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Atorvastatin accelerates clearance of lipoprotein remnants generated by activated brown fat to further reduce hypercholesterolemia and atherosclerosis

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ABSTRACT

Background and aims: Activation of brown adipose tissue (BAT) reduces both hyperlipidemia and atherosclerosis by increasing the uptake of triglyceride-derived fatty acids by BAT, accompanied by formation and clearance of lipoprotein remnants. We tested the hypothesis that the hepatic uptake of lipoprotein remnants generated by BAT activation would be accelerated by concomitant statin treatment, thereby further reducing hypercholesterolemia and atherosclerosis.

Methods: APOE*3-Leiden.CETP mice were fed a Western-type diet and treated without or with the selective β3-adrenergic receptor (AR) agonist CL316,243 that activates BAT, atorvastatin (statin) or both.

Results: β3-AR agonism increased energy expenditure as a result of an increased fat oxidation by activated BAT, which was not further enhanced by statin addition. Accordingly, statin treatment neither influenced the increased uptake of triglyceride-derived fatty acids from triglyceride-rich lipoprotein-like particles by BAT nor further lowered plasma triglyceride levels induced by β3-AR agonism. Statin treatment increased the hepatic uptake of the formed cholesterol-enriched remnants generated by β3-AR agonism. Consequently, statin treatment further lowered plasma cholesterol levels. Importantly, statin, in addition to β3-AR agonism, also further reduced the atherosclerotic lesion size as compared to β3-AR agonism alone, without altering lesion severity and composition.

Conclusions: Statin treatment accelerates the hepatic uptake of remnants generated by BAT activation, thereby increasing the lipid-lowering and anti-atherogenic effects of BAT activation in an additive fashion. We postulate that, in clinical practice, combining statin treatment with BAT activation is a promising new avenue to combat hyperlipidemia and cardiovascular disease.

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1. Introduction

Cardiovascular diseases (CVD) are the number one cause of death in the Western society [1]. The main underlying pathology of CVD is atherosclerosis, for which hyperlipidemia is a major risk factor [2]. Statins are currently the main strategy to lower plasma nonHDL-C levels in patients with elevated risk for CVD. Although statins reduce total cholesterol (TC) levels by approximately 30% [3], they only prevent 25-45% of all cardiovascular events [1], urging the need for additional therapies.

Brown adipose tissue (BAT) was discovered in rodents as an important player in lipid metabolism [4], and was more recently shown to also contribute to lipid metabolism in humans [5]. BAT is physiologically activated by cold, resulting in release of noradrenalin from sympathetic nerve endings within BAT, which subsequently binds to the β3-adrenergic receptor (β3-AR), present on the membrane of brown adipocytes [6]. Upon activation of brown and beige adipocytes, TG-derived fatty acids (FA) are released from the numerous intracellular lipid droplets and directed towards the mitochondria. Here, they are either oxidized and used for oxidative phosphorylation [6] or to allosterically activate uncoupling protein 1 (UCP1) [7], which uncouples the oxidative phosphorylation from ATP synthase, resulting in the generation of heat instead of ATP. As a consequence of intracellular FA combustion, the intracellular lipid stores become depleted and are subsequently replenished by the uptake of TG-derived FA after lipoprotein lipase (LPL)-mediated lipolysis of TG-rich lipoproteins (TRL) (i.e. VLDL and chylomicrons) [8]. In fact, activated BAT can take up such large amounts of FA that it normalizes plasma TG levels in a hypertriglyceridemic mouse model [4].

We have recently reported that BAT activation using the specific β3-AR agonist CL316,243 reduces both hyperlipidemia and atherosclerosis development by increasing the uptake of TG-derived FA from TRL by BAT, and accelerating subsequent formation and hepatic uptake of the cholesterol-enriched lipoprotein remnants in APOE*3-Leiden.CETP (E3L.CETP) mice, a well-established model of human-like lipoprotein metabolism [9]. Of note, an intact apolipoprotein E (apoE)-hepatic LDL receptor (LDLR) pathway is crucial for hepatic uptake of cholesterol-enriched remnants [9]. Since statins increase the LDLR-mediated uptake of lipoproteins remnants [10], we hypothesized that statin treatment increases the lipid-lowering and anti-atherogenic effects of BAT activation by accelerating the clearance of TRL remnants generated by BAT activation.

To test our hypothesis, we treated hyperlipidemic E3L.CETP mice without or with the β3-AR agonist CL316,243, atorvastatin, or a combination of both. We showed that statin treatment accelerated the hepatic uptake of cholesterol-enriched lipoprotein remnants as generated by β3-AR agonism. Consequently, statin treatment further increased the lipid-lowering and anti-atherogenic effects induced by β3-AR agonism.

2. Materials and methods

2.1. Animals and treatment

Hemizygous APOE*3-Leiden (E3L) mice were crossbred with homozygous human cholesteryl ester transfer protein (CETP) transgenic mice to generate heterozygous E3L.CETP mice [11]. In all studies described below, 10-12 week old female E3L.CETP mice were housed under standard conditions in conventional cages with a 12-hour light/dark cycle, at room temperature (22 °C), and with ad libitum access to food and water.

During all studies, mice were fed a Western-type diet (WTD; AB diets, Woerden, The Netherlands) containing 15% cacao butter, 1% corn oil and 0.15% (w/w) cholesterol (composition of the diet is listed in Supplemental Table 1). After a run-in period of 3 weeks, mice were randomized into 2 groups that received WTD supplemented without or with atorvastatin (statin; 0.0036%, w/w). After an additional run-in period of 3 weeks, mice in each treatment group were randomized into 2 groups and additionally treated with vehicle (PBS) or the β3-adrenergic receptor (AR) agonist CL316,243 (Tocris Bioscience Bristol, United Kingdom; 20 μg/mouse; subcutaneous injections between 14.00 and 16.00 h). This resulted in the following 4 treatment groups: (1) vehicle (symbol: -), (2) CL316,243 (β), (3) statin (s), and (4) statin + CL316,243 (s+β).

For the atherosclerosis study, mice were treated with CL316,243 5 times weekly for 9 weeks. For the other (short-term) studies, mice were treated with CL316,243 daily for 2 weeks. Food intake was monitored 3 times a week. Both body weight and body composition (body fat and lean mass; EchoMRI-100; EchoMRI, Houston, TX, USA) were monitored weekly. All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and have received approval from the Animal Ethical Committee (Leiden University Medical Center, Leiden, The Netherlands).

2.2. Indirect calorimetry and physical activity

Indirect calorimetry was performed in fully automatic metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany) during the fourth week of treatment. After 1 day of acclimatization, O_2 consumption (VO_2), CO_2 production (VCO_2) and caloric intake were measured for 4 consecutive days [12]. Total energy expenditure (EE) was calculated from the VO_2 and VCO_2, and carbohydrate and fat oxidation rates were calculated from VO_2 and VCO_2 as described previously [13]. Physical activity was measured using infrared sensor frames.

2.3. Plasma lipid parameters

Blood was collected from the tail vein of 4 h fasted mice into sodium heparinized capillaries. Capillaries were placed on ice and centrifuged, and plasma was assayed for TG and TC using enzymatic kits from Roche Diagnostics (Mannheim, Germany). To measure HDL-C levels, apoB-containing lipoproteins were precipitated from plasma with 20% polyethylene glycol 6000 in 200 mmol/L glycine buffer (pH 10), and TC was measured in the supernatant as described above. Plasma nonHDL-C levels were calculated by subtraction of HDL-C from TC levels.

2.4. Plasma PCSK9

Plasma PCSK9 concentration was measured using a commercially available ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.5. In vivo plasma decay and organ uptake of TRL-like particles

TRL-like particles (45 nm), double-labeled with glycerol tri [³H] oleate ([³H]TO) and [¹⁴C]cholesteryl oleate ([¹⁴C]CO), were prepared and characterized as described previously [14]. Mice were fasted for 4 h and injected ($t = 0$) intravenously with 200 μL of VLDL-like particles (1.0 mg TG per mouse). Blood samples were taken from the tail vein at $t = 2$, 5, 10 and 15 min after injection to determine the plasma clearance of [³H]TO and [¹⁴C]CO. Plasma volumes were calculated as $0.04706 \times$ body weight (g) as previously determined from ¹²⁵I-BSA clearance studies [15]. After taking the last blood sample, mice were killed by cervical dislocation and perfused for 5 min with ice-cold PBS via the heart to remove blood

and non-internalized VLDL-like particles from the organs. Subsequently, organs and tissues were isolated, dissolved overnight at 56 °C in Tissue Solubilizer (Amersham Biosciences, Roosendaal, The Netherlands), and ^3H and ^{14}C activity were quantified. Uptake of [^3H]TO- and [^{14}C]CO-derived radioactivity by organs and tissues is expressed per gram wet tissue weight.

2.6. Hepatic lipid composition

At the end of the study, mice were killed by CO_2 inhalation. Blood was drawn by cardiac puncture for serum isolation. Subsequently, mice were perfused with ice-cold PBS and organs and tissues were weighed and collected for further analysis. Pieces of liver were collected to extract liver lipids as previously described [16]. In brief, small liver pieces (approx. 50 mg) were homogenized in ice-cold methanol (10 μL). After centrifugation, lipids were extracted by addition of 1800 μL $CH_3OH:CHCl_3$ (1:3 vol for volume [v/v]) to 45 μL homogenate. After vigorous vortexing and phase separation by centrifugation (14,000 rpm; 15 min at room temperature), the organic phase was dried and dissolved in 2% Triton X-100 in water. TG, TC (Roche Diagnostics, Mannheim, Germany) and PL concentrations (Instruchemie, Delfzijl, The Netherlands) were measured using commercial kits. Liver lipids were expressed as nmol/mg protein as determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

2.7. Gene expression analysis

RNA was extracted from snap-frozen mouse tissues (approx. 25 mg) using Tripure RNA Isolation reagent (Roche) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega) for qRT-PCR according to the manufacturer's instructions to produce cDNA. mRNA expression was normalized to *β2-microglobulin* and *36b4* mRNA expression and expressed as fold change compared to vehicle-treated mice using the ΔΔCT method. The primer sequences used are listed in Supplemental Table 2.

2.8. Western blotting

Pieces of snap-frozen mouse tissues (approx. 50 mg) were lysed, protein was isolated, and Western blots for LDLR and LRP1 were performed as previously described [17]. Primary antibodies and dilutions are listed in Supplemental Table 3. Protein content was corrected for a control mix on each blot and for the housekeeping protein tubulin. Bands were visualized by increased chemiluminescence and quantified using Image Lab.

2.9. Histology

After necropsy, interscapular BAT (iBAT) was removed, fixed in 4% paraformaldehyde, dehydrated in 70% EtOH and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (HE) using standard protocols. The area of intracellular lipid droplets in BAT was quantified using ImageJ Software (version 1.47).

2.10. Atherosclerosis quantification

Hearts were collected and fixed in phosphate-buffered 4% formaldehyde, embedded in paraffin and cross-sectioned (5 μm) throughout the aortic root area, starting from the appearance of open aortic valve leaflets. Per mouse, 4 sections with 50 μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxine-saffron for histological analysis. Lesions were categorized for severity according to the guidelines of the American Heart Association adapted for mice [18]. Various types of lesions were discerned: no lesions, mild lesions (types 1-3) and severe lesions (types 4-5). Rat monoclonal anti-mouse antibody MAC3 (1:1000; BD Pharmingen, San Diego, CA, USA) was used to quantify macrophage area. Monoclonal mouse antibody M0851 (1:800; Dako, Heverlee, The Netherlands) against smooth muscle cell (SMC) actin was used to quantify the SMC area. Sirius Red staining was used to quantify the collagen area. Lesion area and composition were analysed using ImageJ Software (version 1.47).

2.11. Statistical analysis

Differences between groups were determined using one-way ANOVA with the LSD *post hoc* test. When the variances within the treatment groups were not equal between the different treatment groups, a Kruskal-Wallis test was performed followed by Mann-Whitney. Probability values less than 0.05 were considered statistically significant. Data are presented as mean ± SEM. All statistical analyses were performed with the SPSS 20.0 software package for Windows (SPSS, Chicago, IL USA).

3. Results

3.1. Statin treatment does not influence β3-AR agonism-induced energy expenditure and fat oxidation

E3L.CETP mice were fed a Western-type diet and treated with vehicle (symbol: -), the selective β3-AR agonist CL316,243 (β), atorvastatin (s) or the combination of atorvastatin and the β3-AR agonist (s+β) for 9 weeks. None of the treatments significantly influenced food intake (Fig. 1A) or body weight (Fig. 1B). Statin treatment combined with β3-AR agonism slightly elevated body lean mass as compared to vehicle (+7%, $p<0.01$; Fig. 1C). β3-AR agonism alone, and in combination with statin treatment, reduced total body fat mass (both approx. −40%, $p<0.001$; Fig. 1D) as well as gonadal (g)WAT weight (both approx. −60%, $p<0.001$; Supplemental Fig. 1A) and subcutaneous (s)WAT weight (both approx. −40%, $p<0.001$; Supplemental Fig. 1A) as compared to vehicle. Statin treatment did not influence these β3-AR agonism-mediated effects. Housing of mice in fully-automated metabolic cages indicated that β3-AR agonism alone, and in combination with statin treatment, reduced fat mass by increasing energy expenditure (EE) (both +15%, $p<0.001$; Fig. 1E), while physical activity was unaffected (Fig. 1F). This increased EE was caused by increased fat oxidation (both approx. +45%, $p<0.05$; Fig. 1G) rather than glucose oxidation (Fig. 1H), which is fully compatible with increased BAT activity. This was further supported by more in depth analysis, which revealed a steep increase in EE and fat oxidation directly after daily CL316,243 injection (Supplemental Fig. 1B and C). Statin treatment did not influence the β3-AR agonism-mediated enhanced EE and fat oxidation (Fig. 1E and G and Supplemental Fig. 1B and C). Statin treatment thus did not influence the increased energy expenditure *per se* or as induced by β3-AR agonism.

3.2. Statin treatment does not influence β3-AR agonism-induced BAT activity

We confirmed that β3-AR agonism increased BAT activity by analyzing the lipid droplet content from histological staining of interscapular (i)BAT (Fig. 1I). β3-AR alone, and in combination with statin treatment, reduced lipid droplet content in iBAT as compared
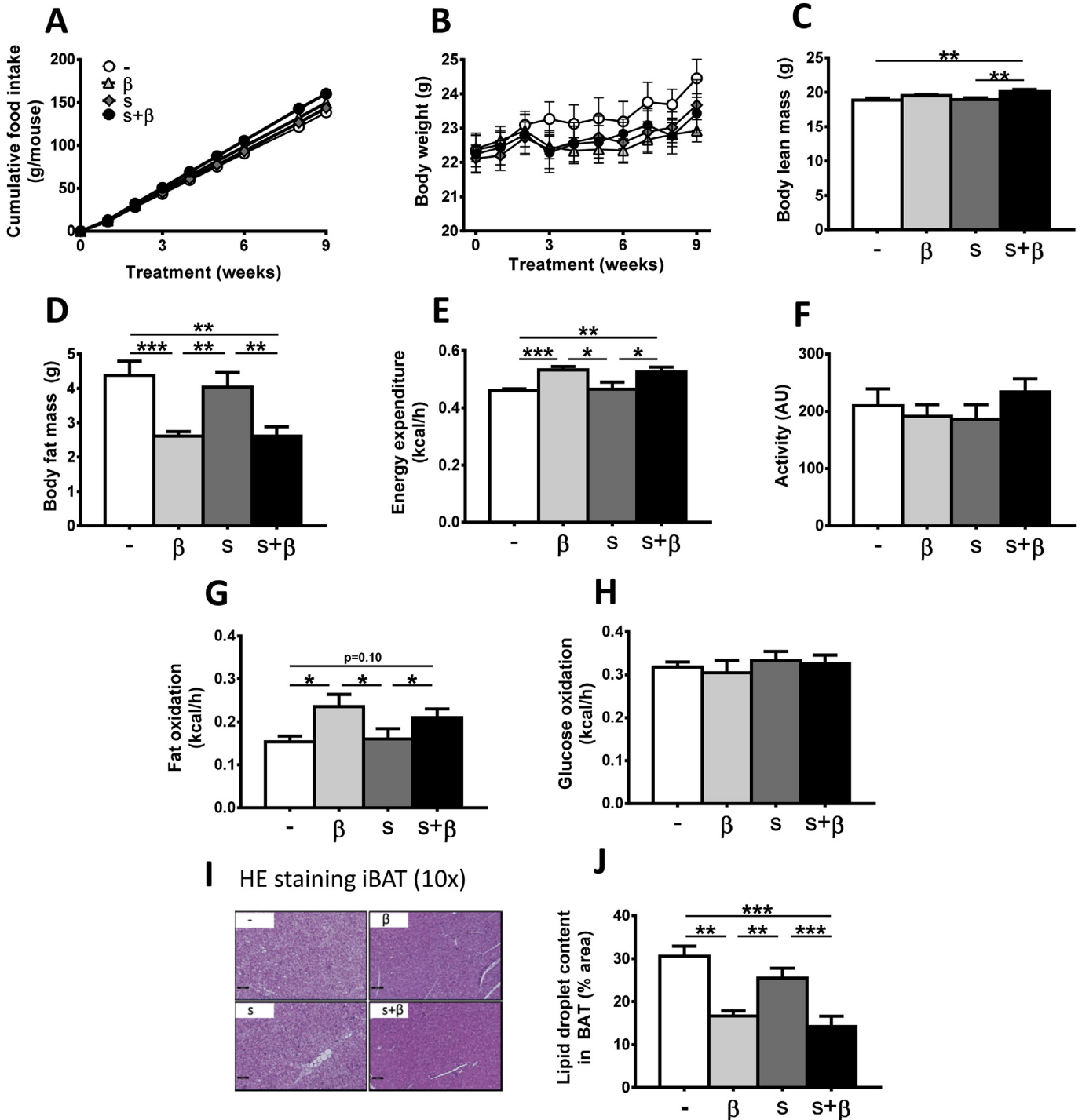
Fig. 1. The effect of β3-AR agonism, statin treatment and statin treatment combined with β3-AR agonism on fat mass, energy expenditure and BAT activity.
(A) Cumulative food intake and (B) body weight. (C) Body lean mass and (D) body fat mass after 9 weeks (n = 15-16/group). (E) Energy expenditure, (F) physical activity levels, (G) fat oxidation, and (H) glucose oxidation after 4 weeks (n = 11-12/group). (I) Representative pictures of haematoxylin-eosin (HE) staining in interscapular (i)BAT. Scale bar, 100 mm (for 10x original magnification). (J) Quantification of the lipid droplet-positive area in BAT (n = 7-8/group). -, vehicle; β, β3-AR agonism; s, statin; s+β, combination. Values are means ± SEM. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

to vehicle (both approx. −50%, $p<0.001$; Fig. 1J), which points to more activated BAT. Statin treatment neither influenced the lipid droplet content in iBAT nor the effect of β3-AR agonism. Thus, statin treatment did not activate BAT *per se*, and did not influence β3-AR agonism-mediated BAT activation.

3.3. Statin treatment in addition to β3-AR agonism additively decreases plasma nonHDL-cholesterol

Next, we assessed the effect of statin treatment in addition to β3-AR agonism on lipid and lipoprotein metabolism. β3-AR

agonism alone largely reduced plasma TG levels as compared to vehicle (−62%, $p<0.001$; Fig. 2A). Statin treatment combined with β3-AR agonism also effectively reduced plasma TG as compared to vehicle (−36%; $p<0.01$) and statin treatment alone (−46%,

$p<0.001$). Moreover, both monotherapies reduced plasma total cholesterol (TC) levels as compared to vehicle (both approx. −35%, $p<0.001$; Fig. 2B). Importantly, statin treatment further reduced plasma TC levels as induced by β3-AR agonism alone (−27%,
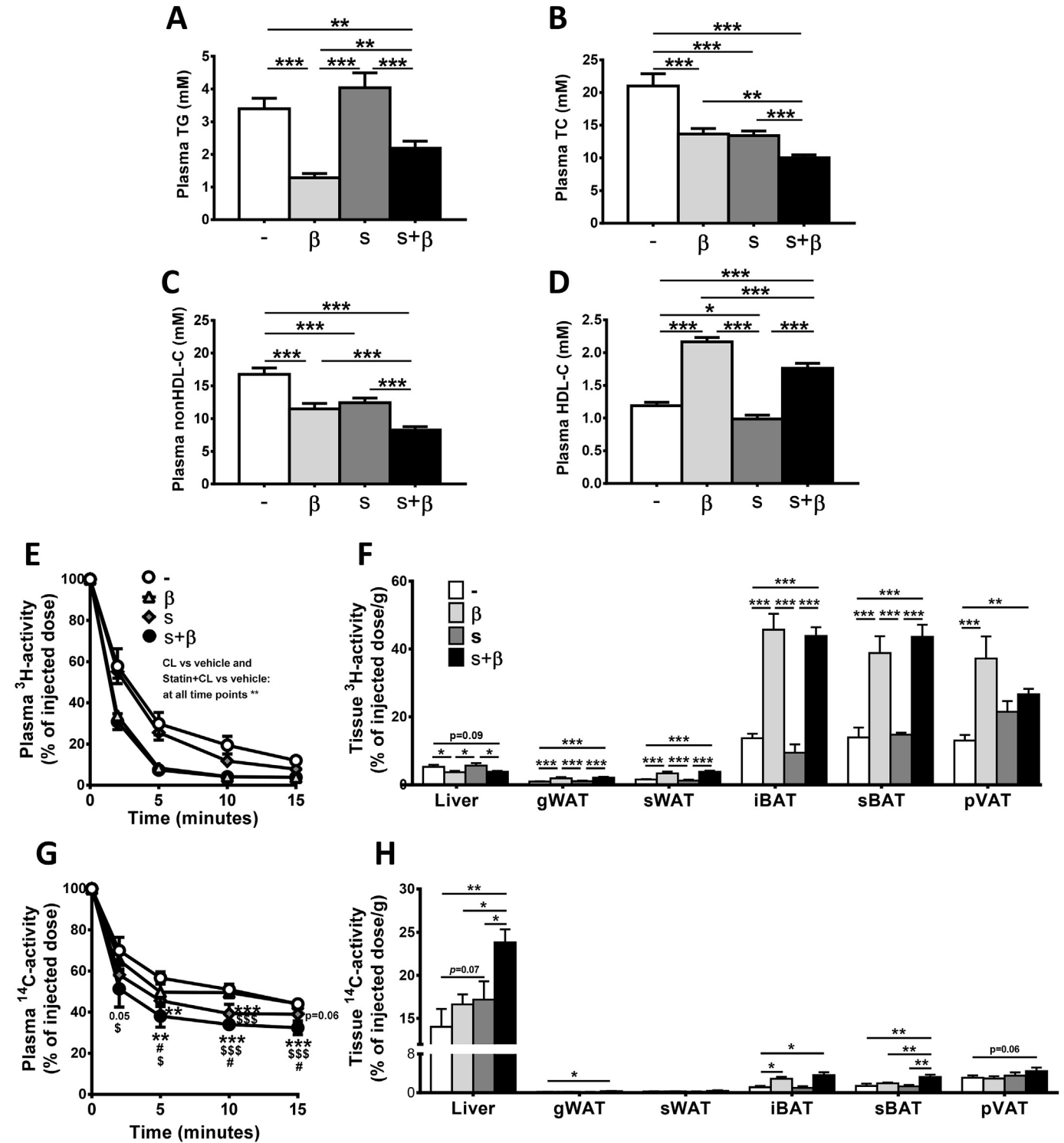
Fig. 2. The effect of β3-AR agonism, statin treatment and statin treatment combined with β3-AR agonism on plasma lipid and lipoprotein metabolism.
Plasma (A) TG, (B) TC, (C) nonHDL-cholesterol (-C) and (D) HDL-C levels (n = 15-16/group). Clearance of (E) ^{3}H-activity and (F) ^{14}C-activity from plasma and uptake of (G) ^{3}H-activity and (H) ^{14}C-activity by organs and tissues (n = 7-8/group). gWAT, gonadal WAT; sWAT, subcutaneous WAT; iBAT, interscapular brown adipose tissue; sBAT, subscapular BAT; pVAT, perivascular adipose tissue. -, vehicle; β, β3-AR agonism; s, statin; s+β, combination. Values are means ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs. control. $^${$p<0.05$; $^{$$$}$$p<0.001$ vs. β3-AR agonism. [#]$p<0.05$ vs. statin.

p<0.01).

To further address the effect of the various treatments on the distribution of cholesterol carried by the pro- and anti-atherogenic lipoprotein classes, we determined plasma HDL-C and nonHDL-C levels (*i.e.* plasma cholesterol carried by apoB-containing lipoproteins). We showed that pro-atherogenic nonHDL-C levels, that were reduced by both monotherapies (approx. −30%, *p*<0.001), were further reduced by statin treatment in addition to β3-AR as compared to vehicle (−51%, *p*<0.001) and the monotherapies (approx. −30%, *p*<0.01) (Fig. 2C). In addition, β3-AR agonism increased HDL-C levels as compared to vehicle (+80%, *p*<0.001; Fig. 2D). Moreover, statin treatment in combination with β3-AR agonism increased HDL-C levels as compared to vehicle (+48%, *p*<0.001) and statin treatment alone (+78%, *p*<0.001). Taken together, these findings demonstrate that statin treatment further reduces the β3-AR agonism-mediated decrease in plasma nonHDL-C levels.

3.4. Statin treatment in addition to β3-AR agonism markedly increases hepatic uptake of cholesterol-enriched remnants

We next assessed whether statin treatment enhances the effect of β3-AR agonism on plasma TRL clearance. Clearance of [³H]TO from plasma, which mainly reflects the uptake of TG-derived FA by LPL-expressing organs, was accelerated after β3-AR agonism alone as compared to vehicle (Fig. 2E). Statin treatment did not accelerate [³H]TO clearance from plasma, neither alone nor in addition to β3-AR agonism. The increased plasma clearance upon β3-AR agonism was due to a massively increased uptake of ³H-activity by all BAT depots (*i.e.* iBAT, subscapular BAT and perivascular adipose tissue; approx. +200%, *p*<0.001; Fig. 2F). The addition of statin did not further enhance the uptake of ³H-activity by the BAT and WAT depots as compared to β3-AR agonism alone.

Clearance of [¹⁴C]CO from plasma, which mainly reflects the uptake of cholesterol-enriched remnant particles by the liver, was significantly increased after statin treatment combined with β3-AR agonism as compared to vehicle (Fig. 2G). This was accompanied by a markedly enhanced uptake of ¹⁴C-activity by the liver upon statin treatment in combination with β3-AR agonism as compared to vehicle (+70%, *p*<0.01; Fig. 2H). Moreover, addition of statin also enhanced the hepatic uptake of ¹⁴C-activity as compared to β3-AR agonism alone (+43%, *p*<0.05). Collectively, these data indicate that statin treatment further lowers plasma nonHDL-C levels as compared to β3-AR agonism alone due to accelerated hepatic uptake of cholesterol-enriched remnant particles.

We next sought to elucidate the mechanism underlying the increased hepatic uptake of cholesterol-enriched remnant particles after statin treatment in addition to β3-AR agonism. Since LDLR is the major receptor responsible for the hepatic uptake of remnants, we measured *Ldlr* mRNA expression and LDLR protein content in the liver. β3-AR agonism reduced *Ldlr* expression (−33%, *p*<0.01; Fig. 3A) as compared to vehicle. Statin treatment alone increased *Ldlr* expression (+33%, *p*<0.01) as compared to vehicle, while statin treatment in addition to β3-AR agonism increased *Ldlr* expression (+34%, *p*<0.05) as compared to β3-AR agonism alone. Total hepatic protein levels of the LDLR were not influenced by any of the treatments (Fig. 3B). Of note, gene expression and protein content of the LDLR-related protein 1 (LRP1) were not influenced by any of the treatments (Fig. 3C and D).

Since LDLR is internalized and degraded by proprotein convertase subtilisin/kexin (PCSK)9, we next focused our attention on PCSK9. Interestingly, β3-AR agonism reduced *Pcsk9* expression of (−45%, *p*<0.01; Fig. 3E) as compared to vehicle. Statin treatment alone increased *Pcsk9* expression (+52%, *p*<0.01) as compared to vehicle, while statin treatment in addition to β3-AR agonism increased *Pcsk9* expression (+64%, *p*<0.05) as compared to β3-AR agonism alone. Moreover, plasma concentration of PCSK9 followed similar patterns (Fig. 3F). These data suggest that statin treatment in addition to β3-AR agonism increased *Ldlr* mRNA expression without increasing the hepatic LDLR protein content, possibly resulting from compensatory PCSK9-mediated LDLR degradation.

3.5. Statin treatment reverses β3-AR agonism-induced hepatic cholesterol accumulation

We next evaluated the effect of increased influx of cholesterol-enriched remnants after statin treatment in addition to β3-AR agonism on hepatic lipid levels. While β3-AR agonism increased the hepatic cholesterol content (+31%, *p*<0.05; Fig. 3G), statin treatment alone largely reduced the hepatic cholesterol content (−38%, *p*<0.001) as compared to vehicle. Importantly, statin treatment in addition to β3-AR agonism reversed the hepatic cholesterol accumulation induced by β3-AR agonism (−57%, *p*<0.001) to a similar low level as with statin treatment alone. While hepatic TG content was not affected (Fig. 3H), statin treatment in addition to β3-AR agonism slightly reduced hepatic phospholipid content as compared to β3-AR agonism alone (−15%, *p*<0.001; Fig. 3I). These data show that statin treatment rescues the liver from β3-AR agonism-mediated cholesterol accumulation.

3.6. Statin treatment in addition to β3-AR agonism further lowers atherosclerosis development

Next, we examined whether statin treatment in addition to β3-AR agonism further reduces atherosclerosis development. To this end, we determined the atherosclerotic lesion area as well as lesion severity and composition in the root of the aortic arch. β3-AR agonism and statin treatment alone reduced atherosclerosis development as compared to vehicle (both approx. −55%, *p*<0.05; Fig. 4A—C). Statin treatment combined with β3-AR agonism diminished atherosclerosis development by −76% as compared to vehicle (*p*<0.001). Importantly, statin treatment in addition to β3-AR agonism even further reduced atherosclerosis development as compared to β3-AR agonism alone (−54%, *p*<0.05). Furthermore, we found that the total plasma cholesterol exposure during the study strongly correlated with the square root (SQRT) transformed lesion area (β = 0.07, R^2 = 0.51, *p*<0.0001; Fig. 4D), demonstrating that the cholesterol exposure is an important predictor of atherosclerosis development under these conditions.

β3-AR agonism alone tended to increase the percentage of mild lesions (+46%, *p*=0.07; Fig. 4E) and to reduce the percentage of severe lesions (−38%, *p*=0.08) as compared to vehicle. Lesion severity was not significantly influenced by statin treatment alone or statin treatment in combination with β3-AR agonism. Macrophage and collagen area within the lesion were not affected by any of the treatments (Fig. 4F and G), but smooth muscle cell area was increased by β3-AR agonism alone (+24%, *p*<0.05; Fig. 4H) as compared to vehicle. Neither of the treatments influenced the stability index (ratio collagen/macrophage area) (Fig. 4I). Together, these findings show that statin treatment in addition to β3-AR agonism further lowers atherosclerotic lesion development without altering lesional composition.

4. Discussion

BAT activation is regarded as a promising strategy to treat hypertriglyceridemia, and more recently also emerged as a tool to alleviate hypercholesterolemia and atherosclerosis development. We reasoned that the effectiveness of BAT activation to reduce
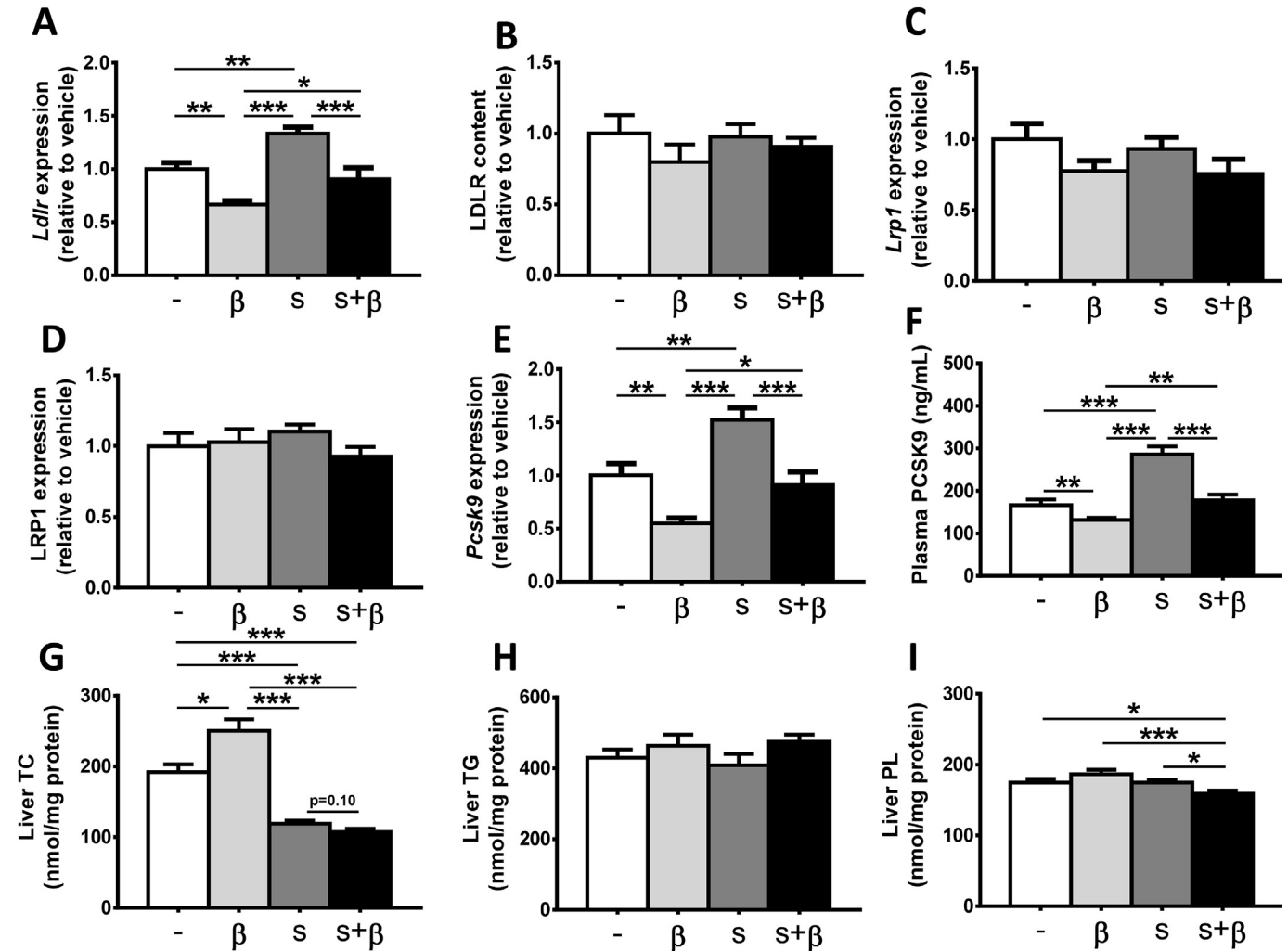
Fig. 3. The effect of β3-AR agonism, statin treatment and statin treatment combined with β3-AR agonism on hepatic lipid levels.
Hepatic mRNA expression of (A) low-density lipoprotein receptor (*Ldlr*), (C) LDLR-related protein (*Lrp*) 1 and (E) proprotein convertase subtilisin/kexin (*Pcsk9*). Hepatic protein content of (B) total LDLR and (D) LRP1, (n = 8/group). PCSK9 concentration in (F) plasma (n = 13–16/group). Liver (J) TC, (K) TG and (L) phospholipid (PL) content (n = 8/group). -, vehicle; β, β3-AR agonism; s, statin; s+β, combination. Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

hypercholesterolemia and atherosclerosis may be limited by the capacity of the liver to internalize lipoprotein remnants. We therefore aimed to accelerate the hepatic clearance of lipoprotein remnants generated by BAT activation via concomitant statin treatment. We investigated whether statin treatment enhances the lipid-lowering and anti-atherogenic effects of BAT activation in dyslipidemic *E3L.CETP* mice. Statin treatment did not influence β3-AR agonism-mediated BAT activation. However, statin treatment in combination with β3-AR agonism indeed further reduced plasma cholesterol levels and atherosclerosis development as compared to β3-AR agonism alone.

We could reproduce previous findings [9] to show that β3-AR agonism increases EE as a result of increased fat oxidation, which is typical for increased BAT activity [19]. Indeed, BAT was activated as shown by reduced lipid droplet content within BAT. Consequently, β3-AR agonism reduced plasma TG levels, accelerated formation and hepatic clearance of cholesterol-enriched lipoprotein remnants and reduced plasma nonHDL-C levels All of these effects also occur when BAT is activated by β3-AR agonism [9] or cold [4,8,9].

The LDLR is the most important hepatic receptor for the uptake of TRL remnants [20] via the interaction with apoE on TRL remnants. Furthermore, LDLR expression is under tight control by PCSK9 which is responsible for LDLR degradation [21]. The expression of *Ldlr* and *Pcsk9* was reduced after β3-AR agonism, which may be due to a compensatory downregulation by *Srebp2* in response to elevated hepatic cholesterol levels. Despite these effects on gene expression, LDLR protein content was not changed. The precise mechanism by which β3-AR agonism increases the hepatic uptake of TRL remnants remains to be elucidated, but we hypothesize that TRL remnants, formed after lipolytic processing by activated BAT, rapidly acquire apoE. These apoE-enriched TRL remnants have a strong affinity for LDLR and are therefore cleared faster from the circulation compared to TRL remnants that acquire less apoE due to slow lipolysis [22].

In addition, BAT activation increased plasma HDL-C levels, which is in accordance with other studies in humans [23] and mice [24]. The increased HDL-C levels are likely the direct consequence of enhanced LPL-mediated hydrolysis of TRL [8,9]. This process results in the generation of phospholipid-rich surface remnants that can bind lipid-poor apoAI [25]. The transport of surface remnants from TRLs to lipid-poor apoAI is mediated by phospholipid transfer protein (PLTP) [26] and precedes maturation of discoidal HDL into small HDL [27]. Although we did not measure HDL-C
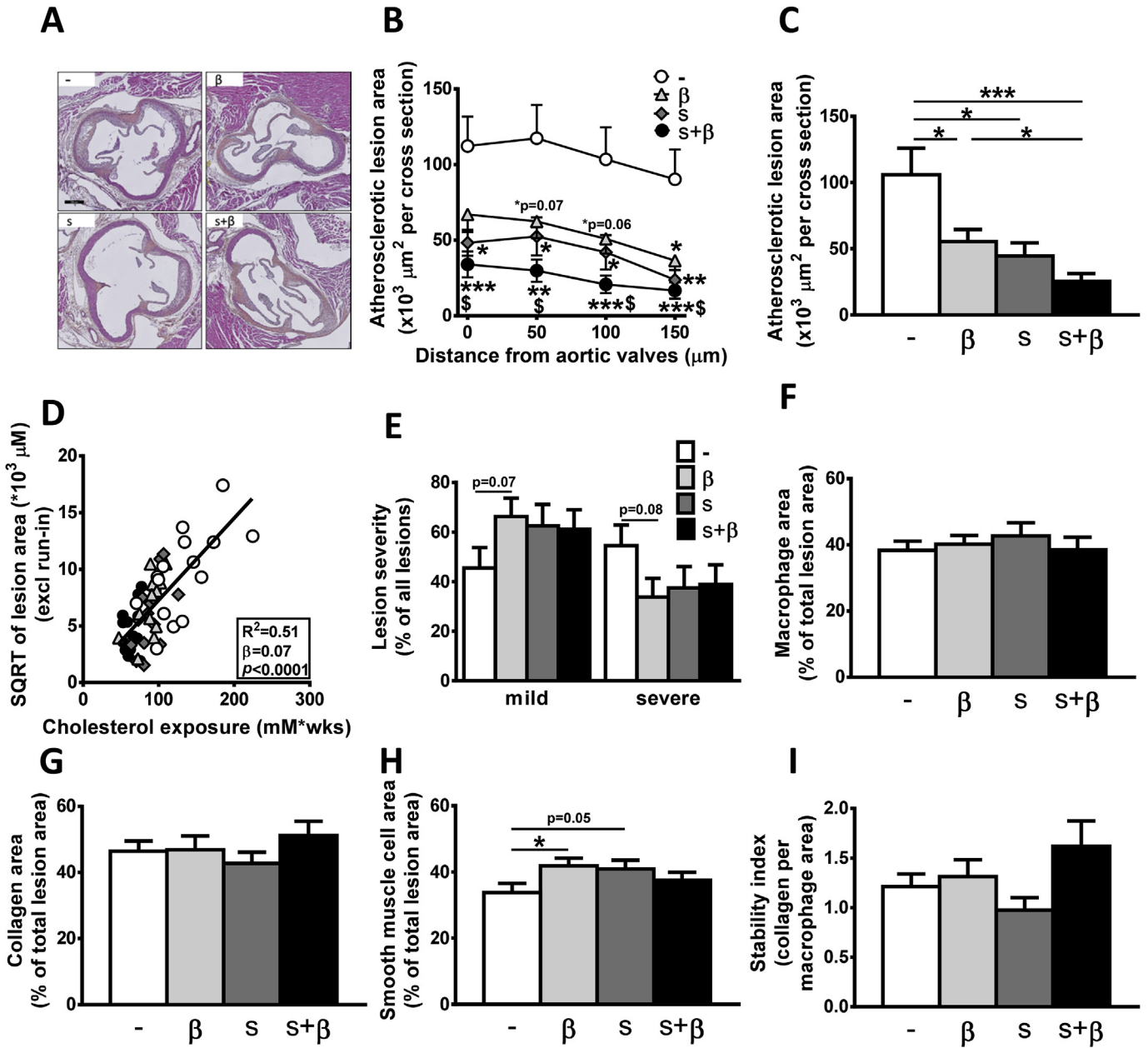
Fig. 4. The effect of β3-AR agonism, statin treatment and statin treatment combined with β3-AR agonism on atherosclerosis development.
(A) Representative pictures of the aortic root. Scale bar, 100 μm. (B) Lesion area as a function of distance from the appearance of open valves and (C) mean atherosclerotic lesion area.
(D) The square root (SQRT) of the atherosclerotic lesion area plotted against the plasma TC exposure during the 9-week treatment period. Linear regression analysis was performed.
(E) Lesion severity as well as (F) macrophage, (G) collagen and (H) smooth muscle cell area within the lesions. (I) The stability (n = 13-15/group). -, vehicle; β, β3-AR agonism; s, statin; s+β, combination. Values are means ± SEM. $*p<0.05$; $***p<0.001$.

functionality in the current study, we previously showed that BAT activation also improved HDL functionality as indicated by increased cholesterol efflux capacity *in vivo* [24] and *ex vivo* [23]. The increased HDL functionality may be related to the above described mechanism as small HDL particles are potent acceptors of cholesterol via ABCA1 [28] and, importantly, this may have contributed to the anti-atherogenic effect of BAT activation that was observed in the current and previous studies [9,24].

Statin treatment alone reduced plasma nonHDL-C levels accompanied by reduced atherosclerosis development, both of which have been described previously [29]. Statin treatment likely lowers nonHDL-C by enhancing hepatic uptake of cholesterol-enriched remnants, as we have demonstrated using TRL-mimicking particles. Despite increased *Ldlr* mRNA expression, statin treatment did not increase total LDLR protein content in the liver. This is in line with previous preclinical studies, which showed no effect of statin treatment on total hepatic LDLR protein content, but rather increased degradation of LDLR [30,31]. This points towards increased turnover of LDLR, since total LDLR protein content was not changed, which may increase the flux by which TRL remnants are taken up by the liver. Statin treatment also increased hepatic and circulating PCSK9 levels, which potentially may have prevented an increase in total hepatic LDLR protein content due to increased PSCK9-mediated breakdown of LDLR. As expected, statin treatment alone did not modulate fat oxidation, BAT histology and TG-derived FA uptake by BAT. Taken together, this indicates that
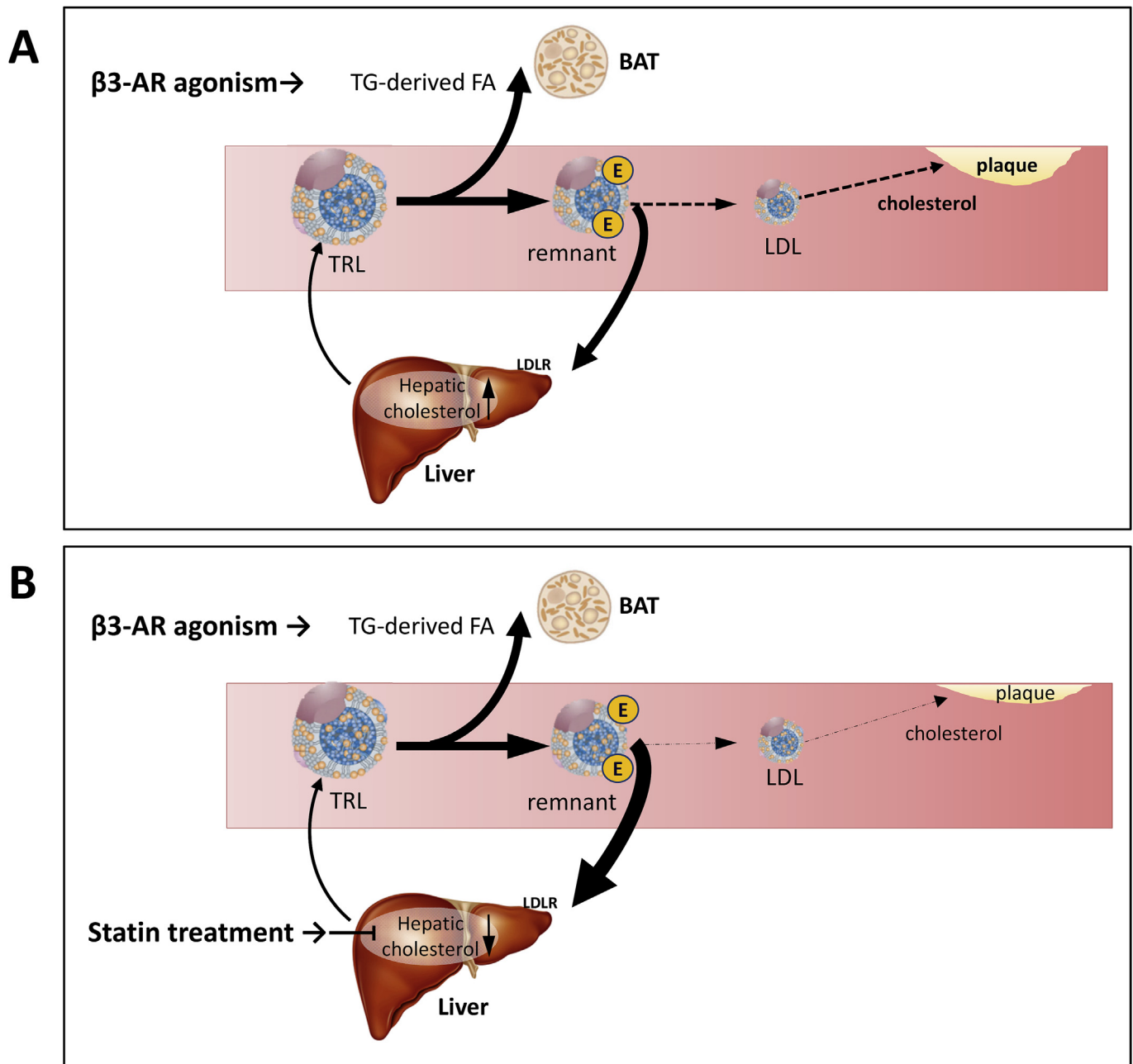
Fig. 5. Current working model explaining the effect of statin treatment in addition to BAT activation on alleviating hyperlipidemia and atherosclerosis.
(A) BAT activation reduces plasma TG by largely increasing the uptake of TRL-TG-derived FA by BAT. During lipolysis of TRL by BAT, cholesterol-enriched TRL remnants are generated that are subsequently taken up by interaction of apoE (E) with LDLR on hepatocytes. (B) Statin treatment in addition to BAT activation increases the hepatic uptake of cholesterol-enriched TRL remnants, thereby further alleviating hyperlipidemia and atherosclerosis development, but also reducing hepatic cholesterol levels. The arrows in both panels show the effect of the treatment on the depicted processes as compared to vehicle.

statin treatment *per se* does not modulate BAT activity but rather impacts on hepatic clearance of TRL remnants.

Statin treatment in addition to β3-AR agonism did not additionally enhance EE or fat oxidation, and did not further reduce plasma TG levels. Importantly, statin treatment further increased the hepatic uptake of cholesterol-enriched lipoprotein remnants as compared to β3-AR agonism alone, which resulted in a further reduction of plasma nonHDL-C levels as compared to β3-AR agonism alone. This is likely related to an increased LDLR functionality, as discussed above. Moreover, enrichment of TRL remnants with apoE, due to accelerated lipolysis of TRLs by activated BAT as

described above, may have contributed to the increased hepatic uptake of TRL remnants. The hypothesized interaction between increased LDLR functionality and apoE-enriched TRL remnants may explain why statin treatment in addition to β3-AR agonism further increased the hepatic uptake of TRL remnants as compared to the monotherapies. Statin treatment in addition to β3-AR agonism also increased HDL-C levels as compared to vehicle. This increase was a little attenuated compared to β3-AR agonism alone, which may be related to the slight reduction in HDL-C after statin treatment. However, the overall increase in HDL-C as compared to vehicle likely contributed to the anti-atherogenic effect of the combination

therapy. Interestingly, statin treatment reversed the hepatic cholesterol accumulation induced by β3-AR agonism to similar levels as statin treatment alone, which is probably a direct consequence of HMG-CoA reductase inhibition. In line with our hypothesis, statin treatment in addition to β3-AR agonism further protected from atherosclerosis, the most important and clinically relevant read-out of the study, as compared to β3-AR agonism alone. Altogether, this indicates that statin treatment in addition to BAT activation is additive to the lipid-lowering and anti-atherogenic effect of β3-AR agonism alone (for working model see Fig. 5).

Elucidating the combined effects of statin treatment with BAT activation on hyperlipidemia and atherosclerosis is of high clinical relevance, given that statins are the first-line therapy for patients with hyperlipidemia that have proven efficacy on CVD. Human BAT is also metabolically active [5] and uses lipids as substrate [5,32], even in obese individuals and elderly [32]. This suggests that BAT activation in humans will likely improve lipid levels as well. Indeed, long term BAT activation by means of cold exposure lowers LDL-C levels in hypercholesterolemic patients [33]. Interestingly, it was recently demonstrated that high BAT activity is associated with a reduced risk of CVD events [34], supporting that BAT activation in humans is a promising therapeutic tool to lower CVD risk. Activation of the β3-AR may be a potent therapy to directly activate BAT, especially since a single dose of the β3-AR agonist mirabegron, currently on the market for overactive bladder, enhances BAT activity and increases EE in humans [35].

In conclusion, we showed that statin treatment in addition to BAT activation further lowers hypercholesterolemia and atherosclerosis development as compared to BAT activation alone as the consequence of an increased hepatic uptake of cholesterol-enriched remnants. The mode of hepatic uptake appears to be complex and more research is necessary to unravel the underlying mechanism(s). Future studies should elucidate whether adding BAT activation to statin therapy further alleviates hyperlipidemia and CVD risk in humans compared to statin therapy alone.

Conflict of interest

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

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Author contributions

G.H., P.C.N.R., J.F.P.B. and M.R.B. conceived and designed the experiments. G.H., Y.W., I.M.M, E.G., H.G.K., S.M.v.d.B. and E.H.P performed the experiments. G.H. and A.D.vD. analysed the data. G.H., A.K.G., H.M.G.P., P.C.N.R., J.F.P.B. and M.R.B were involved in interpretation of the data. G.H., P.C.N.R., J.F.P.B. and M.R.B wrote the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2017.10.030.

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