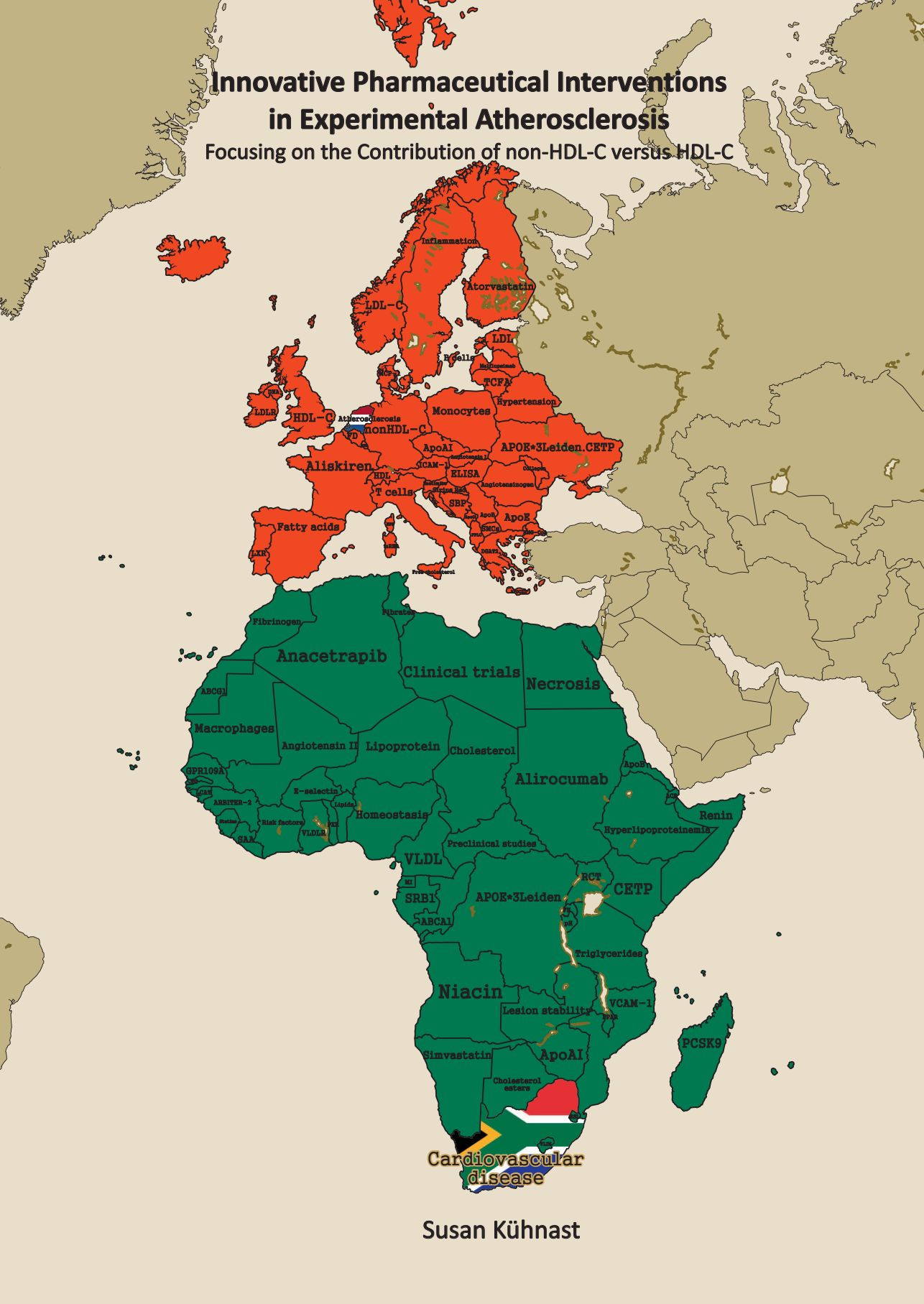
Innovative Pharmaceutical Interventions in Experimental Atherosclerosis

Focusing on the Contribution of non-HDL-C versus HDL-C



Susan Kühnast

Innovative Pharmaceutical Interventions in Experimental Atherosclerosis:
Focusing on the Contribution of non-HDL-C versus HDL-C

Colophon

ISBN: 978-94-6108-977-9

Cover Design: It's Ink, Parys, South Africa
Layout and printing: Gildeprint, Enschede

The printing of this thesis was kindly supported by:
Daan Traas fonds
TNO, Metabolic Health Research

Innovative Pharmaceutical Interventions in Experimental Atherosclerosis:
Focusing on the Contribution of non-HDL-C versus HDL-C

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 11 juni 2015
klokke 11:15 uur

door

Susan Kühnast

geboren te Ermelo, Zuid-Afrika
in 1980

Promotiecommissie

Promotor:	Prof. Dr. J.W. Jukema
Co-promotores:	Dr. J.M.G. Princen (TNO, Leiden)
	Dr. J.W.A. van der Hoorn (TNO, Leiden)
Overige leden:	Prof. Dr. Ir. L.M. Havekes
	Prof. Dr. P.C.N. Rensen
	Prof. Dr. E.S.G. Stroes (AMC, Amsterdam)
	Prof. Dr. J.A. Kuivenhoven (UMCG, Groningen)

The studies presented in this thesis were performed at the Gaubius Laboratory of TNO, Leiden, the Netherlands. The research was in part financially supported by Novartis, Sanofi-Aventis and Regeneron.

Table of Contents

Chapter 1 **7**
General introduction.

Chapter 2 **21**
Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden.CETP transgenic mice with or without treatment with atorvastatin. *J Hypertens. 2012 Jan;30(1):107-16.*

Chapter 3 **43**
Niacin reduces atherosclerosis development in APOE*3Leiden.CETP mice mainly by reducing non-HDL-cholesterol. *PLoS One. 2013 Jun 19;8(6):e66467.*

Chapter 4 **69**
Anacetrapib reduces progression of atherosclerosis, mainly by reducing non-HDL-cholesterol, improves lesion stability and adds to the beneficial effects of atorvastatin. *Eur Heart J. 2015 Jan 1;36(1):39-50.*

Chapter 5 **93**
Anacetrapib reduces (V)LDL-cholesterol by inhibition of CETP activity and reduction of plasma PCSK9. *Submitted.*

Chapter 6 **117**
Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances. the effects of a statin. *J Lipid Res. 2014 Oct;55(10):2103-12.*

Chapter 7 **143**
Innovative pharmaceutical interventions in cardiovascular disease: focusing on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising - A systematic review and meta-analysis of relevant preclinical studies and clinical trials. *Accepted – Eur J Pharmacol.*

Chapter 8 **179**
General discussion and future perspectives

Summary **195**
Samenvatting **199**
List of Publications **203**
Curriculum Vitae **205**
Dankwoord **207**

CHAPTER 1

General Introduction

Cardiovascular disease (CVD) causes a global burden with a death rate of 17.3 million per year which is rapidly inclining to an estimated 23.6 million in 2030.[1] Atherosclerosis, a chronic inflammatory disease of multifactorial origin that may ultimately lead to stenosis or atherothrombosis,[2, 3] is a dominant contributor to the development of CVD.[4] It is characterized by the development of atherosclerotic lesions consisting of activated endothelial cells, lipid accumulation, leukocytes, macrophages, foam cells, connective-tissue elements, calcified regions and necrotic cores.[3, 5] The progressive decrease in lumen size caused by the development of these lesions was previously described as the culprit that led to cardiovascular events.[3] However, it is now believed that this is attributable to a decrease in plaque stability, which may lead to rupture followed by thrombus formation on the ruptured plaques.[6] To this end, an unstable lesion is characterized by a thin, collagen-poor fibrous cap, decreased smooth muscle cells, increased macrophage infiltration and a large necrotic core.[5] This type of vulnerable lesion is referred to as a thin-cap fibroatheroma.[7] From a pharmaceutical perspective, several risk factors are currently being targeted in the fight against the development of atherosclerosis and the prevalence of CVD. These include amongst others; hypertension and high blood cholesterol, more specifically high low-density lipoprotein-cholesterol (LDL-C) and low high-density lipoprotein-cholesterol (HDL-C).[8]

1. Hypertension

Uncontrolled hypertension is the leading risk factor for CVD.[1] Numerous factors including; age, ethnicity, family history, genetic factors, lower education and socioeconomic status, obesity, smoking, sleep apnea and dietary factors contribute to the development of hypertension and several of these factors are modifiable. Nonetheless, according to statistics from the American Heart Association, the prevalence of high blood pressure, defined as a systolic blood pressure of ≥140 mmHg and a diastolic blood pressure of ≥90 mmHg, or taking anti-hypertensive medication, or being diagnosed with hypertension on at least two occasions, was as high as 33% for adults aged ≥20 years (extrapolated to 2010 using data from the National Health and Nutrition Examination Survey (NHANES) 2007 to 2010).[8] The World Health Organization reported that globally elevated blood pressure caused 51% of stroke deaths and 45% of coronary heart disease (CHD) deaths.[1] The inefficiency of the current treatment regimens to reduce these numbers is ascribed not only to lack of adherence, but also to failure of treatment strategies to fully neutralize all mechanisms involved in hypertension, as well as the activation of feedback mechanisms that counteract the treatment effects on blood pressure.[9] Therefore, the development of additional anti-hypertensive treatment options beyond the current 'gold standard' therapies, such as selective calcium channel blockers, β-blockers, diuretics, angiotensin converting enzyme

inhibitors (ACEi) and angiotensin II type I receptor blockers (ARBs),[10] is needed to more effectively treat hypertension.

The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in the regulation of blood pressure. Renin secreted by the kidneys cleaves angiotensinogen produced by the liver to angiotensin I which is converted by angiotensin converting enzyme to angiotensin II. Angiotensin II, in turn binds to angiotensin II receptors leading to arterial vasoconstriction, other tubular and glomerular effects, as well as inflammation, hypertrophy and fibrosis.[10] Current treatment strategies that disrupt the RAAS, such as ACEi and ARBs result in compensatory increases in angiotensin I or II, as well as in plasma renin activity (PRA).[11] The latter may have further implications since renin was shown to exert angiotensin I-independent effects by binding to (pro)renin receptors.[10] Moreover, increased PRA has been associated with increased mortality rates as a result of myocardial infarction (MI) and renal failure.[12] The development of direct renin inhibitors emerged as a potential treatment strategy to more effectively inhibit the RAAS at the point of origin and at its rate-limiting step.[13] Aliskiren is the first orally active direct renin inhibitor approved for the treatment of hypertension.[13] The extent to which aliskiren, administered as monotreatment and in combination with current 'gold standard' treatment strategies in various patient populations, can provide clinical benefit remains to be elucidated.

2. High blood cholesterol

Cholesterol is a hydrophobic molecule that serves as a structural component in plasma membranes and as a precursor for the synthesis of steroid hormones and bile acids.[14, 15] Cholesterol is transported through the circulation in five major classes of lipoprotein particles; chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and HDL.[15] Chylomicrons transport dietary lipids after uptake and secretion from the intestines and VLDL is secreted from the liver to deliver triglycerides and cholesterol to other tissues.

2.1 Low-density lipoprotein-cholesterol

In 1913, Nikolai N. Anitschkow first described the involvement of cholesterol in the development of atherosclerosis when rabbits fed a high-cholesterol diet developed human-like arterial lesions.[16] The recent 100th year anniversary of this discovery is worth commemorating given that serum cholesterol contained in LDL particles is now well recognized as a primary causal risk factor for CHD as evidenced by experimental, epidemiological and genetic studies.[17]

The American Heart Association reported a prevalence of hypercholesterolemia defined by TC levels ≥200 mg/dL of 43.4% and by LDL-C levels ≥130 mg/dL of 31.1% (extrapolated to 2010 using data from NHANES 2007 to 2010).[8] The prevalence of high TC and LDL-C levels in 2009/2010 was considerably lower compared to 1999/2000, most likely attributable to statin use.[1] Statins reduce LDL-C up to 55% by inhibiting hydroxy-3-methyl-glutaryl-CoA reductase, a rate limiting step in cholesterol biosynthesis.[18] Intervention trials provided ample evidence that the lowering of LDL-C with statin therapy contributes to a reduction of CHD[19-21] and recent trials indicated that intensive lipid-lowering with statins may be more beneficial in risk reduction than less intensive (or standard) therapy.[19] According to results from the latter meta-analysis, every 1 mmol/L (40 mg/dL) reduction in LDL-C was associated with a 22% reduction in the risk of major vascular events, suggesting that a 2-3 mmol/L reduction in LDL-cholesterol (LDL-C) would correspond with a 40-50% reduction in events. Nonetheless, there remains a substantial residual risk despite statin treatment which warrants the development of other treatment options to better protect against CVD, especially in combination with statins.

2.1.1 Approved LDL-C-lowering treatment strategies beyond statins

Despite not sharing the success rate of statin treatment, other LDL-C-lowering treatment strategies have been approved for clinical use. These include: bile acid-binding resins, cholesterol absorption inhibitors, niacin, peroxisome proliferator-activated receptor (PPAR)-α agonists and PPAR-γ agonists.[17] In fact, recently the cholesterol absorption inhibitor, ezetimibe was the first compound shown to add to the effect of a statin on CVD outcome (http://newsroom.heart.org/news/cholesterol-lowering-drug-with-different-action-adds-to-statins-reduction-of-cardiovascular-risk).

Bile acid-binding resins and cholesterol absorption inhibitors were developed to inhibit cholesterol absorption in the intestine from food and bile.[22] Resins reduce the efficiency of cholesterol absorption by binding bile acids and therewith decreasing intestinal solubilization of lipids, and by binding bile acids resins also increase bile acid synthesis from the precursor cholesterol.[23] The cholesterol absorption inhibitor, ezetimibe limits cholesterol absorption by blocking the function of the transporter Niemann pick C-1-like 1 (NPC1L1). The benefits of niacin on plasma lipids were first reported in 1955 and led to the development of niacin for therapeutic purposes.[24] The lipid-lowering effects of niacin is ascribed to decreased free fatty acid (FFA) flux from adipose tissue to the liver, although this reduction in FFAs is followed by a rebound effect. Another mechanism described by which niacin decreases lipids is by decreasing TG synthesis.[25] PPARs are nuclear transcription factors involved in the regulation of target gene expression and their effects on glucose and lipid metabolism were utilized to develop PPAR agonists for the treatment of hyperglycemia and dyslipidemia.[26, 27] PPAR-α activation decreases lipids by increasing lipoprotein lipase-mediated lipolysis,

VLDL remnants clearance and β-oxidation.[28] PPAR-γ agonists mainly mediate glucose homeostasis,[26] but pioglitazone also weakly activates PPAR-α and, therefore, also has minor effects on lipid metabolism.[29]

2.1.2. Emerging LDL-C-lowering treatment strategies beyond statins

Several other approaches to lower LDL-C are currently being investigated in clinical trials, including amongst others: apolipoprotein B inhibition by for example antisense oligonucleotides (ASOs), microsomal triglyceride transport protein (MTP) inhibitors, proprotein convertase subtilisin kexin type 9 (PCSK9) inhibition by for instance monoclonal antibodies, gene-silencing or vaccines and PPAR-δ agonists.[17] Some of these treatment strategies have already been approved in certain countries.

The ASO against apolipoprotein B, mipomersen inhibits the synthesis of apolipoprotein B by binding to the messenger RNA coding for apolipoprotein B-100 and the MTP inhibitor, lomitapide inhibits the transfer of triglycerides to apolipoprotein B during formation of a mature VLDL particle within hepatocytes. Both compounds, therefore, decrease LDL-C by reducing VLDL production and secretion.[17] PCSK9 is a serine protease responsible for LDL receptor (LDLR) degradation.[30] Interestingly, the upregulation of the LDLR after statin treatment is accompanied by an upregulation of PCSK9 which in turn promotes LDLR degradation.[31-33] PCSK9 inhibition has, therefore, emerged as a promising new strategy to lower LDL-C, especially in combination with statins. PPAR-δ agonists also improve atherogenic lipid profiles by modifying cell fuel preference from glucose to lipids[34] and a reduction in cholesterol absorption via NPC1L1 has been described as another possible mechanism.[35]

2.2 High-density lipoprotein-cholesterol

In the 1970s, Miller & Miller hypothesized that a reduction in plasma HDL concentration may accelerate the development of atherosclerosis and ischemic heart disease by impairing cholesterol clearance from the arterial wall.[36] Besides its major role in reverse cholesterol transport, HDL has also been described to have anti-inflammatory, anti-oxidant, anti-platelet and vasodilatory properties.[37] Although the original hypothesis referred to HDL particle concentration which could not be measured at the time,[37] epidemiological studies consistently reported an inverse association between CHD risk and HDL-C.[38-40] Results from 4 prospective epidemiologic studies indicated that an increase of 1 mg/dL (0.03 mM) in HDL-C was associated with a 2-3% reduction in CHD risk.[41] However, data from genetic studies do not support a causal relationship between increased HDL-C and reduced risk of MI[42, 43] and evidence from large clinical trials is lacking.

The American Heart Association revealed a prevalence of HDL-C levels ≤40 mg/dL of 21.8% (extrapolated to 2010 using data from NHANES 2007 to 2010). Whereas currently

no clinical trial has demonstrated beneficial effects of HDL-C-raising therapies, several HDL-targeting therapies are still being investigated in clinical trials.

2.2.1. HDL-C-raising treatment strategies beyond statins

Treatment strategies approved for the treatment of hyperlipidemia, such as niacin and PPAR-α agonists (fibrates), that mainly decreases triglycerides with a small LDL-C-lowering effect, also increases HDL-C. Other therapies in clinical development primarily aimed to increase HDL-C include CETP inhibitors, scavenger receptor B-I (SR-BI) inhibitors and apolipoprotein A-I inducers.[44] In addition, the effects of novel treatment strategies specifically targeting HDL are currently being investigated in clinical trials, including reconstituted and delipidated HDL, as well as HDL mimetics, apolipoprotein A-I mimetic peptides and recombinant human lecithin cholesterol acyltransferase (LCAT).

Niacin is described to increase HDL-C by increasing apolipoprotein A-I lipidation and by decreasing apolipoprotein A-I removal,[25, 45] and PPAR-α agonists are shown to increase HDL-C by increasing apolipoprotein A-I/II expression and cholesterol efflux from macrophages.[26] In 1989, markedly increased HDL-C led to the discovery of the first mutation in the CETP gene in two Japanese subjects.[46] CETP facilitates the transfer of cholesteryl esters from atheroprotective HDL to atherogenic (V)LDL and has become a target to increase HDL-C.[47] The HDL-C-raising effects of niacin and PPAR-α agonists are also ascribed to a reduction in CETP.[48, 49] The PPAR-γ agonist, pioglitazone increases HDL-C by weakly activating PPAR-α.[29] PPAR-δ agonists also increase HDL-C and this effect is ascribed to possible mechanisms involving apolipoprotein A-II and ABCA1.[35] The development of reconstituted and delipidated HDL, as well as HDL mimetics, apolipoprotein A-I mimetic peptides and recombinant human lecithin cholesterol acyltransferase (LCAT) have emerged as potential approaches to improve reverse cholesterol transport.[50] In addition, the therapeutic use of recombinant apolipoprotein A-I Milano originated from the observation that carriers of this mutation have low levels of HDL-C without increased atherosclerosis as observed in patients with hypoalhalipoproteinemia.[51, 52] It remains to be elucidated whether these novel treatment strategies may provide additional clinical benefit beyond current therapies.

3. Experimental model for human-like lipoprotein metabolism and atherosclerosis

To investigate the effects of innovative pharmaceutical interventions in experimental atherosclerosis, we used the APOE*3Leiden.CETP mouse model. While normal wild-type mice have a very rapid clearance of apolipoprotein B-containing lipoproteins, APOE*3Leiden.CETP mice have impaired clearance of apolipoprotein B-containing lipoproteins and

mimic the slow clearance observed in humans, particularly in patients with familial dysbetalipoproteinemia (FD).[53] The APOE*3Leiden mouse was initially developed as an animal model for FD or type III hyperlipoproteinemia, which is characterized by elevated levels of cholesterol and an increased ratio of cholesterol to triglycerides in the VLDL and IDL fractions, resulting in the appearance of β-VLDL particles.[53, 54] Similar to FD patients, APOE*3Leiden and APOE*3Leiden.CETP mice carry a major part of plasma cholesterol in the VLDL and VLDL-remnant particles, leading to the formation of β-VLDL particles, which further increases after cholesterol feeding. These mice respond in a similar way to statins as humans with decreases in the apolipoprotein B-containing lipoproteins up to 55%. In addition, APOE*3Leiden.CETP mice express human CETP under control of its natural flanking regions,[55] a crucial gene involved in HDL metabolism and implicated in the mechanisms by which most therapies modulate HDL.[49] These mice develop diet-induced atherosclerosis and respond to TC/LDL-C-lowering and HDL-C-raising drugs in a human-like manner[45, 48, 56-58] and are, therefore, a suitable model to study the effects of innovative pharmaceutical interventions on lipid and lipoprotein metabolism and atherosclerosis development.

4. Outline of this thesis

The research described in this thesis investigated the effects of innovative pharmaceutical interventions in experimental atherosclerosis. Statin treatment is currently the first line of defense against CVD. However, the treatment of CVD remains suboptimal due to; (i) a residual risk that persists after statin treatment, (ii) failure for some patients to reach LDL-C targets despite statin treatment, and (iii) lack of adherence to statin treatment as a result of statin intolerance. We, therefore investigated the effects of novel treatment strategies administered as monotreatment, but also in combination with statin treatment on atherosclerosis development in APOE*3.Leiden.CETP mice, a well-established model for lipid metabolism and atherosclerosis.

Hypertension is a leading risk factor for CVD and is associated with the development of atherosclerosis. Aliskiren is the first commercially available, orally active, direct renin inhibitor approved for the treatment of hypertension. In **chapter 2**, we investigated the effects of aliskiren administered as monotreatment and in combination with atorvastatin on systolic blood pressure, total cholesterol, inflammation markers and atherosclerotic lesion size and composition in APOE*3Leiden.CETP mice.

Cholesterol contained in LDL particles is well recognized as a primary causal risk factor for CHD as evidenced by experimental, epidemiological and genetic data. Furthermore, intervention trials provided ample evidence that the lowering of LDL-C contributes to a reduction in CHD. However, despite the fact that epidemiological studies consistently

reported an inverse association between HDL-C and CHD risk, the benefits of raising HDL-C remain less defined. In chapter 3 to 6, we investigated the effects of novel lipid-modifying treatment strategies, i.e. LDL-C lowering and/or HDL-C-raising compounds on atherosclerosis development in the APOE*3Leiden.CETP mouse model, since these mice respond to both LDL-C-lowering and HDL-C-raising compounds in a human-like manner.

The benefits of niacin on plasma lipids were first described in 1955 and led to the development of niacin for therapeutic purposes. In **chapter 3**, we evaluated the effects of niacin alone and in combination with simvastatin on plasma lipid levels and atherosclerotic lesion size and composition. To further explore the mechanism by which niacin reduces atherosclerosis, we performed additional VLDL production and clearance, as well as reverse cholesterol transport experiments. We also conducted statistical analyses to assess the contribution of the LDL-C-lowering versus HDL-C-raising effects of niacin on the inhibition of atherosclerosis.

CETP is involved in lipoprotein metabolism by facilitating the transfer of cholesterol esters from atheroprotective HDL to atherogenic (V)LDL. In **chapter 4**, we investigated the effects of a broad dose range of the novel CETP inhibitor anacetrapib on CETP activity, lipid levels, atherosclerotic lesion size and composition and HDL function. In addition, we examined possible additive/synergistic effects of anacetrapib on top of atorvastatin. We also performed statistical analyses to evaluate whether the effects of anacetrapib and atorvastatin on atherosclerosis development could be explained by either a decrease in non-HDL-C or an increase in HDL-C or both. Since lowering of non-HDL-C was a major determinant of lesion size, we investigated the mechanism by which anacetrapib decreases (V)LDL-C levels in **chapter 5**.

PCSK9 is a serine protease responsible for LDLR degradation. The upregulation of the LDLR after statin treatment is accompanied by an upregulation of PCSK9 which in turn promotes LDLR degradation. Inhibition of PCSK9 is, therefore, a potential novel strategy in the treatment against CVD, especially in combination with statin treatment. In **chapter 6**, we investigated the effects of 2 dosages of the fully human, monoclonal antibody, alirocumab alone and in combination with atorvastatin on hepatic LDLR protein levels, hepatic and plasma lipid levels, atherosclerosis development and plaque morphology.

In **chapter 7**, we reviewed the effects of established and novel treatment strategies, specifically targeting HDL, on inhibition of atherosclerosis development in animals expressing CETP, a crucial gene involved in HDL metabolism and implicated in the mechanisms by which most therapies modulate HDL. In addition, we conducted a meta-analysis to evaluate the potential effects of these treatment strategies on the prevention of clinical events in randomized controlled trials. In this systematic review and meta-analysis of preclinical studies and clinical trials, we focused specifically on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising on inhibition of atherosclerosis and the prevention of CVD.

The results obtained in these studies and their clinical relevance are discussed in the General discussion and future perspectives in **chapter 8**.

References

1. Laslett LJ, Alagona P, Jr., Clark BA, 3rd, Drozda JP, Jr., Saldivar F, Wilson SR, Poe C and Hart M. The worldwide environment of cardiovascular disease: prevalence, diagnosis, therapy, and policy issues: a report from the American College of Cardiology. *Journal of the American College of Cardiology*. 2012;60:S1-49.
2. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *The New England journal of medicine*. 2013;368:2004-13.
3. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine*. 2005;352:1685-95.
4. Galkina E and Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annual review of immunology*. 2009;27:165-97.
5. Libby P and Sasiela W. Plaque stabilization: Can we turn theory into evidence? *The American journal of cardiology*. 2006;98:26P-33P.
6. Finn AV, Nakano M, Narula J, Kolodgie FD and Virmani R. Concept of vulnerable/unstable plaque. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:1282-92.
7. Moreno PR. The high-risk thin-cap fibroatheroma: a new kid on the block. *Circulation Cardiovascular interventions*. 2009;2:500-2.
8. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D and Turner MB. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation*. 2014;129:e28-e292.
9. Monge M, Lorthioir A, Bobrie G and Azizi M. New drug therapies interfering with the renin-angiotensin-aldosterone system for resistant hypertension. *Journal of the renin-angiotensin-aldosterone system : JRAAS*. 2013;14:285-9.
10. Paulis L and Unger T. Novel therapeutic targets for hypertension. *Nature reviews Cardiology*. 2010;7:431-41.
11. Rajagopalan S, Bakris GL, Abraham WT, Pitt B and Brook RD. Complete renin-angiotensin-aldosterone system (RAAS) blockade in high-risk patients: recent insights from renin blockade studies. *Hypertension*. 2013;62:444-9.
12. Jensen C, Herold P and Brunner HR. Aliskiren: the first renin inhibitor for clinical treatment. *Nature reviews Drug discovery*. 2008;7:399-410.
13. Friedrich S and Schmieder RE. Review of direct renin inhibition by aliskiren. *Journal of the renin-angiotensin-aldosterone system : JRAAS*. 2013;14:193-6.
14. Rader DJ, Cohen J and Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *Journal of Clinical Investigation*. 2003;111:1795-1803.
15. National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*. 2002;106:3143-421.
16. Steinberg D. In celebration of the 100th anniversary of the lipid hypothesis of atherosclerosis. *Journal of lipid research*. 2013;54:2946-9.
17. Ridker PM. LDL cholesterol: controversies and future therapeutic directions. *Lancet*. 2014;384:607-17.
18. Tobert JA. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nature reviews Drug discovery*. 2003;2:517-26.
19. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J and Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *The Lancet*. 2010;376:1670-1681.

20. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R and Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins. *The Lancet*. 2005;366:1267-1278.
21. Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R and Baigent C. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *The Lancet*. 2012;380:581-590.
22. Burnett JR and Huff MW. Cholesterol absorption inhibitors as a therapeutic option for hypercholesterolaemia. *Expert opinion on investigational drugs*. 2006;15:1337-51.
23. Princen HMG, Post SM and Twisk J. Regulation of bile acid biosynthesis. *Curr Pharm Des*. 1997;3:59-84.
24. Carlson LA. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine*. 2005;258:94-114.
25. Kamanna VS, Ganji SH and Kashyap ML. Recent advances in niacin and lipid metabolism. *Current opinion in lipidology*. 2013;24:239-45.
26. Jandeleit-Dahm KAM, Calkin A, Tikellis C and Thomas M. Direct antiatherosclerotic effects of PPAR agonists. *Current opinion in lipidology*. 2009;20:24-29.
27. Lalloyer F and Staels B. Fibrates, glitazones, and peroxisome proliferator-activated receptors. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:894-9.
28. Bijland S, Pieterman EJ, Maas AC, van der Hoorn JW, van Erk MJ, van Klinken JB, Havekes LM, van Dijk KW, Princen HM and Rensen PC. Fenofibrate increases very low density lipoprotein triglyceride production despite reducing plasma triglyceride levels in APOE*3-Leiden.CETP mice. *The Journal of biological chemistry*. 2010;285:25168-75.
29. Sakamoto J, Kimura H, Moriyama S, Odaka H, Momose Y, Sugiyama Y and Sawada H. Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. *Biochemical and biophysical research communications*. 2000;278:704-11.
30. Horton JD, Cohen JC and Hobbs HH. PCSK9: a convertase that coordinates LDL catabolism. *Journal of lipid research*. 2009;50 Suppl:S172-7.
31. Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L and Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:1454-9.
32. Mayne J, Dewpura T, Raymond A, Cousins M, Chaplin A, Lahey KA, Lahaye SA, Mbikay M, Ooi TC and Chretien M. Plasma PCSK9 levels are significantly modified by statins and fibrates in humans. *Lipids in health and disease*. 2008;7:22.
33. Careskey HE, Davis RA, Alborn WE, Troutt JS, Cao G and Konrad RJ. Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *Journal of lipid research*. 2008;49:394-8.
34. Furnsinn C, Willson TM and Brunmair B. Peroxisome proliferator-activated receptor-delta, a regulator of oxidative capacity, fuel switching and cholesterol transport. *Diabetologia*. 2007;50:8-17.
35. Ehrenborg E and Skogsberg J. Peroxisome proliferator-activated receptor delta and cardiovascular disease. *Atherosclerosis*. 2013;231:95-106.
36. Miller GJ and Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet*. 1975;1:16-9.
37. Kingwell BA, Chapman MJ, Kontush A and Miller NE. HDL-targeted therapies: progress, failures and future. *Nature reviews Drug discovery*. 2014;13:445-64.
38. Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A and Zukel WJ. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation*. 1977;55:767-772.
39. Miller NE, Thelle DS, Forde OH and Mjos OD. The Tromso heart-study. High-density lipoprotein and coronary heart-disease: a prospective case-control study. *Lancet*. 1977;1:965-8.

40. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA : the journal of the American Medical Association*. 2009;302:1993-2000.
41. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR, Bangdiwala S and Tyroler HA. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*. 1989;79:8-15.
42. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Holm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart A, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett MS, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki ML, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PI, Klungel OH, Maitland-van der Zee AH, Peters BJ, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VH, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WM, Boer JM, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, Konig IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, Schrezenmeir J, Schreiber S, Schafer A, Danesh J, Blankenberg S, Roberts R, McPherson R, Watkins H, Hall AS, Overvad K, Rimm E, Boerwinkle E, Tybjaerg-Hansen A, Cupples LA, Reilly MP, Melander O, Mannucci PM, Ardissino D, Siscovick D, Elosua R, Stefansson K, O'Donnell CJ, Salomaa V, Rader DJ, Peltonen L, Schwartz SM, Altshuler D and Kathiresan S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet*. 2012;380:572-580.
43. Haase CL, Tybjaerg-Hansen A, Qayyum AA, Schou J, Nordestgaard BG and Frikke-Schmidt R. LCAT, HDL cholesterol and ischemic cardiovascular disease: a Mendelian randomization study of HDL cholesterol in 54,500 individuals. *The Journal of clinical endocrinology and metabolism*. 2012;97:E248-56.
44. Remaley AT, Norata GD and Catapano AL. Novel concepts in HDL pharmacology. *Cardiovascular research*. 2014;103:423-8.
45. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
46. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H and et al. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*. 1989;342:448-51.
47. Barter PJ and Rye KA. Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk. *Journal of lipid research*. 2012;53:1755-66.
48. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.
49. Chapman MJ, Le Goff W, Guerin M and Kontush A. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *European heart journal*. 2010;31:149-64.
50. Balder JW, Staels B and Kuivenhoven JA. Pharmacological interventions in human HDL metabolism. *Current opinion in lipidology*. 2013;24:500-9.
51. Franceschini G, Sirtori CR, Capurso A, 2nd, Weisgraber KH and Mahley RW. A-IMilano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *The Journal of clinical investigation*. 1980;66:892-900.

52. Sirtori CR, Calabresi L, Franceschini G, Baldassarre D, Amato M, Johansson J, Salvetti M, Monteduro C, Zulli R, Muiesan ML and Agabiti-Rosei E. Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study. *Circulation*. 2001;103:1949-54.
53. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR and Havekes LM. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *The Journal of clinical investigation*. 1991;88:643-55.
54. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM and Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *The Journal of biological chemistry*. 1993;268:10540-5.
55. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
56. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1706-21.
57. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
58. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.

CHAPTER 2

Aliskiren Inhibits Atherosclerosis Development and Improves Plaque Stability in APOE*3Leiden.CETP Transgenic Mice with or without Treatment with Atorvastatin

Susan Kühnast[1,2], José W.A. van der Hoorn[1,2], Anita M. van den Hoek[1], Louis M. Havekes[1,3], Gene Liau[4], J. Wouter Jukema[2], Hans M.G. Princen[1]

[1]TNO - Metabolic Health Research, Leiden, The Netherlands;
[2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[3]Dept. of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands;
[4]Novartis Institutes for BioMedical Research, Cambridge, MA, USA

J Hypertens. 2012 Jan;30(1):107-16.

Abstract

Objective Aliskiren is the first commercially available, orally active, direct renin inhibitor approved to treat hypertension. The renin-angiotensin system has been shown to be a significant contributor to the development of hypercholesterolemia-induced atherosclerosis. The aim of this study was to evaluate the anti-atherosclerotic and plaque stabilization effects of aliskiren alone and in combination with atorvastatin.

Methods APOE*3Leiden.CETP mice (n=14-17/group) were fed a Western-type diet (containing 0.25% cholesterol) alone or were treated with either aliskiren (15 mg/kg/day), atorvastatin (3.6 mg/kg/day) or a combination of aliskiren and atorvastatin. Effects on systolic blood pressure (SBP), total cholesterol, inflammation markers and atherosclerotic size and composition were assessed.

Results Aliskiren reduced SBP (-19%, P<0.001) and atorvastatin reduced total cholesterol (-24%, P<0.001). Atherosclerotic lesion area was reduced by aliskiren (-40%, P<0.01), atorvastatin (-61%, P<0.001) and the combination treatment (-69%, P<0.001). Aliskiren alone and together with atorvastatin decreased the number of T cells in the aortic root area (-60%, P<0.01; -41%, P<0.05), as well as macrophage (-64%, P<0.001; -72%, P<0.001) and necrotic area (-52%, P=0.071; -84%, P<0.001) in the lesion. Atorvastatin alone and together with aliskiren decreased monocyte adherence (-43%, P<0.05; -51%, P<0.01) and monocyte chemoattractant protein-1 (both -36%, P<0.01). The combination treatment decreased the number of lesions (-17%, P<0.05) and E-selectin (-17%, P<0.05).

Conclusion Aliskiren inhibited atherosclerosis development and improved plaque stability alone and in combination with atorvastatin, possibly via a mechanism involving T cells. These results suggest a potential benefit of using aliskiren in a clinical setting, particularly in combination with statin treatment.

Keywords APOE*3Leiden.CETP mice, aliskiren, atorvastatin, hypertension, atherosclerosis, plaque stability

Introduction

Atherosclerosis is a chronic inflammatory disease of multifactorial origin that may ultimately lead to stenosis or thrombosis.[1, 2] It is characterized by the development of atherosclerotic lesions consisting of activated endothelial cells, inflamed smooth muscle cells (SMCs), lipid accumulation, leukocytes, macrophages, foam cells, connective-tissue elements, calcified regions and necrotic cores.[2-4]

It is well known that hypertension is associated with increased cardiovascular risk and progression of atherosclerosis.[5-7] Endothelial dysfunction occurs secondary to hypertension and/or hypercholesterolemia in the early stages of atherogenesis.[8] The effects of increased renin-angiotensin system (RAS) activity on both blood pressure and the vascular endothelium contribute to target organ damage and enhance cardiovascular risk. These effects include vasoconstriction and remodeling of the resistance vessels.[9] Angiotensin II is considered a contributor to the development of hypercholesterolemia-induced atherosclerosis.[10, 11] It was found to be involved in inflammation, migration, proliferation and growth[7, 12] by regulating adhesion molecule expression, as well as cytokine, chemokine and growth factor secretion.[13] RAS activity is not only found in the circulation, but local RAS components have also been detected in several tissues, including cardiovascular tissues.[14, 15] Furthermore, RAS activity was implicated in cholesterol synthesis, oxidation of low-density lipoprotein (LDL) molecules, production of reactive oxygen species and SMC proliferation, as well as monocyte activation and adhesion to the endothelium.[8] Beneficial effects of RAS blockers, including angiotensin converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) on atherosclerosis development have been observed in animal and human studies.[16-18] The blockage of RAS appears to be an important factor in atherosclerotic plaque stabilization.[7]

RAS blockade by ACEi and ARBs may not be optimal due to the activation of feedback mechanisms that result in increased plasma renin activity (PRA).[19, 20] Increased PRA has been associated with four to six times higher mortality rates as a result of myocardial infarctions and renal failure.[19] Furthermore, a large longitudinal analysis recently revealed additional side effects in patients with ARB + ACEi therapy.[21] Direct renin inhibitors (DRIs) have been suggested to be more effective than ACEi and ARBs in the prevention or reversal of target organ damage and cardiovascular events[12] by blocking the RAS at the point of origin and at its rate-limiting step.[9, 22] Furthermore, DRIs may exhibit less adverse effects compared to other RAS blockers.[19, 23] Aliskiren is the first commercially available, orally active, non-peptide-like renin inhibitor approved for the treatment of hypertension. It inhibits the catalytic activity of renin by binding to the active site of renin. The blockade of renin with aliskiren may inhibit the feedback effects observed with ACEi and ARBs and thereby, provide a more effective blockage of the RAS.[12] Clinical trials, including the ALLAY (Aliskiren in Left-Ventricular Hypertrophy),[24] the ALOFT (Aliskiren Observation of Heart

Failure Treatment)[25] and the AVOID (Aliskiren in the Evaluation of Proteinuria In Diabetes) studies,[26] have shown beneficial effects of aliskiren on various markers of organ damage. A decrease in atherosclerosis development with aliskiren monotreatment has been observed in experimental studies.[7, 15, 27] However, the effect of aliskiren on major clinical endpoints is not yet known.

To mimic the clinical situation, we have evaluated the effects of aliskiren alone or in combination with the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor, atorvastatin, as statins may be considered as standard treatment for cardiovascular disease (CVD). The purpose of this study is, therefore, to investigate the anti-atherosclerotic effects of the renin inhibitor aliskiren, alone and in combination with atorvastatin, as well as its effects on plaque composition in APOE*3Leiden.CETP transgenic mice. These mice have a human-like lipoprotein profile and develop atherosclerosis when fed a Western-type diet (WTD).[28] They also respond to treatment with anti-atherosclerotic drugs in a similar way as humans and are, therefore, a suitable model for studying the effects of drugs on hyperlipidemia and atherosclerosis.[29-33]

Methods

Mice

Female heterozygous APOE*3Leiden.CETP transgenic mice, 8-12 weeks of age, from the specific pathogen free breeding stock at TNO-Biosciences (Leiden) were used. APOE*3Leiden mice, characterized by an enzyme-linked immunosorbent assay (ELISA) for human apoE, were crossbred with cholesteryl ester transfer protein (CETP) transgenic mice that express human CETP under control of its natural flanking regions.[28] Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO).

Experimental design and diets

Mice were fed on a regular chow until 8-12 weeks of age. During a 3-week run-in period, all mice received a semi-synthetic modified WTD, containing 0.25% cholesterol, 15% saturated fat and 40% sucrose (all w/w, final concentration). This resulted in moderately elevated plasma cholesterol levels of 16.2 ± 3.1 mmol/l. After matching into four groups of 14-17 mice each based on age, body weight and plasma lipid levels, the mice received WTD alone (control group), or were treated with aliskiren (15 mg/kg/day), or with 0.0036% (w/w) atorvastatin (3.6 mg/kg/day), or with aliskiren (15 mg/kg/day) plus atorvastatin (3.6 mg/kg/day) for a period of 14 weeks. Aliskiren (provided by Novartis Institutes for Biomedical Research) was dissolved in 0.9% saline and administered by osmotic mini-pumps from

Alzet (model 2006 for first 6 weeks and thereafter model 1004 twice for 4 weeks) placed subcutaneously on the back of the animals. Mini-pumps loaded with PBS were placed in the control group and in the atorvastatin group. Atorvastatin was administered by admixture to the diet. The atorvastatin dosage was increased from 0.0018% w/w to 0.0036% w/w (3.6 mg/kg/day) after 3 weeks as a result of a non-significant reduction in total cholesterol levels (-10%, P=0.084). The animals received food and water *ad libitum*. Body weight and food intake were monitored throughout the study. The study was performed under blinded conditions.

Plasma total cholesterol levels, lipoprotein profile and markers of inflammation

After a 4-h fasting period, EDTA plasma was collected every 3-4 weeks (Sarstedt, Nümbrecht, Germany). Plasma total cholesterol levels (Roche Diagnostics, No-1489437) were measured by a standard enzymatic method. After 8 weeks of treatment, pooled lipoprotein profiles for total cholesterol and phospholipids were measured by fast protein liquid chromatography.[34] E-selectin levels and monocyte chemoattractant protein-1 (MCP-1) levels (R&D Systems Inc., USA) were determined by ELISA at sacrifice according to manufacturer's instructions. Fibrinogen levels were determined by ELISA, using rabbit anti-rat fibrin monomer immunoglobulin G as capture antibody and peroxidase-conjugated goat anti-mouse fibrinogen immunoglobulin G (Nordic, Tilburg, The Netherlands) as detection antibody. Mouse plasma with gravimetrically determined fibrinogen content was used for calibration.

Systolic blood pressure

To evaluate the effect of aliskiren, the systolic blood pressure (SBP) was measured in all groups after 10 and 13 weeks of treatment by cuff-tail method using the Non-Invasive Blood Pressure Monitor (Columbus Instruments, OH, USA). For each mouse, the blood pressure was measured three times during one session.[34]

Histological assessment of atherosclerosis

After the 14-week treatment period, all the mice were sacrificed and hearts were isolated, formalin fixed and embedded in paraffin. They were then sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch. Once the aortic root was identified by the appearance of aortic valve leaflets, serial cross sections (5 µm thick with intervals of 50 µm) were mounted on 3-aminopropyl-triethoxy-silane-coated slides.[35] These sections were stained with hematoxylin-phloxine-saffron (HPS) for histological analysis. For each mouse, the lesion area was measured in four subsequent sections. Each section consisted of three segments. The average lesion area per cross section was then calculated for each mouse.

For determination of atherosclerotic lesion size and severity, the lesions were classified into five categories according to the American Heart Association (AHA).[36] I) early fatty streak: up to ten foam cells in the intima with no other changes, II) regular fatty streak: ten or more foam cells in the intima with no other changes, III) mild plaque: a fibrotic cap and the presence of foam cells in the media, IV) moderate plaque: progressed lesions with an affected media, but without loss of architecture in the media, V) severe plaque: the media is severely affected and broken elastic fibers, cholesterol clefts, calcification and necrosis are frequently observed.[28, 37] Per mouse, the percentage of all lesions found in the respective categories was calculated. The total lesion area, number of lesions and undiseased segments were calculated per cross section. Lesion severity as a percentage of lesion area was also determined. Type I-III lesions were classified as mild lesions and type IV-V lesions were classified as severe lesions.

Mouse monocytes and T cells were immunostained with rabbit anti-mouse AIA31240 (1: 1000; Accurate Chemical and Scientific, New York, USA) and rat anti-human CD3 (1: 500; AbD Serotec, Oxford, UK) which cross-reacts with mouse CD3, respectively. Macrophage area was measured after immunostaining with rat anti-mouse Mac-3 (1: 50; BD Pharmingen, the Netherlands). Collagen content in the plaque was quantified morphometrically after sirius red staining. Mouse SMCs were immunostained with mouse anti-human alpha actin (1: 800; DAKO, Glostrup, Denmark) which cross-reacts with mouse alpha actin. In each segment used for lesion quantification, the number of monocytes adhering to the endothelium and the number of T cells in the aortic root area were counted and the endothelium length and the total aortic root area were measured. The length of the endothelium did not differ between groups and there were no correlation between the number of T cells and the total aortic root area per group. We, therefore, calculated the average number of monocytes and T cells per cross section. Macrophage and collagen area were measured in the severe lesions (type IV-V) and calculated per cross section and as a percentage of lesion area.[37] In addition, SMC area in the superficial part of the severe lesions,[34] as well as necrotic area of the severe lesions (type IV-V) were determined per cross section and as a percentage of lesion area.

Statistical analysis

Significance of differences between the groups was calculated parametrically by analysis of variance (ANOVA) followed by *post hoc* analysis using the least significant difference (LSD) test. Variables with a non-Gaussian distribution were logarithmically transformed. Due to heterogeneity between groups, the variables, macrophage content (% of lesion area) and necrotic area (% of lesion area) were analyzed by ANOVA using Brown-Forsynthe for overall between groups test and the Dunnett's T3 test for *post hoc*. Differences in lesion area were corrected for blood pressure by analysis of covariance (ANCOVA). The treatment group was the independent variable and blood pressure was the covariate. All groups were compared

to the control group and the combination group was compared to the atorvastatin group. Values are presented as means ± standard deviations (SD). A P-value <0.05 was considered statistically significant. In figures: *P<0.05, **P<0.01, ***P<0.001 as compared to the control group and #P<0.05, ##P<0.01, ###P<0.001 as compared to the atorvastatin group.[34]

Results

Aliskiren reduced blood pressure and atorvastatin lowered plasma cholesterol in APOE*3Leiden.CETP mice

The APOE*3Leiden.CETP mice have SBP similar to wild-type control animals, thus atherosclerosis development is not driven by hypertension in this model. However, to verify the blood pressure-lowering effect of aliskiren, SBP was measured on two occasions during the study. Aliskiren treatment and the combination treatment reduced SBP by -19% (P<0.001) and by -15% (P<0.01), respectively, as compared to the control (103 ± 10 mmHg). The combination treatment showed a -20% (P<0.001) reduction when compared to atorvastatin treatment alone (**Table 1**). To confirm the cholesterol-reducing effect of atorvastatin, plasma total cholesterol levels were measured and total cholesterol exposure (mmol/l * time in weeks) was calculated for each mouse. The control group had an average total cholesterol level of 15.5 ± 2.1 mmol/l, which was reduced by atorvastatin treatment alone and in combination with aliskiren treatment (both -24%, P<0.001). Similar reductions in total cholesterol exposure were seen in the respective groups. Lipoprotein profiling revealed that the reduction in total cholesterol was mainly confined to the very low-density lipoprotein (VLDL)/LDL fractions (data not shown). Aliskiren, in turn, did not affect plasma lipid levels when compared to the control group.

Table 1 The effect of aliskiren, atorvastatin and a combination of aliskiren and atorvastatin on plasma total cholesterol levels and systolic blood pressure (SBP) over a treatment period of 14 weeks.

	Average total cholesterol (mmol/l)	Total cholesterol exposure (mmol/l * weeks)	Average SBP (mmHg)
Control	15.5 ± 2.1	263 ± 36	103 ± 10
Aliskiren	14.4 ± 4.1	248 ± 38	84 ± 10 ***
Atorvastatin	11.8 ± 3.0 ***	207 ± 35 ***	109 ± 12
Aliskiren + atorvastatin	11.8 ± 2.1 ***	196 ± 28 ***	88 ± 12 **###

Values are means ± SD (n=14-17 per group). **P<0.01 and ***P<0.001 as compared to control, ###P<0.001 as compared to atorvastatin.

Aliskiren, atorvastatin and the combination treatment reduced atherosclerosis development

After the 14-week treatment period, the mice were sacrificed to assess the effect of the treatments on atherosclerotic lesion development in the aortic root. Representative photomicrographs of atherosclerotic lesions are illustrated in **Figure 1**. First, the number of lesions was counted per cross section (4.0 ± 0.7 for the control) revealing no effect of treatment with aliskiren or atorvastatin alone, whereas the combination treatment decreased the number of lesions by -17% ($P<0.05$; **Figure 2A**). The total lesion area per cross section was 233 ± 136 *1000 μm^2 in the control group (**Figure 2B**). In contrast to the number of lesions, total lesion area was reduced by aliskiren (-40%, $P<0.01$), atorvastatin (-61%, $P<0.001$) and the combination treatment (-69%, $P<0.001$), as compared to the control group. The combination treatment did not significantly differ from atorvastatin treatment alone. Lesion severity was also analyzed for each mouse, in which type I-III lesions represent mild lesions and type IV-V lesions represent severe lesions. This showed that approximately 66% of the lesions in the control group were severe lesions in comparison to 50% in the aliskiren group ($P<0.05$), 46% in the atorvastatin group ($P<0.01$) and 56% in the combination group (N.S.; **Figure 2C**). Additionally, the percentage of undiseased segments was increased by atorvastatin (+331%, $P<0.001$) and the combination treatment (+426%, $P<0.001$), as compared to the control (3.6 ± 7.4%; **Figure 2D**).

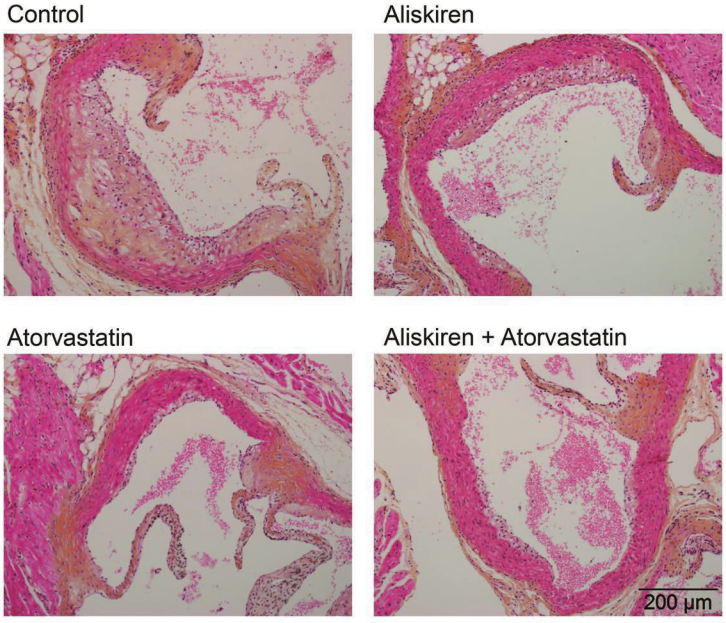


Figure 1 Representative photomicrographs of atherosclerotic lesions in the cross section of the aortic root area in the four groups (hematoxylin-phloxine-saffron staining).

We further analyzed whether aliskiren had anti-atherosclerotic properties beyond its blood pressure-lowering qualities. We calculated, using an ANCOVA (with blood pressure as covariate), that after adjusting for blood pressure the reduction in lesion area remained significant for all groups (P<0.05; P<0.01; P<0.001, respectively; data not shown). This indicated that aliskiren had beneficial effects, other than blood pressure-lowering alone. Therefore, we further explored the nature of these effects in more detail, focusing on inflammatory routes.

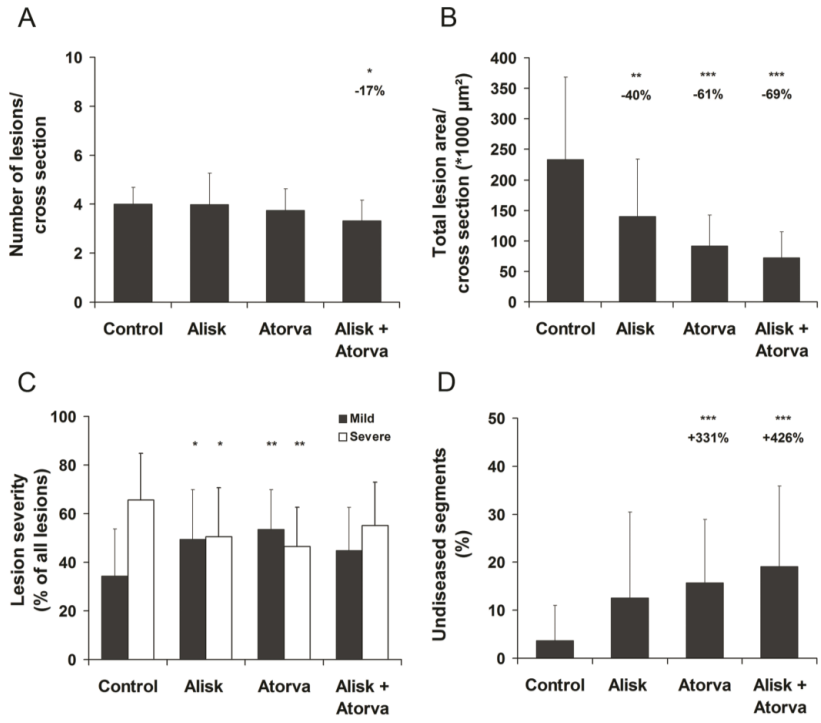


Figure 2 The effect of aliskiren, atorvastatin and a combination of aliskiren and atorvastatin on atherosclerosis development in aortic root area. The number of lesions (A) and total lesion area per cross section (B), as well as the lesion severity as percentage of all lesions (C) and the percentage of undiseased segments (D) were determined after 14 weeks of treatment. Lesion severity was classified as mild (type I-III lesions) and severe (type IV-V lesions).
Alisk, aliskiren; Atorva, atorvastatin; *P<0.05, **P<0.01 and ***P<0.001 as compared to control.

Aliskiren reduced functional markers of inflammation

As a result of the crucial role of inflammation in the development of atherosclerosis, fibrinogen levels, reflecting a general systemic inflammatory state, as well as the adhesion molecule, E-selectin and the pro-inflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1) were measured after 14 weeks of treatment (**Table 2**). Fibrinogen levels were not

affected by any of the treatments, indicating absence of changes in systemic inflammatory status. When compared to the control group (55 ± 11 ng/ml), the only significant difference in E-selectin levels was found in the combination group (-17%, P<0.05), revealing synergistic effects of aliskiren as this was a reduction of -18% (P<0.05) as compared to the atorvastatin group. MCP-1 levels were significantly reduced by -36% (P<0.01) in both the atorvastatin group and the combination group when compared to the control group (92 ± 21 pg/ml).

Table 2 The effect of aliskiren, atorvastatin and a combination of aliskiren and atorvastatin on plasma inflammation markers.

	E-selectin (ng/ml)	**MCP-1 (pg/ml)**	**Fibrinogen (mg/ml)**
Control	55 ± 11	92 ± 21	2.1 ± 0.5
Aliskiren	62 ± 17	91 ± 44	2.0 ± 0.7
Atorvastatin	56 ± 13	59 ± 24 **	2.3 ± 0.9
Aliskiren + atorvastatin	46 ± 7 *#	59 ± 25 **	2.1 ± 0.7

The parameters were measured at the end of the study after 14 weeks of treatment. Values are means ± SD (n=14-17 per group). *P<0.05 and **P<0.01 as compared to control, #P<0.05 as compared to atorvastatin.

As a functional measurement of vessel wall inflammation, monocyte adherence to the activated endothelium, as well as T cell abundance in the aortic root area were assessed. **Figure 3** illustrates representative photomicrographs of the monocyte (**Figure 3A**) and T cell (**Figure 3B**) stainings, respectively. The average number of monocytes and T cells per cross section for the control group were 6.1 ± 3.6 and 14.9 ± 9.5, respectively (**Table 3**). The atorvastatin and combination group showed a reduction in monocytes of -43% (P<0.05) and -51% (P<0.01), respectively, whereas aliskiren alone did not affect monocyte adherence. More interesting, aliskiren did affect the abundance of T cells in the aortic root area when administered alone and together with atorvastatin (-60%, P<0.01; -41%, P<0.05, respectively). Atorvastatin only tended to reduce the amount of T cells (P=0.084). Taken together, these data indicate anti-inflammatory effects of aliskiren via a reduction in T cell abundance and atorvastatin via a reduction in monocyte adherence. The anti-inflammatory effect may be enhanced by the combination treatment via a dampening of endothelial activation as reflected by decreased plasma E-selectin levels.

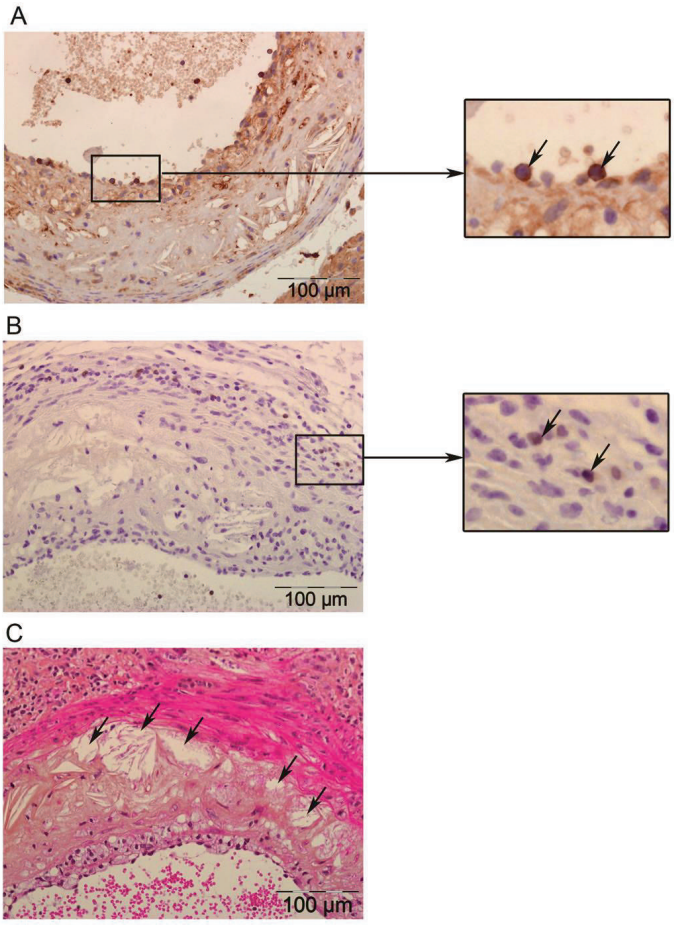


Figure 3 Representative photomicrographs of mouse monocytes and T cells after immunostaining with anti-mouse AIA31240 (A) and anti-human CD3 (B), respectively, as well as necrotic area (indicated by arrows) after hemotoxylin-phloxine-saffron (HPS) staining (C).

Table 3 The effect of aliskiren, atorvastatin and a combination of aliskiren and atorvastatin on functional markers of vessel wall inflammation.

	Number of monocytes/ cross section	**Number of T cells/ cross section**
Control	6.1 ± 3.6	14.9 ± 9.5
Aliskiren	5.9 ± 3.7	6.0 ± 4.5 **
Atorvastatin	3.5 ± 2.3 *	7.6 ± 4.4 p=0.084
Aliskiren + atorvastatin	3.0 ± 2.5 **	8.9 ± 6.7 *

The number of monocytes adhering to the vascular endothelium and T cells in the aortic root area were counted and calculated per cross section. Values are means ± SD (n=14-17 per group). *P<0.05 and **P<0.01 as compared to control.

Aliskiren alone and in combination with atorvastatin improved plaque stability

The composition of all lesions measured was assessed to evaluate the effects of aliskiren, atorvastatin and the combination of aliskiren and atorvastatin on plaque stability. To this end, macrophage and necrotic area as destabilization components and SMC area in the cap, as well as collagen area as stabilization components were measured in the severe lesions (type IV-V) and calculated per cross section and as a percentage of the lesion area. Necrotic area was assessed after HPS staining (**Figure 3C**). **Figure 4** illustrates representative photomicrographs of macrophage content and SMC content in the cap after immunostaining with anti-mouse Mac-3 and anti-human alpha actin, respectively and collagen content after sirius red staining.

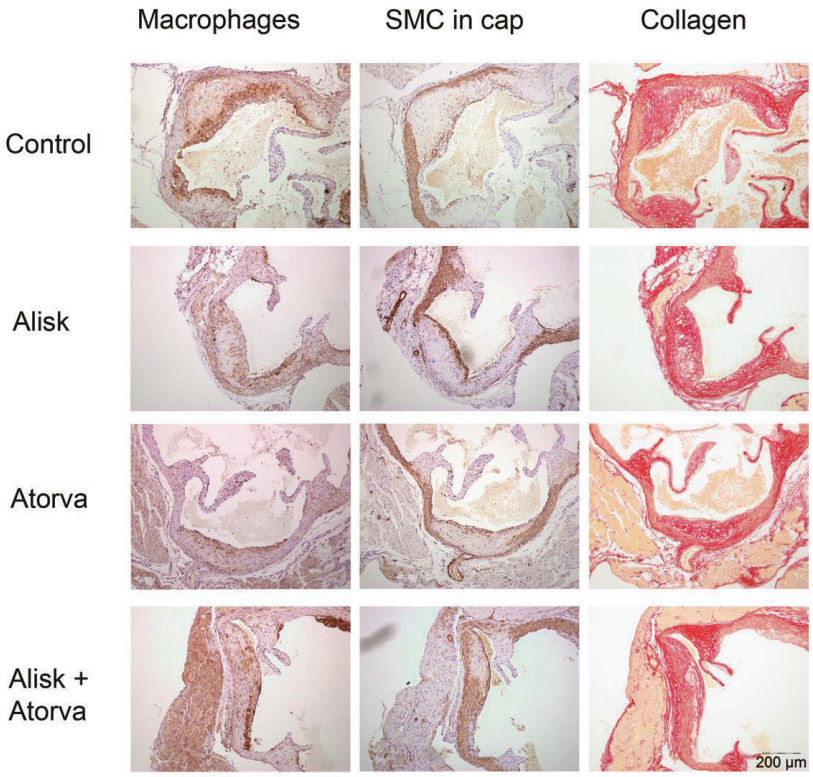


Figure 4 Representative photomicrographs of macrophage content and SMC content in the cap after immunostaining with anti-mouse Mac-3 and anti-human alpha actin, respectively, and collagen content after sirius red staining.
Alisk, aliskiren; Atorva, atorvastatin.

Figure 5 demonstrates the effect of treatment on the various components of lesion composition per cross section of the severe lesions (type IV-V). For the control group, the macrophage area and necrotic area in the severe lesions were 29.7 ± 20.3 *1000 μm^2 and 11.1 ± 7.9 *1000 μm^2, respectively. Aliskiren, atorvastatin and the combination treatment reduced macrophage (-64%, P<0.001; -70%, P<0.001; -72%, P<0.001, respectively; **Figure 5A**) and necrotic area (-52%, P=0.071; -68%, P<0.01; -84%, P<0.001, respectively; **Figure 5B**). The combination treatment tended to reduce necrotic area to a greater extent than atorvastatin treatment alone (-50%, P=0.094). There were no significant differences in SMC area in the cap compared to the control (3.7 ± 2.6 *1000 μm^2; **Figure 5C**).

After correcting for lesion size, aliskiren reduced macrophage content (-40%, P<0.01), the combination treatment reduced necrotic area (-54%, P<0.05) and atorvastatin and the combination treatment increased SMC content in the cap (+89%, P<0.01; +188%, P<0.001, respectively). A significant difference between the atorvastatin and the combination group (+52%, P<0.05) indicates a synergistic effect of the combination treatment on SMC content in the cap. No changes in collagen content were detected between groups (data not shown).

The stabilization/destabilization ratio of the severe lesions was calculated by dividing the sum of the SMC area in the cap and the collagen area by the sum of the macrophage and necrotic area (**Figure 5D**). All treatments increased the stability of the lesions (+62%, P<0.05; +75%, P<0.05; +109%, P<0.01, respectively). Similar increases in lesion stability were found after correcting for lesion size (data not shown). Therefore, aliskiren, atorvastatin and the combination treatment improved plaque stability. This effect of aliskiren was most potent when combined with atorvastatin as evidenced by a reduction in necrotic area, as well as by an increase in SMC content in the cap.

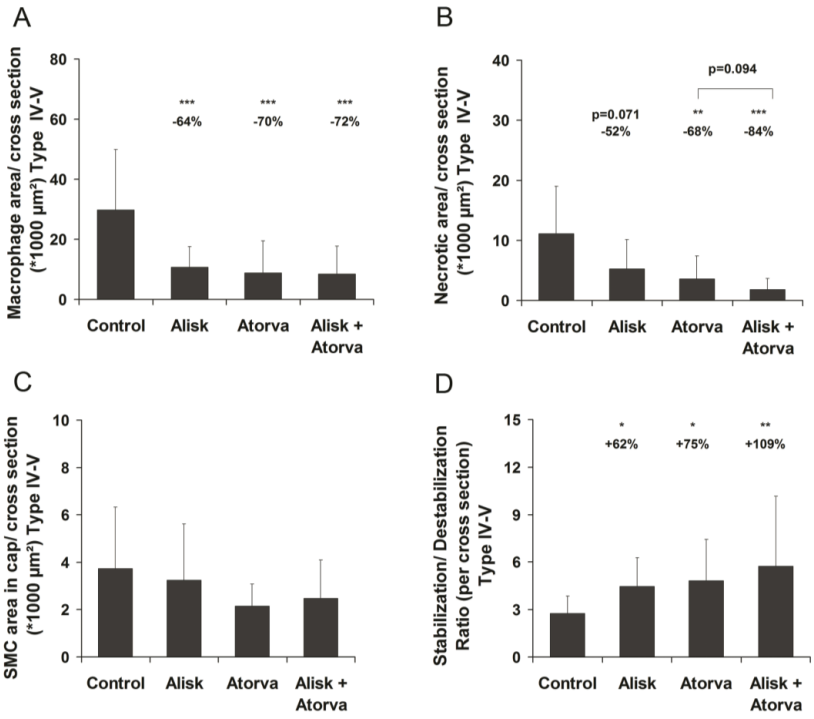


Figure 5 The atherosclerosis development was further analyzed by measuring the lesion composition of the severe lesions (type IV-V). A) Macrophage area per cross section. B) Necrotic area per cross section. C) SMC area in the cap per cross section. D) Lesion stability of the severe lesions (type IV-V) determined by the ratio of the stabilization factors (SMCs in the cap and collagen) to the destabilization factors (macrophages and necrotic area).
Alisk, aliskiren; Atorva, atorvastatin; *P<0.05, **P<0.01 and ***P<0.001 as compared to control.

Discussion

In the present study, the anti-atherosclerotic effects of aliskiren alone and in combination with atorvastatin were evaluated in APOE*3Leiden.CETP mice. Aliskiren reduced SBP (~10-20 mmHg) and atorvastatin reduced total cholesterol levels (~20-30%) in our study, which are both in accordance with findings from previous clinical trials.[23, 38] We demonstrated that aliskiren reduced lesion size and severity and improved stability of the plaque, as illustrated by a reduction in macrophage content. The combination of aliskiren and atorvastatin was the most potent therapy in reducing the number and size of the lesions, as well as markers of inflammation and in improving plaque stability as evidenced by a reduction in macrophage and necrotic area, as well as by an increase in SMC content in the cap.

Hypertension is an important risk factor for the development of atherosclerosis. In accordance, numerous animal studies have found a decrease in atherosclerosis with or without a decrease in blood pressure after treatment with various RAS blockers, including ACEi and ARBs.[6] Possible mechanisms by which RAS blockers may reduce atherosclerosis development described in animal and human studies include inhibition of oxidative stress, endothelial dysfunction and inflammation.[13, 39] Nonetheless, changes in blood pressure as a result of RAS manipulation appear to have a consistent, direct effect on the size of the lesions.[6] Aliskiren reduced blood pressure in our study, which may be a possible mechanism for the reduction in atherosclerosis development. However, it should be noted that the APOE*3Leiden.CETP mice used in this study did not have elevated blood pressure and that the atherosclerosis development is not driven by hypertension in this model. Moreover, the reduction in atherosclerosis development observed by aliskiren remained after correcting for blood pressure. We also demonstrated that other mechanisms besides blood pressure-lowering, such as anti-inflammatory effects, were most likely involved in the reduction of atherosclerosis development observed after aliskiren treatment. In line with our study, aliskiren showed anti-atherosclerotic effects in Watanabe heritable hyperlipidemic (WHHL) rabbits and in ldlr-/- mice beyond its blood pressure-lowering effects,[15, 27] confirming the involvement of other mechanisms. In these studies, aliskiren was administered in higher dosages compared to our study. In addition to monotreatment, we also administered aliskiren in combination with a statin, which is considered as a standard treatment in prevention of CVD and is, therefore, of clinical relevance. According to our data, aliskiren did not significantly enhance the inhibitory activity of atorvastatin on lesion size. However, a synergistic reduction in the number of lesions was found after the combination treatment. This suggests that the combination of aliskiren and atorvastatin was particularly effective in inhibiting early lesion formation, a process in which vascular inflammation plays an essential role.

Thus, to explore the mechanism behind the atherosclerosis protective effect of aliskiren in the current study, various inflammatory routes were assessed. The combination treatment showed a synergistic reduction in E-selectin, a marker of vascular inflammation. Aliskiren treatment alone had no effect on circulating MCP-1 levels, nor did it add to the reduction observed with atorvastatin treatment. The number of monocytes adhering to the endothelium, determined as a functional marker of vessel wall inflammation, confirmed these findings. Previously, reductions in vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and MCP-1 levels were described at substantially higher dosages of aliskiren and plasma cholesterol levels (21 mmol/l versus 15 mmol/l in the present study), leading to a more inflammatory-driven model.[27] The lower dosage of aliskiren and a less inflammatory-driven animal model used in our study may provide possible explanations for the absence of these observations regarding certain markers of

vascular inflammation. However, we found that aliskiren treatment alone and together with atorvastatin treatment reduced T cell abundance in the aortic root area. The participation of T cells in atherosclerotic lesion growth and destabilization has been extensively described in the literature, in which recent evidence also suggests that T cells may be involved in hypertension. The precise mechanism is still unknown.[40] T cells have been shown to express angiotensin II receptors.[41] It was also suggested, although not proven, that the presence of activated T cells in a perivascular distribution may cause a local effect of cytokines that may alter endothelial function.[40] In our study, we assessed the total number of T cells using a general marker for T cells, namely CD3. We, therefore, determined the abundance of T cells in the aortic root area and not the number of activated T cells in the vessel wall.

The presence of inflammation in the lesions plays a crucial role in plaque instability. On the basis of postmortem examination, it is evident that an acute myocardial infarction is provoked by sudden rupture of vulnerable plaques followed by thrombosis.[42] A vulnerable lesion is characterized by a thin, collagen-poor fibrous cap, decreased SMCs, increased macrophage infiltration and a large necrotic core.[3] This type of lesion is referred to as a thin-cap fibroatheroma.[43] Patients with unstable plaque have higher incidents of new coronary events. The therapeutic target has, therefore, shifted from enlargement of the lumen towards stabilization of the plaque.[44] Therefore, additional to the lesion area, we also investigated the composition of the plaque by performing histological analyses.

All treatments reduced macrophage and necrotic area as evidenced by data from the current study. After correcting for lesion size, aliskiren treatment alone reduced macrophage content and when combined with atorvastatin, also increased SMC content in the cap of the more severe lesions. In addition, the combination treatment also reduced the necrotic area. The protective effects of aliskiren on plaque stability were further supported by an increase in the stabilization/destabilization ratio. Taken together, we demonstrated that aliskiren can enhance plaque stability and that the combination treatment enhanced the effects of atorvastatin alone, demonstrating beneficial effects of the combination treatment over both monotreatments. There seems to be some apparent inconsistency in the literature regarding the effects of aliskiren on lesion composition. Lu *et al.*[15] found that renin inhibition by aliskiren reduced atherosclerotic lesion development in ldlr-/- mice without major alterations in cellular composition. In contrast, Nussberger *et al.*[7] demonstrated that aliskiren can preferentially inhibit plaque vulnerability in a severe apoe-/- mouse model as illustrated by both an increase in SMC content, as well as a decrease in macrophage content. However, in the same publication, aliskiren had no effect on SMC content when SMCs already comprise a substantial portion of the atherosclerotic plaque in a second, less severe animal model.[7] Our results further suggest that aliskiren in combination with atorvastatin is most effective at enhancing plaque stability by replacing necrotic core with smooth muscle-containing fibrous lesion.

In this study, we demonstrated the beneficial effects of aliskiren on atherosclerosis development and plaque stability alone and in combination with atorvastatin in a pre-clinical model of CVD, possibly via a mechanism involving T cells. These results suggest a potential benefit of using aliskiren in a clinical setting, particularly in combination with statin treatment. The effect of aliskiren on cardiovascular endpoints awaits further clinical trial results.

Acknowledgements
Erik Offerman, Ria van den Hoogen, Simone van der Drift-Droog, Anita van Nieuwkoop and Ronald van der Sluis are thanked for their outstanding technical assistance.

Funding
J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001 D032). Novartis Institutes for BioMedical Research is gratefully acknowledged for supporting this study in part by an unconditional grant.

Disclosures
This study was supported in part by an unconditional grant from Novartis Institutes for BioMedical Research. G.L. was an employee of Novartis Institutes for BioMedical Research. There are no further conflicts of interest.

References

1. Libby P, Okamoto Y, Rocha VZ and Folco E. Inflammation in Atherosclerosis. *Circulation Journal*. 2010;74:213-220.
2. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine*. 2005;352:1685-95.
3. Libby P and Sasiela W. Plaque stabilization: Can we turn theory into evidence? *The American journal of cardiology*. 2006;98:26P-33P.
4. Galkina E and Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annual review of immunology*. 2009;27:165-97.
5. Jankowski P, Bilo G and Kawecka-Jaszcz K. The pulsatile component of blood pressure: its role in the pathogenesis of atherosclerosis. *Blood pressure*. 2007;16:238-45.
6. Lu H, Cassis LA and Daugherty A. Atherosclerosis and arterial blood pressure in mice. *Current drug targets*. 2007;8:1181-9.
7. Nussberger J, Aubert JF, Bouzourene K, Pellegrin M, Hayoz D and Mazzolai L. Renin inhibition by aliskiren prevents atherosclerosis progression: comparison with irbesartan, atenolol, and amlodipine. *Hypertension*. 2008;51:1306-11.
8. Ferrario CM, Richmond RS, Smith R, Levy P, Strawn WB and Kivlighn S. Renin-angiotensin system as a therapeutic target in managing atherosclerosis. *American journal of therapeutics*. 2004;11:44-53.
9. Pimenta E and Oparil S. Role of aliskiren in cardio-renal protection and use in hypertensives with multiple risk factors. *Therapeutics and clinical risk management*. 2009;5:459-64.
10. Daugherty A, Rateri DL, Lu H, Inagami T and Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation*. 2004;110:3849-57.
11. Wassmann S, Czech T, van Eickels M, Fleming I, Bohm M and Nickenig G. Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation*. 2004;110:3062-7.
12. Wiggins KJ and Kelly DJ. Aliskiren: a novel renoprotective agent or simply an alternative to ACE inhibitors? *Kidney international*. 2009;76:23-31.
13. Montecucco F, Pende A and Mach F. The renin-angiotensin system modulates inflammatory processes in atherosclerosis: evidence from basic research and clinical studies. *Mediators of inflammation*. 2009;2009:752406.
14. Stanton A. Potential of renin inhibition in cardiovascular disease. *Journal of the renin-angiotensin-aldosterone system : JRAAS*. 2003;4:6-10.
15. Lu H, Rateri DL, Feldman DL, Jr RJ, Fukamizu A, Ishida J, Oesterling EG, Cassis LA and Daugherty A. Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice. *The Journal of clinical investigation*. 2008;118:984-93.
16. Grote K, Drexler H and Schieffer B. Renin-angiotensin system and atherosclerosis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2004;19:770-3.
17. Hammoud RA, Vaccari CS, Nagamia SH and Khan BV. Regulation of the renin-angiotensin system in coronary atherosclerosis: a review of the literature. *Vascular health and risk management*. 2007;3:937-45.
18. Unger T and Stoppelhaar M. Rationale for double renin-angiotensin-aldosterone system blockade. *The American journal of cardiology*. 2007;100:25J-31J.
19. Jensen C, Herold P and Brunner HR. Aliskiren: the first renin inhibitor for clinical treatment. *Nature reviews Drug discovery*. 2008;7:399-410.
20. Menard J and Azizi M. The difficult conception, birth and delivery of a renin inhibitor: controversies around aliskiren. *Journal of hypertension*. 2007;25:1775-82.
21. McAlister FA, Zhang J, Tonelli M, Klarenbach S, Manns BJ and Hemmelgarn BR. The safety of combining angiotensin-converting-enzyme inhibitors with angiotensin-receptor blockers in elderly patients: a population-based longitudinal analysis. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*. 2011;183:655-62.

22. O'Brien E. Aliskiren: a renin inhibitor offering a new approach for the treatment of hypertension. *Expert opinion on investigational drugs*. 2006;15:1269-77.
23. Frampton JE and Curran MP. Aliskiren: a review of its use in the management of hypertension. *Drugs*. 2007;67:1767-92.
24. Solomon SD, Appelbaum E, Manning WJ, Verma A, Berglund T, Lukashevich V, Cherif Papst C, Smith BA and Dahlof B. Effect of the direct Renin inhibitor aliskiren, the Angiotensin receptor blocker losartan, or both on left ventricular mass in patients with hypertension and left ventricular hypertrophy. *Circulation*. 2009;119:530-7.
25. McMurray JJ, Pitt B, Latini R, Maggioni AP, Solomon SD, Keefe DL, Ford J, Verma A and Lewsey J. Effects of the oral direct renin inhibitor aliskiren in patients with symptomatic heart failure. *Circulation Heart failure*. 2008;1:17-24.
26. Parving HH, Persson F, Lewis JB, Lewis EJ and Hollenberg NK. Aliskiren combined with losartan in type 2 diabetes and nephropathy. *The New England journal of medicine*. 2008;358:2433-46.
27. Imanishi T, Tsujioka H, Ikejima H, Kuroi A, Takarada S, Kitabata H, Tanimoto T, Muragaki Y, Mochizuki S, Goto M, Yoshida K and Akasaka T. Renin inhibitor aliskiren improves impaired nitric oxide bioavailability and protects against atherosclerotic changes. *Hypertension*. 2008;52:563-72.
28. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
29. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.
30. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
31. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
32. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
33. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1706-21.
34. van der Hoorn JW, Kleemann R, Havekes LM, Kooistra T, Princen HM and Jukema JW. Olmesartan and pravastatin additively reduce development of atherosclerosis in APOE*3Leiden transgenic mice. *Journal of hypertension*. 2007;25:2454-62.
35. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M and Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arteriosclerosis, thrombosis, and vascular biology*. 1996;16:926-33.
36. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD and Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis, thrombosis, and vascular biology*. 1995;15:1512-31.
37. Delsing DJM, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit ECM, Gijbels MJJ, van der Laarse A, Jukema JW, Havekes LM and Princen HMG. Acyl-CoA:Cholesterol Acyltransferase Inhibitor Avasimibe Reduces Atherosclerosis in Addition to Its Cholesterol-Lowering Effect in ApoE*3-Leiden Mice. *Circulation*. 2001;103:1778-1786.

38. Malhotra HS and Goa KL. Atorvastatin: an updated review of its pharmacological properties and use in dyslipidaemia. *Drugs*. 2001;61:1835-81.
39. Werner C, Baumhakel M, Teo KK, Schmieder R, Mann J, Unger T, Yusuf S and Bohm M. RAS blockade with ARB and ACE inhibitors: current perspective on rationale and patient selection. *Clinical research in cardiology : official journal of the German Cardiac Society*. 2008;97:418-31.
40. Bu DX and Lichtman AH. T cells and blood vessels: costimulation turns up the pressure. *Circulation*. 2010;122:2495-8.
41. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C and Harrison DG. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *The Journal of experimental medicine*. 2007;204:2449-60.
42. Finn AV, Nakano M, Narula J, Kolodgie FD and Virmani R. Concept of vulnerable/unstable plaque. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:1282-92.
43. Moreno PR. The high-risk thin-cap fibroatheroma: a new kid on the block. *Circulation Cardiovascular interventions*. 2009;2:500-2.
44. Halvorsen B, Otterdal K, Dahl TB, Skjelland M, Gullestad L, Oie E and Aukrust P. Atherosclerotic plaque stability--what determines the fate of a plaque? *Progress in cardiovascular diseases*. 2008;51:183-94.

CHAPTER 3

Niacin Reduces Atherosclerosis Development in APOE*3Leiden.CETP Mice Mainly by Reducing non-HDL-cholesterol

Susan Kühnast[*1,2,5], Mieke C. Louwe[*3,5], Mattijs M. Heemskerk[4,5], Elsbet J. Pieterman[1], Jan B. van Klinken[4,5], Sjoerd A.A. van den Berg[4,5], Johannes W.A. Smit[3,5], Louis M. Havekes[1,3,5], Patrick C.N. Rensen[3,5], José W.A. van der Hoorn[1,2,5], Hans M.G. Princen[†1], J. Wouter Jukema[†2]

[1]TNO - Metabolic Health Research, Leiden, The Netherlands;
[2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[3]Dept. of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands;
[4]Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands;
[5]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

[*]S.K. and M.C.L. contributed equally
[†]H.M.G.P. and J.W.J. share equal responsibility for the manuscript

PLoS One. 2013 Jun 19;8(6):e66467

Abstract

Objective Niacin potently lowers triglycerides, mildly decreases LDL-cholesterol, and largely increases HDL-cholesterol. Despite evidence for an atheroprotective effect of niacin from previous small clinical studies, the large outcome trials, AIM-HIGH and HPS2-THRIVE did not reveal additional beneficial effects of niacin (alone or in combination with laropiprant) on top of statin treatment. We aimed to address this apparent discrepancy by investigating the effects of niacin without and with simvastatin on atherosclerosis development and determine the underlying mechanisms, in APOE*3Leiden.CETP mice, a model for familial dysbetalipoproteinemia (FD).

Methods and Results Mice were fed a Western-type diet containing cholesterol without or with niacin (120 mg/kg/day), simvastatin (36 mg/kg/day) or their combination for 18 weeks. Similarly as in FD patients, niacin reduced total cholesterol by -39% and triglycerides by -50%, (both $P<0.001$). Simvastatin and the combination reduced total cholesterol (-30%; -55%, $P<0.001$), whereas the combination revealed a greater reduction compared to simvastatin (-36%, $P<0.001$). Niacin decreased total cholesterol and triglycerides primarily by increasing VLDL clearance. Niacin increased HDL-cholesterol (+28%, $P<0.01$) and mildly increased reverse cholesterol transport. All treatments reduced monocyte adhesion to the endothelium (-46%; -47%, $P<0.01$; -53%, $P<0.001$), atherosclerotic lesion area (-78%; -49%, $P<0.01$; -87%, $P<0.001$) and severity. Compared to simvastatin, the combination increased plaque stability index [(SMC + collagen)/ macrophages] (3-fold, $P<0.01$). Niacin and the combination reduced T cells in the aortic root (-71%, $P<0.01$; -81%, $P<0.001$). Lesion area was strongly predicted by non-HDL-cholesterol ($R^2=0.69$, $P<0.001$) and to a much lesser extent by HDL-cholesterol ($R^2=0.20$, $P<0.001$).

Conclusion Niacin decreases atherosclerosis development mainly by reducing non-HDL-cholesterol with modest HDL-cholesterol-raising and additional anti-inflammatory effects. The additive effect of niacin on top of simvastatin is mostly dependent on its non-HDL-cholesterol-lowering capacities. These data suggest that clinical beneficial effects of niacin are largely dependent on its ability to lower LDL-cholesterol on top of concomitant lipid-lowering therapy.

Keywords APOE*3Leiden.CETP mice, niacin, simvastatin, atherosclerosis, plaque stability, non-HDL-cholesterol

Introduction

The beneficial effects of niacin, also known as nicotinic acid or vitamin B3, on plasma lipids and lipoproteins were first described in the 1950s.[1] According to a meta-analysis of 30 randomized controlled trials, niacin potently reduced triglycerides (TG) by ~15-30% and increased HDL-cholesterol (HDL-C) by ~10-25%, while mildly reducing plasma total cholesterol (TC) by ~5-15% and LDL-cholesterol (LDL-C) by ~5-20%, suggesting an atheroprotective effect.[2] Whereas previous small clinical studies supported this notion,[3-6] the recent large outcome trials, AIM-HIGH and HPS2-THRIVE failed to reveal additional beneficial effects of niacin on top of statin treatment.[7, 8]

In patients with atherosclerotic disease or those at risk for atherosclerotic disease due to dyslipidemia, the primary goal of lipid-modifying therapy is the lowering of LDL-C.[9] To this end, statins are currently the standard treatment for cardiovascular disease (CVD) resulting in a 25-45% risk reduction for cardiovascular events.[10] However, a substantial residual risk for adverse cardiovascular outcomes remains with statin therapy.[3, 11] Moreover, despite maximally tolerated statin treatment, some patients cannot reach LDL-C goals. This high risk population together with statin intolerant patients verify the need for another LDL-C-lowering agent to (further) reduce LDL-C levels.[12] Treatment of low HDL-C is currently considered as a secondary lipid target in the reduction of cardiovascular risk,[3] since low HDL-C is an independent risk factor for CVD.[2, 13] Considering the current treatment options, the question remains whether to further reduce LDL-C or to increase HDL-C in addition to LDL-C-lowering.[12] Therefore, due to both its non-HDL-C-lowering and HDL-C-raising properties, niacin was considered an attractive candidate for further cardiovascular risk reduction in addition to statin therapy.

Indeed, an initial small clinical study suggested that the addition of niacin to statin treatment may cause potentially clinical significant reductions in relative risk of cardiovascular events.[6] Recently, a number of relatively small secondary prevention studies (ARBITER-2,[4] ARBITER-3[5] and ARBITER-6-HALTS[3]) have shown reduced progression and even regression of atherosclerosis with combination treatment of niacin and statins compared to statins alone, as measured by carotid artery intima-media thickness as a surrogate for clinical endpoints. Magnetic resonance imaging results from another study confirmed the reduction in carotid atherosclerosis with niacin in statin-treated patients.[14] These clinical data were corroborated by recent observations that niacin reduced atherosclerosis development, independent of lipid-lowering or HDL-C-elevation, in hyperlipidemic LDL receptor knockout mice on a high fat diet containing 1.5% cholesterol.[15] Despite these promising data, the large outcome trial, AIM-HIGH, addressing the effect of niacin on top of aggressive LDL-C-lowering treatment, has recently been prematurely terminated due to futility.[8] In accordance, the much larger HPS2-THRIVE trial failed to reveal additional risk reduction of cardiovascular events with

extended-release (ER)-niacin/laropiprant in combination with statin treatment as compared to statin monotreatment.[7] ER-niacin[2] and ER-niacin/laropiprant combination treatment[16] are more tolerable formulations that have been developed due to a reluctance to use niacin for clinical treatment as a result of extreme flushing as a side effect.[17]

In the present study, we aimed to address the seeming discrepancy between the beneficial effects of niacin in initial clinical trials,[3-6, 14] as well as in LDL receptor knockout mice, a model irresponsive to the lipid-modulating effects of niacin,[15] and the lack of effect of niacin on top of statin treatment on reduction of cardiovascular events in the AIM-HIGH[8] and HPS2-THRIVE trials.[7] Therefore, we evaluated the effects of niacin without and with simvastatin on atherosclerosis development and investigated the underlying mechanisms and contributing factors in APOE*3Leiden.CETP mice. This is a well-established mouse model for familial dysbetalipoproteinemia (FD) with human-like lipoprotein metabolism and atherosclerosis development. These mice respond to the lipid-lowering effects of both niacin[18] and statins, e.g. atorvastatin,[19] as well as the HDL-C-raising effect of niacin.[18]

Methods

Animals, diets and experimental design

Female APOE*3Leiden.CETP transgenic mice,[20] expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions, were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water during the experiment unless indicated otherwise. Body weight (BW) and food intake were monitored during the entire study. To increase plasma cholesterol levels up to ~12 mmol/L, 8-12 week-old mice were fed a semi-synthetic cholesterol-rich diet, containing 15% (w/w) cacao butter and 0.1% cholesterol (Western-type diet; Hope Farms, Woerden, The Netherlands) for 3 weeks. After matching based on age, BW, TC, TG and HDL-C, mice (n=15 per group) received a control Western-type diet (WTD) without or with 0.1% (w/w) niacin (120 mg/kg/day), 0.03% (w/w) simvastatin (36 mg/kg/day) or their combination for 18 weeks. During the treatment period, the effects of treatment on plasma lipids, lipoprotein profiles, CETP activity and CETP mass were assessed at the indicated time points.

The dose of simvastatin targeted a 30-35% reduction in TC and that of niacin a 20-30% increase in HDL-C. While we achieved these targets, it should be noted that the dose of simvastatin was 3 times higher than the maximum dose used in the clinic taking into account a 10 times faster metabolism in mice. For niacin, the dose was comparable to that in patients, about 1 g/day. At the end of the experiment, all animals were sacrificed by CO_2 inhalation. Liver and white adipose tissue (WAT) were isolated to assess CETP expression (n=6-8 per group) and hearts were isolated to assess atherosclerosis development (n=15 per

group). Separate additional experiments were performed to evaluate the effects of niacin on VLDL production and clearance, as well as reverse cholesterol transport (RCT). Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO).

Plasma lipids and lipoprotein profiles

After a 4 h fast, blood was collected via tail vein bleeding and plasma was isolated. Plasma TC, HDL-C after precipitation of apoB-containing lipoproteins using $MnCl_2$[21] and TG were determined individually using enzymatic kits, 1489437 and 1488872 (both from Roche diagnostics), according to the manufacturer's protocols. After 4 and 18 weeks of treatment, pooled lipoprotein profiles for TC were measured by fast protein liquid chromatography (FPLC).[20]

VLDL production and clearance experiments

APOE*3Leiden.CETP mice (11-14 weeks of age) were fed a WTD containing 0.1% cholesterol for 3 weeks. Upon subsequent matching according to plasma TC and TG levels, mice received the cholesterol-containing WTD without or with 0.1% (w/w) niacin for an additional 3 weeks[18] and VLDL production and clearance were determined as described.[22]

Plasma was obtained via tail vein bleeding in heparin microvettes for randomization (Sarstedt, Germany) and in chilled paraoxon-coated capillary tubes to prevent *ex vivo* TG hydrolysis for VLDL production and clearance, and assayed for TG and TC using commercially available kits, 1488872 and 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively.

For the VLDL production experiment, 6 control and 6 niacin-treated mice were fasted for 4 h. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche) and 0.31 mg/kg fentanyl (Janssen-Cilag). At t=0 min, blood was taken via tail bleeding and mice were injected intravenously with 100 µL PBS containing 100 µCi Trans^{35}S label to measure *de novo* apoB synthesis. After 30 min, the mice received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg BW as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG. Additional blood samples were taken 15, 30, 60, and 90 min after tyloxapol injection and used for determination of plasma TG concentration. After 120 min, the mice were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation. Incorporation of ^{35}S-label was measured in the VLDL fraction as marker of *de novo* apoB synthesis.

For the VLDL clearance experiment, glycerol tri[^{3}H]oleate (triolein, TO)- and [1α,2α(n)-^{14}C]cholesteryl oleate (CO)-double labeled VLDL-like emulsion particles (80 nm) were used.[23] In short, radiolabeled emulsions were obtained by adding 100 µCi of [^{3}H]TO and 10

μCi of [^{14}C]CO to 100 mg of emulsion lipids before sonication (isotopes obtained from GE Healthcare, Little Chalfont, U.K.). APOE*3Leiden.CETP mice (5 control and 5 niacin-treated mice) were fasted for 4 h, sedated as described above, and injected intravenously with the radiolabeled emulsion particles (1.0 mg TG in 200 μL PBS). Blood was taken from the tail vein to determine the content of [^{3}H]TO and [^{14}C]CO in serum at 2, 5, 10 and 15 min after emulsion injection. Fifteen min after injection, plasma was collected by orbital puncture and mice were sacrificed by cervical dislocation. Organs were harvested and saponified to determine uptake of radioactivity derived from [^{3}H]TO and [^{14}C]CO by various organs.[22]

Endogenous CETP activity, CETP mass and CETP mRNA expression analysis

Plasma endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP, Roar Biomedical, New York, NY) as described.[19] CETP activity was calculated as nmol cholesteryl ester transfer/mL plasma/h. Plasma CETP mass was determined by using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).[18] Total RNA was extracted from liver and white adipose tissue (WAT) using an RNA isolation kit according to manufacturer's specifications (Macherey-Nagel, Düren, Germany). Total RNA concentrations were measured with Nanodrop. One μg of RNA was reversed-transcribed to cDNA with iScriptcDNA Synthesis kit (Bio-Rad) and purified with Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany). Real-time PCR (RT-PCR) was carried out on an iQ5 PCR detection system (Bio-Rad) using Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). Hypoxanthine-guanine phosporibosyltransferase (HPRT) and acidic ribosomal phosphoprotein PO (36B4) were used as the standard housekeeping genes and expression levels were normalized to these housekeeping genes. Primer sequences are listed in Table S1.

Histological assessment of atherosclerosis

After isolation, hearts were fixed in formalin, embedded in paraffin and cross-sectioned (5 μm) throughout the aortic root area. For each mouse, four sections at intervals of 50 μm were used for quantitative and qualitative assessment of the atherosclerotic lesions after staining with hematoxylin-phloxine-saffron. For determination of severity of atherosclerosis, the lesions were classified into five categories: I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, and V) severe plaque according to the American Heart Association classification.[19, 24] Lesion severity as a percentage of the number of lesions was calculated. To this end, type I-III lesions were classified as mild lesions and type IV-V lesions were classified as severe lesions. Total lesion area and number of lesions per cross section, as well as the percentage undiseased segments, were calculated. In each segment used for lesion quantification, the number of monocytes adhering to the endothelium and the

numbers of T cells in the total aortic root area were counted after immunostaining with AIA 31240 (1:1000; Accurate Chemical and Scientific, New York, New York, USA) and CD3 (1:500; AbD Serotec, Oxford, UK), respectively. Macrophage content of the lesions was measured after immunostaining with Mac-3 (1:50; BD Pharmingen, the Netherlands). In addition, sirius red staining was used to quantify the collagen content in the plaque[25] and the antibody alpha actin (1:800; DAKO, Glostrup, Denmark) was used to quantify the smooth muscle cell (SMC) content.[26] Stained areas were measured using Cell D imaging software (Olympus Soft Imaging Solutions).

Reverse cholesterol transport experiment

16 recipient APOE*3Leiden.CETP mice (10-12 weeks of age) were fed a WTD containing 0.1% cholesterol for a run-in period of 3 weeks after which they were subdivided into 2 groups according to age, BW, TC, TG and HDL-C. After matching, mice (n=8 per group) received a control cholesterol-containing WTD without or with 0.1% (w/w) niacin (120 mg/kg/day) for 3 weeks.

6 donor APOE*3Leiden.CETP mice (10-12 weeks of age) fed a WTD containing 0.1% cholesterol for 3 weeks were injected intraperitoneally with 1 mL solution of 3% thioglycollate to induce an inflammatory response. Three days after the injection, mice were injected intraperitoneally with approximately 300 µCi [^{3}H]-cholesterol together with 100 µg/mL acetylated LDL. Mice were sacrificed 1 h later by CO_2 inhalation. [^{3}H]-cholesterol-labeled macrophages were collected from the 6 donor mice by peritoneal lavage. These macrophages were washed twice with cold PBS and injected intraperitoneally into the 16 recipient APOE*3Leiden.CETP mice. Each recipient mouse received 2.8 x 10^6 [^{3}H]-cholesterol-labeled macrophages containing 7.8 x 10^6 dpm [^{3}H]-cholesterol. Mice were individually caged for 48 h in order to collect feces and sacrificed by CO_2 inhalation. ^{3}H-activity was determined in the plasma, liver and feces. The *in vivo* RCT experiment was based on methods previously described.[27, 28]

Statistical analysis

Significance of differences between the groups was calculated non-parametrically using a Kruskal-Wallis test followed by a Mann-Whitney U-test for independent samples. We performed a univariate analysis of variance (ANOVA) to investigate the role of TC, non-HDL-C and HDL-C exposure as contributing factors in lesion development. A two-way analysis of covariance (ANCOVA) was performed to test for group differences in lesion area, monocyte adhesion, T cell abundance and macrophage area after correcting for HDL-C and non-HDL-C exposure. SPSS 17.0 for Windows (SPSS, Chicago, USA) was used for statistical analysis. All groups were compared to the control group and the combination group was also compared to the simvastatin group. Values are presented as means ± SD. P-values <0.05

were considered statistically significant. In the figures, the symbol * is used to compare to the control group, and # to compare to the simvastatin group.

Results

Niacin, simvastatin and their combination reduce plasma total cholesterol and triglycerides and niacin increases HDL-C in APOE*3Leiden.CETP mice

To verify the lipid-lowering effect of niacin alone and in combination with simvastatin, we measured plasma TC (**Figure 1A**), TG (**Figure 1B**) and HDL-C (**Figure 1C**) levels during the study. The Western-type diet resulted in an average TC of 13.4 ± 1.7 mmol/L, TG of 4.3 ± 1.4 mmol/L and HDL-C of 0.65 ± 0.13 mmol/L (control group). TC and TG levels were reduced by niacin (-39%, $P<0.001$; -50%, $P<0.001$), simvastatin (-30%, $P<0.001$; -19%, NS) and the combination (-55%, $P<0.001$; -52%, $P<0.001$). The combination reduced TC to a greater extent than simvastatin alone (-36%, $P<0.001$). Niacin increased HDL-C by +28% ($P<0.01$) as compared to the control, whereas the combination increased HDL-C by +14% ($P<0.05$) as compared to simvastatin monotreatment. Niacin alone resulted in higher HDL-C than the combination ($P<0.001$). The reductions in plasma TC induced by niacin, simvastatin and the combination were confined to apoB-containing lipoproteins as measured after lipoprotein separation by FPLC (**Figure 1D**).

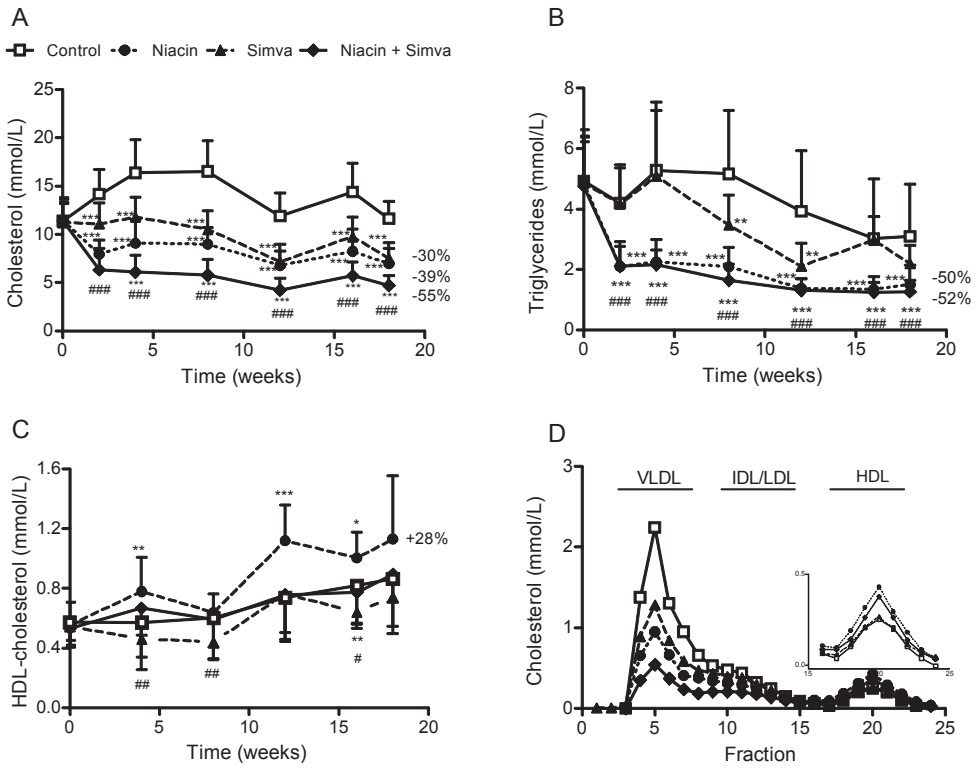


Figure 1 Effect of niacin, simvastatin and their combination on plasma lipid levels**.** Plasma total cholesterol (A), triglycerides (B) and HDL-cholesterol levels were measured at various time points throughout the study. The average HDL-cholesterol levels were calculated for all the treatment groups (C). Lipoproteins were separated by FPLC and cholesterol was measured in the fractions after 18 weeks of treatment (D).
Simva, simvastatin; values are means ± SD; n=15 per group; **P<0.01 and ***P<0.001 as compared to control; #P<0.05 and ###P<0.001 as compared to niacin + simvastatin.

Niacin reduces apoB-containing lipoprotein cholesterol by modestly increasing VLDL clearance without affecting VLDL production

To determine by which mechanism the level of apoB-containing lipoprotein cholesterol is decreased, first the VLDL-TG production was assessed after injection of Tran^{35}S label and tyloxapol. VLDL-TG production did not differ between controls and niacin-treated mice (**Figure 2A**; control 6.1 ± 0.7 µmol/mL/h versus niacin 6.2 ± 0.8 µmol/mL/h; P=0.94). In addition, the apoB production rate, as measured by incorporation of ^{35}S-activity in the VLDL fraction (**Figure 2B**; control 2.9 ± 0.5 µmol/mL/h versus niacin 2.9 ± 0.4 µmol/mL/h; P=0.94) and VLDL-apoB lipidation (control 1.3 ± 0.4 nmol/100 dpm versus niacin 1.4 ± 0.3 nmol/100 dpm; P=0.69) did not differ between groups.

We then examined the clearance and uptake of [^{3}H]TO and [^{14}C]CO-labeled VLDL-like emulsion particles. Despite lack of statistical power due to unexpected loss of mice, there

was a trend towards a faster plasma clearance rate of [^{3}H]TO (**Figure 2C**; control $t_{1/2}$ = 6.4 ± 2.2 min versus niacin $t_{1/2}$ = 4.9 ± 0.9 min; P=0.19) and a significantly faster initial [^{3}H]TO clearance in the first 5 min after niacin treatment (P<0.05). Tissue-specific ^{3}H-accumulation did not differ between groups, although there was a tendency (P=0.07) for a higher ^{3}H-accumulation in the spleen from the niacin-treated mice (**Figure 2D**). This was accompanied by a non-significant increase in the plasma clearance rate of [^{14}C]CO (control $t_{1/2}$ = 11.6 ± 5.5 min versus niacin $t_{1/2}$ = 7.1 ± 1.7 min; P=0.12) with no differences in ^{14}C-accumulation in the various organs between groups (data not shown).

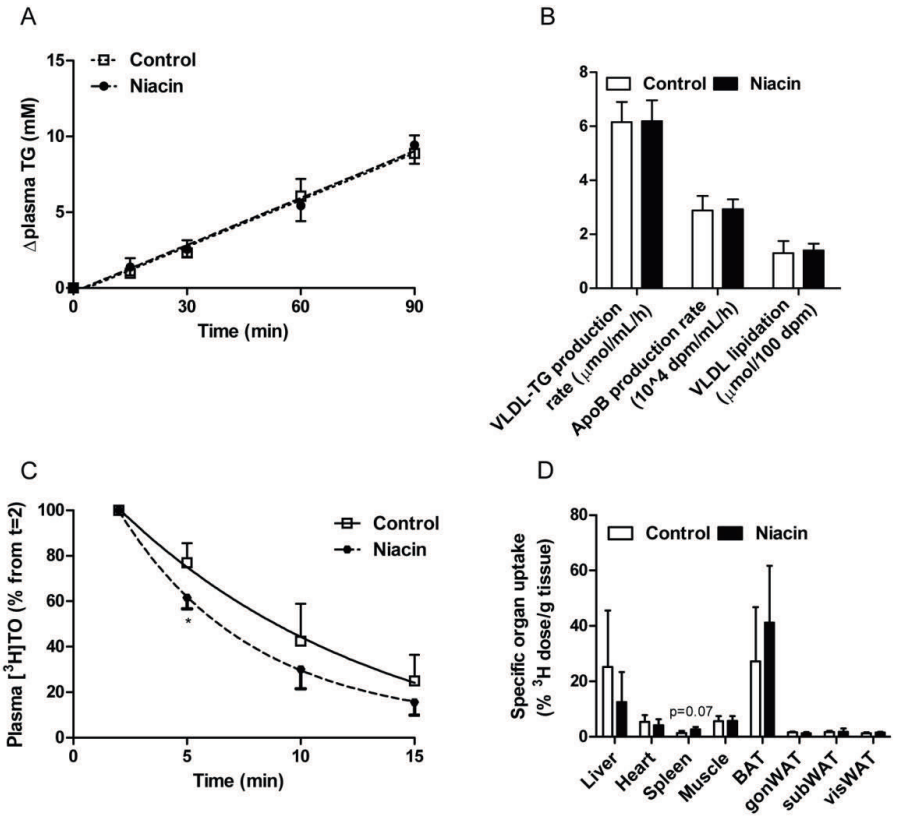


Figure 2 Effect of niacin on VLDL production and clearance. To determine VLDL production, mice were injected with Trans^{35}S label and tyloxapol and the accumulation of TG in plasma (A) and the production rate of VLDL-TG and apoB, as well as VLDL lipidation, defined as the ratio of VLDL-TG/apoB, were determined (B). To determine VLDL clearance, mice were injected with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-labeled VLDL-like emulsion particles. Plasma ^{3}H-activity was determined as percentage of the initial dose (C), and uptake of ^{3}H-activity by various organs was determined as percentage of the injected dose per gram wet tissue (D).
BAT, brown adipose tissue; gonWAT, gonadal white adipose tissue; subWAT, subcutaneous white adipose tissue; visWAT, visceral white adipose tissue; values are means ± SD; n=6 per group for VLDL production and n=3-5 per group for VLDL clearance; *P<0.05 as compared to control.

Niacin, simvastatin and their combination reduce plasma CETP activity, CETP mass and niacin alone and together with simvastatin reduces hepatic CETP gene expression

In a previous study, we showed that niacin increased HDL-C by decreasing hepatic CETP expression and plasma CETP concentration.[18] To verify this, we measured plasma CETP activity and mass and hepatic CETP mRNA expression after 4 and/or 18 weeks of treatment (**Table 1**). Niacin, which most prominently increased HDL-C, reduced the average plasma CETP activity by -21% (P<0.01) and mass by -22% (P<0.01). Simvastatin reduced CETP activity and mass by -25% and -37%, respectively (both P<0.001) without affecting HDL-C levels. The combination reduced CETP activity (-34%; P<0.001) and mass (-48%; P<0.001) to an even higher extent. Previously, we demonstrated that this reduction was due to reduced CETP mRNA expression in the liver. In line with these results, we found that niacin alone and in combination with simvastatin tended to reduce hepatic CETP expression to -76% (P=0.072) and -58% (P=0.059), respectively. Besides the liver, WAT is considered as a major source of CETP and since WAT is a target of niacin,[29] we also determined the effect of all treatments on CETP mRNA expression in WAT. Niacin did not decrease CETP expression in WAT (data not shown), which is consistent with a recent study in CETP transgenic mice.[30] For the control group, a 125 times lower relative CETP expression was measured in WAT compared to the liver (data not shown). A mild correlation was found between hepatic CETP expression and plasma CETP mass (R^2=0.25, P=0.006), whereas CETP expression in WAT did not correlate with plasma CETP mass (R^2=0.02, P=0.45) (data not shown). Taken together, we concluded that the liver is the major determinant for circulating CETP levels in this model, which were affected by all the treatments.

Table 1 The effect of niacin, simvastatin and their combination on plasma CETP activity after 4 and 18 weeks of treatment, as well as plasma CETP mass and hepatic CETP expression after 18 weeks of treatment.

	Average plasma CETP activity (nmol/mL/h)	Plasma CETP mass (µg/mL)	Hepatic CETP expression (% of control)
Control	64.3 ± 11.4	21.3 ± 3.4	100 ± 30
Niacin	50.6 ± 6.6 **	16.6 ± 3.5 **	76 ± 23 P=0.072
Simva	48.1 ± 8.4 ***	13.4 ± 3.5 ***	76 ± 25
Niacin + Simva	42.6 ± 8.9 *** P=0.081	11.0 ± 2.2 *** P=0.050	58 ± 33 P=0.059

CETP, cholesteryl ester transfer protein; Simva, simvastatin. Values are means ± SD (n=15 per group for plasma CETP activity and mass and n=6-8 per group for hepatic CETP expression). **P<0.01 and ***P<0.001 as compared to control.

Niacin alone and in combination with simvastatin reduces atherosclerosis development to a greater extent than simvastatin treatment alone

After 18 weeks of treatment, we measured the effect of niacin, with and without simvastatin on atherosclerosis development in the aortic root. **Figure 3** illustrates representative images of atherosclerotic lesions for each group. We determined the number of lesions per cross section (**Figure 4A**), the lesion severity as a percentage of all lesions (**Figure 4B**), the percentage undiseased segments (**Figure 4C**) and the total lesion area per cross section (**Figure 4D**). To determine lesion severity as a percentage of all lesions, type I-III lesions were classified as mild lesions and type IV-V lesions were classified as severe lesions.

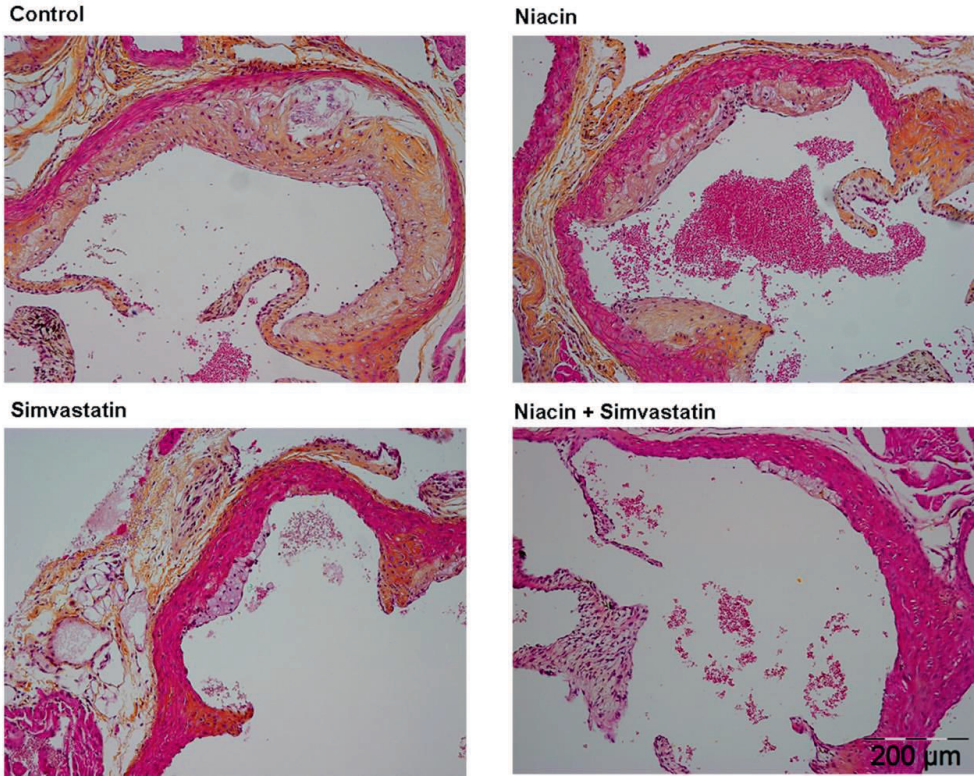


Figure 3 Effect of niacin, simvastatin and their combination on plaque morphology. Representative images of hematoxylin-phloxine-saffron-stained atherosclerotic lesions in a cross section of the aortic root area for the control group (A), niacin group (B), simvastatin group (C) and the combination group (D) after 18 weeks of treatment.

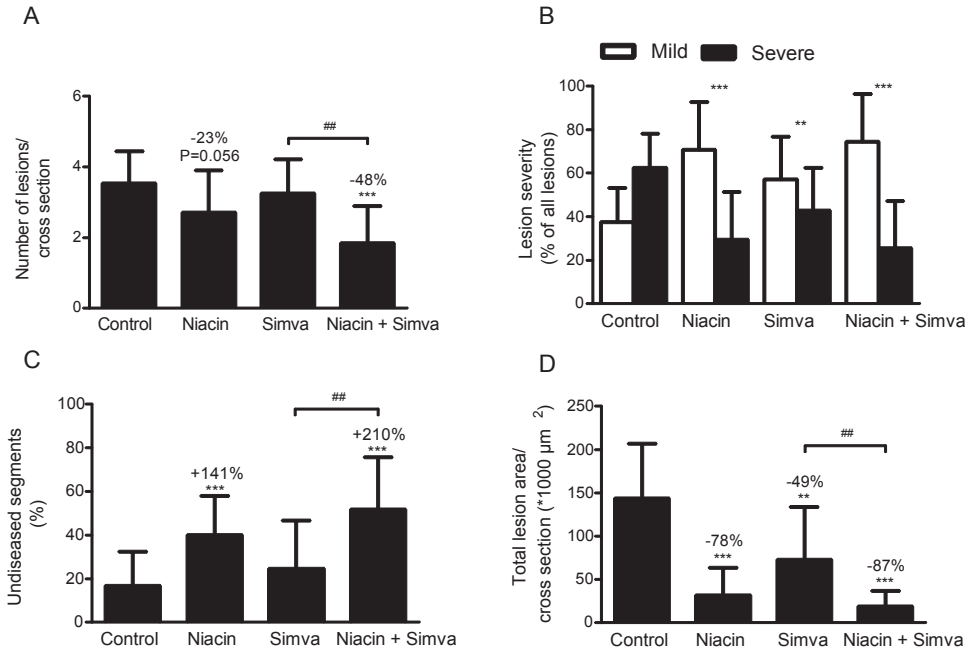


Figure 4 Effect of niacin, simvastatin and their combination on atherosclerosis development in aortic root area. After 18 weeks of treatment, number of lesions (A), lesion severity (B), percentage undiseased segments (C) and total lesion area (D) were determined per cross section. Lesion severity was classified as mild (type I-III) and severe (type IV-V) lesions.
Simva, simvastatin; values are means ± SD; n=15 per group; **P<0.01 and ***P<0.001 as compared to control; ##P<0.01 as compared to niacin + simvastatin.

In the control group, a fair amount of atherosclerosis developed with 3.5 ± 0.9 lesions per cross section, of which 62 ± 16% were severe and only 17 ± 16% of the segments were undiseased. The total lesion area was 144 ± 63 x10^3 µm^2 per cross section. When compared to the control, niacin reduced the number of lesions (-23%; P=0.056), attenuated lesion severity (P<0.001) and increased the percentage undiseased segments (+141%, P<0.001). Furthermore, niacin strongly decreased the total lesion area by -78% (P<0.001). Simvastatin alone was less effective as it only reduced lesion severity (P<0.01) and total lesion area (-49%, P<0.01). The combination had potent inhibiting effects on lesion development, as evidenced by reductions in lesion number (-48%, P<0.001), severity (P<0.001) and area (-87%, P<0.001), and by an increase in the percentage undiseased segments (+210%, P<0.001). Furthermore, the percentage undiseased segments, as well as the reduction in the lesion number and area was greater after the combination compared to simvastatin alone (-44%; +110%; -74%, all P<0.01). These results showed that niacin monotreatment was very potent in inhibiting atherosclerotic lesion development in APOE*3Leiden.CETP mice and that niacin added to the atherosclerosis-reducing effects of simvastatin.

Niacin improves lesion stability index and decreases functional markers of vascular inflammation

After investigation of lesion morphology, we analyzed the treatment effects on plaque composition. For all lesions, the macrophage area as destabilization factor (**Figure 5A**), as well as SMC (**Figure 5B**) and collagen area (data not shown) as stabilization factors were calculated per cross section. The macrophage, SMC and collagen area per cross section in the control group were 24.3 ± 8.6 $x10^3$ μm^2, 4.6 ± 2.3 $x10^3$ μm^2 and 64.2 ± 37.0 $x10^3$ μm^2, respectively. All treatments reduced the macrophage (-73%, -52% and -90%; all $P<0.001$) and the SMC area (-66%, $P<0.01$; -50%, $P<0.01$; -79%, $P<0.001$). As a measure of the lesion stability index, the ratio of collagen and SMC area (i.e. stabilization factors) to macrophage area (i.e. destabilization factor) was determined for all lesions (data not shown). The lesion stability ratio for the control group was 2.7 ± 1.4. Combination treatment tended to increase this ratio by +201% ($P=0.085$).

After finding indications for more stable lesions, we specifically focused on the composition of the more severe lesions, which are considered to be the most vulnerable lesions. Additionally, we corrected for the lesion area. Thus, we measured the macrophage (**Figure 5C**), SMC (**Figure 5D**) and collagen content (data not shown) as a percentage of lesion area. The severe lesions in the control group consisted of 20% macrophages, 4% SMC and 45% collagen (latter data not shown). Niacin alone and in combination with simvastatin reduced the relative macrophage content by -18% (NS) and -54% ($P<0.001$), respectively and increased the relative SMC content by +97% ($P<0.01$) and +102% ($P<0.05$), respectively. The combination was superior to simvastatin monotreatment in stabilizing the plaque as seen by a reduction in macrophage content (-54%; $P<0.05$) and an increase in SMC content (+79%; $P=0.057$). There were no significant differences in collagen content between groups (data not shown). The lesion stability index was also calculated for the severe lesions based on the relative area (**Figure 5E**). Combination treatment increased this ratio by +371% ($P<0.01$) compared to the control (2.6 ± 1.0) and to a greater extent compared to simvastatin ($P<0.05$) alone.

As functional markers of vessel wall inflammation, the number of monocytes adhering to the activated endothelium (**Figure 6A**) and T cells in the aortic root area (**Figure 6B**) were counted and calculated per cross section. In the control group, 3.8 ± 1.2 adhering monocytes and 11.3 ± 8.3 T cells were present. A marked reduction of adhering monocytes of more than -45% ($P<0.01$; $P<0.01$ and $P<0.001$, respectively) was found in all treatment groups, whereas T cells abundance was reduced by niacin alone (-71%, $P<0.01$) and in combination with simvastatin (-81%, $P<0.001$).

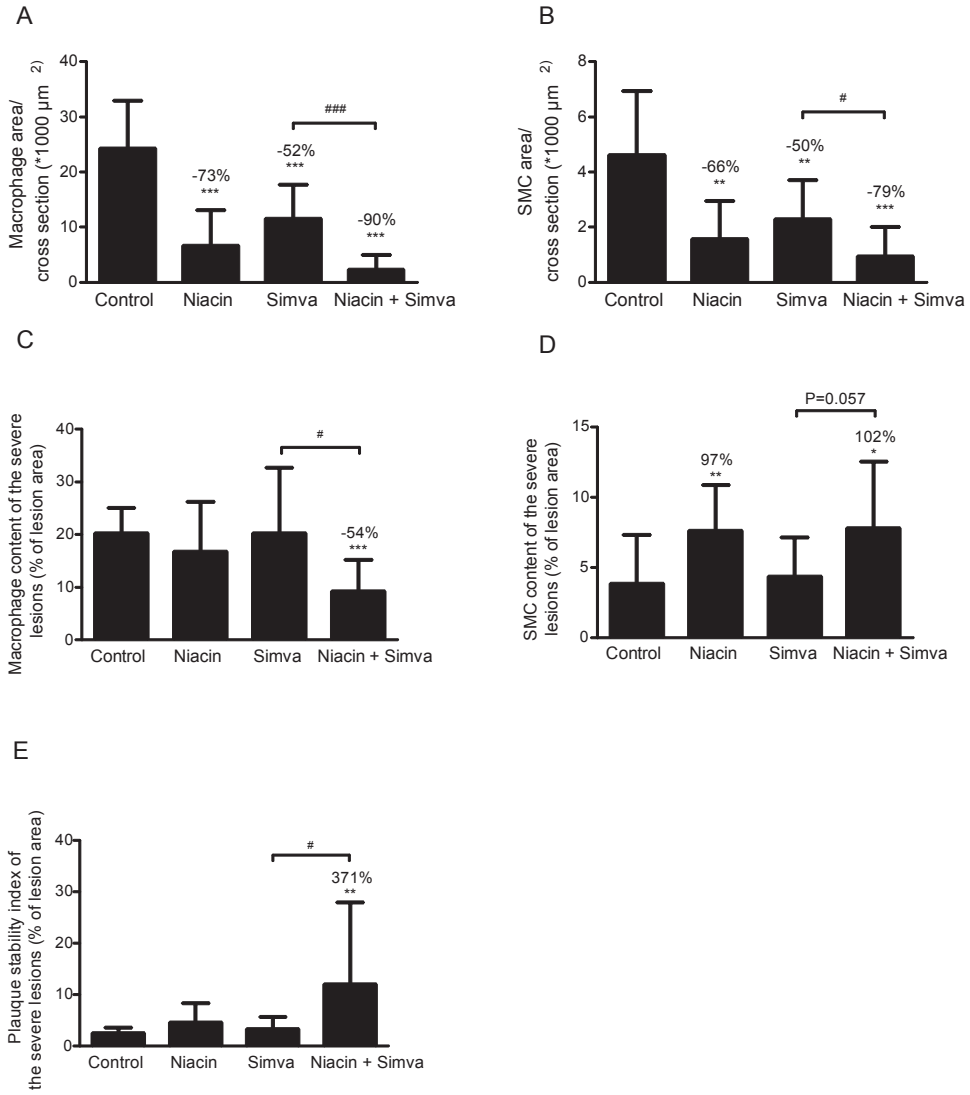


Figure 5 Effect of niacin, simvastatin and their combination on lesion composition. Macrophage area (A) and SMC area (B) were determined for all lesions and calculated per cross section. To correct for lesion size, macrophage content (C), SMC content (D), as well as plaque stability index (ratio of collagen and SMC content to macrophage content) (E) were also calculated as a percentage of lesion area, specifically in severe lesions (Type IV-V).
Simva, simvastatin; SMC, smooth muscle cells; values are means ± SD; n=15 per group; *P<0.05, **P<0.01 and ***P<0.001 as compared to control; #P<0.05, and ###P<0.001 as compared to niacin + simvastatin.

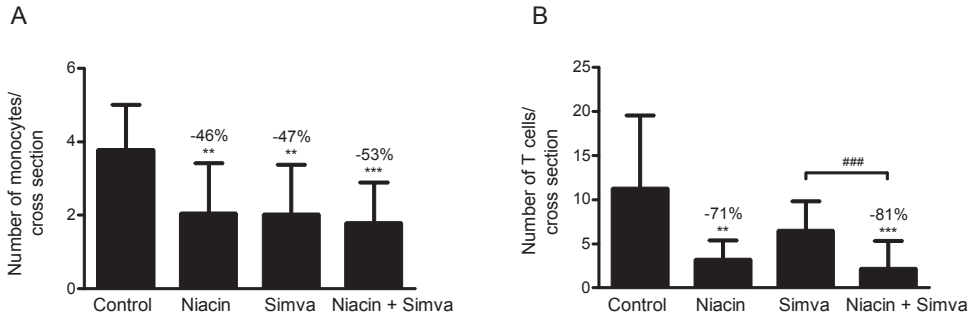


Figure 6 Effect of niacin, simvastatin and their combination on monocyte adhesion and T cell number. The number of monocytes adhering to the endothelium (A) and the number of T cells in the aortic root area (B) were determined per cross section.
Simva, simvastatin; values are means ± SD; n=15 per group; **P<0.01; ***P<0.001 as compared to control; ###P<0.001 as compared to niacin + simvastatin.

Niacin reduces atherosclerosis progression primarily by reducing non-HDL-cholesterol

In addition to inflammation, plasma cholesterol is certainly a strong determinant for atherosclerosis progression. We evaluated whether the anti-atherogenic effect of niacin and simvastatin could be explained by the reduction in plasma TC (**Figure 7**). Since atherosclerotic lesion area showed a quadratic dependence on plasma TC exposure, lesion area was transformed using a square root transformation. Lesion area was strongly predicted by plasma TC exposure (R^2=0.70, P<0.001; **Figure 7A**), and non-HDL-C exposure (R^2=0.69, P<0.001; **Figure 7B**) and to a much lesser extent by HDL-C exposure (R^2=0.20, P<0.001; **Figure 7C**). Together, non-HDL-C and HDL-C exposure accounted for 71% of the variability in lesion area and predicted the lesion area independent of each other (P<0.001 and P<0.05, respectively). Importantly, the effects of niacin and simvastatin on lesion area were lost after correcting for both HDL-C and non-HDL-C exposure (P=0.16; P=0.61, respectively). Furthermore, no effect of niacin and simvastatin on monocyte adhesion was found after correcting for non-HDL-C exposure (P=0.50; P=0.20, respectively). However, niacin decreased the square-root transformed macrophage area and T cell abundance even after correcting for non-HDL-C exposure (both P<0.01), whereas simvastatin did not (P=0.12; P=0.26, respectively).

Collectively, these data are compatible with a mechanism that niacin and simvastatin mainly decreased atherosclerotic lesion development via a reduction of non-HDL-C with an additional effect of HDL-C-elevation for niacin, while a direct effect on lesion macrophages and T cell abundance may contribute to the anti-atherogenic effect of niacin, but not simvastatin.

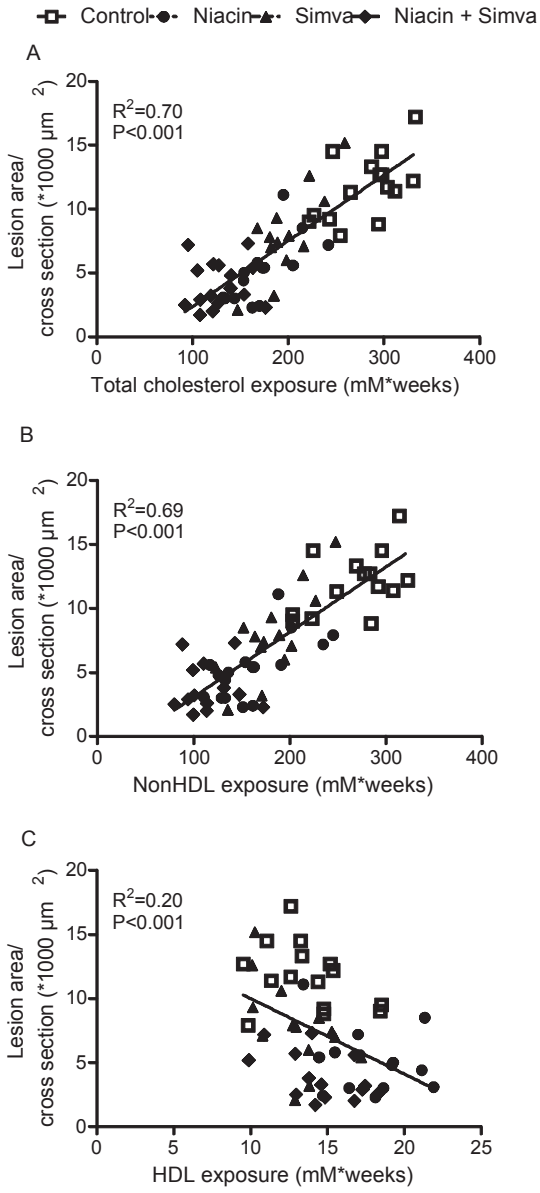


Figure 7 Correlation between plasma cholesterol exposure and lesion area. The square root of the lesion area was plotted against total cholesterol exposure (A), non-HDL-cholesterol exposure (B) and HDL-cholesterol exposure (C). Linear regression analyses were performed.
Simva, simvastatin; n=15 per group.

Niacin mildly increases reverse cholesterol transport

To investigate the possible mechanism by which the HDL-C-raising effect of niacin may contribute to its anti-atherogenic effect, we performed an *in vivo* RCT experiment. After 3 weeks of treatment, mice were injected with [^{3}H]-cholesterol-labeled macrophages. Forty eight hours after injection, plasma total ^{3}H-activity tended to be decreased (-36%, P=0.065), whereas ^{3}H-activity in the HDL fraction was increased after niacin treatment (+155%, P<0.01; **Figure 8A**). In addition, niacin increased ^{3}H-activity in the liver (+33%; P<0.05) and tended to increase fecal ^{3}H-activity (+26%; P=0.065; **Figure 8B**).

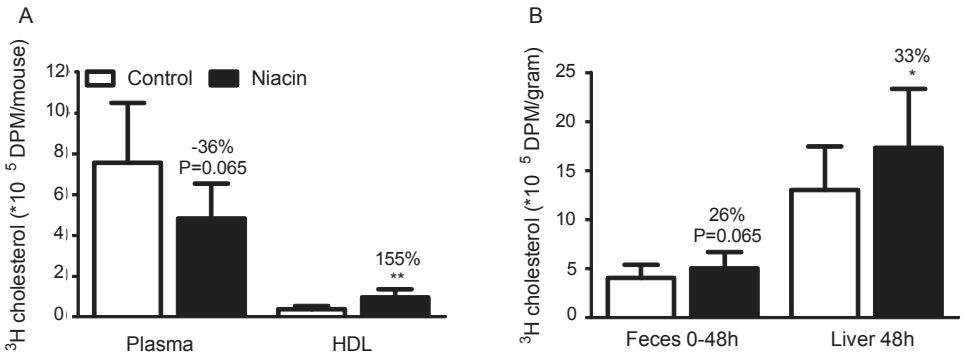


Figure 8 Effect of niacin on reverse cholesterol transport. [^{3}H]-cholesterol-labeled macrophages were injected in control and niacin-treated mice and ^{3}H activity was determined in plasma and HDL (A) and the liver 48 h after injection, as well as in feces collection between 0-48 h after injection (B).
Values are means ± SD; n=8 per group; *P<0.05 and ** P<0.01 as compared to control.

Discussion

In this study, we aimed to address the discrepancy between the beneficial effects of niacin in initial clinical trials[3-6, 14] and the lack of effect of niacin on top of statin treatment on reduction of cardiovascular events in the AIM-HIGH[8] and HPS2-THRIVE[7] trials by investigating the effects of niacin without and with simvastatin on atherosclerosis development and determining the underlying mechanisms in APOE*3Leiden.CETP mice, a mouse model for familial dysbetalipoproteinemia (FD). We demonstrated that niacin decreased atherosclerosis development mainly by reducing non-HDL-C with a modest HDL-C-raising and anti-inflammatory effect and that the additive effect of niacin on top of simvastatin was mostly dependent on its non-HDL-C-lowering capacities.

First, we showed that niacin and simvastatin both reduced plasma lipid levels. Niacin reduced (V)LDL-C and (V)LDL-TG and increased HDL-C, whereas simvastatin mainly reduced (V)LDL-C. Combination treatment of niacin and simvastatin reduced non-HDL-C more

effectively as compared to simvastatin alone. In our study, the reduction in plasma TC after niacin treatment alone and in combination with simvastatin was greater compared to recent clinical trials. The APOE*3Leiden mouse was initially developed as an animal model for FD or type III hyperlipoproteinemia, which is characterized by elevated levels of cholesterol and an increased ratio of cholesterol to TG in the VLDL and intermediate density lipoprotein (IDL) fractions, resulting in the appearance of β-VLDL particles.[31, 32] Similarly as in FD patients, in APOE*3Leiden and APOE*3Leiden.CETP mice as a model for mixed dyslipoproteinemia, a major part of plasma cholesterol is contained in the VLDL and VLDL-remnant particles, leading to formation of β-VLDL particles, which further increases after cholesterol feeding. Whereas niacin reduces plasma TC by ~5-15%, LDL-C by ~5-20% and TG by ~15-30% in patients with hyperlipidemia,[2] in two small studies in FD patients, niacin decreased TC by 23-50% and TG by 43-62%[33, 34] with -56% reduction of VLDL-C and -48% reduction of VLDL-TG.[34] Thus, the extent of lipid-lowering observed with niacin in APOE*3Leiden.CETP mice is comparable to that of FD patients.

Since plasma VLDL-TG and apoB levels are determined by the balance between VLDL production and clearance, we evaluated their individual contribution. VLDL production was not affected in niacin-treated mice, neither was apoB production nor lipidation of the VLDL particle. However, a modest effect of niacin on VLDL clearance was observed. The mechanism behind the lipid-lowering effect of niacin has been generally ascribed to a reduced hepatic VLDL production, as a result of decreased free fatty acid (FFA) flux from WAT after inhibition of hormone sensitive lipase. However, an initially decreased FFA flux is followed by a rebound effect with increased release of FFAs.[1, 35] In humans, contradicting data describe that niacin decreased VLDL production without affecting VLDL clearance[36-38] and on the other hand that niacin enhanced clearance of apoB without affecting production.[39] The latter study is in line with our results, which implicate VLDL clearance rather than VLDL production as the possible mechanism by which niacin reduces apoB.

The niacin-induced increase in HDL-C in the present study may be attributed to a decrease in hepatic and plasma CETP leading to an inhibition of HDL delipidation as previously described.[18] As similar effects on CETP levels and activity were observed after simvastatin treatment, without affecting HDL, different mechanisms are likely involved in either treatment. Interestingly, a decreased macrophage content accompanying decreased hepatic cholesterol accumulation as a result of niacin's lipid-lowering effect was recently proposed as a mechanism by which niacin decreases hepatic CETP expression.[30]

An important observation from our study is that niacin decreases atherosclerosis progression and adds to the anti-atherogenic effect of simvastatin, in particular regarding its enhancing effect on plaque stability. Niacin decreased lesion number, severity and area, and increased the percentage undiseased segments. Moreover, niacin improved lesion composition by reducing the macrophage content and increasing the SMC content.

Importantly, the combination treatment increased the plaque stability index, defined as the ratio of SMC and collagen to macrophage area, as compared to either niacin or simvastatin alone.

It is interesting to speculate on the mechanism(s) underlying the anti-atherogenic effect of niacin. In the APOE*3Leiden.CETP mouse model, statistical analyses revealed that the effects of niacin and simvastatin were largely explained by their reduction in non-HDL-C, as evidenced by a strong correlation between plasma non-HDL-C and lesion area. The fact that the combination treatment reduced non-HDL-C beyond the level reached by simvastatin alone can thus largely explain why niacin added to the anti-atherosclerotic effect of simvastatin. Though, HDL-C also appeared to predict lesion area independent of non-HDL-C, albeit that the predictive value of HDL-C was much smaller than that of non-HDL-C. The HDL-C-raising effect of niacin may, therefore, have contributed to some extent to the anti-atherosclerotic activity of niacin.

To explore the contribution of the niacin-induced increase in HDL-C to the reduction of atherosclerosis, we investigated the functionality of HDL by performing an RCT experiment. From this experiment, we conclude that the effect of niacin on RCT may partially contribute to, but is not the driving force behind its anti-atherogenic effects. This is in accordance with our statistical correlations, which showed non-HDL-C to be a much stronger contributor to atherogenesis. Although HDL-C contributed to some extent, we observed that niacin's attenuating effect on atherosclerosis development in APOE*3Leiden.CETP mice, fed a Western-type diet with 0.1% cholesterol, is largely explained by its lipid-lowering effect. At first sight, this seems to contradict a recent report showing that niacin reduced atherosclerosis development in LDL receptor-deficient mice under conditions that left plasma cholesterol levels unaffected.[15] In that mouse model, the atheroprotective effects of niacin were mainly explained by impaired homing macrophage recruitment to atherosclerotic plaques and by promoting cholesterol efflux from macrophages by upregulation of ABCG1. However, it should be noted that those mice were fed a high fat diet containing as much as 1.5% cholesterol. A previous study from our laboratory showed that dietary cholesterol induced dose-dependent marked inflammation in mice,[40] where the liver switches to an inflammatory, pro-atherosclerotic state as reflected by a strong increase in serum amyloid A levels at dietary cholesterol levels exceeding 0.5%. Previously, Lukasova *et al*.[15] evaluated the anti-atherogenic effect of niacin under highly inflammatory conditions, at which the anti-inflammatory properties of niacin may become dominant and may not necessarily reflect the mode of action for niacin under mild cholesterol intake as used in the present study.

It should be noted that we also obtained evidence that niacin exerted anti-inflammatory effects in our mouse model under milder dietary conditions. Firstly, niacin reduced monocyte adhesion and macrophage area of the atherosclerotic lesions. In fact, niacin reduced macrophage area independent of non-HDL-C, whereas simvastatin did not. These

data not only corroborate the findings in LDL receptor-deficient mice,[15] but also the recent observations that niacin reduced collar-inflicted vascular inflammation and inhibited intima-media neutrophil recruitment in New Zealand White rabbits independent of changes in plasma lipids.[41] Secondly, we observed that niacin, but not simvastatin, strongly reduced the number of T cells in the aortic root area, which are involved in the progression of atherosclerosis.[42] The reduction was independent of non-HDL-C exposure, suggesting the anti-inflammatory effect observed was brought about by niacin, instead of HDL-derived. This is in accordance with a study where niacin inhibited monocyte chemotactic protein 1 (MCP-1), RANTES and fractalkine in adipocytes. These chemokines contribute to the recruitment of T cells and macrophages. WAT is known to express the GPR109A receptor and has the ability to contribute to both systemic and local (perivascular) inflammation associated with atherosclerosis.[29, 43]

Although initial clinical studies showed that niacin reduced atherosclerosis development in combination with statins[3-5] and reduced the relative risk of cardiovascular events,[6] results from the large outcome trials, AIM-HIGH and HPS2-THRIVE did not confirm earlier findings.[8, 44, 45] In order to test the HDL hypothesis, the AIM-HIGH investigators minimized the differences in LDL levels between the groups. Patients enrolled in the trial were subjected to aggressive LDL-C-lowering treatment, aimed at LDL-C of 40-80 mg/dL (1.03-2.07 mmol/L), reaching mean baseline LDL-C of 71 mg/dL (1.84 mmol/L) and HDL-C of 35 mg/dL (0.91 mmol/L).[46] A modest increase in HDL-C was observed in the placebo group, resulting in a 4-5 mg/dL (0.10-0.13 mmol/L) difference in HDL-C between groups. This, together with the aggressive LDL-C-lowering may have given rise to insufficient power to detect a reduction in events.[45] Unexpectedly, the much larger outcome trial, HPS2-THRIVE failed to reveal further cardiovascular risk reduction when adding ER-niacin/laropiprant to vigorous statin treatment, plus if required ezetimibe, as compared to statin/(ezetimibe) monotreatment.[7] Furthermore, there was a significant increase in non-fatal serious adverse events and drop-out rate in the ER-niacin/laropiprant-treated patients. The lack of inclusion criteria for HDL-C resulted in a baseline HDL-C of >40 mg/dL (1.14 mmol/L) with a low baseline LDL-C level of <70 mg/dL (1.64 mmol/L). Patient stratification revealed that baseline HDL-C levels did not predict efficacy of niacin and that indeed, only in patients with high LDL-C, niacin reduced major cardiovascular events.

In conclusion, our results show that niacin decreases atherosclerosis development mainly by reducing non-HDL-C with modest HDL-C-raising and additional anti-inflammatory effects. The additive effect of niacin on top of simvastatin is mostly dependent on its non-HDL-C-lowering capacities. These data suggest that clinical beneficial effects of niacin are largely dependent on its ability to lower LDL-C on top of concomitant lipid-lowering therapy and may explain the failure of niacin in the clinical outcome trials.

Acknowledgement

We thank Erik Offerman, Ria van den Hoogen, Annemarie Maas, Simone van der Drift-Droog, Karin Toet, Wim van Duyvenvoorde and Amanda Pronk for their excellent technical assistance.

Funding

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project PREDICCt (grant 01C-104), and was supported by the Netherlands Heart Foundation, Dutch Diabetes Research Foundation and Dutch Kidney Foundation. PCNR is an Established Investigator of the Dutch Heart Foundation (grant 2009T038). The funders had no role in the study design, data collection, decision to publish or preparation of the manuscript.

Disclosures

JWJ received research grants from and was speaker on (CME accredited) meetings sponsored by Astellas, Astra-Zeneca, Biotronik, Boston Scientific, Daiichi Sankyo, Lilly, Genzyme, Medtronic, Merck-Schering-Plough, Pfizer, Orbus Neich, Novartis, Roche, Servier, Sanofi Aventis, the Netherlands Heart Foundation, the Interuniversity Cardiology Institute of the Netherlands and the European Community Framework KP7 Programme.

References

1. Carlson LA. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine*. 2005;258:94-114.
2. Birjmohun RS, Hutten BA, Kastelein JJ and Stroes ES. Efficacy and safety of high-density lipoprotein cholesterol-increasing compounds: a meta-analysis of randomized controlled trials. *Journal of the American College of Cardiology*. 2005;45:185-97.
3. Taylor AJ, Villines TC, Stanek EJ, Devine PJ, Griffen L, Miller M, Weissman NJ and Turco M. Extended-release niacin or ezetimibe and carotid intima-media thickness. *The New England journal of medicine*. 2009;361:2113-22.
4. Taylor AJ, Sullenberger LE, Lee HJ, Lee JK and Grace KA. Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) 2: a double-blind, placebo-controlled study of extended-release niacin on atherosclerosis progression in secondary prevention patients treated with statins. *Circulation*. 2004;110:3512-7.
5. Taylor AJ, Lee HJ and Sullenberger LE. The effect of 24 months of combination statin and extended-release niacin on carotid intima-media thickness: ARBITER 3. *Current medical research and opinion*. 2006;22:2243-50.
6. Brown BG, Zhao XQ, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, Marino EK, Bolson EL, Alaupovic P, Frohlich J and Albers JJ. Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *The New England journal of medicine*. 2001;345:1583-92.
7. HPS2-THRIVE Collaborative Group. HPS2-THRIVE randomized placebo-controlled trial in 25 673 high-risk patients of ER niacin/laropiprant: trial design, pre-specified muscle and liver outcomes, and reasons for stopping study treatment. *European heart journal*. 2013;34:1279-91.
8. Sharma M. Combination therapy for dyslipidemia. *Current opinion in cardiology*. 2011;26:420-3.
9. Sanford M and Curran MP. Niacin extended-release/simvastatin. *Drugs*. 2008;68:2373-86.
10. Blumenthal RS and Michos ED. The HALTS trial--halting atherosclerosis or halted too early? *The New England journal of medicine*. 2009;361:2178-80.
11. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA and Skene AM. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *The New England journal of medicine*. 2004;350:1495-504.
12. Michos ED, Sibley CT, Baer JT, Blaha MJ and Blumenthal RS. Niacin and statin combination therapy for atherosclerosis regression and prevention of cardiovascular disease events: reconciling the AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome With Low HDL/High Triglycerides: Impact on Global Health Outcomes) trial with previous surrogate endpoint trials. *Journal of the American College of Cardiology*. 2012;59:2058-64.
13. Bruckert E, Labreuche J and Amarenco P. Meta-analysis of the effect of nicotinic acid alone or in combination on cardiovascular events and atherosclerosis. *Atherosclerosis*. 2010;210:353-61.
14. Lee JM, Robson MD, Yu LM, Shirodaria CC, Cunnington C, Kylintireas I, Digby JE, Bannister T, Handa A, Wiesmann F, Durrington PN, Channon KM, Neubauer S and Choudhury RP. Effects of high-dose modified-release nicotinic acid on atherosclerosis and vascular function: a randomized, placebo-controlled, magnetic resonance imaging study. *Journal of the American College of Cardiology*. 2009;54:1787-94.
15. Lukasova M, Malaval C, Gille A, Kero J and Offermanns S. Nicotinic acid inhibits progression of atherosclerosis in mice through its receptor GPR109A expressed by immune cells. *The Journal of clinical investigation*. 2011;121:1163-73.
16. McKenney J, Bays H, Koren M, Ballantyne CM, Paolini JF, Mitchel Y, Betteridge A, Kuznetsova O, Sapre A, Sisk CM and Maccubbin D. Safety of extended-release niacin/laropiprant in patients with dyslipidemia. *Journal of clinical lipidology*. 2010;4:105-112 e1.
17. Meyers CD, Kamanna VS and Kashyap ML. Niacin therapy in atherosclerosis. *Current opinion in lipidology*. 2004;15:659-65.
18. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester

transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
19. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
20. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
21. van der Hoorn JW, Jukema JW, Havekes LM, Lundholm E, Camejo G, Rensen PC and Princen HM. The dual PPARalpha/gamma agonist tesaglitazar blocks progression of pre-existing atherosclerosis in APOE*3Leiden.CETP transgenic mice. *British journal of pharmacology*. 2009;156:1067-75.
22. Bijland S, Pieterman EJ, Maas AC, van der Hoorn JW, van Erk MJ, van Klinken JB, Havekes LM, van Dijk KW, Princen HM and Rensen PC. Fenofibrate increases very low density lipoprotein triglyceride production despite reducing plasma triglyceride levels in APOE*3-Leiden.CETP mice. *The Journal of biological chemistry*. 2010;285:25168-75.
23. Rensen PC, Herijgers N, Netscher MH, Meskers SC, van Eck M and van Berkel TJ. Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. *Journal of lipid research*. 1997;38:1070-84.
24. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD and Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis, thrombosis, and vascular biology*. 1995;15:1512-31.
25. Delsing DJM, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit ECM, Gijbels MJJ, van der Laarse A, Jukema JW, Havekes LM and Princen HMG. Acyl-CoA:Cholesterol Acyltransferase Inhibitor Avasimibe Reduces Atherosclerosis in Addition to Its Cholesterol-Lowering Effect in ApoE*3-Leiden Mice. *Circulation*. 2001;103:1778-1786.
26. van der Hoorn JW, Kleemann R, Havekes LM, Kooistra T, Princen HM and Jukema JW. Olmesartan and pravastatin additively reduce development of atherosclerosis in APOE*3Leiden transgenic mice. *Journal of hypertension*. 2007;25:2454-62.
27. Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH and Rader DJ. Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation*. 2006;113:90-7.
28. Niesor EJ, Magg C, Ogawa N, Okamoto H, von der Mark E, Matile H, Schmid G, Clerc RG, Chaput E, Blum-Kaelin D, Huber W, Thoma R, Pflieger P, Kakutani M, Takahashi D, Dernick G and Maugeais C. Modulating cholesteryl ester transfer protein activity maintains efficient pre-beta-HDL formation and increases reverse cholesterol transport. *Journal of lipid research*. 2010;51:3443-54.
29. Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K and Offermanns S. PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nature medicine*. 2003;9:352-5.
30. Li Z, Wang Y, van der Sluis RJ, van der Hoorn JW, Princen HM, Van Eck M, Van Berkel TJ, Rensen PC and Hoekstra M. Niacin reduces plasma CETP levels by diminishing liver macrophage content in CETP transgenic mice. *Biochemical pharmacology*. 2012;84:821-9.
31. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR and Havekes LM. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *The Journal of clinical investigation*. 1991;88:643-55.
32. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM and Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *The Journal of biological chemistry*. 1993;268:10540-5.

33. Carlson LA and Oro L. Effect of treatment with nicotinic acid for one month on serum lipids in patients with different types of hyperlipidemia. *Atherosclerosis*. 1973;18:1-9.
34. Hoogwerf BJ, Bantle JP, Kuba K, Frantz ID, Jr. and Hunninghake DB. Treatment of type III hyperlipoproteinemia with four different treatment regimens. *Atherosclerosis*. 1984;51:251-9.
35. Kamanna VS and Kashyap ML. Mechanism of action of niacin. *The American journal of cardiology*. 2008;101:20B-26B.
36. Fabbrini E, Mohammed BS, Korenblat KM, Magkos F, McCrea J, Patterson BW and Klein S. Effect of fenofibrate and niacin on intrahepatic triglyceride content, very low-density lipoprotein kinetics, and insulin action in obese subjects with nonalcoholic fatty liver disease. *The Journal of clinical endocrinology and metabolism*. 2010;95:2727-35.
37. Grundy SM, Mok HY, Zech L and Berman M. Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. *Journal of lipid research*. 1981;22:24-36.
38. Wang W, Basinger A, Neese RA, Shane B, Myong SA, Christiansen M and Hellerstein MK. Effect of nicotinic acid administration on hepatic very low density lipoprotein-triglyceride production. *American journal of physiology Endocrinology and metabolism*. 2001;280:E540-7.
39. Lamon-Fava S, Diffenderfer MR, Barrett PH, Buchsbaum A, Nyaku M, Horvath KV, Asztalos BF, Otokozawa S, Ai M, Matthan NR, Lichtenstein AH, Dolnikowski GG and Schaefer EJ. Extended-release niacin alters the metabolism of plasma apolipoprotein (Apo) A-I and ApoB-containing lipoproteins. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:1672-8.
40. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der Greef J, van Ommen B and Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome biology*. 2007;8:R200.
41. Wu BJ, Yan L, Charlton F, Witting P, Barter PJ and Rye KA. Evidence that niacin inhibits acute vascular inflammation and improves endothelial dysfunction independent of changes in plasma lipids. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:968-75.
42. Foks AC, Frodermann V, ter Borg M, Habets KL, Bot I, Zhao Y, van Eck M, van Berkel TJ, Kuiper J and van Puijvelde GH. Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis*. 2011;218:53-60.
43. Digby JE, McNeill E, Dyar OJ, Lam V, Greaves DR and Choudhury RP. Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. *Atherosclerosis*. 2010;209:89-95.
44. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K and Weintraub W. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. *The New England journal of medicine*. 2011;365:2255-67.
45. Giugliano RP. Niacin at 56 years of age--time for an early retirement? *The New England journal of medicine*. 2011;365:2318-20.
46. AIM-HIGH Investigators. The role of niacin in raising high-density lipoprotein cholesterol to reduce cardiovascular events in patients with atherosclerotic cardiovascular disease and optimally treated low-density lipoprotein cholesterol: baseline characteristics of study participants. The Atherothrombosis Intervention in Metabolic syndrome with low HDL/high triglycerides: impact on Global Health outcomes (AIM-HIGH) trial. *American heart journal*. 2011;161:538-43.

Supporting Information

Table S1 RT-PCR primer sequences

Gene	Forward primer	Reverse primer
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
CETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA

HPRT, hypoxanthine-guanine phosporibosyltransferase; 36B4, acidic ribosomal phosphoprotein PO; CETP, cholesteryl ester transfer protein.

CHAPTER 4

Anacetrapib Reduces Progression of Atherosclerosis, Mainly by Reducing non-HDL-cholesterol, Improves Lesion Stability and Adds to the Beneficial Effects of Atorvastatin

Susan Kühnast[*1,2,5], Sam J.L. van der Tuin[*3,5], José W.A. van der Hoorn[1,2,5], Jan B. van Klinken[4,5], Branko Simic[6], Elsbet Pieterman[1], Louis M. Havekes[1,3,5], Ulf Landmesser[7], Thomas F. Lüscher[7], Ko Willems van Dijk[3,4,5], Patrick C.N. Rensen[3,5], J. Wouter Jukema[2], Hans M.G. Princen[1]

[1]TNO - Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands;
[2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[3]Dept. of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands;
[4]Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands;
[5]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands;
[6]Center for Molecular Cardiology, Campus Schlieren, University of Zurich, Switzerland;
[7]University Heart Center, Department of Cardiology, University Hospital Zurich, Switzerland

*S.K. and S.J.L.v.d.T. contributed equally

Eur Heart J. 2015 Jan 1;36(1):39-50

Abstract

Objective The residual risk that remains after statin treatment supports the addition of other LDL-C-lowering agents and has stimulated the search for secondary treatment targets. Epidemiological studies propose HDL-C as a possible candidate. Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from atheroprotective HDL to atherogenic (V) LDL. The CETP inhibitor anacetrapib decreases (V)LDL-C by ~15-40% and increases HDL-C by ~40-140% in clinical trials. We evaluated the effects of a broad dose range of anacetrapib on atherosclerosis and HDL function, and examined possible additive/synergistic effects of anacetrapib on top of atorvastatin in APOE*3Leiden.CETP mice.

Methods and Results Mice were fed a diet without or with ascending dosages of anacetrapib (0.03; 0.3; 3; 30 mg/kg/d), atorvastatin (2.4 mg/kg/d) alone or in combination with anacetrapib (0.3 mg/kg/d) for 21 weeks. Anacetrapib dose-dependently reduced CETP activity (-59% to -100%, P<0.001), thereby decreasing non-HDL-C (-24% to -45%, P<0.001) and increasing HDL-C (+30% to +86%, P<0.001). Anacetrapib dose-dependently reduced atherosclerotic lesion area (-41% to -92%, P<0.01) and severity, increased plaque stability index and added to the effects of atorvastatin by further decreasing lesion size (-95%, P<0.001) and severity. Analysis of covariance showed that both anacetrapib (P<0.05) and non-HDL-C (P<0.001), but not HDL-C (P=0.76), independently determined lesion size.

Conclusion Anacetrapib dose-dependently reduces atherosclerosis, and adds to the anti-atherogenic effects of atorvastatin, which is mainly ascribed to a reduction in non-HDL-C. In addition, anacetrapib improves lesion stability.

Keywords cholesteryl ester transfer protein, non-HDL-cholesterol, HDL-cholesterol, HDL function, atherosclerosis, anacetrapib, atorvastatin

Introduction

Intervention trials provide ample evidence that lowering of low-density lipoprotein-cholesterol (LDL-C) contributes to a reduction in cardiovascular (CV) risk.[1-3] However, the residual risk that remains after statin treatment, as well as failure for some patients to reach recommended LDL-C targets despite statin treatment, support the addition of other LDL-C-lowering agents and also stimulate the search for secondary treatment targets.[3, 4] Prospective epidemiological studies propose high-density lipoprotein (HDL)-C as a potential target.[5] Cholesteryl ester transfer protein (CETP) plays an important role in lipid metabolism by facilitating the transfer of cholesteryl esters from atheroprotective HDL to atherogenic (V)LDL in exchange for triglycerides (TG), and inhibition of CETP activity has been proposed as a therapeutic way to increase HDL-C levels.[6-11]

In mouse models for atherosclerosis, CETP expression aggravated atherosclerosis development.[12, 13] Most but not all studies in rabbits and mice showed that CETP inhibition reduced atherosclerosis development.[14-19] However, torcetrapib failed to enhance the anti-atherogenic effects of atorvastatin and induced a pro-inflammatory, vulnerable plaque phenotype in APOE*3Leiden.CETP mice.[19] In the large clinical outcome trial (ILLUMINATE), torcetrapib increased the risk of major CV events and mortality despite a 72% increase in HDL-C and a 25% reduction in LDL-C.[20] The unexpected detrimental effects were ascribed to either an off-target blood pressure effect or the possible generation of dysfunctional HDL particles.[20] The much less potent CETP inhibitor dalcetrapib increased HDL-C by 31% to 40% with a minimal reduction in LDL-C, but did not translate into clinical benefit and resulted in premature termination of the dal-OUTCOMES trial.[21] Nonetheless, other CETP inhibitors are currently in clinical development. Amongst these, anacetrapib and evacetrapib have remarkable lipid-modulating abilities without the unwanted blood pressure effect as observed with torcetrapib.[22] In phase II trials, anacetrapib (10 to 300 mg) decreased LDL-C by ~15% to 40% and increased HDL-C by ~40% to 140% and evacetrapib (30 to 500 mg) decreased LDL-C by ~15% to 35% and increased HDL-C by ~50% to 130%.[23, 24]

To elucidate whether pharmacological CETP inhibition is anti-atherogenic and to what extent this is due to its LDL-C-lowering and HDL-C-raising abilities, we evaluated the effects of partial to full inhibition of CETP activity with a broad dose range of anacetrapib monotreatment on lipid modulation, atherosclerosis development and HDL functionality in APOE*3Leiden.CETP mice. Secondly, to mimic clinical intervention trials where dyslipidemic patients also receive statin treatment, we examined the possible additive/synergistic effects of anacetrapib on top of atorvastatin treatment in this well-established model for lipoprotein metabolism and atherosclerosis. These mice respond in a human-like manner to lipid-modulating interventions, including LDL-C-lowering[19, 25] and HDL-C-raising drugs.[19, 26, 27]

Methods

Animals and diet

Female APOE*3-Leiden.CETP transgenic mice[13] (n=105) were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water. Body weight and food intake were monitored during the study. At the age of 9 to 16 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 15% cacao butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch, 6.2% cellulose (Western-type diet, WTD; AB-Diets, Woerden, the Netherlands) and 0.1% cholesterol (SigmaAldrich, Zwijndrecht, the Netherlands) for a run-in period of 5 weeks. Animals were matched based on body weight, total cholesterol (TC), TG, HDL-C and age (n=15 per group) and received a control Western-type diet (WTD) without or with incremental dosages of anacetrapib (0.03; 0.3; 3 and 30 mg/kg/d; Dalton Chemical Laboratories Inc., Canada), atorvastatin (2.4 mg/kg/d) or a combination of atorvastatin (2.4 mg/kg/d) and anacetrapib (0.3 mg/kg/d) for a treatment period of 21 weeks. All animals were sacrificed by CO_2 inhalation and hearts were isolated to assess atherosclerosis development. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO).

Plasma lipids, lipoprotein profile, endogenous cholesteryl ester transfer protein activity, cholesteryl ester transfer protein concentration and serum amyloid A

After 4 h fasting, blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated cups via tail vein bleeding and plasma was isolated every 2 to 4 weeks. To measure HDL-C, apoB-containing particles were precipitated from diluted plasma (15 µL previously frozen plasma + 15 µl PBS) by adding 5 µL of 20% polyethylene glycol (PEG) in 200 mM glycine buffer (pH10). This mixture was incubated for 5 min at 25˚C and centrifuged at 6000 rpm for 5 min at 25˚C. Thirty µL of supernatant was mixed with 20 µl of 20% PEG in 200 mM glycine buffer and incubated for 5 min at 25˚C and centrifuged at 6000 rpm for 20 min at 25˚C. TC was measured in the supernatant to determine plasma HDL-C levels. Plasma TC and TG were determined individually using enzymatic kits (cat. no. 1458216 and cat. no. 1488872, Roche Diagnostics) according to manufacturer's protocol, and average plasma TC, TG, non-HDL-C and HDL-C levels were calculated. The distribution of cholesterol over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) as previously described.[13]

Plasma endogenous CETP activity and CETP concentration were determined as previously described.[28] Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (Roar Biomedical, New York, USA), according to manufacturer's protocol. CETP activity was calculated as nmol

cholesteryl ester transfer/mL plasma/h. Plasma CETP concentration (ALPCO Diagnostics, Salem, USA) and serum amyloid A (SAA; Tridelta development Ltd, Maynooth, Ireland) were measured by ELISA according to manufacturer's instructions.

Atherosclerosis quantification

After sacrifice, hearts were isolated and fixed in formalin, embedded in paraffin and cross-sectioned (5 μm) throughout the entire aortic root area. Cross sections were stained with hematoxylin-phloxine-saffron for histological analysis. Each cross section consisted of three segments separated by aortic valve leaflets and for each mouse four cross sections were used to assess atherosclerotic lesion area and severity. The lesions were classified into five categories according to the American Heart Association classification: I) Early fatty streaks: Up to ten foam cells in the intima with no other changes; II) Regular fatty streaks: Ten or more foam cells in the intima with no other changes; III) Mild plaque: A fibrotic cap and the presence of foam cells in the media; IV) Moderate plaque: More progressed lesions with an affected media, but without loss of architecture of the media; V) Severe plaque: The media is severely affected. Broken elastic fibers, cholesterol clefts, calcification and necrosis are frequently observed. Total lesion area, the number of lesions per cross section, as well as the percentage undiseased segments were determined as previously described.[28, 29] To determine lesion severity, the type I-III lesions were classified as mild lesions and the type IV-V lesions were classified as severe lesions. Images were taken with the Olympus BX51 microscope and lesion areas were measured using Cell D imaging software (Olympus Soft Imaging Solutions).

The severe lesions (type IV-V) were further analyzed to assess lesion composition after immunostaining with mouse anti-human alpha actin (1:800; Monosan, Uden, The Netherlands) for smooth muscle cells (SMCs), and rat anti-mouse Mac-3 (1:50; BD Pharmingen, the Netherlands) for macrophages followed by sirius red staining for collagen. Necrotic area and cholesterol clefts, monocyte adhesion to the endothelium, and the calculation of plaque stability index (defined as the ratio of collagen and SMC area as stabilization factors to macrophage and necrotic area as destabilization factors) were determined as previously described.[28, 29] All parameters of lesion composition were calculated per cross section and as a percentage of lesion area. Images of the lesions were taken with the Olympus BX40 microscope with Nuance 2 multispectral imaging system, and stained areas were quantified using Image J software. Evaluation of atherosclerosis development was performed under blinded conditions.

HDL functionality assays

Isolation of HDL

HDL from control and treated mice was isolated by sequential ultracentrifugation (d = 1.063–1.21 g/mL) according to the method of Havel *et al*.[30] using solid potassium bromide (Merck) for density adjustment as described previously.[31]

Vascular cell adhesion molecule detection by cell western

Human arterial endothelial cells (HAECs) (P7) were incubated with isolated HDL for 12 hours and treated with TNF-alpha (R&D Systems) for 4 hours. Cells were fixed in 3.7% formaldehyde, washed, blocked and incubated overnight with vascular cell adhesion molecule (VCAM-1) antibody (R&D Systems). Cells were washed and secondary antibody anti-goat (Odyssey Licor) with Draq-5 for normalization (680CW) was added. After incubation, cells were washed and fluorescence was measured.[32]

Apoptotic cell death inhibition in a cellular system

HAECs (P7) were treated with an apoptosis-inducing agent in the presence and absence of isolated HDL. Cells were lysed and apoptosis was detected using a DNA fragmentation assay (Cell Death Detection $ELISA_{PLUS}$) according to the supplier's protocol (Roche Applied Science, 11 774 425 001). In short, supernatant was placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase (POD) antibodies. Plates were washed to remove unbound components. The amount of nucleosome retained in the immunocomplex was quantitatively photometrically determined with ABTS as peroxidase substrate.

Statistical Analysis

Significance of differences between the groups was calculated non-parametrically using a Kruskal-Wallis test for independent samples, followed by a Mann-Whitney U-test for independent samples. An analysis of covariance (ANCOVA) was performed to test for group differences in lesion area with HDL-C and non-HDL-C exposure as covariates. To test whether collinearity was present between the explanatory variables, we calculated the variance inflation factor (VIF) and the condition index (CI). Values of VIF > 5 and values of CI > 10 were used as a cutoff for collinearity.[33, 34]

SPSS 17.0 for Windows was used for statistical analysis. All groups were compared with the control group and the combination group was compared with the atorvastatin group. Bonferroni-Holm's method was used to determine the level of significance in the case of multiple comparisons. Values are presented as means ± SD. P-values <0.05 were considered statistically significant.

Results

Anacetrapib, atorvastatin and their combination decrease cholesteryl ester transfer protein activity despite an increase in cholesteryl ester transfer protein concentration

To assess the extent to which an ascending dose range of anacetrapib inhibits CETP, we measured CETP activity after 8 weeks of treatment and CETP concentration after 21 weeks of treatment (**Table 1**). Anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) reduced CETP activity by -59% to -100% (P<0.001) and increased plasma CETP concentration by +11% (NS) to +29% (P<0.001). Both CETP activity and concentration were decreased by atorvastatin alone (-29% and -24%, P<0.001) and in combination with 0.3 mg/kg/d anacetrapib (-84% and -23%, P<0.001). Thus, adding anacetrapib to atorvastatin further reduced CETP activity (-78%, P<0.001) without affecting CETP concentration when compared with atorvastatin.

Table 1 Effect of anacetrapib, atorvastatin and their combination on the cholesteryl ester transfer protein activity after 8 weeks of treatment and cholesteryl ester transfer protein concentration after 21 weeks of treatment

	Plasma CETP activity (nmol/mL/h)		Plasma CETP concentration (µg/mL)	
Control	66.8 ± 10.1		15.7 ± 1.2	
0.03 mg/kg/d anacetrapib	27.1 ± 7.1 ***	(-59%)	17.4 ± 3.1	(+11%)
0.3 mg/kg/d anacetrapib	6.3 ± 4.8 ***	(-91%)	19.9 ± 2.4 ***	(+27%)
3 mg/kg/d anacetrapib	0.7 ± 3.3 ***	(-99%)	20.2 ± 3.1 ***	(+29%)
30 mg/kg/d anacetrapib	0.0 ± 2.3 ***	(-100%)	18.5 ± 4.1	(+18%)
Atorvastatin	47.5 ± 8.2 ***	(-29%)	11.9 ± 2.3 ***	(-24%)
Atorvastatin + 0.3 mg/kg/d anacetrapib	10.5 ± 8.3 *** ###	(-84%)	12.1 ± 2.1 ***	(-23%)

***P<0.001 when compared with control; ###P<0.001 when compared with atorvastatin. Data are presented as means ± SD (% inhibition or increase when compared with the control); n=15 per group.

Anacetrapib alone and in combination with atorvastatin reduces plasma non-HDL-cholesterol and increases HDL-cholesterol

During the study, plasma lipids were measured every 2 to 4 weeks and average plasma TC (**Figure 1A**), TG (**Figure 1B**), non-HDL-C (**Figure 1C**) and HDL-C (**Figure 1D**) were calculated. In the control group, the Western-type diet resulted in an average plasma TC of 10.8 ± 1.1 mmol/L, TG of 1.8 ± 0.5 mmol/L, non-HDL-C of 9.5 ± 1.1 mmol/L and HDL-C of 1.2 ± 0.2 mmol/L. When compared with the control, anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) decreased TC (-19%; -25%; -27% and -31%, P<0.001 for all) mainly by decreasing non-HDL-C (-24%; -36%; -42% and -45%, P<0.001 for all). In addition, anacetrapib monotreatment increased HDL-C (+30%; +60%; +86% and +86%, P<0.001 for all) and decreased TG (-21%, P=0.07; -22%, P=0.06; -19%, NS and -27%, P<0.01).

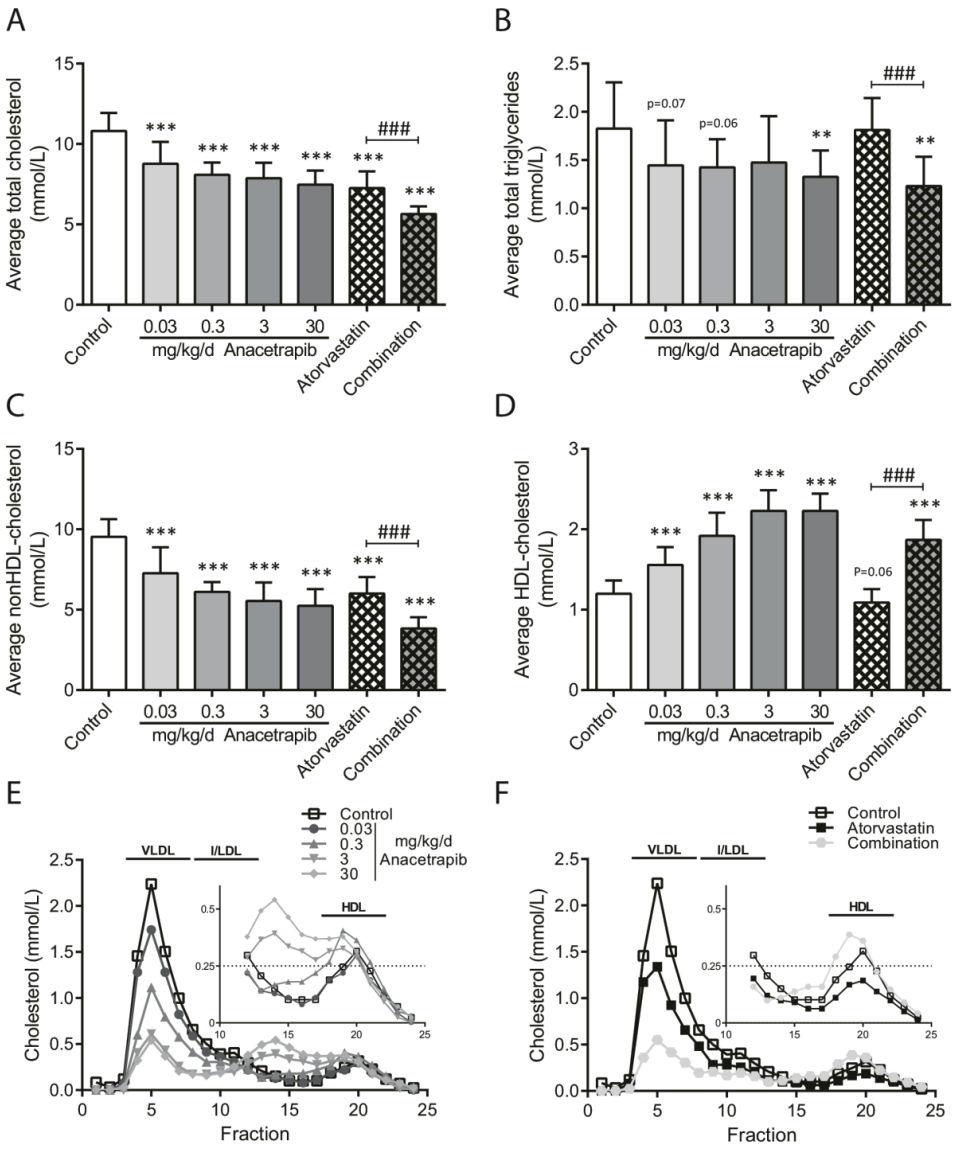


Figure 1. Effect of anacetrapib, atorvastatin and their combination on total cholesterol, triglycerides, non-HDL-cholesterol and HDL-cholesterol levels. Plasma total cholesterol (A), triglycerides (B) non-HDL-cholesterol (C) and HDL-cholesterol (D) were measured throughout the study and average levels were calculated. Lipoprotein profiles for cholesterol were assessed by FPLC lipoprotein separation to study effects of anacetrapib alone (E) and in combination with atorvastatin (F) after 18 weeks of treatment.
**P<0.01, ***P<0.001 when compared with control; ###P<0.001 when compared with atorvastatin. Data are presented as means ± SD (n=15 per group).

Atorvastatin decreased TC (-33%, P<0.001) by decreasing non-HDL-C (-37%, P<0.001) and with no effect on HDL-C and TG. The combination treatment decreased TC (-48%, P<0.001), non-HDL-C (-60%, P<0.001) and TG (-33%, P<0.01) and increased HDL-C (+56%, P<0.001). Anacetrapib enhanced the lipid-modifying effects of atorvastatin with greater reductions in TC (-22%, P<0.001), non-HDL-C (-36%, P<0.001) and TG (-32%, P<0.001) and a greater increase in HDL-C (+72%, P<0.001) when comparing the combination treatment with atorvastatin monotreatment. Lipoprotein profiles confirmed the lipid-modifying effects of anacetrapib and revealed the formation of larger HDL particles, as observed previously[19] after treatment with higher dosages of anacetrapib (3 and 30 mg/kg/d; **Figure 1E and 1F**).

Atorvastatin in combination with anacetrapib reduces atherosclerosis progression to a greater extent than atorvastatin alone

After 21 weeks of treatment, the effects of anacetrapib, atorvastatin and their combination on the progression of atherosclerosis were assessed in the aortic root area as illustrated by representative images in **Figure 2**. The number of lesions (**Figure 3A**), lesion area (**Figure 3B**), undiseased segments (**Figure 3C**) and lesion severity (**Figure 3D**) were assessed as previously described.[28, 29]

For the control group, 4.1 ± 0.6 lesions per cross section developed with a total lesion area of 169 ± 51 $*10^3$ μm^2. Approximately 71% of these lesions were severe lesions (type IV-V) and only 5% of the segments were undiseased. Anacetrapib monotreatment (0.03; 0.3; 3 and 30 mg/kg/d) dose-dependently reduced lesion area (-41%, P<0.01; -72%; -86% and -92%, P<0.001 for all) and the number of lesions and improved lesion severity as indicated by less severe lesions (down to 15%, P<0.001) and more undiseased segments (up to 46%, P<0.001). Atorvastatin monotreatment reduced total lesion area (-63%, P<0.001) and improved lesion severity without affecting the number of lesions and undiseased segments. When compared with the control, the combination treatment further decreased total lesion area (-95%, P<0.001), the number of lesions (-41%, P<0.01), and lesion severity and increased the percentage undiseased segments. When compared with atorvastatin monotreatment, the combination treatment decreased total lesion area (-87%, P<0.001), the number of lesions (-34%, P=0.06) and severity and increased the percentage undiseased segments to a greater extent, indicative of an additional effect of anacetrapib on top of the statin.

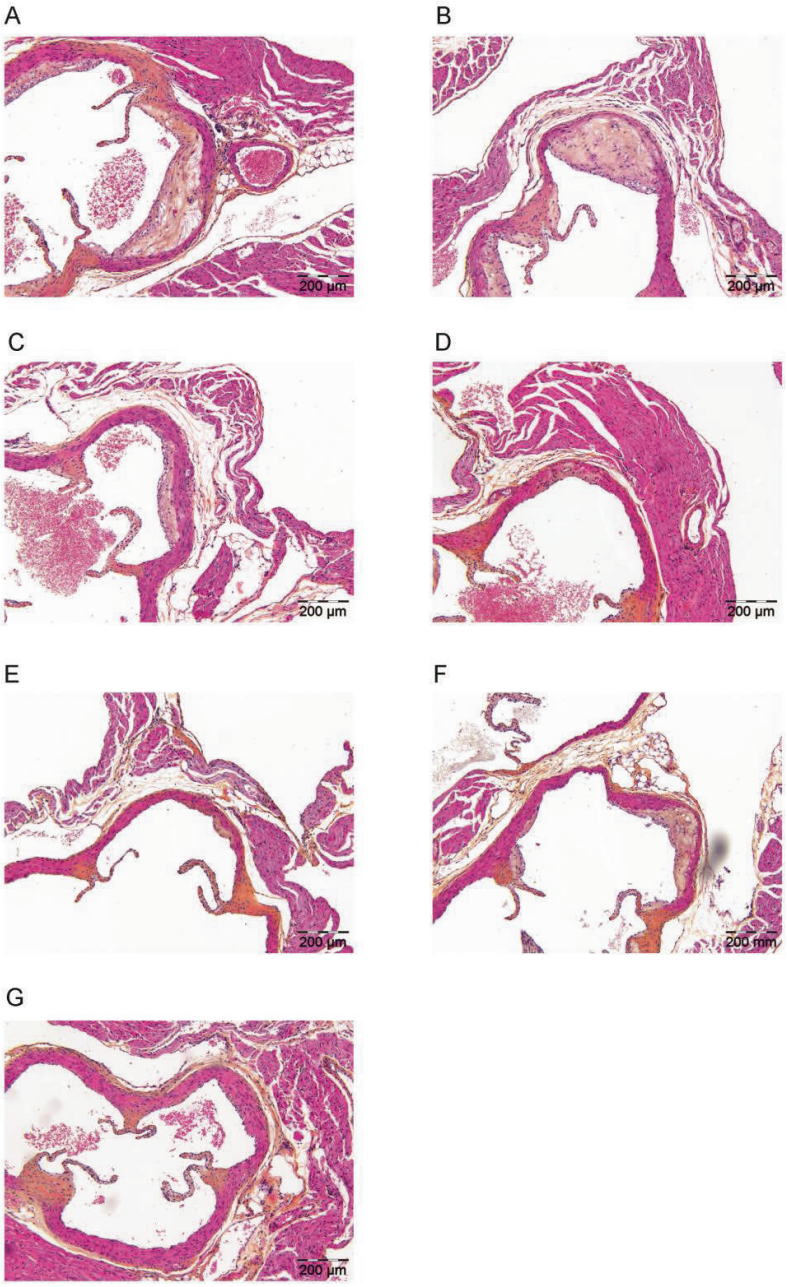


Figure 2. Effect of anacetrapib, atorvastatin and their combination on plaque morphology. Representative images of hematoxylin-phloxine-saffron-stained atherosclerotic lesions in a cross section of the aortic root area for the control group (A), 0.03 mg/kg/d anacetrapib (B), 0.3 mg/kg/d anacetrapib (C), 3 mg/kg/d anacetrapib (D), 30 mg/kg/d anacetrapib (E), atorvastatin group (F) and the combination group (G) after 21 weeks of treatment.

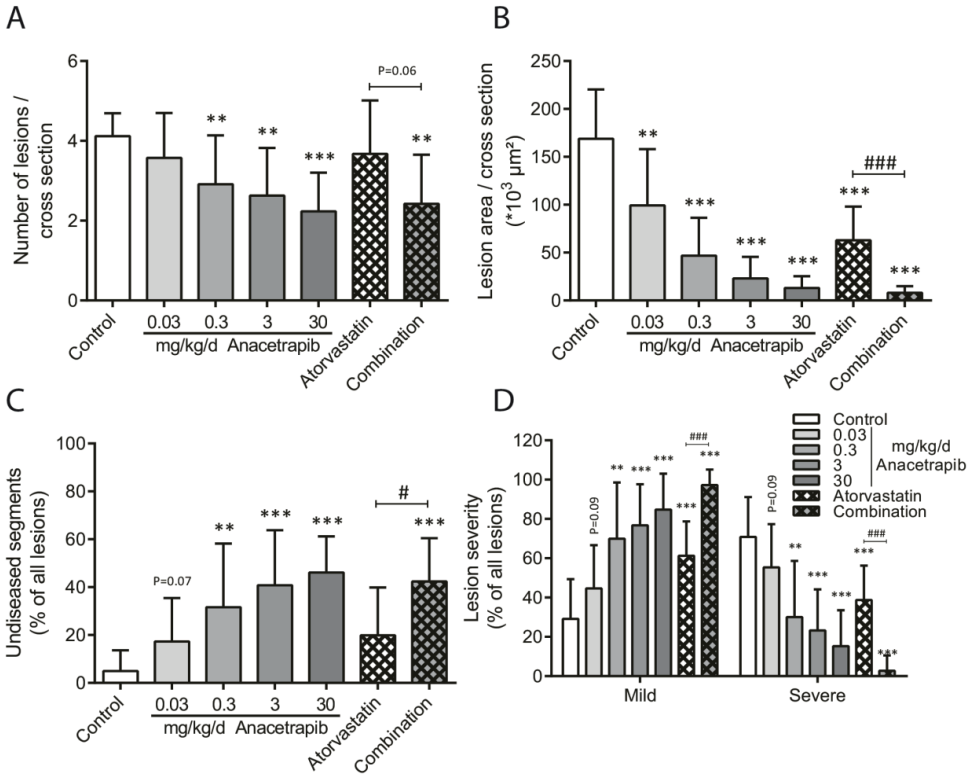


Figure 3. Effect of anacetrapib, atorvastatin and their combination on atherosclerosis development in the aortic root area. The number of lesions per cross section (A), total lesion area per cross section (B), the percentage undiseased segments (C) and lesion severity as a percentage of all lesions (D) were determined after 21 weeks of treatment. Lesion severity was classified as mild (type I-III) and severe (type IV-V) lesions.
**P<0.01, ***P<0.001 when compared with control; #P<0.05, ###P<0.001 when compared with atorvastatin. Data are presented as means ± SD (n=15 per group).

Anacetrapib, atorvastatin and their combination improve lesion stability

In addition to atherosclerotic lesion size and severity, we assessed the number of monocytes adhering to the endothelium as a functional marker for vascular inflammation (**Figure 4A**). Adhering monocytes per cross section in the control group (i.e. 4.1 ± 2.6) were reduced by the higher dosages of anacetrapib (-60%, P<0.01 and -61%, P<0.01), as well as by atorvastatin alone and in combination with anacetrapib (-48%, P<0.05 and -78%, P<0.001). When compared with atorvastatin, the combination treatment reduced the number of monocytes to a greater extent (-57%, P<0.01). In addition, we analyzed the composition of the severe lesions (type IV-V), since these lesions are considered to be most vulnerable and prone to rupture. All parameters of lesion composition were calculated per cross section as absolute values and as a percentage of lesion area. To this end, collagen content (**Figure**

4B) and SMC content in the cap (**Figure 4C**) were considered as stabilization factors and macrophage content (**Figure 4D**) and necrotic content (**Figure 4E**) were considered as destabilization factors. The severe lesions in the control group consisted of approximately 54% collagen, 6% SMCs in the cap, 10% macrophages and 4% necrosis. The lesion stability index for the control group presented as the ratio of stabilization to destabilization factors was 4.9 ± 2.0 (**Figure 4F**).

When corrected for lesion area, the two higher dosages of anacetrapib (3 and 30 mg/kg/d) revealed a more stable plaque phenotype by increasing collagen content (+21%, P<0.001 and +28%, P<0.001) and SMC content in the cap (+120%, P<0.01 and +119%, P<0.05) and by decreasing macrophage (-53%, P=0.06 and -60%, P=0.05) and necrotic (-73%, P<0.001 and -46%, P<0.05) content. This is reflected by an increase in lesion stability index in these two treatment groups (+427%, P<0.001 and +366%, P<0.01). Atorvastatin in combination with anacetrapib tended to increase the SMC content in the cap (+194%, P=0.07) and decreased necrotic content (-96%, P<0.05) with no effect on lesion stability index. However, it should be noted that there were almost no lesions in the combination group and only two mice that received the combination treatment of anacetrapib and atorvastatin developed severe lesions.

Anacetrapib does not affect HDL function

To explore the contribution of the anacetrapib-induced increase in HDL-C to the reduction of atherosclerosis, we investigated the endothelial-vasoprotective properties of HDL, in particular with respect to anti-inflammatory and anti-apoptotic properties in cultured arterial endothelial cells. HDL isolated from anacetrapib-treated mice had no effect on pro-inflammatory cytokine-induced VCAM-1 expression (**Figure 5A**) or on apoptotic cell death (**Figure 5B**).

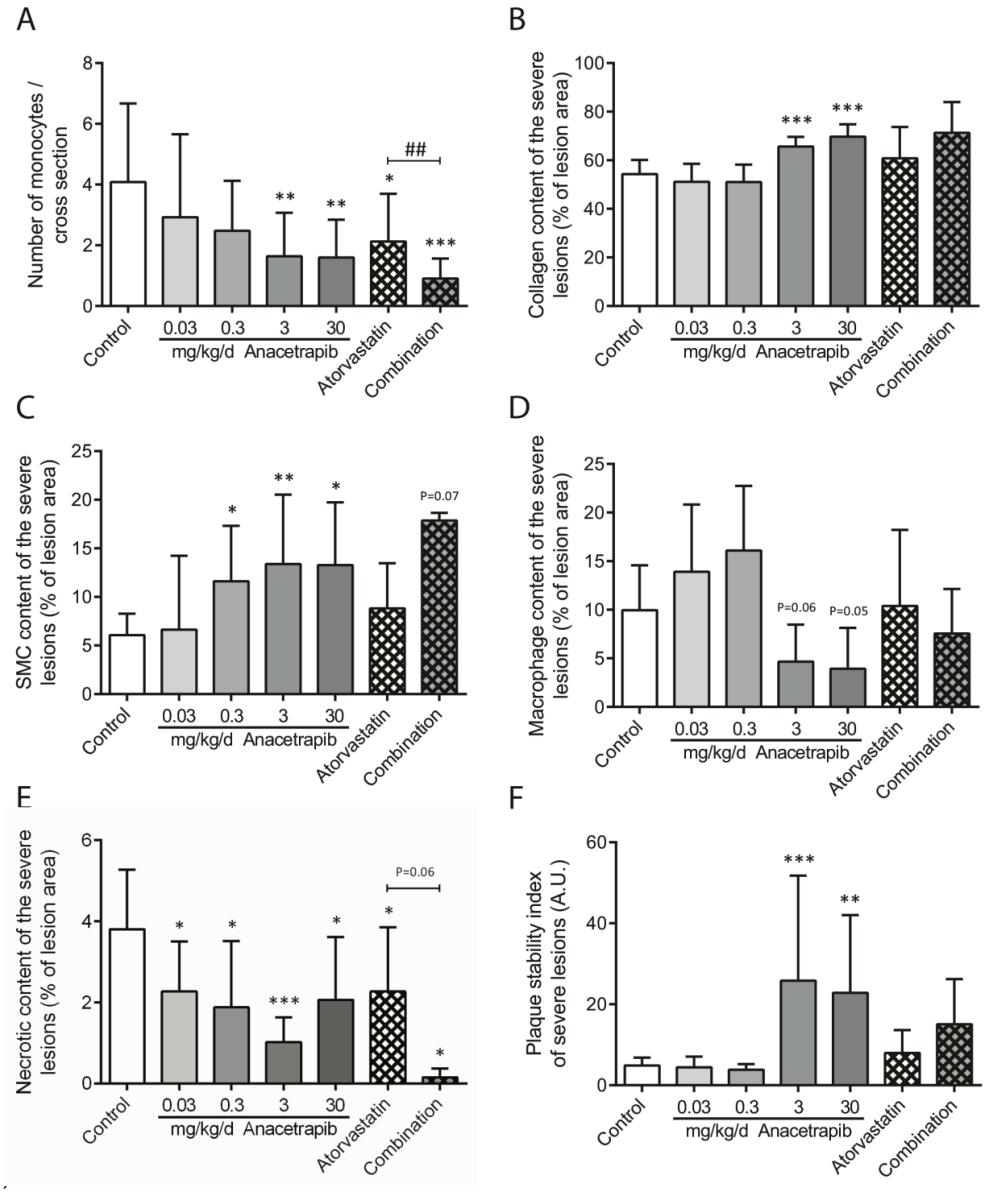


Figure 4. Effect of anacetrapib, atorvastatin and their combination on lesion composition. The number of monocytes adhering to the vascular endothelium per cross section (A) was calculated. In the severe lesions (type IV and V), collagen content (B) and SMC content in the cap (C) were determined as stabilization factors and macrophage content (D) and necrotic content (E) were determined as destabilization factors, all as a percentage of lesion area. The plaque stability index was calculated as the ratio of the stabilization factors to the destabilization factors (F).
*P<0.05, **P<0.01, ***P<0.001 when compared with control; ##P<0.01 when compared with atorvastatin. Data are presented as means ± SD (n=15 per group).

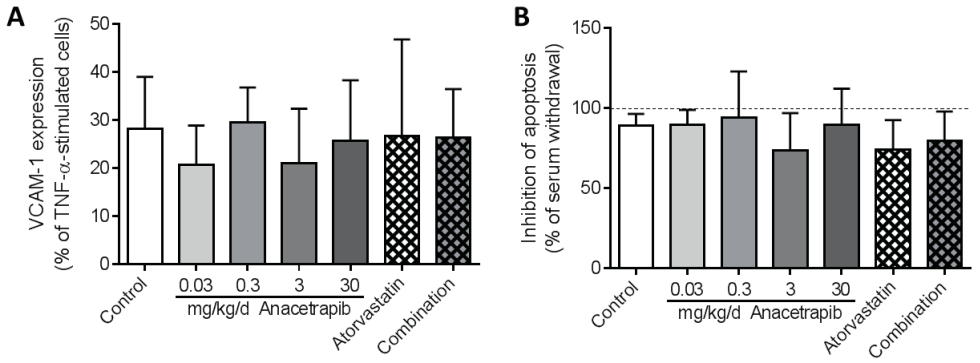


Figure 5. Effect of anacetrapib, atorvastatin and their combination on endothelial-vasoprotective properties of HDL, in particular pro-inflammatory cytokine-induced VCAM-1 expression (A) and apoptotic cell death (B).
Data are presented as means ± SD.

Anacetrapib reduces atherosclerosis progression primarily by reducing non-HDL-cholesterol exposure

We evaluated whether the effects of anacetrapib and atorvastatin on atherosclerosis development could be explained by either an increase in HDL-C or a decrease in non-HDL-C or both. Lesion area was normalized by cubic root transformation (lesion area $^{(1/3)}$). Univariate regression analysis showed that lesion area was predicted by TC (**Figure 6A**), mainly non-HDL-C (**Figure 6B**) and to a lesser extent by HDL-C (**Figure 6C**). Analysis of covariance (ANCOVA) showed that both anacetrapib treatment, at the dosages of 3 and 30 mg/kg/d ($P<0.05$) and non-HDL-C ($P<0.001$), but not HDL-C ($P=0.76$), independently determined lesion size. Importantly, the variance inflation factors of HDL-C and non-HDL-C (VIF = 4.42 and 3.18 respectively) and the condition index (CI = 4.43) did not exceed the threshold for collinearity between the explanatory variables. Collectively, these data are compatible with a mechanism that anacetrapib mainly decreases atherosclerotic lesion development via a reduction of non-HDL-C with an additional effect by the compound itself at the higher doses (**Figure 7**).

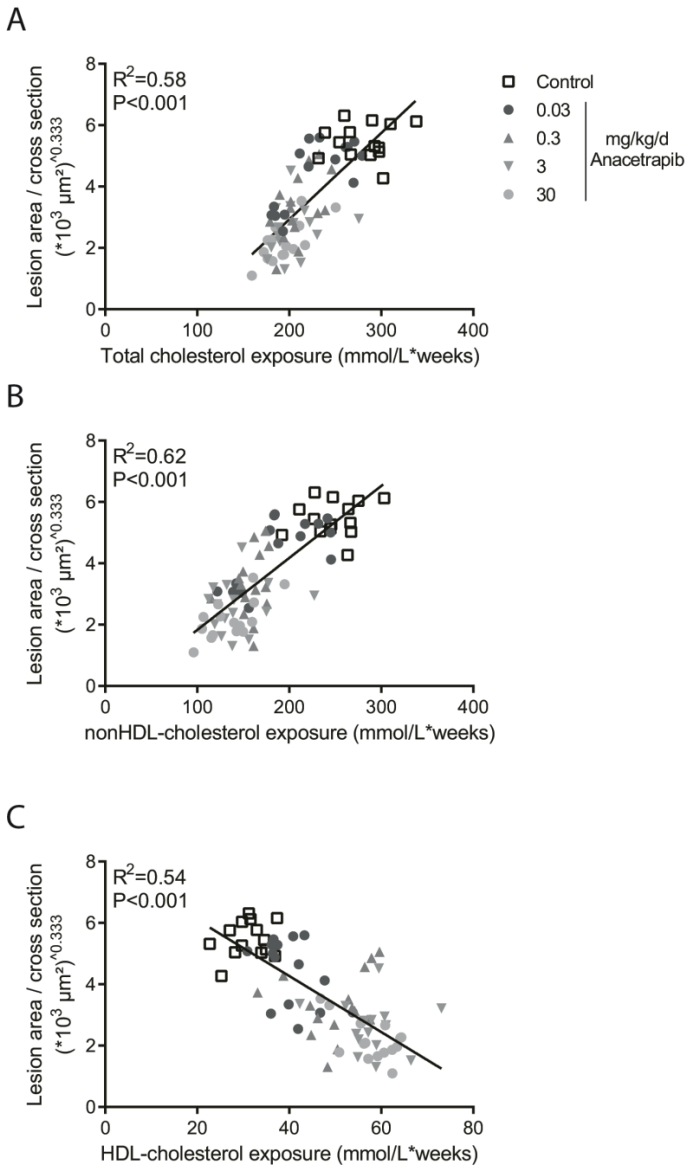


Figure 6. Correlation between plasma cholesterol exposure and lesion area. Linear regression analyses were performed on the cubic root of lesion area plotted against total cholesterol exposure (A), non-HDL-cholesterol exposure (B) and HDL-cholesterol exposure (C).

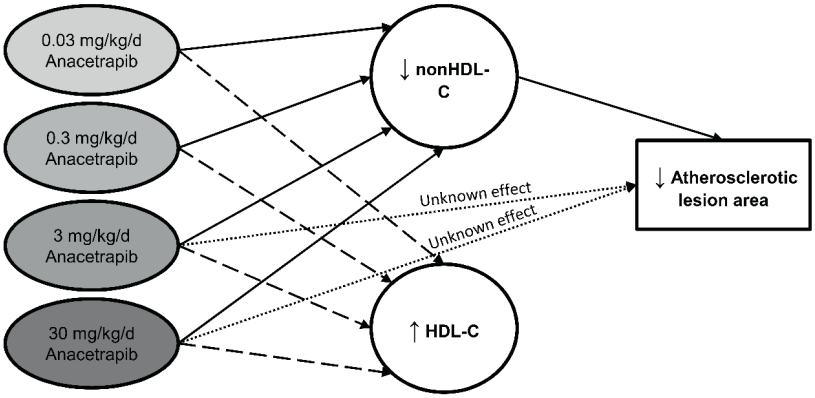


Figure 7. Hypothetical scheme of factors contributing to the effect of anacetrapib on atherosclerotic lesion area as suggested by statistical analyses. An analysis of covariance (ANCOVA) was performed to test for group differences in lesion area with HDL-C and non-HDL-C exposure as covariates. HDL-C was not an independent predictor of lesion area when non-HDL-C was included as covariate, suggesting that the effect of anacetrapib on atherosclerosis development was mainly mediated through the reduction of non-HDL-C. The higher dosages of anacetrapib (3 and 30 mg/kg/d) also revealed an effect on atherosclerosis that was independent of non-HDL-C, but this effect was not explained by the increase in HDL-C.

Anacetrapib slightly increased serum amyloid A as a marker of inflammation

To assess the effect of anacetrapib on general inflammatory status, we measured plasma SAA levels, a systemic inflammatory marker after 16 weeks of treatment (**Figure 8**). Plasma SAA levels in the control group were 1.6 ± 0.5 µg/mL. When compared with the control, 0.3 mg/kg/d anacetrapib tended to increase SAA levels (+37%, P=0.07). No effects on body weight (gain) and food intake were noted with any of the treatments (data not shown).

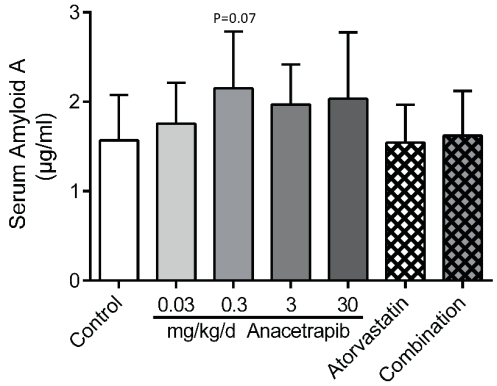


Figure 8. The effects of anacetrapib, atorvastatin and their combination on plasma SAA levels were measured after 16 weeks of treatment.
*P<0.05 when compared with control. Data are presented as means ± SD (n=15 per group)

Discussion

The present study is the first intervention study in a mouse model for atherosclerosis designed to investigate the effects of the CETP inhibitor, anacetrapib alone and in combination with atorvastatin on the progression of atherosclerosis, lesion stability and HDL function. In clinical trials, the effectiveness of novel treatment regimes in CVD is only being tested in patients on a statin background which makes this study unique in also evaluating the effects of anacetrapib monotreatment. In APOE*3Leiden.CETP mice, a broad dose range of anacetrapib dose-dependently reduced atherosclerosis development. This effect was mainly ascribed to the reduction in non-HDL-C despite a remarkable increase in HDL-C and without affecting HDL functionality. Anacetrapib improved lesion stability when given at a higher dose (3 and 30 mg/kg/d). In addition, a moderate dose of anacetrapib (0.3 mg/kg/d) added to the anti-atherogenic effects of atorvastatin.

In our study, incremental dosages of 0.03 to 30 mg/kg/d anacetrapib dose-dependently decreased CETP activity by > 60%, decreased non-HDL-C by 24% to 45% and increased HDL-C by 30% to 86%. These lipid-altering effects are comparable to findings from phase I, II and III clinical trials. In phase I trials, an anacetrapib-induced reduction in CETP activity of > 60% was accompanied by dose-dependent LDL-C-lowering and HDL-C-raising effects both in healthy subjects[35] and in patients with dyslipidemia.[36] In line with our results, these studies also report an increase in CETP concentration possibly due to the formation of an inactive complex between CETP and HDL.[37] In a phase II trial, 8 weeks of treatment with ascending dosages of anacetrapib monotreatment (10 to 300 mg/d) reduced LDL-C by 16% to 39% and increased HDL-C by 44% to 139%. Similar to our study, the addition of anacetrapib to atorvastatin produced incremental LDL-C reductions.[23]

The present study in APOE*3Leiden.CETP mice demonstrates that total blockage of CETP does not reveal adverse effects on the clinical endpoint when compared with partial blockage: anacetrapib dose-dependently reduced the progression of atherosclerosis and increased plaque stability whereas the anti-atherogenic effects of atorvastatin were enhanced in combination with a moderate dose of anacetrapib.

Inconsistent data have been reported on the effect of other CETP inhibitors on atherosclerosis development in animals expressing CETP. In rabbits, dalcetrapib reduced atherosclerosis in one study with no effect in another study.[15, 17] In contrast to the human situation,[21] the reduction in atherosclerosis after dalcetrapib treatment was accompanied by a 40% to 50% decrease in non-HDL-C together with an increase in HDL-C.[15] In mice, torcetrapib monotreatment decreased atherosclerosis.[19] However, unlike the present study, these effects were not enhanced in combination with atorvastatin in the same mouse model.[19] In rabbits, torcetrapib treatment decreased atherosclerosis where aortic lesion area correlated with TC/HDL-C ratio.[18] This could suggest a possible anti-atherogenic role

of increased HDL-C or other pleiotropic effects of HDL. However, in the APOE*3Leiden.CETP mouse model, statistical analyses revealed that HDL-C was not an independent predictor of lesion area when non-HDL-C was included as covariate, suggesting that the effect of anacetrapib on atherosclerosis development was mainly mediated through the reduction of non-HDL-C. The higher dosages of anacetrapib (3 and 30 mg/kg/d) also revealed an effect on atherosclerosis that was independent of non-HDL-C, but this effect was not explained by the increase in HDL-C and could point to other hitherto unknown (off target) effects of anacetrapib.

Besides atherosclerotic lesion size, lesion composition should also be taken into consideration given that in the human situation, a vulnerable lesion consisting of more macrophages, a large necrotic core and a thin, collagen-poor, fibrous cap is more prone to rupture.[38] Previously, our group showed that torcetrapib produced a pro-inflammatory, unstable plaque phenotype as seen by increased monocyte adherence to the vascular endothelium and consequently increased macrophage content of the lesions.[19] In the present study, anacetrapib decreased monocyte adherence and improved lesion composition as shown by an increase in stabilization factors (collagen and SMC content) and a decrease in destabilization factors (macrophage and necrotic content). The inconsistencies can be ascribed to the off-target activation of the renin-angiotensin-aldosterone system (RAAS) and blood pressure effect of torcetrapib.[39] Indeed, in our mouse model for atherosclerosis, torcetrapib also increased aldosterone levels in plasma.[19]

The large phase III DEFINE trial was designed to further assess the efficacy and tolerability of anacetrapib in statin-treated patients with or at risk for coronary heart disease.[40] Anacetrapib (100 mg/d) decreased LDL-C by 40% and increased HDL-C by 138% with an acceptable safety profile and no indication for an increase in CV events. In fact, *post hoc* analyses suggested a reduction in CV endpoints. These initial data provided a rationale for conducting a larger clinical endpoint trial of pharmacological CETP inhibition despite the conflicting outcomes of genetic CETP deficiency and the ILLUMINATE trial.[20] In view of the detrimental effects of torcetrapib in the ILLUMINATE trial, anacetrapib was thoroughly screened and revealed minimal side effects without any indication for an off-target pressure effect.[23, 35, 36]

Despite the absence of reported side effects of anacetrapib, there are some concerns about target-related side effects due to formation of large buoyant cholesterol-rich HDL-2 particles after CETP inhibition,[41, 42] which may be dysfunctional with regard to their endothelial-vasoprotective effects and consequently their atheroprotective properties[10, 37, 43, 44] and that this may have contributed to the failure of torcetrapib.[20] In the present study, we investigated the effects of HDL isolated from control and anacetrapib-treated mice on parameters of vascular inflammation and function. HDL from anacetrapib-treated mice did not suppress cytokine-induced adhesion molecule expression or cell apoptosis in

endothelial cells. This is in line with results from recent studies where no differences were observed in the effect of HDL from control or anacetrapib-treated hamsters and humans on inflammatory markers (adhesion molecule expression, monocyte chemotactic protein-1 secretion, monocyte adhesion, NFkB activation and cytokine mRNAs) in endothelial cells[45] and macrophages.[46] Importantly, although anacetrapib treatment did not improve the anti-inflammatory and anti-apoptotic effects of HDL, it also did not adversely affect these functions of HDL. In addition, we found no effect of anacetrapib on serum paraoxonase 1 (PON-1) activity and the aortic content of reactive oxygen species (ROS) (data not shown). Formation of large cholesterol-rich HDL-2 particles in CETP-deficiency or after CETP inhibition has also been suggested to affect the cholesterol efflux capacity of these particles.[41, 42] Although we did not address this in the present study, data from literature consistently indicate that the cholesterol efflux capacity is not impaired but improved. HDL from CETP-deficient patients displayed enhanced ability to promote cholesterol efflux from macrophages in an ABCG1-dependent manner.[41] In humans, anacetrapib-treated HDL showed increased ABCA1- and ABCG1-mediated cholesterol efflux capacity.[46] Collectively, these data indicate that CETP inhibition does not result in formation of dysfunctional HDL with regard to its atheroprotective properties as assessed by *ex vivo* (cell) assays.

It should be noted that in the DEFINE trial, a non-significant 18% increase in C-reactive protein, a marker of inflammation, after anacetrapib treatment was found.[40] In the present study, the inflammatory marker, SAA was slightly elevated after anacetrapib treatment, but this effect was alleviated when anacetrapib was given in combination with atorvastatin.

The effects of two other CETP inhibitors, DRL-17822 and TA-8995 (DEZ-001), as well as a vaccine against CETP, ATH03, are being tested in phase I/II clinical development. In large phase III clinical trials, the effects of 100 mg anacetrapib (REVEAL) and 130 mg evacetrapib (ACCELERATE) in patients on standard statin treatment on CV outcomes are currently being investigated and results are expected in 2016/17.[44] The outcome of these trials will resolve the unanswered questions regarding possible beneficial effects of pharmacological CETP inhibition and may give additional insight into the HDL-hypothesis and the contribution of HDL and non-HDL to CV endpoints.

Acknowledgement
We would like to thank Erik Offerman (TNO), Wen Liang (TNO/LUMC) and Jasmin Manz (University of Zurich) for their excellent technical assistance.

Funding
This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project PREDICCt (KOWVD, grant 01C-104), and supported by the Dutch Heart Foundation, Dutch Diabetes Research Foundation and Dutch Kidney Foundation and Netherlands Consortium for Systems Biology. We acknowledge the support from 'the Netherlands CardioVascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centers, the Netherlands Organization for Health Research and Development and the Royal Netherlands Academy of Sciences' for the GENIUS project 'Generating the best evidence-based pharmaceutical targets for atherosclerosis' (CVON2011-19). PCNR is an Established Investigator of the Netherlands Heart Foundation (2009T038).

Disclosures
JWJ received research grants from and was speaker on (CME accredited) meetings sponsored by Amgen, Astellas, Astra-Zeneca, Biotronik, Boston Scientific, Daiichi Sankyo, Lilly, Genzyme, Medtronic, Merck-Schering-Plough, Pfizer, Orbus Neich, Novartis, Roche, Servier, Sanofi Aventis, the Netherlands Heart Foundation, the Interuniversity Cardiology Institute of the Netherlands and the European Community Framework KP7 Programme.

References

1. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R and Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins. *The Lancet*. 2005;366:1267-1278.
2. Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R and Baigent C. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *The Lancet*. 2012;380:581-590.
3. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J and Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *The Lancet*. 2010;376:1670-1681.
4. Davidson MH, Maki KC, Pearson TA, Pasternak RC, Deedwania PC, McKenney JM, Fonarow GC, Maron DJ, Ansell BJ, Clark LT and Ballantyne CM. Results of the National Cholesterol Education (NCEP) Program Evaluation ProjecT Utilizing Novel E-Technology (NEPTUNE) II survey and implications for treatment under the recent NCEP Writing Group recommendations. *The American journal of cardiology*. 2005;96:556-63.
5. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA : the journal of the American Medical Association*. 2009;302:1993-2000.
6. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J and Mabuchi H. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*. 1989;342:448-451.
7. Boekholdt SM and Thompson JF. Natural genetic variation as a tool in understanding the role of CETP in lipid levels and disease. *Journal of lipid research*. 2003;44:1080-1093.
8. Inazu A, Brown ML, Hesler CB, Agellon LB, Koizumi J, Takata K, Maruhama Y, Mabuchi H and Tall AR. Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *The New England journal of medicine*. 1990;323:1234-8.
9. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Holm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart A, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett MS, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki ML, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PI, Klungel OH, Maitland-van der Zee AH, Peters BJ, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VH, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WM, Boer JM, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, Konig IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, Schrezenmeir J, Schreiber S, Schafer A, Danesh J, Blankenberg S, Roberts R, McPherson R, Watkins H, Hall AS, Overvad K, Rimm E, Boerwinkle E, Tybjaerg-Hansen A, Cupples LA, Reilly MP, Melander O, Mannucci PM, Ardissino D, Siscovick D, Elosua R, Stefansson K, O'Donnell CJ, Salomaa V, Rader DJ, Peltonen L, Schwartz SM, Altshuler D and Kathiresan S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet*. 2012;380:572-580.
10. Karalis I, Rensen PC and Jukema JW. Journey through cholesteryl ester transfer protein inhibition: from bench to bedside. *Circulation Cardiovascular quality and outcomes*. 2013;6:360-6.
11. Barter PJ and Rye KA. Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk. *Journal of lipid research*. 2012;53:1755-66.

12. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL and Tall AR. Increased Atherosclerosis in ApoE and LDL Receptor Gene Knock-Out Mice as a Result of Human Cholesteryl Ester Transfer Protein Transgene Expression. *Arteriosclerosis, thrombosis, and vascular biology*. 1999;19:1105-1110.
13. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
14. Sugano M. Effect of Antisense Oligonucleotides against Cholesteryl Ester Transfer Protein on the Development of Atherosclerosis in Cholesterol-fed Rabbits. *Journal of Biological Chemistry*. 1998;273:5033-5036.
15. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K and Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000;406:203-7.
16. Rittershaus CW, Miller DP, Thomas LJ, Picard MD, Honan CM, Emmett CD, Pettey CL, Adari H, Hammond RA, Beattie DT, Callow AD, Marsh HC and Ryan US. Vaccine-Induced Antibodies Inhibit CETP Activity In Vivo and Reduce Aortic Lesions in a Rabbit Model of Atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2000;20:2106-2112.
17. Huang Z, Inazu A, Nohara A, Higashikata T and Mabuchi H. Cholesteryl ester transfer protein inhibitor (JTT-705) and the development of atherosclerosis in rabbits with severe hypercholesterolaemia. *Clinical science*. 2002;103:587-94.
18. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH and Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *Journal of lipid research*. 2007;48:1263-72.
19. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
20. 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 and Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine*. 2007;357:2109-22.
21. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, Chaitman BR, Holme IM, Kallend D, Leiter LA, Leitersdorf E, McMurray JJ, Mundl H, Nicholls SJ, Shah PK, Tardif JC and Wright RS. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *The New England journal of medicine*. 2012;367:2089-99.
22. Johns DG, Duffy J, Fisher T, Hubbard BK and Forrest MJ. On- and off-target pharmacology of torcetrapib: current understanding and implications for the structure activity relationships (SAR), discovery and development of cholesteryl ester-transfer protein (CETP) inhibitors. *Drugs*. 2012;72:491-507.
23. Bloomfield D, Carlson GL, Sapre A, Tribble D, McKenney JM, Littlejohn TW, 3rd, Sisk CM, Mitchel Y and Pasternak RC. Efficacy and safety of the cholesteryl ester transfer protein inhibitor anacetrapib as monotherapy and coadministered with atorvastatin in dyslipidemic patients. *American heart journal*. 2009;157:352-360 e2.
24. Nicholls SJ, Brewer HB, Kastelein JJ, Krueger KA, Wang MD, Shao M, Hu B, McErlean E and Nissen SE. Effects of the CETP inhibitor evacetrapib administered as monotherapy or in combination with statins on HDL and LDL cholesterol: a randomized controlled trial. *JAMA : the journal of the American Medical Association*. 2011;306:2099-2109.
25. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
26. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.

27. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
28. Kuhnast S, Louwe MC, Heemskerk MM, Pieterman EJ, van Klinken JB, van den Berg SA, Smit JW, Havekes LM, Rensen PC, van der Hoorn JW, Princen HM and Jukema JW. Niacin Reduces Atherosclerosis Development in APOE*3Leiden.CETP Mice Mainly by Reducing NonHDL-Cholesterol. *PloS one*. 2013;8:e66467.
29. Kuhnast S, van der Hoorn JW, van den Hoek AM, Havekes LM, Liau G, Jukema JW and Princen HM. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden. CETP transgenic mice with or without treatment with atorvastatin. *Journal of hypertension*. 2012;30:107-16.
30. Havel RJ, Eder HA and Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *The Journal of clinical investigation*. 1955;34:1345-1353.
31. Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, Chroni A, Yonekawa K, Stein S, Schaefer N, Mueller M, Akhmedov A, Daniil G, Manes C, Templin C, Wyss C, Maier W, Tanner FC, Matter CM, Corti R, Furlong C, Lusis AJ, von Eckardstein A, Fogelman AM, Luscher TF and Landmesser U. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. *The Journal of clinical investigation*. 2011;121:2693-708.
32. Simic B, Hermann M, Shaw SG, Bigler L, Stalder U, Dorries C, Besler C, Luscher TF and Ruschitzka F. Torcetrapib impairs endothelial function in hypertension. *European heart journal*. 2012;33:1615-1624.
33. Menard S. *Applied Logistic Regression Analysis: Sage University Series on Quantitative Applications in the Social Sciences.* Thousand Oaks, CA: Sage; 1995.
34. Besley DA. A guide to using the collinearity diagnostics. *Computer Science in Economics and Management*. 1991;4:33-50.
35. Krishna R, Bergman AJ, Jin B, Fallon M, Cote J, Van Hoydonck P, Laethem T, Gendrano IN, 3rd, Van Dyck K, Hilliard D, Laterza O, Snyder K, Chavez-Eng C, Lutz R, Chen J, Bloomfield DM, De Smet M, Van Bortel LM, Gutierrez M, Al-Huniti N, Dykstra K, Gottesdiener KM and Wagner JA. Multiple-dose pharmacodynamics and pharmacokinetics of anacetrapib, a potent cholesteryl ester transfer protein (CETP) inhibitor, in healthy subjects. *Clinical pharmacology and therapeutics*. 2008;84:679-83.
36. Krishna R, Anderson MS, Bergman AJ, Jin B, Fallon M, Cote J, Rosko K, Chavez-Eng C, Lutz R, Bloomfield DM, Gutierrez M, Doherty J, Bieberdorf F, Chodakewitz J, Gottesdiener KM and Wagner JA. Effect of the cholesteryl ester transfer protein inhibitor, anacetrapib, on lipoproteins in patients with dyslipidaemia and on 24-h ambulatory blood pressure in healthy individuals: two double-blind, randomised placebo-controlled phase I studies. *The Lancet*. 2007;370:1907-1914.
37. Gutstein DE, Krishna R, Johns D, Surks HK, Dansky HM, Shah S, Mitchel YB, Arena J and Wagner JA. Anacetrapib, a novel CETP inhibitor: pursuing a new approach to cardiovascular risk reduction. *Clinical pharmacology and therapeutics*. 2012;91:109-22.
38. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *The New England journal of medicine*. 2013;368:2004-13.
39. Vergeer M, Bots ML, van Leuven SI, Basart DC, Sijbrands EJ, Evans GW, Grobbee DE, Visseren FL, Stalenhoef AF, Stroes ES and Kastelein JJ. Cholesteryl ester transfer protein inhibitor torcetrapib and off-target toxicity: a pooled analysis of the rating atherosclerotic disease change by imaging with a new CETP inhibitor (RADIANCE) trials. *Circulation*. 2008;118:2515-22.
40. Cannon CP, Shah S, Dansky HM, Davidson M, Brinton EA, Gotto AM, Stepanavage M, Liu SX, Gibbons P, Ashraf TB, Zafarino J, Mitchel Y and Barter P. Safety of anacetrapib in patients with or at high risk for coronary heart disease. *The New England journal of medicine*. 2010;363:2406-15.
41. Matsuura F, Wang N, Chen W, Jiang XC and Tall AR. HDL from CETP-deficient subjects shows enhanced ability to promote cholesterol efflux from macrophages in an apoE- and ABCG1-dependent pathway. *The Journal of clinical investigation*. 2006;116:1435-42.

42. Krauss RM, Wojnooski K, Orr J, Geaney JC, Pinto CA, Liu Y, Wagner JA, Luk JM, Johnson-Levonas AO, Anderson MS and Dansky HM. Changes in lipoprotein subfraction concentration and composition in healthy individuals treated with the CETP inhibitor anacetrapib. *Journal of lipid research*. 2012;53:540-7.
43. Rosenson RS, Brewer HB, Jr., Davidson WS, Fayad ZA, Fuster V, Goldstein J, Hellerstein M, Jiang XC, Phillips MC, Rader DJ, Remaley AT, Rothblat GH, Tall AR and Yvan-Charvet L. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. *Circulation*. 2012;125:1905-19.
44. Ishigami M, Yamashita S, Sakai N, Arai T, Hirano K, Hiraoka H, Kameda-Takemura K and Matsuzawa Y. Large and cholesteryl ester-rich high-density lipoproteins in cholesteryl ester transfer protein (CETP) deficiency can not protect macrophages from cholesterol accumulation induced by acetylated low-density lipoproteins. *Journal of Biochemistry*. 1994;116:257-262.
45. Han S, Levoci L, Fischer P, Wang SP, Gagen K, Chen Y, Xie D, Fisher T, Ehrhardt AG, Peier AM and Johns DG. Inhibition of cholesteryl ester transfer protein by anacetrapib does not impair the anti-inflammatory properties of high density lipoprotein. *Biochimica et biophysica acta*. 2013;1831:825-33.
46. Yvan-Charvet L, Kling J, Pagler T, Li H, Hubbard B, Fisher T, Sparrow CP, Taggart AK and Tall AR. Cholesterol efflux potential and antiinflammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:1430-8.
47. Clinical Trials database. A service of the U.S. National Institutes of Health. Available from: http://www.clinicaltrials.gov [Accessed January 9, 2014].

CHAPTER 5

Anacetrapib Reduces (V)LDL-Cholesterol by Inhibition of CETP Activity and Reduction of Plasma PCSK9

Susan Kühnast[1,3,6],*, Sam J.L. van der Tuin[4,6],*, Jimmy F.P. Berbée[4,6], Lars Verschuren[2], Elsbet J. Pieterman[1], Louis M. Havekes[1,4,6], José W.A. van der Hoorn[1,3,6], Patrick C.N. Rensen[4,6], J. Wouter Jukema[3,6], Hans M.G. Princen[1], Ko Willems van Dijk[4,5,6], Yanan Wang[4,5,6]

[1]TNO - Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands
[2]TNO - Microbiology and Systems Biology, Zeist, The Netherlands
[3]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[4]Dept. of Medicine, Div. of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands;
[5]Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands;
[6]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Centre Leiden, The Netherlands;

*S.K. and S.J.L.v.d.T. contributed equally

Submitted

Abstract

Objectives Recently, we showed in APOE*3-Leiden.CETP mice that anacetrapib attenuated atherosclerosis development by reducing (V)LDL-C rather than by raising HDL-C. Here, we investigated the mechanism by which anacetrapib reduces (V)LDL-C and whether this effect was dependent on the inhibition of CETP.

Approach and Results APOE*3-Leiden.CETP mice were fed a Western type diet alone or supplemented with anacetrapib (30 mg/kg bw/d). Microarray analyses of livers revealed down regulation of the cholesterol biosynthesis pathway (P<0.001) and predicted down regulation of sterol regulatory element-binding protein-1 and -2 controlled pathways (z-score -2.56 and z-score -2.90, respectively; both P<0.001). These data suggest increased supply of cholesterol to the liver. We found that hepatic proprotein convertase subtilisin/kexin type 9 *(Pcsk9)* expression was decreased (-28%, P<0.01) accompanied by decreased plasma PCSK9 levels (-47%, P<0.001), and increased hepatic LDL receptor protein content (+64%, P<0.01). Consistent with this, anacetrapib increased the clearance and hepatic uptake (+25%, P<0.001) of [^{14}C]cholesteryl oleate-labeled VLDL-mimicking emulsion particles. In APOE*3-Leiden mice that do not express CETP, anacetrapib still decreased (V)LDL-C and plasma PCSK9 levels, indicating that these effects were independent of CETP inhibition.

Conclusions Anacetrapib reduces (V)LDL-C by two mechanisms: 1) inhibition of CETP activity, resulting in remodelled VLDL particles that are more susceptible to hepatic uptake and 2) a CETP-independent reduction of plasma PCSK9 levels that has the potential to increase LDL receptor-mediated hepatic remnant clearance.

Keywords CETP, Cholesterol/Metabolism, Drug therapy/Hypolipidemic drugs, LDL/Metabolism, Lipids, Lipoproteins/Metabolism, PCSK9

Introduction

High plasma levels of (very) low-density lipoprotein [(V)LDL]-cholesterol (C) and triglycerides (TG), as well as low levels of high-density lipoprotein (HDL)-C are important risk factors for cardiovascular diseases. The standard treatment for the reduction of cardiovascular disease risk is statin therapy aiming to reduce plasma (V)LDL-C. However, a substantial residual risk remains despite of statin treatment. This has prompted the search for secondary treatment targets.[1, 2] Prospective epidemiological studies indicate HDL-C as a potential target.[3] The ratio of plasma (V)LDL-C to HDL-C is to a great extent affected by cholesteryl ester transfer protein (CETP). CETP facilitates the transfer of cholesteryl esters from HDL to (V)LDL in exchange for TG.[4] In several mouse models, including C57Bl/6, *Ldlr*$^{-/-}$ and APOE*3-Leiden (E3L) transgenic mice, CETP expression aggravates the development of atherosclerosis.[5-7] Although human studies have shown conflicting results with regard to the association between CETP-deficiency and decreased cardiovascular disease risk,[8, 9] CETP inhibition is actively pursued as a potential strategy to reduce this risk.[10] This has led to the development of pharmacological CETP inhibitors, such as torcetrapib, dalcetrapib, anacetrapib and evacetrapib.

In clinical trials, torcetrapib, anacetrapib and evacetrapib have been shown to increase HDL-C (up to +72%[11];+139%[12]; +129%,[13] respectively) and to reduce LDL-C (down to -25%[11]; -40%[12]; -36%,[13] respectively); whereas dalcetrapib only increased HDL-C (up to +40%).[14] Although torcetrapib showed favourable effects on the lipoprotein profile, it failed in phase III clinical development due to increased risk of major cardiovascular events and mortality. These adverse effects were ascribed to an off-target effect[11] and pro-inflammatory lesions.[15] A large phase III clinical trial with dalcetrapib was prematurely terminated, due to a lack of clinical benefit.[14] Nonetheless, the effects of anacetrapib and evacetrapib on cardiovascular outcomes are currently being evaluated in phase III clinical trials.[16] Neither compound shows increased blood pressure as observed with torcetrapib[12, 13] and both compounds are more potent in increasing HDL-C and reducing LDL-C as compared to torcetrapib and dalcetrapib.

Recently, we have shown that anacetrapib treatment increased HDL-C and reduced (V) LDL-C and TG, and dose-dependently reduced atherosclerotic lesion size and severity in APOE*3-Leiden.CETP (E3L.CETP) mice, a well-established mouse model for hyperlipidemia and atherosclerosis with a human-like lipoprotein metabolism.[17-19] Analysis of covariance showed that the effect on lesion size was mainly explained by a reduction in (V)LDL-C.[20] However, the mechanism by which anacetrapib reduces plasma (V)LDL-C and TG is not fully understood. To elucidate this, we performed microarrays on the livers from this latter study, identifying pathways affected by anacetrapib. To confirm physiological consequences of these identified pathways, we performed a VLDL production experiment and studied the clearance of VLDL-mimicking emulsion particles. By using E3L mice with or without CETP expression,[7] we also determined whether these effects of anacetrapib were CETP-dependent.

Material and Methods

RNA isolation, microarray and qPCR validation

Liver pieces were obtained from a previous experiment performed by Kühnast and Van der Tuin *et al.*,[20] investigating the effects of anacetrapib on atherosclerosis in female E3L.CETP mice. In this study, mice were treated with a semi-synthetic cholesterol-rich diet, containing 15% (w/w) cacao butter, 1% corn oil and 0.1% cholesterol (Western-type diet; AB-Diets, Woerden, the Netherlands) with or without anacetrapib (30 mg/kg bw/d) for 21 weeks. Total RNA was extracted from these liver pieces using the Nucleospin RNAII kit (Macherey-Nagel) according to manufacturer's protocol. The microarray, including quality control, RNA labelling, hybridization and data extraction was performed by ServiceXS B.V. (Leiden, The Netherlands).

To perform real time quantitative PCR (qPCR) for validation, RNA quality was verified by the lab-on-a-chip method using Experion™ RNA StdSens analyses kit (Bio-Rad). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and qPCR was performed using a CFX96™ Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized to Beta-2 microglobulin and hypoxanthine-guanine phosphoribosyltransferase. Relative expression was calculated as compared to the control group using Bio-Rad CFX Manager™ software 3.0 (Bio-Rad).

Microarray data analyses

The probe-level background subtracted expression values were used as input for lumi package21 of the R/Bioconductor (http://www.bioconductor.org; http://www.r-project.org) to perform quality control and a quantile normalization. Unexpressed probes ($p>0.01$ in all experiments) were removed from further analyses. Differentially expressed probes were identified using the limma package of R/Bioconductor.22 The calculated P-values <0.05 were used as a threshold for pathway analyses using Ingenuity Pathway Analysis suite (www.ingenuity.com, accessed 2013). Upstream regulator analysis was performed using the Ingenuity Pathway Analysis software. This analysis determines the activation state of transcription factors based on the observed differential gene expression and results in an overlap p-value and activation z-score for each transcription factor in the Ingenuity Pathway Analysis knowledgebase. The overlap p-value indicates the significance of the overlap between the known target genes of a transcription factor and the differentially expressed genes measured in an experiment. The activation z-score indicates activation (positive z-score) or inhibition (negative z-score) of a particular transcription factor. An activation z-score >2 or <2 indicates significant activation or inhibition of a pathway or process.

Experimental set-up

To investigate the effects of anacetrapib on VLDL production and clearance, female E3L[23] and E3L.CETP[7] transgenic mice, 8-10 weeks of age, were fed a Western-type diet for a run-in period of 3-4 weeks. They were then matched based on plasma total cholesterol (TC), HDL-C, TG, body weight and age into two groups receiving either no treatment (control) or anacetrapib (30 mg/kg bw/d) for four weeks after which VLDL production (E3L.CETP only) or clearance (E3L and E3L.CETP) was determined. After both experiments the mice were sacrificed by CO_2 asphyxiation. The mice were housed under standard conditions with a 12 hour light-dark cycle and had free access to food and water during the experiment. Body weight and food intake were monitored during the study. The Institutional Ethics Committee for Animal Procedures from the Leiden University Medical Center, Leiden, The Netherlands, approved the protocol.

Plasma lipid measurements

Blood was collected after a 4 hour fasting period in heparin-coated capillaries via tail vein bleeding and plasma was isolated. TC and TG were determined using enzymatic kits (Roche) according to manufacturer's protocol. To measure HDL-C, apoB-containing particles were precipitated from plasma with 20% polyethylene glycol 6000 (Sigma Aldrich) in 200 mM glycine buffer (pH 10) and HDL-C was measured in the supernatant.[20]

Hepatic LDLr protein and plasma PCSK9 measurements

Snap-frozen mouse livers were lysed in ice-cold lysis buffer containing 50 mM Hepes (pH 7.6), 50 mM NaF, 50 mM KCl, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM β-glycerophosphate, 1 mM sodium vanadate, 1% NP40 and protease inhibitors cocktail (Roche). Thereafter, protein level was determined using the BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Laemmli buffer (Sigma-Aldrich) was added to samples containing equal amounts of protein. Samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride. Blots were incubated with goat-anti-mouse-LDL receptor (1:1000, AF2255, R&D Systems) and mouse-anti-α-Tubulin (1:1000, T5168, Sigma-Aldrich) and subsequently incubated with the appropriate secondary antibody. Bands were visualized by enhanced chemiluminescence with Pierce ECL 2 substrate following manufacturer's protocol and quantified using Image J software as previously described.[24] Plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) was measured by using ELISA (MCP900, R&D Systems) according to manufacturer's instructions.

Hepatic VLDL-TG and VLDL-apoB production analyses

Mice (n= 8/9 per group) were anesthetized with 6.25 mg/kg Acepromazine (Alfasan), 6.25 mg/kg Midazolam (Roche) and 0.31 mg/kg Fentanyl (Janssen-Cilag) after a 4-hour fast. A basal blood sample was taken from the tail and the mice received an intravenous injection of 100 µl PBS containing 100 µCi Tran^{35}S label (MP Biomedicals) via the tail vein. After 30 min, animals received an intravenous injection of Tyloxapol (Triton WR-1339, Sigma-Aldrich; 500 mg/kg body weight), as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG.[25] At indicated time points up to 90 min after Tyloxapol injection, blood was taken and plasma TG concentration was determined. After 120 min, mice were sacrificed and blood was collected for isolation of the VLDL fraction by density gradient ultracentrifugation.[26] Tran^{35}S-activity was measured in the VLDL fraction and VLDL-apoB production rate was calculated as dpm/h.[27]

Clearance analysis of VLDL-mimicking emulsion particles

Glycerol tri(9,10(n)[^{3}H]oleate ([^{3}H]TO) and [1α,2α (n)-^{14}C]cholesteryl oleate ([^{14}C]CO) double-radiolabeled VLDL-mimicking emulsion particles (mean diameter 80 nm) were prepared as previously described.[28] After a 4 hour fast, particles were injected via the tail vein in conscious mice (n= 8/9 per group). At 2, 5, 10, and 15 min post-injection, blood was taken to determine the plasma decay of [^{3}H]TO and [^{14}C]CO. Plasma volumes were calculated as 0.04706 x body weight (g) as described.[29] Mice were sacrificed after 15 min, perfused with ice-cold PBS with 0.1% heparin (v/v), and organs were harvested to determine tissue specific [^{3}H]TO and [^{14}C]CO uptake. Subsequently, organs were dissolved overnight at 56°C in Tissue Solubilizer (Amersham Biosciences), and quantified for ^{3}H and ^{14}C activity. Uptake of [^{3}H]TO- and [^{14}C]CO-derived radioactivity by the organs was calculated as dose per organ after correction for organ weight.

Statistical analysis

Significance of differences between the groups was calculated non-parametrically using a Mann-Whitney U test. All reported p-values are two-tailed, and p-values of less than 0.05 were considered statistically significant.

Results

Pathway analyses predict down regulation of sterol regulatory element-binding protein-1 and -2 controlled pathways by anacetrapib.

To determine the effects of anacetrapib treatment on hepatic gene expression in E3L.CETP mice,[20] microarray analyses were performed. A total of 95 genes (FDR P<0.05; Supplemental Table I) were differentially expressed between control and anacetrapib-treated female mice of which 46 genes were up regulated and 49 genes were down regulated. To gain insight into affected biological processes, a gene-set enrichment analysis was performed using the Ingenuity Pathway Analysis suite (as described in material and methods). This analysis showed that the cholesterol biosynthesis pathway was significantly affected (**Table 1**). *In silico* prediction of transcription factor activity (**Table 2**), based on the differentially expressed genes, predicted inhibition of genes regulated by sterol regulatory element-binding protein (SREBP) 1 (P<0.001; z-score -2.90) and SREBP-2 (P<0.001; z-score -2.56), which are key regulators of cholesterol synthesis. Furthermore, anacetrapib activated genes regulated by nuclear receptor subfamily 1, group I, member 2 (P<0.001; z-score +2.75) and member 3 (P<0.001; z-score +2.94). Both nuclear receptors function as sensors of endobiotic and xenobiotic substances. These data indicate that anacetrapib reduces cholesterol biosynthesis and activates a xenobiotic response.

Table 1: Significantly regulated pathways.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Superpathway of Cholesterol Biosynthesis	10.50	0.24
PXR/RXR Activation	6.32	0.09
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	6.00	0.22
Bupropion Degradation	5.39	0.16
LPS/IL-1 Mediated Inhibition of RXR Function	5.28	0.04
Cholesterol Biosynthesis I	4.65	0.23
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	4.65	0.23
Cholesterol Biosynthesis III (via Desmosterol)	4.65	0.23
Isoleucine Degradation I	4.54	0.21
Mevalonate Pathway I	4.54	0.21

Female E3L.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg bw/d) for 22 weeks. RNA was isolated from liver tissue and a microarray analysis was performed. Selected differentially expressed genes (95 genes, see Table I) were used as input for pathway analysis through ingenuity pathway analysis suite.

Table 2: *In silico* prediction of transcription factor activity based on the expression changes of known target genes

Upstream Regulator	Molecule Type	Activation State	Z-score	p-value of overlap
Sterol regulatory element-binding protein 2 (SREBP2)	transcription regulator	Inhibited	-2.56	1.65E-13
Sterol regulatory element-binding protein 1 (SREBP1)	transcription regulator	Inhibited	-2.90	6.56E-12
Nuclear receptor subfamily 1, group I, member 1 (NR1I3)	ligand-dependent nuclear receptor	Activated	2.94	9.27E-12
Peroxisome proliferator-activated receptor alpha (PPARA)	ligand-dependent nuclear receptor	-	-0.24	6.74E-10
Nuclear receptor subfamily 1, group I, member 2 (NR1I2)	ligand-dependent nuclear receptor	Activated	2.75	6.01E-09
PXR ligand-PXR-Retinoic acid-RXRα	complex	Activated	2.42	1.34E-08
CAR ligand-CAR-Retinoic acid-RXRα	complex	Activated	2.22	1.71E-08
Ncoa-Nr1i3-Rxra	complex	Activated	2.00	6.83E-07
NAD-dependent deacetylase sirtuin-2 (SIRT2)	transcription regulator	Inhibited	-2.00	6.83E-07
Ncoa-Nr1i2-Rxra	complex	Activated	2.00	8.63E-07

To determine the activation status of transcription factors an upstream regulator analysis was performed. The z-score indicates activation (positive) or inhibition (negative).

Anacetrapib decreases hepatic and circulating PCSK9, and increases hepatic LDLr protein in E3L.CETP mice

In addition to effects on cholesterol biosynthesis and xenobiotic metabolism, microarray analyses showed a decrease in the expression of *Pcsk9* mRNA, a downstream target of the SREBP-2 pathway (Supplemental Figure I),[30] in the liver of anacetrapib-treated E3L.CETP mice (-78%, P<0.05; Supplemental Table I), which was confirmed by qPCR (-27%, P<0.01; **Figure 1A**). In accordance, anacetrapib reduced plasma PCSK9 levels (-47%, P<0.01; **Figure 1B**). Since PCSK9 plays an important role in the degradation of intracellular LDL receptor (LDLr),[31-33] hepatic LDLr mRNA expression and protein levels were measured. Anacetrapib did not affect the hepatic *Ldlr* expression (**Figure 1C**), but did increase hepatic LDLr protein levels (+64%, P<0.05; **Figure 1D**). The decrease in plasma PCSK9 levels and increase in LDLr suggest an increased capability of the liver to take up lipoprotein remnants.

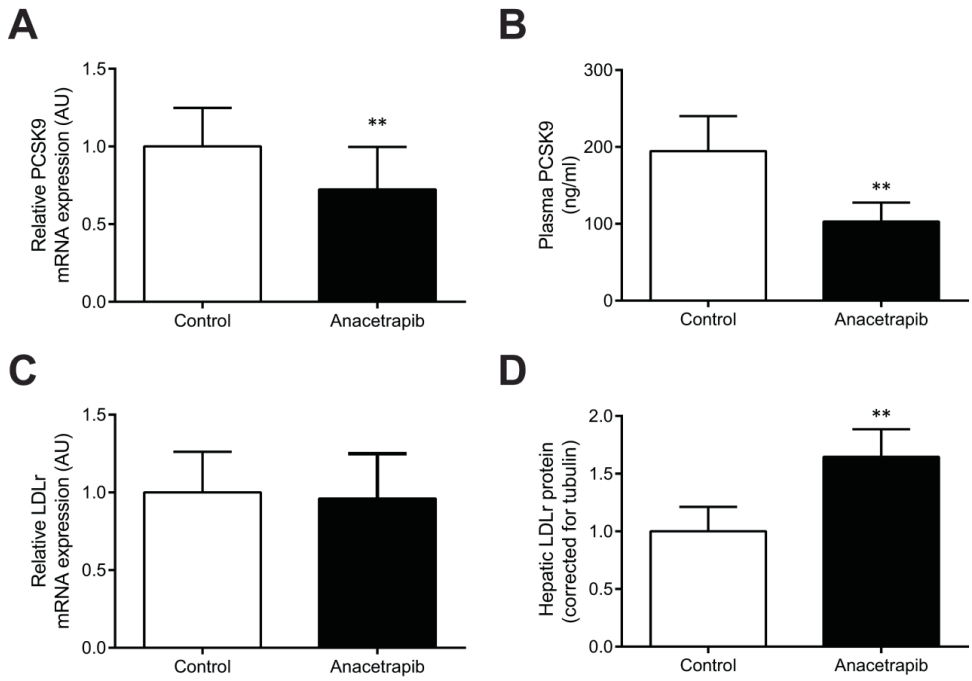


Figure 1. Anacetrapib decreases PCSK9 mRNA expression and plasma levels and increases hepatic LDLr protein levels in E3L.CETP mice.

Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 21 weeks, blood was collected for plasma PCSK9 levels and livers for mRNA expression. Hepatic PCSK9 mRNA expression (A) and plasma levels (B). Hepatic LDLr mRNA (C) and protein (D) levels.

Data are presented as means ± SD (n= 14/15 per group). * P<0.05, ** P<0.01, ***P<0.001 when compared with control group.

Anacetrapib does not affect triglyceride metabolism, but increases lipoprotein remnant clearance by the liver in E3L.CETP mice

To further investigate the effects of anacetrapib on lipoprotein metabolism, we performed a new experiment with female E3L.CETP mice fed a Western-type diet with or without anacetrapib for 4 weeks. Plasma lipid and lipoprotein levels were decreased to the same extent as shown in **Figure 2A** (data not shown). Anacetrapib treatment did not affect the VLDL-TG production rate (**Figure 2B**), the VLDL-^{35}S-apoB production rate (**Figure 2C**) nor the ratio of VLDL-TG to VLDL-apoB production rate (**Figure 2D**), indicating no changes in number or composition of newly synthesized VLDL particles.

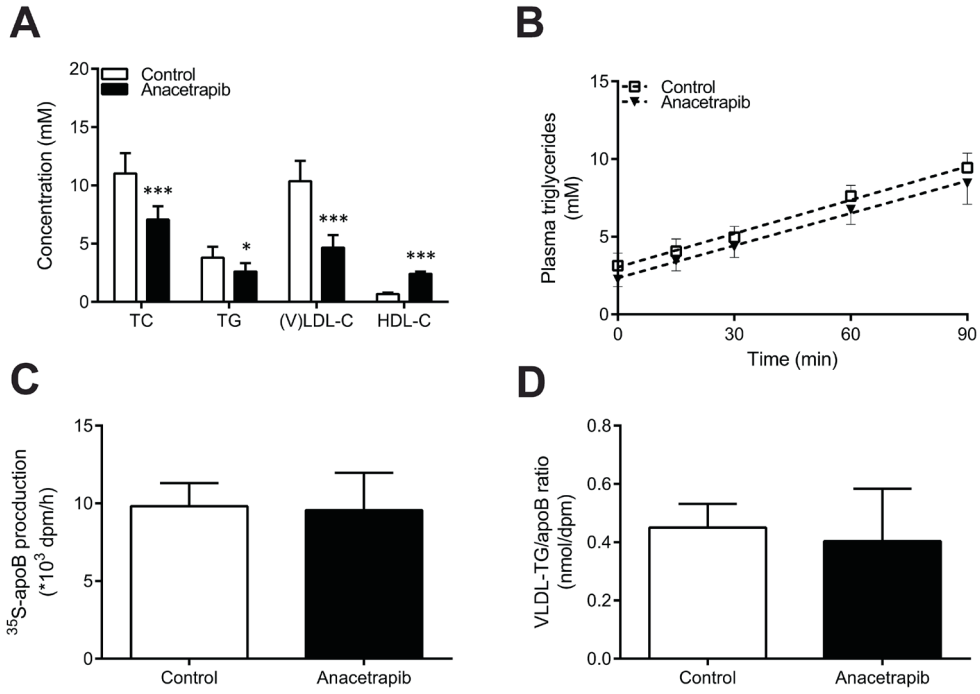


Figure 2. Anacetrapib does not affect hepatic VLDL-TG production in E3L.CETP mice.
Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks, blood was collected by tail bleeding after 4h fasting, and plasma TC, TG, (V)LDL-C and HDL-C were determined (A). After treatment, hepatic VLDL production was assessed (B). ^{35}S-apoB production was determined by scintillation counting of the isolated VLDL fraction (C) and the VLDL-TG production rate to VLDL-apoB production rate ratio was calculated (D).
Data are presented as means ± SD (n= 8/9 per group). * P<0.05, ***P<0.001 when compared with control group.

The effect of anacetrapib on VLDL clearance was assessed by an intravenous injection of glycerol tri(9,10(n)[^{3}H]oleate ([^{3}H]TO) and [1α,2α (n)-^{14}C]cholesteryl oleate ([^{14}C]CO) double-radiolabeled VLDL-mimicking emulsion particles. At indicated time points, blood was taken to determine clearance from plasma. After 15 min, mice were sacrificed and organs were harvested to determine tissue specific uptake of radioactivity-derived from [^{3}H]TO and [^{14}C] CO. We observed no effects on the plasma clearance (**Figure 3A**) or the tissue specific uptake of [^{3}H]TO-derived activity (**Figure 3B**). However, anacetrapib increased the plasma clearance of the [^{14}C]CO label of the VLDL-mimicking emulsion particles (**Figure 3C**), decreased plasma half-life of [^{14}C]CO (-56%, P<0.001; **Figure 3C** inlay) and increased the uptake of [^{14}C]CO by the liver (+25%, P<0.001; **Figure 3D**). Since these particles reflect the behaviour of VLDL,[34] these results indicate that anacetrapib increases the uptake of lipoprotein remnants by the liver.

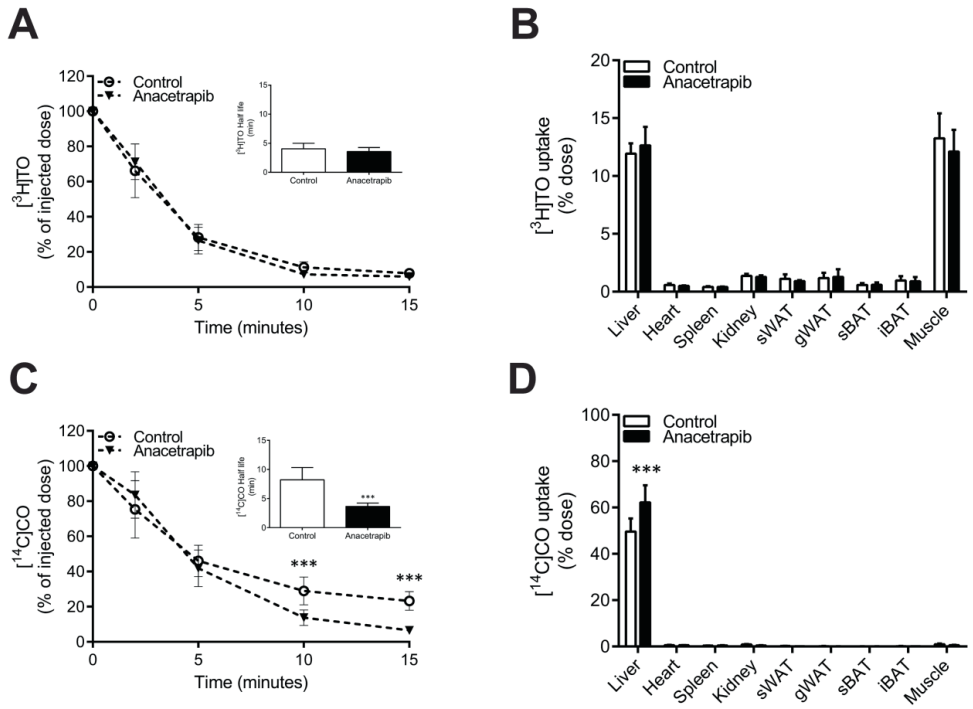


Figure 3. Anacetrapib increases lipoprotein remnant clearance by the liver in E3L.CETP mice.
Female E3L.CETP mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks. Mice received an injection with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-double labelled VLDL-mimicking emulsion particles. Blood was drawn at the indicated time points and ^{3}H and ^{14}C plasma decay (A and C) and tissue specific activity (B and D) were determined. The inlay in figure A and C show plasma half-life.
Data are presented as means ± SD (n= 8/9 per group). ***P<0.001 when compared with control group.

Anacetrapib decreases (V)LDL-C and PCSK9 levels in E3L mice

To determine whether the effects of anacetrapib on (V)LDL metabolism were dependent on CETP inhibition, similar experiments were performed in female E3L mice that do not express CETP. Notably, anacetrapib reduced TC (-17%, $P<0.05$) and (V)LDL-C (-20%, $P<0.05$; **Figure 4A**) levels in E3L mice without CETP, concomitantly with a decrease in hepatic *Pcsk9* expression and plasma PCSK9 levels (-37%, $P<0.05$; **Figure 4B**). These data clearly show that anacetrapib has a CETP-independent lipid-lowering effect. Comparing E3L with E3L.CETP mice, anacetrapib increased the particle clearance in E3L.CETP mice to the similar level as observed in E3L mice without anacetrapib (**Figure 3C and 4C**). Anacetrapib showed no additional effects on the plasma clearance of [^{3}H]TO and [^{14}C]CO (**Figure 4C and 4E**) labels of the VLDL-mimicking emulsion particles, nor the tissue specific uptake of [^{3}H]TO and [^{14}C]CO (**Figure 4D and 4F**) in E3L mice.

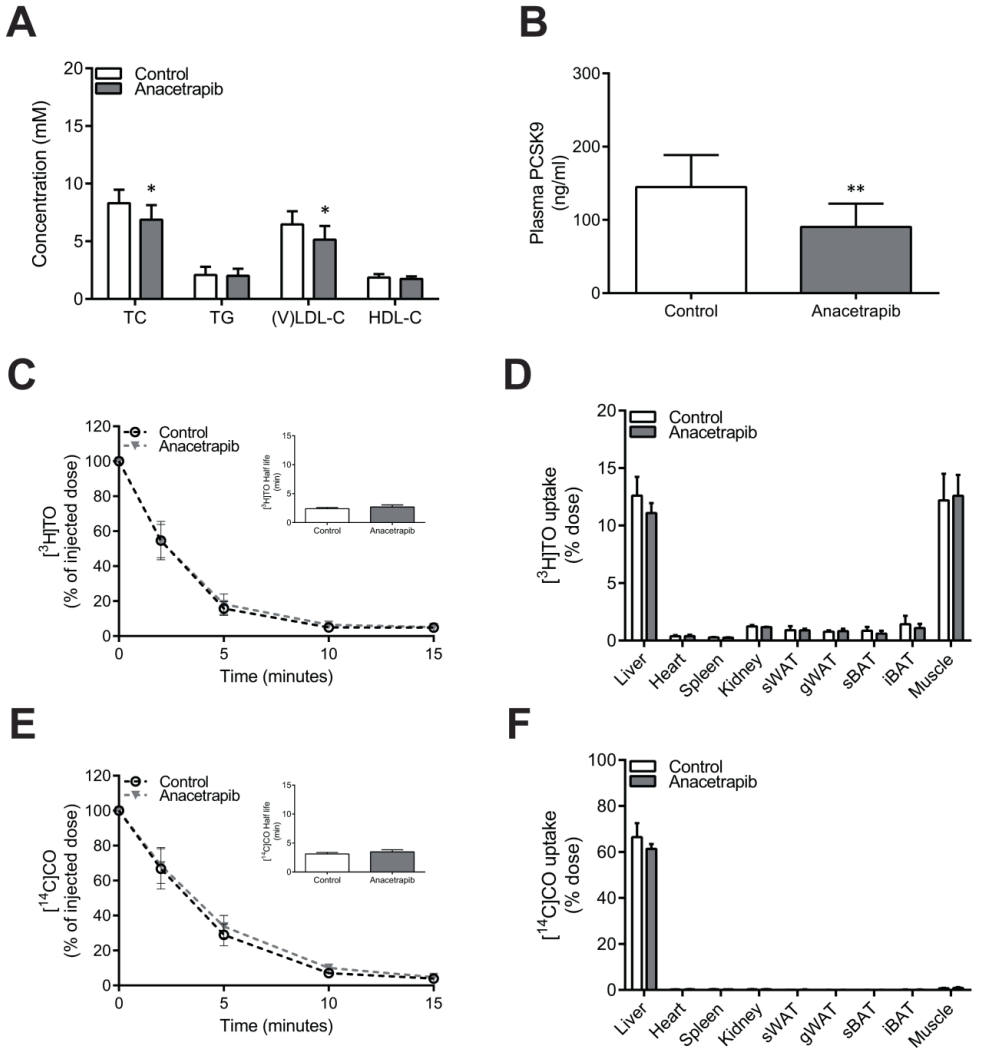


Figure 4. Anacetrapib decreases (V)LDL-C and plasma PCSK9 levels, without affecting lipoprotein remnant clearance in E3L mice.

Female E3L mice were fed a WTD with or without anacetrapib (30 mg/kg bw/d) for 4 weeks, blood was collected by tail bleeding after 4h fasting, and plasma TC, TG, (V)LDL-C, HDL-C (A) and plasma PCSK9 levels (B) were determined. After treatment mice received an injection with glycerol tri[^{3}H]oleate- and [^{14}C]cholesteryl oleate-double labelled VLDL-mimicking emulsion particles. Blood was drawn at the indicated time points and ^{3}H and ^{14}C plasma decay (C and E) and tissue specific activity (D and F) were determined. The inlay in figure A and E show plasma half-life.

Data are presented as means ± SD (n= 8/9 per group). *P<0.05 when compared with control group.

Discussion

In this study, we investigated the mechanism by which anacetrapib reduces plasma (V) LDL-C and whether these effects are dependent on CETP. In E3L.CETP mice, anacetrapib decreased gene expression of cholesterol biosynthesis pathways in the liver, most probably via inhibition of *Srebp-1* and/or *Srebp-2* signalling. In addition, we identified two important processes by which anacetrapib increases cholesterol clearance. First, anacetrapib increased cholesterol clearance by the liver, without affecting VLDL-TG production rate and clearance in E3L.CETP mice. Secondly, in a CETP-independent manner, anacetrapib decreased hepatic *Pcsk9* expression and plasma PCSK9 levels. In E3L mice that do not express CETP, anacetrapib decreased (V)LDL-C and plasma PCSK9 levels. However, no effects on cholesterol or VLDL-TG clearance were detected. These results indicate that CETP inhibition results in remodelled particles that are more susceptible for hepatic clearance.

The observed reduction in plasma PCSK9 levels after anacetrapib treatment is in accordance with recent findings in rhesus macaques.[35] Here, we demonstrate that this effect is independent of CETP inhibition as this was also observed in E3L mice without CETP. A recent study in C57Bl/6 mice also confirmed a CETP-independent decrease in plasma PCSK9 levels by anacetrapib.[36] Pathway analyses of the gene expression data predicted that anacetrapib decreases liver cholesterol synthesis by reducing SREBP-2 regulated pathways. It is known that SREBP-2 is the principal nuclear transcription factor for the regulation of hepatic *Pcsk9* expression.[30] Therefore, the reduction of plasma PCSK9 levels by anacetrapib may be attributed to the reduction of SREBP-2 pathway.

Accumulating evidence shows that inhibiting PCSK9 is an effective strategy to reduce LDL-C both in preclinical and clinical studies.[37, 38] This effect is attributed to a reduction of hepatic LDLr degradation and a subsequent increase of LDL remnant clearance. Our results showed an increase in hepatic LDLr protein levels in E3L.CETP mice after 21 weeks of anacetrapib in parallel with a decrease in plasma PCSK9 levels. Partially in contrast, we found that after 4 weeks of anacetrapib treatment in E3L mice with or without CETP expression, anacetrapib did not change hepatic LDLr protein levels (data not shown) despite a clear reduction of plasma PCSK9 levels (Figure 4B). This discrepancy of anacetrapib affecting plasma PCSK9 levels but not hepatic LDLr protein levels might be due to the duration of the treatment i.e. 4 weeks versus 21 weeks of treatment. Indeed, in the study in C57Bl/6 mice receiving anacetrapib for 1 week, anacetrapib reduced both plasma PCSK9 and hepatic LDLr protein levels,[36] suggesting a treatment time-dependent effect of anacetrapib on PCSK9 and LDLr. Although the effects of anacetrapib in E3L.CETP mice closely resemble the effects in humans,[12, 20] the effects of anacetrapib on plasma PCSK9 and hepatic LDLr protein levels in humans remain to be determined.

The comparison of the clearance rates of VLDL remnants in E3L mice with and without CETP (Figures 3C and 4C) indicates that the presence of CETP results in a decreased remnant particle clearance. This implies that inhibiting the activity of CETP alone is sufficient to increase lipoprotein remnant clearance in E3L.CETP mice. The main activity of CETP is to transfer cholesteryl esters from HDL to (V)LDL in exchange for TG. Apparently, this lipoprotein remodelling activity of CETP renders the (V)LDL less susceptible to clearance. In E3L.CETP mice, anacetrapib treatment results in an increase of plasma apolipoprotein E levels (+59%, P<0.001, data not shown), which is indicative for lipoprotein remodelling. Interestingly, anacetrapib treatment in humans has also been shown to increase plasma apolipoprotein E levels.[12]

Although the direct effects of CETP inhibition on lipoprotein remnant clearance in humans have not been described, treatment with the CETP-inhibitor torcetrapib has been shown to increase the fractional catabolic rate of both VLDL-apoE[39] and VLDL-apoB100.[40] However, the potentially increased catabolism of (V)LDL and thus anti-atherogenic properties were clearly not sufficient to offset or overrule the adverse side effects of torcetrapib.

Microarray analyses also revealed that anacetrapib activates genes regulated by the nuclear receptor subfamily 1, group I (NR1I), members 2 and 3. These transcription factors function as sensors of both toxic and xenobiotic exogenous compounds and toxic products derived from endogenous metabolism, and activate pathways to eliminate these products.[41] In our study, activation of these receptors indicated that anacetrapib was recognized as a xenobiotic product by the liver. It is not yet known whether this has consequences beyond activation of elimination pathways and whether this will have clinical implications. Phase I/II clinical studies evaluating the effects of anacetrapib reported an acceptable side-effect profile.[12, 42-44] However, it should be noted that a non-significant increase in C-reactive protein was found after anacetrapib treatment.[42] We also found elevated SAA levels in anacetrapib-treated E3L.CETP mice.[20]

In the present study, we present evidence that anacetrapib reduces (V)LDL-C by two mechanisms: 1) inhibition of CETP activity, resulting in remodelled lipoproteins that are more susceptible to clearance and 2) a CETP-independent reduction of plasma PCSK9 levels that has the potential to increase LDLr-mediated clearance. This reduction in (V)LDL-C is the crucial factor mediating the atheroprotective effects of anacetrapib in E3L.CETP mice.[20] Whether the additional beneficial effects of anacetrapib on top of a statin translate into clinical benefit in humans will be elucidated in the current phase III REVEAL trial.[16]

Acknowledgements
We thank Trea Streefland, Sander Kooijman and Hetty Sips (Dept. Medicine, Div. Endocrinology, LUMC) for their excellent technical assistance.

Sources of Funding
This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project PREDICCt (grant 01C-104) to KWvD. PCNR is an Established Investigator of the Dutch Heart Foundation (2009T038).

Disclosures
JWJ received research grants from and was speaker on (CME accredited) meetings sponsored by Amgen, Astellas, Astra-Zeneca, Biotronik, Boston Scientific, Daiichi Sankyo, Lilly, Genzyme, Medtronic, Merck-Schering-Plough, Pfizer, Orbus Neich, Novartis, Roche, Servier, Sanofi Aventis, the Netherlands Heart Foundation, the Interuniversity Cardiology Institute of the Netherlands and the European Community Framework KP7 Programme.
PCNR received a grant from MSD and consults Astellas.

References

1. Cholesterol Treatment Trialists C, Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J and Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet*. 2010;376:1670-81.
2. Davidson MH, Maki KC, Pearson TA, Pasternak RC, Deedwania PC, McKenney JM, Fonarow GC, Maron DJ, Ansell BJ, Clark LT and Ballantyne CM. Results of the National Cholesterol Education (NCEP) Program Evaluation ProjecT Utilizing Novel E-Technology (NEPTUNE) II survey and implications for treatment under the recent NCEP Writing Group recommendations. *Am J Cardiol*. 2005;96:556-63.
3. Emerging Risk Factors C, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA*. 2009;302:1993-2000.
4. Tall AR. Plasma cholesteryl ester transfer protein. *J Lipid Res*. 1993;34:1255-74.
5. Marotti KR, Castle CK, Boyle TP, Lin AH, Murray RW and Melchior GW. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature*. 1993;364:73-5.
6. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL and Tall AR. Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler Thromb Vasc Biol*. 1999;19:1105-10.
7. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler Thromb Vasc Biol*. 2006;26:2552-9.
8. de Grooth GJ, Zerba KE, Huang SP, Tsuchihashi Z, Kirchgessner T, Belder R, Vishnupad P, Hu B, Klerkx AH, Zwinderman AH, Jukema JW, Sacks FM, Kastelein JJ and Kuivenhoven JA. The cholesteryl ester transfer protein (CETP) TaqIB polymorphism in the cholesterol and recurrent events study: no interaction with the response to pravastatin therapy and no effects on cardiovascular outcome: a prospective analysis of the CETP TaqIB polymorphism on cardiovascular outcome and interaction with cholesterol-lowering therapy. *J Am Coll Cardiol*. 2004;43:854-7.
9. Ridker PM, Pare G, Parker AN, Zee RY, Miletich JP and Chasman DI. Polymorphism in the CETP gene region, HDL cholesterol, and risk of future myocardial infarction: Genomewide analysis among 18 245 initially healthy women from the Women's Genome Health Study. *Circ Cardiovasc Genet*. 2009;2:26-33.
10. Karalis I, Rensen PC and Jukema JW. Journey through cholesteryl ester transfer protein inhibition: from bench to bedside. *Circ Cardiovasc Qual Outcomes*. 2013;6:360-6.
11. 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 and Brewer B. Effects of torcetrapib in patients at high risk for coronary events. The New England journal of medicine. 2007;357:2109-22.
12. Bloomfield D, Carlson GL, Sapre A, Tribble D, McKenney JM, Littlejohn TW, 3rd, Sisk CM, Mitchel Y and Pasternak RC. Efficacy and safety of the cholesteryl ester transfer protein inhibitor anacetrapib as monotherapy and coadministered with atorvastatin in dyslipidemic patients. *Am Heart J*. 2009;157:352-360 e2.
13. Nicholls SJ, Brewer HB, Kastelein JJ, Krueger KA, Wang MD, Shao M, Hu B, McErlean E and Nissen SE. Effects of the CETP inhibitor evacetrapib administered as monotherapy or in combination with statins on HDL and LDL cholesterol: a randomized controlled trial. *JAMA*. 2011;306:2099-109.
14. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, Chaitman BR, Holme IM, Kallend D, Leiter LA, Leitersdorf E, McMurray JJ, Mundl H, Nicholls SJ, Shah PK, Tardif JC and Wright RS. Effects of dalcetrapib in patients with a recent acute coronary syndrome. The New England journal of medicine. 2012;367:2089-99.

15. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
16. Clinical trial database. A service of the U.S. National Institutes of Health. *Available from: http://wwwclinicaltrialsgov* Accessed September 29, 2014.
17. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
18. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *J Lipid Res*. 2007;48:1763-71.
19. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arterioscler Thromb Vasc Biol*. 2008;28:2016-22.
20. Kuhnast S, van der Tuin SJ, van der Hoorn JW, van Klinken JB, Simic B, Pieterman E, Havekes LM, Landmesser U, Luscher TF, Willems van Dijk K, Rensen PC, Jukema JW and Princen HM. Anacetrapib reduces progression of atherosclerosis, mainly by reducing non-HDL-cholesterol, improves lesion stability and adds to the beneficial effects of atorvastatin. *Eur Heart J*. 2014.
21. Du P, Kibbe WA and Lin SM. lumi: a pipeline for processing Illumina microarray. *Bioinformatics*. 2008;24:1547-8.
22. Wettenhall JM and Smyth GK. limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics*. 2004;20:3705-6.
23. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM and Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol Chem*. 1993;268:10540-5.
24. Kuhnast S, van der Hoorn JW, Pieterman EJ, van den Hoek AM, Sasiela WJ, Gusarova V, Peyman A, Schafer HL, Schwahn U, Jukema JW and Princen HM. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. *J Lipid Res*. 2014;55:2103-12.
25. Aalto-Setala K, Fisher EA, Chen X, Chajek-Shaul T, Hayek T, Zechner R, Walsh A, Ramakrishnan R, Ginsberg HN and Breslow JL. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. *J Clin Invest*. 1992;90:1889-900.
26. Redgrave TG, Roberts DC and West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem*. 1975;65:42-9.
27. Li X, Catalina F, Grundy SM and Patel S. Method to measure apolipoprotein B-48 and B-100 secretion rates in an individual mouse: evidence for a very rapid turnover of VLDL and preferential removal of B-48- relative to B-100-containing lipoproteins. *J Lipid Res*. 1996;37:210-20.
28. Rensen PC, van Dijk MC, Havenaar EC, Bijsterbosch MK, Kruijt JK and van Berkel TJ. Selective liver targeting of antivirals by recombinant chylomicrons--a new therapeutic approach to hepatitis B. *Nat Med*. 1995;1:221-5.
29. Jong MC, Rensen PC, Dahlmans VE, van der Boom H, van Berkel TJ and Havekes LM. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. *J Lipid Res*. 2001;42:1578-85.
30. Jeong HJ, Lee HS, Kim KS, Kim YK, Yoon D and Park SW. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J Lipid Res*. 2008;49:399-409.
31. Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, Asselin MC, Hamelin J, Varret M, Allard D, Trillard M, Abifadel M, Tebon A, Attie AD, Rader DJ, Boileau C, Brissette L, Chretien M, Prat A and Seidah NG. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J Biol Chem*. 2004;279:48865-75.

32. Park SW, Moon YA and Horton JD. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J Biol Chem*. 2004;279:50630-8.
33. Maxwell KN and Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc Natl Acad Sci U S A*. 2004;101:7100-5.
34. Rensen PC, Herijgers N, Netscher MH, Meskers SC, van Eck M and van Berkel TJ. Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. *J Lipid Res*. 1997;38:1070-84.
35. Roddy TP, McLaren DG, Chen Y, Xie D, Dunn K, Kulick A, Szeto D, Forrest G, Albanese K, Donnelly M, Gai C, Gewain A, Lederman H, Jensen KK, Ai X, Vachal P, Akinsanya KO, Cleary MA, Previs SF, Dansky HM and Johns DG. Effects of anacetrapib on plasma lipids, apolipoproteins and PCSK9 in healthy, lean rhesus macaques. *Eur J Pharmacol*. 2014;740:410-6.
36. Dong B, Singh AB, Fung C, Kan K and Liu J. CETP inhibitors downregulate hepatic LDL receptor and PCSK9 expression in vitro and in vivo through a SREBP2 dependent mechanism. *Atherosclerosis*. 2014;235:449-62.
37. Norata GD, Tibolla G and Catapano AL. Targeting PCSK9 for hypercholesterolemia. *Annual review of pharmacology and toxicology*. 2014;54:273-93.
38. Stein EA and Raal F. Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of PCSK9. *Annual review of medicine*. 2014;65:417-31.
39. Millar JS, Brousseau ME, Diffenderfer MR, Barrett PH, Welty FK, Cohn JS, Wilson A, Wolfe ML, Nartsupha C, Schaefer PM, Digenio AG, Mancuso JP, Dolnikowski GG, Schaefer EJ and Rader DJ. Effects of the cholesteryl ester transfer protein inhibitor torcetrapib on VLDL apolipoprotein E metabolism. *J Lipid Res*. 2008;49:543-9.
40. Millar JS, Brousseau ME, Diffenderfer MR, Barrett PH, Welty FK, Faruqi A, Wolfe ML, Nartsupha C, Digenio AG, Mancuso JP, Dolnikowski GG, Schaefer EJ and Rader DJ. Effects of the cholesteryl ester transfer protein inhibitor torcetrapib on apolipoprotein B100 metabolism in humans. *Arterioscler Thromb Vasc Biol*. 2006;26:1350-6.
41. Timsit YE and Negishi M. CAR and PXR: the xenobiotic-sensing receptors. *Steroids*. 2007;72:231-46.
42. Cannon CP, Shah S, Dansky HM, Davidson M, Brinton EA, Gotto AM, Stepanavage M, Liu SX, Gibbons P, Ashraf TB, Zafarino J, Mitchel Y and Barter P. Safety of anacetrapib in patients with or at high risk for coronary heart disease. The New England journal of medicine. 2010;363:2406-15.
43. Krishna R, Anderson MS, Bergman AJ, Jin B, Fallon M, Cote J, Rosko K, Chavez-Eng C, Lutz R, Bloomfield DM, Gutierrez M, Doherty J, Bieberdorf F, Chodakewitz J, Gottesdiener KM and Wagner JA. Effect of the cholesteryl ester transfer protein inhibitor, anacetrapib, on lipoproteins in patients with dyslipidaemia and on 24-h ambulatory blood pressure in healthy individuals: two double-blind, randomised placebo-controlled phase I studies. *Lancet*. 2007;370:1907-14.
44. Krishna R, Bergman AJ, Jin B, Fallon M, Cote J, Van Hoydonck P, Laethem T, Gendrano IN, 3rd, Van Dyck K, Hilliard D, Laterza O, Snyder K, Chavez-Eng C, Lutz R, Chen J, Bloomfield DM, De Smet M, Van Bortel LM, Gutierrez M, Al-Huniti N, Dykstra K, Gottesdiener KM and Wagner JA. Multiple-dose pharmacodynamics and pharmacokinetics of anacetrapib, a potent cholesteryl ester transfer protein (CETP) inhibitor, in healthy subjects. *Clin Pharmacol Ther*. 2008;84:679-83.

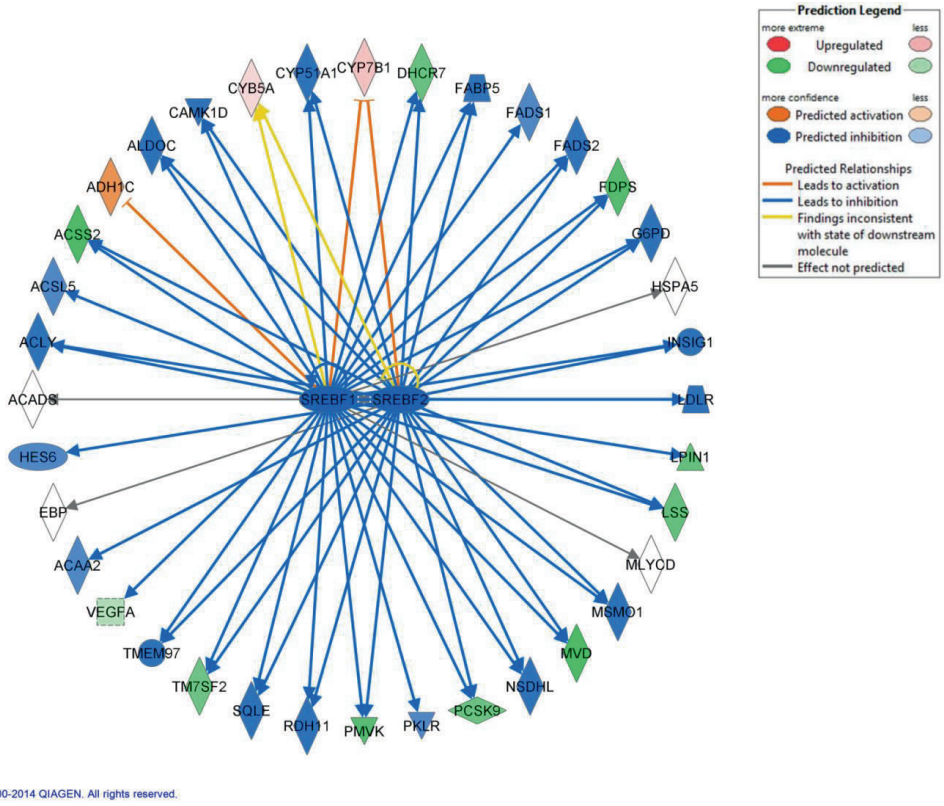
Supplemental Table I: Differentially expressed genes after 22 weeks of anacetrapib treatment.

Gene Name	Gene Symbol	Fold Change	Adjusted P-value
cytochrome P450, family 3, subfamily a, polypeptide 11	*Cyp3a11*	5.649	2.52E-08
cytochrome P450, family 2, subfamily b, polypeptide 10	*Cyp2b10*	103.604	2.09E-07
cytochrome P450, family 3, subfamily a, polypeptide 25	*Cyp3a25*	3.724	2.81E-05
cytochrome P450, family 2, subfamily c, polypeptide 29	*Cyp2c29*	10.133	2.93E-05
cytochrome P450, family 2. subfamily c, polypeptide 37	*Cyp2c37*	2.427	1.71E-04
sodium channel, nonvoltage-gated 1 alpha	*Scnn1a*	-3.941	2.43E-04
cytochrome P450, family 2, subfamily c, polypeptide 50	*Cyp2c50*	2.412	3.25E-04
aldehyde dehydrogenase family 1, subfamily A1	*Aldh1a1*	3.939	1.36E-03
cathepsin A	*Ctsa*	-4.641	1.36E-03
phospholipase A1 member A	*Pla1a*	-2.357	1.36E-03
leucine rich repeat containing 14B	*Lrrc14b*	5.494	1.80E-03
transmembrane protein 33	*Tmem33*	1.839	1.80E-03
breast cancer anti-estrogen resistance 1	*Bcar1*	2.217	2.60E-03
ectonucleoside triphosphate diphosphohydrolase 5	*Entpd5*	2.268	4.21E-03
glypican 1	*Gpc1*	5.006	5.04E-03
heme binding protein 1	*Hebp1*	-3.970	5.04E-03
transmembrane 7 superfamily member 2	*Tm7sf2*	-4.176	6.35E-03
7-dehydrocholesterol reductase	*Dhcr7*	-4.797	6.93E-03
cytochrome c oxidase subunit VIIa 1	*Cox7a1*	-4.018	7.26E-03
hosphomevalonate kinase	*Pmvk*	-6.690	7.84E-03
phosphate cytidylyltransferase 2, ethanolamine	*Pcyt2*	-2.142	8.66E-03
acetyl-Coenzyme A acetyltransferase 2	*Acat2*	-5.139	1.00E-02
heat shock protein 1	*Hspb1*	4.339	1.00E-02
serine dehydratase	*Sds*	-3.830	1.00E-02
cytochrome P450, family 2, subfamily d, polypeptide 9	*Cyp2d9*	9.285	1.06E-02
choline phosphotransferase 1	*Chpt1*	2.217	1.09E-02
RIKEN cDNA 9130409I23 gene	*9130409I23Rik*	7.743	1.11E-02
cytochrome P450, family 2, subfamily c, polypeptide 55	*Cyp2c55*	10.108	1.24E-02
cytochrome P450, family 7, subfamily b, polypeptide 1	*Cyp7b1*	2.965	1.24E-02
GM2 ganglioside activator protein	*Gm2a*	-1.804	1.24E-02
interleukin 11 receptor, alpha chain 1	*Il11ra1*	-2.312	1.31E-02
farnesyl diphosphate synthetase	*Fdps*	-7.399	1.35E-02
NFKB inhibitor interacting Ras-like protein 2	*Nkiras2*	-2.175	1.35E-02
PDZK1 interacting protein 1	*Pdzk1ip1*	-6.021	1.35E-02
low density lipoprotein receptor-related protein associated protein 1	*Lrpap1*	-2.775	1.55E-02

testis expressed gene 264	*Tex264*	1.913	1.55E-02
proprotein convertase subtilisin/kexin type 9	*Pcsk9*	-4.460	1.58E-02
C-type lectin domain family 4, member b1	*Clec4b1*	2.807	1.64E-02
nuclear receptor binding protein 2	*Nrbp2*	-2.046	1.73E-02
olfactory receptor 194	*Olfr194*	4.830	1.76E-02
predicted gene 5922	*Gm5922*	7.427	1.84E-02
RIKEN cDNA 0610012H03 gene	*0610012H03Rik*	2.458	1.94E-02
DEAQ RNA-dependent ATPase	*Dqx1*	-3.873	1.94E-02
cytochrome b-5	*Cyb5*	1.863	2.07E-02
replication factor C (activator 1) 5	*Rfc5*	-2.150	2.37E-02
vitelline membrane outer layer 1 homolog (chicken)	*Vmo1*	-1.908	2.67E-02
fasciculation and elongation protein zeta 2 (zygin II)	*Fez2*	1.651	2.73E-02
vomeronasal 1 receptor 63	*Vmn1r63*	4.944	2.73E-02
proteasome (prosome, macropain) 26S subunit, ATPase, 4	*Psmc4*	1.646	2.73E-02
proteasome (prosome, macropain) subunit, beta type 7	*Psmb7*	1.689	2.75E-02
cysteine conjugate-beta lyase 1	*Ccbl1*	-2.433	2.75E-02
ubiquitin-conjugating enzyme E2F (putative)	*Ube2f*	1.853	2.76E-02
RIKEN cDNA 1700034O15 gene	*1700034O15Rik*	3.533	3.02E-02
RIKEN cDNA 4931406C07 gene	*4931406C07Rik*	2.043	3.02E-02
glycine-N-acyltransferase	*Glyat*	1.651	3.02E-02
six transmembrane epithelial antigen of prostate 2	*Steap2*	-2.563	3.02E-02
TLC domain containing 1	*Tlcd1*	-2.980	3.02E-02
zinc finger, AN1-type domain 2A	*Zfand2a*	1.990	3.02E-02
cleft lip and palate associated transmembrane protein 1	*Clptm1*	-1.859	3.14E-02
interleukin 1 receptor accessory protein	*Il1rap*	-1.884	3.14E-02
annexin A6	*Anxa6*	-1.747	3.32E-02
cDNA sequence BC021614	*BC021614*	-2.597	3.32E-02
branched chain aminotransferase 2, mitochondrial	*Bcat2*	-2.513	3.32E-02
prune homolog (Drosophila)	*Prune*	1.950	3.47E-02
reversion-inducing-cysteine-rich protein with kazal motifs	*Reck*	-2.113	3.47E-02
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	*Abcc3*	3.802	3.50E-02
biliverdin reductase B (flavin reductase (NADPH))	*Blvrb*	1.968	3.50E-02
lipin 1	*Lpin1*	-5.103	3.50E-02
upstream transcription factor 2	*Usf2*	-2.389	3.50E-02
vascular endothelial growth factor A	*Vegfa*	-1.824	3.50E-02
cleavage and polyadenylation specific factor 1	*Cpsf1*	-2.048	3.56E-02
carboxylesterase 2A	*Ces2a*	4.824	3.59E-02

Gene Name	Gene Symbol	Fold Change	Adjusted P-value
PHD finger protein 2	*Phf2*	-2.171	4.17E-02
argininosuccinate synthetase 1	*Ass1*	-2.753	4.23E-02
histone cluster 1, H2bk	*Hist1h2bk*	1.871	4.25E-02
aminolevulinic acid synthase 1	*Alas1*	3.070	4.64E-02
interferon, alpha-inducible protein 27 like 2B	*Ifi27l2b*	3.620	4.64E-02
lanosterol synthase	*Lss*	-5.130	4.64E-02
MACRO domain containing 1	*Macrod1*	-2.309	4.64E-02
mevalonate (diphospho) decarboxylase	*Mvd*	-9.687	4.64E-02
RAN GTPase activating protein 1	*Rangap1*	2.048	4.64E-02
epoxide hydrolase 1, microsomal	*Ephx1*	2.595	4.80E-02
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	*Emr1*	3.535	4.91E-02
hypoxanthine guanine phosphoribosyl transferase	*Hprt*	2.247	4.93E-02
RIKEN cDNA 1600016N20 gene	*1600016N20Rik*	-2.797	4.95E-02
valosin containing protein	*Vcp*	1.573	4.95E-02
acid phosphatase, prostate	*Acpp*	-11.171	4.98E-02
acyl-CoA synthetase short-chain family member 2	*Acss2*	-9.253	4.98E-02
coagulation factor XII (Hageman factor)	*F12*	-1.529	4.98E-02
family with sequence similarity 213, member A	*Fam213a*	-1.987	4.98E-02
myotubularin related protein 11	*Mtmr11*	-3.126	4.98E-02
neurocalcin delta	*Ncald*	2.432	4.98E-02
phospholipid transfer protein	*Pltp*	-3.541	4.98E-02
pyrroline-5-carboxylate reductase-like	*Pycrl*	-1.688	4.98E-02
small G protein signaling modulator 1	*Sgsm1*	-3.306	4.98E-02

Female E3L.CETP mice were fed a Western-type diet with or without anacetrapib (30 mg/kg bw/d) for 22 weeks. RNA was isolated from liver tissue and a microarray analysis was performed. Fold change is the change in expression for anacetrapib versus control treatment.



Supplemental Figure I. Regulation of sterol regulatory element-binding protein gene (*Srebf*) -1 and *Srebf*-2-related genes by anacetrapib.
Molecular network showing *Srebf-1 and* -2-related gene regulation of anacetrapib. The molecular network consists of genes that are differentially expressed after anacetrapib treatment (cut off: P<0.01) and are coloured red if upregulated or green if downregulated (cut off: FDR<0.05). An *in silico* prediction based on the differentially expressed target genes was performed to indicate *Srebf-1 and -2* activation state. Predicted regulation of genes is indicated by orange if they are predicted to be upregulated and blue if predicted to be downregulated.

CHAPTER 6

Alirocumab Inhibits Atherosclerosis, Improves the Plaque Morphology, and Enhances the Effects of a Statin

Susan Kühnast[1,2,3*], José W.A. van der Hoorn[1,2,3*], Elsbet J. Pieterman[1], Anita M. van den Hoek[1], William J. Sasiela[4], Viktoria Gusarova[4], Anusch Peyman[5], Hans-Ludwig Schäfer[5], Uwe Schwahn[5], J. Wouter Jukema[2], Hans M.G. Princen[1]

[1]TNO - Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands;
[2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[3]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands;
[4]Regeneron Pharmaceuticals Inc., Tarrytown, NY, USA;
[5]Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany

*S.K. and J.W.A.v.d.H. contributed equally

J Lipid Res. 2014 Oct;55(10):2103-12

Abstract

Objective Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition is a potential novel strategy for treatment of CVD. Alirocumab is a fully human PCSK9 monoclonal antibody in phase III clinical development. We evaluated the anti-atherogenic potential of alirocumab in APOE*3Leiden.CETP mice.

Methods Mice received a Western-type diet and were treated with alirocumab (3 or 10 mg/kg, weekly subcutaneous dosing) alone and in combination with atorvastatin (3.6 mg/kg/d) for 18 weeks.

Results Alirocumab alone dose-dependently decreased total cholesterol (-37%; -46%, P<0.001) and triglycerides (-36%; -39%, P<0.001) and further decreased cholesterol in combination with atorvastatin (-48%; -58%, P<0.001). Alirocumab increased hepatic LDL receptor protein levels, but did not affect hepatic cholesterol and triglyceride content. Fecal output of bile acids and neutral sterols was not changed. Alirocumab dose-dependently decreased atherosclerotic lesion size (-71%; -88%, P<0.001) and severity and enhanced these effects when added to atorvastatin (-89%; -98%, P<0.001). Alirocumab reduced monocyte recruitment and improved the lesion composition by increasing the smooth muscle cell and collagen content and decreasing the macrophage and necrotic core content.

Conclusion Alirocumab dose-dependently decreases plasma lipids and, as a result, atherosclerosis development, and enhances the beneficial effects of atorvastatin in APOE*3Leiden.CETP mice. In addition, alirocumab improves plaque morphology.

Keywords APOE*3Leiden.CETP mice, PCSK9, alirocumab, atorvastatin, atherosclerosis

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease involved in LDL metabolism.[1] PCSK9, previously known as neutral apoptosis regulated convertase, is mainly expressed in the liver, kidney, and intestines.[2, 3]

Besides familial hypercholesterolemia (FH) caused by *ldlr* mutations and familial defective apolipoprotein B100 caused by *apob* mutations,[1] autosomal dominant hypercholesterolemia can be caused by gain-of-function mutations of the *pcsk9* gene, now commonly referred to as FH3.[3, 4] Conversely, loss-of-function mutations of the *pcsk9* gene were associated with a reduction in LDL-cholesterol (LDL-C) and protection against coronary heart disease.[5, 6] In addition, familial hypobetalipoproteinemia related to loss-of-function mutations of *pcsk9* resulted in very low plasma levels of LDL-C, attributed to an increased clearance rate of LDL.[7]

Several studies have confirmed that PCSK9 is responsible for targeting the LDL receptor (LDLR) for lysosomal degradation in the liver by preventing recycling of the receptor to the cell membrane after internalization of the LDL-bound LDLR.[4] PCSK9 interacts with the LDLR on the cell membrane, after which the LDLR-PCSK9 complex is internalized and travels through the endosome to the lysosome for degradation.[8] In a study in mice, adenovirus-mediated expression of PCSK9 increased plasma LDL-C levels, which was associated with decreased hepatic LDLR protein, although LDLR mRNA levels were unaffected.[9] On the contrary, mice lacking PCSK9 have decreased plasma LDL-C as a result of increased hepatic LDLR levels.[10] A recent study in wild-type, APOE-/-, and LDLR-/- mice with or without expression of PCSK9 revealed a direct relationship between PCSK9 and atherosclerosis development, mainly mediated via the LDLR, and suggests that PCSK9 inhibition will be beneficial in reducing atherosclerosis.[11]

Although statins remain the most effective treatment option for CVD, there remains a substantial persistent cardiovascular risk and, despite statin treatment, some patients cannot reach the recommended LDL-C target.[12, 13] Recent outcome studies and *post hoc* analyses indicate that therapeutic regimens that further lower LDL-C lead to further reductions in cardiovascular events[14-16] and, consequently, cholesterol management guidelines have evolved to more rigorous goals.[17-19] The upregulation of the LDLR after statin treatment is accompanied by an upregulation of PCSK9, which in turn promotes LDLR degradation.[20-22] In humans, the ~35% to 50% decrease in LDL-C after atorvastatin treatment (10 to 40 mg) was accompanied by a ~7% to 35% increase in circulating PCSK9 levels.[21, 22] Inhibition of PCSK9 is, therefore, a potential novel strategy for treatment of CVD, specifically in combination with statin treatment. Several approaches to inhibit PCSK9, including monoclonal antibodies, gene silencing, and mimetic peptides, are currently being investigated.[4] The anti-PCSK9 monoclonal antibody, alirocumab is a lead compound in this class and is currently being tested in phase III clinical trials.

Alirocumab, also known as SAR236553/REGN727, is a fully human, monoclonal antibody that lowers plasma LDL-C in normocholesterolemic volunteers[23] and hypercholesterolemic patients on stable statin dose.[24, 25] In patients with hypercholesterolemia, alirocumab in combination with low- and high-dose atorvastatin decreased LDL-C to a greater extent than titration to high-dose atorvastatin, and considerably more patients who received the combination treatments reached LDL-C goals of <100 mg/dL or <70 mg/dL compared with patients who received atorvastatin treatment alone.[26] Phase II trials demonstrated reductions in LDL-C of 40% to 72% across a dose range of 50 to 150 mg administered every 2 weeks, and of 32% to 48% with doses 200 to 300 mg administered every 4 weeks.[23-25]

The aim of this study was to investigate the effects of two dosages of alirocumab alone and in combination with atorvastatin on plasma lipids, atherosclerosis development and lesion composition in APOE*3Leiden.CETP mice.[27] This is a well-established model for hyperlipidemia and atherosclerosis with all features of familial dysbetalipoproteinemia (FD) in humans, which is characterized by accumulation of remnant lipoproteins and an increased VLDL-cholesterol to LDL-C ratio.[28] APOE*3Leiden mice have an impaired clearance of (V)LDL and increased triglycerides (TG) levels and are thereby mimicking the slow clearance observed in humans, in contrast to normal wild-type mice which have a very rapid clearance of apoB-containing lipoproteins.[29] The lipoprotein profile in APOE*3Leiden.CETP mice reflects that of FD patients with a similar response to lipid-modifying therapies,[30] including statins,[31] fibrates,[32] niacin,[33] and cholesteryl ester transfer protein inhibitors.[34] This is illustrated by a comparable reduction in cholesterol in all apoB-containing lipoprotein subfractions with statin treatment.[35] We hypothesized that alirocumab alone could reduce progression of atherosclerosis and add to the atheroprotective effects of atorvastatin. Inhibition of atherosclerosis by atorvastatin in APOE*3Leiden.CETP mice has been observed previously.[34, 36]

Methods

Animals

Ninety female APOE*3Leiden.CETP transgenic mice (9 to 13 weeks of age),[27] expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions, were used. During the study, mice were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research.

Experimental design

Mice received a semi-synthetic cholesterol-rich diet, containing 15% (w/w) cacao butter and 0.15% cholesterol (Western-type diet [WTD]; Hope Farms, Woerden, The Netherlands) for a run-in period of 3 weeks to increase plasma total cholesterol (TC) levels up to ~15 mmol/l. Body weight and food intake were monitored regularly during the study. After matching based on body weight, plasma TC, TG and age, mice (n=15 per group) received a WTD alone or were treated with two dosages of alirocumab (3 or 10 mg/kg) alone or in combination with atorvastatin (3.6 mg/kg/d) for 18 weeks, and an arm with atorvastatin alone was added. Alirocumab (provided by Regeneron) was administered via weekly subcutaneous injections and atorvastatin was added to the diet. We aimed for a reduction in TC of about 20% to 30% with the dose of atorvastatin. At the end of the treatment period all animals were sacrificed by CO_2 inhalation. Livers and hearts were isolated to assess hepatic LDLR protein levels, lipid content, atherosclerosis development, and plaque composition.

Plasma lipids, lipoprotein analysis and measurement of alirocumab levels

Plasma was isolated from blood collected in EDTA-coated cups via tail vein bleeding after a 4-h fast every 2 to 4 weeks. Plasma TC and TG were determined using enzymatic kits according to the manufacturer's protocols (cat. no. 1458216 and cat. no. 1488872, respectively; Roche/Hitachi) and average plasma TC and TG levels were calculated by total exposure over number of weeks. Lipoprotein profiles for TC were measured after lipoprotein separation by fast protein liquid chromatography (FPLC) after 4, 12, and 18 weeks of treatment.[27] Alirocumab levels were measured by a human Fc enzyme-linked immunosorbent assay.

Hepatic LDLR protein levels

Liver tissues were homogenized in lysis buffer (50 mM Tris-HCL [pH=7.4], 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40 [Igepal], 1mM EDTA, protease inhibitor cocktail [complete, Roche], 1 mM PMSF, 1 mM Na3VO4) and then centrifuged at 6500 rpm at 4˚C for 30 min. Protein concentration in cell lysates was determined by bicinchonic acid protein assay (Thermo Scientific) according to manufacturer's instructions. 50 µg of protein lysates were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore). Blots were subjected to goat anti-mouse LDLR from R&D Systems and rabbit anti-goat horseradish peroxidase (HRP) from AbD Serotec or mouse anti-α-Tubulin from Sigma and horse anti-mouse HRP from Cell Signaling Technologies (according to the manufacturer's instructions); blots were developed with West Femto Super Signal ECL (Thermo Scientific) and subjected to the Chemi-Doc-it imaging system. Intensities of protein bands were quantified using Image J software.

Hepatic lipid analysis and fecal excretion of bile acids and neutral sterols

Liver tissue samples were homogenized in phosphate-buffered saline, and the protein content was measured. Lipids were extracted, separated by high-performance thin-layer chromatography on silica gel plates, and analyzed with TINA2.09 software (Raytest Isotopen Messgeräte Straubenhardt, Germany), as previously described.[37]

Mice were housed at five mice per cage, and feces were collected during two consecutive periods of 72 h and 48 h, respectively. Aliquots of lyophilized feces were used for determination of neutral and acidic sterol content by gas-liquid-chromatography, as previously described.[38]

Histological assessment of atherosclerosis

Hearts were isolated, fixed in formalin, and embedded in paraffin. They were then sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch. Once the aortic root was identified by the appearance of aortic valve leaflets and smooth muscle cells (SMCs) instead of collagen-rich tissue, serial cross sections (5 μm thick with intervals of 50 μm) were taken and mounted on aminopropyl-triethoxy-silane (APES)-coated slides. These sections were stained with hematoxylin-phloxine-saffron (HPS) for histological analysis. For each mouse, atherosclerosis was measured in four subsequent cross sections. Each section consisted of three segments. The average total lesion area per cross section was then calculated.[36, 39] For determination of lesion severity the lesions were classified into five categories according to the American Heart Association classification[40]: 0) no lesion I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, and V) severe plaque. The percentage of each lesion type was calculated, and type I-III lesions were classified as mild lesions and type IV-V lesions were classified as severe lesions.[36, 39] To determine the total plaque load in the thoracic aorta, perfusion-fixed aortas (from the aortic origin to the diaphragm) were cleaned of extravascular fat, opened longitudinally, pinned en face, and stained for lipids with oil red O as described previously.[41] Data were normalized for analyzed surface area and expressed as a percentage of the stained area. Photos/images were taken with the Olympus BX51 microscope and lesion areas were measured using Cell D imaging software (Olympus Soft Imaging Solutions).

In the aortic root, lesion composition was determined for the severe lesions (type IV-V) as a percentage of lesion area after immunostaining with anti-human α-actin (1:800; Monosan, Uden, The Netherlands) for SMCs, and anti-mouse Mac-3 (1:25; BD Pharmingen, the Netherlands) for macrophages followed by sirius red staining for collagen. Necrotic area and cholesterol clefts were measured in macrophage/collagen staining.[36, 39, 42] In each segment used for lesion quantification, the number of monocytes adhering to the endothelium and the number of T cells in the total aortic root area were counted after immunostaining with AIA 31240 antibody (1:1000; Accurate Chemical and Scientific, New York, New York, USA) and CD3 (1:500; AbD Serotec, Oxford, UK), respectively. Rat anti-

mouse CD54 antibody, GTX76543 (GeneTex, Inc., San Antonio, TX, USA) was used for immunostaining of intercellular adhesion molecule 1 (ICAM-1).[43] Photos/images of the lesions were taken with the Olympus BX40 microscope with Nuance 2 multispectral imaging system, and stained areas were quantified using Image J software.

Flow cytometric analysis

After 8 weeks of treatment, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples and were sorted into GR-1+ (neutrophils/granulocytes), GR-1- (lymphocytes/monocytes), CD3+ (T cells), CD19+ (B cells) and CD11b+/Ly6C^{low} and CD11b+/Ly6C^{hi} (monocytes) cells using flow cytometric analysis. The following conjugated monoclonal antibodies were used from Becton Dickinson: GR-1 FITC, CD3 PerCpCy5-5, CD19 V450, CD11b APC and Ly6C PE-Cy7.

Statistical analysis

Significance of differences between the groups was calculated non-parametrically using a Kruskal-Wallis test for independent samples, followed by a Mann-Whitney U-test for independent samples. Linear regression analyses were used to assess correlations between variables. Since the atherosclerotic lesion area showed a quadratic dependence on plasma cholesterol exposure, it was transformed using square root transformation.

IBM SPSS Statistics 20 for Windows (SPSS, Chicago, USA) was used for statistical analysis. All groups were compared with the control group and with the atorvastatin group, and 3 mg/kg alirocumab was compared with 10 mg/kg alirocumab either with or without atorvastatin. Values are presented as means ± SD. Bonferroni-Holm's method was used to determine the level of significance in the case of multiple comparisons. P-values <0.05 were considered statistically significant. In figures, significant effects after correction for multiple comparisons are indicated by * to compare with the control group, † to compare with the atorvastatin group and ‡ to compare 3 mg/kg alirocumab with 10 mg/kg alirocumab.

Results

Alirocumab and atorvastatin monotreatment and their combination decrease plasma TC and TG in APOE*3Leiden.CETP mice

Alirocumab binds both human and mouse PCSK9 with high affinity (Kd=0.58nM and 2.6nM, respectively, at pH 7.4 and 25°C) as determined by surface plasmon resonance. Circulating alirocumab levels were detected in all groups administered alirocumab and ranged between 5 to 12 μg/ml (3 mg/kg dose) and 12 to 30 μg/ml (10 mg/kg dose) during the study. No immune response was observed as evidenced by stable efficacy throughout the study. After 18 weeks of treatment, the APOE*3Leiden.CETP mice on a cholesterol-containing WTD

(control group) reached average plasma TC and TG levels of 16.2 ± 1.8 mmol/l and 2.9 ± 0.6 mmol/l, respectively (**Figure 1A and 1B**). Compared with the control, alirocumab decreased average plasma TC (-37%, P<0.001; -46%, P<0.001) and TG (-36%, P<0.001; -39%, P<0.001) and further decreased TC in combination with atorvastatin (-48%, P<0.001; -58%, P<0.001). Compared with atorvastatin, both combination treatments decreased TC (-36%, P<0.001; -48%, P<0.001) and TG (-39%, P<0.001; -50%, P<0.001) to a greater extent than atorvastatin alone. The (higher) reductions in TC (at the higher dose) after alirocumab alone (-14%, P<0.01; 3 mg/kg alirocumab vs. 10 mg/kg alirocumab) and in combination with atorvastatin (-19%, P<0.001; 3 mg/kg alirocumab + atorvastatin vs. 10 mg/kg alirocumab + atorvastatin) were dose-dependent and sustained during the study. TC reductions after alirocumab (**Figure 1C**), atorvastatin and their combination (**Figure 1D**) were confined to non-HDLs.

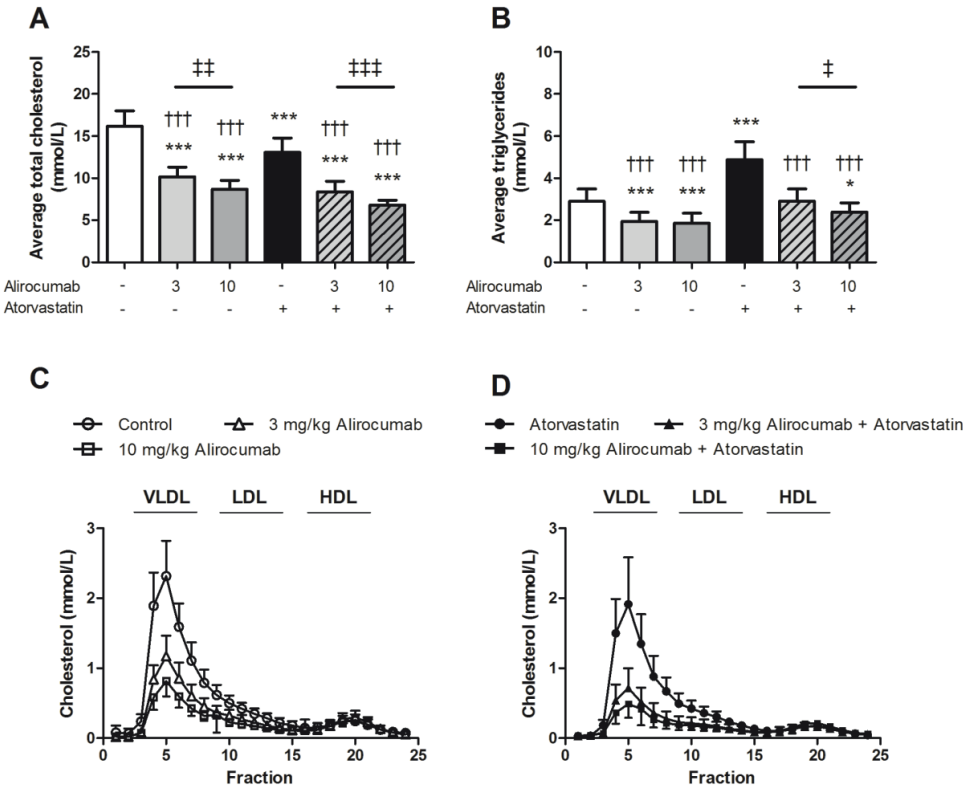


Figure 1 Effect of alirocumab, atorvastatin, and their combination on average plasma TC (A) and TG (B) levels as measured throughout the 18-week study. Lipoprotein profiles for cholesterol were assessed by FPLC lipoprotein separation to study effects of alirocumab alone (C) and in combination with atorvastatin (D).
*P<0.05, ***P<0.001 as compared with control; †††P<0.001 as compared with atorvastatin; ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.001 for 3 mg/kg alirocumab compared with 10 mg/kg alirocumab (n=15 per group).

Alirocumab without and with atorvastatin decreases plasma lipids by reducing LDLR degradation

Hepatic LDLR protein levels were measured to verify whether PCSK9 inhibition by alirocumab decreases plasma lipids by rescuing LDLR degradation (**Figure 2**). Hepatic LDLR protein levels were increased after alirocumab treatment alone (+80%, P<0.05; +133%, P<0.01) and together with atorvastatin (+98%, P<0.01; +178%, P<0.05). Compared with atorvastatin alone, both the combination treatments increased LDLR protein levels to a greater extent (+71%, P<0.01; +140%, P<0.05). An inverse correlation between LDLR protein levels and plasma TC confirms the involvement of the LDLR in lowering of TC by alirocumab (R^2=0.50, P<0.001).

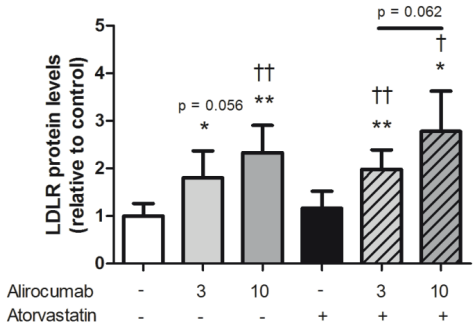


Figure 2 Effect of alirocumab, atorvastatin, and their combination on hepatic LDLR protein levels. *P<0.05, **P<0.01 as compared with control; †P<0.05, ††P<0.01 as compared with atorvastatin (n=8 per group).

Alirocumab does not affect hepatic lipids and fecal bile acid and neutral sterol excretion

To evaluate the consequences of alirocumab-induced alterations in lipoprotein metabolism on hepatic lipid metabolism and excretion into feces, we determined liver lipids and fecal excretion of bile acids and neutral sterols. Alirocumab did not affect the hepatic content of cholesterol and TG, whereas atorvastatin and the combination treatments led to significant reductions in hepatic cholesteryl esters (-48%, P<0.05; -41%, P<0.05 and -44%, P=0.28, respectively) as compared with the control group, without a change in hepatic TG (**Table 1**). Fecal output of bile acids and neutral sterols was not changed by the treatments (**Table 2**). These data indicate that despite the greater influx of cholesterol from the plasma compartment hepatic cholesterol homeostasis is maintained during alirocumab and statin treatment in mice.

Table 1 Effect of alirocumab, atorvastatin and their combination on liver lipids.

	Liver lipids (µg/mg protein)		
	FC	**CE**	**TG**
Control	11.6 ± 1.6	50.6 ± 14.0	119.2 ± 33.3
3 mg alirocumab	11.2 ± 1.4	48.2 ± 8.2 †	117.7 ± 21.6
10 mg alirocumab	11.4 ± 2.0	53.9 ± 10.4 †††	142.1 ± 43.0
Atorvastatin	9.5 ± 0.9	26.2 ± 4.8 *	90.6 ± 28.5
3 mg alirocumab + atorvastatin	10.4 ± 1.8	29.6 ± 5.8 *	103.5 ± 36.8
10 mg alirocumab + atorvastatin	10.7 ± 1.2	28.3 ± 9.0	109.8 ± 28.8

FC, free cholesterol; CE, cholesterol esters. *P<0.05 as compared with control; †P<0.05, †††P<0.001 as compared with atorvastatin

Table 2 Effect of alirocumab, atorvastatin and their combination on neutral sterol and bile acid excretion.

	Neutral sterol excretion (µmol/100 g mouse/day)	**Bile acid excretion (µmol/100 g mouse/day)**
Control	25.8 ± 5.5	13.5 ± 3.3
3 mg alirocumab	20.4 ± 6.2	14.3 ± 2.7
10 mg alirocumab	21.6 ± 5.6	12.4 ± 3.2
Atorvastatin	30.3 ± 6.5	10.7 ± 2.4
3 mg alirocumab + atorvastatin	28.6 ± 6.0	11.4 ± 2.2
10 mg alirocumab + atorvastatin	27.5 ± 4.4	12.7 ± 1.6

Alirocumab dose-dependently reduces atherosclerosis development and enhances the atheroprotective effects of atorvastatin

Effects of alirocumab on atherosclerosis development in the absence and presence of atorvastatin were assessed in the aortic root and arch after 18 weeks of treatment. Representative images of atherosclerotic lesions as illustrated in **Figure 3** show that alirocumab, atorvastatin, and their combination reduced lesion progression. To confirm a reduction in atherosclerosis development, we determined lesion area per cross section (**Figure 4A**), and calculated lesion severity (**Figure 4C**). For the control group, total lesion area was 278 ± 89 x 10^3 µm^2 per cross section. Alirocumab dose-dependently decreased atherosclerotic lesion size (-71%, P<0.001; -88%, P<0.001) and dose-dependently enhanced the effects of atorvastatin (-89%, P<0.001; -98%, P<0.001) as compared with the control. Mice treated with alirocumab alone and in combination with atorvastatin had more lesion-free sections and fewer severe (type IV-V) lesions compared with the control. Atorvastatin alone decreased lesion size (-35%, P<0.05) and reduced severity to a lesser extent with no effect on the percentage undiseased segments. When compared with atorvastatin monotreatment, the combinations further decreased lesion size (-82%, P<0.001; -97%, P<0.001) and increased the percentage undiseased segments.

To evaluate the effect of alirocumab treatment on lesion development at another spot along the aorta prone to development of atherosclerosis, plaque surface in the aortic arch was measured (**Figure 4B**). At this site, lesion development is delayed as compared with the aortic root.[41] In line with the effects on atherogenesis in the aortic origin, both doses of alirocumab together with atorvastatin (-73%, P<0.05; -73%, P<0.05) reduced the total plaque area**.**

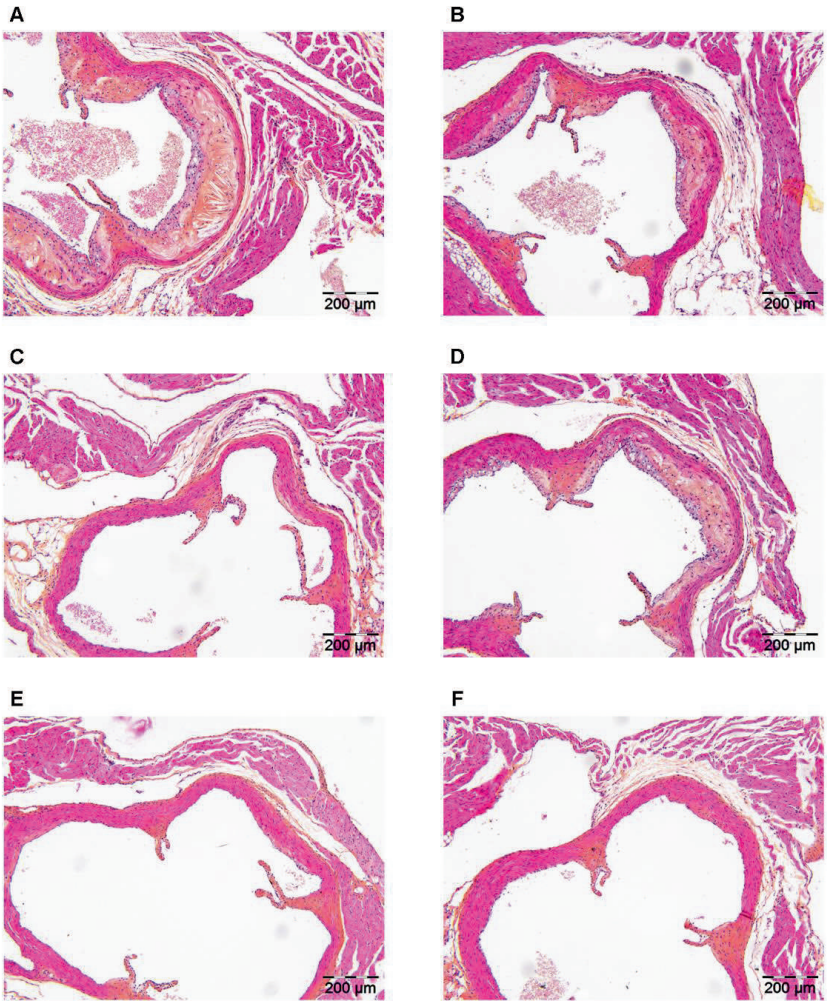


Figure 3 Effect of alirocumab, atorvastatin, and their combination on plaque morphology. Representative images of HPS-stained atherosclerotic lesions in a cross section of the aortic root area for the control (A), 3 mg/kg alirocumab (B), 10 mg/kg alirocumab (C), atorvastatin (D), 3 mg/kg alirocumab + atorvastatin (E) and 10 mg/kg alirocumab + atorvastatin (F) groups, respectively, after 18 weeks of treatment.

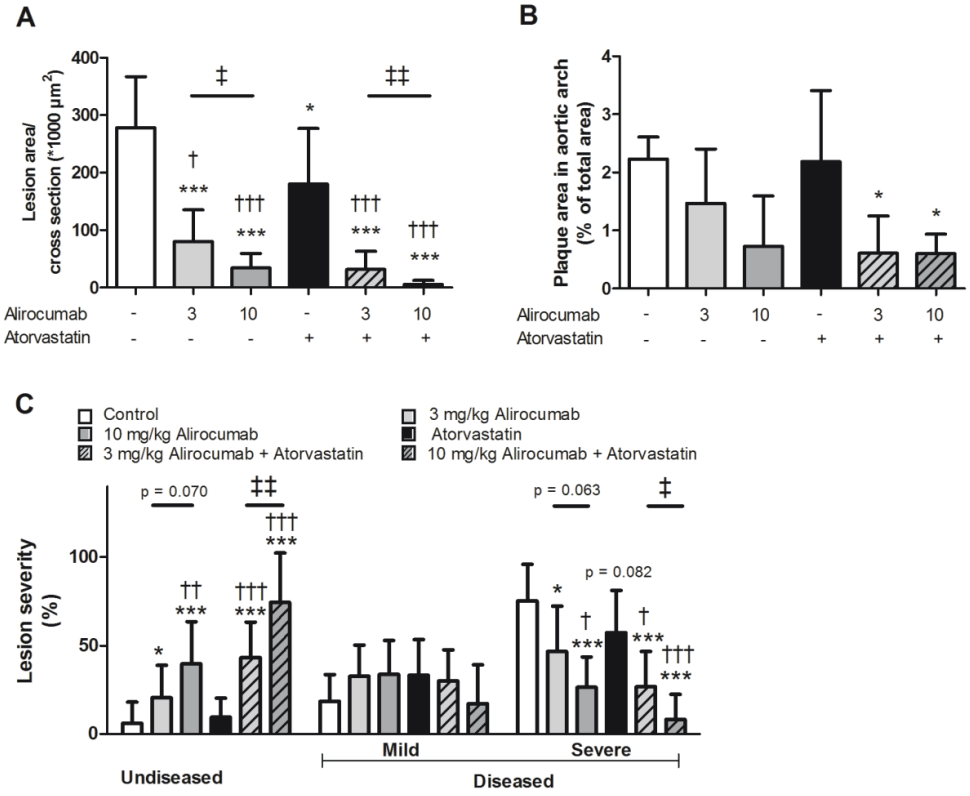


Figure 4 Effect of alirocumab, atorvastatin, and their combination on atherosclerosis development in aortic root and arch. After 18 weeks of treatment, the total lesion area per cross section was assessed (A). The total plaque load in the aortic arch was analyzed after oil red O-staining (B). Lesion severity was assessed and categorized as no lesions, mild (type I-III) lesions and severe (type IV-V) lesions (C). Data are expressed as percentage of the stained area.
*P<0.05, ***P<0.001 as compared with control; †P<0.05, ††P<0.01, †††P<0.001 as compared with atorvastatin; ‡P<0.05, ‡‡P<0.01 for 3 mg/kg alirocumab compared with 10 mg/kg alirocumab (n=15 per group in the root area and n=6-7 in the arch).

We evaluated whether the anti-atherogenic effect of alirocumab and atorvastatin could be explained by the reduction in plasma TC. A strong correlation between plasma TC levels and atherosclerotic lesion area in the aortic root was observed (R^2=0.84, P<0.001; **Figure 5**), indicating an important role of cholesterol in the development of atherosclerosis.

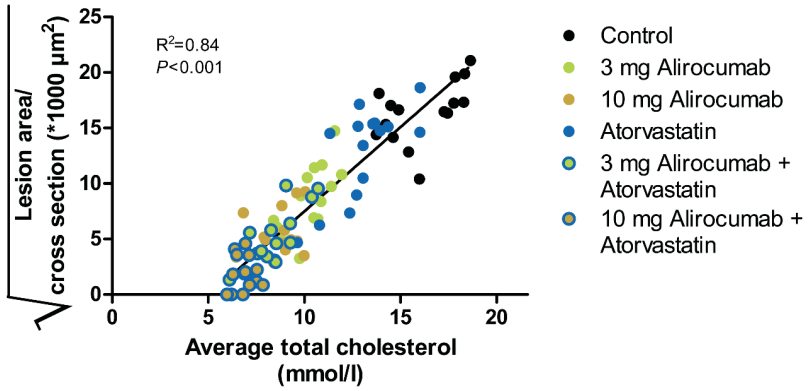


Figure 5 Correlation between average plasma TC and atherosclerotic lesion area. The square root of the lesion area was plotted against average TC. Linear regression analysis was performed.

Alirocumab improves plaque morphology

After investigating lesion morphology, we analyzed treatment effects on plaque composition in the severe lesions (type IV-V), as shown by representative images in **Figure 6**. To illustrate that a pro-inflammatory plaque phenotype is not always dependent on the size of the lesions, we included representative images of similar size lesions for the control group and the alirocumab group. Lesion macrophage area plus lesion necrotic core area (including cholesterol clefts), were quantified as pro-inflammatory factors (**Figure 7A**), whereas SMCs in the fibrotic cap and collagen area were quantified as fortifying factors (**Figure 7B**). All were expressed as a percentage of total lesion area. Lesions in the control group consisted of 10.3% macrophages, 4.8% necrotic core and cholesterol clefts, 3.1% SMCs in the cap and 48.4% collagen. Alirocumab (10 mg/kg) alone and in combination with atorvastatin reduced the pro-inflammatory factors as compared with control (-37% P<0.001; -73% P<0.001) and with atorvastatin treatment (-35% P<0.001; -72% P<0.001). Fortifying factors were increased by 10 mg/kg alirocumab + atorvastatin as compared with control (+29% P<0.001) and dose-dependently as compared with atorvastatin treatment (+29% P<0.05; +40% P<0.001).

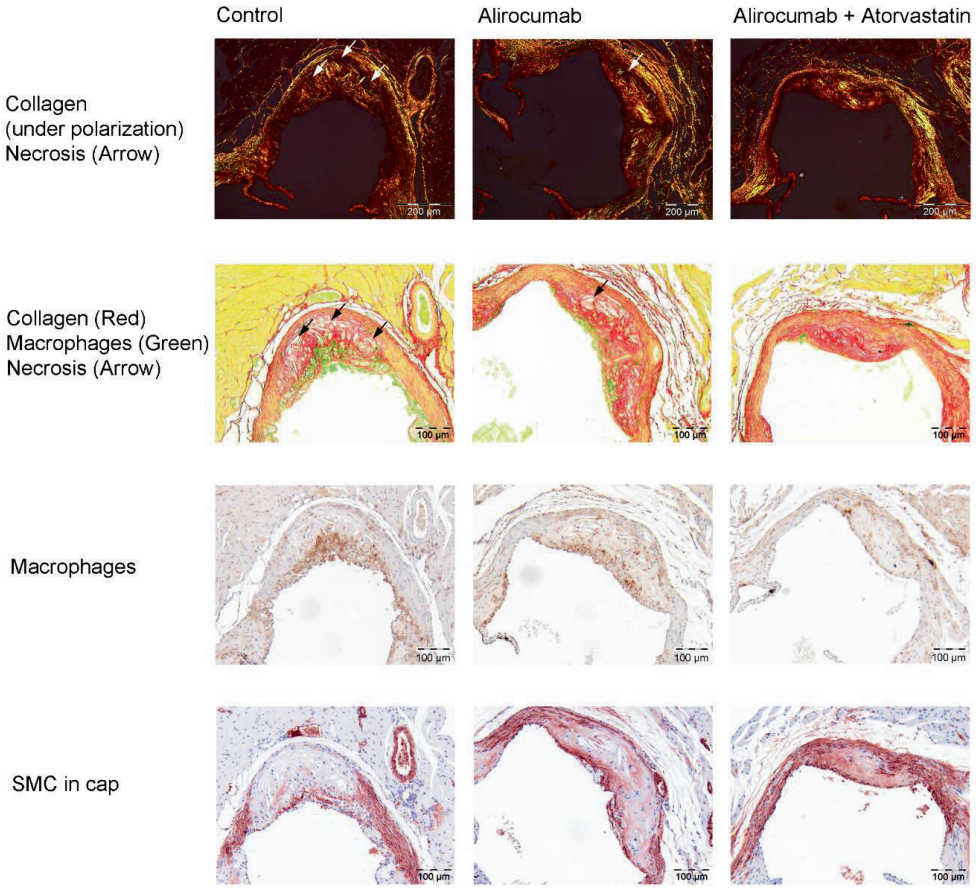


Figure 6 Effect of alirocumab, atorvastatin, and their combination on lesion composition. Representative images of immunostaining with Mac-3 for macrophages followed by sirius red staining for collagen and α-actin for SMCs for the control and after 18 weeks of treatment with alirocumab alone and in combination with atorvastatin.

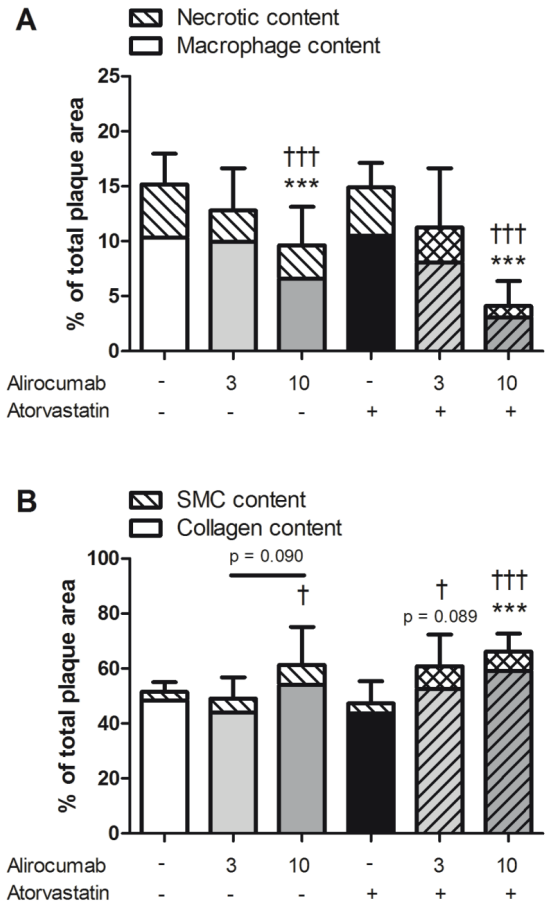


Figure 7 Effect of alirocumab, atorvastatin, and their combination on lesion composition. Macrophage and necrotic content, including cholesterol clefts as pro-inflammatory factors (A), and SMC and collagen content as fortifying factors (B), were determined in the severe (type IV-V) lesions after correcting for lesion size.
**P<0.01, ***P<0.001 as compared with control; †P<0.05, ††P<0.01, †††P<0.001 as compared with atorvastatin (n=15 per group).

Alirocumab reduces monocyte and T cell recruitment

As a functional marker of vessel wall inflammation, the number of monocytes adhering to the activated endothelium (**Figure 8A**) and the number of T cells in the aortic root area (**Figure 8B**) were counted and calculated per cross section. In the control group, 5.7 ± 4.2 adhering monocytes and 16.7 ± 7.7 T cells were present. When administered alone and together with atorvastatin, the higher dose of alirocumab (10 mg/kg) decreased the adhering monocytes (-57%, P<0.05; -82%, P<0.001) and the abundance of T cells (-37%, P<0.05; -62%, P<0.001). To further underline the mechanism by which alirocumab reduced monocyte adherence, we assessed endothelial ICAM-1 expression by immunohistochemistry (**Figure 8C**). For the

control, 39% of the endothelium was positive for ICAM-1 compared with 19% (P<0.001) after 10 mg/kg alirocumab monotreatment and 16% (P<0.001) when given in combination with atorvastatin. The reduction in monocyte adherence was, therefore, corroborated by a reduction in adhesion molecule expression in endothelial cells after alirocumab treatment alone and in combination with atorvastatin.

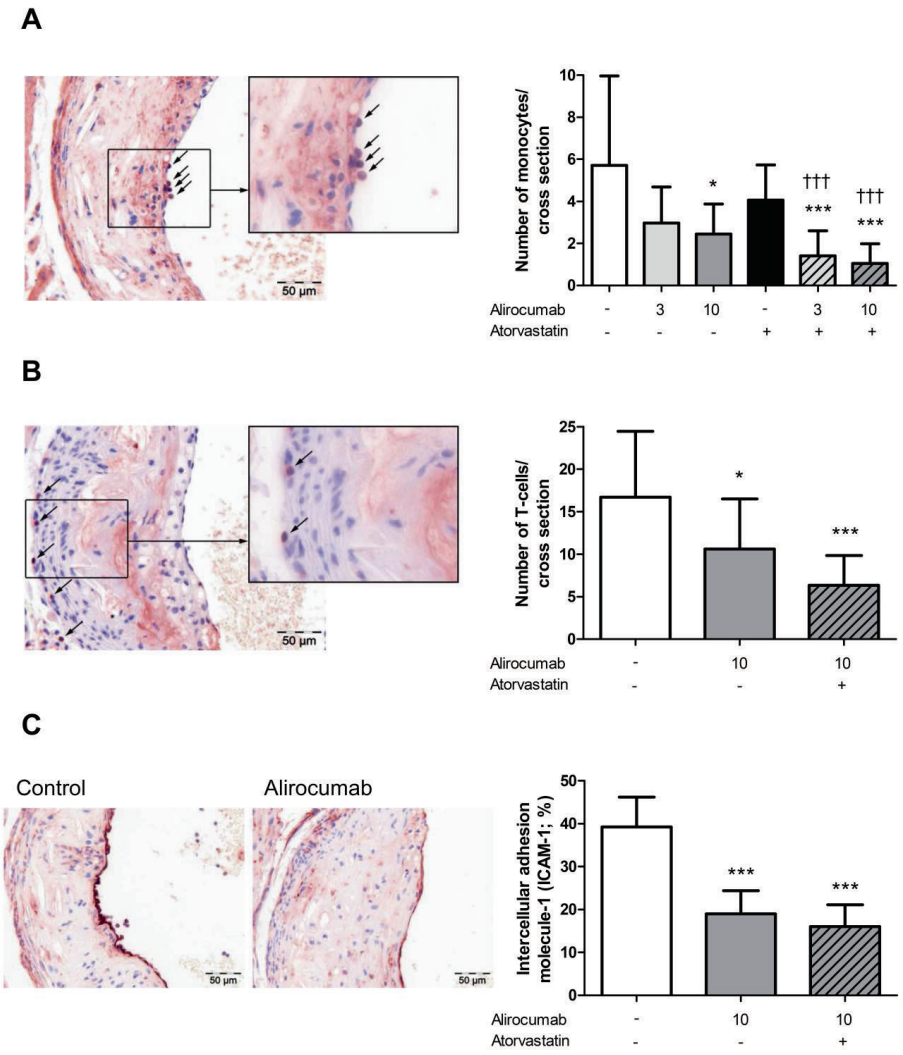


Figure 8 Effect of alirocumab, atorvastatin, and their combination on markers of vascular inflammation. The number of monocytes adhering to the endothelium (A) and the number of T cells in the aortic root area (B) were determined per cross section. In addition, ICAM-1 was determined as percentage of the stained area (C). Representative images are included.
*P<0.05, ***P<0.001 as compared with control; †††P<0.001 as compared with atorvastatin (n=15 per group).

Alirocumab reduces circulating monocytes

The effects of alirocumab alone and in combination with atorvastatin on white blood cell count was assessed by flow cytometry (**Table 3**). Interestingly, alirocumab alone and together with atorvastatin reduced granulocytes/neutrophils (-20%, P<0.05; -34%, P<0.01) and monocytes (-28%, P<0.05; -39%, P<0.01) when expressed as a percentage of the PBMC population. More specifically, alirocumab alone and in combination with atorvastatin tended to decrease pro-inflammatory Ly6C^{hi} (-8%, N.S.; -19%, P<0.001) and increase anti-inflammatory Ly6C^{low} (+12%, N.S.; +35%, P<0.001) monocytes. Therefore, the effect of alirocumab on vascular recruitment and adhesion of monocytes may be augmented by a reduction in circulating monocytes.

Table 3 Effect of alirocumab, atorvastatin, and their combination on white blood cell count as assessed by flow cytometric analysis after 8 weeks of treatment.

	Control	10 mg/kg Alirocumab	Atorvastatin	10 mg/kg Alirocumab + Atorvastatin
Neutrophils/granulocytes (% of PBMC population)	8.9 ± 2.4	7.1 ± 2.0 * †	5.1 ± 1.6 ***	5.9 ± 2.0 **
Lymphocytes/monocytes (% of PBMC population)	91.1 ± 2.4	92.9 ± 2.0 * †	94.9 ± 1.6 ***	94.1 ± 2.0 **
▪ **T cells** (% of PBMC population)	22.9 ± 4.6	21.4 ± 5.0 †	17.0 ± 4.8 **	18.5 ± 4.5 *P*=0.054
▪ **B cells** (% of PBMC population)	63.9 ± 8.5	66.3 ± 15.0	69.5 ± 17.4 **	66.9 ± 2.0 **
▪ **Monocytes** (% of PBMC population)	12.3 ± 5.0	8.9 ± 2.5 * †††	5.3 ± 2.4 ***	7.5 ± 2.8 ** †
▪ ***CD11b+ Ly6C^{hi}*** (% of monocytes)	62.2 ± 8.5	57.5 ± 8.4	51.2 ± 6.4 **	50.2 ± 4.1 ***
▪ ***CD11b+ Ly6C^{low}*** (% of monocytes)	35.6 ± 7.7	40.0 ± 8.0 †	47.8 ± 6.3 ***	48.0 ± 3.5 ***

PBMC, peripheral blood mononuclear cells. *P<0.05, **P<0.01, ***P<0.001 as compared with control; †P<0.05, †††P<0.001 as compared with atorvastatin (n=15 per group)

Safety aspects of alirocumab

No effects on body weight (gain) and food intake were noted in any treatment group as compared with the control group (data not shown). The 10 mg/kg dose of alirocumab on top of atorvastatin treatment led to a reduction in liver weight as compared with the control group after 18 weeks of treatment (-20%, P<0.05, respectively), whereas monotreatment did not have an effect on liver weight. Plasma aspartate transaminase and alanine transaminase were measured in all animals after 16 weeks of treatment (**Table 4**).

Table 4 Safety aspects of alirocumab**.**

	Safety aspects		
	Plasma ALT (U/L)	**Plasma AST (U/L)**	**Liver weight (g)**
Control	91.6 ± 75.6	223.0 ± 189.6	1.23 ± 0.25
3 mg alirocumab	67.1 ± 42.7	189.4 ± 108.7	1.18 ± 0.25
10 mg alirocumab	153.0 ± 122.6	426.3 ± 309.2 *	1.38 ± 0.43
Atorvastatin	59.8 ± 24.3	217.0 ± 76.5	1.05 ± 0.12
3 mg alirocumab + atorvastatin	54.5 ± 25.6	171.7 ± 66.1	1.02 ± 0.17
10 mg alirocumab + atorvastatin	51.0 ± 23.7	157.6 ± 29.2 †	0.99 ± 0.10 *

ALT, alanine transaminase; AST, aspartate transaminase. *P<0.05, as compared with control, †P<0.05 as compared with atorvastatin

Discussion

The PCSK9 monoclonal antibody alirocumab has been shown to strongly lower LDLC and non-HDL-cholesterol (HDL-C) alone and on top of statin treatment,[23-26] and is currently in phase III clinical development, which includes a large CVD outcome trial in hypercholesterolemic patients with relatively recent acute coronary syndrome treated with high-dose statins.[44] It should be realized that the effectiveness of alirocumab on cardiovascular endpoints will only be assessed in patients who also receive statins. Therefore, the present study was designed to investigate the effects of alirocumab on atherosclerosis development, alone and in combination with atorvastatin. Taken together, we have shown that alirocumab dose-dependently decreases plasma cholesterol and reduces progression of atherosclerosis. Moreover, alirocumab improves lesion morphology and composition, and enhances the beneficial effects of atorvastatin in APOE*3Leiden.CETP mice. This is the first study to show that a monoclonal antibody to PCSK9 reduces atherosclerosis development.

Rescue of LDLR from intracellular degradation was verified by an increase in hepatic LDLR protein levels after alirocumab treatment. Consequently, alirocumab decreased TC (-37% to -46%) and TG (-36% to -39%) by a reduction in non-HDLs. The dose-dependent reduction in TC after alirocumab treatment was enhanced in combination with atorvastatin (-48% to -58%). These results support an improvement in cholesterol management by adding alirocumab to statin treatment. The dose-dependent cholesterol-lowering effects in our study are in accordance with results from phase I and phase II clinical trials.[23-26] In phase I trials, alirocumab administered as a single ascending dose (50 to 250 mg) in healthy subjects, and as multiple doses (50 to 150 mg) in statin-treated FH patients, decreased LDL-C by 33% to 46% and by 39% to 61%, respectively.[23] Results from the latter study indicate an additive effect of alirocumab on statin treatment, since similar reductions were observed with alirocumab monotherapy and in statin-treated patients.

In a phase II trial in patients with hypercholesterolemia, addition of 50 to 150 mg alirocumab every 2 weeks to 10 to 40 mg/d atorvastatin decreased LDL-C by 40% to 72% and TC by 23% to 45%.[25] In a phase II trial in patients with FH, addition of 150 mg of alirocumab every 2 weeks to a stable dose of statin, with or without ezetimibe, decreased LDL-C by 68% and TC by 44%.[24] A multicenter phase II trial confirmed these findings with a 66% to 73% reduction in LDL-C and a 41% to 47% reduction in TC when adding alirocumab to either 10 or 80 mg/d atorvastatin in hypercholesterolemic patients.[26] In the present study, a similar additive cholesterol-lowering effect on top of atorvastatin treatment (-36% to -48% reductions in TC as compared with atorvastatin alone) was found. The clinical trials also provide evidence for additional modest reductions in TGs and modest increases in HDL-C. However, baseline TG levels were low in the latter studies, which may explain the larger effect on TG found in our study.

A higher (V)LDL clearance increases liver cholesterol exposure and may result in changes in hepatic cholesterol content and/or fecal excretion of cholesterol or, after its conversion, bile acids. However, alirocumab did not lead to hepatic lipid accumulation, whereas atorvastatin and the combination treatments significantly reduced cholesteryl ester content without changes in hepatic TG. Intriguingly, fecal output of bile acids and neutral sterols remained unchanged by the treatments. In line with our data, full absence of PCSK9 was recently reported not to be associated with hepatic lipid accumulation or fecal excretion of cholesterol.[45] However, contrasting data in PCSK9 -/- mice demonstrated increased LDL-C excretion via the transintestinal cholesterol excretion pathway and subsequently mildly increased fecal neutral sterol loss, with unfortunately no data on fecal bile acid loss.[46] As opposed to PCSK9 inhibition by alirocumab in the present study, lack of PCSK9 was reported to increase fecal bile acid output.[45] These data indicate that, despite the greater influx of cholesterol from the plasma compartment into the liver, hepatic cholesterol homeostasis is maintained, although the precise mechanism remains to be established.

The lipid-modifying effects of PCSK9 inhibition provide indications for an atheroprotective effect. This notion is supported by data from a study where mice expressing high levels of PCSK9 had significantly more aortic cholesterol ester accumulation, and developed severe aortic lesions, compared with wild-type and PCSK9 knockout mice when fed an atherogenic diet.[11] In the same study, no differences were found in LDLR-deficient mice expressing no, normal, or high PCSK9 levels, suggesting that PCSK9 modulates atherosclerosis mainly via the LDLR. However, to date, the atheroprotective effect of pharmacological PCSK9 inhibition has not been investigated. Our study demonstrates for the first time that inhibition of serum PCSK9 with the monoclonal antibody, alirocumab decreases plasma lipid levels and as a result reduces atherosclerosis development, as evidenced by a reduction in atherosclerotic lesion size and severity in the aortic root area and arch. This dose-dependent inhibitory effect of alirocumab on lesion size was strongly enhanced in combination with atorvastatin,

where a considerable number of animals did not develop any severe lesions. Although pleiotropic effects of statin treatment may contribute to the reduction in CVD risk,[47] results from our study emphasize the importance of cholesterol-lowering per se in treatment of CVD. In our study, the effects of alirocumab alone and in combination with atorvastatin on lesion area were mainly predicted by the reduction in plasma TC levels as illustrated by the strong association ($R^2 = 0.84$) between TC levels and the lesion area.

In the present study, alirocumab, atorvastatin, and their combination reduced the circulating granulocytes/neutrophils and monocytes, in particular pro-inflammatory Ly6C^{hi} monocytes. Ly6C^{hi} monocytes are proposed to be precursors of pro-inflammatory M1 macrophages[48] and studies in mice have shown that hypercholesterolemia induces Ly6C^{hi} monocytosis.[49] In addition, alirocumab decreased endothelial expression of ICAM-1 and consequently reduced monocyte adhesion to the vascular endothelium. In hypercholesterolemia, modified lipoproteins induce endothelium activation, thereby mediating the arrest and transmigration of circulating monocytes into the subendothelial space where they differentiate into macrophages.[48]

In addition to monocyte adhesion, alirocumab reduced other markers of vascular inflammation, including T cell abundance in the aortic root area, as well as macrophage and necrotic content and cholesterol clefts of the lesions. Cholesterol crystals have been shown to be particularly pro-inflammatory and to trigger local and systemic inflammatory responses.[50, 51] Moreover, increased macrophage content and a large necrotic core, as well as a thin, collagen-poor fibrous cap and decreased SMCs, are important characteristics of a vulnerable lesion that is prone to rupture.[47] Alirocumab alone and in combination with atorvastatin reduced vascular inflammation and strongly improved the plaque morphology.

The present study is a progression/prevention study which may pose as a potential limitation with respect to translation to the clinic where patients with existing lesions are often treated. Nonetheless, data from this study may also suggest beneficial effects on markers of atherosclerosis by reducing TC with alirocumab in the human situation where new lesions are formed alongside existing plaques.

PCSK9 has received a considerable amount of attention in the last decade as a possible target for treatment of CVD, and several approaches to inhibit the protein are currently being investigated.[4] Efficacy and safety of alirocumab will be further investigated in large phase III clinical outcome trials, in patients with FH and in high cardiovascular risk patients with hypercholesterolemia on lipid-modifying therapy within the ODYSSEY program.[44] These trials will reveal whether PCSK9 inhibition with alirocumab translates into clinical benefit.

Acknowledgements

Erik Offerman, Anita van Nieuwkoop, Simone van der Drift-Droog and Marijke Voskuilen (TNO) are thanked for their excellent technical assistance. Editorial support was provided by Rob Campbell of Prime Medica Ltd. (Knutsford, Cheshire, UK), supported by Sanofi-Aventis and Regeneron.

Funding

The study was sponsored by Sanofi-Aventis and Regeneron.

Disclosures

JWJ received research grants from and was speaker on (CME-accredited) meetings sponsored by Amgen, Astellas, Astra-Zeneca, Biotronik, Boston Scientific, Daiichi Sankyo, Lilly, Genzyme, Medtronic, Merck-Schering-Plough, Pfizer, Orbus Neich, Novartis, Roche, Servier, Sanofi-Aventis, the Netherlands Heart Foundation, the Interuniversity Cardiology Institute of the Netherlands, and the European Community Framework KP7 Programme. WJS and VG are employees of Regeneron. AP, HS and US are employees of Sanofi-Aventis.

References

1. Horton JD, Cohen JC and Hobbs HH. Molecular biology of PCSK9: its role in LDL metabolism. *Trends in biochemical sciences*. 2007;32:71-7.
2. Seidah NG and Prat A. The biology and therapeutic targeting of the proprotein convertases. *Nature Reviews Drug Discovery*. 2012;11:367-383.
3. Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villeger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG and Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature genetics*. 2003;34:154-6.
4. Lambert G, Sjouke B, Choque B, Kastelein JJ and Hovingh GK. The PCSK9 decade. *Journal of lipid research*. 2012;53:2515-24.
5. Benn M, Nordestgaard BG, Grande P, Schnohr P and Tybjaerg-Hansen A. PCSK9 R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease: 3 independent studies and meta-analyses. *Journal of the American College of Cardiology*. 2010;55:2833-42.
6. Cohen JC, Boerwinkle E, Mosley TH, Jr. and Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *The New England journal of medicine*. 2006;354:1264-72.
7. Cariou B, Ouguerram K, Zair Y, Guerois R, Langhi C, Kourimate S, Benoit I, Le May C, Gayet C, Belabbas K, Dufernez F, Chetiveaux M, Tarugi P, Krempf M, Benlian P and Costet P. PCSK9 dominant negative mutant results in increased LDL catabolic rate and familial hypobetalipoproteinemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:2191-7.
8. Lagace TA, Curtis DE, Garuti R, McNutt MC, Park SW, Prather HB, Anderson NN, Ho YK, Hammer RE and Horton JD. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. *The Journal of clinical investigation*. 2006;116:2995-3005.
9. Maxwell KN and Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:7100-5.
10. Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA and Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:5374-9.
11. Denis M, Marcinkiewicz J, Zaid A, Gauthier D, Poirier S, Lazure C, Seidah NG and Prat A. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. *Circulation*. 2012;125:894-901.
12. Davidson MH, Maki KC, Pearson TA, Pasternak RC, Deedwania PC, McKenney JM, Fonarow GC, Maron DJ, Ansell BJ, Clark LT and Ballantyne CM. Results of the National Cholesterol Education (NCEP) Program Evaluation ProjecT Utilizing Novel E-Technology (NEPTUNE) II survey and implications for treatment under the recent NCEP Writing Group recommendations. *The American journal of cardiology*. 2005;96:556-63.
13. Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R and Baigent C. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *The Lancet*. 2012;380:581-590.
14. Cannon CP, Braunwald E, McCabe CH, Rader DJ, Rouleau JL, Belder R, Joyal SV, Hill KA, Pfeffer MA and Skene AM. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *The New England journal of medicine*. 2004;350:1495-504.
15. LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J and Wenger NK. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *The New England journal of medicine*. 2005;352:1425-1435.
16. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT and Glynn RJ.

Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *The New England journal of medicine*. 2008;359:2195-2207.
17. Grundy SM, Cleeman JI, Merz CN, Brewer HB, Jr., Clark LT, Hunninghake DB, Pasternak RC, Smith SC, Jr. and Stone NJ. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. *Journal of the American College of Cardiology*. 2004;44:720-32.
18. Reiner Z, Catapano AL, De Backer G, Graham I, Taskinen MR, Wiklund O, Agewall S, Alegria E, Chapman MJ, Durrington P, Erdine S, Halcox J, Hobbs R, Kjekshus J, Filardi PP, Riccardi G, Storey RF and Wood D. ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). *European heart journal*. 2011;32:1769-818.
19. Expert Dyslipidemia Panel and Grundy SM. An International Atherosclerosis Society Position Paper: global recommendations for the management of dyslipidemia. *Journal of clinical lipidology*. 2013;7:561-5.
20. Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L and Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:1454-9.
21. Mayne J, Dewpura T, Raymond A, Cousins M, Chaplin A, Lahey KA, Lahaye SA, Mbikay M, Ooi TC and Chretien M. Plasma PCSK9 levels are significantly modified by statins and fibrates in humans. *Lipids in health and disease*. 2008;7:22.
22. Careskey HE, Davis RA, Alborn WE, Troutt JS, Cao G and Konrad RJ. Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *Journal of lipid research*. 2008;49:394-8.
23. Stein EA, Mellis S, Yancopoulos GD, Stahl N, Logan D, Smith WB, Lisbon E, Gutierrez M, Webb C, Wu R, Du Y, Kranz T, Gasparino E and Swergold GD. Effect of a monoclonal antibody to PCSK9 on LDL cholesterol. *The New England journal of medicine*. 2012;366:1108-18.
24. Stein EA, Gipe D, Bergeron J, Gaudet D, Weiss R, Dufour R, Wu R and Pordy R. Effect of a monoclonal antibody to PCSK9, REGN727/SAR236553, to reduce low-density lipoprotein cholesterol in patients with heterozygous familial hypercholesterolaemia on stable statin dose with or without ezetimibe therapy: a phase 2 randomised controlled trial. *The Lancet*. 2012;380:29-36.
25. McKenney JM, Koren MJ, Kereiakes DJ, Hanotin C, Ferrand AC and Stein EA. Safety and efficacy of a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 serine protease, SAR236553/REGN727, in patients with primary hypercholesterolemia receiving ongoing stable atorvastatin therapy. *Journal of the American College of Cardiology*. 2012;59:2344-53.
26. Roth EM, McKenney JM, Hanotin C, Asset G and Stein EA. Atorvastatin with or without an antibody to PCSK9 in primary hypercholesterolemia. *The New England journal of medicine*. 2012;367:1891-900.
27. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
28. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR and Havekes LM. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *The Journal of clinical investigation*. 1991;88:643-55.
29. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH and Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *The Journal of clinical investigation*. 1994;93:1403-10.
30. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1706-21.

31. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
32. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.
33. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
34. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
35. Kawashiri MA, Kobayashi J, Nohara A, Noguchi T, Tada H, Nakanishi C, Inazu A, Mabuchi H and Yamagishi M. Impact of bezafibrate and atorvastatin on lipoprotein subclass in patients with type III hyperlipoproteinemia: result from a crossover study. *Clinica chimica acta; international journal of clinical chemistry*. 2011;412:1068-75.
36. Kuhnast S, van der Hoorn JW, van den Hoek AM, Havekes LM, Liau G, Jukema JW and Princen HM. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden. CETP transgenic mice with or without treatment with atorvastatin. *Journal of hypertension*. 2012;30:107-16.
37. Post SM, Zoeteweij JP, Bos MH, de Wit EC, Havinga R, Kuipers F and Princen HM. Acyl-coenzyme A:cholesterol acyltransferase inhibitor, avasimibe, stimulates bile acid synthesis and cholesterol 7alpha-hydroxylase in cultured rat hepatocytes and in vivo in the rat. *Hepatology*. 1999;30:491-500.
38. Post SM, de Crom R, van Haperen R, van Tol A and Princen HM. Increased fecal bile acid excretion in transgenic mice with elevated expression of human phospholipid transfer protein. *Arteriosclerosis, thrombosis, and vascular biology*. 2003;23:892-7.
39. Delsing DJM, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit ECM, Gijbels MJJ, van der Laarse A, Jukema JW, Havekes LM and Princen HMG. Acyl-CoA:Cholesterol Acyltransferase Inhibitor Avasimibe Reduces Atherosclerosis in Addition to Its Cholesterol-Lowering Effect in ApoE*3-Leiden Mice. *Circulation*. 2001;103:1778-1786.
40. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD and Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis, thrombosis, and vascular biology*. 1995;15:1512-31.
41. Verschuren L, Kleemann R, Offerman EH, Szalai AJ, Emeis SJ, Princen HM and Kooistra T. Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:161-7.
42. Kuhnast S, Louwe MC, Heemskerk MM, Pieterman EJ, van Klinken JB, van den Berg SA, Smit JW, Havekes LM, Rensen PC, van der Hoorn JW, Princen HM and Jukema JW. Niacin Reduces Atherosclerosis Development in APOE*3Leiden.CETP Mice Mainly by Reducing NonHDL-Cholesterol. *PloS one*. 2013;8:e66467.
43. Verschuren L, de Vries-van der Weij J, Zadelaar S, Kleemann R and Kooistra T. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. *Journal of lipid research*. 2009;50:301-11.
44. Stein EA and Swergold GD. Potential of proprotein convertase subtilisin/kexin type 9 based therapeutics. *Current atherosclerosis reports*. 2013;15:310.
45. Parker RA, Garcia R, Ryan CS, Liu X, Shipkova P, Livanov V, Patel P and Ho SP. Bile acid and sterol metabolism with combined HMG-CoA reductase and PCSK9 suppression. *Journal of lipid research*. 2013;54:2400-9.

46. Le May C, Berger JM, Lespine A, Pillot B, Prieur X, Letessier E, Hussain MM, Collet X, Cariou B and Costet P. Transintestinal cholesterol excretion is an active metabolic process modulated by PCSK9 and statin involving ABCB1. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33:1484-93.
47. Libby P and Sasiela W. Plaque stabilization: Can we turn theory into evidence? *The American journal of cardiology*. 2006;98:26P-33P.
48. Moore KJ, Sheedy FJ and Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nature reviews Immunology*. 2013;13:709-21.
49. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R and Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *The Journal of clinical investigation*. 2007;117:195-205.
50. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *The New England journal of medicine*. 2013;368:2004-13.
51. Abela GS. Cholesterol crystals piercing the arterial plaque and intima trigger local and systemic inflammation. *Journal of clinical lipidology*. 2010;4:156-64.

CHAPTER 7

Innovative Pharmaceutical Interventions in Cardiovascular Disease: Focusing on the Contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising

A systematic review and meta-analysis of relevant preclinical studies and clinical trials

Susan Kühnast[1,2,3], Marta Fiocco[4,5], José W.A. van der Hoorn[1,2,3], Hans M.G. Princen[1*], J. Wouter Jukema[2*]

[1]TNO - Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands;
[2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands;
[3]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands;
[4]Dept. of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands;
[5]Mathematical Institute, Leiden University, Leiden, The Netherlands

[*]HMGP and JWJ contributed equally

Accepted – Eur J Pharmacol.

Abstract

Introduction Cholesterol contained in LDL particles is well recognized as a primary causal risk factor for cardiovascular disease. However, despite consistent epidemiological evidence for an inverse association between HDL-C and coronary heart disease, clinical trials aimed at raising HDL-C (AIM-HIGH, HPS2-THRIVE, dal-OUTCOMES) failed to meet their primary goals. This systematic review and meta-analysis investigated the effects of established and novel treatment strategies, specifically targeting HDL, on inhibition of atherosclerosis in cholesteryl ester transfer protein-expressing animals, and the prevention of clinical events in randomized controlled trials.

Methods and Results Linear regression analyses using data from preclinical studies revealed associations for TC and non-HDL-C and lesion area (R^2=0.258, P=0.045; R^2=0.760, P<0.001), but not for HDL-C (R^2=0.030, P=0.556). In clinical trials, non-fatal myocardial infarction risk was significantly less in the treatment group with pooled odd ratios of 0.87 [0.81; 0.94] for all trials and 0.85 [0.78; 0.93] after excluding some trials due to off-target adverse events, whereas all-cause mortality was not affected (OR 1.05 [0.99-1.10]). Meta-regression analyses revealed a trend towards an association between between-group differences in absolute change from baseline in LDL-C and non-fatal myocardial infarction (P=0.066), whereas no correlation was found for HDL-C (P=0.955).

Discussion We conclude that the protective role of lowering LDL-C and non-HDL-C is well-established. The contribution of raising HDL-C on inhibition of atherosclerosis and the prevention of cardiovascular disease remains undefined and may be dependent on the mode of action of HDL-C-modification. Nonetheless, treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may be worth exploring.

Keywords HDL-C-raising pharmaceutical interventions; preclinical studies; randomized controlled trials; systematic review; meta-analysis

Content

1. Introduction
2. Methods
2.1 Preclinical studies
    2.1.1 Literature search strategy
    2.1.2 Study selection
    2.1.3 Quality assessment and data extraction
    2.1.4 Data presentation and analysis
    2.1.5 Statistical analysis
2.2 Randomized controlled clinical trials
    2.2.1 Literature search strategy
    2.2.2 Study selection
    2.2.3 Quality assessment and data extraction
    2.2.4 Data presentation and analysis
    2.2.5 Statistical analysis
3. Results
3.1 Preclinical studies
    3.1.1 Reference screening
    3.1.2 Study design and baseline lipid levels
    3.1.3 Effect of lipid-modifying treatment strategies on atherosclerosis development
    3.1.4 The association between TC/non-HDL-C/HDL-C and atherosclerotic lesion area
3.2 Randomized controlled clinical trials
    3.2.1 Reference screening
    3.2.2 Trial design and baseline characteristics
    3.2.3 Effect of lipid-modifying treatment strategies on clinical outcomes
    3.2.4 Effect of treatment on the prevention of non-fatal myocardial infarction in patient populations with low versus high baseline LDL-C
    3.2.5 The association between LDL-C/HDL-C and non-fatal myocardial infarction
4. Discussion
4.1 Treatment strategies aimed at raising HDL-C and atherosclerosis: preclinical studies
    4.1.1 Niacin
    4.1.2 Peroxisome proliferator-activated receptor (PPAR) agonists
        4.1.2.1 PPAR-α agonists
        4.1.2.2 PPAR-γ agonists
        4.1.2.1 PPAR-α/γ agonists
    4.1.3 Cholesteryl ester transfer protein (CETP) inhibition

4.1.4 Scavenger receptor B-I (SR-BI) inhibitor and ATP-binding cassette A1 (ABCA1) degradation inhibitors
4.2 Treatment strategies aimed at raising HDL-C and cardiovascular disease: randomized controlled clinical trials
4.2.1 The effect of treatment on clinical outcomes in patient populations with high baseline LDL-C
4.2.2 The effect of treatment on clinical outcomes in patient populations with low baseline LDL-C
4.2.3 The effect of treatment on clinical outcomes in trials excluded from the meta-analyses due to serious off-target cardiovascular adverse events
4.3 Novel treatment strategies specifically targeting HDL on atherosclerosis: preclinical studies and clinical trials
4.3.1 Purified, reconstituted and delipidated HDL
4.3.2 Apolipoprotein A-I Milano
4.3.3 Apolipoprotein mimetic peptides
4.3.4 Apolipoprotein A-I inducer
4.4 Limitations
4.5 Current status and future perspectives
5. Conclusion

1. Introduction

In 1913, Nikolai N. Anitschkow first described the involvement of cholesterol in atherosclerosis development when rabbits fed a high-cholesterol diet developed human-like arterial lesions.[1] The recent 100th year anniversary of this discovery is worth commemorating given that serum cholesterol contained in low-density lipoprotein (LDL) particles is now well recognized as a primary causal risk factor for cardiovascular disease as evidenced by experimental, epidemiological and genetic studies.[2] Indeed, intervention trials with statin therapy confirmed a reduced incidence of coronary heart disease as a consequence of cholesterol-lowering[3, 4] and recent trials indicated that intensive lipid-lowering with statins may be more beneficial in risk reduction than less intensive (or standard) therapy.[5] According to results from the latter meta-analysis, every 1 mmol/L (40 mg/dL) reduction in LDL-C was associated with a 22% reduction in the risk of major vascular events suggesting that a 2-3 mmol/L reduction in LDL-cholesterol (LDL-C) would correspond with a 40-50% reduction in events. However, treatment of cardiovascular disease remains suboptimal due to (i) the residual risk that persists after statin treatment,[6] (ii) failure for some patients to reach LDL-C targets despite statin treatment,[7] and (iii) lack of adherence as a result of statin intolerance.[8] Therefore, the search for secondary treatment targets is warranted.

In the 1970s, Miller & Miller hypothesized that a reduction in plasma high-density lipoprotein (HDL) concentration may accelerate the development of atherosclerosis and ischemic heart disease by impairing cholesterol clearance from the arterial wall.[9] Besides its major role in reverse cholesterol transport, HDL has also been described to have anti-inflammatory, anti-oxidant, anti-platelet and vasodilatory properties.[10] Although the original hypothesis referred to HDL particle concentration which could not be measured at the time,[10] epidemiological studies consistently reported an inverse association between coronary heart disease risk and HDL-C.[11-13] Results from 4 prospective epidemiologic studies indicated that an increase of 1 mg/dL (0.03 mM) in HDL-C was associated with a 2-3% reduction in coronary heart disease risk.[14]

Several therapeutic approaches aimed at raising HDL-C levels have since been investigated. However, undisputed proof for causality of low HDL-C in cardiovascular disease is lacking and clinical trials aimed at raising HDL-C to prevent disease (AIM-HIGH, HPS2-THRIVE, dal-OUTCOMES) have failed to meet their primary goals.[15-17] In addition, data from Mendelian randomization studies showed that genetic variants related to altered plasma HDL-C *per se* were not associated with risk of myocardial infarction,[18, 19] and that despite an inverse correlation, HDL-C and myocardial infarction risk are not causally related. Nonetheless, numerous therapeutic strategies aimed at raising HDL-C or improving HDL function are still under investigation in preclinical studies and clinical trials.

This systematic review investigated the effects of established and novel treatment strategies, specifically targeting HDL, on inhibition of atherosclerosis development in cholesteryl ester transfer protein (CETP)-expressing animals, since CETP is a crucial gene involved in HDL metabolism and implicated in the mechanisms by which most therapies modulate HDL.[20] In addition, we conducted a meta-analysis to evaluate the potential effects of these treatment strategies on the prevention of clinical events in randomized controlled trials, focusing specifically on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising.

2. Methods

The study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines using a PRISMA checklist.[21]

2.1 Preclinical studies

2.1.1 Literature search strategy

To identify relevant preclinical studies, we performed a computerized search of PUBMED and EMBASE. The search was restricted to studies published in the English-language from January 1975 to current. The following treatment strategies, specifically targeting HDL were included in the search: niacin; peroxisome proliferator-activated receptor (PPAR)-α agonists (fibrates); PPAR-γ agonist (glitazones); PPAR-α/γ agonists (glitazars); PPAR-δ agonists; CETP inhibitors; liver X receptor (LXR) agonists; micro RNAs; reconstituted HDL and apolipoprotein A-I-based compounds. We focused on preclinical studies evaluating the effects of these treatment strategies on atherosclerosis development in CETP-expressing animals. Statins were not included in the search criteria since the effects of statins on HDL/apolipoprotein A-I in relation to clinical outcomes were recently extensively reviewed.[22] Additional studies were identified by searching bibliographies from relevant studies and additional review articles.

2.1.2 Study selection

To investigate the role of HDL-C-raising treatment strategies on atherosclerosis development, we included preclinical studies that reported an increase in HDL-C. The effect of reconstituted HDL and apolipoprotein A-I-based compounds were described regardless of an effect on lipids, since several studies revealed protection against atherosclerosis with no change in plasma lipids. Studies were excluded if the relevant compound was used in a control group. All studies were screened to eliminate irrelevant studies by title and abstract. Remaining records were screened based on a review of the full text.

2.1.3 Quality assessment and data extraction

The following data were extracted from relevant preclinical studies: study design (compound, animal model, sex, diet, run-in phase, group size, dose and treatment phase), baseline and on-treatment serum/plasma total cholesterol (TC) and HDL-C levels, as well as atherosclerotic lesion area. The data were extracted by one author (SK) and thoroughly checked by another author (JWAvdH). Disagreements between authors were resolved by consensus.

2.1.4 Data presentation and analysis

To evaluate the effects of lipid-modifying treatment strategies on atherosclerosis, the percentage difference in atherosclerotic lesion area (gain) between the control and the treatment group was reported for all preclinical studies. Plasma/serum TC and HDL-C levels were retrieved for all time points reported in these studies. If not reported, non-HDL-C levels were calculated (TC – HDL-C). TC, non-HDL-C and HDL-C levels were standardized by converting mmol/L to mg/dL by multiplying by 38.67. Where possible, the percentage difference in TC, non-HDL-C and HDL-C exposure (duration of intervention in weeks x cholesterol levels) between the control and the treatment groups were calculated from the retrieved data and correlated with the between-group percentage difference in atherosclerotic lesion area. Reconstituted HDL and apolipoprotein A-I-based treatment strategies were not included in the correlations, but described in the discussion section due to different mechanisms of action.

2.1.5 Statistical analysis

Linear regression analyses were used to assess the association between the percentage difference in TC, non-HDL-C and HDL-C exposure and atherosclerotic lesion area between the control and the treatment groups.

2.2 Randomized controlled clinical trials

2.2.1 Literature search strategy

To find relevant randomized controlled clinical trials, we performed a search of PUBMED including studies from clinicaltrials.gov, a clinical trial registry and results database, and EMBASE. We included the same restrictions and treatment strategies as previously described for preclinical studies (see 2.1.1). The search involved clinical trials reporting major cardiovascular events and we searched for phase II, III and IV clinical trials, multicenter, randomized controlled trials and meta-analyses. Additional studies were identified by searching bibliographies from relevant trials, as well as meta-analyses and review articles. The study authors were not contacted regarding the retrieval of unpublished data.

2.2.2 Study selection

Inclusion criteria used for the selection of clinical trials for pooled meta-analyses were as follows:

- A randomized placebo-controlled trial design
- Patients with type 2 diabetes or cardiovascular disease or patients at risk of developing cardiovascular disease
- A trial sample size of ≥ 200 participants in each study arm
- A mean follow up duration of ≥ 1 year
- Pharmaceutical HDL-C-raising agents
- At least two of the following clinical outcomes: all-cause mortality, coronary heart disease mortality, non-fatal myocardial infarction and stroke

After discarding irrelevant records based on title and abstract, relevant articles were selected based on full text screening.

2.2.3 Quality assessment and data extraction

Study design (compound, study population, follow up duration and sample size), baseline characteristics (age, sex, BMI, history of cardiovascular disease, diabetes, hypertension, smoking, myocardial infarction, stroke, angina, revascularization, heart failure, peripheral vascular disease and previous statin use), baseline and on-treatment TC, HDL-C, LDL-C and non-HDL-C levels, as well as the occurrence of clinical events (all-cause mortality, coronary heart disease mortality, non-fatal myocardial infarction and stroke) were obtained from selected clinical trials. The data were extracted by one author (SK) and thoroughly checked by another author (JWAvdH). Disagreements between authors were resolved by consensus.

2.2.4 Data presentation and analysis

We performed 4 separate meta-analyses to analyze the effects of treatment on the prevention of all-cause mortality, coronary heart disease mortality, non-fatal myocardial infarction and stroke in randomized controlled trials. These 4 meta-analyses were repeated after excluding a number of trials using compounds with serious off-target cardiovascular adverse events. These include trials with torcetrapib and aleglitazar of which clinical development was stopped due to adverse effects, as well as pioglitazone which was shown to increase heart failure.[23-28] Other primary endpoint data were not included in this meta-analysis, because of different composite endpoints for the various trials. In addition, we performed 2 meta-analyses to assess the effects of treatment on the prevention of non-fatal myocardial infarction in patients with low versus high baseline LDL-C by dividing the remaining trials into 2 subgroups using LDL-C levels of 100 mg/dL as the cut-off. In fact, the trials with lower baseline LDL-C levels concern patients on statin treatment (60%-100% of the subjects). In this regard, patients in the ACCORD trial had an average LDL-C of 100.6

mg/dL, and 60% of these patients received statin treatment at trial entry. We, therefore, included this study in the subgroup analysis of patients with low baseline LDL-C.[29]

Baseline and on-treatment TC, LDL-C, non-HDL-C and HDL-C levels were standardized by converting mmol/L to mg/dL by multiplying by 38.67. When lipid data were presented for multiple time points, we reported results from the longest follow up period. To determine the between-group differences in lipid changes, we calculated the difference between the absolute and the percentage change from baseline in the control group and the treatment group. Meta-regression analyses were performed to assess the potential association between the between-group differences in absolute and percentage change from baseline in LDL-C, as well as HDL-C and the occurrence of non-fatal myocardial infarction.

2.2.5 Statistical analysis

A random effects model was employed to pool clinical trial-specific odds ratios in order to estimate an overall odds ratio and its associated confidence intervals. Inverse variance method which gives more weight to larger trials was used to pool outcomes for different trials. The overall effects corresponding to a fixed and random effects model are reported together in the same forest plot along with their confidence intervals. The sizes of the square boxes on the forest plots are proportional to the total number of patients in the selected trials. An overall test on heterogeneity between studies was performed for each separate meta-analysis (value I-squared in figures). To estimate the between-study variance, which is represented as 'tau' in the forest plots, DerSimonian-Laird's method has been employed.[30] The log-transformed odd ratios for myocardial infarction was modeled as a linear function of the between-group differences in absolute and percentage change from baseline in LDL-C, as well as HDL-C by employing meta-regression. All statistical analyses were performed using R version 2.18. (http://cran.rproject.org/).

3. Results

3.1 Preclinical studies

3.1.1 Reference screening

The computerized search identified 967 records of which 119 duplicates were removed. The remaining 848 records were screened based on title and abstract and an additional 729 records were excluded. After reviewing 119 full text articles, 92 irrelevant records were removed and the results of 29 preclinical studies, including 2 studies that were not identified in the original search, were included in the systematic review (**Figure 1**).

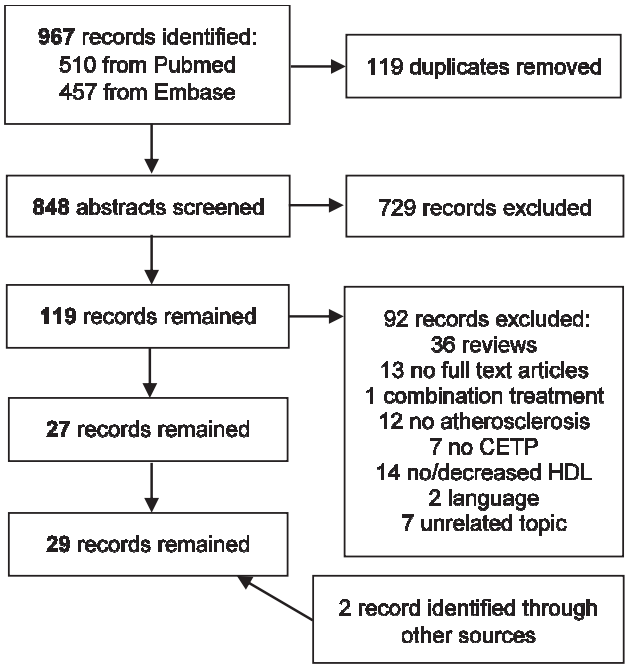


Figure 1 An outline of the systematic search conducted to identify relevant preclinical studies for the systematic review.

3.1.2 Study design and baseline lipid levels

The study design and baseline lipid levels for the selected preclinical studies are summarized in **Table 1**. In these studies, the effects of niacin,[31, 32] PPAR-α agonists,[33, 34] PPAR α/γ agonist,[35] CETP antisense,[36] CETP vaccines,[37-40] CETP inhibitors,[41-45] SR-BI inhibitor,[46] ABCA1 degradation inhibitors,[47] purified or reconstituted HDL,[48-51] apolipoprotein A-I Milano[52-57] and apolipoprotein A-I mimetic peptide[58, 59] on atherosclerosis development were investigated in APOE*3Leiden.CETP and *ldlr*$^{+/-}$.CETP mice, New Zealand White (NZW), Japanese White (JW) and Watanabe heritable hyperlipidemic (WHHL) rabbits, as well as F1B hamsters. Whereas several studies were designed to detect a reduction in the progression of atherosclerosis, a number of lipid-modifying treatment studies[33-35] and most purified and reconstituted HDL and apolipoprotein A-I-based studies investigated the effects of treatment on existing atherosclerosis in a regression set-up. All animals received a cholesterol-containing diet except for WHHL rabbits, which spontaneously develop atherosclerosis. In a number of studies, atherosclerosis was induced by collar placement, electric and balloon injury/denudation.[33, 51-57] The effects of PPAR-δ agonists[60] and miR-33 antagonism[61] on atherosclerosis development were investigated in animals that do not express CETP and according to or knowledge have not been tested in an animal model with a more human-like

lipoprotein metabolism. Studies of LXR and FXR agonists in CETP-expressing animals were not included in this review, since no increase in HDL-C was observed except for the study by Srivastava *et al.* evaluating the anti-atherosclerotic activities of PPAR-α, PPAR-γ and LXR agonist (T0901317) in F1B hamsters.[34]

3.1.3 Effect of lipid-modifying treatment strategies on atherosclerosis development

All lipid-modifying treatment strategies decreased atherosclerotic lesion area, except dalcetrapib.[42] In this study, the treatment also failed to reduce TC levels despite an increase in HDL-C.

Table 1 Design and baseline lipid levels of the selected preclinical studies.

Study	**Year**	**Compound**	**Animal model**	**Sex**	**Diet**	**Run-in phase** (weeks)	**Group size** C/T	**Dose** (mg/kg/d)	**Treatment phase** (weeks)	**Baseline TC/*LDL-C*** (mg/dL)	**Baseline HDL-C** (mg/dL)
Parwaresch	1978	Nicotinic acid Xantinol-nicotinate* β-pyridylcarbinol* Pirozadil*	NZW rabbits	M	Diet + 3% cholesterol	0	26/19 26/33 26/28 26/19	50 50 50 50	12	958.0	19.0
Kühnast	2013	Niacin	E3L.CETP mice	F	WTD + 0.1% cholesterol	3	15/15	120	18	440.8	21.3
Corti	2007	Fenofibrate	NZW rabbits	M	Diet + 0.2% cholesterol	39: Induction after 4 & 13	6/6	ns	26	*736.7*	57.3
van der Hoorn	2009	Tesaglitazar	E3L.CETP mice	F	WTD + 0.3% or 0.1% cholesterol	15: 11 high + 4 low cholesterol	16/16	10 µg	8: low cholesterol	421.5	ns
Srivastava	2011	Fenofibrate	F1B hamsters	ns	High fat high cholesterol	2 11	11/11 10/10	100 100	13	ns	ns
Sugano	1998	CETP antisense oligonucleotides	JW rabbits	M	Chow + 0.3% cholesterol	8	6/6	30 µg/kg 2x/week	8	270.0	19.0
Rittershaus	2000	CETP vaccine	NZW rabbits	ns	Chow + 0.25% cholesterol	0	12/12	Week 1, 5, 8, 16, 22	32: high cholesterol from 16	ns	ns
Gaofu	2005	CETP vaccine	NZW rabbits	F	Chow + 0.25% cholesterol	0	6/6	Week 1, 5, 10, 16, 22	26: high cholesterol from 12	130.7	7.0
Mao	2006	CETP vaccine	NZW rabbits	F	Diet + 0.5g cholesterol	0	5/5	Week 1, 3, 7, 11, 16, 22	26: high cholesterol from 11	130.7	7.0
Jun	2012	CETP vaccine	NZW rabbits	M	Diet + 0.5% cholesterol	0	8/8	Week 0, 2, 4, 6, 8	25: high cholesterol from 9	ns	ns
Okamoto	2000	Dalcetrapib	JW rabbits	M	Chow + 0.2% cholesterol	5	10/10	255	26	274.0	17.5
Huang	2002	Dalcetrapib	JW rabbits	M	Chow + 0.25% cholesterol	4	10/8 10/10	100 300	12	406.0	21.0
Morehouse	2007	Torcetrapib	NZW rabbits	M	Diet + 0.2% cholesterol	5 days	23/24	90 to 60	16: 3 dose finding	122.0	57.0 (after 1 week)
De Haan	2008	Torcetrapib	E3L.CETP mice	F	WTD + 0.25% cholesterol	4	15/15	12	14	653.5	ns
Kühnast	2014	Anacetrapib	E3L.CETP mice	F	WTD + 0.1% cholesterol	5	15/15 15/15 15/15 15/15	0.03 0.3 3 30	21	514.3	41.4
Masson	1990	SRBI inhibitor, ITX5061	*ldlr*$^{+/-}$ mice AAV CETP	ns	Diet + 1.25% cholesterol	0	10/10	0.037%	18	509.0	54.1
Arakawa	2009	ABCA1 degradation inhibitors, spiroquinone and diphenoquinone	NZW rabbits	M	Diet + 0.5% cholesterol	0	8/8 8/8	25 25	8	ns	19.0

Badimon	1990	HDL-VHDL plasma fraction	NZW rabbits	ns	Chow + 0.5% cholesterol	9	7/7	50 mg HDL/VHDL protein (1x/week)	4	1559.0	ns
Mezdour	1995	Reconstituted apoAI-containing HDL ± reconstituted TG-rich lipoprotein	JW rabbits	M	Diet + 0.5% cholesterol	0	7/6 7/4	18 mg apoAI-rHDL + Intralipid 18 mg apoAI-rHDL + 2.5 ml rTRL + 4 mg apoE (1x/week)	8	58.0	25.0
Miyazaki	1995	Purified rabbit apoAI	NZW rabbits	M	Chow ± 0.5% cholesterol	9:w cholesterol 15:w cholesterol	14/14 8/8 8/8	40 mg apoAI/week 1 mg apoAI/2 days 40 mg apoAI/week	4: w cholesterol 9: w/o cholesterol 9: w/o cholesterol	2500.0 2000.0 2000.0	ns ns ns
Nicholls	2005	Reconstituted apoAI-containing HDL or rabbit HDL	NZW rabbits	M	Chow + 0.2% cholesterol	16: Induction after 1	15/6 15/5 15/8	2x 25 mg apoAI + PLPC 2x 25 mg apoAI + DPPC Rabbit HDL	5 days (treatment on day 1 and 3)	483.4	ns
Ameli	1994	Recombinant apoAI Milano	NZW rabbits	M	Diet + 1% cholesterol	18 days	4/8	40 mg $apoAI_M$ + PC-C (/2 days for 10 days)	4: Induction 4 days after start of treatment	606.0	24.0
Soma	1995	Recombinant apoAI Milano dimer	NZW rabbits	M	Diet + 1% cholesterol	3: Induction after 3 weeks	5/5 5/5	40 mg $apoAI_M$ + EPC (at -5, -3, -1, 1, 3 days) 40 mg $apoAI_M$ + EPC (at 0, 2, 4, 6, 8 days)	10 days	930.0	ns
Chiesa	2002	Recombinant apoAI Milano	NZW rabbits	M	Diet + 1.5% cholesterol	13: Induction at start	5/5 5/5 5/5	250 mg $apoAI_M$ + DPPC 500 mg $apoAI_M$ + DPPC 1000 mg $apoAI_M$ +DPPC	3 days	ns	ns
Parolini	2008	Recombinant apoAI Milano (ETC-216)	NZW rabbits	M	Diet + 1.5% cholesterol	13: Induction at start	5/6 5/4 5/6 5/5 5/5	5 mg/kg $apoAI_M$ 10 mg/kg $apoAI_M$ 20 mg/kg $apoAI_M$ 40 mg/kg $apoAI_M$ 150 mg/kg $apoAI_M$ (5x every 4 days)	4 (until 1 week after last infusion)	ns	ns
Ibanez	2008	Recombinant apoAI Milano (ETC-216)	NZW rabbits	M	Diet + 0.2% cholesterol	39: Induction after 1 & 12	18/22	75 mg/kg $apoAI_M$ (2x every 4 days)	1 (until 4 days after last infusion)	ns	ns
Ibanez	2012	Recombinant apoAI Milano (ETC-216) or apo A-I wild type	NZW rabbits	M	Diet + 0.2% cholesterol	39: Induction after 1 & 12	5/5 5/5	75 mg/kg $apoAI_M$ 75 mg/kg $apoAI_{WT}$ (2x every 4 days)	1 (until 4 days after last infusion)	ns	ns
van Lenten	2007	ApoAI mimetic peptide (D-4F/L-4F)	NZW rabbits	F	Diet + 1% cholesterol	4	15/15 15/15	10 mg/kg/d D-4F 10 mg/kg/d L-4F	4	1963.0	ns
Iwata	2011	ApoAI mimetic peptide (ETC-642)	WHHL-MI rabbits	ns	Chow	0	8/5 8/8	15 mg/kg $apoAI_{MP}$ 50 mg/kg $apoAI_{MP}$ (2x/week)	12	1098.0	12.0

*nicotinic acid derivatives; AA- balloon inj, Abdominal Aorta balloon denudation injury; apo A-I, apolipoprotein A-I; apo $A\text{-}I_M$, apolipoprotein A-I Milano; apo $A\text{-}I_{MP}$, apolipoprotein mimetic peptide; apo $A\text{-}I_{WT}$, apolipoprotein A-I wild type; CA- collar, carotid artery - non-occlusive collar placement; CA- electric inj, Carotid Artery – electric perivascular injury; CETP, cholesteryl ester transfer protein; chol, cholesterol; DPPC, 1,2-dipalmitoyl phosphatidylcholine; E3L.CETP, apoE*3Leiden.CETP; EPC, egg phosphatidylcholine; F, female; HDL-VHDL, high-density lipoprotein-very high-denstiy lipoprotein; JW, Japanese White; *Idlr*$^{+/-}$AAV CETP, low desity lipoprotein receptor heterozygous mice injected with adeno-associated virus CETP; M, male; ns, not specified; NZW, New Zealand White; PC-C, phosphatidylcholine-choline; PLPC, 1-palmitoyl-2-linoleoyl phosphatidylcholine; rHDL, reconstituted HDL; rTRL, reconstituted triglyceride-rich lipoprotein; SRBI, scavenger receptor B-I; TG, triglyceride; WHHL-MI, Watanabe-heritable hyperlipidemic myocardial infarction; WTD, western-type diet

3.1.4 The association between TC/non-HDL-C/HDL-C and atherosclerotic lesion area

To further explore the atheroprotective role of non-HDL-C and HDL-C, a linear regression model was employed to study the association between the between-group percentage difference in TC, non-HDL-C and HDL-C exposure and atherosclerotic lesion area (**Figure 2**). Whereas TC and non-HDL-C associated with lesion area (R^2=0.258, P=0.045 and R^2=0.760, P<0.001, respectively), no correlation was found for HDL-C (R^2=0.030, P=0.556). After excluding an extreme data point (400% increase in HDL-C), both TC and non-HDL-C strongly correlated with lesion area (R^2=0.695, P<0.001 and R^2=0.818, P<0.001, respectively), but the association was still much less apparent for HDL-C (R^2=0.155, P=0.183).

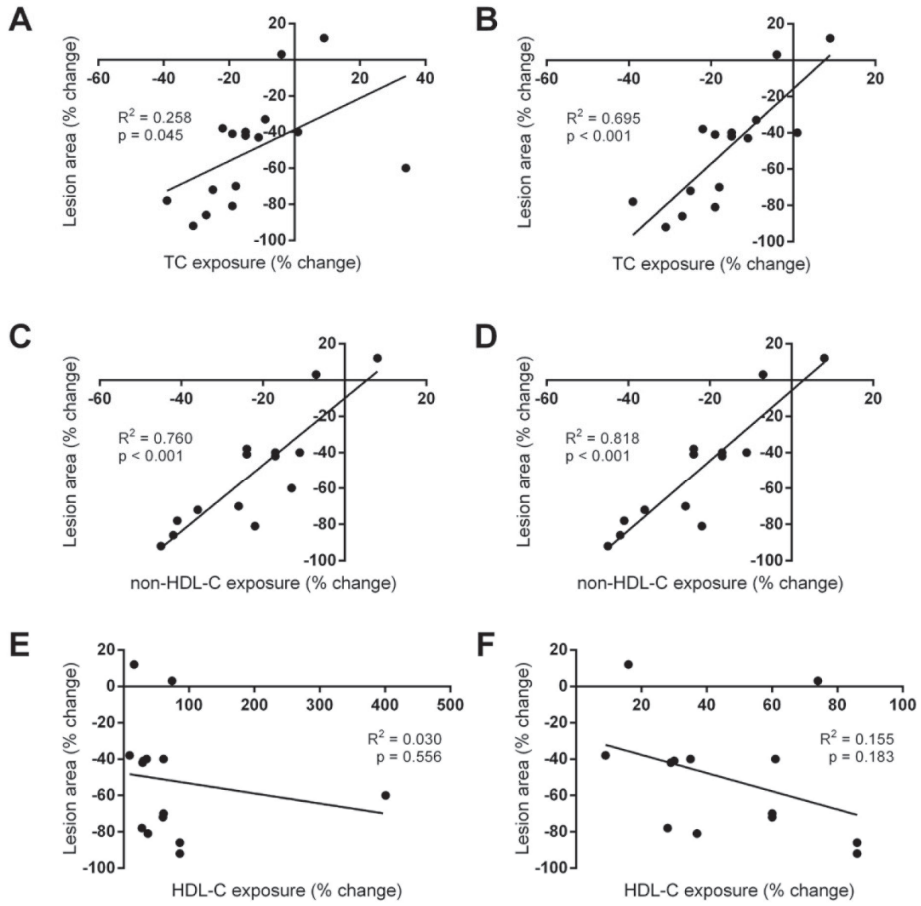


Figure 2 The correlation between percentage differences in plasma TC (A and B), non-HDL-C (C and D), as well as HDL-C (E and F) exposure and atherosclerotic lesion area between the control and treatment groups for all preclinical studies (A, C, E) and after excluding an extreme data point (400% increase in HDL-C) (B, D, F), respectively.

3.2 Randomized controlled clinical trials

3.2.1 Reference screening

The titles and abstracts of 629 records excluding 147 duplicates that were identified in the computerized search were reviewed and 287 records were removed. After retrieving 195 full text articles, 181 records were removed due to failure to meet the inclusion criteria. Together with an additional 8 articles that were identified from the bibliographies of relevant trials, meta-analysis and review articles, a total number of 22 randomized controlled trials were included in the meta-analysis (**Figure 3**).

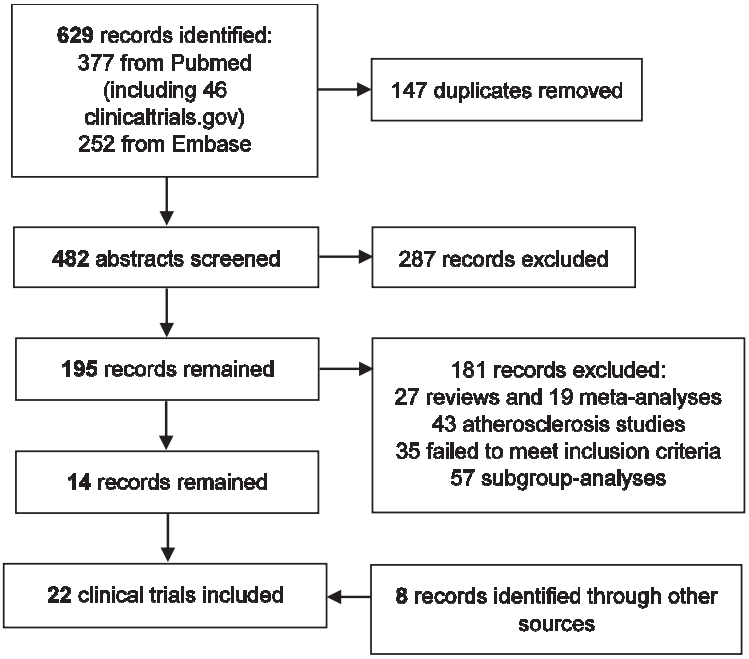


Figure 3 An outline of the systematic search conducted to identify relevant randomized controlled clinical trials for the meta-analysis.

3.2.2 Trial design and baseline characteristics

The trial design and baseline characteristics for the 22 randomized controlled trials that met the inclusion criteria are reviewed in **Table 2**. These trials evaluated the effects of PPAR-α agonists,[29, 62-72] niacin,[15, 16, 72] CETP inhibitors,[17, 23-26, 73] as well as PPAR-γ[27] and PPAR-α/γ agonists[28] on clinical outcomes. The 22 trials enrolled a total number of 121 666 patients: 61 093 in the control group and 60 573 in the treatment group. The mean duration of follow up was 3.8 years. The CDP trial investigated the effects of both clofibrate and niacin in two separate groups and we described this trial as two separate entities.[72]

Table 2 Design and baseline characteristics of the selected randomized controlled clinical trials.

Trial	**Year**	**Compound**	**Study population**	**Follow up** (yrs)	**Sample size** Total (Control/ Treatment)	**Age** (yrs)	**Men** (%)	**CVD** (%)	**Diabetes** (%)	**Previous statin use** (%)	**Baseline LDL-C** (mg/dL)	**Baseline HDL-C** (mg/dL)
CDP (clofibrate)	1975	Clofibrate	CHD	6.2	3892 2789/1103	ns	100	100	40	none	ns	ns
WHO	1978	Clofibrate	Healthy men	5.3	10627 5296/5331	46	100	0	0	none	ns	ns
DIS	1991	Clofibric acid	NIDDM	5.0	761 382/379	46	56	ns	100	ns	ns	ns
HHS	1987	Gemfibrozil	Dyslipidemia	5.0	4081 2030/2051	47	100	0	3	none	188.7	47.4
HHS Frick	1993	Gemfibrozil	Dyslipidemia with CHD symptoms	5.0	628 317/311	49	100	ns	ns	ns	188.3	46.4
VA-HIT	1999	Gemfibrozil	CHD	5.1	2531 1267/1264	64	100	100	25	ns	111.5	32.0
BIP	2000	Bezafibrate	CAD	6.2	3090 1542/1548	60	91	100	10	ns	148.5	34.6
LEADER	2002	Bezafibrate	Lower extremity arterial disease	4.6	1568 785/783	68	100	100	17	ns	131.5	46.0
DAIS	2001	Fenofibrate	T2D ± CHD	3.3	418 211/207	57	73	48	100	ns	131.7	39.8
FIELD	2005	Fenofibrate	T2D ± CVD	5.0	9795 4900/4895	62	63	22	100	0	118.7	42.5
ACCORD	2010	Fenofibrate	T2D + CVD or high risk for CVD	4.7	5518 2753/2765	62	69	37	100	60	100.6	38.1
FIRST	2014	Fenofibric acid	Dyslipidemia	2.0	682 342/340	61	68	22	50	63	84.3	39.9
CDP (niacin)	1975	Niacin	CHD	6.2	3908 2789/1119	ns	100	100	39	none	ns	ns
AIM-HIGH	2011	Niacin	CVD	3.0	3414 1696/1718	64	85	100	34	94	74.1	34.7

HPS2-THRIVE	2014	Niacin	CVD	3.9	25673 12835/12838	65	83	100	32	76	63.5	44.0
RADIANCE 1	2007	Torcetrapib	FH	2.0	904 454/450	46	49	ns	3	ns	138.7	52.4
RADIANCE 2	2007	Torcetrapib	Dyslipidemia	1.8	752 375/377	57	64	ns	21	ns	100.5	47.6
ILLUMINATE	2007	Torcetrapib	CVD or high risk for CVD or T2D	1.5	15067 7534/7533	61	78	ns	44	ns	79.8	48.6
ILLUSTRATE	2007	Torcetrapib	CHD	2.0	1188 597/591	57	70	100	21	91	83.7	45.6
DEFINE	2010	Anacetrapib	CHD or high risk for CHD	1.5	1623 812/811	63	77	55	53	99	81.8	40.5
dal-OUTCOMES	2012	Dalcetrapib	CHD + recent ACS	2.6	15871 7933/7938	60	81	100	24	97	76.1	42.4
PROactive	2005	Pioglitazone	T2D + macro vascular disease	2.9	5238 2633/2605	62	66	100	100	43	ns	ns
AleCardio	2014	Aleglitazar	T2D + recent ACS	2.0	7226 3610/3616	61	73	100	100	93	79.5	42.0

ACS, acute coronary syndrome; CAD, coronary artery disease; CHD, coronary heart disease; CVD, cardiovascular disease; FH, familial hypercholesterolemia; NIDDM, non-insulin dependent diabetes mellitus; ns, not specified; T2D, type 2 diabetes

3.2.3 Effect of lipid-modifying treatment strategies on clinical outcomes

All 22 trials were included in the analysis of all-cause mortality. Trials that did not report coronary heart disease mortality, non-fatal myocardial infarction and stroke were excluded from the analyses. The occurrence of all-cause mortality tended to be more frequent after lipid-modifying treatment as compared to the control with pooled odds ratios obtained by employing a random effects model of 1.05 [0.99; 1.10] for all studies and 1.04 [0.99; 1.10] after excluding a number of trials due to serious off-target adverse events (data not shown). The risk of non-fatal myocardial infarction was significantly less in the treatment group for an analysis of all trials (**Figure 4**) and after excluding a number of trials due to off-target adverse events (data not shown). A significant heterogeneity was observed for non-fatal myocardial infarction (I^2=40.8%, P=0.025). However, the pooled odds ratio obtained by employing a random effects model was 0.87 [0.81; 0.94] for all trials and 0.85 [0.78; 0.93] after excluding trials with off-target effects. No significant differences were observed in the occurrence of coronary heart disease mortality or stroke between the control and treatment groups (data not shown).

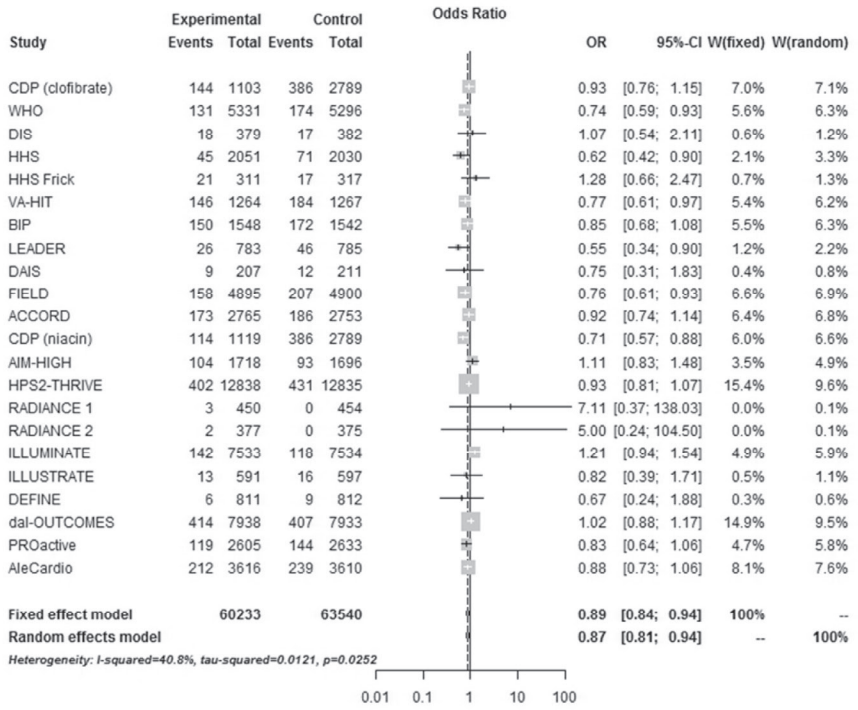


Figure 4 Forest plot for the effects of lipid-modifying treatment strategies, specifically targeting HDL-C, other than statins (PPAR-α agonists, niacin, CETP inhibitors, PPAR-γ and PPAR-α/γ agonists) on the occurrence of non-fatal myocardial infarction for all trials, demonstrating a significant risk reduction for treated subjects.

3.2.4 Effect of treatment on the prevention of non-fatal myocardial infarction in patient populations with low versus high baseline LDL-C

To assess the effects of lipid-modifying treatment on the prevention of non-fatal myocardial infarction in patients with high baseline LDL-C[62, 63, 65-69] versus low baseline LDL-C,[15-17, 29, 73] we performed 2 separate meta-analyses by dividing the remaining trials into 2 subgroups using LDL-C levels of 100 mg/dL as the cut-off. In patients with high baseline LDL-C, the occurrence of non-fatal myocardial infarction was significantly lower after lipid-modifying treatment as compared to patients in the control group (0.77 [0.68; 0.86]; **Figure 5A**). However, lipid-modifying treatment strategies failed to prevent the occurrence of non-fatal myocardial infarction in patients with low baseline LDL-C (0.97 [0.89; 1.06]; **Figure 5B**).

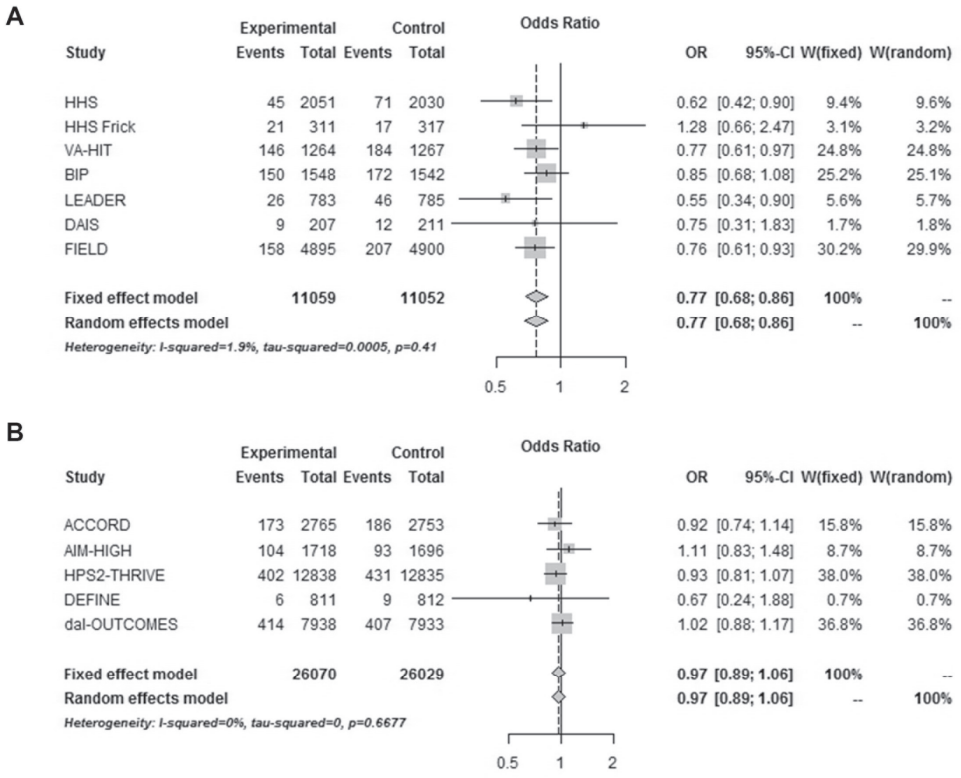


Figure 5 Forest plots for the effects of lipid-modifying treatment strategies, specifically targeting HDL-C, other than statins (PPAR-α agonists, niacin and CETP inhibitors) on the occurrence of non-fatal myocardial infarction in patient populations with baseline LDL-C > 100 mg/dL (A) and baseline LDL-C < 100 mg/dL (B), only revealing a significant risk reduction for treated subjects with baseline LDL-C > 100 mg/dL.

3.2.5 The association between LDL-C/HDL-C and non-fatal myocardial infarction

Meta-regression analyses revealed a trend towards an association between between-group differences in absolute change from baseline in LDL-C and non-fatal myocardial infarction (P=0.066; **Figure 6A**), whereas no correlation was found for HDL-C (P=0.955; **Figure 6B**).

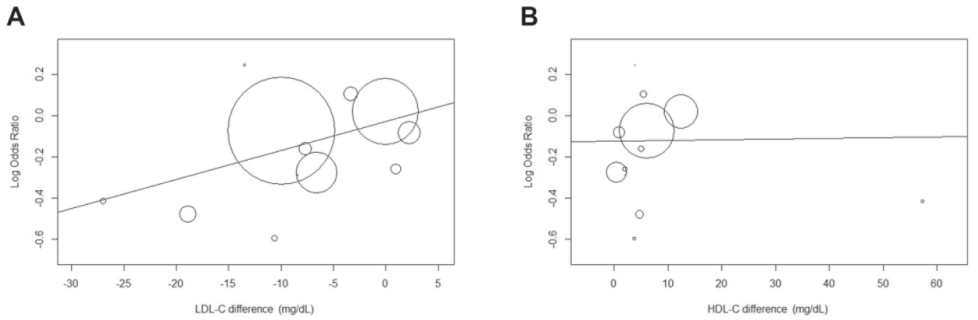


Figure 6 The association between absolute between-group differences in LDL-C and HDL-C change from baseline (mg/dL) and non-fatal myocardial infarction (log[OR]) in randomized controlled trials involving lipid-modifying treatment strategies, specifically targeting HDL-C, other than statins (PPAR-α agonists, niacin and CETP inhibitors), demonstrating a trend toward a positive correlation for LDL-C (P=0.066) and no correlation for HDL-C (P=0.955).

4. Discussion

The current systematic review and meta-analysis confirm the importance of cholesterol-lowering in the treatment of atherosclerosis and cardiovascular disease. However, based on data from preclinical studies and clinical trials, the protective effect of raising HDL-C is less defined and may be dependent on the mode of action of HDL-C-modification.

4.1 Treatment strategies aimed at raising HDL-C and atherosclerosis: preclinical studies

This systematic review investigated the effects of established and novel treatment strategies other than statins, specifically targeting HDL, on inhibition of atherosclerosis in animals expressing CETP. Most rodent models for atherosclerosis, for example *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, lack this crucial gene involved in HDL metabolism.[74] APOE*3Leiden.CETP mice express human CETP under control of its natural flanking regions.[75] These mice have impaired clearance of apolipoprotein B-containing lipoproteins and mimic the slow clearance observed in humans, particularly in patients with familial dysbetalipoproteinemia.[76] The APOE*3Leiden.CETP mice develop diet-induced atherosclerosis and respond to lipid-lowering and HDL-C-raising drugs in a human-like manner.[32, 44, 45, 77-82] Rabbits and hamsters naturally express CETP and develop diet-induced atherosclerosis. Nonetheless, it should be noted that cholesterol-fed rabbits develop lesions that predominantly consist of foam cells and do not represent

advanced lesions as observed in humans.[58] WHHL rabbits, a LDL receptor-deficient model for familial hypercholesterolemia spontaneously develop severe atherosclerotic lesions that are morphologically more similar to human lesions.[83] Hamsters are not a widely used as a model for atherosclerosis since it takes considerable time to develop atherosclerosis, and the lesions have a spotty-like appearance covering the whole aorta.

Using data from relevant animal studies that studied the effects of lipid-modifying treatment on atherosclerosis development as described below, we found significant correlations between both TC and non-HDL-C exposure and atherosclerosis (between-group percentage difference), however, there was no significant association between HDL-C and atherosclerosis.

4.1.1 Niacin

The benefits of niacin on plasma lipids was first described in 1955 and led to the development of niacin for therapeutic purposes.[84] Several mechanisms have been proposed for the beneficial effects of niacin on lipid metabolism. These include decreased free fatty acid flux from adipose tissue to the liver, decreased TG synthesis, increased apolipoprotein A-I lipidation and decreased apolipoprotein A-I removal, as well as inhibition of CETP.[20, 80, 85] In addition, niacin also exerts anti-inflammatory and anti-oxidant effects independent of lipid-lowering.[85]

In NZW rabbits, the increase in HDL-C/LDL-C ratio after treatment with different nicotinic acid derivatives resulted in a reduction in aortic surface area.[31] In APOE*3Leiden.CETP mice, the reduction in atherosclerotic lesion area was mostly accounted for by a decrease in non-HDL-C, although to some extent HDL-C predicted lesion area independent of non-HDL-C.[32] In the latter study, the combination of niacin and simvastatin reduced non-HDL-C beyond the level reached by simvastatin monotreatment and largely explained why niacin added to the anti-atherosclerotic effects of simvastatin.

4.1.2 Peroxisome proliferator-activated receptor (PPAR) agonists

PPARs are transcription factors involved in regulation of target gene expression. The effects of PPAR isoforms (α, γ) on glucose and lipid metabolism have led to the development of PPAR agonists for the treatment of hyperglycemia and dyslipidemia.[86, 87]

4.1.2.1 PPAR-α agonists

PPAR-α activation by fibrates increases lipoprotein lipase-mediated lipolysis, VLDL remnants clearance, β-oxidation,[88] apolipoprotein A-I/II expression and cholesterol efflux from macrophages.[86, 87] In addition, the HDL-C-raising effects of fibrates were ascribed to a reduction in CETP.[20, 79] Fibrates also inflict direct anti-oxidant and anti-inflammatory effects.[86, 89]

In NZW rabbits and F1B hamsters, fenofibrate reduced atherosclerosis progression and induced regression.[33, 34] In the rabbit model, the beneficial effects of fenofibrate were ascribed to an increase in HDL-C or potential pleiotropic effects of fibrates, since no significant change in LDL-C was observed.

4.1.2.2 PPAR-γ agonists

PPAR-γ agonists (glitazones) mainly mediate glucose homeostasis,[86] but pioglitazone also weakly activates PPAR-α which explains the small increase in HDL-C.[90] Similar to fibrates, glitazones exert anti-inflammatory and anti-oxidant effects independent of its metabolic activities. The atheroprotective effects of glitazones in preclinical studies were mostly independent of lipid modulation and according to our knowledge no studies reported an increase in HDL-C.

4.1.2.3 PPAR-α/γ agonists

PPAR-α/γ agonists (glitazars) were developed to more effectively improve lipid and glucose metabolism.[86] In APOE*3Leiden.CETP mice, tesaglitazar prevented progression of pre-existing atherosclerosis.[35] A strong reduction in non-HDL-C was observed, as well as an increase in HDL-C. The latter was not accompanied by a rise in apolipoprotein A-I, suggesting an increase in particle size rather than the number of particles.

4.1.3 Cholesteryl ester transfer protein (CETP) inhibition

In 1989, markedly increased HDL-C led to the discovery of the first mutation in the CETP gene in two Japanese subjects.[91] CETP facilitates the transfer of cholesteryl esters from atheroprotective HDL to atherogenic V(LDL) and has become a target to increase HDL-C.[92]

In an early study, antisense oligonucleotides against CETP suppressed atherosclerosis with a reduction in LDL-C and a small increase in HDL-C in JW rabbits.[36] Several studies have confirmed a reduction of lesion development after treatment with anti-CETP vaccines in NZW rabbits[37-40] and small molecule inhibitors, torcetrapib and anacetrapib in NZW rabbits[43] and APOE*3Leiden.CETP mice.[44, 45] In the rabbit study, aortic lesion area correlated with TC/HDL-C ratio and a trend toward an inverse correlation between HDL-C and lesion size was found. It should be noted, however, that torcetrapib produced a pro-inflammatory, unstable plaque phenotype possibly related to an increase in aldosterone levels in APOE*3Leiden.CETP mice,[44] the latter is in line with data from clinical studies.[23] Inconsistent data have been reported on the atheroprotective effects of dalcetrapib in JW rabbits[41, 42] where a reduction in atherosclerosis was only found when accompanied by a decrease in non-HDL-C levels, the latter of which is in contrast to clinical findings.[41] In the study by Huang *et al.*, despite no effect of treatment on lesion size, TC and non-HDL-C, but not HDL-C correlated with lesion area.[42] In APOE*3Leiden.CETP mice treated with anacetrapib, HDL-C inversely correlated

with lesion area, however, only non-HDL-C and not HDL-C independently determined lesion size.[45]

4.1.4 Scavenger receptor B-I (SR-BI) inhibitor and ATP-binding cassette A1 (ABCA1) degradation inhibitors

SR-BI mediates selective uptake of HDL-cholesterol esters by the liver and cholesterol efflux from other tissues. The SR-BI inhibitor, ITX5061 increased HDL-C without increasing (V) LDL-C and reduced atherosclerotic lesion area in *ldlr*$^{+/-}$ mice expressing CETP.[46] ATP-binding cassette (ABC) transporter, ABCA1 plays an important role in cholesterol efflux by mediating cholesterol transport to lipid-poor apolipoprotein A-I.[10] In rabbits, pharmacological inhibition of ABCA1 degradation increased HDL-C and reduced atherosclerotic lesion area.[47]

4.2 Treatment strategies aimed at raising HDL-C and cardiovascular disease: randomized controlled clinical trials

The efficacy and cost-effectiveness of statins have assured its partaking in randomized controlled trials involving high risk patients. This has led to a noticeable decrease in baseline LDL-C when compared to previous trials. It is not surprising that in relevant clinical trials involving patients with a baseline LDL-C of < 100 mg/dL, on average 82% of the patients were on prior statin treatment. In a meta-analysis involving 8 statin trials and 38 153 participants, HDL-C and apolipoprotein A-I levels, as well as the increase in apolipoprotein A-I were associated with reduced cardiovascular risk, however no association was found for the increase in HDL-C.[22] This is in line with results from the current meta-analysis with other lipid-modulating therapies where we observed no association between between-group differences in absolute or percentage change from baseline in HDL-C and non-fatal myocardial infarction, whereas a trend toward an association between absolute change from baseline in LDL-C levels and non-fatal myocardial infarction was found.

4.2.1 The effect of treatment on clinical outcomes in patient populations with high baseline LDL-C

Niacin reduced non-fatal myocardial infarction, whereas clofibrate/clofibric acid did not protect against cardiovascular disease in coronary heart disease patients in the CDP trial [72] and in newly diagnosed diabetic patients in the DIS trial.[70] In the WHO trial, clofibrate decreased non-fatal myocardial infarction in healthy subjects.[71] LDL-C and HDL-C levels were not measured in these earlier trials. Gemfibrozil, a fibrate that decreases LDL-C and increases HDL-C, reduced non-fatal myocardial infarction in dyslipidemic patients in the HHS trial[66] and in coronary heart disease patients in the VA-HIT trial,[69] but failed to affect clinical outcomes in patients with suspected heart disease that were excluded from the original HHS trial.[67] The reason for failure in the latter trial was ascribed to lack of power and

heterogeneity. In the VA-HIT trial, baseline LDL-C levels were much lower as compared to other trials without statins whereby no decrease in LDL-C was reported during the trial. In the BIP trial, bezafibrate had favorable effects on both LDL-C and HDL-C, but the reduction in cardiovascular events did not reach significance in patients with coronary artery disease,[62] whereas bezafibrate significantly reduced non-fatal myocardial infarction in patients with lower extremity arterial disease in the LEADER trial.[68] The angiographic DAIS trial that investigated the effects of fenofibrate in diabetic patients was not designed to detect differences in events,[63] but showed comparable reductions in clinical endpoints to that of post-hoc analyses in subgroups with diabetes.[66, 69] In the FIELD trial, fenofibrate reduced non-fatal myocardial infarction in diabetic patients.[65] Fenofibrate decreased LDL-C in both trials, but the increase in HDL-C was not apparent at study closure in the FIELD trial. It should be noted that during the BIP, LEADER and FIELD trials, significantly more patients in the placebo group received lipid-modifying drugs, mostly statins. This could have contributed to the unexpected reduction in the cumulative probability of the primary endpoint after placebo treatment in the BIP trial, especially given the decline in LDL-C towards the end of the trial.

4.2.2 The effect of treatment on clinical outcomes in patient populations with low baseline LDL-C

The ACCORD and the FIRST trials were the only 2 trials that investigated the effects of a fibrate in combination with a statin in patients with diabetes[29] and dyslipidemia.[64] At the end of these trials, fenofibrate/fenofibric acid did not significantly affect LDL-C, had a small effect on HDL-C and failed to reduce cardiovascular outcomes. However, fenofibrate treatment in patient with high baseline TG and low baseline HDL-C appeared to be beneficial in post-hoc analysis.[29]

Despite a decrease in LDL-C and an increase in HDL-C, the lack of efficacy of niacin in cardiovascular disease patients ascribed to insufficient power led to the premature termination of the AIM-HIGH trial.[15] However, in the much larger HPS2-THRIVE study, niacin-laropiprant also failed to reduce the risk of cardiovascular events in high risk patients.[16] A potential adverse effect of laropiprant on the clinical outcome cannot not be fully excluded.

In the dal-OUTCOMES trial, dalcetrapib, a CETP inhibitor which only raises HDL-C without affecting LDL-C had no effect on cardiovascular events in patients with recent acute coronary syndrome and although not significant, the 0.6 mmHg rise in systolic blood pressure and 18% increase in C-reactive protein certainly warrants attention, specifically with regards to other CETP inhibitors currently in clinical development.[17] In the DEFINE trial, treatment with anacetrapib showed a non-significant 18% increase in C-reactive protein levels without affecting blood pressure and within the power limits of this trial, anacetrapib did not reveal similar adverse cardiovascular effects as torcetrapib.[73]

In aggregate, none of the studies with (reasonably) well-treated patients using statins showed additional beneficial effects on top of statin treatment with different classes of intervention (fenofibrate/fenofibric acid, niacin and dalcetrapib), perhaps with the exception of patients with high TG and low HDL levels at baseline. This indicates that using the current therapeutic options patients are treated well and that more powerful treatment modalities are needed to lower the residual cardiovascular risk.

4.2.3 The effect of treatment on clinical outcomes in trials excluded from the meta-analyses due to serious off-target cardiovascular adverse events

In the ILLUMINATE trial, torcetrapib favorably affected both LDL-C and HDL-C, but increased mortality most likely due to an off-target increase in aldosterone and blood pressure.[23] Interestingly, post-hoc analysis revealed lower risk of cardiovascular events in patients with a higher increase in HDL/apolipoprotein A-I from baseline to 1-3 month of treatment. Three imaging studies, the ILLUSTRATE, RADIANCE 1 and RADIANCE 2 trials also reported more serious clinical adverse events (cardiovascular and blood pressure-related events) after torcetrapib treatment in patients with coronary disease, familial hypercholesterolemia and dyslipidemia, although these trials were underpowered to detect differences in events.[24-26]

Pioglitazone and aleglitazar increased both LDL-C and HDL-C and non-significantly reduced nonfatal myocardial infarction in diabetic patients in the PROactive[27] and the AleCardio trials [28]. In both trials, however, more patients in the treatment group suffered from heart failure, indicating adverse off-target effects. Development of other PPAR-α/γ agonists, muraglitazar and tesaglitazar were also stopped due to adverse events.[93]

4.3 Novel treatment strategies specifically targeting HDL on atherosclerosis: preclinical studies and clinical trials

4.3.1 Purified, reconstituted and delipidated HDL

Badimon *et al*. demonstrated that administration of homologous HDL fraction not only inhibited aortic fatty streak formation, but also induced lesion regression in NZW rabbits [48]. Purified rabbit apolipoprotein A-I administration to NZW rabbits reduced aortic fatty streak progression without inducing regression.[50] In these studies, the lack of plasma lipid modification and the reduction in aortic lipid accumulation suggested a direct role of HDL and/or apolipoprotein A-I on the vessel wall, possibly via an increase in reverse cholesterol transport. However, injection of reconstituted HDL failed to protect against fatty streak development and did not reduce aortic cholesterol content in JW rabbits.[49] Results from a more recent study in NZW rabbits show that native or reconstituted HDL infusion reduced atherosclerotic lesion area and improved lesion stability to a similar extent as statins.[51]

In humans, infusions of reconstituted HDL, CSL-111 reduced atheroma volume from baseline, but not versus placebo in the ERASE trial,[94] whereas another small study revealed

a significant reduction in lipid content in the plaque after CSL-111 infusion versus placebo.[95] Both studies showed improved plaque characteristics. The reinfusion of delipidated plasma HDL, another potential approach to improve reverse cholesterol transport, non-significantly reduce atheroma volume in humans.[96]

4.3.2 Apolipoprotein A-I Milano

The therapeutic use of recombinant apolipoprotein A-I Milano originated from the observation that carriers of this mutation have low levels of HDL-C without increased atherosclerosis as observed in patients with hypoalhalipoproteinemia,[97, 98] possibly due to accelerated binding and dissociation from lipids.[99] In NZW rabbits, short-term administration of apolipoprotein A-I Milano reduced atherosclerosis progression,[52, 53, 55] induced rapid regression and improved plaque stability.[54, 56, 57] Interestingly, apolipoprotein A-I Milano showed similar effects on aortic cholesterol content with a greater reduction in intimal macrophage content as compared to phospholipid carrier alone.[52] Other mechanisms besides reverse cholesterol transport were, therefore, suggested, including anti-oxidant, anti-inflammatory and vasodilatory effects. In another study, HDL Milano and HDL wild-type showed similar reductions in reverse cholesterol transport as evidenced by a reduction in aortic cholesterol content and up-regulation of ABCA1 and SRBI.[56]

In clinical trials, recombinant apolipoprotein A-I Milano, ETC-216 demonstrated rapid regression of atherosclerosis as seen by a reduction in atheroma volume from baseline[100] that was characterized by rapid remodeling with consequently no effect on lumen volume.[101]

4.3.3 Apolipoprotein mimetic peptides

In NZW rabbits, the apolipoprotein AI mimetic peptides, D-4F and L-4F decreased atherosclerotic lesion area and reported a greater predictive value of inflammation markers as opposed to HDL-C levels.[59] In WHHL rabbits, infusion of apolipoprotein A-I mimetic peptide/phospholipid complexes inhibited the progression of atherosclerosis mainly due to changes in LDL charge and by converting small, dense LDL into large, buoyant LDL.[58] According to our knowledge, the effects of other apolipoprotein A-I mimetic peptides, 6F and 5A, as well as an apolipoprotein E-derived HDL mimetic peptide, ATI-5261 were only investigated in animals lacking CETP.[102, 103]

In the CHI-SQUARE clinical trial, the HDL mimetic, CER-001 failed to reduce atheroma volume when compared with placebo and although not powered for clinical outcomes, revealed no differences in endpoints between groups.[104]

4.3.4 Apolipoprotein A-I inducer

In preclinical development, the effects of the apolipoprotein A-I inducer, RVX-208 on atherosclerosis were investigated in an animal model lacking CETP.[105]

In the ASSURE trial, RVX-208 failed to reduce atheroma volume in statin-treated patients.[103]

Based on the present data, reconstituted HDL and apolipoprotein A-I-based treatment strategies seem promising in the protection against atherosclerosis development and cardiovascular disease.

4.4 Limitations

To specifically investigate the role of non-HDL-C/LDL-C versus HDL-C, we pooled the effects of different compounds with different mechanisms of action. It is possible that these compounds also have different anti-atherosclerotic properties that are independent of their lipid-modifying effects. In addition, the studies that did not report lipid levels necessary to perform the analyses were excluded. Most compounds affected both LDL-C and HDL-C and it is, therefore, difficult to truly determine the contribution of each separate lipid fraction. In our preclinical studies with niacin[32] and anacetrapib,[45] we have tried to address this issue by performing statistical analyses (analysis of covariance) which suggested that anacetrapib mainly decreased atherosclerotic lesion development via a reduction in non-HDL-C, whereas the increase in HDL-C with niacin contributed to some extent to the reduction of atherosclerosis progression.

4.5 Current status and future perspectives

Niacin and fibrates have been clinically available for many years. If indeed the baseline LDL-C levels in recent clinical trials were too low to detect reductions in clinical outcomes and after careful consideration of the reported adverse events in these trials, niacin and fibrates may still be feasible treatment options for certain patient populations, such as statin-intolerant patients, patients with familial hypercholesterolemia and dysbetalipoproteinemia, and patients with different forms of hypertriglyceridemia. In fact, a more potent PPAR-α agonist, K-877 is currently being investigated in phase II/III clinical trials.[106] Despite failure of torcetrapib and dalcetrapib, the CETP inhibitors, anacetrapib and evacetrapib are currently being investigated in phase III clinical trials (the REVEAL and ACCELERATE trials) and TA-8995 (DEZ-001) is in phase II clinical development (clinicaltrials.gov).

Apolipoprotein A-I Milano (ETC-216, now MDCO-216) had manufacturing problems which limited its development.[107] However, the compound is still in development. Phase II trials investigating the effects of reconstituted HDL and apolipoprotein A-I mimetic peptide infusions (CSL-112, CER-001, APL-180 (L-4F)) are ongoing (clinicaltrials.gov). Other apolipoprotein mimetic peptides, 5A, 6F and ATI-5261 are currently in preclinical development.[102, 103] Another infusion therapy with recombinant human lecithin cholesterol acyltransferase (LCAT), ACP-501 recently passed a phase I trial (clinical trials.gov) and

was shown to increase HDL-C in patients with coronary artery disease.[103] LCAT esterifies cholesterol, thereby converting small lipid-poor nascent HDL into larger spherical HDL and may play a role in reverse cholesterol transport [102]. A small molecule activator of LCAT in hamsters increased HDL-C, HDL particle size, plasma apolipoprotein A-I level and plasma cholesteryl ester (CE) to free cholesterol ratio and significantly reduced VLDL-C.[108] In addition, phase II trials investigating the effects of an apolipoprotein A-I inducer (RVX208/ RVX000222) were recently completed (clinicaltrials.gov).

Other compounds in clinical development not yet discussed in this review due to lack of studies in CETP-expression animals or lack of efficacy (no increase in HDL-C), include PPAR-δ agonists, LXR agonists and miR-33 antagonism. Data from early phase II trials suggest that treatment with PPAR-δ agonists, GW501516 and MBX-8025 may be beneficial in patients with metabolic dysfunction.[60] The clinical development of agonists of the transcription factor LXR is hindered due to its undesired effects on de novo lipogenesis and induction of CETP expression. The LXR agonist, LXR-623 also revealed central nervous system-related adverse events in phase I.[102] Inhibition of miR33 improved ABCA1 expression and increased plasma HDL levels in preclinical studies and clinical trials should follow soon.[103] Additional HDL-targeting compounds in preclinical development include endothelial lipase inhibitors and antisense oligonucleotides targeting CETP.[10]

5. Conclusion

According to results from the current systematic review and meta-analysis, as well as supporting evidence obtained from the literature, we conclude that the protective role of lowering LDL-C and non-HDL-C is well-established, although occasionally LDL-C lowering compounds have failed due to (off-target) side effects. The contribution of raising HDL-C on inhibition of atherosclerosis and the prevention of cardiovascular disease remains undefined and may be dependent on the mode of action of HDL-C-modification. Nonetheless, treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may be worth exploring.

Disclosures

JWJ received research grants from and was speaker on (CME accredited) meetings sponsored by Amgen, Astellas, Astra-Zeneca, Biotronik, Boston Scientific, Daiichi Sankyo, Lilly, Genzyme, Medtronic, Merck-Schering-Plough, Pfizer, Orbus Neich, Novartis, Roche, Servier, Sanofi Aventis, the Netherlands Heart Foundation, the Interuniversity Cardiology Institute of the Netherlands and the European Community Framework KP7 Programme. None of the other authors has any conflict of interest regarding this paper.

References

1. Steinberg D. In celebration of the 100th anniversary of the lipid hypothesis of atherosclerosis. *Journal of lipid research*. 2013;54:2946-9.
2. Ridker PM. LDL cholesterol: controversies and future therapeutic directions. *Lancet*. 2014;384:607-17.
3. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R and Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins. *The Lancet*. 2005;366:1267-1278.
4. Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, Halsey J, Qizilbash N, Peto R and Collins R. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths. *The Lancet*. 2007;370:1829-1839.
5. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J and Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *The Lancet*. 2010;376:1670-1681.
6. Libby P. The forgotten majority: unfinished business in cardiovascular risk reduction. *Journal of the American College of Cardiology*. 2005;46:1225-8.
7. Davidson MH, Maki KC, Pearson TA, Pasternak RC, Deedwania PC, McKenney JM, Fonarow GC, Maron DJ, Ansell BJ, Clark LT and Ballantyne CM. Results of the National Cholesterol Education (NCEP) Program Evaluation ProjecT Utilizing Novel E-Technology (NEPTUNE) II survey and implications for treatment under the recent NCEP Writing Group recommendations. *The American journal of cardiology*. 2005;96:556-63.
8. Bitzur R, Cohen H, Kamari Y and Harats D. Intolerance to statins: mechanisms and management. *Diabetes care*. 2013;36 Suppl 2:S325-30.
9. Miller GJ and Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet*. 1975;1:16-9.
10. Kingwell BA, Chapman MJ, Kontush A and Miller NE. HDL-targeted therapies: progress, failures and future. *Nature reviews Drug discovery*. 2014;13:445-64.
11. Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A and Zukel WJ. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation*. 1977;55:767-772.
12. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA : the journal of the American Medical Association*. 2009;302:1993-2000.
13. Miller NE, Thelle DS, Forde OH and Mjos OD. The Tromso heart-study. High-density lipoprotein and coronary heart-disease: a prospective case-control study. *Lancet*. 1977;1:965-8.
14. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR, Bangdiwala S and Tyroler HA. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*. 1989;79:8-15.
15. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K and Weintraub W. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. *The New England journal of medicine*. 2011;365:2255-67.
16. Landray MJ, Haynes R, Hopewell JC, Parish S, Aung T, Tomson J, Wallendszus K, Craig M, Jiang L, Collins R and Armitage J. Effects of extended-release niacin with laropiprant in high-risk patients. *The New England journal of medicine*. 2014;371:203-12.
17. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, Chaitman BR, Holme IM, Kallend D, Leiter LA, Leitersdorf E, McMurray JJ, Mundl H, Nicholls SJ, Shah PK, Tardif JC and Wright RS. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *The New England journal of medicine*. 2012;367:2089-99.

18. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Hólm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart AFR, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett M-S, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki M-L, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PIW, Klungel OH, Maitland-van der Zee A-H, Peters BJM, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VHM, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WMM, Boer JMA, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, König IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, Schrezenmeir J, Schreiber S, Schäfer A, Danesh J, Blankenberg S, Roberts R, McPherson R, Watkins H, Hall AS, Overvad K, Rimm E, Boerwinkle E, Tybjaerg-Hansen A, Cupples LA, Reilly MP, Melander O, Mannucci PM, Ardissino D, Siscovick D, Elosua R, Stefansson K, O'Donnell CJ, Salomaa V, Rader DJ, Peltonen L, Schwartz SM, Altshuler D and Kathiresan S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *The Lancet*. 2012;380:572-580.
19. Haase CL, Tybjaerg-Hansen A, Qayyum AA, Schou J, Nordestgaard BG and Frikke-Schmidt R. LCAT, HDL cholesterol and ischemic cardiovascular disease: a Mendelian randomization study of HDL cholesterol in 54,500 individuals. *The Journal of clinical endocrinology and metabolism*. 2012;97:E248-56.
20. Chapman MJ, Le Goff W, Guerin M and Kontush A. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *European heart journal*. 2010;31:149-64.
21. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J and Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ*. 2009;339:b2700.
22. Boekholdt SM, Arsenault BJ, Hovingh GK, Mora S, Pedersen TR, Larosa JC, Welch KM, Amarenco P, Demicco DA, Tonkin AM, Sullivan DR, Kirby A, Colhoun HM, Hitman GA, Betteridge DJ, Durrington PN, Clearfield MB, Downs JR, Gotto AM, Jr., Ridker PM and Kastelein JJ. Levels and changes of HDL cholesterol and apolipoprotein A-I in relation to risk of cardiovascular events among statin-treated patients: a meta-analysis. *Circulation*. 2013;128:1504-12.
23. 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 and Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine*. 2007;357:2109-22.
24. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP and Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *The New England journal of medicine*. 2007;356:1304-16.
25. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT and Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *The New England journal of medicine*. 2007;356:1620-30.
26. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE and Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *The Lancet*. 2007;370:153-160.
27. Dormandy JA, Charbonnel B, Eckland DJA, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefèbvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Korányi L, Laakso M, Mokáň M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Škrha J, Smith U and Tatoň J. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *The Lancet*. 2005;366:1279-1289.

28. Lincoff AM, Tardif JC, Schwartz GG, Nicholls SJ, Ryden L, Neal B, Malmberg K, Wedel H, Buse JB, Henry RR, Weichert A, Cannata R, Svensson A, Volz D and Grobbee DE. Effect of aleglitazar on cardiovascular outcomes after acute coronary syndrome in patients with type 2 diabetes mellitus: the AleCardio randomized clinical trial. *JAMA : the journal of the American Medical Association*. 2014;311:1515-25.
29. Ginsberg HN, Elam MB, Lovato LC, Crouse JR, 3rd, Leiter LA, Linz P, Friedewald WT, Buse JB, Gerstein HC, Probstfield J, Grimm RH, Ismail-Beigi F, Bigger JT, Goff DC, Jr., Cushman WC, Simons-Morton DG and Byington RP. Effects of combination lipid therapy in type 2 diabetes mellitus. *The New England journal of medicine*. 2010;362:1563-74.
30. DerSimonian R and Laird N. Meta-analysis in clinical trials. *Controlled clinical trials*. 1986;7:177-88.
31. Parwaresch MR, Haacke H and Mader C. Efficacy of hypolipidemic treatment in inhibition of experimental atherosclerosis: the effect of nicotinic acid and related compounds. *Atherosclerosis*. 1978;31:395-401.
32. Kuhnast S, Louwe MC, Heemskerk MM, Pieterman EJ, van Klinken JB, van den Berg SA, Smit JW, Havekes LM, Rensen PC, van der Hoorn JW, Princen HM and Jukema JW. Niacin Reduces Atherosclerosis Development in APOE*3Leiden.CETP Mice Mainly by Reducing NonHDL-Cholesterol. *PloS one*. 2013;8:e66467.
33. Corti R, Osende J, Hutter R, Viles-Gonzalez JF, Zafar U, Valdivieso C, Mizsei G, Fallon JT, Fuster V and Badimon JJ. Fenofibrate induces plaque regression in hypercholesterolemic atherosclerotic rabbits: in vivo demonstration by high-resolution MRI. *Atherosclerosis*. 2007;190:106-13.
34. Srivastava RA. Evaluation of anti-atherosclerotic activities of PPAR-alpha, PPAR-gamma, and LXR agonists in hyperlipidemic atherosclerosis-susceptible F(1)B hamsters. *Atherosclerosis*. 2011;214:86-93.
35. van der Hoorn JW, Jukema JW, Havekes LM, Lundholm E, Camejo G, Rensen PC and Princen HM. The dual PPARalpha/gamma agonist tesaglitazar blocks progression of pre-existing atherosclerosis in APOE*3Leiden.CETP transgenic mice. *British journal of pharmacology*. 2009;156:1067-75.
36. Sugano M. Effect of Antisense Oligonucleotides against Cholesteryl Ester Transfer Protein on the Development of Atherosclerosis in Cholesterol-fed Rabbits. *Journal of Biological Chemistry*. 1998;273:5033-5036.
37. Jun L, Jie L, Dongping Y, Xin Y, Taiming L, Rongyue C, Jie W and Jingjing L. Effects of nasal immunization of multi-target preventive vaccines on atherosclerosis. *Vaccine*. 2012;30:1029-37.
38. Mao D, Kai G, Gaofu Q, Zheng Z, Li Z, Jie W, Jingjing L and Rongyue C. Intramuscular immunization with a DNA vaccine encoding a 26-amino acid CETP epitope displayed by HBc protein and containing CpG DNA inhibits atherosclerosis in a rabbit model of atherosclerosis. *Vaccine*. 2006;24:4942-50.
39. Gaofu Q, Jun L, Xiuyun Z, Wentao L, Jie W and Jingjing L. Antibody against cholesteryl ester transfer protein (CETP) elicited by a recombinant chimeric enzyme vaccine attenuated atherosclerosis in a rabbit model. *Life sciences*. 2005;77:2690-702.
40. Rittershaus CW, Miller DP, Thomas LJ, Picard MD, Honan CM, Emmett CD, Pettey CL, Adari H, Hammond RA, Beattie DT, Callow AD, Marsh HC and Ryan US. Vaccine-Induced Antibodies Inhibit CETP Activity In Vivo and Reduce Aortic Lesions in a Rabbit Model of Atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2000;20:2106-2112.
41. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K and Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000;406:203-7.
42. Huang Z, Inazu A, Nohara A, Higashikata T and Mabuchi H. Cholesteryl ester transfer protein inhibitor (JTT-705) and the development of atherosclerosis in rabbits with severe hypercholesterolaemia. *Clinical science*. 2002;103:587-94.
43. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH and Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *Journal of lipid research*. 2007;48:1263-72.
44. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not

reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
45. Kuhnast S, van der Tuin SJ, van der Hoorn JW, van Klinken JB, Simic B, Pieterman E, Havekes LM, Landmesser U, Luscher TF, Willems van Dijk K, Rensen PC, Jukema JW and Princen HM. Anacetrapib reduces progression of atherosclerosis, mainly by reducing non-HDL-cholesterol, improves lesion stability and adds to the beneficial effects of atorvastatin. *European heart journal*. 2014;doi:10.1093/eurheartj/ehu319.
46. Masson D, Koseki M, Ishibashi M, Larson CJ, Miller SG, King BD and Tall AR. Increased HDL cholesterol and apoA-I in humans and mice treated with a novel SR-BI inhibitor. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:2054-60.
47. Arakawa R, Tsujita M, Iwamoto N, Ito-Ohsumi C, Lu R, Wu CA, Shimizu K, Aotsuka T, Kanazawa H, Abe-Dohmae S and Yokoyama S. Pharmacological inhibition of ABCA1 degradation increases HDL biogenesis and exhibits antiatherogenesis. *Journal of lipid research*. 2009;50:2299-305.
48. Badimon JJ, Badimon L and Fuster V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *The Journal of clinical investigation*. 1990;85:1234-41.
49. Mezdour H, Yamamura T, Nomura S and Yamamoto A. Exogenous supply of artificial lipoproteins does not decrease susceptibility to atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis*. 1995;113:237-46.
50. Miyazaki A, Sakuma S, Morikawa W, Takiue T, Miake F, Terano T, Sakai M, Hakamata H, Sakamoto Y, Natio M and et al. Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits. *Arteriosclerosis, thrombosis, and vascular biology*. 1995;15:1882-8.
51. Nicholls SJ, Cutri B, Worthley SG, Kee P, Rye KA, Bao S and Barter PJ. Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis in rabbits. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2416-21.
52. Ameli S, Hultgardh-Nilsson A, Cercek B, Shah PK, Forrester JS, Ageland H and Nilsson J. Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits. *Circulation*. 1994;90:1935-1941.
53. Soma MR, Donetti E, Parolini C, Sirtori CR, Fumagalli R and Franceschini G. Recombinant apolipoprotein A-IMilano dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits. *Circulation research*. 1995;76:405-11.
54. Chiesa G. Recombinant Apolipoprotein A-IMilano Infusion Into Rabbit Carotid Artery Rapidly Removes Lipid From Fatty Streaks. *Circulation research*. 2002;90:974-980.
55. Parolini C, Marchesi M, Lorenzon P, Castano M, Balconi E, Miragoli L, Chaabane L, Morisetti A, Lorusso V, Martin BJ, Bisgaier CL, Krause B, Newton RS, Sirtori CR and Chiesa G. Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo assessment by intravascular ultrasound and magnetic resonance imaging. *Journal of the American College of Cardiology*. 2008;51:1098-103.
56. Ibanez B, Giannarelli C, Cimmino G, Santos-Gallego CG, Alique M, Pinero A, Vilahur G, Fuster V, Badimon L and Badimon JJ. Recombinant HDL(Milano) exerts greater anti-inflammatory and plaque stabilizing properties than HDL(wild-type). *Atherosclerosis*. 2012;220:72-7.
57. Ibanez B, Vilahur G, Cimmino G, Speidl WS, Pinero A, Choi BG, Zafar MU, Santos-Gallego CG, Krause B, Badimon L, Fuster V and Badimon JJ. Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration: magnetic resonance imaging study in an experimental model of atherosclerosis. *Journal of the American College of Cardiology*. 2008;51:1104-9.
58. Iwata A, Miura S, Zhang B, Imaizumi S, Uehara Y, Shiomi M and Saku K. Antiatherogenic effects of newly developed apolipoprotein A-I mimetic peptide/phospholipid complexes against aortic plaque burden in Watanabe-heritable hyperlipidemic rabbits. *Atherosclerosis*. 2011;218:300-7.
59. Van Lenten BJ, Wagner AC, Navab M, Anantharamaiah GM, Hama S, Reddy ST and Fogelman AM. Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits. *Journal of lipid research*. 2007;48:2344-53.

60. Ehrenborg E and Skogsberg J. Peroxisome proliferator-activated receptor delta and cardiovascular disease. *Atherosclerosis*. 2013;231:95-106.
61. Hennessy EJ and Moore KJ. Using microRNA as an alternative treatment for hyperlipidemia and cardiovascular disease: cardio-miRs in the pipeline. *Journal of cardiovascular pharmacology*. 2013;62:247-54.
62. Bezafibrate Infarction Prevention Study Group. Secondary Prevention by Raising HDL Cholesterol and Reducing Triglycerides in Patients With Coronary Artery Disease : The Bezafibrate Infarction Prevention (BIP) Study. *Circulation*. 2000;102:21-27.
63. Diabetes Atherosclerosis Intervention Study Investigators. Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. *The Lancet*. 2001;357:905-910.
64. Davidson MH, Rosenson RS, Maki KC, Nicholls SJ, Ballantyne CM, Mazzone T, Carlson DM, Williams LA, Kelly MT, Camp HS, Lele A and Stolzenbach JC. Effects of fenofibric acid on carotid intima-media thickness in patients with mixed dyslipidemia on atorvastatin therapy: randomized, placebo-controlled study (FIRST). *Arteriosclerosis, thrombosis, and vascular biology*. 2014;34:1298-306.
65. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, Forder P, Pillai A, Davis T, Glasziou P, Drury P, Kesaniemi YA, Sullivan D, Hunt D, Colman P, d'Emden M, Whiting M, Ehnholm C and Laakso M. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. *The Lancet*. 2005;366:1849-1861.
66. Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, Huttunen JK, Kaitaniemi P, Koskinen P, Manninen V and et al. Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. *The New England journal of medicine*. 1987;317:1237-45.
67. Frick MH, Heinonen OP, Huttunen JK, Koskinen P, Manttari M and Manninen V. Efficacy of gemfibrozil in dyslipidaemic subjects with suspected heart disease. An ancillary study in the Helsinki Heart Study frame population. *Annals of medicine*. 1993;25:41-5.
68. Meade T, Zuhrie R, Cook C and Cooper J. Bezafibrate in men with lower extremity arterial disease: randomised controlled trial. *BMJ*. 2002;325:1139.
69. Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ and Wittes J. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *The New England journal of medicine*. 1999;341:410-8.
70. Hanefeld M, Fischer S, Schmechel H, Rothe G, Schulze J, Dude H, Schwanebeck U and Julius U. Diabetes Intervention Study. Multi-intervention trial in newly diagnosed NIDDM. *Diabetes care*. 1991;14:308-17.
71. WHO Study Investigators. A co-operative trial in the primary prevention of ischaemic heart disease using clofibrate. Report from the Committee of Principal Investigators. *British heart journal*. 1978;40:1069-118.
72. The Coronary Drug Project Research Group. Clofibrate and niacin in coronary heart disease. *JAMA : the journal of the American Medical Association*. 1975;231:360-81.
73. Cannon CP, Shah S, Dansky HM, Davidson M, Brinton EA, Gotto AM, Stepanavage M, Liu SX, Gibbons P, Ashraf TB, Zafarino J, Mitchel Y and Barter P. Safety of anacetrapib in patients with or at high risk for coronary heart disease. *The New England journal of medicine*. 2010;363:2406-15.
74. Tani M, Matera R, Horvath KV, Hasan TS, Schaefer EJ and Asztalos BF. The influence of apoE-deficiency and LDL-receptor-deficiency on the HDL subpopulation profile in mice and in humans. *Atherosclerosis*. 2014;233:39-44.
75. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.

76. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR and Havekes LM. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *The Journal of clinical investigation*. 1991;88:643-55.
77. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1706-21.
78. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
79. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.
80. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
81. Kuhnast S, van der Hoorn JW, Pieterman EJ, van den Hoek AM, Sasiela WJ, Gusarova V, Peyman A, Schafer HL, Schwahn U, Jukema JW and Princen HM. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. *Journal of lipid research*. 2014;55:2103-12.
82. Kuhnast S, van der Hoorn JW, van den Hoek AM, Havekes LM, Liau G, Jukema JW and Princen HM. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden. CETP transgenic mice with or without treatment with atorvastatin. *Journal of hypertension*. 2012;30:107-16.
83. Buja LM, Kita T, Goldstein JL, Watanabe Y and Brown MS. Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. *Arteriosclerosis*. 1983;3:87-101.
84. Carlson LA. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine*. 2005;258:94-114.
85. Kamanna VS, Ganji SH and Kashyap ML. Recent advances in niacin and lipid metabolism. *Current opinion in lipidology*. 2013;24:239-45.
86. Jandeleit-Dahm KAM, Calkin A, Tikellis C and Thomas M. Direct antiatherosclerotic effects of PPAR agonists. *Current opinion in lipidology*. 2009;20:24-29.
87. Lalloyer F and Staels B. Fibrates, glitazones, and peroxisome proliferator-activated receptors. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:894-9.
88. Bijland S, Pieterman EJ, Maas AC, van der Hoorn JW, van Erk MJ, van Klinken JB, Havekes LM, van Dijk KW, Princen HM and Rensen PC. Fenofibrate increases very low density lipoprotein triglyceride production despite reducing plasma triglyceride levels in APOE*3-Leiden.CETP mice. *The Journal of biological chemistry*. 2010;285:25168-75.
89. Kooistra T, Verschuren L, de Vries-van der Weij J, Koenig W, Toet K, Princen HM and Kleemann R. Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2322-30.
90. Sakamoto J, Kimura H, Moriyama S, Odaka H, Momose Y, Sugiyama Y and Sawada H. Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. *Biochemical and biophysical research communications*. 2000;278:704-11.
91. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H and et al. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*. 1989;342:448-51.
92. Barter PJ and Rye KA. Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk. *Journal of lipid research*. 2012;53:1755-66.
93. Wilding JP. PPAR agonists for the treatment of cardiovascular disease in patients with diabetes. *Diabetes, obesity & metabolism*. 2012;14:973-82.

94. Tardif JC, Gregoire J, L'Allier PL, Ibrahim R, Lesperance J, Heinonen TM, Kouz S, Berry C, Basser R, Lavoie MA, Guertin MC and Rodes-Cabau J. Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA : the journal of the American Medical Association*. 2007;297:1675-82.
95. Shaw JA, Bobik A, Murphy A, Kanellakis P, Blombery P, Mukhamedova N, Woollard K, Lyon S, Sviridov D and Dart AM. Infusion of reconstituted high-density lipoprotein leads to acute changes in human atherosclerotic plaque. *Circulation research*. 2008;103:1084-91.
96. Waksman R, Torguson R, Kent KM, Pichard AD, Suddath WO, Satler LF, Martin BD, Perlman TJ, Maltais JA, Weissman NJ, Fitzgerald PJ and Brewer HB, Jr. A first-in-man, randomized, placebo-controlled study to evaluate the safety and feasibility of autologous delipidated high-density lipoprotein plasma infusions in patients with acute coronary syndrome. *Journal of the American College of Cardiology*. 2010;55:2727-35.
97. Franceschini G, Sirtori CR, Capurso A, 2nd, Weisgraber KH and Mahley RW. A-IMilano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *The Journal of clinical investigation*. 1980;66:892-900.
98. Sirtori CR, Calabresi L, Franceschini G, Baldassarre D, Amato M, Johansson J, Salvetti M, Monteduro C, Zulli R, Muiesan ML and Agabiti-Rosei E. Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study. *Circulation*. 2001;103:1949-54.
99. Franceschini G, Vecchio G, Gianfranceschi G, Magani D and Sirtori CR. Apolipoprotein AIMilano. Accelerated binding and dissociation from lipids of a human apolipoprotein variant. *The Journal of biological chemistry*. 1985;260:16321-5.
100. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC and Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA : the journal of the American Medical Association*. 2003;290:2292-300.
101. Nicholls SJ, Tuzcu EM, Sipahi I, Schoenhagen P, Crowe T, Kapadia S and Nissen SE. Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein A-I Milano. *Journal of the American College of Cardiology*. 2006;47:992-7.
102. Balder JW, Staels B and Kuivenhoven JA. Pharmacological interventions in human HDL metabolism. *Current opinion in lipidology*. 2013;24:500-9.
103. Remaley AT, Norata GD and Catapano AL. Novel concepts in HDL pharmacology. *Cardiovascular research*. 2014;103:423-8.
104. Tardif JC, Ballantyne CM, Barter P, Dasseux JL, Fayad ZA, Guertin MC, Kastelein JJ, Keyserling C, Klepp H, Koenig W, L'Allier P L, Lesperance J, Luscher TF, Paolini JF, Tawakol A and Waters DD. Effects of the high-density lipoprotein mimetic agent CER-001 on coronary atherosclerosis in patients with acute coronary syndromes: a randomized trial. *European heart journal*. 2014;35:3277-86.
105. Jahagirdar R, Zhang H, Azhar S, Tobin J, Attwell S, Yu R, Wu J, McLure KG, Hansen HC, Wagner GS, Young PR, Srivastava RA, Wong NC and Johansson J. A novel BET bromodomain inhibitor, RVX-208, shows reduction of atherosclerosis in hyperlipidemic ApoE deficient mice. *Atherosclerosis*. 2014;236:91-100.
106. van Capelleveen JC, Brewer HB, Kastelein JJ and Hovingh GK. Novel therapies focused on the high-density lipoprotein particle. *Circulation research*. 2014;114:193-204.
107. Rached FH, Chapman MJ and Kontush A. An overview of the new frontiers in the treatment of atherogenic dyslipidemias. *Clinical pharmacology and therapeutics*. 2014;96:57-63.
108. Chen Z, Wang SP, Krsmanovic ML, Castro-Perez J, Gagen K, Mendoza V, Rosa R, Shah V, He T, Stout SJ, Geoghagen NS, Lee SH, McLaren DG, Wang L, Roddy TP, Plump AS, Hubbard BK, Sinz CJ and Johns DG. Small molecule activation of lecithin cholesterol acyltransferase modulates lipoprotein metabolism in mice and hamsters. *Metabolism*. 2012;61:470-81.

CHAPTER 8

General Discussion and Future Perspectives

Cardiovascular disease (CVD) is the leading cause of death worldwide despite the successful development of several pharmaceutical interventions of which statin therapy is the dominating lipid-lowering treatment option.[1] However, the treatment of CVD remains suboptimal due to; (i) a residual risk that persists after statin treatment,[2] (ii) failure for some patients to reach low-density lipoprotein-cholesterol (LDL-C) targets despite statin treatment,[3] and (iii) lack of adherence to statin treatment as a result of amongst others statin intolerance.[4] Atherosclerosis, a chronic inflammatory disease of multifactorial origin,[5, 6] is a dominant contributor to the development of CVD.[7] The research described in this thesis investigated the effects of innovative pharmaceutical interventions in experimental atherosclerosis, targeting hypertension and high blood cholesterol, more specifically high LDL-C and low high-density lipoprotein-cholesterol (HDL-C), as risk factors for CVD. We used APOE*3Leiden.CETP mice which express human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions.[8] These mice have impaired clearance of apolipoprotein B-containing lipoproteins and mimic the slow clearance observed in humans, particularly in patients with familial dysbetalipoproteinemia (FD).[9] The APOE*3Leiden.CETP mouse model is a well-established model for lipid and lipoprotein metabolism and atherosclerosis, because these mice; (i) develop diet-induced atherosclerosis, (ii) have a human-like lipoprotein metabolism and, (iii) respond in a human-like manner to lipid-modifying treatment strategies, including LDL-C-lowering and HDL-C-raising compounds.[10-15]

In view of the fact that hypertension is a leading risk factor for CVD and associated with the development of atherosclerosis,[16, 17] we investigated the anti-atherosclerotic effects of aliskiren, the first commercially available, orally active, direct renin inhibitor approved for the treatment of hypertension in **chapter 2**.[18] In this study in APOE*3Leiden.CETP mice, we demonstrated beneficial effects of aliskiren on atherosclerosis development and plaque stability when administered alone and in combination with atorvastatin. Aliskiren reduced systolic blood pressure and additionally reduced atherosclerotic lesion size and severity. Interestingly, the reduction in atherosclerosis development observed by aliskiren remained after correcting for blood pressure, suggesting that aliskiren had anti-atherosclerotic properties beyond its blood pressure-lowering qualities. Aliskiren also improved plaque stability as evidenced by a decrease in macrophage and necrotic area, as well as by an increase in SMC content in the cap, possibly via a mechanism involving T cells. The combination of aliskiren and atorvastatin was more potent in reducing atherosclerotic lesion size, as well as markers of inflammation and in improving plaque stability. Clinical trials, including the ALLAY (Aliskiren in Left-Ventricular Hypertrophy),[19] the ALOFT (Aliskiren Observation of Heart Failure Treatment)[20] and the AVOID (Aliskiren in the Evaluation of Proteinuria In Diabetes) trials[21] reported beneficial effects of aliskiren on various markers of organ damage. However, aliskiren in combination with angiotensin converting enzyme inhibitors (ACEi) and angiotensin II type I receptor blockers (ARBs) failed to provide additional

cardiovascular benefit in diabetic patients at high risk of developing cardiovascular and renal complications in the ALTITUDE trial[22] and in patients hospitalized for heart failure in the ASTRONAUT trial.[23] Both these trials reported more adverse events, i.e. renal dysfunction, hyperkalemia and hypotension. In contrast, results from the prematurely terminated APOLLO trial that investigated the cardiovascular protective effects of aliskiren monotreatment and in combination with hydrochlorothiazide and amlodipine in elderly patients[24] and the AQUARIUS trial that evaluated the effects of aliskiren on coronary atherosclerosis in patients with prehypertension, revealed potential for CVD reduction.[25] The latter trial reported a non-significant trend towards a reduction in atheroma volume from baseline after aliskiren treatment. The ongoing ATMOSPHERE trial (NCT00853658) evaluating the efficacy and safety of aliskiren and aliskiren + enalapril combination treatment in patients with chronic heart failure will provide additional insight into the protective role of aliskiren and results are expected in 2015.

Cholesterol contained in LDL particles is well recognized as a primary causal risk factor for coronary heart disease (CHD) as evidenced by experimental, epidemiological and genetic data.[26] Furthermore, intervention trials provided ample evidence that the lowering of LDL-C contributes to a reduction in CHD.[27-29] However, despite the fact that epidemiological studies consistently reported an inverse association between HDL-C and CHD risk,[30-32] the benefits of raising HDL-C remain less defined. In Chapter 3 to 6, we investigated the effects of novel lipid-modifying treatment strategies, i.e. LDL-C-lowering and/or HDL-C-raising compounds on atherosclerosis development in the APOE*3Leiden.CETP mouse model, since these mice respond to both LDL-C-lowering and HDL-C-raising compounds in a human-like manner.

The benefits of niacin on plasma lipids were first described in 1955 and led to the development of niacin for therapeutic purposes.[33] In **chapter 3**,[34] we aimed to address the discrepancy between the beneficial effects of niacin in initial clinical trials[35-38] and the lack of effect of niacin on top of statin treatment on the reduction of CVD events in the large AIM-HIGH and HPS2-THRIVE clinical trials[39, 40] by evaluating the effects of niacin alone and in combination with simvastatin on plasma lipid levels and atherosclerotic lesion size and composition. We demonstrated that niacin reduced (V)LDL-C and (V)LDL-TG and that the increase in HDL-C may be attributable to a decrease in hepatic and plasma CETP. Importantly, the extent of lipid-lowering observed with niacin in our study in E3L.CETP mice was comparable to that of FD patients.[34, 41, 42] Moreover, we showed that niacin decreased atherosclerosis development mainly by reducing non-HDL-C with a modest HDL-C-raising and additional anti-inflammatory effects. We demonstrated that the additive effect of niacin on top of simvastatin was mostly dependent on its non-HDL-C-lowering capacities. Based on these findings and results from a reverse cholesterol transport (RCT) experiment, we conclude that the effects of niacin on HDL-C and HDL functionality may partially contribute to, but is not the driving force behind its anti-atherogenic effects observed in our study.

Therefore, data from our study suggested that clinical beneficial effects of niacin are largely dependent on its ability to lower (V)LDL-C on top of concomitant lipid-lowering therapy and may explain the failure of niacin in the AIM-HIGH and HPS2-THRIVE trials in hyperlipidemic patients subjected to aggressive LDL-C-lowering treatment with limited effects of niacin on (V)LDL-C.[39, 40]

In 1989, markedly increased HDL-C led to the discovery of the first mutation in the CETP gene in two Japanese subjects.[43] CETP facilitates the transfer of cholesteryl esters from atheroprotective HDL to atherogenic V(LDL) and has become a target to increase HDL-C. This has led to the development of several small molecule CETP inhibitors, including amongst others torcetrapib, dalcetrapib, anacetrapib and evacetrapib. However, despite favorable effects on both LDL-C and HDL-C, torcetrapib increased mortality in the large phase III ILLUMINATE trial, most likely due to an off-target increase in aldosterone which leads to activation of RAAS and an increase in blood pressure,[44] potentially resulting in a more vulnerable plaque phenotype.[13] In the large phase III dal-OUTCOMES trial, dalcetrapib which only raises HDL-C without affecting LDL-C had no effect on cardiovascular events in patients with recent acute coronary syndrome (ACS) and although not significant, the 0.6 mmHg rise in systolic blood pressure and 18% increase in C-reactive protein certainly warrants attention, specifically with regards to other CETP inhibitors that are currently in clinical development.[45] In **chapter 4**,[46] we investigated the effects of a broad dose range of the novel CETP inhibitor, anacetrapib on CETP activity, lipid levels, atherosclerotic lesion size and composition, as well as HDL function and we examined possible additive/synergistic effects of anacetrapib on top of atorvastatin. In our study, anacetrapib dose-dependently reduced CETP activity, thereby decreasing non-HDL-C and increasing HDL-C. These lipid-altering effects were comparable to findings from clinical trials.[47-49] Moreover, anacetrapib dose-dependently reduced atherosclerosis development. This effect was mainly ascribed to a reduction in non-HDL-C despite a remarkable increase in HDL-C and without affecting HDL functionality. Interestingly, anacetrapib itself also contributed to the reduction of the lesion size by a hitherto unknown mechanism. In addition, anacetrapib improved lesion stability when given at a higher dose and a moderate dose anacetrapib added to the anti-atherogenic effects of atorvastatin. In phase II clinical trials, neither anacetrapib nor evacetrapib showed the side effects observed with torcetrapib treatment and both compounds were more potent in reducing LDL-C and increasing HDL-C when compared to torcetrapib and dalcetrapib.[49-51] The effects of anacetrapib and evacetrapib on clinical outcomes are currently being investigated in the large phase III REVEAL (NCT01252953) and ACCELERATE (NCT01687998) clinical trials and results are expected in 2016/2017. We further explored the mechanism by which anacetrapib reduces (V)LDL-C and whether this effect is dependent on the inhibition of CETP activity in **chapter 5**. In this study, we showed that anacetrapib reduces (V)LDL-C by increasing hepatic remnant uptake via two

mechanisms; (i) inhibition of CETP activity, resulting in remodelled VLDL particles that are more susceptible to hepatic uptake, and (ii) a CETP-independent reduction in plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) level that has the potential to increase LDL receptor (LDLR)-mediated hepatic remnant clearance. According to our knowledge, there are currently no clinical data describing the mechanism by which anacetrapib reduces non-HDL-C/LDL-C. However, a reduction in plasma PCSK9 levels after anacetrapib treatment was in accordance with findings in rhesus macaques[52] and results from our study confirmed a CETP-independent decrease in plasma PCSK9 levels by anacetrapib as previously observed in C57BL/6 mice.[53]

In 2003, Abifadel *et al.* identified two French families with autosomal dominant hypercholesterolemia caused by mutations in PCSK9.[54] PCSK9 is a serine protease responsible for LDLR degradation by preventing the recycling of the receptor to the cell membrane after internalization.[55] The upregulation of the LDLR after statin treatment is accompanied by an upregulation of PCSK9 which in turn promotes LDLR degradation.[56-58] Inhibition of PCSK9 is, therefore, a potential novel strategy in the treatment against CVD, especially in combination with statin treatment. We, therefore investigated the effects of 2 dosages of the fully human, monoclonal antibody against PCSK9, alirocumab alone and in combination with atorvastatin on hepatic LDLR protein levels and hepatic cholesterol metabolism, plasma lipid levels, atherosclerosis development and plaque morphology in **chapter 6**.[59] In this study, alirocumab dose-dependently increased hepatic LDLR protein levels without changes in hepatic cholesterol and TG levels and consequently decreased plasma cholesterol levels and reduced the development of atherosclerosis. Moreover, alirocumab improved lesion morphology and composition and enhanced the beneficial effects of a mild dose of atorvastatin. The anti-atherosclerotic effect was strongly dependent on the reduction of plasma TC levels, indicating that the majority of the effect was brought about by cholesterol-lowering leaving limited/no space for other potential (pleiotropic) effects. This is the first study to show that a monoclonal antibody to PCSK9 reduces atherosclerosis development. It should be noted that this is a progression/prevention study which may pose as a potential limitation with respect to translation to the clinic where patients with existing lesions are often treated. Nonetheless, data from this study may also suggest beneficial effects on markers of atherosclerosis by reducing TC with alirocumab in the human situation where new lesions are formed alongside existing plaques. The dose-dependent cholesterol-lowering effects observed in our study were in accordance with results from phase I and II clinical trials.[60-62] No significant safety issues emerged from these short term trials. Results from phase III trials within the ODYSSEY programs will provide further insight regarding the long term efficacy, safety and tolerability of alirocumab in patients with familial hypercholesterolemia and in high CVD risk patients with hypercholesterolemia on lipid-modifying therapy.[63] The large ODYSSEY Outcomes trial (NCT01663402) evaluating the effects of alirocumab on the

occurrence of cardiovascular events in patients with relatively recent ACS treated with high-dose statins will reveal whether PCSK9 inhibition with alirocumab translates into clinical benefit and results are expected in 2018.

In **chapter 7**, we reviewed the effects of established and novel treatment strategies, specifically targeting HDL, other than statins on inhibition of atherosclerosis development in preclinical studies in animals expressing CETP, a crucial gene involved in HDL metabolism and implicated in the mechanisms by which most therapies modulate HDL.[64] In addition, we conducted a meta-analysis to evaluate the potential effects of these treatment strategies on the prevention of clinical events in randomized controlled trials. In the systematic review and meta-analysis, we focused specifically on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising on inhibition of atherosclerosis and the prevention of CVD. Using data from relevant preclinical studies, we found significant correlations between both TC and non-HDL-C exposure and atherosclerosis, however, there was no significant association between HDL-C exposure and atherosclerosis. The meta-analysis of relevant clinical trials revealed no association between the absolute or percentage increase in HDL-C and non-fatal myocardial infarction, whereas a trend toward an association between the absolute decrease in LDL-C levels and non-fatal myocardial infarction was found. This is in line with results from a recent meta-analysis involving only statin trials (8 trials with 38 153 participants), showing that HDL-C and apolipoprotein A-I levels, as well as the increase in apolipoprotein A-I were associated with reduced cardiovascular risk, however no association was found for the increase in HDL-C.[65] The effects of other novel treatment strategies specifically targeting HDL, including reconstituted and delipidated HDL, as well as HDL mimetics, apolipoprotein A-I mimetic peptides, recombinant apolipoprotein A-I Milano and recombinant human lecithin cholesterol acyltransferase (LCAT) seem promising in the protection against atherosclerosis development and cardiovascular disease based on data from preclinical studies[66-77] and clinical trials.[78-81] In these studies, the lack of plasma lipid modification suggests a direct role of HDL and/or apolipoprotein A-I, possibly via an increase in reverse cholesterol transport and supports the view that HDL function rather than HDL-C may have a causal relation to atherprotection.[82]

Thus, according to results from our systematic review and meta-analysis, as well as supporting evidence obtained from the literature, it is evident that the protective role of lowering LDL-C and non-HDL-C is well-established, although occasionally LDL-C lowering compounds have failed due to (off-target) side effects. The contribution of raising HDL-C on inhibition of atherosclerosis and the prevention of cardiovascular disease remains undefined and may be dependent on the mode of action of HDL-C-modification. Nonetheless, treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may be worth exploring.

In conclusion, the research described in this thesis provides evidence for anti-atherogenic effects of several innovative pharmaceutical interventions that are currently being investigated in clinical trials, specifically targeting hypertension and hypercholesterolemia as risk factors for CVD. Our results further support additional benefit of these treatment strategies in combination with statin treatment which is currently the 'gold standard' therapy for the treatment of CVD. Most of these lipid-modifying treatment strategies affect both LDL-C and HDL-C and we demonstrate that the beneficial effects of these treatment strategies predominantly derive from their non-HDL-C/LDL-C-lowering abilities. Nonetheless, results from preclinical studies and clinical trials support the notion that treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may also inhibit the development of atherosclerosis and reduce the prevalence of CVD.

References

1. Laslett LJ, Alagona P, Jr., Clark BA, 3rd, Drozda JP, Jr., Saldivar F, Wilson SR, Poe C and Hart M. The worldwide environment of cardiovascular disease: prevalence, diagnosis, therapy, and policy issues: a report from the American College of Cardiology. *Journal of the American College of Cardiology*. 2012;60:S1-49.
2. Libby P. The forgotten majority: unfinished business in cardiovascular risk reduction. *Journal of the American College of Cardiology*. 2005;46:1225-8.
3. Davidson MH, Maki KC, Pearson TA, Pasternak RC, Deedwania PC, McKenney JM, Fonarow GC, Maron DJ, Ansell BJ, Clark LT and Ballantyne CM. Results of the National Cholesterol Education (NCEP) Program Evaluation ProjecT Utilizing Novel E-Technology (NEPTUNE) II survey and implications for treatment under the recent NCEP Writing Group recommendations. *The American journal of cardiology*. 2005;96:556-63.
4. Bitzur R, Cohen H, Kamari Y and Harats D. Intolerance to statins: mechanisms and management. *Diabetes care*. 2013;36 Suppl 2:S325-30.
5. Libby P, Okamoto Y, Rocha VZ and Folco E. Inflammation in Atherosclerosis. *Circulation Journal*. 2010;74:213-220.
6. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine*. 2005;352:1685-95.
7. Galkina E and Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annual review of immunology*. 2009;27:165-97.
8. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM and Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:2552-9.
9. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR and Havekes LM. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *The Journal of clinical investigation*. 1991;88:643-55.
10. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:1706-21.
11. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM and Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *Journal of lipid research*. 2007;48:1763-71.
12. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC and Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2016-22.
13. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM and Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008;117:2515-22.
14. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM and Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008;197:57-63.
15. van den Hoek AM, van der Hoorn JW, Maas AC, van den Hoogen RM, van Nieuwkoop A, Droog S, Offerman EH, Pieterman EJ, Havekes LM and Princen HM. APOE*3Leiden.CETP transgenic mice as model for pharmaceutical treatment of the metabolic syndrome. *Diabetes, obesity & metabolism*. 2014;16:537-44.
16. Jankowski P, Bilo G and Kawecka-Jaszcz K. The pulsatile component of blood pressure: its role in the pathogenesis of atherosclerosis. *Blood pressure*. 2007;16:238-45.
17. Lu H, Cassis LA and Daugherty A. Atherosclerosis and arterial blood pressure in mice. *Current drug targets*. 2007;8:1181-9.

18. Kuhnast S, van der Hoorn JW, van den Hoek AM, Havekes LM, Liau G, Jukema JW and Princen HM. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden. CETP transgenic mice with or without treatment with atorvastatin. *Journal of hypertension*. 2012;30:107-16.
19. Solomon SD, Appelbaum E, Manning WJ, Verma A, Berglund T, Lukashevich V, Cherif Papst C, Smith BA and Dahlof B. Effect of the direct Renin inhibitor aliskiren, the Angiotensin receptor blocker losartan, or both on left ventricular mass in patients with hypertension and left ventricular hypertrophy. *Circulation*. 2009;119:530-7.
20. McMurray JJ, Pitt B, Latini R, Maggioni AP, Solomon SD, Keefe DL, Ford J, Verma A and Lewsey J. Effects of the oral direct renin inhibitor aliskiren in patients with symptomatic heart failure. *Circulation Heart failure*. 2008;1:17-24.
21. Parving HH, Persson F, Lewis JB, Lewis EJ and Hollenberg NK. Aliskiren combined with losartan in type 2 diabetes and nephropathy. *The New England journal of medicine*. 2008;358:2433-46.
22. Parving HH, Brenner BM, McMurray JJ, de Zeeuw D, Haffner SM, Solomon SD, Chaturvedi N, Persson F, Desai AS, Nicolaides M, Richard A, Xiang Z, Brunel P and Pfeffer MA. Cardiorenal end points in a trial of aliskiren for type 2 diabetes. *The New England journal of medicine*. 2012;367:2204-13.
23. Gheorghiade M, Bohm M, Greene SJ, Fonarow GC, Lewis EF, Zannad F, Solomon SD, Baschiera F, Botha J, Hua TA, Gimpelewicz CR, Jaumont X, Lesogor A and Maggioni AP. Effect of aliskiren on postdischarge mortality and heart failure readmissions among patients hospitalized for heart failure: the ASTRONAUT randomized trial. *JAMA: the journal of the American Medical Association*. 2013;309:1125-35.
24. Teo KK, Pfeffer M, Mancia G, O'Donnell M, Dagenais G, Diaz R, Dans A, Liu L, Bosch J, Joseph P, Copland I, Jung H, Pogue J and Yusuf S. Aliskiren alone or with other antihypertensives in the elderly with borderline and stage 1 hypertension: the APOLLO trial. *European heart journal*. 2014;35:1743-51.
25. Nicholls SJ, Bakris GL, Kastelein JJ, Menon V, Williams B, Armbrecht J, Brunel P, Nicolaides M, Hsu A, Hu B, Fang H, Puri R, Uno K, Kataoka Y, Bash D and Nissen SE. Effect of aliskiren on progression of coronary disease in patients with prehypertension: the AQUARIUS randomized clinical trial. *JAMA : the journal of the American Medical Association*. 2013;310:1135-44.
26. Ridker PM. LDL cholesterol: controversies and future therapeutic directions. *Lancet*. 2014;384:607-17.
27. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J and Collins R. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *The Lancet*. 2010;376:1670-1681.
28. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R and Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins. *The Lancet*. 2005;366:1267-1278.
29. Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R and Baigent C. The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *The Lancet*. 2012;380:581-590.
30. Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A and Zukel WJ. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation*. 1977;55:767-772.
31. Miller NE, Thelle DS, Forde OH and Mjos OD. The Tromso heart-study. High-density lipoprotein and coronary heart-disease: a prospective case-control study. *Lancet*. 1977;1:965-8.
32. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA : the journal of the American Medical Association*. 2009;302:1993-2000.

33. Carlson LA. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine*. 2005;258:94-114.
34. Kuhnast S, Louwe MC, Heemskerk MM, Pieterman EJ, van Klinken JB, van den Berg SA, Smit JW, Havekes LM, Rensen PC, van der Hoorn JW, Princen HM and Jukema JW. Niacin Reduces Atherosclerosis Development in APOE*3Leiden.CETP Mice Mainly by Reducing NonHDL-Cholesterol. *PloS one*. 2013;8:e66467.
35. Taylor AJ, Sullenberger LE, Lee HJ, Lee JK and Grace KA. Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) 2: a double-blind, placebo-controlled study of extended-release niacin on atherosclerosis progression in secondary prevention patients treated with statins. *Circulation*. 2004;110:3512-7.
36. Taylor AJ, Lee HJ and Sullenberger LE. The effect of 24 months of combination statin and extended-release niacin on carotid intima-media thickness: ARBITER 3. *Current medical research and opinion*. 2006;22:2243-50.
37. Taylor AJ, Villines TC, Stanek EJ, Devine PJ, Griffen L, Miller M, Weissman NJ and Turco M. Extended-release niacin or ezetimibe and carotid intima-media thickness. *The New England journal of medicine*. 2009;361:2113-22.
38. Lee JM, Robson MD, Yu LM, Shirodaria CC, Cunnington C, Kylintireas I, Digby JE, Bannister T, Handa A, Wiesmann F, Durrington PN, Channon KM, Neubauer S and Choudhury RP. Effects of high-dose modified-release nicotinic acid on atherosclerosis and vascular function: a randomized, placebo-controlled, magnetic resonance imaging study. *Journal of the American College of Cardiology*. 2009;54:1787-94.
39. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K and Weintraub W. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. *The New England journal of medicine*. 2011;365:2255-67.
40. Landray MJ, Haynes R, Hopewell JC, Parish S, Aung T, Tomson J, Wallendszus K, Craig M, Jiang L, Collins R and Armitage J. Effects of extended-release niacin with laropiprant in high-risk patients. *The New England journal of medicine*. 2014;371:203-12.
41. Carlson LA and Oro L. Effect of treatment with nicotinic acid for one month on serum lipids in patients with different types of hyperlipidemia. *Atherosclerosis*. 1973;18:1-9.
42. Hoogwerf BJ, Bantle JP, Kuba K, Frantz ID, Jr. and Hunninghake DB. Treatment of type III hyperlipoproteinemia with four different treatment regimens. *Atherosclerosis*. 1984;51:251-9.
43. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H and et al. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature*. 1989;342:448-51.
44. 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 and Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine*. 2007;357:2109-22.
45. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, Chaitman BR, Holme IM, Kallend D, Leiter LA, Leitersdorf E, McMurray JJ, Mundl H, Nicholls SJ, Shah PK, Tardif JC and Wright RS. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *The New England journal of medicine*. 2012;367:2089-99.
46. Kuhnast S, van der Tuin SJ, van der Hoorn JW, van Klinken JB, Simic B, Pieterman E, Havekes LM, Landmesser U, Luscher TF, Willems van Dijk K, Rensen PC, Jukema JW and Princen HM. Anacetrapib reduces progression of atherosclerosis, mainly by reducing non-HDL-cholesterol, improves lesion stability and adds to the beneficial effects of atorvastatin. *European heart journal*. 2014;doi:10.1093/eurheartj/ehu319.
47. Krishna R, Bergman AJ, Jin B, Fallon M, Cote J, Van Hoydonck P, Laethem T, Gendrano IN, 3rd, Van Dyck K, Hilliard D, Laterza O, Snyder K, Chavez-Eng C, Lutz R, Chen J, Bloomfield DM, De Smet M, Van Bortel LM, Gutierrez M, Al-Huniti N, Dykstra K, Gottesdiener KM and Wagner JA. Multiple-dose pharmacodynamics and pharmacokinetics of anacetrapib, a potent cholesteryl ester transfer protein (CETP) inhibitor, in healthy subjects. *Clinical pharmacology and therapeutics*. 2008;84:679-83.

48. Krishna R, Anderson MS, Bergman AJ, Jin B, Fallon M, Cote J, Rosko K, Chavez-Eng C, Lutz R, Bloomfield DM, Gutierrez M, Doherty J, Bieberdorf F, Chodakewitz J, Gottesdiener KM and Wagner JA. Effect of the cholesteryl ester transfer protein inhibitor, anacetrapib, on lipoproteins in patients with dyslipidaemia and on 24-h ambulatory blood pressure in healthy individuals: two double-blind, randomised placebo-controlled phase I studies. *The Lancet*. 2007;370:1907-1914.
49. Bloomfield D, Carlson GL, Sapre A, Tribble D, McKenney JM, Littlejohn TW, 3rd, Sisk CM, Mitchel Y and Pasternak RC. Efficacy and safety of the cholesteryl ester transfer protein inhibitor anacetrapib as monotherapy and coadministered with atorvastatin in dyslipidemic patients. *American heart journal*. 2009;157:352-360 e2.
50. Johns DG, Duffy J, Fisher T, Hubbard BK and Forrest MJ. On- and off-target pharmacology of torcetrapib: current understanding and implications for the structure activity relationships (SAR), discovery and development of cholesteryl ester-transfer protein (CETP) inhibitors. *Drugs*. 2012;72:491-507.
51. Nicholls SJ, Brewer HB, Kastelein JJ, Krueger KA, Wang MD, Shao M, Hu B, McErlean E and Nissen SE. Effects of the CETP inhibitor evacetrapib administered as monotherapy or in combination with statins on HDL and LDL cholesterol: a randomized controlled trial. *JAMA: the journal of the American Medical Association*. 2011;306:2099-2109.
52. Roddy TP, McLaren DG, Chen Y, Xie D, Dunn K, Kulick A, Szeto D, Forrest G, Albanese K, Donnelly M, Gai C, Gewain A, Lederman H, Jensen KK, Ai X, Vachal P, Akinsanya KO, Cleary MA, Previs SF, Dansky HM and Johns DG. Effects of anacetrapib on plasma lipids, apolipoproteins and PCSK9 in healthy, lean rhesus macaques. *European journal of pharmacology*. 2014;740:410-6.
53. Dong B, Singh AB, Fung C, Kan K and Liu J. CETP inhibitors downregulate hepatic LDL receptor and PCSK9 expression in vitro and in vivo through a SREBP2 dependent mechanism. *Atherosclerosis*. 2014;235:449-62.
54. Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villeger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG and Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature genetics*. 2003;34:154-6.
55. Lambert G, Sjouke B, Choque B, Kastelein JJ and Hovingh GK. The PCSK9 decade. *Journal of lipid research*. 2012;53:2515-24.
56. Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L and Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24:1454-9.
57. Mayne J, Dewpura T, Raymond A, Cousins M, Chaplin A, Lahey KA, Lahaye SA, Mbikay M, Ooi TC and Chretien M. Plasma PCSK9 levels are significantly modified by statins and fibrates in humans. *Lipids in health and disease*. 2008;7:22.
58. Careskey HE, Davis RA, Alborn WE, Troutt JS, Cao G and Konrad RJ. Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *Journal of lipid research*. 2008;49:394-8.
59. Kuhnast S, van der Hoorn JW, Pieterman EJ, van den Hoek AM, Sasiela WJ, Gusarova V, Peyman A, Schafer HL, Schwahn U, Jukema JW and Princen HM. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. *Journal of lipid research*. 2014;55:2103-12.
60. Stein EA, Gipe D, Bergeron J, Gaudet D, Weiss R, Dufour R, Wu R and Pordy R. Effect of a monoclonal antibody to PCSK9, REGN727/SAR236553, to reduce low-density lipoprotein cholesterol in patients with heterozygous familial hypercholesterolaemia on stable statin dose with or without ezetimibe therapy: a phase 2 randomised controlled trial. *The Lancet*. 2012;380:29-36.
61. McKenney JM, Koren MJ, Kereiakes DJ, Hanotin C, Ferrand AC and Stein EA. Safety and efficacy of a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 serine protease, SAR236553/REGN727, in patients with primary hypercholesterolemia receiving ongoing stable atorvastatin therapy. *Journal of the American College of Cardiology*. 2012;59:2344-53.

62. Roth EM, McKenney JM, Hanotin C, Asset G and Stein EA. Atorvastatin with or without an antibody to PCSK9 in primary hypercholesterolemia. *The New England journal of medicine*. 2012;367:1891-900.
63. Stein EA and Swergold GD. Potential of proprotein convertase subtilisin/kexin type 9 based therapeutics. *Current atherosclerosis reports*. 2013;15:310.
64. Chapman MJ, Le Goff W, Guerin M and Kontush A. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *European heart journal*. 2010;31:149-64.
65. Boekholdt SM, Arsenault BJ, Hovingh GK, Mora S, Pedersen TR, Larosa JC, Welch KM, Amarenco P, Demicco DA, Tonkin AM, Sullivan DR, Kirby A, Colhoun HM, Hitman GA, Betteridge DJ, Durrington PN, Clearfield MB, Downs JR, Gotto AM, Jr., Ridker PM and Kastelein JJ. Levels and changes of HDL cholesterol and apolipoprotein A-I in relation to risk of cardiovascular events among statin-treated patients: a meta-analysis. *Circulation*. 2013;128:1504-12.
66. Badimon JJ, Badimon L and Fuster V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *The Journal of clinical investigation*. 1990;85:1234-41.
67. Mezdour H, Yamamura T, Nomura S and Yamamoto A. Exogenous supply of artificial lipoproteins does not decrease susceptibility to atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis*. 1995;113:237-46.
68. Miyazaki A, Sakuma S, Morikawa W, Takiue T, Miake F, Terano T, Sakai M, Hakamata H, Sakamoto Y, Natio M and et al. Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits. *Arteriosclerosis, thrombosis, and vascular biology*. 1995;15:1882-8.
69. Nicholls SJ, Cutri B, Worthley SG, Kee P, Rye KA, Bao S and Barter PJ. Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis in rabbits. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2416-21.
70. Ameli S, Hultgardh-Nilsson A, Cercek B, Shah PK, Forrester JS, Ageland H and Nilsson J. Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits. *Circulation*. 1994;90:1935-1941.
71. Soma MR, Donetti E, Parolini C, Sirtori CR, Fumagalli R and Franceschini G. Recombinant apolipoprotein A-IMilano dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits. *Circulation research*. 1995;76:405-11.
72. Chiesa G. Recombinant Apolipoprotein A-IMilano Infusion Into Rabbit Carotid Artery Rapidly Removes Lipid From Fatty Streaks. *Circulation research*. 2002;90:974-980.
73. Parolini C, Marchesi M, Lorenzon P, Castano M, Balconi E, Miragoli L, Chaabane L, Morisetti A, Lorusso V, Martin BJ, Bisgaier CL, Krause B, Newton RS, Sirtori CR and Chiesa G. Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo assessment by intravascular ultrasound and magnetic resonance imaging. *Journal of the American College of Cardiology*. 2008;51:1098-103.
74. Ibanez B, Giannarelli C, Cimmino G, Santos-Gallego CG, Alique M, Pinero A, Vilahur G, Fuster V, Badimon L and Badimon JJ. Recombinant HDL(Milano) exerts greater anti-inflammatory and plaque stabilizing properties than HDL(wild-type). *Atherosclerosis*. 2012;220:72-7.
75. Ibanez B, Vilahur G, Cimmino G, Speidl WS, Pinero A, Choi BG, Zafar MU, Santos-Gallego CG, Krause B, Badimon L, Fuster V and Badimon JJ. Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration: magnetic resonance imaging study in an experimental model of atherosclerosis. *Journal of the American College of Cardiology*. 2008;51:1104-9.
76. Van Lenten BJ, Wagner AC, Navab M, Anantharamaiah GM, Hama S, Reddy ST and Fogelman AM. Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits. *Journal of lipid research*. 2007;48:2344-53.
77. Iwata A, Miura S, Zhang B, Imaizumi S, Uehara Y, Shiomi M and Saku K. Antiatherogenic effects of newly developed apolipoprotein A-I mimetic peptide/phospholipid complexes against aortic plaque burden in Watanabe-heritable hyperlipidemic rabbits. *Atherosclerosis*. 2011;218:300-7.

78. Tardif JC, Gregoire J, L'Allier PL, Ibrahim R, Lesperance J, Heinonen TM, Kouz S, Berry C, Basser R, Lavoie MA, Guertin MC and Rodes-Cabau J. Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA: the journal of the American Medical Association*. 2007;297:1675-82.
79. Shaw JA, Bobik A, Murphy A, Kanellakis P, Blombery P, Mukhamedova N, Woollard K, Lyon S, Sviridov D and Dart AM. Infusion of reconstituted high-density lipoprotein leads to acute changes in human atherosclerotic plaque. *Circulation research*. 2008;103:1084-91.
80. Waksman R, Torguson R, Kent KM, Pichard AD, Suddath WO, Satler LF, Martin BD, Perlman TJ, Maltais JA, Weissman NJ, Fitzgerald PJ and Brewer HB, Jr. A first-in-man, randomized, placebo-controlled study to evaluate the safety and feasibility of autologous delipidated high-density lipoprotein plasma infusions in patients with acute coronary syndrome. *Journal of the American College of Cardiology*. 2010;55:2727-35.
81. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC and Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA : the journal of the American Medical Association*. 2003;290:2292-300.
82. Rader DJ and Hovingh GK. HDL and cardiovascular disease. *Lancet*. 2014;384:618-25.

Summary

Samenvatting

List of Publications

Curriculum Vitae

Dankwoord

Summary

Cardiovascular disease (CVD) is the leading cause of death worldwide despite the successful development of several pharmaceutical interventions of which statin therapy is the dominating lipid-lowering treatment option. Atherosclerosis, a chronic inflammatory disease of multifactorial origin, is a dominant contributor to the development of CVD. The research described in this thesis investigated the effects of innovative pharmaceutical interventions in experimental atherosclerosis, targeting hypertension and high blood cholesterol, more specifically high low-density lipoprotein-cholesterol (LDL-C) and low high-density lipoprotein-cholesterol (HDL-C), as risk factors for CVD.

In view of the fact that hypertension is a leading risk factor for CVD and associated with the development of atherosclerosis, we investigated the anti-atherosclerotic effects of aliskiren, the first commercially available, orally active, direct renin inhibitor approved for the treatment of hypertension in **chapter 2**. In this study in APOE*3Leiden.CETP mice, we demonstrated beneficial effects of aliskiren on atherosclerosis development and plaque stability when administered alone and in combination with atorvastatin, possibly via a mechanism involving T cells. These results suggest a potential benefit of using aliskiren in a clinical setting, particularly in combination with statin treatment.

Cholesterol contained in LDL particles is well recognized as a primary causal risk factor for coronary heart disease (CHD) as evidenced by experimental, epidemiological and genetic data and intervention trials. However, despite the fact that epidemiological studies consistently reported an inverse association between HDL-C and CHD risk, the benefits of raising HDL-C remain less defined. In **Chapter 3 to 6**, we investigated the effects of novel lipid-modifying treatment strategies, i.e. LDL-C-lowering and/or HDL-C-raising compounds on atherosclerosis development in the APOE*3Leiden.CETP mouse model, since these mice respond to both LDL-C-lowering and HDL-C-raising compounds in a human-like manner.

The benefits of niacin on plasma lipids were first described in 1955 and led to the development of niacin for therapeutic purposes. In **chapter 3**, we aimed to address the discrepancy between the beneficial effects of niacin in initial clinical trials and the lack of effect of niacin on top of statin treatment on the reduction of CVD events in the large AIM-HIGH and HPS2-THRIVE clinical trials by evaluating the effects of niacin alone and in combination with simvastatin on atherosclerosis development. We showed that niacin decreases atherosclerosis development mainly by reducing non-HDL-C with modest HDL-C-raising and additional anti-inflammatory effects. The additive effect of niacin on top of simvastatin was mostly dependent on its non-HDL-C-lowering capacities. These data suggest that clinical beneficial effects of niacin are largely dependent on its ability to lower LDL-C on top of concomitant lipid-lowering therapy and may explain the failure of niacin in the AIM-HIGH and HPS2-THRIVE trials in patients subjected to aggressive LDL-C-lowering treatment.

In 1989, markedly increased HDL-C led to the discovery of the first mutation in the cholesteryl ester transfer protein (CETP) gene in two Japanese subjects. CETP facilitates the transfer of cholesteryl esters from atheroprotective HDL to atherogenic V(LDL) and has become a target to increase HDL-C. This has led to the development of several small molecule CETP inhibitors. In **chapter 4**, we investigated the effects of a broad dose range of the novel CETP inhibitor, anacetrapib on atherosclerosis development and we examined possible additive/synergistic effects of anacetrapib on top of atorvastatin. In our study, anacetrapib dose-dependently reduced CETP activity, thereby decreasing non-HDL-C and increasing HDL-C. Moreover, anacetrapib dose-dependently reduced atherosclerosis development. This effect was mainly ascribed to a reduction in non-HDL-C despite a remarkable increase in HDL-C and without affecting HDL functionality. In addition, anacetrapib improved lesion stability and added to the anti-atherogenic effects of atorvastatin. We further explored the mechanism by which anacetrapib reduces (V)LDL-C and whether this effect is dependent on the inhibition of CETP activity in **chapter 5**. In this study, we showed that anacetrapib reduces (V)LDL-C by increasing hepatic remnant uptake via two mechanisms: (i) inhibition of CETP activity, resulting in remodelled VLDL particles that are more susceptible to hepatic uptake; and (ii) a CETP-independent reduction in plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) level that has the potential to increase LDL receptor (LDLR)-mediated hepatic remnant clearance.

In 2003, Abifadel et al. identified two French families with autosomal dominant hypercholesterolemia caused by mutations in PCSK9. PCSK9 is a serine protease responsible for LDL receptor (LDLR) degradation by preventing the recycling of the receptor to the cell membrane after internalization. The upregulation of the LDLR after statin treatment is accompanied by an upregulation of PCSK9 which in turn promotes LDLR degradation. Inhibition of PCSK9 is, therefore, a potential novel strategy in the treatment against CVD, especially in combination with statin treatment. We, therefore investigated the effects of 2 dosages of the fully human, monoclonal antibody against PCSK9, alirocumab alone and in combination with atorvastatin on atherosclerosis development in **chapter 6**. In this study, alirocumab dose-dependently increased hepatic LDLR protein levels and consequently decreased plasma cholesterol levels and reduced the development of atherosclerosis. Moreover, alirocumab improved lesion morphology and enhanced the beneficial effects of atorvastatin. The anti-atherosclerotic effect was strongly dependent on the reduction of plasma TC levels, indicating that the majority of the effect was brought about by cholesterol lowering leaving limited/no space for other potential (pleiotropic) effects. This is the first study to show that a monoclonal antibody to PCSK9 reduces atherosclerosis development.

In **chapter 7**, we reviewed the effects of established and novel treatment strategies, specifically targeting HDL, other than statins on inhibition of atherosclerosis development in preclinical studies in animals expressing CETP, a crucial gene involved in HDL metabolism

and implicated in the mechanisms by which most therapies modulate HDL. In addition, we conducted a meta-analysis to evaluate the potential effects of these treatment strategies on the prevention of clinical events in randomized controlled trials. We focused specifically on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising on inhibition of atherosclerosis and the prevention of CVD. According to results from our systematic review and meta-analysis, it is evident that the protective role of lowering LDL-C and non-HDL-C is well-established. The contribution of raising HDL-C on inhibition of atherosclerosis and the prevention of cardiovascular disease remains undefined and may be dependent on the mode of action of HDL-C-modification. Nonetheless, treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may be worth exploring.

In conclusion, the research described in this thesis provides evidence for anti-atherogenic effects of several innovative pharmaceutical interventions that are currently being investigated in clinical trials, specifically targeting hypertension and hypercholesterolemia as risk factors for CVD. Our results further support additional benefit of these treatment strategies in combination with statin treatment which is currently the 'gold standard' therapy for the treatment of CVD. Most of these lipid-modifying treatment strategies affect both LDL-C and HDL-C and we demonstrate that the beneficial effects of these treatment strategies predominantly derive from their non-HDL-C/LDL-C-lowering abilities. Nonetheless, results from preclinical studies and clinical trials support the notion that treatment strategies aimed at improving HDL function and raising apolipoprotein A-I may also inhibit the development of atherosclerosis and reduce the prevalence of CVD.

Samenvatting

Hart- en vaatziekten zijn wereldwijd doodsoorzaak nummer één ondanks succesvolle ontwikkeling van verschillende medicijnen, waarvan statines gelden als gouden standaard voor lipidenverlagende therapie. Atherosclerose is een chronische ontstekingsziekte, van multifactoriële origine, welke een grote bijdrage levert aan de ontwikkeling van hart- en vaatziekten. Het in dit proefschrift beschreven onderzoek bekeek de effecten van innovatieve farmaceutische interventies op experimentele atherosclerose. Deze interventies waren gericht tegen hypertensie of hyperlipidemie en, meer specifiek, tegen een hoog lage dichtheid lipoproteine- cholesterol (LDL-C) niveau en laag hoge dichtheid lipoproteine-cholesterol (HDL-C) niveau, wat risico's zijn voor hart- en vaatziekten.

Gezien het feit dat hypertensie een belangrijke risicofactor voor hart- en vaatziekten is en geassocieerd is met de ontwikkeling van atherosclerose, werden de anti-atherosclerotische effecten van aliskiren onderzocht in **hoofdstuk 2**. Aliskiren is de eerste directe renineremmer op de markt voor de behandeling van hypertensie en is geschikt voor orale toediening. In deze studie werden in APOE*3Leiden.CETP transgene muizen positieve effecten gezien van aliskiren op de ontwikkeling van atherosclerose en de stabiliteit van atherosclerotische lesies. Dit was het geval in monotherapie, wanneer aliskiren alleen werd gegeven, en in combinatietherapie met atorvastatine, mogelijk via een mechanisme waarin T-cellen betrokken zijn. Deze resultaten suggereren een voordeel van het gebruik van aliskiren in de kliniek, met name in combinatie met een statinebehandeling.

Cholesterol verpakt in LDL deeltjes is erkend als een primaire en causale risicofactor voor coronaire hartziekten, bewezen door experimentele, epidemiologische en genetische data en interventie studies. Ondanks het feit dat epidemiologische studies consistent een omgekeerde associatie rapporteren voor HDL-C en coronaire hartziekten, blijven de voordelen van HDL-C verhoging onduidelijk. In **hoofdstuk 3 t/m 6** werden de effecten onderzocht van nieuwe lipidenmodulerende behandelingsstrategieën, zoals LDL-C verlagende en/of HDL-C verhogende stoffen, op de ontwikkeling van atherosclerose in APOE*3Leiden.CETP transgene muizen. Deze muizen zijn responsief voor zowel LDL-C verlagende als HDL-C verhogende stoffen op een manier die lijkt op die van de mens.

De voordelen van niacine op plasmalipiden werden voor het eerst beschreven in 1955 en leidden tot de ontwikkeling van niacine voor therapeutisch gebruik. In **hoofdstuk 3** is getracht de discrepantie uit te leggen tussen de positieve effecten van niacine, gevonden in de eerste klinische studies, en de afwezigheid van een effect van niacine op hart- en vaatziekten wanneer het in combinatie met een statine werd gebruikt in de grote AIM-HIGH en HPS2-THRIVE klinische studies. Hiervoor werden de effecten van niacinebehandeling alleen of in combinatie met simvastatine op atherosclerose ontwikkeling onderzocht. Deze studie liet zien dat niacine de ontwikkeling van atherosclerose vermindert, hoofdzakelijk

door non-HDL-C te verlagen, met milde HDL-C verhogende en additionele anti-inflammatoire effecten. Het additive effect van niacine bovenop simvastatinebehandeling was voornamelijk toe te schrijven aan de non-HDL-C verlaging. Deze data suggereren dat positieve effecten van niacine in de kliniek voor een groot deel afhankelijk zijn van de LDL-C verlagende effecten bovenop de toegepaste lipidenverlagende therapie. Daarmee kunnen deze data het falen van niacine in de AIM-HIGH en HPS2-THRIVE studies verklaren, waarin patiënten een agressieve LDL-C verlagende behandeling ondergingen.

In 1989 leidde een overduidelijk verhoogd HDL-C in twee Japanners tot de ontdekking van de eerste mutatie in het cholesteryl ester transfer proteine (CETP) gen. CETP faciliteert de overdracht van cholesterylester van het atheroprotectieve HDL naar het atherogene V(LDL) en is daardoor een doelwit geworden om HDL-C te verhogen. Dit heeft geleid tot de ontwikkeling van verschillende klein molecuul CETP remmers. In **hoofdstuk 4**, werden de effecten onderzocht van een grote doseringsreeks van de nieuwe CETP remmer anacetrapib op atherosclerose ontwikkeling. Tevens werden mogelijk additieve/synergistische effecten van anacetrapib in combinatie met statinetherapie beoordeeld. In deze studie verlaagde anacetrapib de CETP activiteit dosisafhankelijk en verlaagde daarbij het non-HDL-C en verhoogde het HDL-C. Anacetrapib remde de atherosclerose ontwikkeling dosisafhankelijk. Dit effect werd voornamelijk toegeschreven aan de verlaging in non-HDL-C ondanks een opmerkelijke verhoging van HDL-C, zonder daarbij de functionaliteit van HDL te veranderen. Anacetrapib verbeterde ook de stabiliteit van lesies en had een toegevoegde waarde voor de anti-atherogene effecten van atorvastatine. Vervolgens werd het (V)LDL-C verlagende werkingsmechanisme van anacetrapib onderzocht in **hoofdstuk 5** en werd bekeken of dit effect afhankelijk is van de remming op CETP activiteit. De studie liet zien dat anacetrapib (V)LDL-C verlaagt door het verhogen van de hepatische opname van restpartikels via twee mechanismen: (i) remming van de CETP activiteit, wat resulteert in geremodelleerde VLDL deeltjes, die makkelijker op te nemen zijn door de lever; en (ii) een CETP-onafhankelijke afname in plasma proprotein convertase subtilisin/kexin type 9 (PCSK9) niveau wat de LDL receptor (LDLR)- gemedieerde hepatische restdeeltjes klaring kan verhogen.

In 2003 identificeerde Abifadel et al. twee Franse families met autosomaal dominante hypercholesterolemie, wat veroorzaakt werd door mutaties in het PCSK9 gen. PCSK9 is een serine protease welke LDLR degradatie promoot door recycling van de receptor na internalisatie terug naar het celmembraan te voorkomen. De verhoging van de LDLR expressie na statinebehandeling wordt vergezeld door een verhoging van PCSK9, wat daarop weer de LDLR degradatie bevordert. Remming van PCSK9 is daarom in potentie een nieuwe strategie in de behandeling tegen hart- en vaatziekten, vooral in combinatie met een statinebehandeling. De effecten van 2 verschillende doseringen alirocumab, een volledig humaan monoclonaal antilichaam tegen PCSK9, alleen en in combinatie met atorvastatine op atherosclerose ontwikkeling werden onderzocht in **hoofdstuk 6**. In deze studie verhoogde

alirocumab de hepatische LDLR eiwitexpressie dosisafhankelijk en daardoor verlaagde het de plasma cholesterolwaarden en de ontwikkeling van atherosclerose. Tevens verbeterde alirocumab de morfologie van lesies en versterkte het de positieve effecten van atorvastatine. Het anti-atherosclerotische effect was sterk gecorreleerd aan de verlaging van het plasma totaal cholesterol, wat suggereert dat het effect grotendeels werd veroorzaakt door cholesterolverlaging waarbij geen of weinig ruimte was voor andere mogelijke (pleiotrope) effecten. Dit is de eerste studie die laat zien dat een monoclonaal antilichaam tegen PCSK9 daadwerkelijk atherosclerose ontwikkeling kan remmen.

In **hoofdstuk 7** hebben we de effecten van reeds bestaande en nieuwe behandelingsstrategieën, specifiek gericht op HDL anders dan statines, op atherosclerose ontwikkeling in preklinische studies geëvalueerd. Voor dit systematische overzicht werden alleen studies bekeken waarin dieren werden gebruikt die CETP tot expressie brengen, omdat dit een cruciaal gen is voor het HDL metabolisme en veelal betrokken is in het werkingsmechanisme van de meeste HDL modulerende therapieën. Tevens werd een meta-analyse uitgevoerd om de potentiele effecten van deze behandelingen op de preventie van mortaliteit en morbiditeit in gerandomiseerde gecontroleerde studies te evalueren. Specifiek werd de bijdrage van de non-HDL-C/LDL-C verlaging ten opzichte van HDL-C verhoging op de remming van atherosclerose en de preventie van hart- en vaatziekten onderzocht. De resultaten van dit systematisch overzicht en deze meta-analyse bevestigen dat de beschermende rol van non-HDL-C/ LDL-C verlaging evident is. De bijdrage van HDL-C verhoging op de remming van atherosclerose ontwikkeling en de preventie van hart- en vaatziekten blijft echter onduidelijk en zou afhankelijk kunnen zijn van het werkingsmechanisme van HDL-C modificatie. Niettemin zouden behandelingen gericht op de verbetering van HDL functie en de verhoging van apolipoproteine A-I zeer waardevol kunnen zijn.

In conclusie, het in dit proefschrift beschreven onderzoek levert bewijs voor anti-atherogene effecten van verschillende innovatieve farmaceutische interventies, die momenteel onderzocht worden in klinische studies, specifiek gericht tegen hypertensie en hypercholesterolemie wat risicofactoren zijn voor het ontstaan van hart- en vaatziekten. Deze resultaten onderschrijven de toegevoegde waarde van deze behandelingsstrategieën in combinatie met een statinebehandeling, wat momenteel de gouden standaard is voor de behandeling van hart- en vaatziekten. De meeste van deze lipidenmodulerende stoffen beïnvloeden zowel LDL-C als HDL-C. Onderzoek in dit proefschrift laat zien dat de positieve effecten van deze behandelingen voornamelijk toe te schrijven zijn aan de non-HDL-C/LDL-C verlagende effecten. Niettemin ondersteunen resultaten van preklinische en klinische studies de gedachte dat het verbeteren van HDL functie en het verhogen van apolipoproteine A-I de ontwikkeling van atherosclerose kan remmen en de prevalentie van hart- en vaatziekten kan verminderen.

List of Publications

Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden.CETP transgenic mice with or without treatment with atorvastatin
Susan Kühnast, José W.A. van der Hoorn, Anita M. van den Hoek, Louis M. Havekes, Gene Liau, J. Wouter Jukema, Hans M.G. Princen
J Hypertens. 2012 Jan;30(1):107-16.

Niacin reduces atherosclerosis development in APOE*3Leiden.CETP mice mainly by reducing non-HDL-cholesterol
Susan Kühnast, Mieke C. Louwe, Mattijs M. Heemskerk, Elsbet J. Pieterman, Jan B. van Klinken, Sjoerd A.A. van den Berg, Johannes W.A. Smit, Louis M. Havekes, Patrick C.N. Rensen, José W.A. van der Hoorn, Hans M.G. Princen, J. Wouter Jukema
PLoS One. 2013 Jun 19;8(6):e66467.

Osteoarthritis development is induced by increased dietary cholesterol and can be inhibited by atorvastatin in APOE*3Leiden.CETP mice, a translational model for atherosclerosis
Lobke M. Gierman, Susan Kühnast, Angela Koudijs, Elsbet J. Pieterman, Margreet Kloppenburg, Gerjo J.V.M. van Osch, Vedrana Stojanovic-Susulic, Tom W.J. Huizinga, Hans M.G. Princen, Anne-Marie Zuurmond.
Ann Rheum Dis. 2014 May;73(5):921-7.

Anacetrapib reduces progression of atherosclerosis, mainly by reducing non-HDL-cholesterol, improves lesion stability and adds to the beneficial effects of atorvastatin
Susan Kühnast, Sam J.L. van der Tuin, José W.A. van der Hoorn, Jan B. van Klinken, Branko Simic, Elsbet Pieterman, Louis M. Havekes, Ulf Landmesser, Thomas F. Lüscher, Ko Willems van Dijk, Patrick C.N. Rensen, J. Wouter Jukema, Hans M.G. Princen
Eur Heart J. 2015 Jan 1;36(1):39-50.

Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin
Susan Kühnast, José W.A. van der Hoorn, Elsbet J. Pieterman, Anita M. van den Hoek, William J. Sasiela, Viktoria Gusarova, Anusch Peyman, Hans-Ludwig Schäfer, Uwe Schwahn, J. Wouter Jukema, Hans M.G. Princen
J Lipid Res. 2014 Oct;55(10):2103-12.

Anacetrapib reduces (V)LDL-cholesterol by inhibition of CETP activity and reduction of plasma PCSK9

Susan Kühnast, Sam J.L. van der Tuin, Jimmy F.P. Berbée, Lars Verschuren, Elsbet J. Pieterman, Louis M. Havekes, José W.A. van der Hoorn, Patrick C.N. Rensen, J. Wouter Jukema, Hans M.G. Princen, Ko Willems van Dijk, Yanan Wang

Submitted.

Innovative pharmaceutical interventions in cardiovascular disease: focusing on the contribution of non-HDL-C/LDL-C-lowering versus HDL-C-raising

A systematic review and meta-analysis of relevant preclinical studies and clinical trials

Susan Kühnast, Marta Fiocco, José W.A. van der Hoorn, Hans M.G. Princen, J. Wouter Jukema

Accepted – Eur J Pharmacol.

Curriculum Vitae

Susan Kühnast was born on 8 December 1980 in Ermelo, South Africa. In 1998, she graduated from the Technical High School Vereeniging*. In 1999, she enrolled for a bachelor program at the Potchefstroom University for Christian Higher Education (PU for CHE)[#]. She obtained a BSc degree in Human Movement Science cum laude in 2002, a BSc Honours degree in Biokinetics/Exercise Physiology cum laude in 2003 and a BSc Honours degree in Cardio- and Neurovascular Physiology cum laude in 2004. She received an award for outstanding academic achievements in the Health Science Faculty from the PU for CHE in 2002. Susan worked as a qualified Biokineticist/Exercise Physiologist in her own practice from 2005 until 2009 and during this time she completed her Master studies in Cardiovascular Physiology at the PU for CHE. In 2009, she relocated to the Netherlands and was appointed as a PhD candidate at the department of Cardiology, Leiden University Medical Center (LUMC). During her PhD studies she was sent on secondment to the Netherlands Organization for Applied Research (TNO - Metabolic Health Research) under supervision of Prof. Dr. J.W. Jukema, Dr. J.M.G. Princen and Dr. J.W.A. van der Hoorn. Her PhD research, of which the results are described in this thesis, investigated the effects of innovative pharmaceutical interventions in experimental atherosclerosis. During her PhD studies she received a poster award at the Cardio Vascular Conference in the Netherlands in 2011 and a Travel Award for Young Investigators at the Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions in the USA in 2013. After completion of her thesis in 2015, Susan relocated back to South-Africa where she will pursue a career in heath science.

*Now Vereeniging Gimnasium; [#]Now North-West University

Dankwoord

Ter inleiding van my dankwoord verwys ek graag na stelling 10: “It is not where you go, it is who you know.” Baie dankie vir elkeen se hulp en ondersteuning tydens die totstandkoming van my proefskrif.

Beste Jose, dit was vir my ‘n groot plesier om jou as begeleider te hê. Ek het oneindig respek vir die manier waarop jy alle projekte aanpak. Beste Hans, ek bewonder die vermoë waarop jy enige vraag kan beantwoord met ‘n verwysing na ‘n artikel en hoe jy dan ook die artikel onmiddellik kan vind. Aan prof. Jukema: beste Wouter, jou insette vanuit ‘n kliniese perspektief het gehelp om die gaping tussen prekliniese en kliniese navorsing te oorbrug, en daarvoor doen ons uiteraard prekliniese navorsing. Baie dankie vir julle bydrae en dat ek julle enige tyd kon lastig val met ‘n paar vrae.

Liewe Martine en Petra, baie dankie dat julle bereid is om paranimfs te wees vir iemand wat 14 000 km van Leiden af woon. Petra, jy is so ‘n liewe mens en jy is altyd meer as bereid om te luister en te help waar jy kan. Martine, baie dankie is net nie goed genoeg nie en as ek jou kon bedank vir al jou hulp en ondersteunng met iets wat jy kan eet, dan was obesiteit jou voorland. Maar ook veral dankie vir julle vriendskap wat nie deur afstand geaffekteer word nie.

Beste Rob, Wen, Lobke en Anne. Sonder kamergenote kan geen AIO promoveer nie. Julle het die lewe as AIO interessant gemaak. Baie dankie vir al julle hulp met die laaste voorbereidings en sterkte met toekomstige ekperimente en werksonderhoude.

Liewe Elsbet, behalwe vir die feit dat jy alles weet en dat ek jou veel meer geraadpleeg het as google, mis ek veral die nie-wetenskaplike gesprekke. Beste Erik, toe ek hoor dat jy met pensioen gaan, het ek ook maar besluit om my AIO- periode af te rond.

Beste Mieke en Sam, met stampe en stote het ons saam mooi artikels gepubliseer. Dankie vir die samewerking en sterkte met alle toekomstige projekte. Prof. Rensen en prof. van Dijk, beste Patrick en Ko, dankie vir julle insette wat bygedra het tot die publisering van bogenoemde artikels.

Dr. Fiocco, beste Marta, dankie vir die samewerking wat gelei het tot die ontstaan van ‘n uitgebreide meta-analise.

TNO kollegas: Coen, Roeland, Robert K, Teake, Peter, Anita v d H, Reinout, Anne-Marie Z, Alain, Leslie, Benno, Lex, Esther, Kanita, Lars, Frans, Robert O, Jan, Teun, Ria, Karin, Simone, Anita v N, Wim, Marijke, Annemarie M, Frits, Angela, Carla, Elly, Herma, Koen, Nicole, Sabine, Jessica, Natascha, Joline, Arianne, Marrie, Christa, Annie, Ageeth, Adri, Bea, Pascalle, Kirsty, Suzanna, Marjolein en Renate, baie dankie vir ‘n fantastiese vyf jaar wat ek vir niks sou verruil nie.

LUMC kollegas: Amanda, Bruno, Claudia, Edwin, Hanna, Iliana, Irene, Janine, Janna, Jimmy, Jitske, Lianne, Man-Chi, Mariette, Martiene, Matthijs, Nancy, Padmini, Sander en

Sjoerd, baie dankie vir interessante weeklikse presentasies en prettige naweke in Frankryk. Prof. Havekes: beste Louis, die prettige naweke in Frankryk was natuurlik nie moontlik sonder jou nie. Baie dankie daarvoor, maar ook vir jou kommentaar en advies wat gelei het tot ‘n aantal mooi publikasies.

Beste Karin en Christian, Tanja en Marc-Simon, Deon en Ella, Vinnie, Neill, Linton en Karima, baie dankie vir julle vriendskap tydens die afgelope vyf jaar in Nederland. Liewe Rhonda, Duncan en Olivia, julle is die gom wat die vriende bymekaar hou en julle huis staan altyd oop vir ons. Baie dankie vir julle diepgewortelde vriendskap.

Liewe Marja, Hans en Tante Rietje, ons is bevoorreg om familie soos julle te hê. Beste Ans, jy het ook die afgelope vyf jaar deel geword van my familie. Baie dankie vir al die lekker kuiertjies by julle tuis, waar ‘n paar uur soos ‘n paar vakansiedae voel.

Beste skoonfamilie, Rolf, Lida, Marianne en Deon, dankie vir julle ondersteuning die afgelope vyf jaar. Lida, ek is bevoorreg om ‘n taalpraktisyn in die familie te hê.

Liewe ma, Isabel, en broer, Hennie, ek sê nie genoeg dankie vir alles wat julle vir my beteken nie, selfs al sit ek aan de ander kant van die wêreld. Hierdie proefskrif was verseker nie moontlik sonder julle ondersteuning en besoeke in Nederland nie. Ek is oneindig lief vir julle.

Liewe Alex, baie dankie vir al jou liefde en ondersteuning die afgelope vyf jaar. Ons het al soveel saam meegemaak en elke jaar word net beter. Jy is my beste vriend.