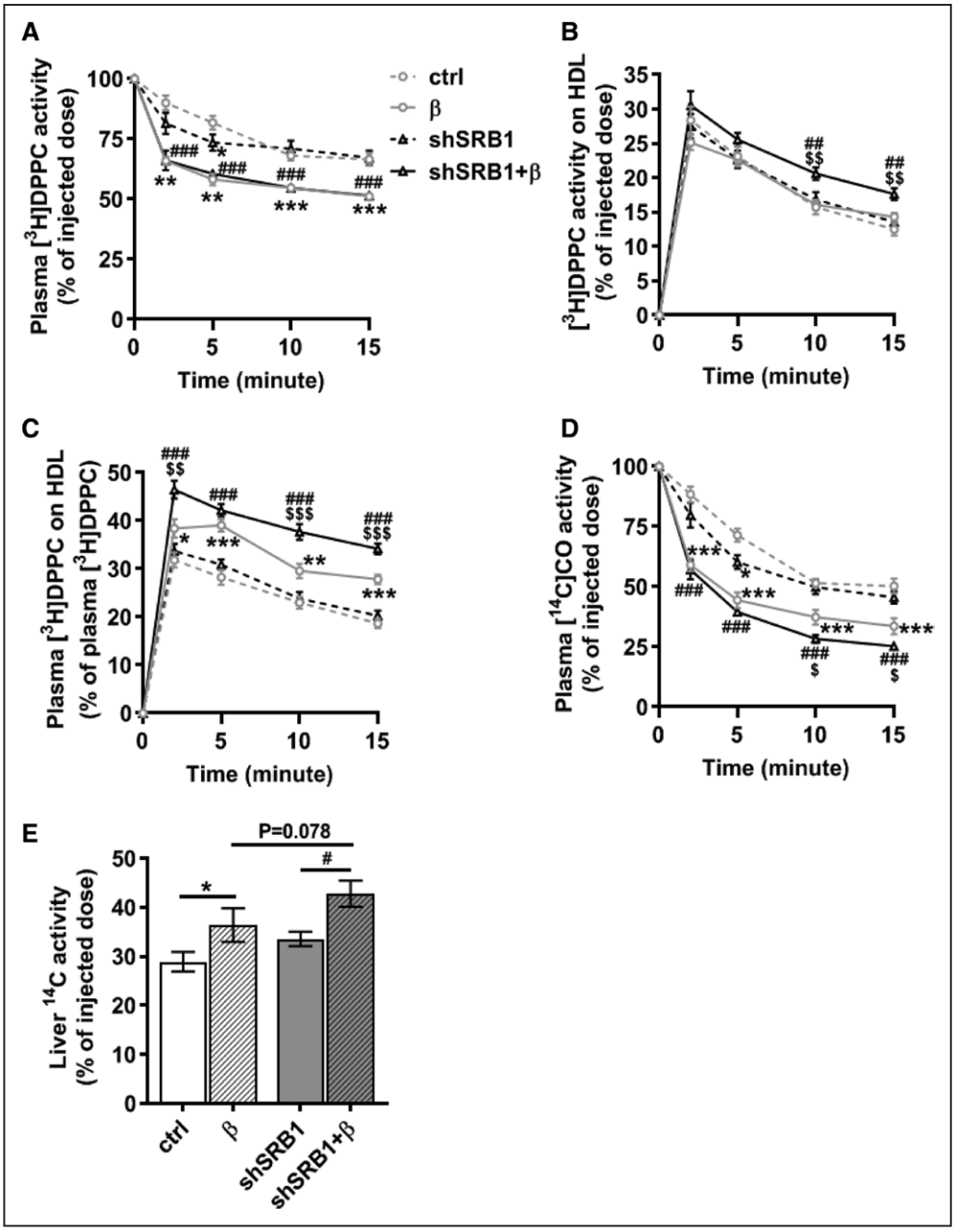

Figure 3. Hepatic SRB1 (scavenger receptor class B type 1) knockdown on top of β3-adrenergic receptor (AR) agonism increases VLDL (very low-density lipoprotein) surface phospholipid transfer and VLDL clearance.
E3L.CETP mice fed a Western-type diet and pretreated with vehicle (ctrl) or an adenoassociated virus serotype 8 loaded with an shRNA-targeting SRB1 (shSRB1) at wk-2 were treated with vehicle or the β3-AR agonist CL316 243 (β) from wk 0. At wk 9, mice were intravenously injected with [^{3}H]dipalmitoylphosphatidylcholine (DPPC) and [^{14}C]cholesteryl oleate ([^{14}C]CO) doubly labeled VLDL-mimicking particles. Plasma samples were collected at indicated time points and clearance of (**A**) [^{3}H]DPPC and (**D**) [^{14}C]CO was determined. HDL (high-density lipoprotein) was isolated to determine (**B**) [^{3}H]DPPC activities on HDL and (**C**) ratio of [^{3}H]DPPC activity of HDL over non-HDL. **E**, Hepatic uptake of [^{14}C]CO was measured after 15 min of particle injection (n=10/11 mice per group). Values are means±SEM. Differences between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test. *P<0.05, **P<0.01, ***P<0.001 vs ctrl; $$P$<0.05, $$$P$<0.01, $$$$P$<0.001 vs β; #$P$<0.05, ##$P$<0.01, ###$P$<0.001 vs shSRB1.

First, we showed that partial knockdown of hepatic SRB1 (40%–50%) in *E3L.CETP* mice increases HDL-C as well as apoAI without an obvious increase in HDL size, indicating an increase in HDL pool size[24] and does not affect the clearance of [^{3}H]COEth-HDL from plasma. This may be somewhat counterintuitive, since SRB1-deficiency on a wild-type background largely increases HDL size in addition to increased HDL-C and delays plasma clearance of [^{3}H]COEth-HDL.[25] It should, however, be noted we previously also showed that cross-breeding of SRB1-deficient mice with CETP-transgenic mice largely prevents the increase in HDL size as well as

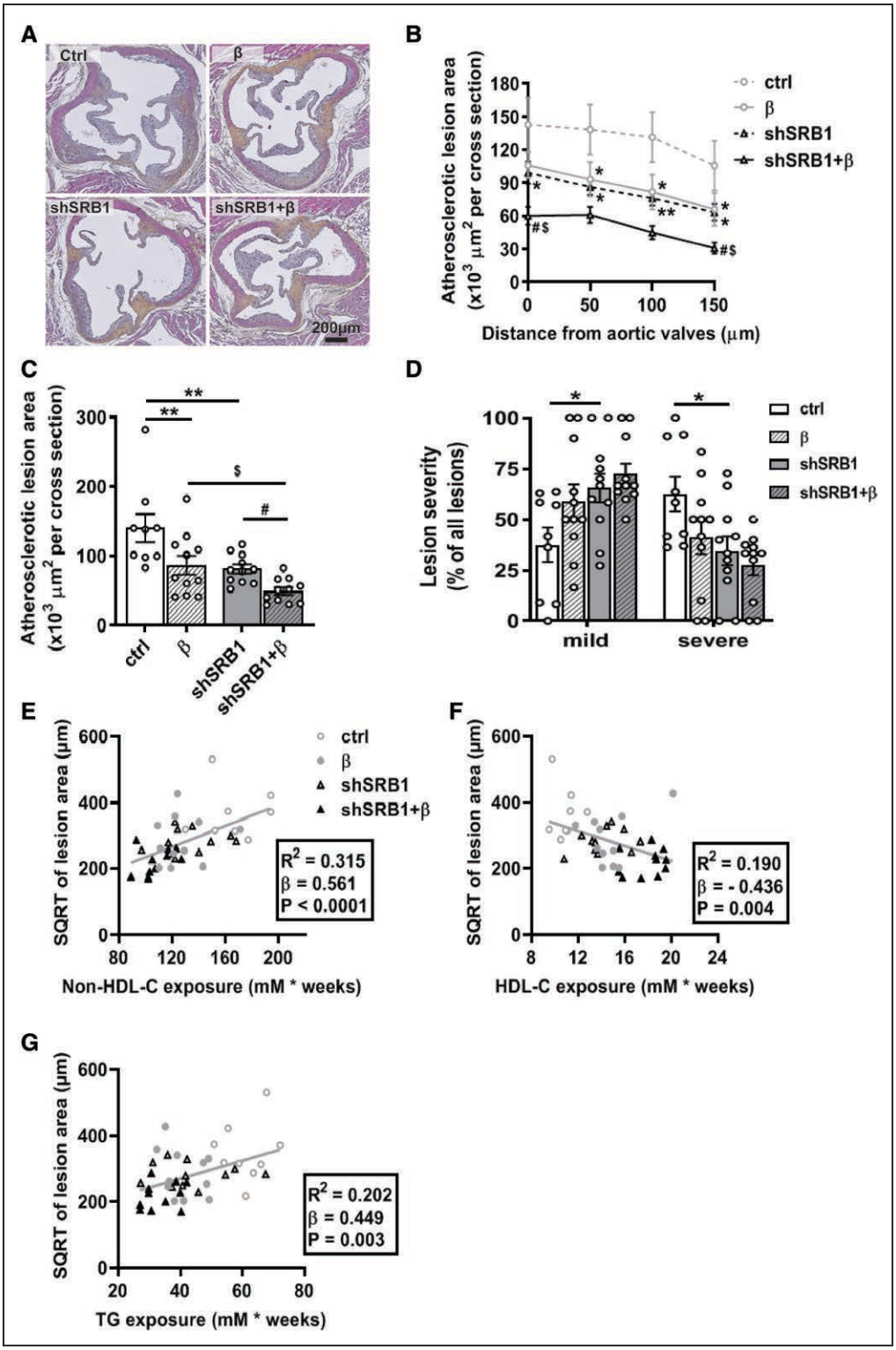

Figure 4. Hepatic SRB1 (scavenger receptor class B type 1) knockdown additively improves β3-adrenergic receptor (AR) agonism-induced attenuation in atherosclerosis development.
E3L.CETP mice fed a Western-type diet and pretreated with vehicle (ctrl) or an adenoassociated virus serotype 8 loaded with an shRNA targeting SRB1 (shSRB1) at wk-2 were treated with vehicle or the β3-AR agonist CL316 243 (β) from wk 0 for 9 wks. **A**, Hearts were collected and cross-sections of the aortic roots were stained with hematoxylin-phloxine-saffron and representative pictures of atherosclerotic lesions of each group are presented. **B**, Plaque lesion area as a function of distance from the appearance of open valves and (**C**) mean atherosclerotic lesion area were calculated. **D**, Lesions were categorized according to lesion severity. The square root (SQRT) of the mean atherosclerotic lesion area was plotted against the plasma (**E**) non-HDL (high-density lipoprotein) cholesterol (-C) exposure, (**F**) HDL-C exposure, and (**G**) triglyceride (TG) exposure (n=7–11 mice per group). Values are means±SEM. Differences between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test. **P*<0.05, ***P*<0.01 vs ctrl; $*P*<0.05 vs β; #*P*<0.05 vs shSRB1.

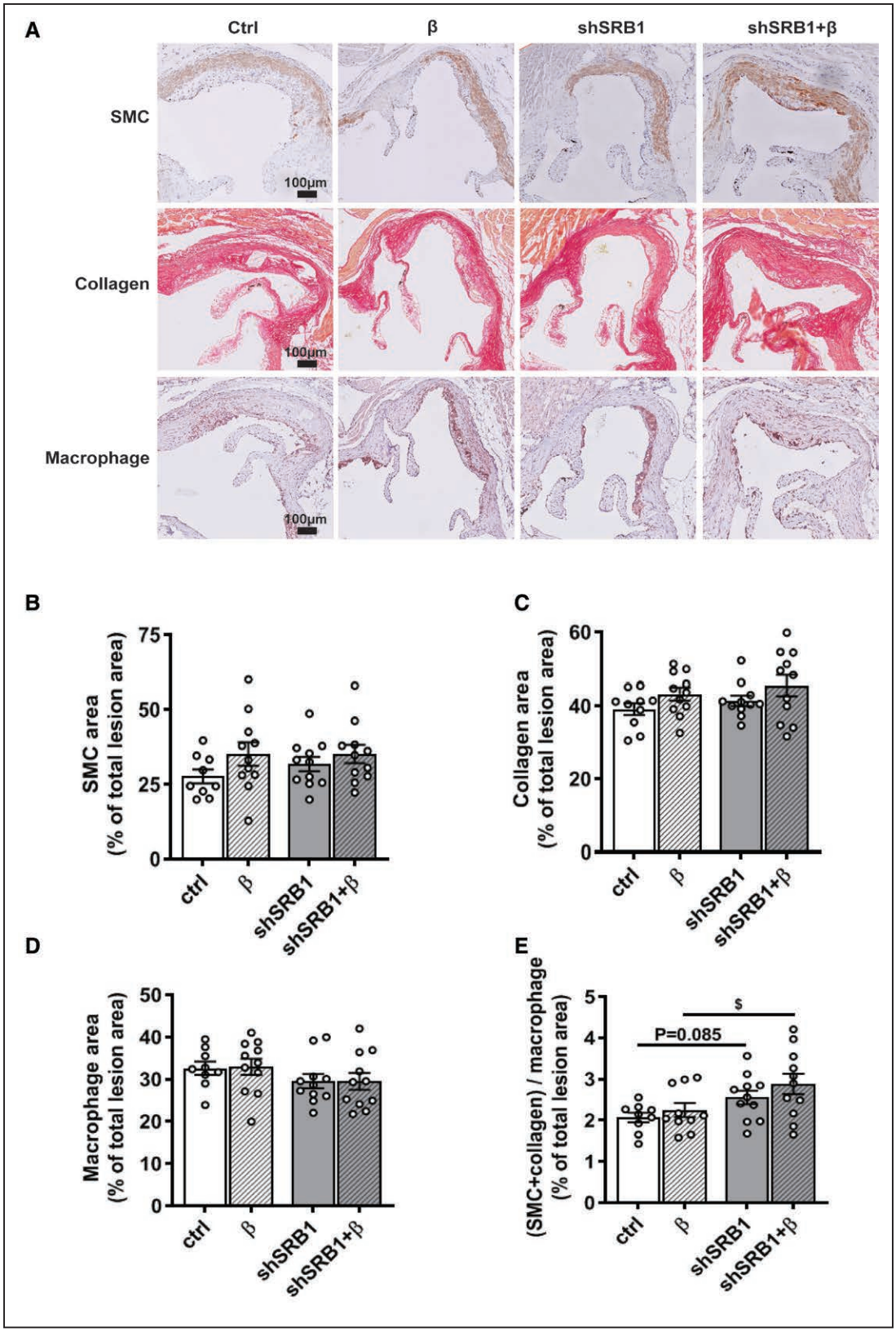

Figure 5. Hepatic SRB1 (scavenger receptor class B type 1) knockdown on top of β3-adrenergic receptor (AR) agonism increases the ratio of smooth muscle cell and collagen area to macrophage area.
E3L.CETP mice fed a Western-type diet and pretreated with vehicle (ctrl) or an adenoassociated virus serotype 8 loaded with an shRNA-targeting SRB1 (shSRB1) at wk-2 were treated with vehicle or the β3-AR agonist CL316 243 (β) from wk 0 for 9 wks. Cross-sections of aortic root were stained for (**A** and **B**) smooth muscle cells (SMCs), (**A** and **C**) collagen, and (**A** and **D**) macrophages, and their relative areas within the lesions were quantified. **E**, The ratio of SMC area and collagen area to macrophage area was calculated (n=9/11 mice per group). Values are means±SEM. Differences between 2 groups were determined using 2-way ANOVA followed by a Fisher LSD post hoc test. $$P<0.05$ vs β.

the delay in plasma decay of [^{3}H]COEth-HDL resulting from SRB1-deficiency, while HDL-C was still increased, in mice fed a Western-type diet.[25] These findings can be explained by CETP-mediated transfer of HDL-CE to apoB (apolipoprotein B)-containing particles, which prevents CE accumulation in HDL and thereby prevents an obvious increase in HDL size, at least judged from FPLC profiling, as well as a delay in HDL-CE clearance. Nevertheless, the precise reason for the increase in ApoAI, as thus HDL pool size, is still unclear and deserves attention in future research.

Hepatic SRB1 knockdown also caused a striking reduction in triglyceride and cholesterol within non-HDL. This may seem surprising given previous findings suggesting that SRB1 facilitates hepatic TRL remnant uptake.[26] Likely, the increased HDL resulting from SRB1 knockdown forms a larger pool of acceptors for TRL surface remnants generated during LPL-mediated lipolysis of VLDL, as we showed by increased transfer of TRL-derived phospholipids to HDL. As a consequence, lipolytic TRL processing is accelerated followed by avid hepatic clearance of TRL remnants via the ApoE-LDLR pathway in *E3L.CETP* mice. Apparently, this effect occurs under conditions of unchanged endogenous lipolytic activity. At the same time, in *E3L.CETP* mice, the build up of cholesterol in HDL induced by partial hepatic SRB1-knockdown can be partially relieved by CETP-mediated transfer of HDL-associated cholesteryl esters to TRL with subsequent clearance via the ApoE-LDLR pathway.[27] This may explain why plasma clearance and hepatic uptake of HDL-cholesteryl ether was not influenced by hepatic SRB1 knockdown. In line with our findings, a genetic variant (P297S) of SCARB1, the gene encoding SRB1 in humans also tended to decrease VLDL-C (−44%, P=0.07) besides raising HDL-C as compared with family controls,[28] and a more recent meta-analysis showed that the SCARB1 rs5888 polymorphism associates with lower plasma triglyceride levels besides higher HDL-C in men.[29] Collectively, our data confirm the relevance of our mouse model for human-like lipid metabolism and are consistent with the notion that hepatic SRBI knockdown improves TRL metabolism in the presence of an intact ApoE-LDLR clearance pathway.

Furthermore, we observed that hepatic SRB1 knockdown attenuated atherosclerosis development in *E3L.CETP* mice. Recently, Huang et al[30] reported that endothelial cell–specific SRB1 deficiency in ApoE$^{-/-}$ mice protects against atherosclerosis development, via blocking endothelial LDL transcytosis without effects on plasma TC, triglyceride, and HDL levels. Such a mechanism seems unlikely to explain the reduction in atherosclerosis observed in our model, as shSRB1-AAV8 specifically downregulated SRB1 expression in the liver and even increased SRB1 protein levels in aortas. At first glance, the reduction of atherosclerosis induced by selective hepatic SRB1 knockdown may seem surprising given that SRB1-deficiency aggravated atherosclerosis development in ApoE$^{-/-}$ mice[12] and in LDLR$^{-/-}$ mice, without lowering non-HDL-C.[13] Likely, these opposing effects of SRB1 on atherosclerosis are explained by the difference in genetic backgrounds between these disease models. As the hepatic ApoE-LDLR clearance pathway for TRL remnants is absent in both ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, SRB1 deficiency does not reduce non-HDL-C and the increased atherosclerosis is likely explained by increased oxidative stress.[25] In favorable contrast, *E3L.CETP* mice have an intact ApoE-LDLR clearance pathway and CETP, which facilitate hepatic clearance of cholesterol from TRL remnants and indirectly from HDL, thereby lowering non-HDL-C-induced atherosclerosis development. In support of our findings, inhibition of hepatic SRB1 by RNA interference also attenuated atherosclerosis in rabbits, an animal model that naturally expresses CETP.[31] Although a rare variant in SCARB1 has been reported to increase the risk for coronary heart disease,[32] studies investigating other SCARB1 mutations did not show either increased atherosclerosis or CAD.[28,33] As large-scale human studies addressing the role of SRB1 in cardiovascular disease are still lacking, the jury on the precise role of SRB1 is still out. Based on our study in *E3L.CETP* mice and previous observations in rabbits, loss-of-function SCARB1 mutations in humans may in fact slow down atherosclerosis progression and could in fact be cardioprotective.

Given that SRB1 deficiency in *E3L.CETP* mice reduces non-HDL-C and attenuates atherosclerosis progression, it is not surprising that SRB1 deficiency improved the atheroprotective effects of brown fat activation by β3-AR agonism. Previously, we demonstrated that brown fat activation protects against atherosclerosis development, which was largely due to accelerated TRL remnant clearance via the ApoE-LDLR pathway.[7] Since SRB1 knockdown apparently evoked the same effect, the combined effect of β3-AR agonism and SRB1 knockdown was an additive reduction in non-HDL-C, which can be further explained since combination treatment resulted in higher hepatic LDL protein levels likely caused by reduced hepatic *Pcsk9* expression. Given the tight relation between non-HDL-C exposure and lesion size, this translated in an additive reduction in atherosclerosis. In this study, we mainly focused on effects on lipid metabolism, while brown fat activation displays additional benefits, including decreased inflammation,[34] decreased oxidative stress,[35] and improved glucose metabolism.[34,36] Further studies will be needed to investigate whether hepatic SRB1 pathway may contribute to those potentially antiatherogenic benefits of brown fat activation.

The findings from the present study are likely of clinical relevance. Brown fat is functional in both healthy and obese individuals,[37,38] long-term brown fat activation by cold exposure reduces low density lipoprotein-cholesterol in hypercholesterolemic patients[39] and brown

fat activity is associated with a reduced risk of CVD events.[40] A series of studies shows that Mirabegron, a β3-AR agonist clinically approved for the treatment of overactive bladder, induces beiging of subcutaneous white adipose tissue and increases brown fat activity in humans. This results in improvement in glucose homeostasis and increase in whole-body energy expenditure, plasma HDL-C levels, as well as other beneficial metabolic biomarkers.[9,41–44] Therefore, pharmacological strategies to activate brown fat in humans may prove to reduce CVD risk. Since hepatic SRB1 knockdown apparently does not interfere with the antiatherogenic effects of brown fat activation, such pharmacological strategies may be equally effective in those individuals with compromised SRB1 function, which would be subject for future studies.

In conclusion, by using a relevant mouse model for human-like lipoprotein metabolism, that is, with an intact ApoE-LDLR clearance pathway, we showed that hepatic SRB1 knockdown not only increases HDL-C but also lowers non-HDL-C and attenuates atherosclerosis development. As such, combined brown fat activation and hepatic SRB1 knockdown additively improve the antiatherogenic properties of brown fat activation.

ARTICLE INFORMATION

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Disclosures

None.

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