

Fibulin-4 deficiency induces thoracic and abdominal aortic wall dilation and altered plaque morphology in apolipoprotein E-deficient mice

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ABSTRACT

Objective: Extracellular matrix degradation plays an important role in aortic aneurysm formation. In Fibulin-4^{R/R} mice, deficiency of the extracellular matrix protein Fibulin-4 induces upregulation of matrix metalloproteinases (MMP) and elastin irregularities, resulting in early thoracic aortic aneurysms. In humans, aneurysms usually develop in the abdominal aorta with increasing age and are often associated with atherosclerosis. To investigate the molecular mechanisms of the interaction between aneurysm formation and atherosclerotic disease we crossbred Fibulin-4^{+R} mice, with minor extracellular matrix (ECM) abnormalities such as increased ECM deposition and slight MMP activation in the thoracic aorta, but without aortic dilation yet, onto an atherosclerotic Apolipoprotein E knockout (ApoE^{-/-}) background.

Approach: Double ApoE^{-/-}/Fibulin-4^{+R} mutant mice were fed a high fat diet (HFD) for 10, 20 or 30 weeks and compared to ApoE^{-/-}/Fibulin-4^{+/+} control mice. MMP activity in the aorta was determined using protease-activatable near-infrared fluorescent probes. Thoracic and abdominal aortic diameters were assessed using high-frequency ultrasound. After sacrifice, atherosclerotic burden in the aorta was evaluated.

Results: Interestingly, after 10 weeks of HFD, ApoE^{-/-}/Fibulin-4^{+R} mice displayed increased MMP activity in the abdominal aorta and after 20 weeks of diet thoracic and abdominal aortic dilations were observed as compared to ApoE^{-/-}/Fibulin-4^{+/+} mice. In addition, ApoE^{-/-}/Fibulin-4^{+R} mice showed increased plaque formation after 10 weeks of HFD and histological plaque analysis showed a distinct plaque architecture. Moreover, part of the ApoE^{-/-}/Fibulin-4^{+R} mice developed symptoms of paralysis between 20 and 30 weeks of HFD and 30% did not survive beyond 30 weeks.

Conclusions: These results indicate that a subtle defect in the extracellular matrix of the aortic wall predisposes to the development of thoracic and abdominal aortic dilation upon atherosclerosis, and induces altered plaque morphology.

INTRODUCTION

Aortic aneurysm and dissections account for 1-2% of all deaths in the developed countries.¹ According to their location, aneurysms can be categorized in two main groups: thoracic aortic aneurysms (TAA) and abdominal aortic aneurysms (AAA). Aneurysms of the thoracic aorta, in particular the aortic arch, are characterized by necrosis of the medial layer of the aortic wall, also called cystic medial necrosis. TAAs usually occur due to a genetic mutation, for example aneurysms in Marfan's disease. Aneurysms of the distal aorta, in particular AAAs, are much more common and are thought to be caused by a multifactorial process.² Interestingly, recent clinical studies report a high frequency of TAAs in patients with aneurysms of the abdominal aorta.^{3, 4} Important risk factors for aortic aneurysm formation are age and atherosclerosis, and these risk factors are similar for patients with aneurysms and those with arterial occlusive disease, which is characterized by narrowing of the arteries due to atherosclerosis.⁵ However, the molecular mechanisms underlying aneurysm formation and the relation with atherosclerosis are largely unknown.

It is known that extracellular matrix degeneration plays an important role in TAA formation. Elastin is a crucial component of the extracellular matrix that is responsible for maintaining vessel wall elasticity.⁶ Fibulin-4 is an extracellular matrix protein, which plays an important role in elastic fiber assembly and function and is a regulatory factor in elastogenesis.^{7, 8} Indeed, Fibulin-4 deficient patients described so far present with TAAs, due to homozygous or compound heterozygous mutations, which usually develop to a severe stage of the disease within the first months or years of their life.⁹⁻¹⁵ A proportion of these patients also presented with abdominal tortuosity and/or dilation on further examination.^{12, 15}

Similar to Fibulin-4 deficient patients, previously developed mutant mice with a systemic 4-fold (Fibulin-4^{R/R}) reduced expression of Fibulin-4 present with aortic wall degeneration and thoracic aortic aneurysm.¹⁶⁻¹⁸ Additionally, they develop impaired vascular contractility and increased arterial stiffness. Interestingly, a 2-fold reduced expression of Fibulin-4, in Fibulin-4^{+R} mice, also induces aortic disease but in a milder form. Although they do not develop aortic aneurysms spontaneously at adult age, they present with aortic wall degeneration, including elastic fiber fragmentation and slightly increased TGF- β signaling.^{17, 19} Additionally, destruction of the extracellular matrix in the aortic wall of these Fibulin-4 mice is associated with increased expression and activation of matrix metalloprotease (MMPs), which are involved in degradation of the extracellular matrix.^{17, 19} Molecular imaging using a near-infrared *in vivo* imaging probe for MMP activity (MMPsense680TM) shows a graded increase in MMP activity in aneurysmal lesions of the aortic arch of Fibulin-4^{+R} and Fibulin-4^{R/R} mice, showing that MMP activity is a leading indicator in these hypomorphic Fibulin-4 mice for aneurysm formation.¹⁹

To study whether and how a primary extracellular matrix defect can be involved in aortic dilation and atherosclerosis, we developed a mouse model in which we combined the subtle defect in the extracellular matrix of the Fibulin-4^{+R} mouse with the most commonly used model for atherosclerosis, the apolipoprotein E knockout (ApoE^{-/-}) mouse. At the age of 9 weeks, the double mutant mice were fed a high fat diet (HFD) to induce atherosclerotic plaque formation. Since MMP-induced elastin and collagen degradation are known to affect atherosclerotic plaque morphology^{20, 21}, we additionally analyzed whether plaque morphology in ApoE^{-/-} mice is affected by Fibulin-4 deficiency. This model mimics the human situation as it combines the clinically observed association between (thoracic and abdominal) aortic dilation and atherosclerosis by combining an atherosclerosis mouse model (ApoE) and a subtle inherited defect present in the aortic wall (Fibulin-4^{+R}) that might predispose these animals for the development of atherosclerosis associated aortic disease.

MATERIAL AND METHODS

Mouse model

Mice containing the Fibulin-4^R allele were generated as previously described.² All mice used were bred in a C57Bl/6J background and were kept in individually ventilated cages to keep them consistently micro-flora and disease free. Fibulin-4^R mice were crossbred with ApoE^{-/-} mice (C57Bl/6J background) to obtain ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice. Female and male ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice were fed either a normal chow diet (Standard CRM (P), Special Diets Services, UK), a HFD containing 16% fat (Purified diet W 4021.06, AB diets Animal Nutrition, Woerden, the Netherlands) or a control fat diet (CFD) containing 5% fat (Purified diet W control 4021.69, AB diets Animal Nutrition, Woerden, the Netherlands) starting at the age of 9 weeks. Hind limb paralysis was observed by dragging of the limbs, and facial paralysis by loss of eye blink reflex, a bulging eye and abnormal vibrissae orientation with fibers flattened posterior against the head. Animals were housed at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the “Animal Welfare Act” of the Dutch government, using the “Guide for the Care and Use of Laboratory Animals” as its standard. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. All animals studied were approved by an independent Animal Ethical Committee (Dutch equivalent of the IACUC).

MMP imaging

Per 25 grams of body weight 2 nmol specific MMP activatable NIRF probes, MMPsense680TM (Perkin Elmer Inc., Akron, Ohio, USA), was injected into the tail vein of anesthetized mice

after 10 and 20 weeks of HFD or normal chow diet. Intact aortas were harvested 24 hours after injection for *ex vivo* fluorescence imaging, and analyzed using the Odyssey Imaging system (LI-COR® Biosciences, Lincoln, Nebraska, USA). Near-infrared images were obtained in the 700 nm channel.

Ultrasound imaging

Animals were sedated with 4% isoflurane and maintained on 1-3% isoflurane for anaesthesia, adjusted to the vital parameters of the mouse (heart rate > 400 bpm, breath rate 30 strokes/min). Mice were placed on a heating pad to maintain body temperature at 37°C. *In vivo* ultrasound imaging of the aortic arch, abdominal aorta and left ventricle (LV) was performed with a Vevo2100 (Visualsonics Inc., Toronto, Canada) using a 40-MHz linear interfaced array transducer (MS550S). B-mode and M-mode images of the aorta were captured. Diameters of the aortic arch were measured from the parasternal window at the level of the ascending aorta. Distensibility of the aortic arch was measured as the systolic to diastolic aortic diameter ratio in M-mode image data (calculated as systolic diameter minus diastolic diameter, divided by the diastolic diameter).

Analysis of plaque area and composition

To quantify the surface area affected by atherosclerosis, aortas were stained with Oil-red-O after 10 and 20 weeks of HFD and macro photographs of *en face* preparations were made (n=minimal 5 mice in each group). The Oil-red-O stained surface areas in the aortic arch, descending and abdominal aorta were quantified using ImageJ (Fiji). Additionally, plaque size and morphology were histologically analyzed after 10 and 20 weeks of HFD (n=minimal 5 mice in each group). The aortas with the branching brachiocephalic artery, left carotid artery and left subclavian artery of mice on 10 weeks of HFD were perfusion fixed with 1% paraformaldehyde after PBS flush, dehydrated and embedded in paraffin. Serial longitudinal sections of the aortic arch and cross sections of the abdominal aorta (5 µm) were prepared for histological analysis. Total plaque size in the inner curvature of the aortic arch and in the brachiocephalic artery was measured on haematoxylin-eosin stained slides using BioPix iQ 2.0 imaging software (BioPix, Göteborg, Sweden). Aortic wall structure and plaque morphology were additionally analyzed by histochemical staining with Resorcin-Fuchsin (elastin). Elastin content was analyzed on elastin stained slides with ImageJ (Fiji).

To further determine differences in plaque phenotype, including lipid content, cryosections were made of mice on 20 weeks HFD. After PBS flush aortic arches were embedded in Tissue-Tek (O.C.T. compound) and serial longitudinal cryosections were made (5 µm). Plaque size in the aortic arch and brachiocephalic artery was quantified on haematoxylin-eosin stained slides using Biopix. Oil-red-O staining was used to determine lipid content of the plaques.

Statistical analysis

All results are expressed as mean \pm SEM (continuous results) or median (lower to upper limit) (aortic arch diameters). The unpaired 2-tailed Student t-test was performed to analyze the specific sample groups for significant differences. A p-value <0.05 was considered to indicate a significant difference between groups. All analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Abdominal aorta of ApoE^{-/-}Fibulin-4^{+R} mice shows increased MMP activity

We have previously shown that extracellular matrix degeneration in Fibulin-4^{+R} and Fibulin-4^{R/R} mice is associated with increased MMP activity in the thoracic aorta.¹⁹ In this study, we tested whether extracellular matrix degeneration in the aortic wall of Fibulin-4 deficient mice could contribute to abdominal aortic lesions. Measurements of MMP activity in the abdominal aorta using the MMPsense probe indeed revealed a mild increased activity of 1.2 fold in Fibulin-4^{+R} abdominal aortas as compared to Fibulin-4^{+/+} mice (Fig. 1A). However, these Fibulin-4^{+R} mice do not yet develop abdominal aortic dilations.

As atherosclerosis can be associated with aortic aneurysms, we next tested whether the induction of atherosclerosis in Fibulin-4^R heterozygous mice, which have minor extracellular matrix defects in the aortic wall, could lead not only to thoracic, but also abdominal aortic dilation. To induce atherosclerosis, Fibulin-4^{+R} mice were crossbred with ApoE^{-/-} mice, which develop atherosclerosis spontaneously after 12 weeks.²²⁻²⁴ Starting from the age of 9 weeks the double mutant ApoE^{-/-}Fibulin-4^{+R} and ApoE^{-/-}Fibulin-4^{+/+} littermate controls were fed a HFD for 10 or 20 weeks to accelerate atherosclerotic plaque formation. ApoE^{+/+}Fibulin-4^{+/+} and ApoE^{+/+}Fibulin-4^{+R} mice on a HFD for 10 or 20 weeks did not develop atherosclerosis and did not show additional vascular abnormalities as compared to Fibulin-4^{+/+} and Fibulin-4^{+R} mice.

Interestingly, after 10 weeks of HFD, MMP activity measurement on whole aortas revealed a strongly increased MMP activity in the abdominal aortas of ApoE^{-/-}Fibulin-4^{+R} mice of 2.7 fold as compared to ApoE^{-/-}Fibulin-4^{+/+} mice, and also compared to ApoE^{-/-}Fibulin-4^{+R} mice on a chow diet, which had a 1.6 fold increase compared to ApoE^{-/-}Fibulin-4^{+/+} mice on chow diet (Fig.1B). This highly increased MMP activity in the abdominal aortas of ApoE^{-/-}Fibulin-4^{+R} mice was further increased after 20 weeks of HFD. ApoE^{-/-}Fibulin-4^{+R} mice on 20 weeks of HFD had a 13 fold increase as compared to ApoE^{-/-}Fibulin-4^{+/+} mice. This indicates that induction of atherosclerosis in ApoE^{-/-}Fibulin-4^{+R} mice enhanced the already slightly increased MMP activity observed in the abdominal aorta of Fibulin-4^{+R} mice, which suggests that abdominal aortic wall lesions worsened progressively in ApoE^{-/-}Fibulin-4^{+R} mice on a HFD.

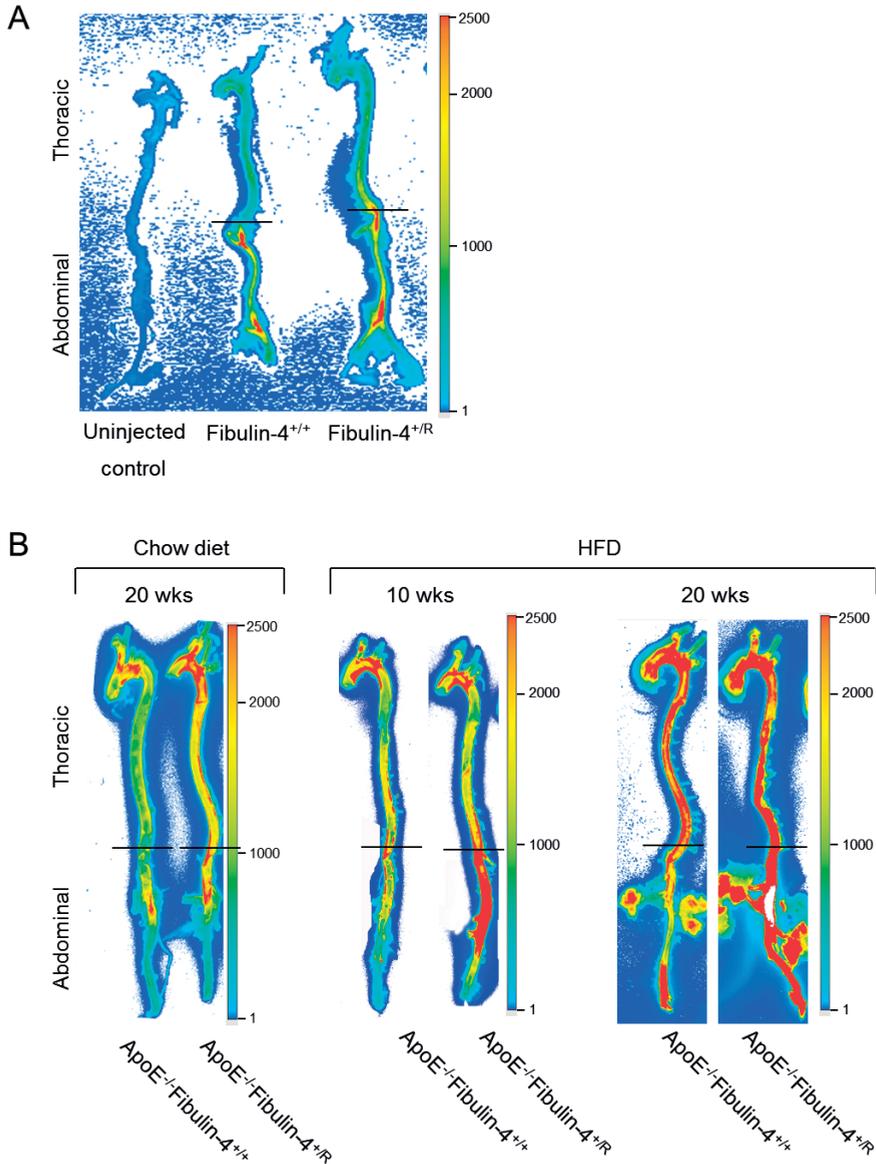


Figure 1. Increased MMP activity in ApoE^{-/-}Fibulin-4^{+/R} abdominal aortas after 10 and 20 weeks of HFD. (A) Ex vivo imaging of isolated aortas shows 1.2 fold higher MMP activity in the abdominal aortas of Fibulin-4^{+/R} mice (n=6) as compared to Fibulin-4^{+/+} abdominal aortas (n=4). (B) Ex vivo imaging of aortas after 10 and 20 weeks of HFD and chow diet shows a further increase in MMP activity in the abdominal aorta of ApoE^{-/-}Fibulin-4^{+/R} mice (n=3) with a 2.7 fold increase after 10 weeks of HFD as compared to ApoE^{-/-}Fibulin-4^{+/+} aortas (n=3), and a 13 fold increase after 20 weeks of HFD in the abdominal aortas of ApoE^{-/-}Fibulin-4^{+/R} mice (n=5) as compared to ApoE^{-/-}Fibulin-4^{+/+} aortas (n=5) as well as compared to ApoE^{-/-}Fibulin-4^{+/R} mice after 20 weeks of chow diet (n=5), which had a 1.6 fold increase compared to ApoE^{-/-}Fibulin-4^{+/+} aortas (n=3). Horizontal lines depict the level of the diaphragm, indicating the transition of the thoracic into the abdominal part of the aorta.

ApoE^{-/-}Fibulin-4^{+R} aortas display increased thoracic and abdominal aortic diameters

To subsequently determine whether the combination of atherosclerosis and an extracellular matrix defect can result in aortic dilation in ApoE^{-/-}Fibulin-4^{+R} mice, we measured thoracic and abdominal aortic diameters using *in vivo* ultrasound imaging of ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice after 10 and 20 weeks of HFD and after 10 or 20 weeks of CFD, which has a similar nutrient composition as HFD, but a lower fat percentage. We also included mice after 20 weeks of chow diet and mice of 9 weeks old (without starting a diet: 0 weeks HFD/CFD) as controls. Interestingly, significantly increased thoracic aortic diameters were observed in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of CFD compared to ApoE^{-/-}Fibulin-4^{+/+} mice, but no difference was observed between the two genotypes on HFD or chow diet (Fig. 2A and Supplemental Fig. 1A). The thoracic aortic diameters of the ApoE^{-/-}Fibulin-4^{+R} mice on 20 weeks CFD were also significantly larger compared to ApoE^{-/-}Fibulin-4^{+R} mice on 10 weeks CFD and ApoE^{-/-}Fibulin-4^{+R} mice on 20 weeks chow diet. ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice on 10 and 20 weeks of HFD displayed similar distributions with large variations in aortic arch diameters, which seemed to show some dilation in both ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice. Since increased aortic arch diameters were also observed in ApoE^{-/-}Fibulin-4^{+/+} mice on HFD, this probably indicates that HFD induces aortic arch dilations in both ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice. Aortic arch diameter measurements at 0 weeks of diet or after 20 weeks of chow diet, showed no difference between ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice. In short, after a subtle increase in fat diet (CFD) ApoE^{-/-}Fibulin-4^{+R} mice developed thoracic aortic dilations compared to ApoE^{-/-}Fibulin-4^{+/+} mice, while a diet with even more fat (HFD) results in a large variation in thoracic aortic diameters between animals in both the ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} group.

Additionally, to determine the effect of atherosclerosis on the stiffness of the aortic wall, we performed calculations on the distensibility of the aortic arch, which is an elasticity index of the aorta and inversely correlates with aortic wall stiffness. A slight reduction was already observed in adult Fibulin-4^{+R} aortas compared to Fibulin-4^{+/+} aortas (Supplemental Fig. 2A). A similar slight reduction was observed in ApoE^{-/-}Fibulin-4^{+R} mice after 10 and 20 weeks of CFD as compared to ApoE^{-/-}Fibulin-4^{+/+} mice (data not shown). The same measurements in ApoE^{-/-}Fibulin-4^{+R} mice after 10 and 20 weeks of HFD revealed a further decreased distensibility as compared to ApoE^{-/-}Fibulin-4^{+/+} mice (Supplemental Fig. 2B). This decrease was significant after 10 weeks of HFD, whereas after 20 weeks of HFD ApoE^{-/-}Fibulin-4^{+/+} aortas also showed a slight decrease in aortic arch distensibility. In conclusion, these results indicate that CFD, probably inducing modest atherosclerosis formation, results in aortic arch dilation in extracellular matrix defective ApoE^{-/-}Fibulin-4^{+R} mice, while a HFD leads to an equal distribution of aortic arch diameters in both ApoE^{-/-}Fibulin-4^{+R} and ApoE^{-/-}Fibulin-4^{+/+} mice, (including aortic arch dilations in some of both). This could point to the fact that under the same conditions Fibulin-4^{+R} animals

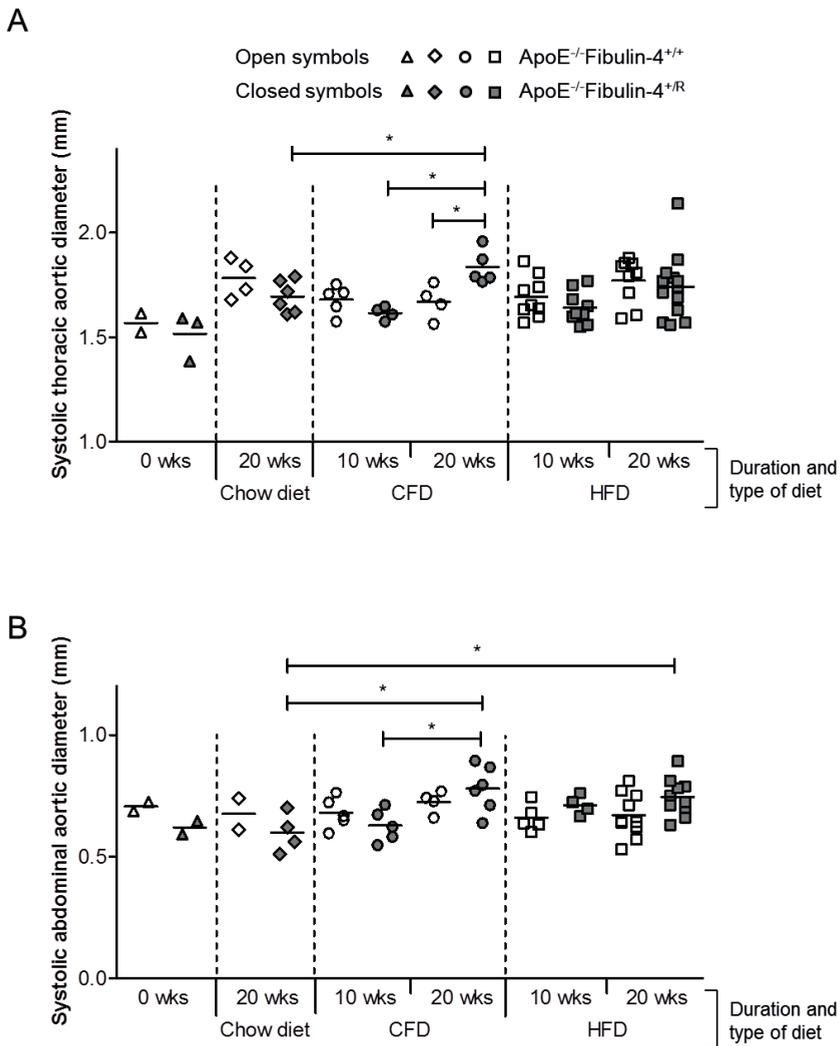


Figure 2. Increased thoracic and abdominal aortic diameters in ApoE^{-/-}Fibulin-4^{+R} mice. (A) Aortic arch diameter measurements by ultrasound imaging in M-mode show significant increased systolic aortic arch diameters in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of CFD (n=5) compared to ApoE^{-/-}Fibulin-4^{+/+} mice after 20 weeks of CFD (n=5), ApoE^{-/-}Fibulin-4^{+R} mice after 10 weeks of CFD (n=5), and compared to ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of chow diet (n=6). Aortic arch diameters after 10 and 20 weeks HFD appear to be equally distributed in ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice, with an increased variation in diameters. No differences are observed in mice fed a chow diet for 0 or 20 weeks. (B) Abdominal aortic measurements at the level of the iliac artery bifurcation also show significantly increased aortic diameters in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of CFD compared to ApoE^{-/-}Fibulin-4^{+R} mice after 10 weeks of CFD and 20 weeks of chow diet. Furthermore, increased abdominal aortic diameters are observed in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of HFD compared to ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of chow diet (*p<0.05). Open symbols indicate aortic diameters of ApoE^{-/-}Fibulin-4^{+/+} mice, closed symbols indicate aortic diameters of ApoE^{-/-}Fibulin-4^{+R} mice.

are more susceptible than Fibulin-4^{+/+} animals to atherosclerosis induction, which is therefore already apparent on a low fat diet. In addition, HFD leads to increased aortic wall stiffness in ApoE^{-/-}Fibulin-4^{+R} mice.

Interestingly, assessment of abdominal aortic diameters at the level of the iliac artery bifurcation showed significantly increased diameters in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks on CFD as compared to 10 weeks on CFD, and also compared to ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks chow diet (Fig. 2B and Supplemental Fig. 1B). Moreover, significantly increased abdominal diameters were observed in ApoE^{-/-}Fibulin-4^{+R} mice on 20 weeks of HFD compared to ApoE^{-/-}Fibulin-4^{+R} mice on 20 weeks of chow diet. Abdominal aortic diameters in ApoE^{-/-}Fibulin-4^{+/+} mice were not increased when compared among different diets and diet durations. Altogether, these data suggest that Fibulin-4 deficient ApoE^{-/-} mice already develop thoracic and abdominal aortic dilation on a low fat diet, while in addition HFD induces abdominal aortic dilation in ApoE^{-/-}Fibulin-4^{+R} mice and increases aortic arch stiffness.

ApoE^{-/-}Fibulin-4^{+R} aortas present increased plaque area in the thoracic and abdominal aorta

Next, we investigated the effect of a primary extracellular matrix defect on atherosclerotic plaque formation after 10 or 20 weeks of HFD feeding. Plaque area was quantified on *en face* preparations of Oil-red-O stained aortas. The descending and abdominal aorta of ApoE^{-/-}Fibulin-4^{+R} mice after 10 weeks of HFD showed significantly increased plaque area as compared to ApoE^{-/-}Fibulin-4^{+/+} mice (Fig. 3A and C). However, the plaque area in the aortic arch was similar between ApoE^{-/-}Fibulin-4^{+R} and ApoE^{-/-}Fibulin-4^{+/+} mice at this age. After 20 weeks of HFD increased plaque area was detected in the aortic arch and abdominal aorta of ApoE^{-/-}Fibulin-4^{+R} mice compared to ApoE^{-/-}Fibulin-4^{+/+} mice, but this was not significant due to large variability in plaque area between ApoE^{-/-}Fibulin-4^{+/+} mice (Fig. 3B and D). This suggests that Fibulin-4 deficiency accelerates plaque formation such that these are present after 10 weeks of HFD, while plaque occurrence after 20 weeks of HFD also increases in ApoE^{-/-}Fibulin-4^{+/+} mice. Interestingly, increased plaque area in individual ApoE^{-/-}Fibulin-4^{+/+} mice significantly correlated with increased aortic arch diameter (Fig. 4), while plaque area in ApoE^{-/-}Fibulin-4^{+R} mice did not correlate with aortic arch diameter. These data indicate that the observed dilation in ApoE^{-/-}Fibulin-4^{+/+} aortas is due to progression of atherosclerosis. However, in ApoE^{-/-}Fibulin-4^{+R} mice this correlation is absent, which is probably due to the fact that aortic dilation is already present at an earlier stage or with mild atherosclerosis, which means that in these mice the extracellular matrix defect is the underlying cause of the aortic dilation. At the same time, these results together indicate that the extracellular matrix defect in ApoE^{-/-}Fibulin-4^{+R} mice contributes to the increase in plaque area observed in thoracic and abdominal aortas.

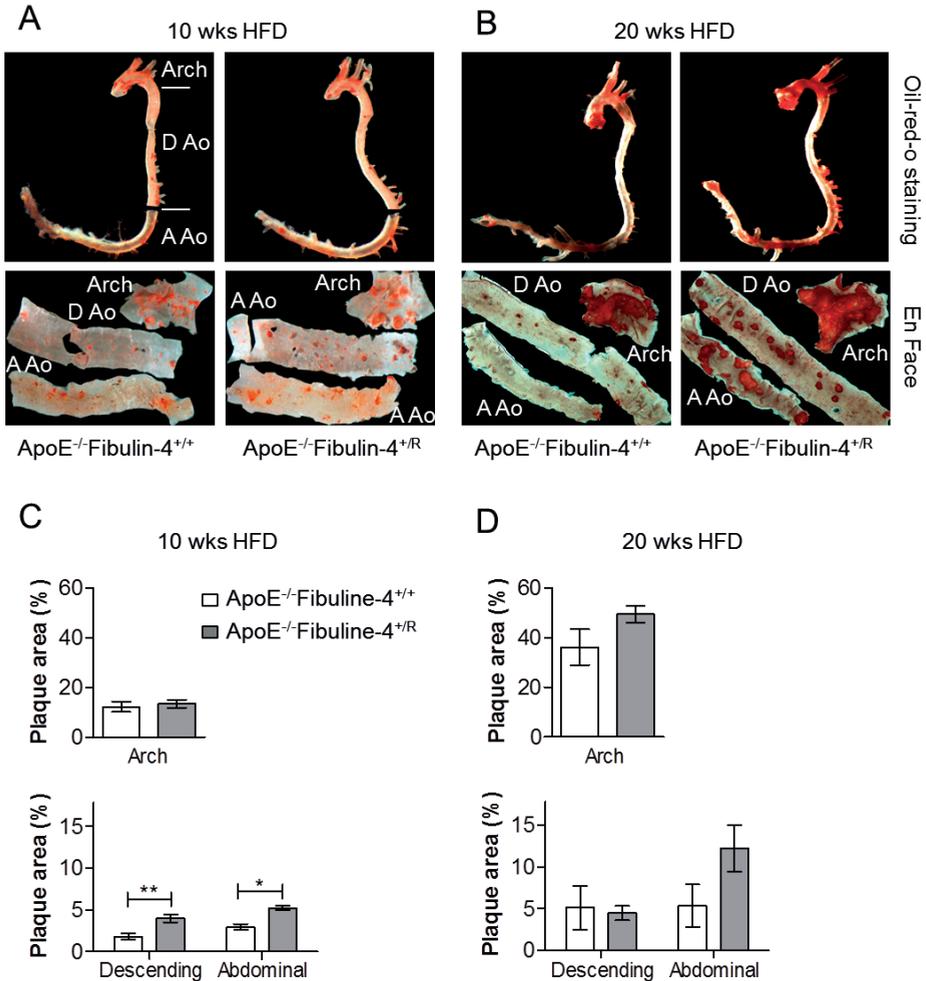


Figure 3. Increased plaque deposition in thoracic and abdominal aortas of ApoE^{-/-}Fibulin-4^{+/-R} mice. Oil-red-O staining and *en face* preparations of ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+/-R} aortas after (A) 10 and (B) 20 weeks of HFD show increased plaque areas in the thoracic and abdominal aortas of ApoE^{-/-}Fibulin-4^{+/-R} mice. Images in B represent aortas from ApoE^{-/-}Fibulin-4^{+/-R} mice, which show increased plaque areas as compared to their littermate ApoE^{-/-}Fibulin-4^{+/+}. Arch= aortic arch, D Ao= descending aorta, A Ao= abdominal aorta. (C) Quantification of the Oil-red-O stained *en face* preparations of ApoE^{-/-}Fibulin-4^{+/-R} aortas after 10 weeks of HFD (n=5) shows significantly increased plaque areas in the descending and abdominal aortas as compared to ApoE^{-/-}Fibulin-4^{+/+} aortas (n=5). (D) After 20 weeks of HFD no significant differences could be observed between ApoE^{-/-}Fibulin-4^{+/-R} mice (n=10) and ApoE^{-/-}Fibulin-4^{+/+} mice (n=5), as some ApoE^{-/-}Fibulin-4^{+/+} aortas also displayed an increase in plaque areas (* p<0.05, **p<0.01).

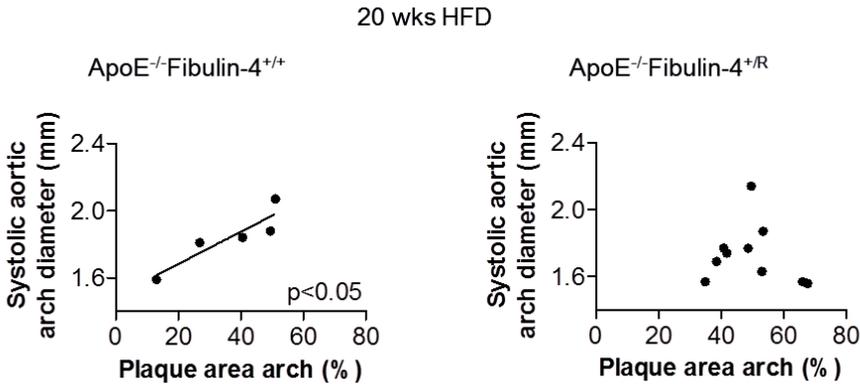


Figure 4. Increased plaque formation correlates with aortic arch dilation in ApoE^{-/-}Fibulin-4^{+/+} mice. After 20 weeks of HFD, ApoE^{-/-}Fibulin-4^{+/+} mice significantly develop increased aortic arch diameters with increased plaque formation, whereas no such correlation is present in ApoE^{-/-}Fibulin-4^{+/-} mice (*p<0.05).

ApoE^{-/-}Fibulin-4^{+/-} aortas show altered plaque morphology

Results of the *en face* Oil-red-O staining of the aortic arches were confirmed by quantification of plaque size on haematoxylin-eosin stained sections of the aortic arch, showing no significantly increased plaque size in ApoE^{-/-}Fibulin-4^{+/-} animals after 10 and 20 weeks of HFD as compared to ApoE^{-/-}Fibulin-4^{+/+} mice. Although a small increase in plaque size and lipid content was observed after 20 weeks of HFD in histological sections of aortic arches of ApoE^{-/-}Fibulin-4^{+/-} mice, this increase was not significant due to the low amount of samples (Supplemental Fig. 3). Interestingly, a different plaque morphology was observed in aortic arches of ApoE^{-/-}Fibulin-4^{+/-} mice after just 10 weeks of HFD; ApoE^{-/-}Fibulin-4^{+/-} aortas showed either 1) partially loose plaques (which will be referred to as disconnected plaques) or 2) plaques grown over existing plaques (which will be referred to as overlying plaques) or 3) a combination of both, in the brachiocephalic artery and in the inner curvature of the aortic arch (Fig. 5A and C). Out of the eight ApoE^{-/-}Fibulin-4^{+/-} animals examined, all displayed either disconnected plaques or overlying plaques, or both, in the brachiocephalic artery, whereas one out of eight ApoE^{-/-}Fibulin-4^{+/+} mice displayed a disconnected plaque and one an overlying plaque (Table 1). To determine whether this altered plaque morphology is associated with elastin abnormalities due to Fibulin-4 deficiency, we performed histological elastin analysis. This revealed a significantly decreased elastin content in plaques of the brachiocephalic artery of ApoE^{-/-}Fibulin-4^{+/-} animals (Fig. 5B). In the inner curvature of the aortic arch, three out of seven ApoE^{-/-}Fibulin-4^{+/-} animals had either a disconnected or an overlying plaque, or both, as compared to one out of eight ApoE^{-/-}Fibulin-4^{+/+} mice with a disconnected plaque, which is the same animal that showed the disconnected plaque in the brachiocephalic artery (Table 1). Plaques of the inner curvature of the aortic arch of ApoE^{-/-}Fibulin-4^{+/-} animals additionally contained a

Table 1. Amount of disconnected and overlying plaques found in the inner curvature of the aortic arch and in the brachiocephalic artery of ApoE^{-/-}Fibulin-4^{+R} mice as compared to ApoE^{-/-}Fibulin-4^{+/+}

Genotype	Inner curvature			Brachiocephalic artery		
	Disconnected plaque	Overlying plaque	Total	Disconnected plaque	Overlying plaque	Total
ApoE ^{-/-} Fibulin-4 ^{+R}	1/8	-/8	1/8	1/8	1/8	2/8
ApoE ^{-/-} Fibulin-4 ^{+/+}	2/7	3/7	3/7	5/8	6/8	8/8

*One arch could not be analyzed

decrease in elastin content (Fig. 5D). Histological analysis on cross-sections of abdominal aortas showed thickening of the abdominal aortic wall with increased spaces between the elastic laminae in ApoE^{-/-}Fibulin-4^{+R} animals after both 10 and 20 weeks of HFD, which is also observed at sites of plaque formation in the abdominal aorta as compared to

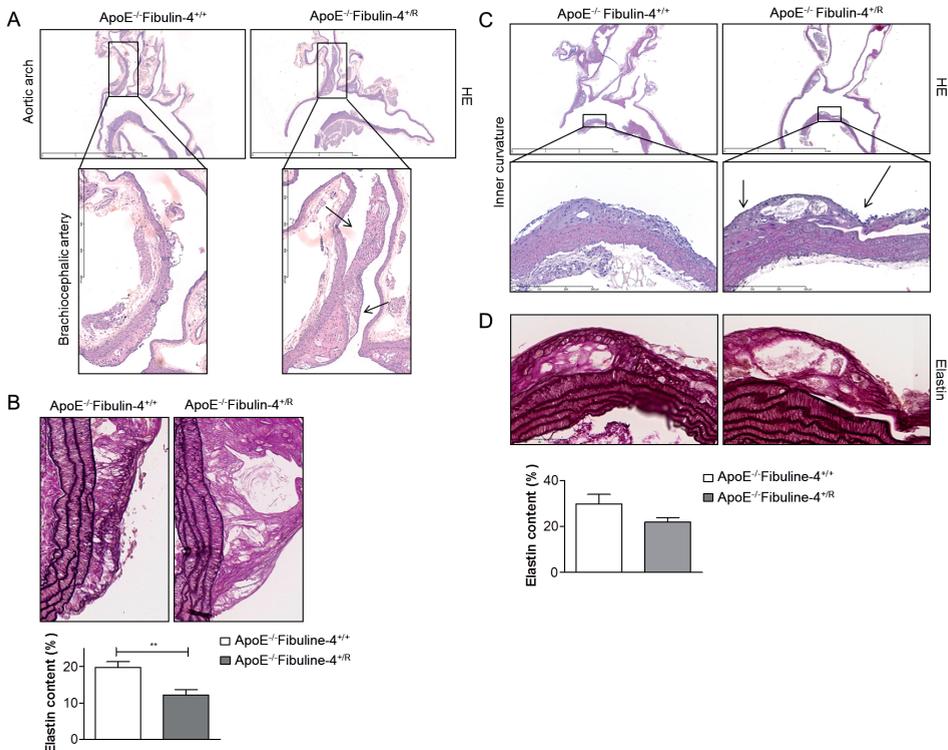


Figure 5. Plaque morphological changes in ApoE^{-/-}Fibulin-4^{+R} aortic arches after 10 weeks of HFD. HE analysis points to more disconnected and overlying plaques in (A) the brachiocephalic artery and (C) inner curvature of the arch of ApoE^{-/-}Fibulin-4^{+R} aortas after 10 weeks of HFD compared to ApoE^{-/-}Fibulin-4^{+/+} aortas. (B and D) Elastin staining and quantification of the elastin content in plaques revealed significantly less elastin in plaques in (B) the brachiocephalic artery of ApoE^{-/-}Fibulin-4^{+R} mice. (D) Plaques of the inner curvature of the aortic arch of ApoE^{-/-}Fibulin-4^{+R} mice also show a tendency towards less elastin (*p < 0.05).

ApoE^{-/-}Fibulin-4^{+/+} animals (Supplemental Fig. 4 and 5). This was also previously observed in the thoracic aortas of homozygous Fibulin-4^{R/R} mice and is associated with degeneration of the aortic wall.¹⁷ No differences in plaque morphology could be observed in the abdominal aorta. Altogether, these results suggest that a deficiency in the extracellular matrix protein Fibulin-4 leads to the formation of morphologically different atherosclerotic plaques in the thoracic aorta.

Decreased survival of atherosclerotic ApoE^{-/-}Fibulin-4^{+R} mice between 20 and 30 weeks of HFD

Interestingly, approximately 30% of ApoE^{-/-}Fibulin-4^{+R} animals that were fed a HFD with the aim to be analyzed at 30 weeks, died suddenly between 20 and 30 weeks on the diet as compared to a 100% survival of ApoE^{-/-}Fibulin-4^{+/+} control animals (Fig. 6). Moreover, symptoms of paralysis were observed after handling of 4 out of 16 ApoE^{-/-}Fibulin-4^{+R} animals that survived between 20 and 30 weeks of CFD or HFD, while none of these symptoms were observed in ApoE^{-/-}Fibulin-4^{+/+} mice (n=9) (Table 2). These results indicate that atherosclerotic Fibulin-4 deficient ApoE^{-/-}Fibulin-4^{+R} animals display a worsened survival outcome, possibly due to atherosclerosis-induced events, which may cause the paralysis symptoms.

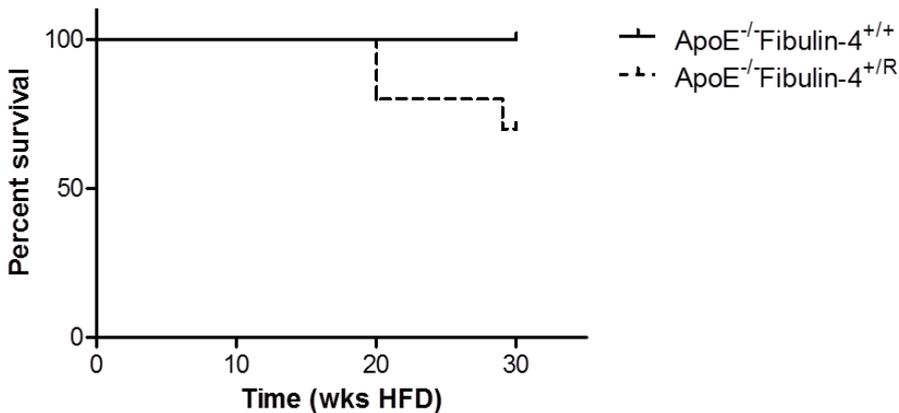


Figure 6. Decreased survival of ApoE^{-/-}Fibulin-4^{+R} mice between 20 and 30 weeks of HFD. After 30 weeks of HFD approximately 30% of ApoE^{-/-}Fibulin-4^{+R} mice (n=10) did not survive as compared to 100% survival of ApoE^{-/-}Fibulin-4^{+/+} mice (n=3).

DISCUSSION

In this study we demonstrate that a genetic defect leading to subtle changes in the extracellular matrix structure of the aortic wall, in combination with atherosclerosis, may predispose for thoracic and abdominal aortic disease, including morphologically altered atherosclerotic plaques, and a worsened survival outcome. Atherosclerosis induction in the double mutant ApoE^{-/-}Fibulin-4^{+R} mice results in increased abdominal MMP activity, thoracic and abdominal aortic dilation and increased thoracic and abdominal plaque formation. Furthermore, ApoE^{-/-}Fibulin-4^{+R} mice display altered plaque morphology and have a reduced survival rate after 20 weeks of HFD. These results indicate that an underlying extracellular matrix defect promotes a bidirectional relation between atherosclerotic disease and aortic wall dilation.

On one side, atherosclerosis induction in Fibulin-4 deficient mice leads to enhanced aortic wall degeneration in Fibulin-4 deficient mice. ApoE^{-/-}Fibulin-4^{+R} mice already develop both thoracic and abdominal aortic wall dilations after 20 weeks on CFD. Most probably 20 weeks of CFD induces mild atherosclerosis since ApoE^{-/-} mice spontaneously develop atherosclerosis after 12 weeks on normal chow diet, which has a lower fat percentage and a different nutrient composition compared to CFD.²²⁻²⁴ Abdominal aortic dilations also occur after 20 weeks of HFD compared to chow diet, indicating that induction of both mild and high atherosclerosis results in increased abdominal aortic dilation in ApoE^{-/-}Fibulin-4^{+R} mice. However, after 20 weeks of HFD both ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice have a wide but equal distribution in thoracic aortic arch diameters. This might be explained by highly increased atherosclerotic plaque formation induced by the HFD, which probably overrules the effects of the extracellular matrix degeneration on the aortic wall.

On the other side the aortic wall degeneration in ApoE^{-/-}Fibulin-4^{+R} mice on HFD leads to increased plaque formation and altered plaque morphology. Ten weeks of HFD induces significantly more atherosclerotic plaques in the thoracic and abdominal aorta of ApoE^{-/-}Fibulin-4^{+R} mice, whereas 20 weeks of HFD induces increased plaque formation in both ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+R} mice. However, a slight increase in plaque formation in the aortic arch and abdominal aorta of ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of HFD can be observed. This suggests that Fibulin-4 deficiency leads to enhanced atherosclerosis progression. In ApoE^{-/-}Fibulin-4^{+/+} mice the increased plaque formation is associated with the observed increased diameters, which is in concordance with previous reports.²⁵ However, ApoE^{-/-}Fibulin-4^{+R} mice show increased plaque formation independent of changes in aortic diameters, probably because this dilation already occurs in an earlier stage at a lower percentage of fat. Histological analyses of aortic plaques of ApoE^{-/-}Fibulin-4^{+R} mice after 10 weeks of HFD show more disconnected plaques, more overlying plaques and less elastin content in plaques compared to ApoE^{-/-}Fibulin-4^{+/+} aortic plaques. The

reduced elastin content in atherosclerotic plaques of ApoE^{-/-}Fibulin-4^{+R} mice is likely to be a consequence of impaired elastogenesis since Fibulin-4 influences crosslinking of elastic fiber by affecting the recruitment of LOX.^{5, 7, 26, 27} A reduction in elastin content can make these plaques less stable. Additionally, ApoE^{-/-}Fibulin-4^{+R} mice show symptoms of paralysis and a reduced survival between 20 and 30 weeks of HFD. The histological observed alterations in plaque morphology together with the observed worsened survival outcome might indicate that Fibulin-4 deficiency increases atherosclerosis-induced events. Whether these events are caused by plaque rupture is unclear. Our data show overlying plaques, disconnected plaques, and plaques with reduced elastin content and high MMP activity in the double mutant ApoE^{-/-}Fibulin-4^{+R} mice. These features coincide with the occurrence of paralysis and reduced survival outcome.

In this respect, it would be interesting to make whole body angiographies of these animals just before they succumb. However, this is complicated due to their unpredictable and sudden death. Strikingly, plaque rupture was observed in another atherosclerotic mouse model with a more severe ECM defect; the ApoE^{-/-}Fibrillin-1^{+/-} mouse.²⁸ Fibrillin-1 is the major structural component of microfibrils, which provide the scaffold for the deposition and crosslinking of elastin. The C1039G mutated Fibrillin-1 mice used in these studies however are different from the Fibulin-4^R mice used here, as the Fibrillin-1 mice spontaneously develop thoracic aneurysms thereby also affecting the hemodynamic parameters. Fragmentation of elastic fibers in these double knockout mice leads to increased vascular stiffness and promoted features of multifocal plaque instability. These mouse models with a structural defect in elastic fibers associated proteins provide insight into the role of extracellular matrix degeneration in the susceptibility for altered plaque morphology and its consequences.

The bidirectional interaction between aortic wall degeneration and atherosclerosis formation in our ApoE^{-/-}Fibulin-4^{+R} mice may lead to a vicious circle, in which the observed increased MMP activity could play a prominent role. The MMPsense probe is activated by MMP2, -3, -9 and -13, of which MMP2 and MMP9 are known to play an important role in extracellular matrix degeneration and in aortic aneurysm formation. Increased MMP activity has indeed been observed in Fibulin-4 deficient mice with these probes.^{17, 19} Furthermore, MMPs, mainly MMP3, -9, -12 and -13, were shown to be involved in different stages of plaque formation.²⁰ Therefore, the highly increased MMP activity observed in ApoE^{-/-}Fibulin-4^{+R} mice might be due to aortic wall degeneration as well as increased atherosclerotic plaques, and might contribute to the altered plaque morphology in these mice.

This ApoE^{-/-}Fibulin-4^{+R} mouse model with diet-induced atherosclerosis shows that subtle manifestations of aberrant elastin formation in heterozygous Fibulin-4^{+R} mice might predispose to thoracic and abdominal aortic disease as well as enhanced atherosclerotic disease. This combined mouse model provides the opportunity to unravel the biological

processes underlying aortic wall degeneration and to identify markers that elucidate key events in the early stages of the pathogenic sequence that might culminate in an aneurysm. In fact, the ApoE^{-/-}Fibulin-4 mouse model therefore indicates that a haploinsufficiency for Fibulin-4 leads to a pathogenic outcome in combination with fat diets, and therefore might resemble patients that experience late-onset 'sporadic' and barely detectable forms of aneurysms. Additionally, this model provides insight in the effect of mild extracellular matrix defects, as observed during aging, on the progression of atherosclerosis.

ACKNOWLEDGEMENT

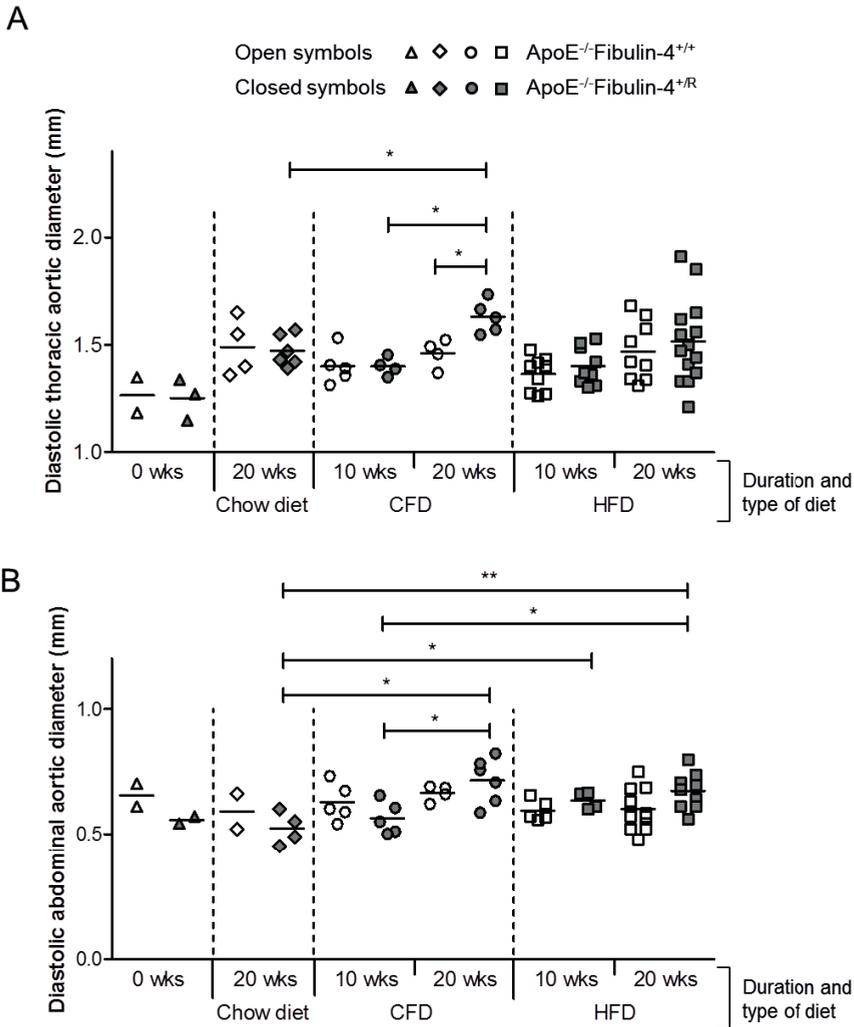
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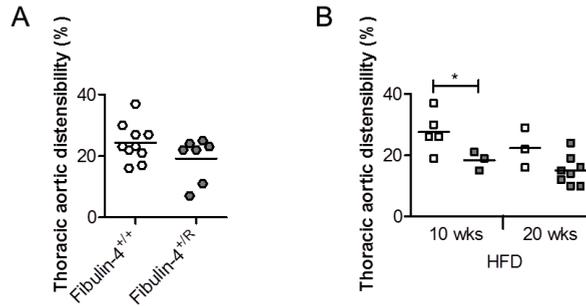
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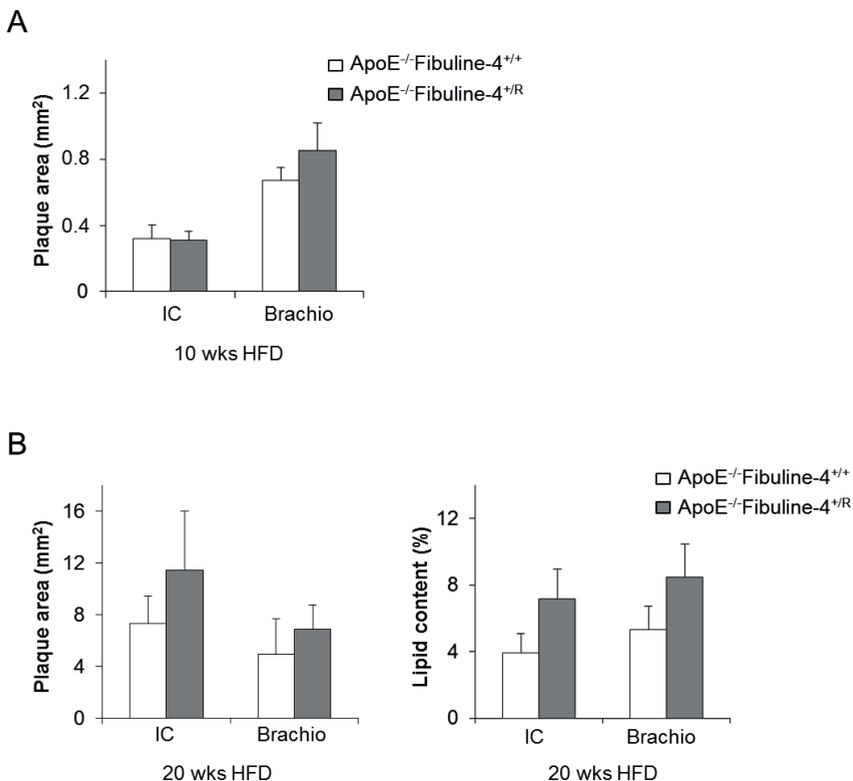
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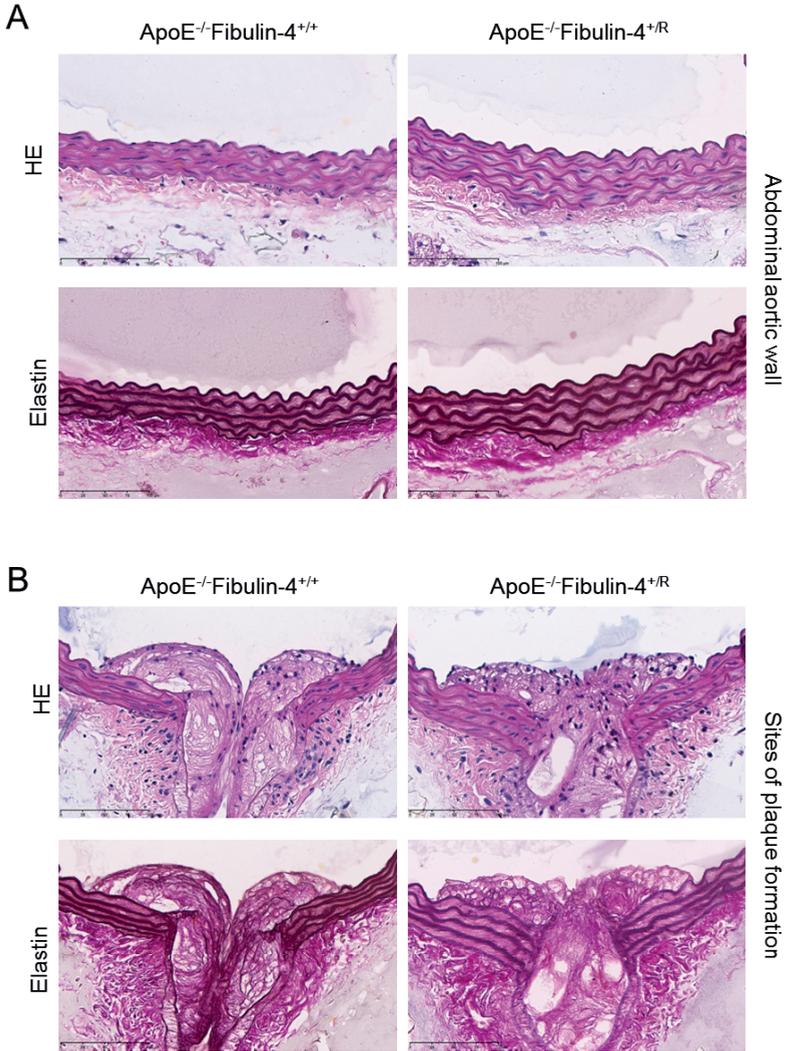
Supplemental Figure 1. Increased diastolic thoracic and abdominal aortic diameters in ApoE^{-/-}Fibulin-4^{+/-} mice. (A) M-mode aortic diameter measurements in diastole by ultrasound imaging show significant dilation of the aortic arches of ApoE^{-/-}Fibulin-4^{+/-} mice after 20 weeks of CFD (n=5) compared to ApoE^{-/-}Fibulin-4^{+/+} mice after 20 weeks of CFD (n=5), ApoE^{-/-}Fibulin-4^{+/-} mice after 10 weeks of CFD (n=5) and ApoE^{-/-}Fibulin-4^{+/-} mice after 20 weeks of chow diet (n=6). Aortic arch diameters after 10 or 20 weeks of HFD seem to be evenly distributed in ApoE^{-/-}Fibulin-4^{+/+} and ApoE^{-/-}Fibulin-4^{+/-} mice, with an increased diameter variation. No differences are observed in mice fed a chow diet for 0 or 20 weeks. (B) Abdominal aortic measurements in diastole show significantly increased diameters in ApoE^{-/-}Fibulin-4^{+/-} mice after 20 weeks of CFD compared to ApoE^{-/-}Fibulin-4^{+/+} mice after 10 weeks of CFD and 20 weeks of chow diet. Furthermore, increased abdominal aortic diameters are observed in ApoE^{-/-}Fibulin-4^{+/-} mice after 10 and 20 weeks of HFD compared to ApoE^{-/-}Fibulin-4^{+/+} mice after 20 weeks of chow diet, and ApoE^{-/-}Fibulin-4^{+/-} mice after 20 weeks of HFD compared to ApoE^{-/-}Fibulin-4^{+/+} mice after 10 weeks of CFD (*p<0.05, **p<0.01). Open symbols indicate aortic diameters of ApoE^{-/-}Fibulin-4^{+/+} mice, closed symbols indicate aortic diameters of ApoE^{-/-}Fibulin-4^{+/-} mice.



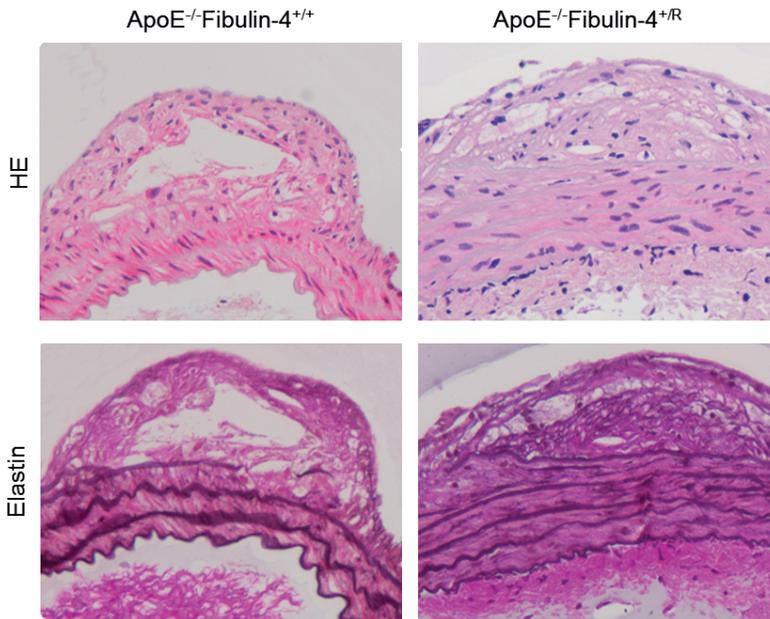
Supplemental Figure 2. Decreased distensibility of aortic arches from ApoE^{-/-}Fibulin-4^{+R} mice. (A) Calculations of aortic wall displacements in M-mode during systole and diastole indicate a slight non-significant reduced distensibility of 15 weeks old Fibulin-4^{+R} aortas on a chow diet compared to Fibulin-4^{+/+} aortas. (B) A significantly reduced distensibility is observed in calculations of aortic wall displacements in B-mode of ApoE^{-/-}Fibulin-4^{+R} (n=5) aortas after 10 weeks of HFD, which further decreases after 20 weeks (n=10) of HFD compared to ApoE^{-/-}Fibulin-4^{+/+} aortas (n=5) (*p<0.05).



Supplemental Figure 3. Quantification of plaque area on histological sections of ApoE^{-/-}Fibulin-4 aortas after 10 and 20 weeks of HFD. (A) Plaque area quantified in the inner curvature of the aortic arch shows no difference between ApoE^{-/-}Fibulin-4^{+R} mice (n=8) and ApoE^{-/-}Fibulin-4^{+/+} mice after 10 weeks of HFD (n=8), while a slight increase is observed in the brachiocephalic artery. (B) Quantified plaque area and percentage lipid content in plaques show a slight increase in ApoE^{-/-}Fibulin-4^{+R} mice after 20 weeks of HFD (n=5) compared to ApoE^{-/-}Fibulin-4^{+R} mice (n=3). IC= inner curvature, Brachio= brachiocephalic artery.



Supplemental Figure 4. Histological analysis of abdominal aortas after 10 weeks of HFD. HE and elastin analysis of the abdominal aorta revealed (A) thickened abdominal aortic wall with increased spaces between the elastic laminae in ApoE^{-/-}Fibulin-4^{+R} mice after 10 weeks of HFD as compared to ApoE^{-/-}Fibulin-4^{+/+} mice, (B) which is also observed at sites of plaque formation.



Supplemental Figure 5. Histological analysis of abdominal aortas after 20 weeks of HFD. HE and elastin analysis of the abdominal aorta after 20 weeks of HFD points to a thickened abdominal aortic wall with increased spaces between the elastic laminae in ApoE^{-/-}Fibulin-4^{+/R} mice as compared to ApoE^{-/-}Fibulin-4^{+/+} mice.