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Poor maternal nutrition programmes a pro-atherosclerotic phenotype in ApoE$^{-/-}$ mice

Heather L. BLACKMORE*[1], Ana V. PIEKARZ*[1], Denise S. FERNANDEZ-TWINN*, John R. MERCER†, Nichola FIGG†, Martin BENNETT† and Susan E. OZANNE*

*Metabolic Research Laboratories, Institute of Metabolic Sciences, Addenbrooke's Hospital, Cambridge, U.K., and †Addenbrookes Centre for Clinical Investigation, Addenbrooke's Hospital, Cambridge, U.K.

ABSTRACT

Numerous animal studies have consistently shown that early life exposure to LP (low-protein) diet programmes risk factors for CVD (cardiovascular disease) such as dyslipidaemia, high BP (blood pressure) and cardiac dysfunction in the offspring. However, studies on the effect of maternal under-nutrition on offspring development of atherosclerosis are scarce. Applying our LP model to the ApoE$^{-/-}$ atherosclerosis-prone mouse model, we investigated the development of atherosclerotic lesions in the aortic root of 6-month-old offspring. In addition, markers of plaque progression including SMA (smooth muscle actin) and Mac3 (macrophage marker 3) were studied. Pregnant dams were fed on a control (20% protein) or on an isocaloric LP diet (8% protein) throughout pregnancy and lactation. After weaning, male offspring were maintained on 20% normal laboratory chow. At 6 months of age, LP offspring showed a significantly greater plaque area ($P < 0.05$) with increased cholesterol clefts and significantly higher indices of DNA damage compared with controls ($P < 0.05$). The expression of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) ($P < 0.05$) and LDL (low-density lipoprotein) receptor in the liver of LP offspring were increased. Furthermore, LP offspring had higher LDL-cholesterol levels ($P < 0.05$) and a trend towards elevated insulin. There were no differences in other lipid measurements and fasting glucose between groups. These observations suggest that early exposure to an LP diet accelerates the development and increases the progression of atherosclerotic lesions in young adult offspring. Future studies are needed to elucidate the specific mechanisms linking *in utero* exposure to a diet low in protein to the development of atherosclerosis.

INTRODUCTION

The DOHaD (Developmental Origins of Health and Disease) hypothesis proposes that insults acting during critical periods of growth and development may have a long-term effect on susceptibility to chronic diseases later in life [1]. The organism responds to these early insults by adapting its phenotype to the environment resulting in alteration of growth, body composition, tissue and organ structure, gene expression and/or metabolism. Although these adaptations are vital for immediate benefit, many of them are permanent and may be disadvantageous when

Key words: apolipoprotein, atherosclerosis, cholesterol, fetal programming, low-protein diet, maternal nutrition, offspring.

Abbreviations: ApoE, apolipoprotein E; ATM, ataxia telangiectasia mutated; BP, blood pressure; CVD, cardiovascular disease; H&E, haematoxylin and eosin; H2AX, histone H2AX; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein; LP, low-protein; NBF, neutral buffered formalin; p-ATM, phospho-ATM; p-H2AX, phospho-H2AX; SMA, smooth muscle actin; VLDL, very-LDL; VSMC, vascular smooth muscle cell.

[1] These authors contributed equally to this work.

Corresponding author: Miss Heather L. Blackmore (email hlb46@cam.ac.uk).

there is a mismatch between the actual environment and the one predicted *in utero* [1–3].

Although many insults can arise during development, maternal under-nutrition is one of the best characterized. For instance, populations exposed to short periods of famine have been studied to investigate the importance of maternal nutrition in mediating the relationship between nutrition *in utero* and future adult disease [4,5]. A cohort exposed *in utero* to famine in early gestation during the Dutch Hunger Winter of 1944–1945 were found to be more likely to develop premature coronary heart disease and presented with a more atherogenic lipid profile than individuals born the year before the famine [4]. In addition, a recent study of individuals exposed to the 1967–1970 Biafran famine showed elevated systolic and diastolic BP (blood pressure) following adjustment for BMI (body mass index) [6]. These observations provide epidemiological evidence that early exposure to under-nutrition during critical periods of growth and development has long-term detrimental effects on cardiovascular health.

Epidemiological studies largely rely on gathering data retrospectively from birth cohorts, providing limited information on potential molecular mechanisms. Therefore experimental animal models have been developed to understand the underlying mechanisms through which nutrition during pre- and early post-natal development could affect susceptibility to adult chronic diseases [7]. The maternal LP (low-protein) rodent model devised by Snoeck et al. [8] is one of the most extensively documented. This model has provided evidence of a direct link between sub-optimal maternal nutrition and alterations in offspring cardiac function and structure. For example, young adult rats exposed to the maternal LP diet during pregnancy and lactation show cardiac abnormalities, including an elevated basal HR (heart rate) and an attenuated β-adrenergic response, suggestive of premature heart failure [9].

There are very few studies in animal models addressing the programming of atherosclerosis [10–13]. This is likely to reflect the inherent difficulties of such studies in rodents, as they are highly resistant to atherosclerosis [14]. In contrast with humans, mice naturally have high levels of HDL (high-density lipoprotein) and low levels of LDL (low-density lipoprotein). Genetically modified mice with disorders in lipid metabolism, such as inactivation of the gene encoding the ApoE (apolipoprotein E) are therefore essential when studying atherosclerosis in rodents [15]. In ApoE$^{-/-}$ mice, cholesterol is largely distributed between VLDL (very-LDL) and IDL (intermediate-density lipoprotein) fractions; however, LDL also forms a major contribution [16].

ApoE$^{-/-}$ mice are considered to be an effective model for studying the pathogenesis of atherosclerosis because they are hypercholesterolaemic and develop spontaneous arterial lesions [17]. We applied our LP model of early sub-optimal nutrition to the ApoE$^{-/-}$ mouse model of atherosclerosis, with the aim of investigating the development and progression of atherosclerotic lesions following maternal and early postnatal under-nutrition. Atherosclerotic plaque deposition in the aortic root of 6-month-old offspring was quantified and markers indicative of plaque progression were studied.

MATERIALS AND METHODS

Animal protocol

All experimental procedures were conducted according to the British Home Office Animals Act, 1986. Pregnant ApoE$^{-/-}$ mice of the C57BL6/J background were housed one per cage, kept in standard environmental conditions (12 h light/12 h dark cycle, room temperature of 23 °C) and fed on either a control diet containing 20 % protein or an isocaloric 8 % LP diet during pregnancy and lactation; the maternal LP diet was supplemented with carbohydrate to match the calorie content of the control diet. Composition of both maternal diets has been published previously [9]. Fresh diet was provided every 2–3 days and distilled water administered *ad libitum*. Maternal body weight and food intake were recorded daily during pregnancy and lactation. Birth occurred on approximately day 20 of pregnancy. This was considered PND0 (postnatal day 0). There was no effect of maternal diet on litter size. At 21 days of age male pups were weaned, housed in litters (three or four pups per cage) and fed standard laboratory chow containing 20 % protein (LAD1; SDS). Animals were maintained on this diet until analysis at 6 months of age.

Heart perfusion–fixation

Male ApoE$^{-/-}$ offspring (6-months old) were fasted overnight, weighed and killed by carbon dioxide overdose. Subsequently, blood was collected followed by *in situ* perfusion–fixation of the heart using a washout phase of 20 ml of PBS, followed by fixation with 20 ml of 10 % NBF (neutral buffered formalin) via the left ventricle. After perfusion–fixation, the heart was post-fixed in 10 % NBF for 24 h and stored in PBS at 4 °C for further analysis.

Histology

Samples stored in PBS were prepared by removal of fat and connective tissue. Subsequently, tissue was processed, paraffin-embedded and cut into 5 μm serial sections. To preserve integrity of the aortic valve region, the entire heart of each animal was sectioned from its apex to the source of the coronary arteries, close to the point of greatest occlusion by plaque. This landmark was used as a basis for which sections were subsequently collected. The aortic root has three valve leaflets and deposition of plaque was measured in each. Sections

were de-paraffinated and processed for staining or immunohistochemistry as described below. A single section of aortic root was used for each of the separate staining protocols per animal.

H&E (haematoxylin and eosin) for lesion size analysis

Sections were stained with H&E (Sigma HHS-32 and E8017) to allow the quantification of atherosclerosis and cholesterol cleft content within the aortic root. Atherosclerotic lesion area was measured for each of the three leaflets of the aortic root. Total plaque area was calculated by summing the three leaflet values for each animal and averaging these values according to group exposure. Imaging software CellD (Olympus) was used to circumscribe the borders of each plaque to generate plaque area (μm^2) as described previously [18,19].

Immunohistochemistry

Specific primary antibodies were used for the following: α-SMA (α-smooth muscle actin) (DAKO M0851, 1:500 dilution), Mac3 (macrophage marker 3) (M3/84; Pharmingen, 1:200 dilution), p-ATM [phospho-ATM (ataxia telangiectasia mutated)] (Cell Signaling Technology 4526, 1:200 dilution) and p-H2AX [phospho-H2AX (histone H2AX)] (Cell Signaling Technology 2577, 1:200 dilution). SMA and Mac3 content was calculated by expressing total positive stain as a percentage of total plaque area. p-ATM and p-H2AX were assessed by counting the total number of positive staining cells per animal, then subsequently expressing this positive staining per 100 000 μm^2 plaque area. Images were captured using a BX51 microscope (Olympus), air-cooled CCD (charge-coupled-device) camera (CoolSnap) and imaging software (CellD and Adobe Photoshop).

Blood collection and analysis

Blood was obtained from each animal at the time of death by cardiac puncture, and tail blood was used for analysis of glucose concentration (OneTouch Ultra; LifeScan). Blood was allowed to clot and centrifuged for 3 min at 8000 *g* and the supernatant serum kept at $-80\,^{\circ}$C for subsequent analysis of lipids (mouse phenotyping facility, Department of Clinical Biochemistry) and fasting insulin (Ultrasensitive mouse insulin EIA kit; Mercodia). We did not directly measure VLDL; however, the concentration of LDL was derived using the Friedwald equation, which includes the VLDL fraction.

Western blotting

Liver-derived protein lysate was resolved by SDS/PAGE (10 % polyacrylamide gels). Proteins were transferred to PVDF membranes (Immobilon-P; Millipore) at 110 mV. Membranes were blocked [3 % low fat milk (Marvel), PBS and 0.1 % Tween] at 4 °C overnight. Primary antibodies to the HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) (Abgent AP6577c), p-HMG-CoA reductase [phospho-HMG-CoA reductase (Ser^{872})] (Millipore 09-356) and LDL receptor (Biosciences Lifesciences ABE1252) were incubated with the membrane for 1 h. Membranes were washed and incubated with HRP (horseradish peroxidase)-conjugated secondary antibody (anti-rabbit) and binding detected using Super Signal West Pico Chemiluminescent substrate (Thermo Scientific 34078) and Amersham Hyperfilm ECL (GE Healthcare).

Statistical analysis

Results represent the number (n) of different litters as only one animal from each litter was used in the analysis. Where data showed a normal Gaussian distribution, results are presented as means $\pm$ S.E.M. and analysed by an independent Student's t test. Data not normally distributed were analysed non-parametrically and are presented as medians (interquartile ranges) or log-transformed and are presented as means [95 % CI (confidence interval)]. Differences were considered significant at $P < 0.05$.

RESULTS

Body weights

Offspring exposed to an LP diet throughout pregnancy and lactation showed no difference in body weight at day 3 when compared with controls. Body weight was significantly reduced in LP offspring at weaning (LP, 6.31 ± 0.24 g, $n = 12$; controls, 7.44 ± 0.37 g, $n = 7$; $P < 0.05$). There was no difference in bodyweight at 6 months of age between groups (LP, 29.3 ± 0.7 g, $n = 12$; controls; 30.8 ± 0.8 g, $n = 6$).

Atherosclerosis

Atherosclerotic plaque development was analysed in the aortic root of both offspring groups at 6 months of age. LP offspring had a greater total plaque area per animal compared with controls ($P < 0.05$; Figure 1). Furthermore, LP offspring had greater total cholesterol cleft containing area compared with control offspring ($P < 0.05$; Figure 2).

Immunohistochemistry

Atherosclerotic plaque phenotype was assessed by immunohistochemistry to quantify VSMC (vascular smooth muscle cell) content, inflammation and DNA damage. LP offspring showed a significant reduction in VSMCs indicated by attenuated SMA positive staining ($P < 0.05$; Table 1). DNA damage marker p-H2AX was significantly increased in the atherosclerotic plaque of LP offspring, compared with controls ($P < 0.05$), whereas inflammatory marker Mac3 and p-ATM were not different between the two groups (Table 1).

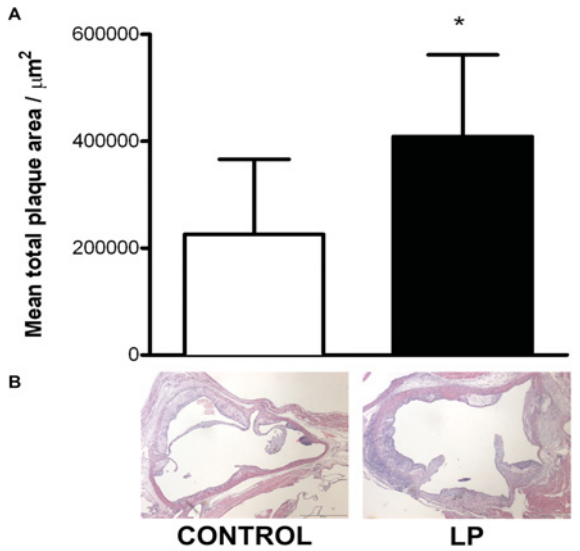

Figure 1 Aortic root sections from 6-month-old offspring stained for H&E and analysed for plaque area

(A) Total plaque area per animal: controls ($n = 6$) and LP offspring ($n = 12$). (B) Representative control and LP H&E stains used for the calculation of plaque area, $^{*}P < 0.05$.

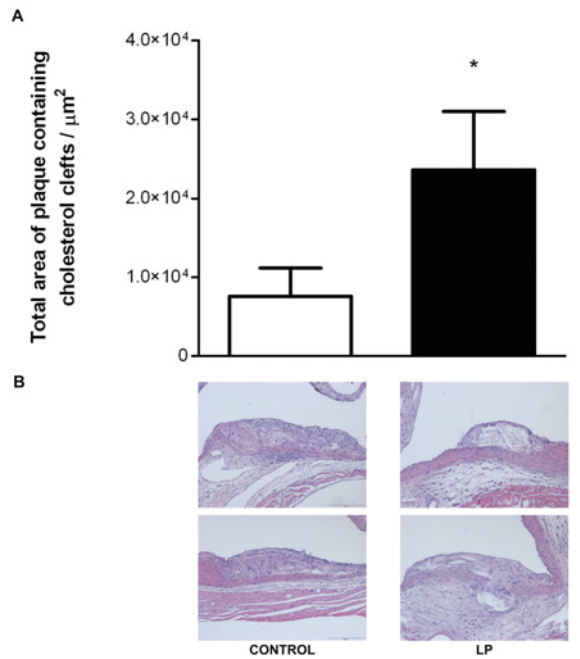

Figure 2 Aortic root sections analysed from 6-month-old offspring stained for total cholesterol cleft content

(A) Total plaque area (μm^2) containing cholesterol clefts per animal: controls ($n = 6$) and LP offspring ($n = 12$). (B) Representative images of cholesterol cleft content in one chosen animal for both control and LP offspring, $^{*}P < 0.05$.

Serum analysis

Total and LDL-cholesterol levels in LP offspring were higher than control offspring at 6 months of age ($P < 0.05$ and $P < 0.01$ respectively; Table 2). LP offspring showed a trend to elevated insulin, whereas no significant differences were observed in other lipid measurement or fasting glucose (Table 2).

Table 1 Characteristics of aortic root plaques of male offspring at 6 months of age

Values are expressed as medians (25th–75th percentile). LP, $n = 11$; Control, $n = 6$. $^{*}P < 0.05$.

Characteristic	Control	LP
SMA staining/plaque area (%)	5.7 (2.7–10.2)	2.8 (1.1–6.2)*
Mac3 staining/plaque area (%)	8.8 (2.3–15.8)	9.2 (4.1–17.0)
p-ATM cells/100 000 μm^2 plaque area	82 (50–133)	105 (61–157)
p-H2AX cells/100 000 μm^2 plaque area	14 (10–47)	36 (28–83)*

Table 2 Serum measurements of male offspring at 6 months of age

Values are expressed as means ± S.E.M. or medians (25th–75th percentile). LP, $n = 6$; Control, $n = 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Measurement	Control	LP
Total cholesterol (mmol/l)	6.73 ± 1.01	9.77 ± 0.78*
LDL-cholesterol (mmol/l)	4.62 ± 0.70	8.65 ± 0.70**
HDL-cholesterol (mmol/l)	0.63 ± 0.12	0.44 ± 0.11
Triacylglycerol (mmol/l)	1.30 ± 0.14	1.52 ± 0.19
Non-esterified fatty acids (μmol/l)	728 (705–1217)	1011 (706–1573)
Glucose (mmol/l)	5.4 ± 0.3	5.9 ± 0.4
Insulin (μg/l)	0.26 (0.15–0.35)	0.49 (0.30–0.90)

Western blotting

Protein expression of the rate-limiting enzyme in the synthesis of cholesterol HMG-CoA reductase was significantly increased in the livers of LP offspring compared with controls, expressed as a percentage of control mean ($P < 0.05$; Figure 3). LDL receptor, responsible for clearance of LDL from the circulation, was elevated in LP offspring (LP, 177 ± 38, $n = 4$; and controls, 100 ± 33, $n = 7$).

DISCUSSION

The main finding of the present study was that exposure to an LP diet early in life accelerates the development and progression of atherosclerotic lesions in young adult offspring. Both human and animal studies have consistently shown that early life exposure to maternal under-nutrition programmes dyslipidaemia [4,13,20], high BP [21,22] and cardiac dysfunction in the offspring [9,23]. However, these are all risk factors for CVD (cardiovascular disease) rather than disease outcome. For instance, it has been shown that the interaction of prenatal under-nutrition with a postnatal atherogenic diet

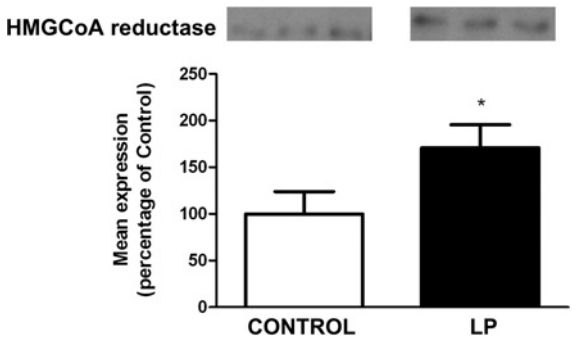

Figure 3 Western blot analysis of liver from 6-month-old offspring probing for hepatic HMG-CoA reductase
Controls ($n = 4$) and LPs ($n = 7$), $^{*}P < 0.05$.

increases atherosclerosis in female ApoE*3 Leiden mice, but has no effect on male offspring [13]. In the present study, we did not expose pregnant dams or their offspring to a high-fat diet in order not to interfere with the spontaneous development of the atherosclerotic lesions. To our knowledge, this is the first study to report the sole effect of impaired maternal nutrition on atherosclerotic plaque development and accelerated progression in the aortic root of ApoE$^{-/-}$ male offspring at 6 months of age.

The atherosclerotic lesion initially forms in the arterial intima and is characterized by intimal thickening arising from cellular and lipid accumulation [24]. In the intima, lipoproteins undergo spontaneous or cell-mediated modifications resulting in fatty streaks. These fatty streaks are not obstructive or clinically significant. However, they can progress to form more complex lesions. The progression to a fibrous cap or fibroatheroma, the characteristic lesion of more advanced atherosclerosis, consists of the development of a cap of smooth muscle cells, collagen and a lipid-rich necrotic core [25]. The fibrous cap is a common site of plaque rupture induced by macrophage and T-cell infiltration combined with apoptosis and shear stress [26]. A plaque containing a thick fibrous cap is considered stable [27,28], whereas unstable or vulnerable lesions prone to rupture are characterized by thinning of the fibrous cap and loss of VSMC content. Loss of VSMCs within the cap has been associated with increased lesion growth and progression [18,19]. In response to sub-optimal maternal nutrition, LP offspring had a significantly greater plaque area compared with controls. Furthermore, the atherosclerotic lesions in these offspring showed reduced SMA positive staining, indicative of VSMC loss and fibrous cap thinning. Increased DNA damage has been observed in human atherosclerotic plaques, therefore we chose to investigate two markers of DNA damage, p-H2AX and p-ATM [29,30]. Increased levels of DNA damage marker p-H2AX staining was observed in the shoulder region of the plaques. These results indicate that the atherosclerotic plaques of LP offspring were not only larger but showed enhanced lesion progression compared with controls.

The role of LDL-cholesterol in the pathogenesis of atherosclerosis is well documented [25]. LP offspring showed elevated total and LDL-cholesterol concentrations compared with controls. Owing to the known role of serum cholesterol in the pathogenesis of atherosclerosis it could be hypothesized that the enhanced atherosclerosis observed in LP offspring is a consequence of their increased serum cholesterol concentrations. Furthermore, expression of hepatic HMG-CoA reductase was also significantly increased in the LP offspring. This enzyme catalyses the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis [31]. Inhibition of HMG-CoA reductase using statins reduces serum LDL-cholesterol and reverses atherosclerotic plaque development [32,33], thereby reducing the risk of cardiovascular events and subsequent mortality [34,35]. The mechanisms underlying the programmed increase in HMG-CoA reductase are unknown; however, it is possible that epigenetic mechanisms are involved. These observations also suggest that the increased LDL-cholesterol levels in LP offspring is the result of programmed changes affecting *de novo* cholesterol biosynthesis.

The observation that LDL receptors were increased in the presence of raised cholesterol levels appears at first paradoxical. The role of the LDL receptor is normally to remove excess cholesterol from the blood [36]. However, it can be argued that the increased LDL receptor levels reflect an unsuccessful compensatory response to normalize serum cholesterol levels. The mechanistic basis of such a compensatory mechanism is unclear.

In conclusion, we have shown maternal under-nutrition through protein deficiency accelerates the development of atherosclerosis in young adult offspring. In particular, we demonstrated that atherosclerotic lesion development and progression are susceptible to alterations in early nutrition. Further studies are needed to elucidate the specific molecular mechanism and pathway by which exposure to under-nutrition early in life leads to the development of more progressive atherosclerotic lesions later in life.

AUTHOR CONTRIBUTION

Heather Blackmore, Ana Piekarz and Denise Fernandez-Twinn carried out the experiments, the data analysis and prepared the paper. Nichola Figg carried out the experiments, and John Mercer analysed the data. Martin Bennett designed the experiments. Susan Ozanne designed the experiments, carried out the data analysis and prepared the paper.

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REFERENCES

1 Barker, D. J. (2007) The origins of the developmental origins theory. J. Intern. Med. **261**, 412–417
2 Eriksson, J. G., Forsen, T., Tuomilehto, J., Winter, P. D., Osmond, C. and Barker, D. J. (1999) Catch-up growth in childhood and death from coronary heart disease: longitudinal study. Br. Med. J. **318**, 427–431
3 Ozanne, S. E. and Hales, C. N. (2004) Lifespan: catch-up growth and obesity in male mice. Nature **427**, 411–412
4 Roseboom, T., de Rooij, S. and Painter, R. (2006) The Dutch famine and its long-term consequences for adult health. Early Hum. Dev. **82**, 485–491
5 Huang, C., Li, Z., Wang, M. and Martorell, R. (2010) Early life exposure to the 1959–1961 Chinese famine has long-term health consequences. J. Nutr. **140**, 1874–1878
6 Hult, M., Tornhammar, P., Ueda, P., Chima, C., Bonamy, A. K., Ozumba, B. and Norman, M. (2010) Hypertension, diabetes and overweight: looming legacies of the Biafran famine. PLoS ONE **5**, e13582
7 Warner, M. J. and Ozanne, S. E. (2010) Mechanisms involved in the developmental programming of adulthood disease. Biochem. J. **427**, 333–347
8 Snoeck, A., Remacle, C., Reusens, B. and Hoet, J. J. (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol. Neonate **57**, 107–118
9 Fernandez-Twinn, D. S., Ekizoglou, S., Wayman, A., Petry, C. J. and Ozanne, S. E. (2006) Maternal low-protein diet programs cardiac beta-adrenergic response and signaling in 3-mo-old male offspring. Am. J. Physiol. Regul. Integr. Comput. Physiol. **291**, R429–R436
10 Norman, J. F. and LeVeen, R. F. (2001) Maternal atherogenic diet in swine is protective against early atherosclerosis development in offspring consuming an atherogenic diet post-natally. Atherosclerosis **157**, 41–47
11 Palinski, W., D'Armiento, F. P., Witztum, J. L., de Nigris, F., Casanada, F., Condorelli, M., Silvestre, M. and Napoli, C. (2001) Maternal hypercholesterolemia and treatment during pregnancy influence the long-term progression of atherosclerosis in offspring of rabbits. Circ. Res. **89**, 991–996
12 Wang, Z., Huang, Z., Lu, G., Lin, L. and Ferrari, M. (2009) Hypoxia during pregnancy in rats leads to early morphological changes of atherosclerosis in adult offspring. Am. J. Physiol. Heart Circ. Physiol. **296**, H1321–H1328
13 Yates, Z., Tarling, E. J., Langley-Evans, S. C. and Salter, A. M. (2009) Maternal undernutrition programmes atherosclerosis in the ApoE*3-Leiden mouse. Br. J. Nutr. **101**, 1185–1194
14 Piedrahita, J. A., Zhang, S. H., Hagaman, J. R., Oliver, P. M. and Maeda, N. (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. **89**, 4471–4475
15 Daugherty, A. (2002) Mouse models of atherosclerosis. Am. J. Med. Sci. **323**, 3–10
16 O'Neill, T. P. (1997) Apolipoprotein E-deficient mouse model of human atherosclerosis. Toxicol. Pathol. **25**, 20–21
17 Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. and Ross, R. (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler. Thromb.: J. Vasc. Biol. **14**, 133–140
18 Clarke, M. C., Figg, N., Maguire, J. J., Davenport, A. P., Goddard, M., Littlewood, T. D. and Bennett, M. R. (2006) Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. Nat. Med. **12**, 1075–1080
19 Clarke, M. C., Littlewood, T. D., Figg, N., Maguire, J. J., Davenport, A. P., Goddard, M. and Bennett, M. R. (2008) Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. Circ. Res. **102**, 1529–1538
20 Lumey, L. H., Stein, A. D., Kahn, H. S. and Romijn, J. A. (2009) Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. Am. J. Clin. Nutr. **89**, 1737–1743
21 Stein, A. D., Zybert, P. A., van der Pal-de Bruin, K. and Lumey, L. H. (2006) Exposure to famine during gestation, size at birth, and blood pressure at age 59 y: evidence from the Dutch Famine. Eur. J. Epidemiol. **21**, 759–765
22 Aihie Sayer, A., Dunn, R., Langley-Evans, S. and Cooper, C. (2001) Prenatal exposure to a maternal low protein diet shortens life span in rats. Gerontology **47**, 9–14
23 Cheema, K. K., Dent, M. R., Saini, H. K., Aroutiounova, N. and Tappia, P. S. (2005) Prenatal exposure to maternal undernutrition induces adult cardiac dysfunction. Br. J. Nutr. **93**, 471–477
24 Libby, P. (2002) Inflammation in atherosclerosis. Nature **420**, 868–874
25 Lusis, A. J. (2000) Atherosclerosis. Nature **407**, 233–241
26 Bjorkerud, S. and Bjorkerud, B. (1996) Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. Am. J. Pathol. **149**, 367–380
27 Iyemere, V. P., Proudfoot, D., Weissberg, P. L. and Shanahan, C. M. (2006) Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. J. Intern. Med. **260**, 192–210
28 Davies, M. J., Richardson, P. D., Woolf, N., Katz, D. R. and Mann, J. (1993) Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. Br. Heart J. **69**, 377–381
29 Mahmoudi, M., Gorenne, I., Mercer, J., Figg, N., Littlewood, T. and Bennett, M. (2008) Statins use a novel Nijmegen breakage syndrome-1-dependent pathway to accelerate DNA repair in vascular smooth muscle cells. Circ. Res. **103**, 717–725
30 Martinet, W., Knaapen, M. W., De Meyer, G. R., Herman, A. G. and Kockx, M. M. (2002) Elevated levels of oxidative DNA damage and DNA repair enzymes in human atherosclerotic plaques. Circulation **106**, 927–932
31 Goldstein, J. L. and Brown, M. S. (1973) Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. Proc. Natl. Acad. Sci. U.S.A. **70**, 2804–2808
32 Shanmugam, N., Roman-Rego, A., Ong, P. and Kaski, J. C. (2010) Atherosclerotic plaque regression: fact or fiction? Cardiovasc. Drugs Ther. **24**, 311–317
33 Morales-Villegas, E. C., Di Sciascio, G. and Briguori, C. (2011) Statins: cardiovascular risk reduction in percutaneous coronary intervention-basic and clinical evidence of hyperacute use of statins. Int. J. Hypertens. **2011**, 904742
34 Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Lorimer, A. R., MacFarlane, P. W., McKillop, J. H. and Packard, C. J. (1995) Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N. Engl. J. Med. **333**, 1301–1307

35 Pedersen, T. R., Kjekshus, J., Berg, K., Haghfelt, T., Faergeman, O., Faergeman, G., Pyorala, K., Miettinen, T., Wilhelmsen, L., Olsson, A. G. and Wedel, H. (2004) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Atheroscler. Suppl. **5**, 81–87

36 Alonso, R., Mata, N., Castillo, S., Fuentes, F., Saenz, P., Muniz, O., Galiana, J., Figueras, R., Diaz, J. L., Gomez-Enterria, P. et al. (2008) Cardiovascular disease in familial hypercholesterolaemia: influence of low-density lipoprotein receptor mutation type and classic risk factors. Atherosclerosis. **200**, 315–321

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