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Modulation of HDL metabolism : studies in APOE*3-Leiden.CETP mice

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Citation

Haan, W. de. (2009, April 16). *Modulation of HDL metabolism : studies in APOE*3-Leiden.CETP mice*. Retrieved from <https://hdl.handle.net/1887/13730>

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Chapter 5

TORCETRAPIB DOES NOT REDUCE ATHEROSCLEROSIS BEYOND ATORVASTATIN AND INDUCES MORE PRO- INFLAMMATORY LESIONS THAN ATORVASTATIN

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Circulation 2008; 117: 2515-2522

Abstract

Background: Although CETP inhibition is regarded as a promising strategy to reduce atherosclerosis by increasing HDL-cholesterol, the CETP inhibitor torcetrapib given on top of atorvastatin had no effect on atherosclerosis and even increased cardiovascular death in the recent ILLUMINATE trial. Therefore, we evaluated the anti-atherogenic potential and adverse effects of torcetrapib in humanized *APOE*3-Leiden.CETP* (*E3L.CETP*) mice.

Methods and Results: *E3L.CETP* mice were fed a cholesterol-rich without drugs or with torcetrapib (12 mg/kg/day), atorvastatin (2.8 mg/kg/day) or both for 14 weeks. Torcetrapib decreased CETP activity both in the absence and presence of atorvastatin (-74% and -73% respectively, $P<0.001$). Torcetrapib decreased plasma cholesterol (-20%, $P<0.01$), albeit to a lesser extent than atorvastatin (-42%, $P<0.001$) or the combination of torcetrapib and atorvastatin (-40%, $P<0.001$). Torcetrapib increased HDL-cholesterol in the absence (+30%) and in the presence (+34%) of atorvastatin. Torcetrapib and atorvastatin alone both reduced atherosclerotic lesion size (-43% and -46%, $P<0.05$), but combination therapy did not reduce atherosclerosis as compared to atorvastatin alone. Remarkably, as compared to atorvastatin, torcetrapib induced enhanced monocyte recruitment and expression of monocyte chemoattractant protein-1 and resulted in lesions of a more inflammatory phenotype, as reflected by an increased macrophage content and reduced collagen content.

Conclusions: CETP inhibition by torcetrapib *per se* reduces atherosclerotic lesion size but does not enhance the anti-atherogenic potential of atorvastatin. However, as compared to atorvastatin, torcetrapib introduces lesions of a less stable phenotype.

Introduction

The cholesteryl ester transfer protein (CETP) is an important regulator of the HDL-C level. CETP is secreted predominantly by the liver and mainly associates with HDL in plasma, where it transports cholesteryl esters (CE) from HDL to (V)LDL in exchange for triglycerides,^{1,2} and thus lowers HDL-C. HDL is atheroprotective as it mediates reverse cholesterol transport (i.e. transport of cholesterol from the vessel wall to the liver) and it has anti-inflammatory, anti-thrombotic and anti-oxidative properties.^{3,4} Therefore, CETP inhibition is regarded as a promising strategy to increase HDL-C levels and to reduce atherosclerosis.² However, the effect of CETP activity on atherosclerosis in humans has not been unequivocally determined. Mutations in the CETP gene that reduce CETP mass and activity (e.g. D442G and Int14 G(+1)>A) lead to elevated HDL-C levels,^{5,6} but the effects of these mutations on atherosclerosis are still in dispute.⁷⁻¹⁰

Torcetrapib, which forms an inactive complex between CETP and HDL² has been the first CETP inhibitor tested in large human trials, in which it was shown to increase HDL-C levels by approx. 60%.¹¹⁻¹³ The resulting HDL particles were able to mediate cellular cholesterol efflux more efficiently.¹⁴ However, the large scale ILLUMINATE trial was stopped prematurely because of an excess of deaths in patients receiving torcetrapib with atorvastatin as compared to those receiving atorvastatin alone, mainly related to cardiovascular events.¹⁵ In addition, the RADIANCE and ILLUSTRATE trials revealed no therapeutic benefit of combining torcetrapib with atorvastatin with respect to atherosclerosis progression as assessed by coronary intima-media thickness (IMT) and intravascular ultrasonography (IVUS) measurements.¹¹⁻¹³

The effect of torcetrapib alone on atherosclerosis, however, has not yet been evaluated in humans, and the mechanism underlying the increased death rate associated with torcetrapib treatment has not been elucidated as yet. Therefore, we now examined the effect of torcetrapib with or without atorvastatin on atherosclerosis development in humanized *APOE*3-Leiden.CETP (E3L.CETP)* transgenic mice.¹⁶ *E3L* mice show a human-like response to lipid-lowering therapies.¹⁷ Cross-breeding with *CETP* transgenic mice, which express human CETP under control of its natural flanking regions, resulted in *E3L.CETP* mice that also respond to HDL-modulating intervention.^{18,19}

Methods

Animals

Human CETP transgenic mice which express CETP under control of its natural flanking regions (strain 5203)²⁰ were obtained from Jackson laboratories (Bar Harbor, MC) and crossbred with *E3L* mice²¹ to obtain *E3L.CETP* mice.¹⁶ All mice used in this study were heterozygous *E3L.CETP* transgenic females on a

C57Bl/6 background. Mice were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. Mice were fed regular chow (Ssniff, Soest, Germany) or a diet with 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, the Netherlands) supplemented with 0.1% or 0.25% (w/w) cholesterol (Sigma) with or without torcetrapib (2R,4S)-4-[[[3,5bis(trifluoromethyl) phenyl]methyl]-(methoxycarbonyl)amino]-2-ethyl-3,4-dihydro-6-trifluoromethyl)-3-phenyl-1(2H)-quinolinecarboxylic acid, ethyl ester (C₂₆H₂₅N₂O₄F₉), (kindly provided by Roche, Basel, Switzerland) and/or atorvastatin ([R-(R*,R*)]-2-(4-fluorophenyl)-beta,delta-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid (C₃₃H₂₄FN₂O₅) (Lipitor). Unless indicated otherwise, blood was drawn after 4 h fasting in EDTA-containing cups by tail bleeding and plasma was isolated. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Single Torcetrapib Treatment

To verify that torcetrapib inhibits CETP activity in *E3L.CETP* mice *in vivo*, mice on a chow diet were given a single intragastric gavage of torcetrapib (0, 1, 3 and 10 mg/kg) in approx. 200 µL of ethanol: solutol: saline 10:10:80 (v:v:v). Blood was drawn before gavage and at 1, 2, 4, 6, 8 and 24 h after gavage. During the first 8 h after the gavage mice were fasted. Plasma was assayed for total CETP activity as described below. Alternatively, mice were fed a diet containing 15% cacao butter with 0.1% or 0.25% cholesterol, and the effect of 10 mg/kg torcetrapib was determined on plasma CETP activity at 2 h after gavage.

Total Plasma CETP Activity, Endogenous CETP Activity and CETP Mass

Total plasma CETP activity was measured as the transfer of [³H]cholesteryl oleate (CO) from LDL to HDL.¹⁶ Briefly, 5 µL (diluted) mouse plasma was incubated with human [³H]CO-labeled LDL and HDL in sodium phosphate buffer containing 5,5'-dithio-bis(2-nitrobenzoic acid) to inhibit lecithin-cholesterol acyltransferase (LCAT) activity. After overnight incubation, LDL was precipitated. The supernatant containing [³H]CO-HDL was counted for ³H activity. CETP activity was calculated as nmol CE transfer/ mL plasma/ h. Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole (NBD)-labeled cholesteryl esters (RB-CETP, Roar Biomedical, NY, USA), as described.²² CETP mass was determined using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).

Long-term Torcetrapib Treatment

To determine the effect of torcetrapib without and with atorvastatin on atherosclerosis development and plasma cholesterol, *E3L.CETP* mice were fed a diet containing 0.25% cholesterol to increase plasma cholesterol levels to ~16 mM. After 4 weeks, mice were randomized into four groups according to their plasma cholesterol levels. Mice were fed a control diet, a diet with atorvastatin (0.0023% ~ 2.8 mg/kg/day), torcetrapib (0.01% ~ 12 mg/kg/day) or both. Blood was drawn one week before randomization and at week 6, 9 and 14 of drug treatment, and was assayed for lipids, CETP mass and activity. After 14 weeks, mice were euthanized and atherosclerosis development was assessed as described below.

Plasma Lipids and Lipoprotein Profiles

Plasma was assayed for cholesterol and phospholipids (PL) using commercially available enzymatic kits according to the manufacturer's protocols (236691, Roche Molecular Biochemicals, Indianapolis IN, USA, and phospholipids B Wako Chemicals, Neuss, Germany, respectively). To determine the lipid distribution over plasma lipoproteins, lipoproteins were separated using fast protein liquid chromatography (FPLC). Plasma was pooled per group and 50 μ L of each pool was injected onto a Superose 6 HR 10/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 μ L/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 μ L were collected and assayed for cholesterol and PL as described above.

Atherosclerosis Quantification

After 14 weeks of drug intervention, mice were sacrificed by CO₂ inhalation. Blood was drawn via cardiac puncture and hearts were isolated. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin and were cross-sectioned (5 μ m) throughout the aortic root area. Per mouse 4 sections with 50 μ m intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxin-saffron (HPS) for histological analysis. Lesions were categorized for severity according to the American Heart system adapted for mice.^{23,24} Various types of lesions were discerned: type 0 (no lesions), type 1-3 (early fatty streak-like lesions containing foam cells) and type 4-5 (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization and/or necrosis). Lesion area was determined using Leica Qwin image analysis software (EIS, Asbury NJ). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used to quantify the macrophage area and the number of monocytes adhering to the endothelium. Sirius Red was used to quantify the collagen area, and the antibody M0851 (1:800, DAKO) against smooth muscle cell actin to quantify the smooth muscle cell area. Monocyte chemoattractant

protein-1 (MCP-1) was detected using goat anti-mouse MCP-1 (M18, 1:300; Santa Cruz Biotechnology, Santa Cruz, Calif).

Statistical Analysis

Data are presented as means $\pm$ SD unless indicated otherwise. Statistical differences were assessed using the Mann Whitney U test. For lesion typing, differences were assessed by the Chi Square test. SPSS 14.0 was used for statistical analysis. Values of $P < 0.05$ was regarded as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Torcetrapib Inhibits CETP Activity in *E3L.CETP* mice

To verify that *E3L.CETP* mice appropriately respond to CETP inhibition, *E3L.CETP* mice on a chow diet received an oral gavage of torcetrapib (1, 3 and 10 mg/kg) or vehicle. As expected, torcetrapib time- and dose-dependently reduced plasma CETP activity, reaching a minimum at 2 h after gavage ($-59 \pm 8\%$, $-83 \pm 4\%$, and $-96 \pm 4\%$; $P < 0.01$). At 3 and 10 mg/kg, significant reductions were still observed after 8 h ($-45 \pm 25\%$ and $-45 \pm 17\%$ respectively; $P < 0.01$) (Fig. 1A). Because cholesterol-feeding of *E3L.CETP* mice increases plasma CETP mass and activity,¹⁶ we next measured the inhibitory capacity of torcetrapib on plasma CETP activity in mice fed a diet without or with 0.1%

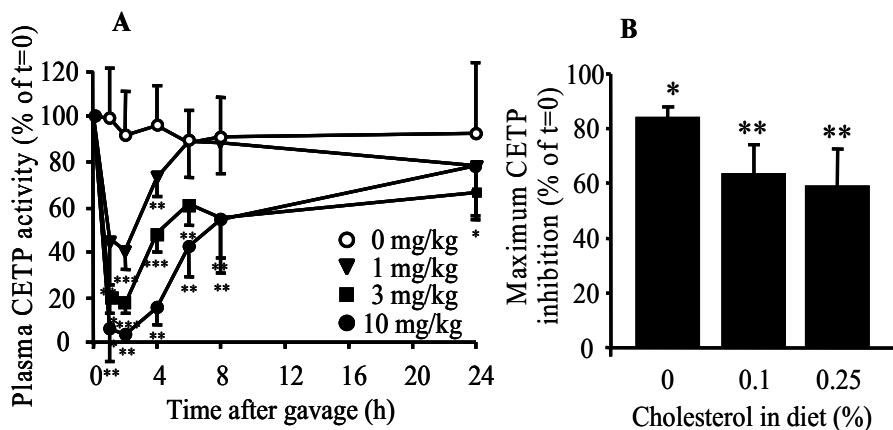


Figure 1. A single dose of torcetrapib inhibits CETP *in vivo*. *E3L.CETP* mice fed a chow diet received the indicated amounts of torcetrapib via intragastric gavage. Blood was drawn at the indicated time points and plasma was assayed for CETP activity (A). *E3L.CETP* mice, fed a chow diet or a diet containing 0.1% and 0.25% cholesterol, received torcetrapib (10 mg/kg) by intragastric gavage and total CETP activity was measured 2 h after gavage (B). Values are means $\pm$ SD (n=4-6); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to the control group.

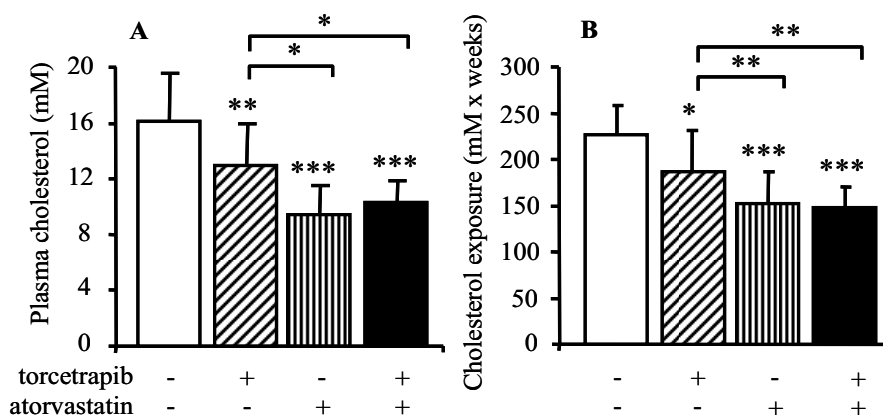


Figure 2. Torcetrapib reduces plasma cholesterol to a lesser extent than atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed for cholesterol (A). Blood was drawn at additional time points (0, 6, 9, and 14 weeks) and TC was measured. Total cholesterol exposure during the study was calculated (B). Values are means $\pm$ SD ($n=14-15$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to the control group.

(w/w) or 0.25% (w/w) cholesterol, which increased plasma CETP activities (3.4-fold and 4.3- fold, respectively). Despite the increase in plasma CETP activity, an oral gavage of torcetrapib (10 mg/kg) still profoundly decreased CETP activity in the presence of 0.1% ($-64 \pm 11\%$; $P < 0.05$) and 0.25% ($-59 \pm 13\%$; $P < 0.05$) cholesterol in the diet (Fig. 1B).

Torcetrapib Reduces Plasma Cholesterol Levels to a Lesser Extent than Atorvastatin

To determine the effect of torcetrapib on plasma lipid levels in the absence or presence of atorvastatin, *E3L.CETP* mice were fed a diet containing 0.25% (w/w) cholesterol without or with torcetrapib and/or atorvastatin. Addition of torcetrapib, atorvastatin or both to the diet did not affect food intake or body weights of *E3L.CETP* mice (not shown). The cholesterol-rich diet resulted in a plasma cholesterol level of 16.1 ± 3.5 mM in the control group. Torcetrapib decreased plasma cholesterol (-20% ; $P < 0.01$) to a lesser extent as compared to atorvastatin (-42% ; $P < 0.001$). The combination of torcetrapib and atorvastatin did not decrease plasma cholesterol further as compared to atorvastatin alone (-40% vs -42%) (Fig. 2A). Since torcetrapib and atorvastatin consistently lowered plasma cholesterol throughout the study, they similarly decreased total cholesterol exposure (Fig. 2B). Thus, torcetrapib alone reduced total cholesterol exposure to a lower extent as compared to atorvastatin and combination therapy (Fig. 2B).

To determine the distribution of lipids over lipoproteins, lipoproteins were fractionated by FPLC and cholesterol and PL were measured in the individual fractions (Fig. 3). Torcetrapib reduced (V)LDL-C (-26%) (Fig. 3A) to a lesser extent than atorvastatin (-42%) (Fig. 3A and 3B), and torcetrapib did not enhance the (V)LDL-C reducing effect of atorvastatin (Fig. 3B). In addition, torcetrapib increased plasma HDL-C levels by +30% in the absence of atorvastatin (Fig. 3A) and by +34% in the presence of atorvastatin, as judged from the cholesterol content of the FPLC fractions 17-22 (Fig. 3B). This torcetrapib-induced increase in HDL-C was paralleled by an increase in PL in the HDL fractions (Fig. 3C and 3D). Despite these increased HDL-C levels, apoAI levels were not altered by torcetrapib treatment (not shown).

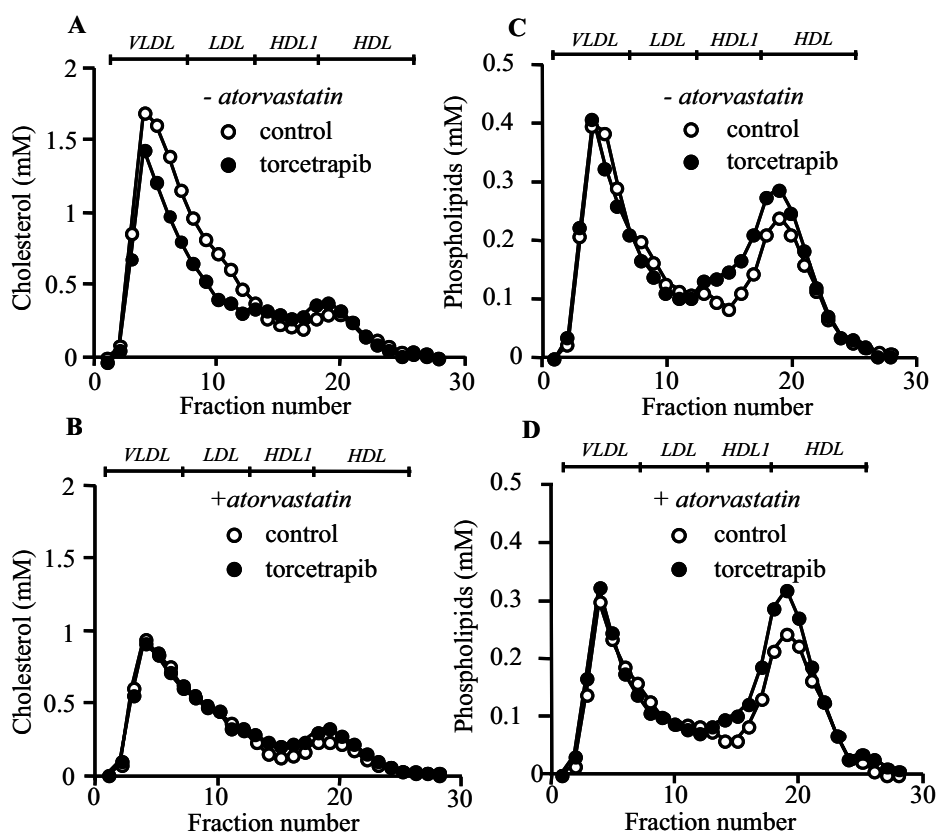


Figure 3. Torcetrapib reduces plasma VLDL and increases HDL levels. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, blood was drawn and plasma was pooled per treatment group (n=14-15). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and phospholipid (C, D).

Torcetrapib Reduces CETP Activity and Increases CETP Mass Whereas Atorvastatin Decreases Both CETP Activity and Mass

Torcetrapib decreased CETP activity efficiently both in the absence (-73%; $P<0.001$) and presence of atorvastatin (-74%; $P<0.001$) (Fig. 4A). Atorvastatin alone also decreased CETP activity, but to a lesser extent (-32%; $P<0.001$). Despite the decreased CETP activity, torcetrapib treatment increased CETP mass (+33%; $P<0.001$). On the contrary, atorvastatin decreased CETP mass (-24%; $P<0.001$), whereas the combination therapy did not significantly affect CETP mass as compared to untreated mice (Fig. 4B). These data are in line with previous observations that torcetrapib increases CETP mass in humans despite the decrease in CETP activity²⁵ and that atorvastatin decreases CETP levels^{26,27} by decreasing CETP expression.¹⁹

Torcetrapib Reduces Atherosclerotic Lesion Severity and Lesion Area but Does Not Enhance the Anti-Atherogenic Effect of Atorvastatin

To determine the effect of torcetrapib on atherosclerosis development in the absence or in the presence of atorvastatin, the 4 groups of mice were euthanized after 14 weeks and atherosclerosis severity and lesion size were measured in the aortic root. Representative pictures of each group are shown in Fig. 5A. As compared to the control group, mice treated with torcetrapib, atorvastatin or both had more lesion-free sections and fewer severe lesions of type 4 to 5. Thus, torcetrapib, atorvastatin and the combination of both reduced lesion severity similarly (Fig. 5B). Accordingly, torcetrapib and atorvastatin alone induced a similar reduction in lesion area (-43% and -46% respectively; $P<0.05$).

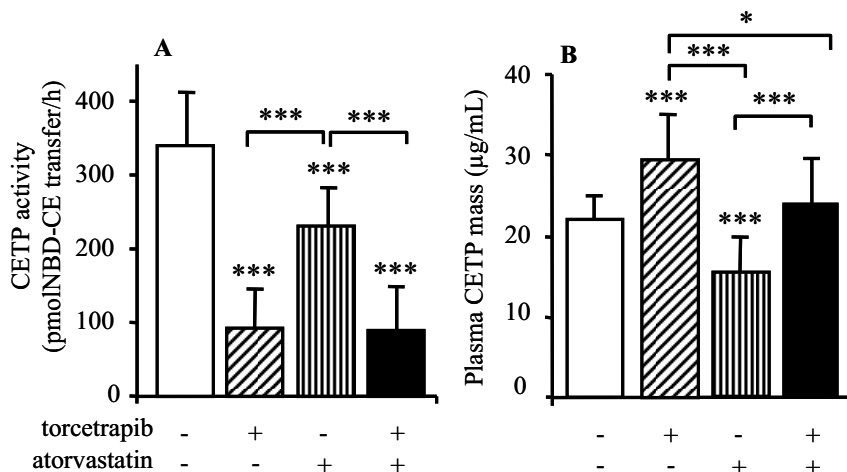


Figure 4. Torcetrapib reduces plasma CETP activity and increases CETP mass. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed endogenous CETP activity (A) and CETP mass (B). Values are means $\pm$ SD (n=14-15); * $P<0.05$, *** $P<0.001$ vs the control group.

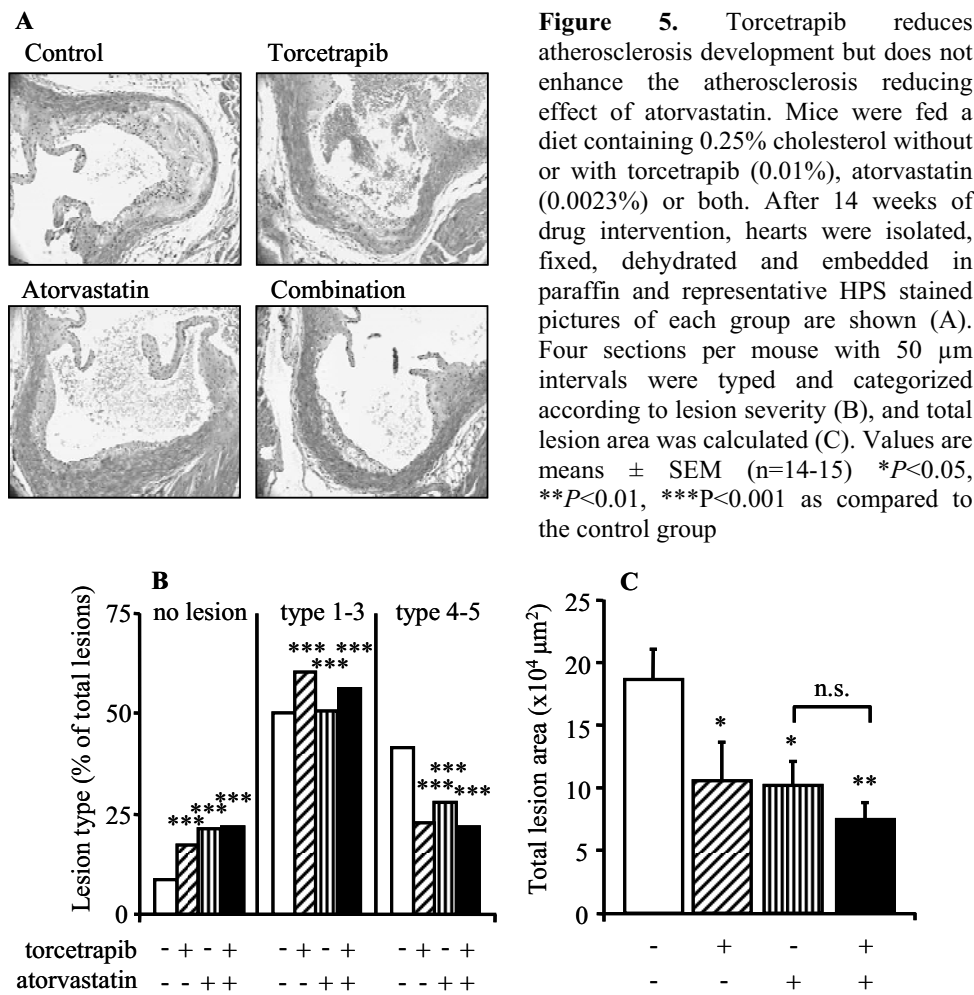


Figure 5. Torcetrapib reduces atherosclerosis development but does not enhance the atherosclerosis reducing effect of atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, hearts were isolated, fixed, dehydrated and embedded in paraffin and representative HPS stained pictures of each group are shown (A). Four sections per mouse with 50 μm intervals were typed and categorized according to lesion severity (B), and total lesion area was calculated (C). Values are means $\pm$ SEM (n=14-15) * P <0.05, ** P <0.01, *** P <0.001 as compared to the control group

Combination treatment also reduced atherosclerosis as compared to the control group (-60%; P <0.001), but did not significantly enhance the atherosclerosis-reducing potency of atorvastatin alone (Fig. 5C).

Torcetrapib Induces Monocyte Recruitment and Results in a More Pro-Inflammatory Lesion Phenotype as Compared to Atorvastatin

We next evaluated the effect of torcetrapib, atorvastatin and the combination of both on monocyte recruitment and lesion composition with respect to the macrophage area, smooth muscle cell area and collagen area. Torcetrapib alone and in combination with atorvastatin increased the adherence of monocytes to the vessel wall as compared to the control and atorvastatin-treated group (Fig 6A). Although torcetrapib did not significantly raise MCP-1 as compared to the control group, torcetrapib significantly increased MCP-1 as compared to

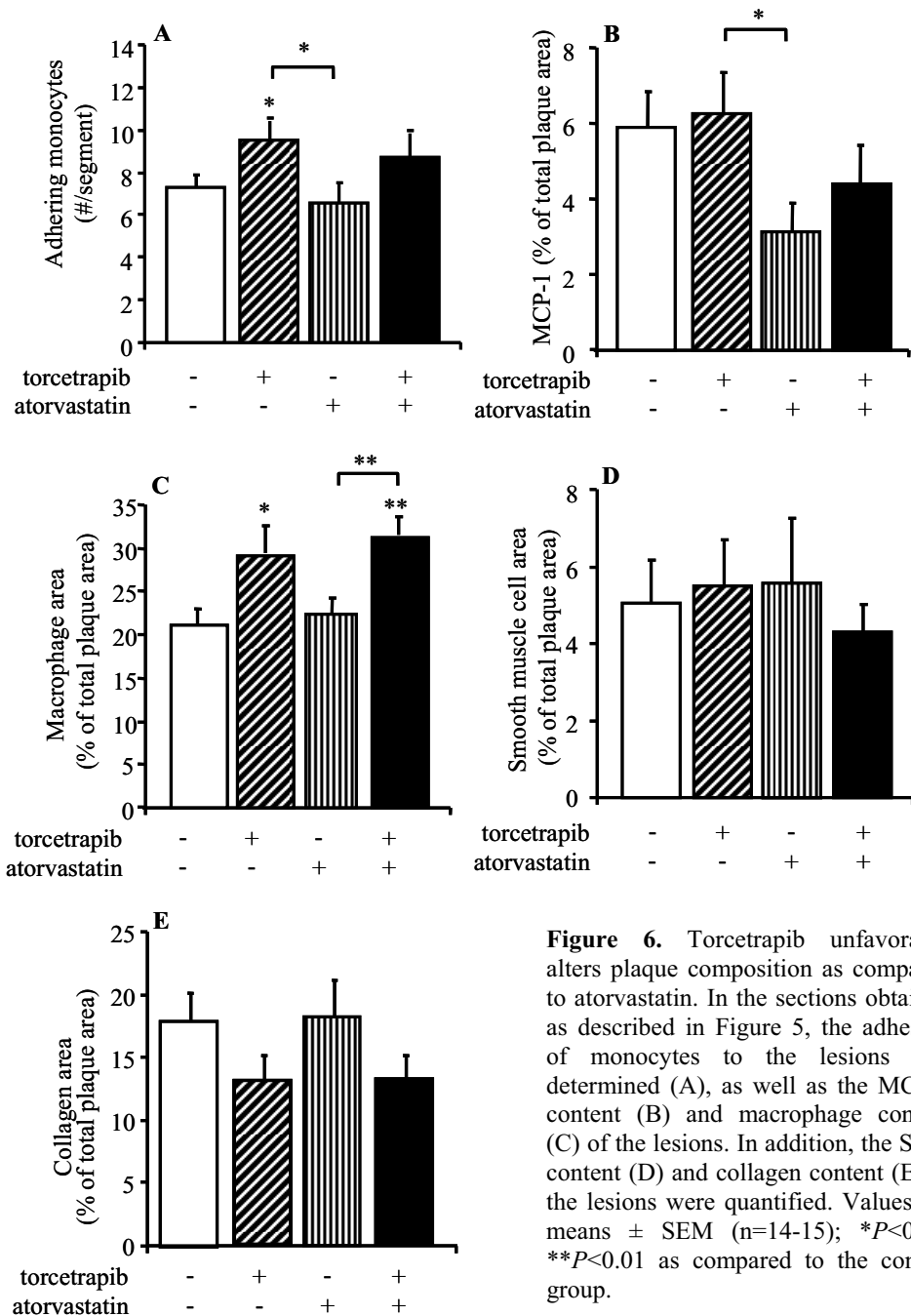


Figure 6. Torcetrapib unfavorably alters plaque composition as compared to atorvastatin. In the sections obtained as described in Figure 5, the adhesion of monocytes to the lesions was determined (A), as well as the MCP-1 content (B) and macrophage content (C) of the lesions. In addition, the SMC content (D) and collagen content (E) of the lesions were quantified. Values are means $\pm$ SEM (n=14-15); * P <0.05, ** P <0.01 as compared to the control group.

atorvastatin (+99%; P <0.05) (Fig. 6B). The increase in adhering monocytes as induced by torcetrapib was accompanied by an increased area of macrophages in the intima (Fig. 6C). Although torcetrapib did not appear to affect the smooth muscle cell content (Fig. 6D), torcetrapib alone and in combination with

atorvastatin tended to decrease the area of collagen ($P=0.14$ and $P=0.13$, resp.) (Fig. 6E). Thus, whereas atorvastatin reduces lesion size without affecting lesion composition as compared to untreated mice, torcetrapib reduces lesion size accompanied by a more pro-inflammatory lesion phenotype, reflected by an increased macrophage-to-collagen ratio, as compared to control-treated mice (+75%) and atorvastatin-treated mice (+67%).

Discussion

Torcetrapib has been shown to markedly raise HDL-C and was, therefore, expected to reduce atherosclerosis in humans. Despite this, the recent RADIANCE, ILLUSTRATE AND ILLUMINATE trials have concluded that torcetrapib was ineffective in reducing atherosclerosis¹¹⁻¹³ and increased clinical event rate.¹⁵ However, it should be realized that the effectiveness of torcetrapib has only been assessed in dyslipidemic patients who also received atorvastatin. Therefore, in the present study we examined the effect of torcetrapib *per se* on atherosclerosis development. In our study we show that torcetrapib alone reduces the progression of atherosclerosis, but does not enhance the anti-atherosclerotic potency of atorvastatin and that torcetrapib results in a more pro-inflammatory lesion phenotype as compared to atorvastatin.

Torcetrapib reduced total cholesterol exposure to a lesser extent (-17%) as compared to atorvastatin (-41%), whereas torcetrapib and atorvastatin equally reduced atherosclerotic lesion size (both ~-45%). Previous diet-induced atherosclerosis studies in mice have consistently demonstrated that atherosclerotic lesion area could generally be reliably predicted from cholesterol exposure (H.M.G. Princen PhD and P.C.N. Rensen PhD, unpublished data, 2007). Therefore, torcetrapib decreased atherosclerosis development more drastically than could be expected based merely on the observed reduction in cholesterol exposure. Since torcetrapib treatment results in increased HDL levels, it is likely that HDL is involved in the atheroprotective effect of torcetrapib. In line with this hypothesis, we have observed previously that *E3L.CETP* mice show a 7-fold increased atherosclerotic lesion area as compared to *E3L* only mice, which was much more than could be expected based on a modest increase in total plasma cholesterol *per se*. In fact, we showed that plasma from *E3L.CETP* mice was less effective in mediating SR-BI-dependent cholesterol efflux than plasma from *E3L* mice, as accompanied by a large reduction in HDL-1.¹⁶ In the present study, we did not detect an effect of torcetrapib on either SR-BI or ABCA1-mediated cholesterol efflux (not shown), possibly related to the relatively mild effect of torcetrapib on the HDL level as compared to total CETP deficiency. We therefore speculate that effects of torcetrapib on other properties of HDL, including its anti-inflammatory, anti-oxidative and/or anti-thrombotic properties may have resulted in the more

prominent reduction in atherosclerotic lesion size than could be expected merely on the basis of a reduction in total cholesterol.

The fact that torcetrapib alone reduced atherosclerosis development is in line with a previous study showing that torcetrapib treatment alone reduces atherosclerosis in rabbits.²⁸ However, we also show that torcetrapib did not significantly enhance the anti-atherogenic potential of atorvastatin. We have evaluated the effects of torcetrapib and atorvastatin in E3L.CETP mice with a relatively high plasma cholesterol level of approx. 16 mM, to avoid the possibility that the combined cholesterol-lowering actions of atorvastatin and torcetrapib would result in a plasma cholesterol level below that required for atherosclerosis development in E3L.CETP mice (*i.e.* 6-8 mM). Despite this limitation, torcetrapib *per se* (*i.e.* without concomitant administration of atorvastatin) may thus have an anti-atherosclerotic effect in humans as well.

From the recent clinical trials, it has become clear that torcetrapib has several adverse effects. The ILLUMINATE trial showed that torcetrapib elevated blood pressure, increased cardiovascular events and increased death rate, mainly related to cardiovascular causes.¹⁵ However, the mechanisms underlying these unexpected adverse effects have not completely been elucidated yet. In the present study, we did not detect a significant effect of torcetrapib on blood pressure, probably because of small experimental groups (data not shown). However, compared with atorvastatin, torcetrapib enhanced monocyte adherence to the vessel wall, enhanced vascular MCP-1 expression, and increased the macrophage area within the lesions. Torcetrapib thus appears to enhance the recruitment of monocytes to the endothelium and transmigration of the monocytes into the intima resulting in an enhanced macrophage content of the plaque, compared with similarly sized lesions resulting from atorvastatin treatment. The observation that torcetrapib tended to reduce the collagen content of the plaque independent of the smooth muscle cell content can be explained by induction of collagen breakdown by macrophages, (*e.g.*, via secretion of metalloproteinases). Although plaque rupture is a rare phenomenon in mice, such inflammatory lesions with a high macrophage to collagen ratio are more unstable and may well have caused an increased incidence of plaque rupture in humans, thereby explaining increased cardiovascular death. It would be interesting to evaluate in future studies whether these effects of torcetrapib are compound-specific or related to its effect on lipoprotein metabolism, by comparison with other CETP inhibitors that are currently under development (*e.g.* JTT-705 and anacetrapib).

Interestingly, recent data from the ILLUMINATE trial indicate that torcetrapib increased plasma aldosterone levels via an as yet unknown mechanism.¹⁵ In addition to increasing blood pressure,²⁹ aldosterone increases atherosclerosis development in mice.³⁰⁻³² This is related to its pro-inflammatory properties including increased MCP-1 expression, increased monocyte infiltration into the coronary artery, increased lipid loading of macrophages, and increased

expression of matrix metalloproteinases.^{29,30} Preliminary data on aldosterone levels in pooled plasma of the various mouse groups indicated that the average aldosterone level is higher in the torcetrapib-treated group (+15%) and combination-treated group (+48%) than in the atorvastatin-treated group. This suggests that the torcetrapib-induced increase in aldosterone levels may causally increase the inflammatory plaque phenotype in mice.

In conclusion, torcetrapib inhibits the progression of atherosclerosis, but does not enhance the anti-atherosclerotic potency of atorvastatin. In addition, as compared to atorvastatin, torcetrapib causes a more pro-inflammatory and unstable lesion phenotype.

Acknowledgments

We thank Dr. C. Maugeais (Hoffman La Roche, Basel, Switzerland) for scientific discussion and E.H. Offerman, C.M. van den Hoogen and A. van Nieuwkoop (TNO-Biosciences, Leiden, The Netherlands) for their excellent technical assistance. This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO, and supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.), the Centre for Medical Systems Biology (CMSB project 115) in the framework of the Netherlands Genomics Initiative (NGI) and the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.). J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (NHS grant 2001D032).

References

1. Ha YC, Calvert GD, Barter PJ. In vivo metabolism of esterified cholesterol and apoproteins in rabbit plasma low density lipoproteins. *Atherosclerosis*. 1979; 34:451-455.
2. Barter PJ and Kastelein JJ. Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. *J.Am.Coll.Cardiol*. 2006; 47:492-499.
3. Chapman MJ. Therapeutic elevation of HDL-cholesterol to prevent atherosclerosis and coronary heart disease. *Pharmacol.Ther*. 2006; 111:893-908.
4. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM, Kastelein JJ, Bittner V, Fruchart JC. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *N.Engl.J.Med*. 2007; 357:1301-1310.
5. Takahashi K, Jiang XC, Sakai N, Yamashita S, Hirano K, Bujo H, Yamazaki H, Kusunoki J, Miura T, Kussie P. A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. *J.Clin.Invest*. 1993; 92:2060-2064.
6. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J.Clin.Invest*. 1994; 94:1872-1882.
7. Moriyama Y, Okamura T, Inazu A, Doi M, Iso H, Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, Komachi Y. A low prevalence of coronary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including

- those with plasma cholesteryl ester transfer protein deficiency. *Prev.Med.* 1998; 27:659-667.
8. Boekholdt SM, Kuivenhoven JA, Wareham NJ, Peters RJ, Jukema JW, Luben R, Bingham SA, Day NE, Kastelein JJ, Khaw KT. Plasma levels of cholesteryl ester transfer protein and the risk of future coronary artery disease in apparently healthy men and women: the prospective EPIC (European Prospective Investigation into Cancer and nutrition)-Norfolk population study. *Circulation.* 2004; 110:1418-1423.
 9. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J.Clin.Invest.* 1996; 97:2917-2923.
 10. Kuivenhoven JA, Jukema JW, Zwiderman AH, de Knijff P, McPherson R, Bruschke AV, Lie KI, Kastelein JJ. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N.Engl.J.Med.* 1998; 338:86-93.
 11. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *N.Engl.J.Med.* 2007; 356:1620-1630.
 12. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *N.Engl.J.Med.* 2007; 356:1304-1316.
 13. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *Lancet.* 2007; 370:153-160.
 14. Yvan-Charvet L, Matsuura F, Wang N, Bamberger MJ, Nguyen T, Rinninger F, Jiang XC, Shear CL, Tall AR. Inhibition of cholesteryl ester transfer protein by torcetrapib modestly increases macrophage cholesterol efflux to HDL. *Arterioscler.Thromb.Vasc.Biol.* 2007; 27:1132-1138.
 15. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N.Engl.J.Med.* 2007; 357:2109-2122.
 16. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler.Thromb.Vasc.Biol.* 2006; 26:2552-2559.
 17. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler.Thromb.Vasc.Biol.* 2007; 27:1706-1721.
 18. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM, Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *J.Lipid Res.* 2007; 48:1763-1771.
 19. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM, Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis.* 2008; 197:57-63.

20. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *J.Clin.Invest.* 1992; 90:1290-1295.
21. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J.Biol.Chem.* 1993; 268:10540-10545.
22. Gautier T, Tietge UJ, Boverhof R, Perton FG, Le Guern N, Masson D, Rensen PC, Havekes LM, Lagrost L, Kuipers F. Hepatic lipid accumulation in apolipoprotein C-I-deficient mice is potentiated by cholesteryl ester transfer protein. *J.Lipid Res.* 2007; 48:30-40.
23. Gijbels MJ, van der CM, van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G. Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis.* 1999; 143:15-25.
24. Volger OL, Mensink RP, Plat J, Hornstra G, Havekes LM, Princen HM. Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice. *Atherosclerosis.* 2001; 157:375-381.
25. Clark RW, Sutfin TA, Ruggeri RB, Willauer AT, Sugarman ED, Magnus-Aryitey G, Cosgrove PG, Sand TM, Wester RT, Williams JA, Perlman ME, Bamberger MJ. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler.Thromb.Vasc.Biol.* 2004; 24:490-497.
26. Guerin M, Lassel TS, Le Goff W, Farnier M, Chapman MJ. Action of atorvastatin in combined hyperlipidemia : preferential reduction of cholesteryl ester transfer from HDL to VLDL1 particles. *Arterioscler.Thromb.Vasc.Biol.* 2000; 20:189-197.
27. van Venrooij FV, Stolk RP, Banga JD, Sijmonsma TP, Van Tol A, Erkelens DW, Dallinga-Thie GM. Common cholesteryl ester transfer protein gene polymorphisms and the effect of atorvastatin therapy in type 2 diabetes. *Diabetes Care* 2003; 26:1216-1223.
28. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH, Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *J.Lipid Res.* 2007; 48:1263-1272.
29. Hirono Y, Yoshimoto T, Suzuki N, Sugiyama T, Sakurada M, Takai S, Kobayashi N, Shichiri M, Hirata Y. Angiotensin II receptor type 1-mediated vascular oxidative stress and proinflammatory gene expression in aldosterone-induced hypertension: the possible role of local renin-angiotensin system. *Endocrinology.* 2007; 148:1688-1696.
30. Hashikabe Y, Suzuki K, Jojima T, Uchida K, Hattori Y. Aldosterone impairs vascular endothelial cell function. *J.Cardiovasc.Pharmacol.* 2006; 47:609-613.
31. Suzuki J, Iwai M, Mogi M, Oshita A, Yoshii T, Higaki J, Horiuchi M. Eplerenone with valsartan effectively reduces atherosclerotic lesion by attenuation of oxidative stress and inflammation. *Arterioscler.Thromb.Vasc.Biol.* 2006; 26:917-921.
32. Keidar S, Hayek T, Kaplan M, Pavlotzky E, Hamoud S, Coleman R, Aviram M. Effect of eplerenone, a selective aldosterone blocker, on blood pressure, serum and macrophage oxidative stress, and atherosclerosis in apolipoprotein E-deficient mice. *J.Cardiovasc.Pharmacol.* 2003; 41:955-963.