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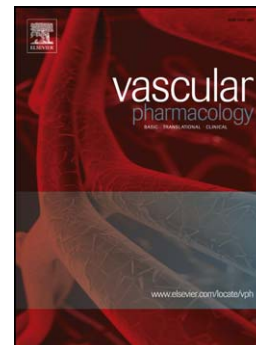
Cholesterol-independent effects of atorvastatin prevent cardiovascular morbidity and mortality in a mouse model of atherosclerotic plaque rupture

Lynn Roth, Miche Rombouts, Dorien M. Schrijvers, Wim Martinet and Guido R.Y. De Meyer

Laboratory of Physiopharmacology, University of Antwerp, Antwerp, Belgium

Address for correspondence: Lynn Roth, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium.

E-mail: lynn.roth@uantwerpen.be

Abstract

Because cholesterol-independent effects of statins are difficult to determine in patients, we studied these pleiotropic effects in apolipoprotein E-deficient (ApoE$^{-/-}$) mice with a mutation in the fibrillin-1 gene (Fbn1$^{C1039G+/-}$). These mice develop exacerbated atherosclerosis and spontaneous plaque ruptures, accompanied by myocardial infarctions (MI) and sudden death. ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice were fed a Western diet (WD). At week 10 of WD, mice were divided in a control (WD), atorvastatin (10mg/kg/day + WD) and cholesterol withdrawal group (cholW, normal chow). The latter was included to compare the effects of atorvastatin with dietary lipid lowering. Fifteen weeks later, the mice were sacrificed.

CholW, but not atorvastatin, reduced plasma cholesterol. Survival increased from 50% to 90% both in cholW and atorvastatin treated mice. CholW as well as atorvastatin treatment increased plaque collagen and fibrous cap thickness, but they did not affect the amount of plaque macrophages and T cells. MMP-2 and MMP-9 activity was significantly lower and the expression of MMP-12, TNF-α and IL-1β was strongly reduced in both treatment groups. Blood monocytes and neutrophils returned to baseline levels (ApoE$^{-/-}$ mice before the onset of atherosclerosis). Importantly, atorvastatin but not cholW significantly reduced coronary stenosis (from 50 to 28 %) and the occurrence of MI (from 43 to 10 %).

In conclusion, independent of cholesterol lowering, atorvastatin significantly reduced mortality, plaque vulnerability and inflammation to the same extent as cholW. In addition, atorvastatin but not cholW reduced coronary stenosis and the occurrence of MI. These data unequivocally illustrate the significance of the pleiotropic effects of atorvastatin in the prevention of cardiovascular morbidity and mortality.

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Introduction

Atherosclerosis is a progressive inflammatory disease of the large and medium-sized arteries, characterized by the formation of plaques in the vessel wall. During the development of the disease, the stability of the atherosclerotic plaque plays a major role. Features of plaque instability are a large necrotic core, a high infiltration of inflammatory macrophages and a thin fibrous cap, composed of few smooth muscle cells (SMCs) and collagen fibres. When a plaque develops such an unstable phenotype, it may easily rupture, followed by thrombosis and clinical complications such as myocardial infarction and stroke.[1-3] Moreover, despite the significant therapeutic advances in cardiology over the past decades, atherosclerotic plaque rupture remains a leading cause of acute cardiovascular death.

A healthy diet is considered the cornerstone of cardiovascular disease prevention, but when lifestyle interventions are not sufficient, lipid lowering through statin therapy is recommended.[4] Statins reduce cholesterol levels via inhibition of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, and are widely used in primary and secondary prevention of cardiovascular disease. Furthermore, it was suggested that statins display effects beyond lipid lowering, which provide additional benefit in the reduction of atherosclerosis. These pleiotropic effects might include improvement of endothelial function, atherosclerotic plaque stabilisation and a reduction in vascular inflammation and oxidative stress.[5-8] However, it is very challenging to directly prove the existence of these pleiotropic effects in clinical trials, because in patients statin treatment results in lipid lowering, which makes it difficult to distinguish cholesterol-dependent and cholesterol–independent effects.[6] Accordingly, the overall benefit of the pleiotropic effects of statins on clinical complications and the outcome of cardiovascular disease remains unclear.

It is known that statins do not have the same cholesterol-lowering effects in mice as compared to humans. For instance, apolipoprotein E deficient (ApoE$^{-/-}$) mice treated with a statin

predominantly do not show a decrease in cholesterol levels.[9] This can be explained by the lack of ApoE, which is one of the ligands for the LDL-receptor and necessary for the clearance of LDL. Thus, studying the cholesterol-independent effects of statins can be done more straightforwardly in mice than in humans. In the present study, ApoE^{-/-} mice with a heterozygous mutation in the fibrillin-1 gene (Fbn1[C1039G+/-]) were used. We previously reported that this unique mouse model shows accelerated plaque progression, spontaneous plaque ruptures, myocardial infarction (MI) and sudden death.[10,11] Therefore, it is an adequate model to investigate the pleiotropic effects of a statin not only on inflammation and atherosclerotic plaque stability, but also on the prevalence of MI and survival. Besides atorvastatin treatment, which affects endogenous cholesterol synthesis, we included a cholesterol withdrawal group (cholW, reduced exogenous cholesterol uptake) to mimic a dietary lifestyle intervention in ApoE^{-/-} Fbn1[C1039G+/-] mice and compared this non-pharmacological approach with statin therapy. It is indisputable that a decrease in total plasma cholesterol and LDL cholesterol results in a very significant reduction of cardiovascular morbidity and mortality.[4,12,13] Thus, by comparing the effects of cholW and statin treatment in ApoE^{-/-} Fbn1[C1039G+/-] mice, we aimed to assess the contribution of the pleiotropic effects to the overall benefit of a statin in preventing MI and sudden death.

Material and methods

Mice

Female ApoE^{-/-} Fbn1[C1039G+/-] mice were fed a Western diet (WD, 4021.90, AB Diets, Woerden, the Netherlands) starting at an age of 6 weeks. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and had free access to water and food. Cases of sudden death were documented. At the end of the experiment (25 weeks of diet), blood samples were obtained from the retro-orbital plexus of anesthetized mice

(ketamine 100mg/kg, xylazine 10mg/kg, i.p.). Subsequently, mice were sacrificed with sodium pentobarbital (250 mg/kg, i.p.). The animal procedures were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and all experiments were approved by the ethics committee of the University of Antwerp.

Cholesterol withdrawal and atorvastatin

At 10 weeks WD, ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice were randomly divided into 3 groups: control mice on WD (n=21), cholesterol withdrawal mice on ND (normal diet; cholW, n=25) and mice receiving WD supplemented with atorvastatin (10mg/kg/day, n=20, Pfizer, New York City, NY) for a period of 15 weeks.

Total plasma cholesterol, lipoproteins and CRP

Analysis of total plasma cholesterol was performed by using a commercially available kit (Randox, Crumlin, UK). Plasma lipoprotein profiles were determined on pooled samples (3 per group) by fast protein liquid chromatography on a Superose 6 column. Plasma C-reactive protein (CRP) was measured using ELISA (MCRP00, R&D systems, Minneapolis, USA).

Echocardiography

Transthoracic echocardiograms were performed on anesthetized mice (sevoflurane; 8% for induction and 4.5% for maintenance, SevoFlo®, Penlon vaporizer) at the start of treatment (10 weeks WD), at 17 weeks WD and at the end of the experiment, using a Toshiba diagnostic ultrasound system (SSA-700A), equipped with a 15 MHz transducer. End-diastolic diameter (EDD) and end-systolic diameter (ESD) were measured and fractional shortening (FS) was calculated.

Flow cytometry

EDTA-treated blood (500µl) was lysed using red blood cell lysing buffer Hybri-Max (Sigma-Aldrich, St. Louis, USA). Subsequently, remaining leukocytes were labelled with the following antibodies (BioLegend, San Diego, USA): APC anti-CD11c (N418), FITC anti-I-A^b (KH74), PerCP anti-CD11b (M1/70), APC anti-CD3ε (145-2C11), FITC anti-NK1.1 (PK136), PE anti-CD115 (AFS98) and PE anti-Gr-1 (RB6-8C5). Labelling occurred in the dark at 4°C in FACS buffer (PBS + 0.1% BSA (Sigma Aldrich) + 0.05% NaN3 (Merck, USA)) containing CD16/32 Fc-receptor blocker (BioLegend). Next, cells were analysed on a BD Accuri C6 cytometer equipped with a blue and red laser (Becton Dickinson, Erembodegem, Belgium). Dead cells were excluded based on forward scatter, side scatter and positive staining for propidium iodide (Invitrogen, Oregon, USA). Data analysis was performed with FCS Express 4 (De Novo Software, Glendale, USA). Data of blood immune cells of $ApoE^{-/-}$ mice (n=8-12) on a normal diet at the age of 6 weeks (before the onset of atherosclerosis) were used as baseline values.

Gel zymography

Thoracic aorta (n=3 per group) was homogenized in RIPA buffer (Sigma). Protein samples (20µg) were loaded on 10% Tris-glycine gels containing 0.1% gelatin (Life Technologies, Carlsbad, CA). After electrophoresis in Novex® Tris-Glycine SDS Running Buffer (Life Technologies Carlsbad, CA), proteins were renatured using Novex® Zymogram Renaturing buffer (Life Technologies Carlsbad, CA). Next, gels were incubated in Novex® Zymogram Developing Buffer (Life Technologies Carlsbad, CA) for 18h at 37°C. Gels were subsequently stained with Coomassie Brilliant Blue (0.5% Coomassie R250, 30% Methanol and 10% acetic acid) for 3 hours, followed by destaining (50% methanol and 10% acetic

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acid). Gelatinolytic activity was identified as a transparent band on a blue-stained background.[14]

Western blotting

Thoracic aorta homogenates (n=3 per group) were denatured for 5 min (in boiling water) in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) supplemented with β-mercaptoethanol. 10μg sample was loaded in each lane on Bolt 4-12% (Bis)-Tris gels (Invitrogen, Carlsbad, CA). After electrophoresis and blotting, membranes were probed with the following primary antibodies: rabbit anti-matrix metalloproteinase-12 (MMP-12, ab52897, Abcam, Cambridge, UK), hamster anti-tumour necrosis factor-α (TNF-α, 1221-00, Genzyme Diagnostics, Boston, MA), rabbit anti-interleukin-1β (IL-1β, ab9722, Abcam, Cambridge, UK) and mouse anti-β-actin (A5441, Sigma). Secondary antibodies were species-appropriate horseradish peroxidase conjugated (Dako, Glostrup, Denmark; MMP-12, β-actin) or avidin/biotin conjugated (Vectastain ABC kit, Burlingame, CA; TNF-α, IL-1β). Immunodetection was performed with SuperSignal West Pico or Femto Substrate (Thermo Fisher Scientific, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). Signals were normalized versus β-actin expression.

Histology

After sudden death or sacrifice of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice, the proximal ascending aorta, the left common carotid artery (LCCA) and the heart were collected. One half of the proximal ascending aorta was embedded in Neg-50 (Thermo Scientific), snap frozen in liquid nitrogen and stored at -80°C. The other half of the proximal ascending aorta, as well as the LCCA and the heart were fixed in 4% formaldehyde (pH 7.4) for 24 hours, dehydrated and embedded in paraffin. Serial cross sections (5 μm) of the proximal ascending aorta, LCCA and the heart

were prepared for histology. Atherosclerotic plaque size, stenosis (plaque size divided by internal elastic lamina area multiplied by 100) and necrotic core (defined as acellular areas with a threshold of 3000μm²)[15] of the proximal ascending aorta were analysed on haematoxylin-eosin (H-E) stained sections. The percentage of plaque neutrophils was determined via diaminobenzidine staining. Collagen content was measured in Sirius red stained sections and collagen type I was quantified under polarized light. The percentage of plaque smooth muscle cells and fibrous cap thickness (the median value of 10 measurements per atherosclerotic plaque) was determined on α-SMC actin (F3777, Sigma, St Louis, MO) stained sections. Elastin content was analysed via orcein staining. The presence of microvessels was analysed on H-E stained sections of the LCCA. The occurrence of myocardial infarctions (defined as large fibrotic areas with infiltration of inflammatory cells), coronary plaques and perivascular fibrosis, measured as the perivascular collagen area divided by the luminal area (PVCA/LA) of 10 coronary arteries per mouse, was analysed on Masson's trichrome stained transversal sections (cut from the middle of the heart to the apex). If plaques were present in the coronary arteries, the plaque size and percentage of stenosis were measured on Masson's trichrome stained sections. Serial cross sections (5 µm) of Neg-50 embedded proximal ascending aorta were prepared for the analysis of plaque macrophages and MMP-13 expression via immunohistochemical staining with a primary antibody against MOMA-2 (MCA519, AbD Serotec, Oxford, UK) and MMP-13 (ab39012, Abcam, Cambridge, UK). The presence of T cells was determined by using an anti-CD3 antibody (ab5690, Abcam, Cambridge, UK). The endothelial coverage and ICAM-1 expression of the endothelial cells (ECs) was measured via immunohistochemical staining with an anti-CD31 (01951A, BD Pharmingen, San Diego, CA) and anti-ICAM-1 (550287, BD Pharmingen, San Diego, CA) antibody, respectively. The length of CD31 positive cells along the luminal border was quantified in a complete cross-section of the proximal ascending aorta and

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expressed as percentage of the total perimeter. ICAM-1 positivity was expressed as percentage of CD31 positivity. All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and were quantified with Image J software (National Institutes of Health, Bethesda, MD).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using SPSS software (version 22, SPSS Inc., Chicago, IL). Statistical tests are specified in the figure legends. Histological data of the proximal ascending aorta only include mice that survived the experiment, because of the high variability in plaque development (due to age difference) in mice that suddenly died. Data on MI and coronary arteries include all mice. Differences were considered significant at $p < 0.05$.

Results

Total plasma cholesterol and lipoproteins

Analysis of total plasma cholesterol revealed a significant decrease in the cholW group as compared to control mice, and, as expected, atorvastatin treated mice did not show lower cholesterol levels (Figure 1A). FPLC analysis revealed a drastically reduced VLDL peak and also lower LDL levels in cholW mice, but not in the atorvastatin treated mice (Figure 1B).

Survival

CholW and atorvastatin treated mice showed an improvement in survival of 40% when compared to controls. Moreover, sudden death in the atorvastatin group initiated at a later time point as compared to cholW and control mice (Figure 1C).

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Plaque size, composition and intra-plaque microvessels

The atherosclerotic plaque size and percentage of stenosis in the proximal ascending aorta were significantly reduced both in the cholW and atorvastatin group (Figure 2A-C). Moreover, the necrotic core was decreased in cholW, but not in atorvastatin treated mice (Table 1). Both treatment groups showed increased fibrous cap thickness and smooth muscle cell content, although the latter only reached significance in the atorvastatin treated mice. Total plaque collagen content was higher after both cholW and atorvastatin treatment, but the collagen type I percentage was not different. Moreover, elastin content was higher in the cholW mice when compared to control and atorvastatin treatment (Table 1, Supplementary figure 1). The amount of macrophages and T cells in the plaque was not different from control mice. Furthermore, plaque neutrophils were almost absent and not influenced by both treatment groups (Table 1, Supplementary figure 1). Endothelial coverage did not differ between cholW, atorvastatin and control mice, but the percentage of ICAM-1 positive endothelial cells was decreased after cholW and atorvastatin treatment when compared to controls (Table 1, Supplementary figure 1).

The percentage of mice showing intra-plaque microvessels in the LCCA was reduced by 75% in both cholW and atorvastatin treated mice (Figure 2D and Table 1).

Inflammation and blood immune cells

Gel zymography indicated a decreased activity of MMP-2 both after cholW and atorvastatin treatment. MMP-9 activity was also slightly lower in both treatment groups (Figure 3A). Western blot analysis of MMP-12 expression revealed an 80% reduction in both cholW and atorvastatin treated mice (Figure 3B and C). Moreover, an extensive decrease of 90% in TNF-α and IL-1β expression was observed (Figure 3B, D and E). Analysis of MMP-13 expression in plaques of the proximal ascending aorta did not reveal significant differences between

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control (4.5±1.1%), cholW (4.6±1.3%) and atorvastatin treated mice (2.4±0.7%), although there seemed to be a downward trend in the latter.

Plasma CRP analysis revealed a significant reduction as a result of cholW (7.9±0.3μg/ml) and atorvastatin (8.2±0.6μg/ml) treatment when compared to control mice (11.9±0.8μg/ml, Figure 3F).

Flow cytometry of blood immune cells showed that cholW and atorvastatin treatment resulted in a normalization of circulating monocytes and neutrophils to baseline levels of ApoE$^{-/-}$ mice at the age of 6 weeks before the onset of atherosclerosis (Figure 4A and D). T cells were increased but dendritic cells were not affected either by cholW or atorvastatin treatment (Figure 4B and C). CholW reduced circulating natural killer (NK) and natural killer T (NKT) cells as compared to the control group (WD), but atorvastatin had no effect on these cell types (Figure 4E and F).

Cardiac function and morphology

CholW and atorvastatin treatment resulted in a decrease in EDD, ESD and an increase in FS at 17 weeks of WD when compared to control mice. At the end of the experiment (25 weeks WD) no effects of treatment were observed (Supplementary figure 2A, B and C). Heart weight divided by body weight was significantly lower in cholW and atorvastatin treated mice (Supplementary figure 2D).

Plaque size and the number of mice showing plaques in the coronary arteries were not different between the two treatment groups and the control mice, but the degree of coronary stenosis was almost halved after atorvastatin treatment (Table 2 and Figure 5A). Coronary perivascular fibrosis was significantly reduced in atorvastatin treated mice, but not in cholW mice (Table 2 and Figure 5A). The percentage of mice that showed MI was reduced in both

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treatment groups, but only reached significance in atorvastatin treated mice (Table 2 and Figure 5B).

Discussion

The existence of pleiotropic effects of statins is still under debate for many reasons. For instance, it is difficult to provide direct evidence for these cholesterol-independent actions in clinical trials and effects of statins, such as decreased inflammation and plaque stability, might also be the result of lipid lowering.[6] Some clinical trials have shown indirect proof for the presence of pleiotropic effects. For instance, the CARE study showed that pravastatin was able to decrease CRP levels, which was not directly correlated with a reduction in cholesterol.[16] These pleiotropic actions of statins are probably the result of an inhibition of the isoprenylation of small GTP-binding proteins such as Rho and Ras, involved in cell proliferation, differentiation, apoptosis, migration and the regulation of gene transcription, leading to an accumulation of their inactive form in the cytoplasm.[6,8]

In the present study, we documented that atorvastatin did not reduce lipid levels in ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice, implicating that we were able to study cholesterol-independent effects of atorvastatin treatment. As expected, cholW resulted in a significant reduction in total plasma cholesterol, VLDL and LDL. Thus, by comparing both treatments we could directly evaluate to which extent the pleiotropic effects of atorvastatin contribute to their anti-atherosclerotic properties, not only on 'surrogate endpoints' such as plaque composition but also on survival and the prevalence of MI.

Survival of ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice was closely monitored during this study and we observed a 40% reduction in mortality both after cholW and atorvastatin treatment. Although it is well-known that cholW and atorvastatin treatment result in increased survival, this effect is mainly attributed to cholesterol lowering.[17] However, in the present study, atorvastatin was

able to increase survival independent of lipid lowering, unequivocally illustrating the significance of the pleiotropic effects of statins.

Another fascinating finding was the anti-inflammatory effect of atorvastatin on monocytes and neutrophils in the circulation, but also on immune cells present in the atherosclerotic plaque. Both cholW and atorvastatin therapy reduced circulating monocytes and neutrophils to baseline levels (ApoE$^{-/-}$ mice on a normal diet before the onset of atherosclerosis). Because a larger density of circulating monocytes is an independent risk factor for coronary artery disease[18] and depletion of neutrophils reduces plaque burden,[19] the decreased blood monocyte and neutrophil pool in the present study reduces atherogenesis. It is important to note that in the case of atorvastatin these effects occurred independently of cholesterol lowering. We also showed a reduction in NK cells and NKT cells in the cholW group but not after atorvastatin treatment. NK and NKT cells can contribute to plaque inflammation via the production of interferon-γ[20,21] Furthermore, NK cells show cytotoxic properties via the release of perforin and granzyme, eventually leading to enhanced atherosclerosis.[22] In the present study, an increase in the percentage circulating T cells was observed both in the cholW and atorvastatin group. Although the measurement comprised the total population of T cells, it is conceivable that regulatory T cells (Tregs) were upregulated. Indeed, Tregs play a protective role in atherosclerosis[23] and statins can enhance their circulating levels[24,25] Overall, it is clear that both cholW and atorvastatin treatment reduced the inflammatory blood profile of ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice. This result was confirmed by an analysis of the plasma CRP concentration, which revealed a very significant reduction of this inflammatory marker after cholW and atorvastatin therapy. In the atherosclerotic plaque, the percentage of ICAM-1 positive endothelial cells was decreased after cholW and atorvastatin treatment, but this was not responsible for a lower percentage of macrophages and T cells. However, we did observe a significant reduction in the expression of inflammatory cytokines TNF-α and IL-1β. These

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inflammatory cytokines are secreted by macrophages, T cells, endothelial cells and SMCs in the atherosclerotic plaque and contribute to plaque instability.[26,27] It has been reported that statins reduce the expression of inflammatory cytokines such as TNF-α[26-28], but in the present study we clearly showed that this effect can be obtained independent of cholesterol lowering. Furthermore, MMPs play an important role in plaque destabilisation.[12,29,30] For instance, degradation of collagen weakens the fibrous cap and loss of elastin results in outward remodelling.[12,29] Accordingly, MMP expression in human atherosclerotic plaques correlates with adverse clinical outcomes.[12] We showed that both cholW and atorvastatin therapy reduced the activity of the gelatinases MMP-2 and MMP-9, the latter less distinct. The amount of MMP-13 in atherosclerotic plaques was not altered, but a very clear reduction in MMP-12 expression was observed as a result of cholW or atorvastatin treatment. The metalloelastase MMP-12 is predominantly produced by macrophages and plays a detrimental role in plaque progression and destabilisation.[29,31,32] For example, plaque size and SMC content was increased and the percentage of macrophages was decreased in ApoE/MMP-12 double knock-out mice.[30] Taken together, we showed that both cholW and atorvastatin therapy, the latter independent of cholesterol lowering, reduced systemic and plaque inflammation via reducing circulating monocytes and neutrophils and suppressing the expression of TNF-α, IL-1β and the levels of MMP-2, MMP-9 and MMP-12. This resulted in smaller, less stenotic atherosclerotic plaques with a more stable plaque phenotype, illustrated by an increased plaque collagen content and a thicker fibrous cap.

Intra-plaque neovascularisation is an important feature of human vulnerable plaques and is correlated with plaque rupture.[12,33] In the present study, cholW and atorvastatin treatment were both responsible for a 75% reduction in the occurrence of intra-plaque microvessels in the LCCA. Previous studies showed that statins can exert anti-angiogenic effects and reduce adventitial neovascularisation,[34,35] but intra-plaque microvessels were absent and could not be

investigated. We presume that the reduced occurrence of microvessels in this study is the result of a decreased expression of TNF-α and IL-1β, since these cytokines can indirectly affect the process of neovascularisation.[26] Furthermore, the reduced levels of MMPs are responsible for less extracellular matrix degradation. As a result, basement membrane sequestered angiogenic factors, such as transforming growth factor-β, are not released and angiogenesis is suppressed.[29]

Importantly, the number of mice showing MI was lower in the cholW and atorvastatin treated group, but only reached statistical significance in the latter. In human trials, statins have shown a very clear benefit on reducing cardiovascular disease endpoints such as MI. However, this has not been described in mouse models. Strikingly, these beneficial effects of atorvastatin occurred independent of cholesterol lowering, which is an important and novel finding. Together with the reduced prevalence of MI, heart weight of ApoE$^{-/-}$ Fbn1$^{C1039G+/-}$ mice was decreased and cardiac function was improved in cholW and atorvastatin treated mice. A significant decrease in EDD (only in cholW) and ESD was seen, combined with an increase in fractional shortening at 17 weeks WD. At 25 weeks WD, this effect on heart function was not observed, which is most likely due to the higher number of control mice that had suddenly died. Interestingly, the coronary arteries of atorvastatin treated, but not cholW, mice showed less stenosis and a reduction in perivascular fibrosis. Because coronary perivascular fibrosis can be caused by post-myocardial ventricular remodelling[36] or an impaired coronary blood flow,[37] the reduced presence of MI and the lower degree of stenosis in the coronary arteries of atorvastatin treated mice probably contributes to this finding.

The results shown in the present study confirm the data available in literature on cholesterol-independent effects of statins in mouse models. For instance, simvastatin and pravastatin can increase fibrous cap thickness, contributing to plaque stability.[38,39] Moreover, atorvastatin has the ability to increase plaque collagen content.[28] Some of these studies also show a reduced

macrophage infiltration,[28,40] which we did not observe. However, we did report reduced MMP expression and plasma CRP concentrations as previously described.[28,41] Atorvastatin has also been shown to decrease the occurrence of adventitial neovessels in ApoE$^{-/-}$ mice.[34] We were able to extend this finding with a reduction of neovessels within atherosclerotic plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice, which is a novel observation. In ApoE$^{-/-}$ mice fat fed for 40 weeks, pravastatin decreased mortality.[38] We also observed a very significant improvement in survival of atorvastatin treated mice, even at an earlier time point. The novelty of the current study is that we investigated both plaque and systemic inflammation to provide a more general view on the cholesterol-independent actions of atorvastatin. Furthermore, the ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mouse model provided us with the opportunity to investigate the role of pleiotropic effects of atorvastatin in the prevention of intraplaque angiogenesis, coronary stenosis, and MI.

The present study contains some limitations. To begin with, we did not include a treatment group with atorvastatin administration in combination with cholesterol withdrawal, which would have allowed the assessment of synergistic effects of statin treatment above and beyond cholesterol lowering. Furthermore, the exact cause of mortality in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice is still unknown, but this study strongly suggests that a heart-related problem is an important contributor, since we observed a decreased occurrence of MI in the atorvastatin treated mice combined with a reduced mortality. Nevertheless, ventricular remodelling and heart failure or arrhythmias cannot be excluded as cause of death. This study showed a higher percentage of circulating T cells both after cholW and atorvastatin treatment, which did not result in a destabilisation of atherosclerotic plaques. Because we were unable to distinguish the specific T cell subtype(s) responsible for this finding, further investigation is needed.

Finally, we have proven the beneficial role of pleiotropic effects of atorvastatin in the prevention of cardiovascular morbidity and mortality. However, it remains unclear whether

other statins exert the same effects, since structural differences among statins can play a role in their clinical efficacy and pleiotropic actions.[42]

In conclusion, we compared atorvastatin therapy with cholW and made the striking observation that atorvastatin treatment, independent of cholesterol lowering, was able to increase survival from 50 to 90%, and to decrease plaque vulnerability and inflammation (MMP-2, MMP-9, MMP-12, TNF-α and IL-1β) to the same extent as cholW. In addition, atorvastatin, but not cholW, reduced coronary stenosis and the occurrence of MI. Accordingly, ventricular remodelling was diminished, indicated by a reduction in heart weight, improved cardiac function and less coronary perivascular fibrosis. Our data not only prove the existence of pleiotropic effects of atorvastatin, but also unequivocally illustrate the significance of these cholesterol-independent effects in the prevention of cardiovascular morbidity and mortality.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure legends

Figure 1: Plasma cholesterol and survival. (A) Total plasma cholesterol levels were significantly lower in cholW mice (n=22) as compared to control (n=10) and atorvastatin (n=17) treated mice (One-way ANOVA, post hoc LSD; ***p<0.001 vs. control, §§§p<0.001 vs. atorvastatin). **(B)** Analysis of cholesterol fractions showed a drastic decrease of VLDL in cholW mice, whereas atorvastatin treated mice revealed no difference with the control group. **(C)** CholW (n=25) and atorvastatin (n=20) treated mice showed a survival of 88% and 90% respectively. These percentages are significantly higher than the 48% survival observed in control mice (n=21, Log-rank test; ***p<0.001 vs. control). Control = 25 weeks WD, cholW = 10 weeks WD + 15 weeks normal diet, atorvastatin = 10 weeks WD + 15 weeks atorvastatin supplemented WD.

Figure 2: Plaque size and stenosis. (A) H-E stained images of atherosclerotic plaques in the proximal ascending aorta clearly showed the decreased plaque development in treated vs. non-treated mice (scale bar = 200μm). **(B)** Plaque size was significantly lower in CholW (n=21) and atorvastatin (n=18) treated mice as compared to control (n=10) mice (One-way ANOVA, post hoc LSD; *p<0.05, **p<0.01 vs. control). **(C)** The percentage stenosis was also reduced in both treatment groups (control n=9, cholW n=18, atorvastatin n=18, One-way ANOVA, post hoc LSD; *p<0.05, ***p<0.001 vs. control, §p<0.05 vs. atorvastatin). **(D)** Histological image (H-E) of the LCCA showing the presence of intra-plaque microvessels (arrowheads) in close proximity of the media (M, scale bar = 20μm). Control = 25 weeks WD, cholW = 10 weeks WD + 15 weeks normal diet, atorvastatin = 10 weeks WD + 15 weeks atorvastatin supplemented WD.

Figure 3: Matrix metalloproteinases, inflammatory cytokines and plasma CRP. **(A)** Gel zymography showed decreased activity of pro MMP-2 in plaques of cholW and atorvastatin treated mice as compared to controls. MMP-9 activity was also lower, but apparently less clear than MMP-2. **(B)** Western blot analysis of MMP-12, TNF-α and IL-1β in atherosclerotic plaques revealed a drastic decrease in both treatment groups. **(C, D and E)** Quantification of Western blot data confirmed that cholW and atorvastatin treatment resulted in a significant decrease of MMP-12, TNF-α and IL-1β expression as compared to control mice (One-way ANOVA, post hoc LSD; ***p<0.001 vs. control). **(F)** Plasma CRP was significantly lower as a result of cholW and atorvastatin treatment (One-way ANOVA, post hoc LSD; ***p<0.001 vs. control). Control = 25 weeks WD, cholW = 10 weeks WD + 15 weeks normal diet, atorvastatin = 10 weeks WD + 15 weeks atorvastatin supplemented WD.

Figure 4: Blood immune cells. **(A)** Circulating monocytes were significantly lower in cholW (n=22) and atorvastatin (n=17) treated mice as compared to controls (n=13). The percentage of monocytes in both treatment groups was even comparable to baseline values (dashed line) of ApoE$^{-/-}$ mice (4.6±0.5%, n=12) on a normal diet before the onset of atherosclerosis. **(B)** CholW (n=22) and atorvastatin (n=17) treatment resulted in a higher percentage of circulating T cells (control n=11, baseline ApoE$^{-/-}$ mice: 16.1±1.4%, n=12). **(C)** The percentage dendritic cells was not affected by cholW (n=22) and atorvastatin (n=17) treatment (control n=13, baseline ApoE$^{-/-}$ mice: 0.5±0.1%, n=10). **(D)** Neutrophils were decreased to baseline levels in both treatment groups (control n=12, cholW n=22, atorvastatin n=17, baseline ApoE$^{-/-}$ mice: 14.0±1.6%, n=12). **(E+F)** Circulating NK and NKT cells were decreased in cholW mice (n=22) as compared to atorvastatin (n=17) and control mice (n=13, baseline ApoE$^{-/-}$ mice: NK 4.7±0.6%, n=12 and NKT 0.4±0.2%, n=8). One-way ANOVA, post hoc LSD; *p<0.05, ***p<0.001 vs. control and §§p<0.01, §§§p<0.001 vs. atorvastatin. Control = 25 weeks WD,

cholW = 10 weeks WD + 15 weeks normal diet, atorvastatin = 10 weeks WD + 15 weeks atorvastatin supplemented WD.

Figure 5: Coronary plaque, perivascular fibrosis and myocardial infarctions. **(A)** Trichrome Masson staining of the coronary arteries showed the presence of plaques and fibrosis. Plaque size was equal between control and treated mice, but the percentage stenosis and perivascular fibrosis was reduced after atorvastatin treatment. L = lumen, P = plaque. **(B)** The presence of myocardial infarctions, defined as large fibrotic areas with infiltration of inflammatory cells, was higher in the control group when compared to cholW and atorvastatin treated mice. Scale bar = 50μm, control = 25 weeks WD, cholW = 10 weeks WD + 15 weeks normal diet, atorvastatin = 10 weeks WD + 15 weeks atorvastatin supplemented WD.

Tables

Table 1: Atherosclerotic plaque composition.

	Control	**CholW**	**Atorvastatin**
Necrotic core (%)	19±3	11±1**,§§	18±2
Macrophages (%)	1.5±0.4	2.5±0.9	1.9±0.4
T cells (%)	0.6±0.2	0.5±0.1	0.5±0.1
Neutrophils (%)	0.06±0.04	0.09±0.05	0.04±0.03
Smooth muscle cells (%)	4.4±0.6	6.1±0.5	6.6±0.6*
Fibrous cap thickness (μm)	8±1	23±2***,§	14±1**
Collagen (%)	62±2	74±1***	69±1**
Collagen type I (%)	4.6±0.7	3.9±0.6	4.2±0.5
Elastin (%)	12±2	21±1***,§§§	14±1
Endothelial coverage (%)	92±3	98±1	95±2
ICAM-1 positive ECs (%)	79±4	58±6**	63±3**
Microvessels LCCA (% of mice)[a]	50	11*	13*

Data from proximal ascending aorta, mean ± SEM, ECs = endothelial cells, control n=7-10, cholW n=18-21, atorvastatin n=15-18, one-way ANOVA, post hoc LSD; *p<0.05, **p<0.01, ***p<0.001 vs. control and §p<0.05, §§p<0.01, §§§p<0.001 vs. atorvastatin.

[a] Percentage of mice showing intra-plaque microvessels in the left common carotid artery (LCCA), Pearson's chi-squared test; *p<0.05 vs. control.

Table 2: Coronary plaque parameters and occurrence of myocardial infarctions.

	Control	CholW	Atorvastatin
Coronary plaque (%)[a]	43	42	45
Coronary plaque size (μm²)	5887±1679	5868±1452	11516±3685
Coronary stenosis (%)	50±6	50±11	28±6*
Perivascular fibrosis (%)	62±4	54±5	44±4*
Myocardial infarctions (%)[b]	43	29	10*

Data are shown as mean ± SEM, control n=9-21, cholW n=10-24, atorvastatin n=8-20, One-way ANOVA, post hoc LSD; *p<0.05 vs. control.

[a] Percentage of mice showing coronary plaques. Pearson's chi-squared test; p>0.05.

[b] Percentage of mice showing myocardial infarctions, Pearson's chi-squared test; *p<0.05 vs. control.
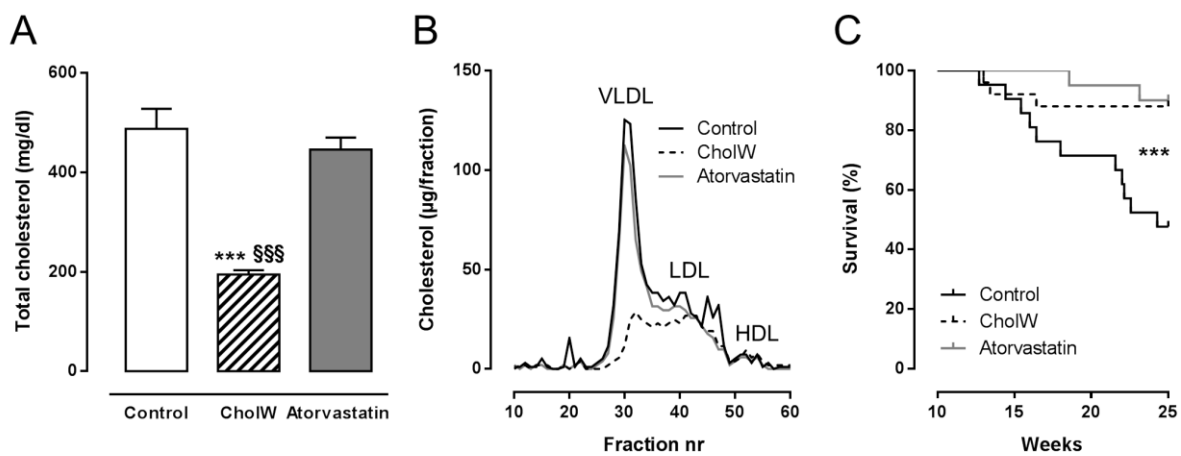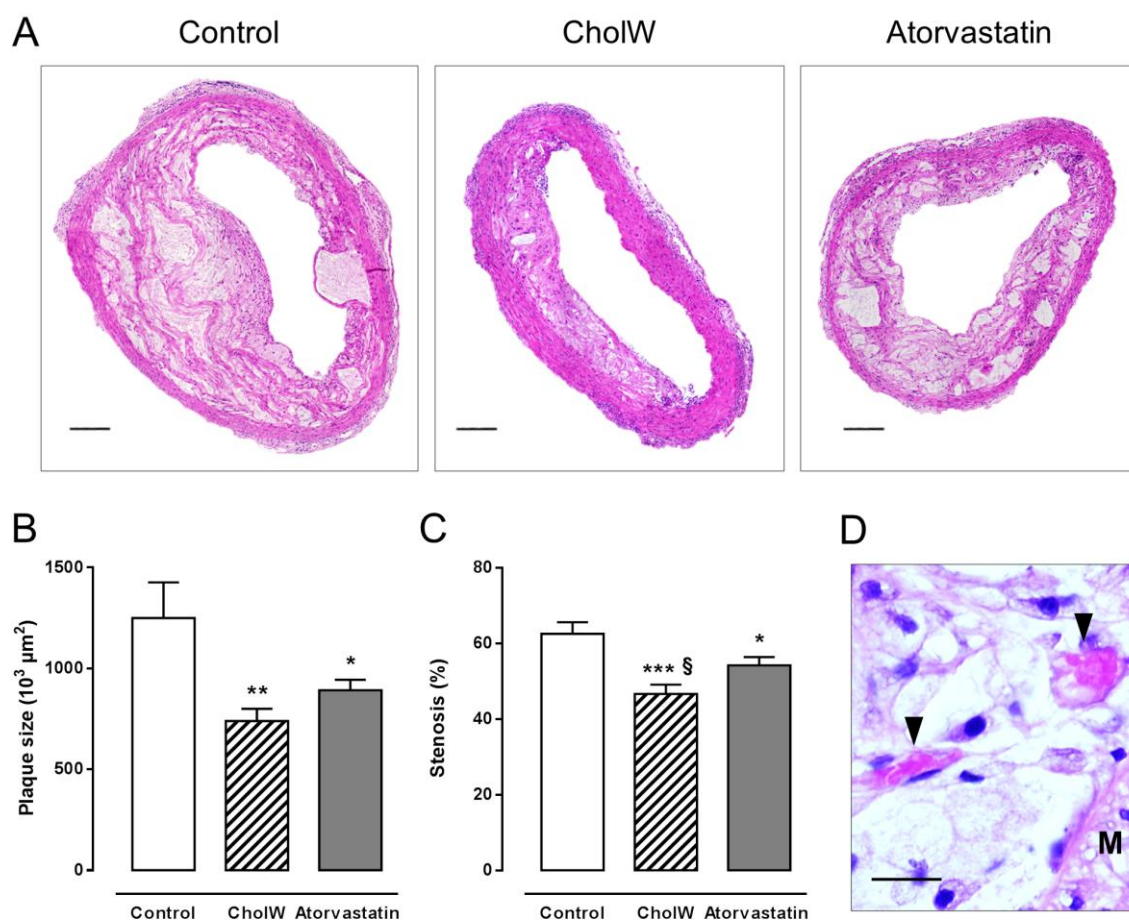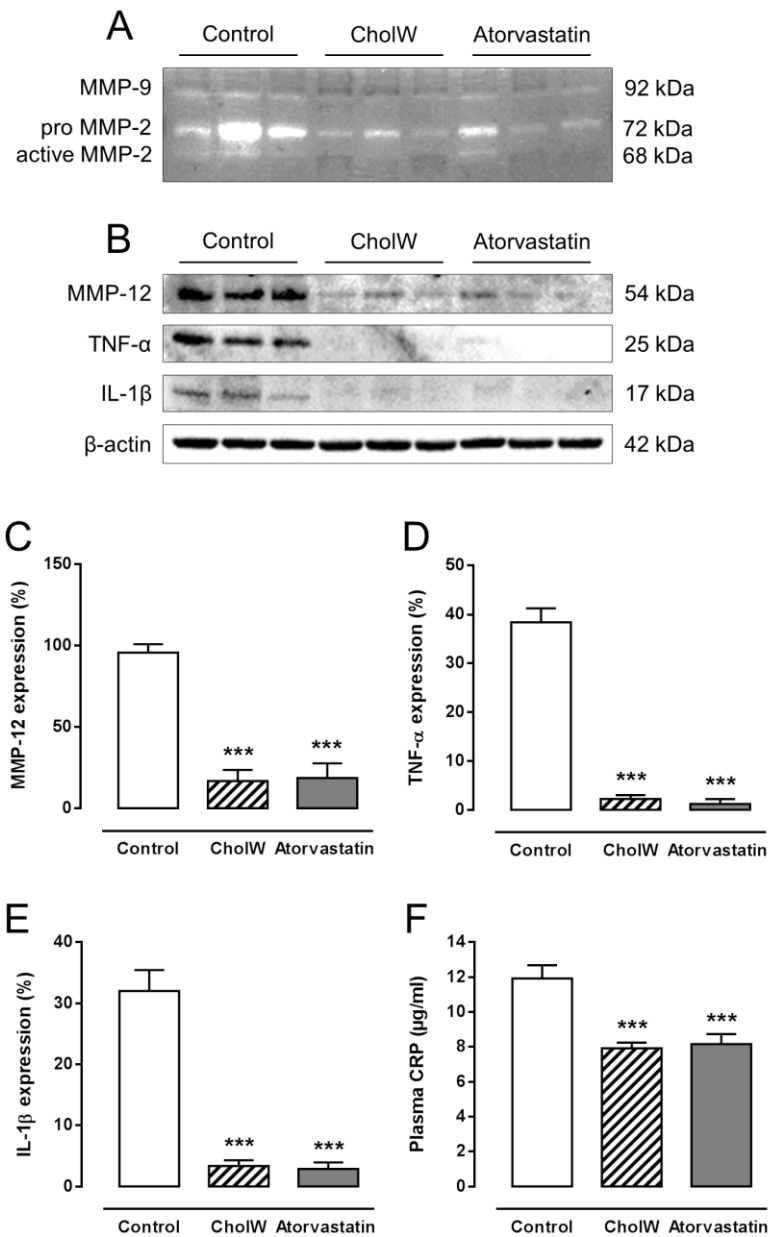
Figure 1

Figure 2

A   Control   CholW   Atorvastatin

B

Plaque size (10^3 µm^2)

Control   CholW   Atorvastatin

C

Stenosis (%)

Control   CholW   Atorvastatin

D

M

Figure 3

Figure 4

Figure 5

A   Control   CholW   Atorvastatin

B

Graphical abstract