CHAPTER 2

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

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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**).

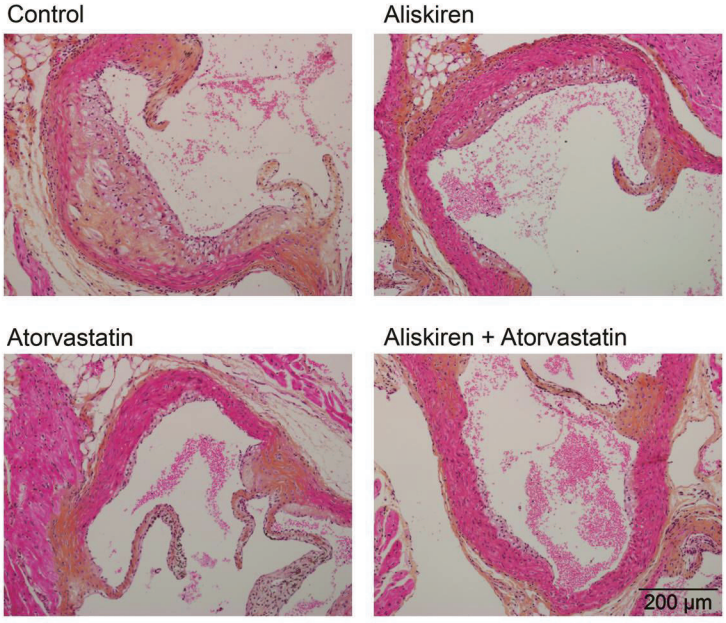

Figure 1 Representative photomicrographs of atherosclerotic lesions in the cross section of the aortic root area in the four groups (hematoxylin-phloxine-saffron staining).

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.

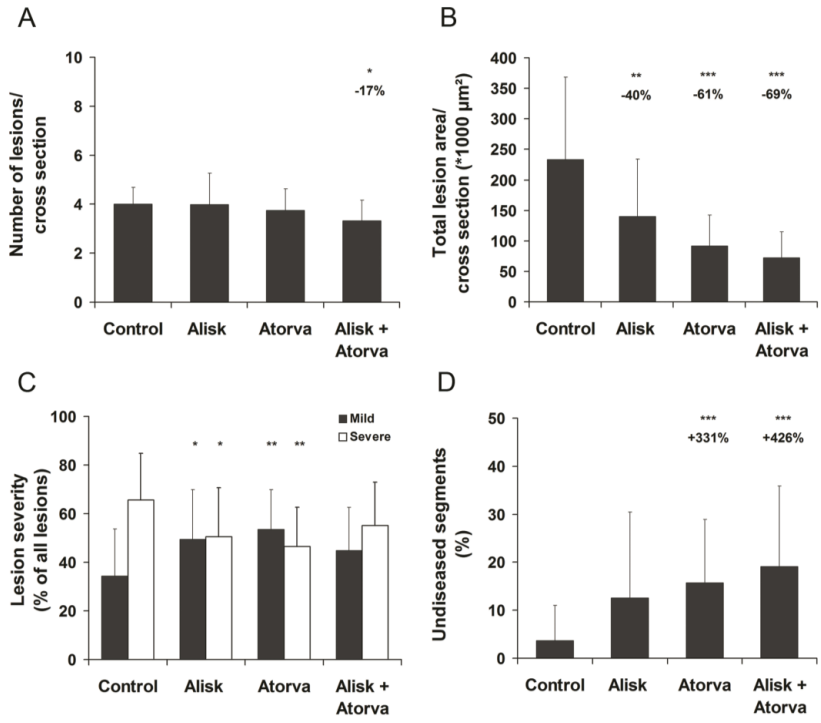

Figure 2 The effect of aliskiren, atorvastatin and a combination of aliskiren and atorvastatin on atherosclerosis development in aortic root area. The number of lesions (A) and total lesion area per cross section (B), as well as the lesion severity as percentage of all lesions (C) and the percentage 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.

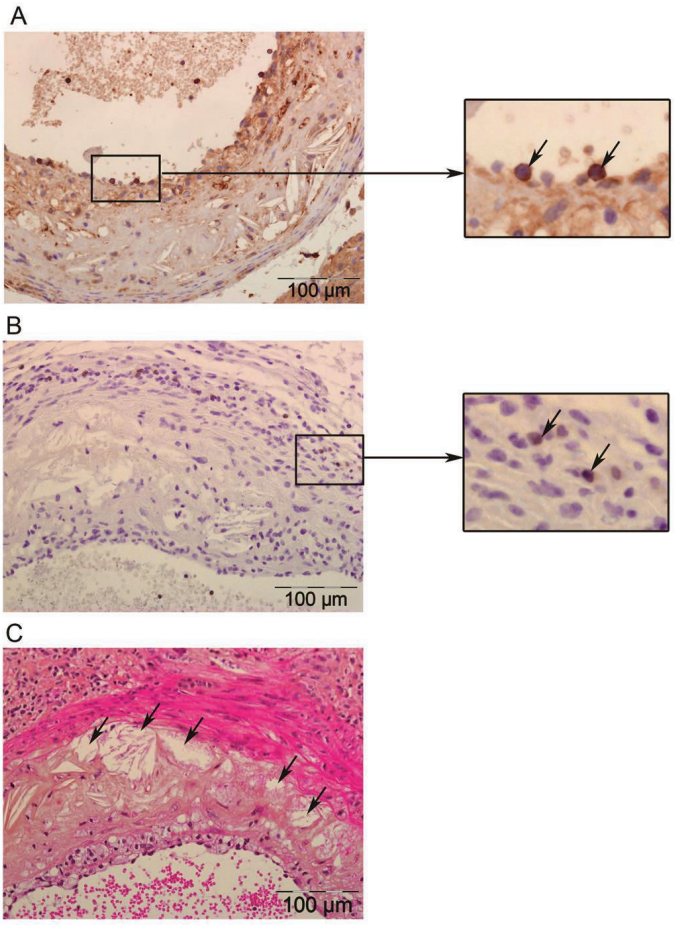

Figure 3 Representative photomicrographs of mouse monocytes and T cells after immunostaining with anti-mouse AIA31240 (A) and anti-human CD3 (B), respectively, as well as necrotic area (indicated by arrows) after hemotoxylin-phloxine-saffron (HPS) staining (C).

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.

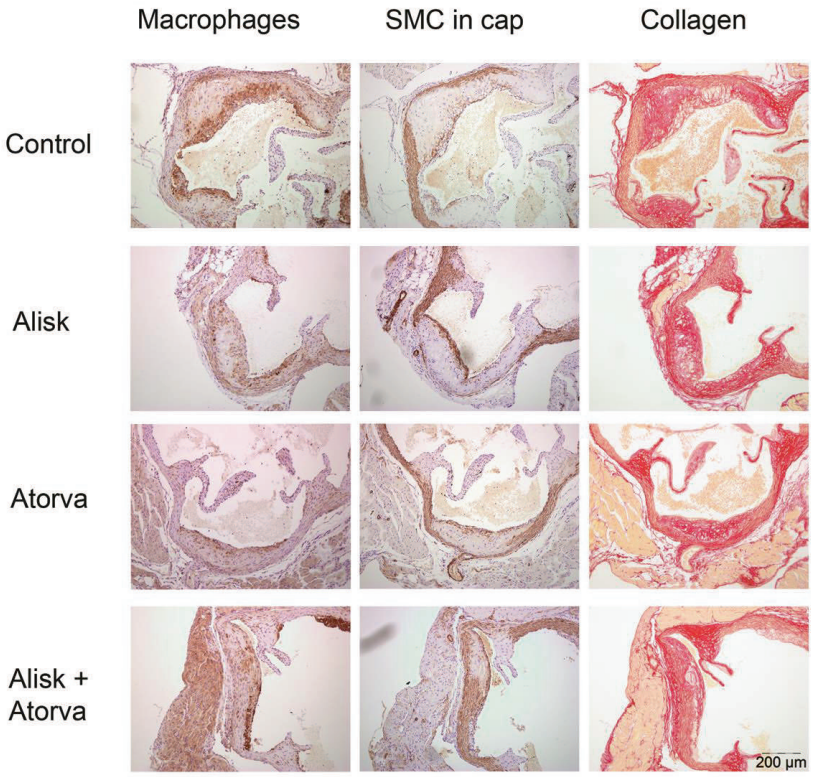

Figure 4 Representative photomicrographs of macrophage content and SMC content in the cap after immunostaining with anti-mouse Mac-3 and anti-human alpha actin, respectively, and collagen content after sirius red staining.
Alisk, aliskiren; Atorva, atorvastatin.

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.

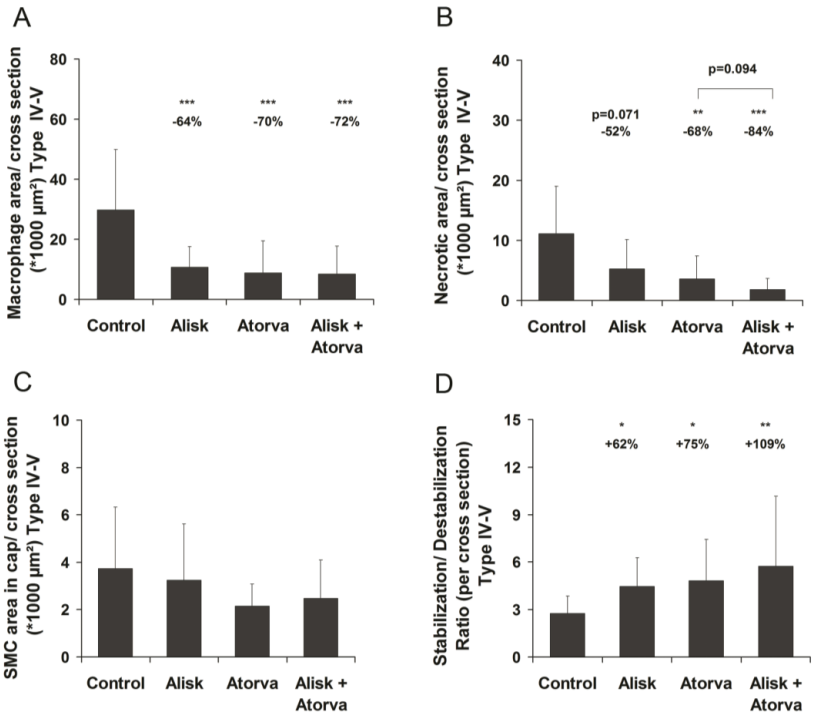

Figure 5 The atherosclerosis development was further analyzed by measuring the lesion composition of the severe lesions (type IV-V). A) Macrophage area per cross section. B) Necrotic area per cross section. C) SMC area in the cap per cross section. D) Lesion stability of the severe lesions (type IV-V) 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.

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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.