The fetal origin of adult atherosclerosis : a study in ApoE and Ldlr mouse models

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Chapter 3

Maternal ApoE-Deficiency Promotes Collar-Induced Neointima Formation in Adult ApoE$^{+/-}$ Offspring Even Without Cholesterol Feeding

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Abstract

We have previously shown that adult apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers exhibit increased susceptibility to cardiovascular disease, as compared to adult apoE$^{+/-}$ offspring from wild-type mothers. In this model, neointimal lesions, as a reflection of cardiovascular disease, were induced by placement of a constrictive collar around the carotid artery in combination with a high-cholesterol diet. The aim of the present study was to investigate whether adult apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers are also more susceptible to neointima formation without feeding a cholesterol-containing diet. On a chow diet, both types of apoE$^{+/-}$ offspring were normocholesterolemic. After placement of a constrictive collar, lesions could not be detected in apoE$^{+/-}$ offspring from wild-type mothers, whereas apoE$^{+/-}$ offspring from apoE$^{-/-}$ did develop neointimal lesions. These neointimal lesions were small and relatively a-cellular. The mesenchymal cells stained positive for α-smooth muscle actin and were negative for the macrophage marker Mac-3. We conclude that intrauterine exposure to maternal apoE-deficiency is sufficient to allow collar-induced neointima formation, even without cholesterol feeding, sustaining the hypothesis that the intrauterine environment poses a major risk factor for adult cardiovascular disease.

Introduction

Although the exact cause of atherosclerosis is unknown, many risk factors have been identified, for example age, gender, smoking, hypertension, hypercholesterolemia, obesity, and lack of physical exercise. These factors accumulate throughout life and operate synergistically, thereby promoting earlier and more severe onset of disease.

In addition to adult onset risk factors for atherosclerosis, prenatal risk factors have been identified. The "fetal origins hypothesis" postulated by Barker[1] proposes that adjustment to an unfavorable maternal environment is beneficial to the developing embryo. However, these adaptations may lead to an increased disease risk as the adult environment differs from the fetal situation. Examples of prenatal risks are fetal undernutrition and maternal hypercholesterolemia. Fetal undernutrition leads to persisting changes in a range of metabolic processes in the fetus thereby enhancing cardiovascular disease risk in adulthood.[2-4] Intrauterine exposure to maternal hypercholesterolemia on the other hand, has been reported to enhance fatty streak formation, as well as lesion size in aortas of fetuses from hypercholesterolemic mothers compared with fetuses from normocholesterolemic mothers.[5] After birth, lesion progression remained accelerated in these children in relation to children from normocholesterolemic mothers.[6]

To study the long-term consequences of intrauterine exposure to environmental maternal atherosclerotic risk factors such as hypercholesterolemia, we have used a genetically homogeneous mouse model. We were able to demonstrate increased endothelial cell activation and vascular injury in heterozygous apolipoprotein E-deficient ($apoE^{+/-}$) fetuses from homozygous apolipoprotein E-deficient ($apoE^{-/-}$) mothers as compared to genetically identical $apoE^{+/-}$ fetuses from wild-type mothers.[7] Combined constrictive collar placement around the left common carotid artery and high-cholesterol feeding in adult $apoE^{+/-}$ offspring severely aggravated neointima formation in $apoE^{+/-}$ offspring from $apoE^{-/-}$ mothers whereas almost no lesions were found in $apoE^{+/-}$ offspring from wild-type mothers. However, it remained unclear whether solely intrauterine exposure to maternal apoE-deficiency was sufficient for increased development of cardiovascular disease in adult life.

Therefore, in this study we investigated whether adult $apoE^{+/-}$ offspring from $apoE^{-/-}$ mothers are also more susceptible for neointima formation in absence of a high-cholesterol diet. We generated genetically identical $apoE^{+/-}$ offspring from normocholesterolemic wild-type mothers, as well as from hypercholesterolemic $apoE^{-/-}$ mothers. Mice heterozygous for the apoE allele are comparable to wild-type mice regarding their lipid levels and their relative resistance to atherosclerosis.[8-10]

Even when fed a diet containing cholesterol, these mice do not develop atherosclerosis spontaneously up to the age of 20 weeks.[7] The probability that apoE$^{+/-}$ mice will develop atherosclerosis spontaneously during their life time is quite low. To investigate the consequences of prenatal priming on the susceptibility for cardiovascular disease, neointimal lesions, as a reflection of cardiovascular disease, were induced by placement of a constrictive collar around the left common artery of both apoE$^{+/-}$ offspring from wild-type mothers and apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. With the collar technique we can mimic the normal aging process and at the same time accelerate the process of atherosclerosis. The high-cholesterol diet that was used in our previous study[7] was replaced by regular chow to maintain normocholesterolemic conditions throughout the experiment.

Methods

Animals

The apoE$^{-/-}$ and wild-type C57Bl/6J mice were purchased from Charles River Laboratories (Maastricht, The Netherlands, import agency for Jackson Laboratories). ApoE$^{-/-}$ mothers were crossed with wild-type fathers and vice versa to generate genetically identical apoE$^{+/-}$ offspring from wild-type as well as from apoE$^{-/-}$ mothers. Female offspring (n=5 each group) were used for experiments. Regular chow and water were provided ad libitum. The Committee on Animal Welfare, Leiden University Medical Center, approved all animal experiments.

Lipid Measurements

Total levels of cholesterol and triglycerides were enzymatically quantified in blood plasma of 4 hour fasted offspring at age 4, 8 and 16 weeks by using commercially available kits (Roche, Almere, The Netherlands). Blood samples were obtained through tail bleeding.

Neointimal Lesion Induction

Neointima formation was induced by placement of a constrictive collar around the left common carotid artery in all apoE$^{+/-}$ animals at the age of 16 weeks, as previously described.[7,11]

Tissue Harvesting and Preparation

Mice were anesthetized 4 weeks after collar placement. Pressure-perfusion (76 mm Hg) was performed through the cardiac left ventricle with sterile PBS for 5 minutes. Both common carotid arteries were harvested and the collar was removed. The carotid arteries were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 6 hours. Fixation was followed by dehydration in series of ethanol and xylene and tissues were paraffin-embedded. Transverse 5-µm sections were cut and serially mounted.

Immunohistochemistry

Routine staining was performed with Mayer's hematoxylin and eosin, Resorcin-Fuchsin for detection of elastin and Sirius red for collagen. Unless indicated otherwise the immunohistochemistry was performed as described earlier.[7,12] In short, for each staining all sections were stained in one batch. The sections were incubated overnight at room temperature with a mouse monoclonal primary antibody against α-smooth muscle actin to identify vascular smooth muscle cells

(1:2000, Sigma Aldrich, Product No. A2547, Zwijndrecht, The Netherlands), a rabbit polyclonal anti-von Willebrand factor (vWF; 1:2000, DAKO, Glostrup, Denmark) and a rat monoclonal anti-CD31 (1:50, PharMingen, Alphen, The Netherlands) to study endothelial cells, and a rat monoclonal Mac-3 (1:400, PharMingen, Alphen, The Netherlands) against macrophages. Goat anti-rabbit biotin conjugate (1:200, Vector, Amsterdam, The Netherlands), goat anti-rat biotin conjugate (1:200, PharMingen) and rabbit anti-mouse peroxidase conjugate (1:200, DAKO) with normal goat and mouse serum diluted in PBS were used as secondary antibodies (1 hour at room temperature). Biotin labeling was followed by incubation with Vectastain ABC (Vector). The CD31 signal was enhanced with a CSA kit (DAKO). The 3-3' diaminobenzidine tetrahydrochloride was used as chromogen and counterstaining was performed with Mayer's hematoxylin.

Morphometry

The Cavalieri principle[13] was used to estimate the volume of the neointimal lesions in the region proximal to the collar. The lesion area was counted in 20 equally spaced sections and the volume calculated. Medial volumes were estimated in 10 equally spaced sections comprising a total length of 350 µm proximal to the collar. As a control, intimal and medial volumes were calculated in contralateral noncompromised carotid arteries.

Statistical Analysis

Data are represented as mean ± SEM. The data on offspring weight and plasma lipid levels were evaluated by general linear model repeated measures. The Fisher's Exact test and ANOVA were used to statistically substantiate the results on neointima formation. The differences were considered to be significant if $P < 0.05$ and the power ≥ 80%.

Results

Body Weight and Lipid Levels

Throughout the experiment, no significant differences were observed between the body weights of apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers and apoE$^{+/-}$ offspring from wild-type mothers (Table 1). Intrauterine exposure to maternal apoE-deficiency did not affect the total plasma cholesterol (Table 2) and triglyceride levels (Table 3) of the offspring.

Table 1. Weight offspring

Offspring	Weight (g)		
	4 weeks	8 weeks	16 weeks
Mat WT	10.3 ± 1.2	19.8 ± 0.6	23.3 ± 0.6
Mat apoE$^{-/-}$	14.5 ± 1.0*	19.9 ± 0.3	24.1 ± 0.2

All data are shown as mean ± SEM. Mat WT indicates apoE$^{+/-}$ offspring from wild-type mothers; Mat apoE$^{-/-}$, apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. $^{*}P = 0.027$ versus Mat WT at 4 weeks.

Table 2. Total plasma cholesterol levels offspring

Offspring	TC (mmol/L)		
	4 weeks	8 weeks	16 weeks
Mat WT	1.30 ± 0.11	1.84 ± 0.20	1.74 ± 0.17
Mat apoE$^{-/-}$	1.54 ± 0.10	1.74 ± 0.12	1.69 ± 0.08

All data are shown as mean ± SEM. TC indicates total plasma cholesterol; TG, total plasma triglycerides; Mat WT, apoE$^{+/-}$ offspring from wild-type mothers; Mat apoE$^{-/-}$, apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. No significant differences were found between the two groups at all time points.

Table 3. Total plasma triglyceride levels offspring

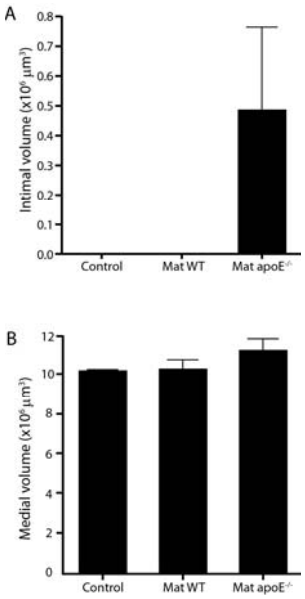
Offspring	TG (mmol/L)		
	4 weeks	8 weeks	16 weeks
Mat WT	0.52 ± 0.08	0.75 ± 0.06	0.55 ± 0.04
Mat apoE$^{-/-}$	0.62 ± 0.04	0.68 ± 0.08	0.68 ± 0.05

All data are shown as mean ± SEM. TC indicates total plasma cholesterol; TG, total plasma triglycerides; Mat WT, apoE$^{+/-}$ offspring from wild-type mothers; Mat apoE$^{-/-}$, apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. No significant differences were found between the two groups at all time points.

In contrast to our previous study in which the apoE$^{+/-}$ offspring were fed a high-cholesterol diet to induce hypercholesterolemia,[7] all mice received regular chow to maintain normocholesterolemia. In this way, the effects of intrauterine exposure to

maternal apoE-deficiency alone on cardiovascular disease risk could be examined. With age, a slight increase in total plasma cholesterol was observed in all animals, but levels remained within normal range.

Neointima Formation



The long-term consequences of intrauterine exposure to maternal apoE-deficiency were determined by studying the response of the vascular wall of both apoE$^{+/-}$ offspring from wild-type mothers and those from apoE$^{-/-}$ mothers after collar placement. Four weeks after collar placement, a significantly different response was observed between the two groups of mice. In 4 out of 5 apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers neointimal lesions were detected whereas no neointimal lesions were found in any apoE$^{+/-}$ offspring from wild-type mothers (4 out of 5 versus 0 out of 5; P = 0.024). The average lesion volume in the apoE$^{+/-}$ mice from apoE$^{-/-}$ mothers was 0.49 ± 0.28x10^6 µm^3 (Figure 1A). In addition, a trend towards an increased medial volume in the proximal region of the collar was observed in apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers (10.33 ± 0.47x10^6 µm^3) compared with those from wild-type mothers (9.43 ± 0.42x10^6 µm^3) and contralateral noncompromised carotid arteries (9.37 ± 0.02x10^6 µm^3, Figure 1B).

Carotid Artery Morphology and Lesion Composition

To characterize the morphology of the adult carotid arteries and the neointimal lesions, several histological and immunohistochemical staining techniques were used. In apoE$^{+/-}$ offspring from wild-type mothers, the region proximal to the collar resembled the normal morphology as observed in noncompromised contralateral arteries (Figure 2A-B, D-E and G-H). In response to collar placement, neointimal lesions of 1 to 2 subendothelial layers developed in apoE$^{+/-}$ progeny from apoE$^{-/-}$ mothers (Figure 2C). One lesion per carotid artery was detected. The lesions were relatively a-cellular. Extensive deposition of the extracellular matrix proteins elastin and collagen was demonstrated (Figure 2F and 2I). Furthermore, thickening of the media and disorganization of the smooth muscle cells were detected in comparison to apoE$^{+/-}$ offspring from wild-type mothers (Figure 2C,F and I). The internal elastic lamina was intact indicating the immaturity of the lesions (Figure 2F).

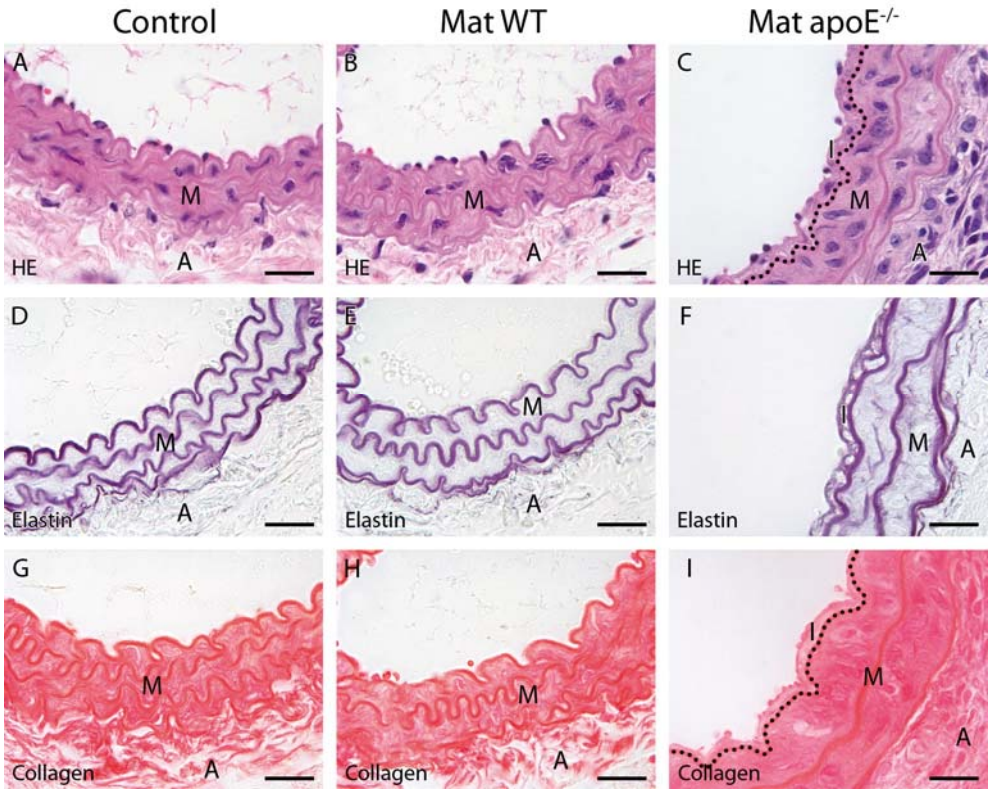


Figure 2. Histological staining. Representative cross-sections from (A,D,G) noncompromised carotid arteries and the region proximal to the collar in apoE$^{+/-}$ mice from (B,E,H) wild-type mothers and (C,F,I) apoE$^{-/-}$ mothers, respectively. Sections were stained with Mayer's (A-C) hematoxylin and eosine, (D-F) Resorcin-Fuchsin and (G-I) Sirius red. The dotted line represents the border between intima and media. I indicates intima; M, media; A, adventitia. Scale bars: 20 µm.

⬅ **Figure 1**. Morphometric analysis of adult carotid arteries 4 weeks after collar placement. (A) Estimation of intimal volume and (B) medial volume of noncompromised (control) and collared carotid arteries of apoE$^{+/-}$ mice from wild-type mothers (Mat WT) and apoE$^{-/-}$ mothers (Mat apoE$^{-/-}$). Data are means ± SEM (n = 5 each).

The CD31 staining revealed an intact endothelial lining in all carotid arteries (Figure 3A-C). The mesenchymal cells in the neointima were negative for CD31. The endothelial cells showed an activated phenotype. In comparison to control arteries in which all endothelial cells stained positively for anti-vWF, increased activation was observed in apoE$^{+/-}$ from wild-type mothers (Figure 3D-E). In apoE$^{+/-}$ mice from apoE$^{-/-}$ mothers increased turnover and upregulation of vWF was seen in endothelial cells in the region proximal to the collar resulting in intense staining for vWF in the neointima (Figure 3F). In addition, vWF was detected in the media and

adventitia in these animals. Besides α-smooth muscle actin staining in the media, as also seen in the noncompromised contralateral arteries en those of apoE$^{+/-}$ offspring from wild-type mothers, additional positivity was observed in the neointima of apoE$^{+/-}$ mice from apoE$^{-/-}$ mothers (Figure 3G-I). Macrophages were present abundantly in the adventitia and only sporadic presence was seen in the neointima and media of apoE$^{+/-}$ mice from apoE$^{-/-}$ mothers, (Figure 3L). The noncompromised control arteries and those of apoE$^{+/-}$ offspring from wild-type mothers were completely devoid of macrophages (Figure 3J-K).

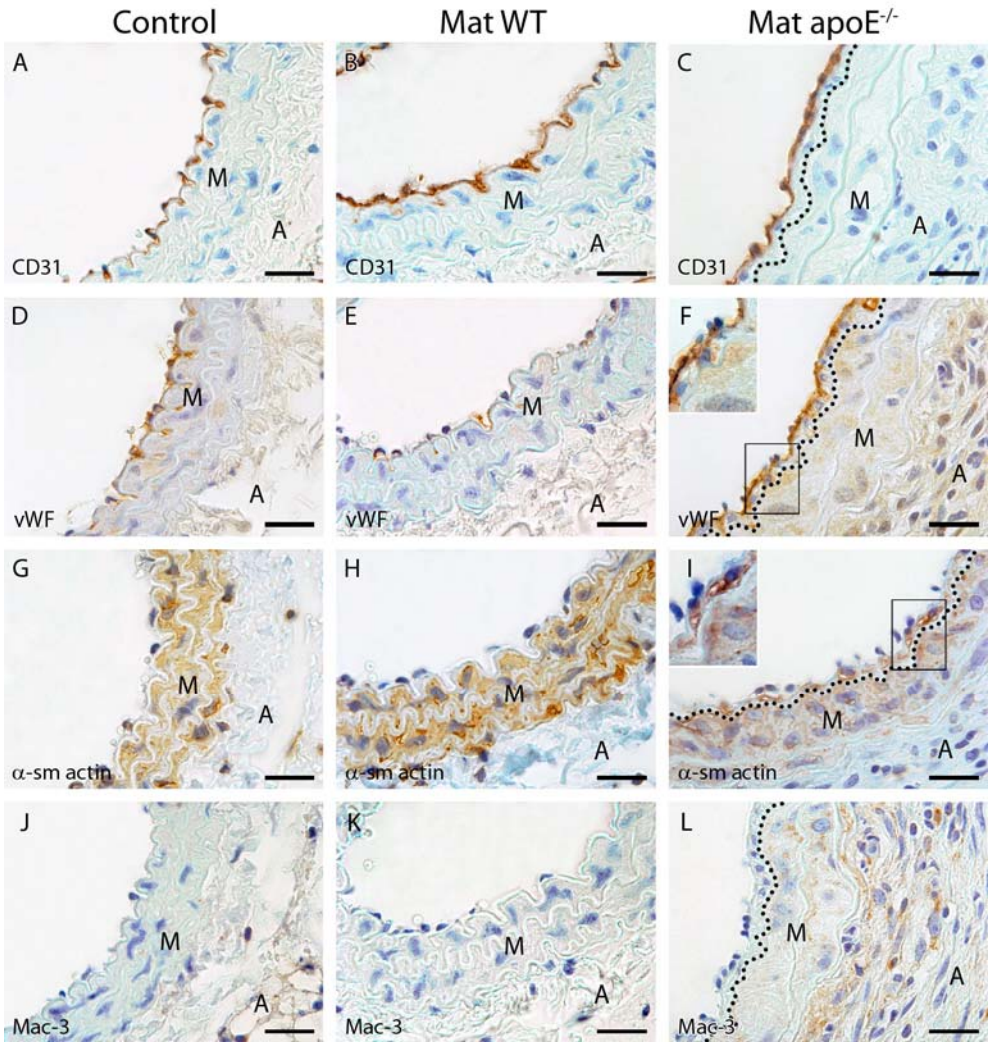


Figure 3. Immunohistological characterization of the vascular wall. Representative cross-sections from (A,D,G,J) noncompromised carotid arteries and the region proximal to the collar in apoE$^{+/-}$ mice from (B,E,H,K) wild-type mothers and (C,F,I,L) apoE$^{-/-}$ mothers, respectively. Sections were stained with (A-C) anti-CD31, (D-F) anti-vWF, (G-I) α-smooth muscle actin (α-sm actin) and (J-L) anti-Mac-3 to identify macrophages. Note the presence of vWF and α-sm actin in the neointima. The dotted line represents the border between intima and media. I indicates intima; M, media; A, adventitia. Scalebars: 20 µm.

Discussion

The present results demonstrate that high-cholesterol feeding is not required in the process of initiation of neointimal lesion development in apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. Obviously, intrauterine exposure to maternal apoE-deficiency has a dramatic effect on atherosclerosis development in adult life. A shift from athero-protective high shear stress levels towards athero-prone low shear stress profiles within the carotid arteries as a result of collar placement was already sufficient to induce neointimal lesion formation in chow-fed normocholesterolemic apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. No lesions were observed in apoE$^{+/-}$ mice from wild-type mothers. These data indicate that the intrauterine environment poses a major risk factor for adult cardiovascular disease and can exert its effects independent of hypercholesterolemia induced by a cholesterol-containing diet.

The neointimal lesions in the carotid arteries of apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers were of substantial size and could be characterized by 1 or 2 subendothelial layers and relative a-cellularity. Whereas large numbers of macrophages were recruited into the adventitia, only sporadic presence of macrophages was seen in the neointima of apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. In our previous study we used an identical approach as in the current study, except for the additional use of a cholesterol-containing diet.[7] In the presence of diet-induced hypercholesterolemia large lesions developed and massive accumulation of macrophages was observed in apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers. Together, these data indicate that high-cholesterol feeding accelerates and aggravates neointimal lesion progression. Lesion progression seems to be accelerated through increased recruitment of inflammatory cells to the vascular wall. It has been reported that rolling of monocytes over the endothelial layer is increased in mice fed a cholesterol-containing diet.[14] Moreover, adhesion molecules like VCAM-1 and P-selectin, are enhanced under these conditions.[15,16] The lack of macrophages in the lesions observed in the current study could be the result of limited active recruitment of inflammatory cells from the circulation. Future studies have to reveal whether the current immature lesions observed in the 5 months old chow-fed apoE$^{+/-}$ offspring from apoE$^{-/-}$ mothers will acquire a large numbers of macrophages, associated fat deposition, and remodeling upon aging.

In addition to hypercholesterolemia induced by high-cholesterol feeding, other postnatal environmental risk factors are likely to promote more severe vascular disease. Therefore, exposure should be limited to maintain a relatively low disease risk throughout life. Awareness and development of risk reducing programs in adult life are also beneficial for a second reason; it reduces prenatal risk factors as well when applied to pregnant women and women in their reproductive period. General monitoring of for example cholesterol levels and

identification of markers of inflammation in relation to apoE polymorphisms may identify a high-risk population of pregnant women. To reduce the cardiovascular disease risk in progeny, lifestyle management programs and dietary intervention should be introduced for these women.

In apoE$^{+/-}$ mice fetuses and neonates from both normocholesterolemic wild-type and hypercholesterolemic apoE$^{-/-}$ mothers no hemodynamically induced atherosclerosis was detected throughout the vasculature. In contrast, in humans fatty streaks already develop in the fetal aorta.[5] Development of these lesions is greatly enhanced in progeny from hypercholesterolemic mothers. In childhood, despite normal cholesterol levels, lesion progression is higher in these children.[6] The apparent distinction in the rate of lesion development between mice and humans may be explained by hemodynamics. In mice, the average wall shear stress in the carotid artery is more than 6 times higher than in humans.[17] Because high shear stress is athero-protective, mice may be more resistant to atherosclerosis than humans. We hypothesize that in human fetuses, the combined effects of epigenetic programming by maternal hypercholesterolemia, and the intrauterine environment almost directly initiate the process of atherosclerosis in the fetal vascular wall. Elucidation of the mechanisms of priming by maternal atherosclerotic risk factors would give more insight into the fetal tissues that are affected and may provide us with early indicators of atherosclerosis susceptibility (reviewed by DeRuiter and colleagues[18]).

In conclusion, intrauterine exposure to maternal apoE-deficiency, through epigenetic programming of the fetus, plays a key role in neointimal lesion initiation in adult offspring, even without high-cholesterol feeding. Hypercholesterolemia induced by high-cholesterol feeding promotes more severe vascular disease. Because there is increasing evidence that prenatal atherosclerotic risk factors are of importance for development of adult vascular disease, they should be taken into account in lifestyle management programs of pregnant women and mothers of the future.

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