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Effects of maternal high-fat diet and statin treatment on bone marrow endothelial progenitor cells in female offsprings fed a similar diet

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2. Abbreviations: EPCs, Endothelial progenitor cells, HF, high fat, HMG-Co-A, 3-hydroxy-3-methylglutaryl-coenzyme A

ABSTRACT

Background: Maternal high-fat (HF) and cholesterol-rich diets increase cardiovascular disease risk in mothers and offsprings, and treatment with statins reduce this risk.

Objectives: We hypothesize that one possible statin-related protective mechanism in pregnant mothers and offsprings fed on HF diet is the reduction in impairment of the vasculogenic element of endothelial regeneration.

Methods: To explore this, virgin C57BL/6 mice (n = 8/group) were fed HF diet (fat- 45% kcal) or standard chow (C; fat- 21% kcal) from weaning and throughout their pregnancy and lactation. Half of the HF group was also given the HMG-Co-A reductase inhibitor pravastatin (S) through their drinking water (5 mg/kg body weight per day) to create HF+S dam group. Offspring from each group were fed HF or C diets from weaning to adulthood, generating respective dam/offspring dietary groups (C/C, HF/HF, HF+S/HF). Body weight, blood pressure and serum lipid profile were measured in offspring at 24 weeks of age, and bone marrow endothelial progenitor cells (EPCs) were cultured.

Results: The results indicate that in the female offsprings, the statin-fed (HF+S/HF) cohort had lower total and low-density lipoprotein cholesterol concentrations, were less obese and hypertensive and showed increased bone marrow EPCs expressing colony numbers ($P < 0.001$) as compared with the HF/HF phenotype.

Conclusions: Our results demonstrate that statin administration in early life to dams fed a HF diet had a significant impact on their female offspring in terms of reduction in cardiovascular risk factors. In addition, statin administration to female offsprings on HF diet during early life was associated with

reduction in circulating C-reactive proteins (CRPs) and an increased bone marrow EPC numbers and colony-forming characteristics.

KEYWORDS: blood pressure, cardiovascular dysfunction, developmental programming, HMG-CoA reductase inhibition, endothelial progenitor cells, C-reactive proteins

INTRODUCTION

Endothelial dysfunction is a common phenomenon that occurs in the metabolic syndrome (1, 2). Evidence suggests that diabetes and hyperlipidemia leads to reduced circulation of blood and bone marrow-derived mononuclear cells, i.e. endothelial progenitor cells (EPCs), thus resulting in endothelial cell dysfunction (3-6). Emerging reports also suggest endothelial dysfunction (i.e. reduced EPC) as a common phenotype in a number of rodent models of both maternal HF and total nutrient restriction in the context of developmental origins of health and disease (DOHaD) models (7, 8).

In response to marked morphological changes in the surrounding mature endothelial cells, EPCs play a critical role in maintaining endothelial function in mature blood vessels by contributing to re-endothelialisation and neovascularisation (9, 10). It is therefore conceivable that the mobilization and differentiation of EPCs are important in this process of adult neovascularization (11-13) and any impairment of this vasculogenic element of endothelial regeneration may account for the progression of endothelial dysfunction (13).

Accumulating evidence also suggests that the number and migratory activity of circulating EPCs inversely correlate with circulating C-reactive protein (CRP) levels (4, 14). The combination of reduced circulating EPCs and increased circulating CRP is known to be associated with metabolic syndrome and high LDL cholesterol (15, 16). Reports have advocated that CRP down-regulates endothelial nitric oxide synthase (eNOS) in a synchronous fashion and destabilizes eNOS mRNA transcription, decreases both basal and stimulated nitric oxide (NO) release (17), up-regulates nuclear factor kappa-B (NF-κB), a key nuclear factor that facilitates the transcription of numerous pro-atherosclerotic genes) (18), and mediates adhesion molecules and LDL uptake (19, 20).

These interrelations among LDL cholesterol, CRP and bone marrow EPC, suggest that CRP-related alteration in progenitor cell number and function in offspring may be induced by maternal HF consumption. Also it would be interesting to study, through net reduction effect on maternal dyslipidemia load, whether statin therapy in dams exerts any advantageous effect on bone marrow EPC in their offspring fed a similar high-fat diet. These hypotheses are supported by several experimental models and *in vivo* studies on ischemic disease patients (21, 22). Although, the precise mechanisms remain unclear, it is known that statin improves endothelial function by activating protein kinase Akt (21), mobilizing EPC (23), reducing senescence, and increasing proliferation of EPC (24).

A major area of DOHaD research provides a novel explanation on disturbances in the maternal metabolism resulting from altered nutrient supply in the mother, a trait that is transmitted to the fetus in the form of structural and functional adaptations during fetal development and throughout life (25-28). Consequently, we developed a mice model (29, 30) for studying the effect of altered maternal nutrients supply in the mother transmitted to the fetus during development. In these studies (29, 30) we reported that statin administration during the second half of pregnancy and lactation in dams consuming a HF

diet reduces metabolic risk factors not only in dams but also in their offspring (31). These favorable effects were more prominent in the offspring of HF mothers who had statin treatment at the time they were weaned, during pregnancy and lactation (31). However, it is not clear what the possible underlying mechanism is. We hypothesize that one such possible protective mechanism in pregnant mothers and offsprings fed on HF diet is the reduction in impairment of the vasculogenic element of endothelial regeneration. We believe that statins reduce the levels of circulating CRPs that may be linked with the pathological mechanisms that regulate bone marrow EPC numbers and their colony-forming properties. Our study was designed to compare the effect of statin with that of other hypercholesterolemic drugs and to use an experimental design that minimizes the differences in body weight and other parameters induced by a high-fat diet before pregnancy as well as during lactation as described in more detail in our previous publications (29-31). In brief, using this model we had previously explored the impact of long-term consumption of a high-fat diet by pregnant mothers. The results indicated that high-fat diet in pregnant mothers predisposes her female offspring to developing a metabolic syndrome-like phenotype in adult life as indicated by chronic elevation of serum cholesterol and blood pressure. In the follow-on study (30) using the same model, we observed that offsprings from high-fat fed dams showed increased adiposity in their femurs in comparison to offsprings from mothers fed standard chow. In particular, female offsprings from mothers fed a high-fat diet exhibited altered trabecular structure, indicative of *in utero* programming. However, the scope covered by all our studies was limited by the availability of funding and other resources, and hence the focus of this study was on metabolic syndrome-like outcomes. However, the use of this type of model to test the effect of treatment with statins does not find direct relevance to clinical practice in view of the existing controversy concerning the potential high risk of teratogenicity to the unborn fetus (32).

MATERIALS AND METHODS

Experimental Protocol

Study part I

The aim of this protocol is to resolve the question whether statins when administered to pregnant mothers on a high-fat diet has any effect on the mechanisms of endothelial regeneration. All of the animal procedures were humane and carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and the study protocol was approved by the University of Southampton Animal Care and Research Ethics Committee. The experimental protocol for study-I is as described on figure 1. Female C57BL/6 mice (Charles River Laboratories, United Kingdom) were maintained under a 12-hour light-dark cycle at constant temperature (25 $\pm$ 2 °C) with food and water supplied *ad libitum*. At 4 weeks of age, the females were randomly allocated to either a control diet of standard laboratory chow (C; 5.3% fat [corn oil], 21.2% protein, 49.2% carbohydrate; Special Diet Services, United Kingdom) or an HF experimental diet (29-31) [2] supplemented with 18% weight/weight animal lard, with additional vitamins and minerals, protein, and choline to correct for the dilution (final composition in percentage of grams [weight/weight]: lard 17.8; casein 26.5; choline chloride 0.3; L-cysteine 0.4; rice starch 28.3; cellulose 6.1; soya oil 4.3; sucrose 10.4; minerals 4.3 and vitamins 1.2; Special Diet Services diet 824053). This HF diet has been used in previous studies (33, 34). At 10 weeks old, the females were time mated and after confirmation of mating (i.e. presence of vaginal plug), were individually housed. From the second half of the pregnancy and throughout lactation, half of the pregnant females on the HF diet were given a water-soluble 3-hydroxy3-

[2] The composition of the diets has been published in the following citations:

29. Elahi MM et al., Hypertension 2008; 51 (4): 939-44
30. Elahi MM et al., Br J Nutr 2009; 102 (4): 514-9
31. Elahi MM et al., Ann Nutr Metab 2013; 62 (3): 250-6

methylglutaryl-coenzyme A reductase inhibitor (pravastatin, Sigma United Kingdom) in their drinking water (HF+S). Pravastatin was dissolved at a concentration that gave a daily dose of 5 mg/kg per day, based on the daily water consumption of pregnant and lactating mice predetermined from our previous study (33). The pregnant dams were allowed to give birth, and the pups were weighed, and litter size was standardized to 8 pups. After weaning (3 weeks postpartum), all female offsprings whose mothers had been fed on diets of C, HF and HF+S, were themselves randomly allocated to be fed on either C, or HF. This generated mother and daughter dietary combinations of C/C, C/HF, HF/HF, HF/C; HF+S/HF and HF+S/C. The female offsprings were monitored until 24 weeks of age in terms of body weights (from 1 week of age onwards to avoid maternal rejection of the pups) and food intake (from weaning). Systolic blood pressure (SBP), biochemical markers (total, LDL and HDL cholesterol) and CRP, and colonies of the bone marrow mononuclear cells were measured up till 24 weeks.

Study part II

In this part of the study, 50% of the HF-fed females were given the water-soluble Pravastatin (Sigma UK; 5 mg/kg/day) in their drinking water. These females were fed the assigned diet and treated with statin through weaning to pregnancy and lactation. After birth, the pups were weighed and litter size was reduced to 8 female pups. From weaning (21 days postpartum), offsprings from the HF and HF+ S dams were fed the same HF diet, and were referred to as the HF/HF and HF+S/HF dietary groups, respectively. Offsprings from the dams fed on the same diet post-weaning were referred to as the C/C group (Figure 1). Colonies of bone marrow-derived mononuclear cells were measured up until 24 weeks.

Tail cuff plethysmography

Systolic arterial pressure was measured by tail cuff plethysmography, as we described previously (29-31). In brief, measurements were conducted in a heated room (34 °C) to get optimal blood pressure (BP) readings and were conducted at the same time during the day (afternoon). All of the animals were accustomed to the procedure for 7 days before each BP measurement session. At least 5 readings were taken from each animal per session with the highest and lowest readings discarded, and the remaining readings were averaged to get a single session value. We took the average BP values from 8 female offspring picked randomly from each of the 8 litters in each treatment group. Tail cuff method has served a valuable role in experimental hypertension research for many years and continues to be useful in study designs. We specifically used this method as it shares some advantages: (1) It is non-invasive and does not require general anaesthesia and surgery; (2) it can be used to obtain repeated measurements of SBP in conscious animals during studies of short or long duration; (3) It requires less expensive equipment than the alternative of radio-telemetry and is also less expensive to operate; and (4) it can be used to screen for systolic hypertension or substantial differences in SBP among large numbers of animals.

Serum lipid profile measurements

A blood sample was drawn by direct heart puncture after anesthetizing the animal with isofluorane and cervical dislocation. We sampled 8 female offspring picked randomly from each of the 8 litters in each treatment group. Total cholesterol, low-density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL) cholesterol in the serum were measured with commercially available kits (Vitros Products) using enzymatic methods and reflectance spectrophotometry, as reported previously (29, 30).

C-reactive protein (CRP) measurement

CRP was measured using a quantitative sensitive double-antibody sandwich enzyme-linked immunosorbent assay. In this assay anti-CRP antibodies were coated to the surfaces of polystyrene microtiter wells. Plasma samples were added and incubated at 37 ^{0}C for 2 hours. After washing, to remove unbound proteins, anti-mouse CRP antibodies conjugated with horseradish peroxidase (HRP) were added. These enzyme-labelled antibodies form complexes with the previously plate-bound CRPs. After another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme is proportional to the concentration of CRPs; thus, the absorbance, at 450 nm, is a measure of the concentration of CRPs in the test sample. The quantity of CRPs in the test sample was interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

Culture and staining

Bone marrow from mice femurs were collected into tubes containing buffered saline (HANKS). 6 ml of diluted sample was layered over 3 ml Lymphoprep in a 15 ml centrifuge tube, centrifuged at 800 x g for 20 minutes at room temperature (approximately 20 ºC). Samples were spun in wells (Lab-Tek ™ II Chamber slide) containing 400 µL of Dulbecco's modified Eagle medium (D-MEM/F-12). Once the centrifugation process was over, mononuclear cells from the interphase were transferred to 15 ml centrifuge tubes containing 10 ml phosphate buffered saline (PBS). The cell suspensions were centrifuged at least twice at 200 g for 5 min at room temperature (25 ºC) with brakes. The supernatants were removed and cells suspended in 200 µL PBS were plated into incubation slides and incubated for an hour at 37 ºC. After an hour, 200 µl of fresh D-MEM/F-12 media were added to each well of the slide and incubated for 48-96 hours. Viable cells were counted using Trypan blue staining (0.4% stock solution).

197

198 **Detection of bone marrow EPCs with double-staining**

199 To each well containing 500 µl of cell suspensions, 25 µl of acetylated low density lipoprotein labelled
200 with 1,1'-deoctadecyl-3,3,3',3'-tetramethyllindo-carbocyanine perchlorate (Dil-Ac-LDL) stain was
201 added and gently mixed once or twice and then the slide was incubated for 4 h at 37 ºC. After 4 h, the
202 chamber slides were examined under the microscope with green filter for red staining (RITC). Following
203 this step, the media from the wells were removed, and the cells were gently washed with PBS three
204 times. Cells were then fixed in 500 µl/well of Paraformaldehyde (3.7% in PBS) on ice for 30 min. After
205 the fixation stage, cells were incubated in 200 µL *Ulex Europaeus* lectin (UEA) solution made up of 10
206 µl of UEA suspended in 990 µl of PBS (stock 1mg/ml) for 1 h at 37 ºC without CO_2 and in the dark. The
207 cells were then gently washed up to 3 times every 5 min in the dark using PBS. The slides were mounted
208 using citifluor and were examined under a microscope using UV purple lens and an inverted coverslip
209 containing a drop of fluid composed of 90% glycerol and 10% PBS.

210

211 **Phase contrast and fluorescence imaging**

212 Phase contrast and fluorescence images were collected using a Zeiss Axiovert 2 inverted microscope
213 with a 5x CP-ACHROMAT/0.12 NA objective. Images were acquired using a SPOT RT colour camera
214 (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer's software. Composite images were
215 assembled in Adobe Photoshop version 8.0.

216

217 **Data analysis**

218 The biochemical and biophysical parameters in the dams were analysed using one-way analysis of
219 variance followed by the Tukey–Kramer comparisons test. All data are expressed as mean values with

their standard errors. P-values < 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS 14.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

The effect of prenatal and postnatal high-fat diet consumption on risk factors for cardiovascular disease in female offsprings

Females offsprings with a dietary profile history of C/HF, HF/C and HF/HF were heavier (Figure 2A), had significantly higher systolic blood pressure (Figure 2B), with increased serum levels of LDL-cholesterol (Figure 2C) and CRP (Figure 2D) than C/C offspring at 24 weeks. Total cholesterol levels were significantly increased in female offsprings on a diet history of HF/C and HF/HF compared with control C/C at 24 weeks. However, the differences in total cholesterol were not observed in female offsprings on a diet history of C/HF as compared with the control C/C diet group (Figure 2E). Short-term statin therapy (second half of pregnancy and lactation) in mothers on prenatal HF diet did not affect systolic blood pressure in female offsprings (Figure 2B). In contrast, short-term pravastatin therapy in mothers given HF diet during pregnancy significantly reduced bodyweight and LDL-cholesterol in female offsprings whether or not they were on the postnatal HF diet. In addition, pravastatin therapy reduced the levels of CRP to negligible in HF+S/C and HF+S/HF female offsprings.

The effect of prenatal and postnatal HF diet consumption on bone marrow mononuclear EPCs in female offsprings

HF diet (prenatal or postnatal) significantly reduced percentage of positively stained bone marrow mononuclear cells, decreased the number of double stained colonies and inhibited the expression of acetylated low-density lipoprotein (Figure 3). Pravastatin treatment to these hypercholesterolemic dams significantly improved and increased the number of bone marrow EPC observed in the culture. Representative photomicrographs of bone marrow EPC colonies stained for endothelial markers Dil-Ac-LDL (red) and lectin (green) are shown in figure 4.

The relationship of hypercholesterolemia with bone marrow mononuclear EPC numbers

Bone marrow mononuclear EPC numbers were inversely correlated to the total cholesterol values (Figure 5A) and LDL-cholesterol (Figure 5B) levels. In contrast, there was no strong correlation between the number of bone marrow mononuclear EPCs and HDL-cholesterol levels ($r = 0.237$, $P > 0.05$). Total cholesterol (standard coefficient = −0.530, $P < 0.001$) and LDL cholesterol (standard coefficient = −0.417, $P < 0.01$) were both independently correlated with lower bone marrow EPC numbers.

The effect of pravastatin treatment to pregnant dams fed a HF diet on bone marrow EPC number and colonies in female offsprings after a post-weaning HF diet

As summarised in figure 6, for female offsprings HF/HF dietary history significantly inhibits bone marrow mononuclear EPC numbers and colonies, thereby affecting key components of angiogenesis and endothelial repair. In contrast, in dams fed on a HF diet, pravastatin treatment started in early life

and continued right through pregnancy appears to protect their female offsprings from HF diet-induced depletion of bone marrow mononuclear EPC numbers and colonies (Figure 6).

DISCUSSION

The present study investigates whether long-term maternal HF diet has an impact on the expression of bone marrow endothelial progenitor cells (double stained endothelial progenitors, EPCs) in their female offsprings even if they were fed a HF or C diet right through their lives, i.e. to study the role of prenatal and postnatal diet on EPCs. To understand potential underlying mechanisms, we studied both the short-term (second half of pregnancy and lactation) and long-term effects (soon after weaning through to pregnancy and lactation) on bone marrow mononuclear EPC numbers and colonies in female offsprings from mothers on a high-fat diet treated with or without pravastatin.

The results demonstrate that: (1) bone marrow EPC numbers and expression in female offspring exposed prenatally or postnatally (C/HF, HF/HF and HF/C) to the HF diet are significantly decreased; (2) treating dams with Pravastatin (in both study protocols) proves beneficial for improving bone marrow EPC colony forming units in their offspring irrespective of their postnatal diet; (3) number of bone marrow EPC is inversely correlated with total cholesterol and LDL-cholesterol levels (4) and LDL-cholesterol is a predictor of bone marrow EPC expression. To date, it has been suggested that statins mobilize EPCs independent of their cholesterol lowering effects. Indeed, findings from our work also support the hypothesis that there is a relationship between statins and improvement in bone marrow EPC numbers in female offsprings with a history of prenatal and postnatal HF+S/HF diet. The effect may be related to the abrogation of CRP-induced inflammation and improvement in the cholesterol profile. Although previous studies have reported the role of statin in improving

287 angiogenesis (21, 22), this had not been observed in studies before in the context of the DOHaD
288 phenomenon.
289
290 Studies and laboratory evidence have identified that EPCs participate in postnatal neovascularization
291 and re-endothelialization (6-37). In, this study we have observed that hypercholesterolemia can
292 decrease bone marrow EPC number and activity. Given the well-established role of EPCs in
293 neovascularisation and re-endothelization, our findings may have identified a possible
294 pathophysiological mechanism related to hypercholesterolemia, i.e., hypercholesterolemia not only
295 impairs endothelial cells directly, but also affects bone marrow EPC numbers and function at the same
296 time. Thus hypercholesterolemia may influence the endothelial repair process and alter the balance
297 between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction,
298 and promote the early progression of coronary artery disease (CAD) in adult offspring.
299
300 On the other hand statins have been developed as lipid-lowering drugs, and are well established to
301 reduce morbidity and mortality from CAD (38). Besides lipid lowering, primary and secondary
302 prevention trials and laboratory investigations established that statins possess favourable effects
303 independent of cholesterol reduction (4, 39). In particular, statins have recently been reported to
304 promote EPC proliferation, migration and cell survival *in vitro* (39-41). A recently performed clinical
305 study demonstrated an increase in EPC number with enhanced migratory activity by statin treatment in
306 patients with stable CAD (40). Results of this work, together with the findings of other investigators
307 suggested a possible mechanism of action for statins in the augmentation and promotion of EPC
308 functional activity.
309

More recently, two groups have documented in animals and human subjects that EPCs contribute up to 25% of endothelial cells in newly formed vessels (41-43). Thus, increasing the number of circulating EPCs by transplantation of hematopoietic stem cells or by injection of *in vitro*-differentiated EPCs has been shown to improve neovascularization of ischemic hind-limbs, accelerate blood flow in diabetic mice (6) and improve cardiac function (36). More importantly, reports suggest that patients with CAD reveal reduced levels and functional impairment of EPCs which correlates with risk factors of CAD (33, 34). One may wonder what would be the mechanisms. It might be due to increased apoptosis of premature progenitor cells or oxidised LDL known to induce apoptotic cell death. It may well be hypercholesterolemia that could interfere with the signalling pathways regulating EPC differentiation or mobilization. The mechanistic effects of statins on EPC in such settings may be related to their impact on increased regional blood perfusion most probably mediated by increased production of endothelial NO (44, 45), or induced EPC differentiation by reducing CRP-mediated inflammation (40-42). Further work is therefore needed to elucidate the underlying mechanisms that may explain why and how high-fat diets have a deleterious effects and cause functional impairment of bone marrow EPCs in mothers and their female offsprings.

CONCLUSION

In conclusion, our work has demonstrated that maternal hypercholesterolemia is associated with reduction in bone marrow EPC numbers and differentiation. It has been suggested that statins mobilize EPCs independent of their cholesterol lowering effects. However, evidence from our work suggests that there is a possible relationship between statins and improvement in bone marrow EPC numbers which may be related to the combination of the abrogation of CRP-induced inflammation and improvement in the cholesterol profile. This may suggest a potential important direction for future

investigations in the developmental origins of CVD. Such studies will expand our understanding of the underlying pathophysiology.

There are some limitations of our work. It is difficult to deduce from our findings that the decrease in the number of bone marrow EPCs in HF/HF is directly influenced by the maternal diet and thus contribute to the defect in postnatal vascular response, i.e. bone marrow EPCs mediated mobilization and endothelial function. There seems to be a discrepancy between the effects statin treatment has in HF/HF regarding blood pressure on the one hand (no effect), and lipids and bone marrow EPC numbers on the other. Whether statins alter endothelial cell phenotype in these animals is not clear. This is an important consideration since mice do not generally develop overt vascular pathology (e.g. atherosclerosis), even with severe obesity models like a high-fat diet. This might point at a different mode of action for blood pressure regulation that deserves further investigations and this might have relevance to understanding of the underlying pathophysiology. Indeed, it is not known whether different target organs are affected (liver, kidney, and vasculature), a question that requires further investigations. In this study, we harvested mononuclear cells from the bone-marrow and not from the peripheral blood. We acknowledge that there are a host of other mechanisms that regulate the entry of bone marrow EPCs into the peripheral blood circulation and therefore levels in bone marrow aspirates may not truly reflect this. Preferably, measurement of levels of circulating EPCs may better provide information about the ability of these cells to translocate to areas within the circulation where vascular repair is needed. In addition, it would have been advantageous to conduct fluorescence-activated cell sorting (FACS) for cultured mononuclear cells to evaluate whether these cells progressed to an EPC-like phenotype rather than relying on the double staining alone to identify them. Our work was limited by shortage of funding and other research resources, and hence we mainly focused on the effect of the high-fat diet and treatment with statins on

endothelial progenitor cells. We acknowledge that there were large quantities of other types of bone marrow cells such as CD14 that could have been investigated more thoroughly.

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STATEMENT OF AUTHORS' CONTRIBUTIONS TO MANUSCRIPT:

- designed research (project conception, development of overall research plan, and study oversight) – ME, BM
- conducted research (hands-on conduct of the experiments and data collection - ME
- provided essential reagents, or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc., necessary for the research) - ME
- analyzed data or performed statistical analysis – ME, BM
- wrote paper (only authors who made a major contribution) – ME, BM
- had primary responsibility for final content - ME
- All authors have read and approved the final manuscript - ME, BM

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FIGURE LEGENDS:

Figure 1: Flow diagram of the experimental protocol.

Figure 2: Comparison of body weight gain (A), blood pressure (B), LDL (C), CRP (D) and cholesterol (E) levels in female offspring (at 24 weeks of age) from control-fed mothers that were then fed a chow diet (C/C) or high-fat diet (C/HF) and from high-fat-fed mothers that were then fed a high-fat diet (HF/HF) or a chow diet (HF/C) and from high-fat-statin fed mothers that were then fed a high-fat diet (HF+S/HF) or a chow diet (HF+S/C). Values are means (n = 8 per group), with standard errors represented by vertical bars, a, b, c and d. Mean values with dissimilar lettering indicated significant differences ($P < 0{\cdot}05$; Tukey–Kramer comparisons test). Statin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on the body weight, blood pressure, LDL levels, CRP levels and cholesterol profile in their offspring.

Figure 3: Comparison of positively stained bone marrow mononuclear cells described as bone marrow endothelial progenitor cells (EPCs) (A) and number of double stained colonies (per 10^6 cells) (B) in female offspring (at 24 weeks of age) from control-fed mothers that were then fed a chow diet (C/C) or high-fat diet (C/HF) and from high-fat-fed mothers that were then fed a high-fat diet (HF/HF) or a chow diet (HF/C) and from high-fat-statin fed mothers that were then fed a high-fat diet (HF+S/HF) or a chow diet (HF+S/C). Values are means (n = 8 per group), with standard errors represented by vertical bars. a, b, c and d. Mean values with dissimilar lettering indicated significant differences ($P < 0{\cdot}05$; Tukey–Kramer comparisons test). Pravastatin treatment in hypercholesterolemic mothers during late

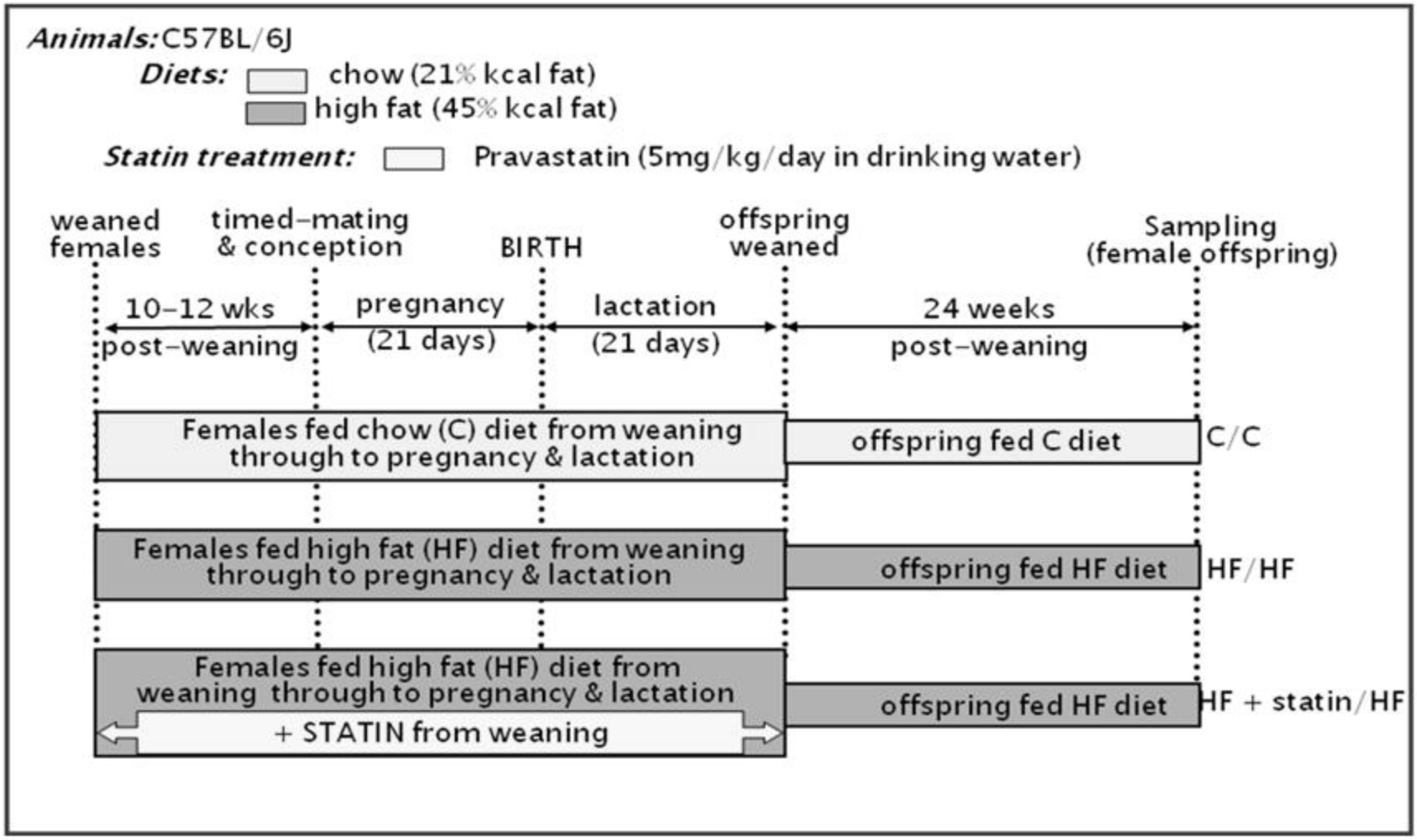
pregnancy and lactation has beneficial effects on the positively stained bone marrow EPCs and the number of double stained colonies in offsprings from mothers on high fat-high cholesterol (HF) diet.

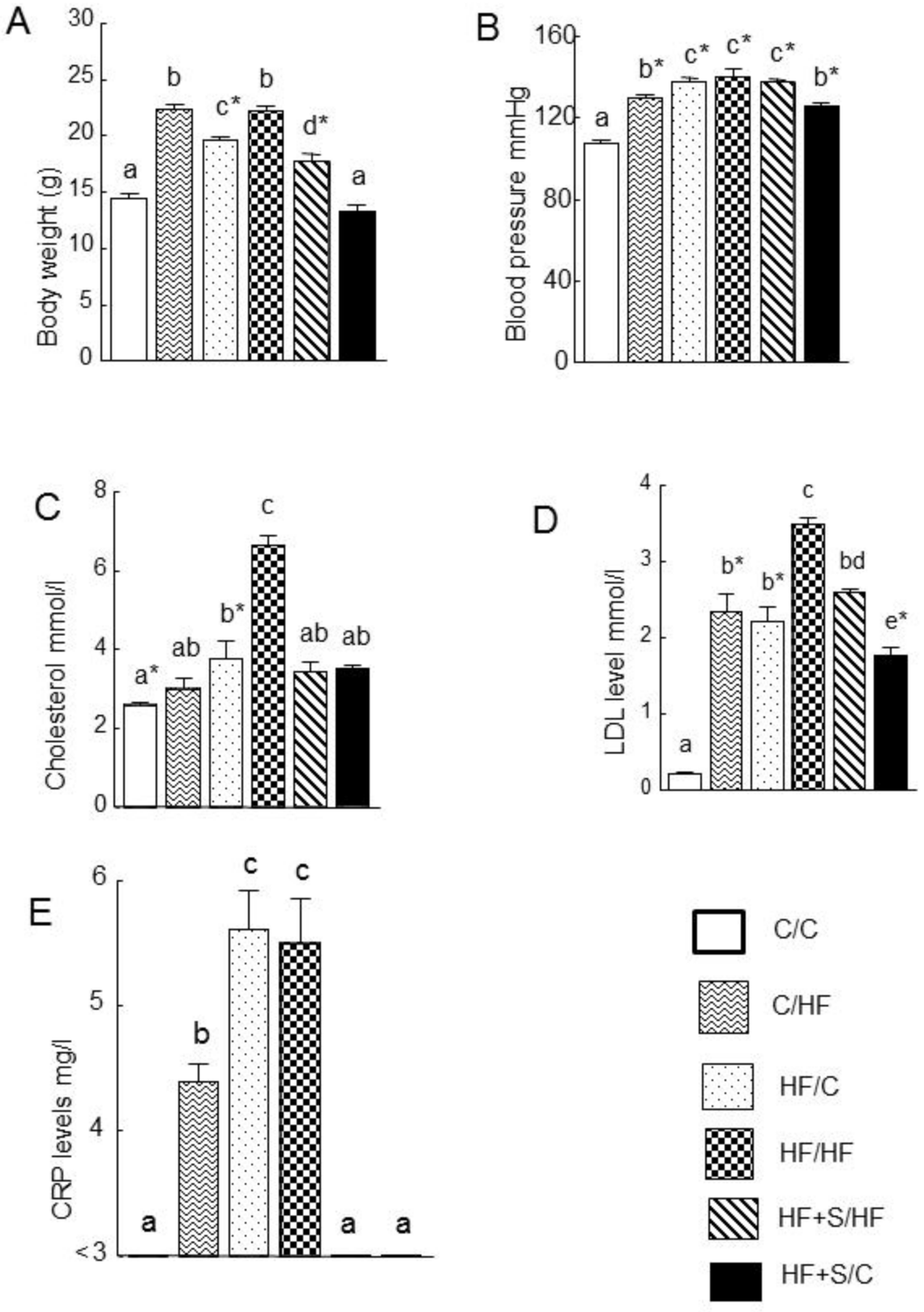
Figure 4: Expression of endothelial markers on bone marrow mononuclear EPCs. Representative photomicrographs of EPC colonies stained for endothelial markers Dil-Ac-LDL (red) and lectin (green). EPC colonies demonstrate reduced staining in HF/HF vs C/C. Statin treatment to HF-fed dams abolished these effects in their offspring. Measurements were made at 24 weeks of age

Figure 5: Correlation between the number of bone marrow EPCs from HF/HF offspring with hypercholesterolemia and total cholesterol (A) and LDL-cholesterol (B) levels. (Age = 24 weeks)

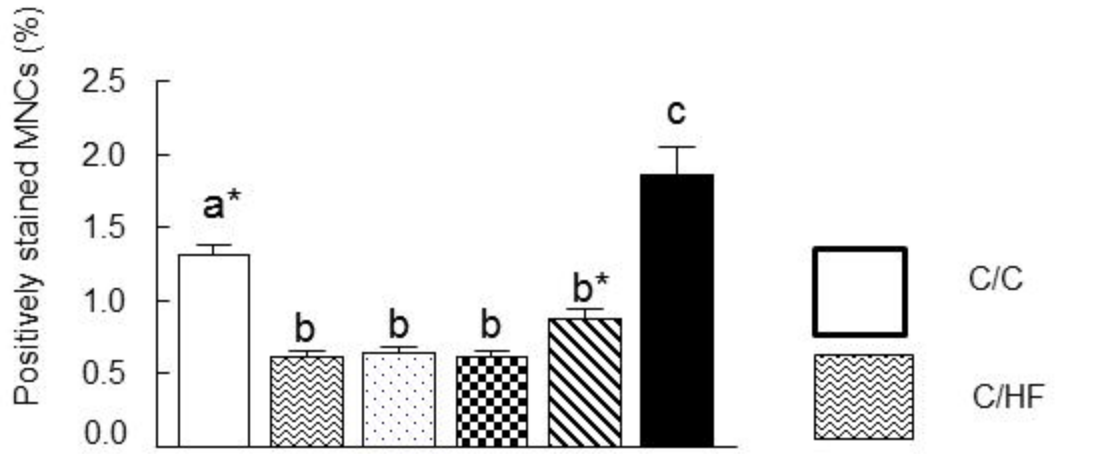
Figure 6: The effect of long-term statin treatment in hypercholesterolemic mothers on bone marrow EPCs: (A) number of stained bone marrow EPC colonies (B) percentage of mononuclear cells that are Fluorescein Isothiocyanate (FITC) labelled. Weaned offsprings were then fed either HF or C diets through adulthood, thus generating the dam/offspring dietary groups of HF/HF, HF-S/HF and C/C (n = 8 per group; Age = 24 weeks).

Figure 1:


Animals: C57BL/6J
Diets: chow (21% kcal fat)
high fat (45% kcal fat)
Statin treatment: Pravastatin (5mg/kg/day in drinking water)
weaned females
timed-mating & conception
BIRTH
offspring weaned
Sampling (female offspring)
10–12 wks post-weaning
pregnancy (21 days)
lactation (21 days)
24 weeks post-weaning
Females fed chow (C) diet from weaning through to pregnancy & lactation
offspring fed C diet
C/C
Females fed high fat (HF) diet from weaning through to pregnancy & lactation
offspring fed HF diet
HF/HF
Females fed high fat (HF) diet from weaning through to pregnancy & lactation
+ STATIN from weaning
offspring fed HF diet
HF + statin/HF


A
Body weight (g)
30
25
20
15
10
5
0
a
b
c*
b
d*
a
B
Blood pressure mmHg
160
120
80
40
0
a
b*
c*
c*
c*
b*
C
Cholesterol mmol/l
8
6
4
2
0
a*
ab
b*
c
ab
ab
D
LDL level mmol/l
4
3
2
1
0
a
b*
b*
c
bd
e*
E
CRP levels mg/l
6
5
4
< 3
a
b
c
c
a
a
C/C
C/HF
HF/C
HF/HF
HF+S/HF
HF+S/C

A


Positively stained MNCs (%)
2.5
2.0
1.5
1.0
0.5
0.0
a*
b
b
b
b*
c
C/C
C/HF
HF/C
HF/HF
HF+S/HF
HF+S/C

B

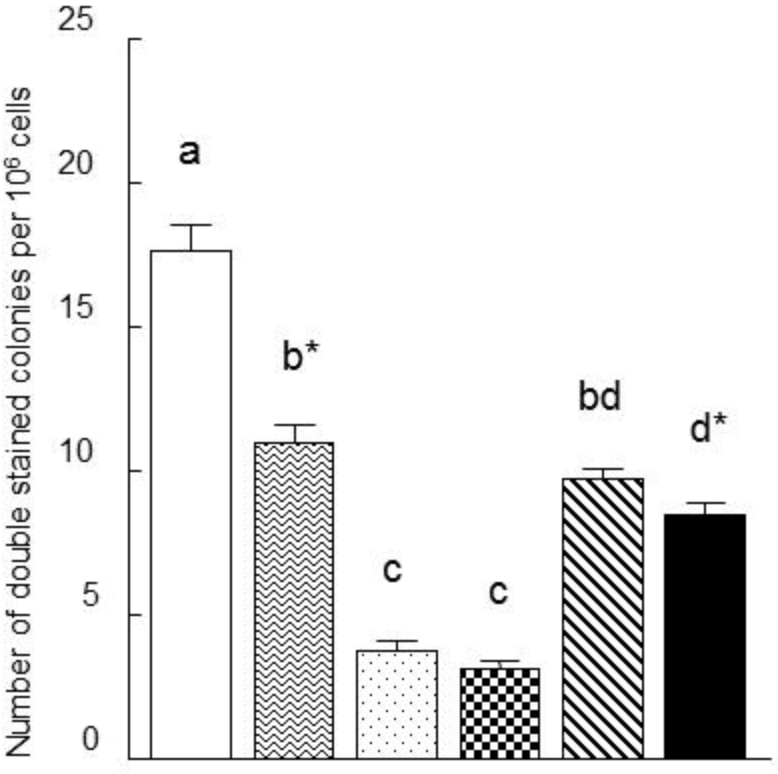

Number of double stained colonies per 10^6 cells
25
20
15
10
5
0
a
b*
c
c
bd
d*

Figure 4

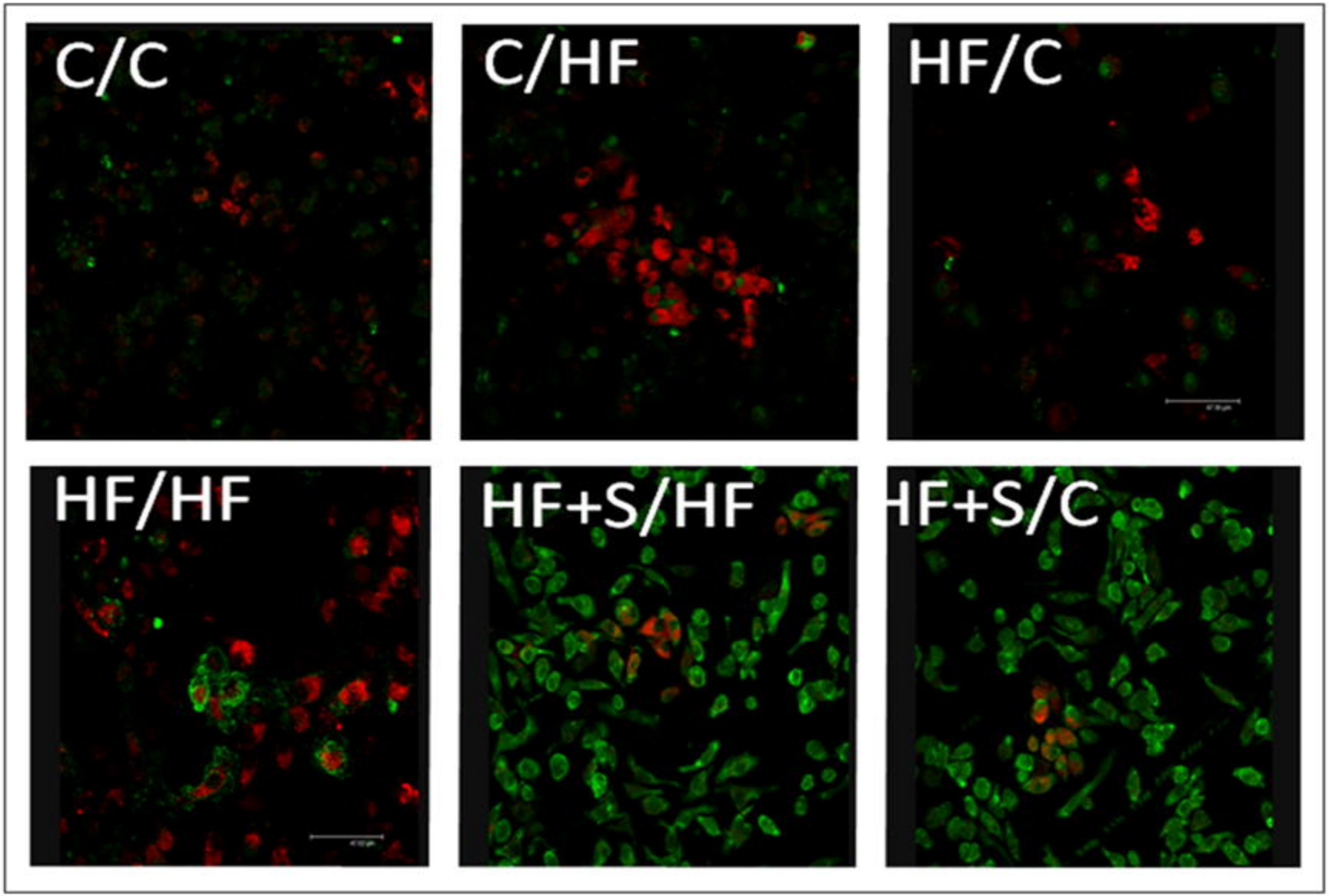


Figure 5

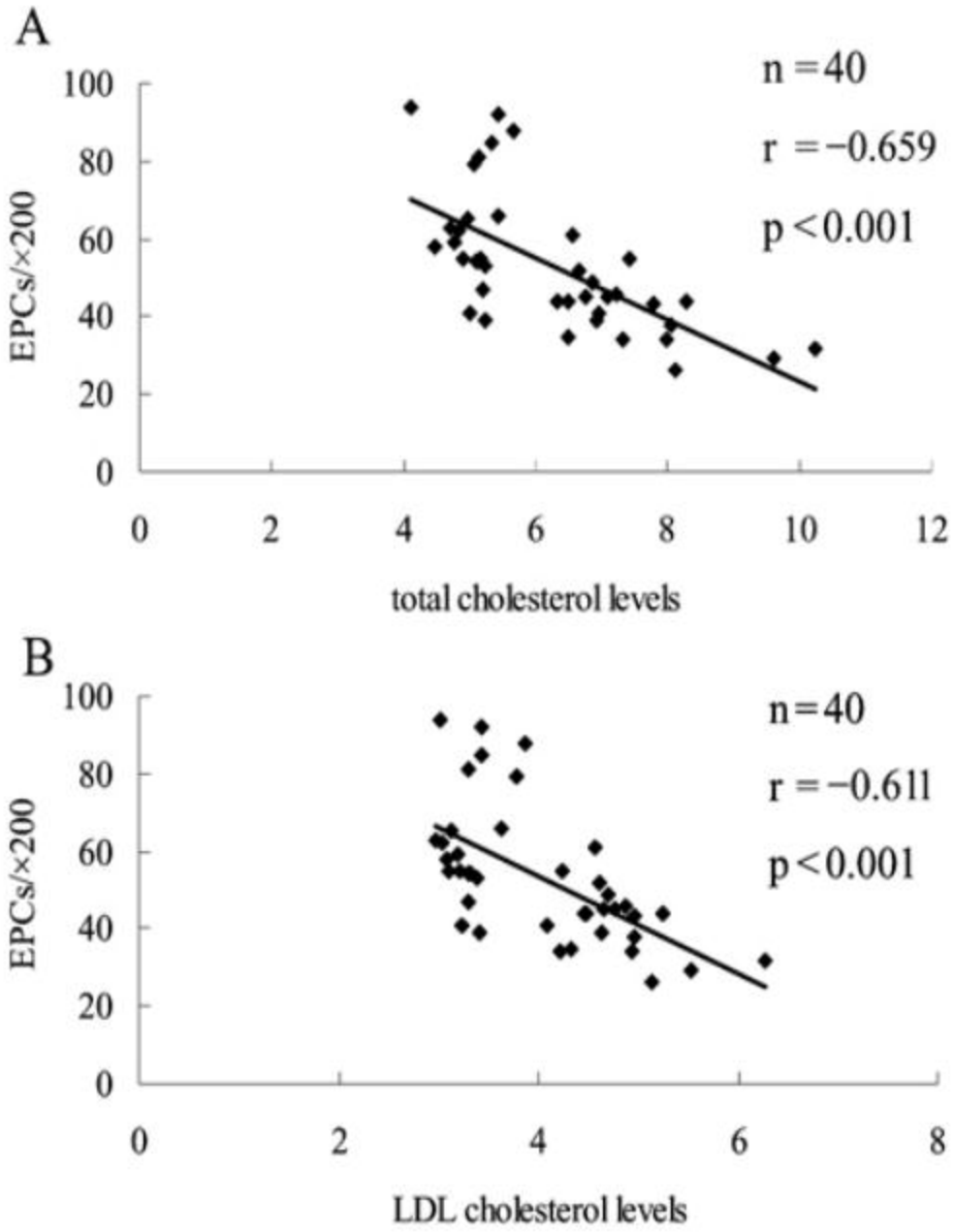
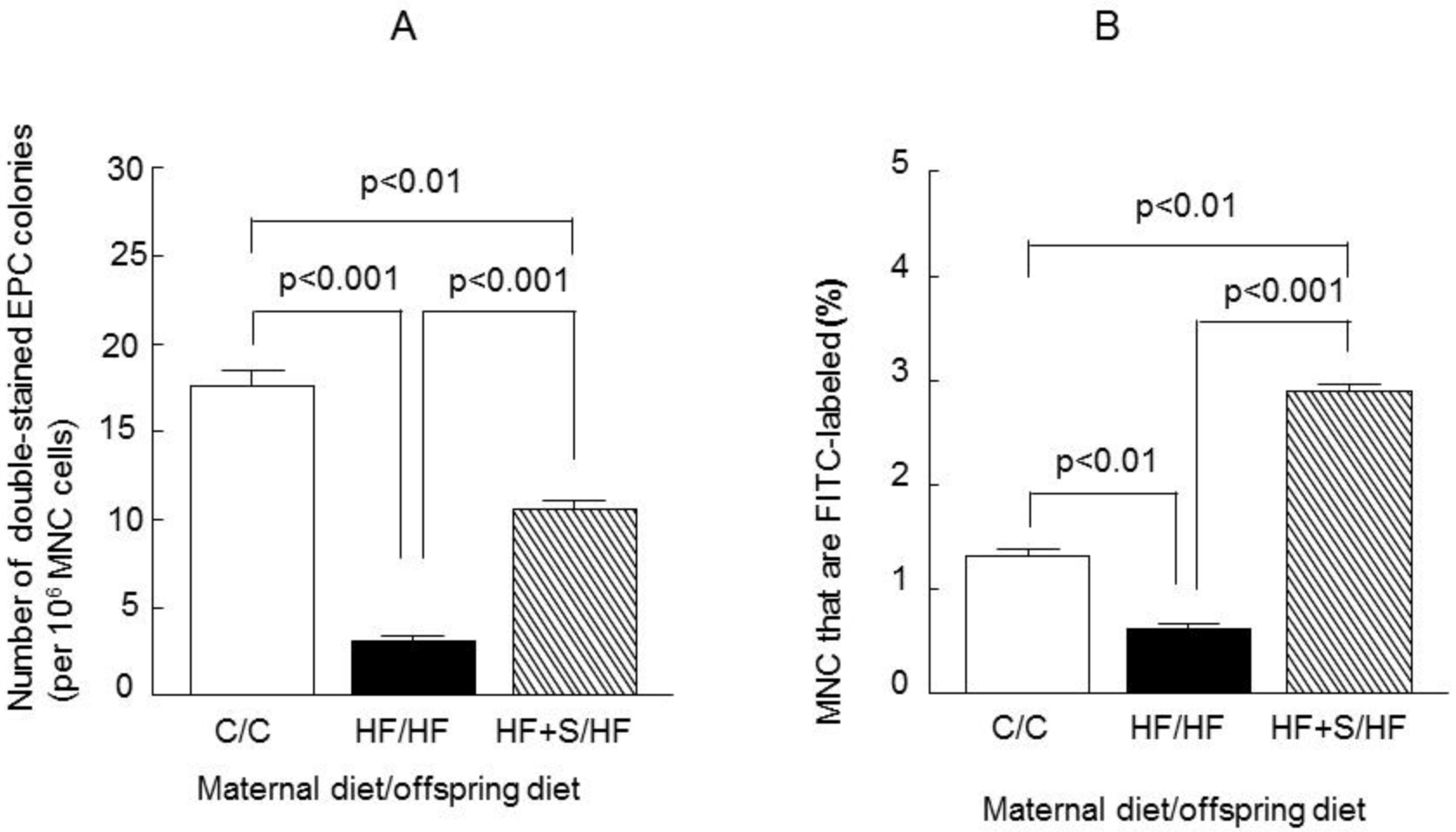

A
n =40
r =−0.659
p <0.001
EPCs/×200
total cholesterol levels
B
n=40
r =−0.611
p <0.001
EPCs/×200
LDL cholesterol levels

Figure 6


A
Number of double-stained EPC colonies (per 10^6 MNC cells)
30
25
20
15
10
5
0
p<0.01
p<0.001
p<0.001
C/C
HF/HF
HF+S/HF
Maternal diet/offspring diet
B
MNC that are FITC-labeled (%)
5
4
3
2
1
0
p<0.01
p<0.001
p<0.01
C/C
HF/HF
HF+S/HF
Maternal diet/offspring diet