European Cells and Materials Vol. 22 2011 (pages 321-332) DOI: 10.22203/eCM.v022a24 ISSN 1473-2262

# ANIMAL MODELS OF MATERNAL NUTRITION AND ALTERED OFFSPRING BONE STRUCTURE – BONE DEVELOPMENT ACROSS THE LIFECOURSE

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## Abstract

It is widely accepted that the likelihood of offspring developing heart disease, stroke, or diabetes in later life, is influenced by the their *in utero* environment and maternal nutrition. There is increasing epidemiological evidence that osteoporosis in the offspring may also be influenced by the mother's nutrition during pregnancy. This review provides evidence from a range of animal models that supports the epidemiological data; suggesting that lifelong bone development and growth in offspring is determined during gestation.

**Keywords:** Animal models, bone, bone development, epidemiology, epigenetics.

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## Introduction

Epidemiological evidence accrued over the last decade indicate that the environment and nutritional status experienced during *in utero*, postnatal as well as early life plays an important role in the aetiology of diseases such as osteoporosis (Cooper *et al*., 1997; Cooper *et al*., 2002; Jordan and Cooper, 2002). This environmental phenomenon often referred to as "programming", describes long-lasting changes in structure and function caused by environmental stimuli acting at critical periods during development. This concept, first detailed by Barker (Barker, 1992; Hales and Barker, 1992), has been informed by epidemiological studies on birth and long-term outcomes in diverse communities around the world, and by laboratory-based research looking at both specific systems and at the basic science underpinning developmental origins. Much of the work, until now, has been directed at cardiovascular and metabolic outcomes (Ozaki *et al*., 2001; Gluckman and Hanson, 2004; Gluckman *et al*., 2005; Gluckman and Hanson, 2006), however evidence is emerging that bone development and growth are programmed, although, interestingly, bone is constantly remodelled throughout life (Baird *et al*., 2011). In particular, maternal nutrition appears to be important in determining skeletal size at maturity. However, to date, there remains a paucity of understanding surrounding the cellular and, critically, molecular mechanisms whereby environmental modulation *in utero* results in an altered skeletal development among the offspring. This review will cover the emerging data from various animal models that have been used in the elucidation of the developmental origins of bone development *across the lifecourse* with correlation as available to the human scenario.

## Nutrition and Bone Development – Linking Cause and Effect

It has been known for several decades that nutrition can permanently alter growth. Furthermore, there is increasing evidence that the timing of a nutritional insult can produce differing effects on skeletal development. Widdowson and McCance (Widdowson and McCance, 1963) demonstrated that if rats were undernourished 3-6 weeks after birth, the animals lost weight compared to control animals. These nutritionally challenged animals remained smaller, even when fed control levels of food. However, when rats 9-12 weeks of age were undernourished, these animals initially lost weight, but surprisingly regained their normal growth trajectory when returned to a normal diet; so-called 'catch

up growth', indeed, their weight gain was observed to exceed the weight of the control group.

Nevertheless, it is not simply a lack of food that can alter growth. Various groups have shown that rats fed a low protein diet from weaning grew for a longer period (Reichling and German, 2000; Miller and German, 1999), but at a slower rate than control animals, and finally attained the same size as control animals. These studies demonstrate an important point that final mass at maturity can be programmed *in utero*, as post-weaning protein restriction does not affect the eventually size of the animal. Hence, there would appear to be a post-natal period where bone development (as assessed by final size and mass) is susceptible to nutritional challenge. Clinically, a long established example of such nutritional influences on bone development is Rickets, where incomplete mineralisation of the organic bone matrix arises as a consequence of poor childhood nutrition. This provides a well-established and documented example of how a post-natal period of nutrition can influence bone development. However, what is not well-established is the influence of the pre-natal maternal nutrition on bone development of the offspring.

Peak bone mass attained at skeletal maturity is a major risk factor for fracture in later life. The evidence to date suggests peak bone mass is partly inherited, however genetic markers only partially explain the differences in an individual's bone mass or fracture risk (Ralston, 1998). Epidemiological studies suggest impaired growth during fetal life, infancy and early childhood is associated with reduced adult bone mass (Cooper *et al*., 1995; Cooper *et al*., 1997; Fall *et al*., 1998; Cooper *et al*., 2002).

It is known that the longitudinal growth of long bones depends on a functional growth plate. Skeletal growth is regulated systemically by growth hormone with important contributions from glucocorticoids and thyroid hormone (Siebler *et al*., 2001). At the growth plate, all three factors converge *via* locally acting IGF-1, which binds to IGF-1 receptors on chondrocytes, stimulating their proliferation. One possibility is that fetal programming may occur *via* the growth hormone neuro-endocrine axis, where alterations could potentially affect growth plate structure and function, possibly through local availability of IGF-1. However, IGF-1 mainly influences cell proliferation. Although bone growth is not simply restricted to the control of skeletal cell proliferation; the main parameters contributing to linear growth are matrix synthesis and cellular enlargement during hypertrophy (Hunziker and Schenk, 1989; Breur *et al*., 1991; Kuhn *et al*., 1996; Farnum *et al*., 2002). This has been studied in the proximal tibial growth plate of 4-week old rats. At this age the animal is in its pubertal growth spurt. In the tibia only 9 % of growth is contributed by proliferation, whereas 32 % is due to matrix synthesis and 59 % as a consequence of chondrocyte enlargement (Wilsman *et al*., 1996). Hence, to understand the possible effects of programming on the bone development requires an understanding of the regulation of longitudinal growth that occurs at the growth plate.

In larger mammals, the growth plates close at skeletal maturity and hence longitudinal growth ceases. However, smaller mammals such as rats and mice maintain a growth plate into old age (Roach *et al*., 2003). In rats the *rate* of growth increases during the first 1 to 5 weeks after birth and subsequently declines until skeletal maturity at approximately 11.5 to 13 weeks of age (Kember, 1973; Hunziker and Schenk, 1989). Therefore, bones continue to grow at a reduced rate until around 26 weeks of age, after which growth virtually ceases and the growth plate height is fixed. Therefore, after 5 weeks of age, the *rate* of growth starts to decline and the height of the growth plate continually decreases (Roach *et al*., 2003). During this time period the greater the height of the growth plate, the lower the level of skeletal maturity. It should be noted that changes do still occur within the growth plate structure after 26 weeks of age (Roach *et al*., 2003) with areas where chondrocyte death occurs resulting in large acellular areas, or 'core' cartilage is resorbed and replaced by bone, or there is direct bone formation by former growth plate chondrocytes.

Growth in the developing offspring also has a significant skeletal stem cell cellular component. Bone formation and subsequent bone growth in the developing offspring depend on the differentiation of bone marrow mesenchymal stem cells into cells of the osteogenic lineage (Tare *et al*., 2008; Tare *et al*., 2010). Therefore, differences in bone quality are likely to be as a consequence of altered regulation of skeletal/mesenchymal stem cell activity. It is this exquisite temporal and spatial process of bone formation that provides a potential mechanism for the programming of the skeletal growth trajectory by dietary protein restriction during intrauterine life.

To help dissect out the mechanisms involved in skeletal programming and the role of nutrition, in general two methods are applied; an isocaloric maternal low protein diet or utilisation of varying degrees of global reduction in nutrition – each will be detailed below. However, it is important to note, that data is now emerging that, even if an isocaloric low protein diet does not affect birth weight significantly in the first generation, an isocaloric low protein diet may affect birth weight in subsequent generations (Stewart *et al*., 1975). Thus it is clear that not only are the offspring of the first generation subject to influence but also the $F_2$ gametes in the female are affected.

## Fetal Growth and Nutrition

A variety of factors is critical for optimum fetal growth such as oxygen, growth factors and protein. Thus, for example, insulin is an important fetal growth hormone and through most of fetal life, amino acids, rather than glucose, determine insulin secretion by β-pancreatic cells (Hales and Barker, 1992). Furthermore, a maternal low protein diet during pregnancy affects islet cells as well as insulin-sensitive organs like liver, muscle, kidney and brain in the offspring (Langley-Evans, 2000). IGF-1 has been shown to play a role in fetal growth with both maternal and fetal IGF-1 regulated by nutrition (Gluckman *et al*., 1996). In a rat model, when mothers were fed 30 % of *ad libitum* diet, maternal IGF-1 levels were observed to be reduced throughout pregnancy and, critically, late gestation fetuses and placental weights were significantly reduced (Woodall *et al*., 1996). In a guinea pig model, mothers were fed a 70

% of *ad libitum* diet and fetal measurements taken on day 40 or a 70 day gestation (Sohlström *et al*., 2001). Maternal IGF-1 and IGF-2 levels were significantly reduced, total fetal mass per dam was reduced by 29 % and placental mass by 23 %. Support for a role of IGF-1 has also been observed in large animal models of development, Thus, in sheep, raising IGF-1 levels in pregnant ewes resulted in increased uptake of amino acids and glucose by the placenta and enhanced glucose delivery to the fetus (Harding *et al*., 1994), whereas in growth restricted fetuses, raising IGF-1 levels produces different effects suggesting altered sensitivity to IGF-1 depending on the cause, duration and severity of growth retardation (Jensen *et al*., 1999).

In the last 10 years, work from the Karsenty Laboratory has shown that bone is an important endocrine organ (Ducy *et al*., 2000; Takeda *et al*., 2002; Hinoi *et al*., 2009; Ferron *et al*., 2010), with leptin, insulin, and osteocalcin all playing important roles in both bone development and energy metabolism. Hence, bone is not simply an organ that is affected by other systems, but bone can also influence energy metabolism and male fertility (in mice) (Oury *et al*., 2011), *via* the action of osteocalcin on testosterone production.

Therefore, there is compelling emerging evidence that modulation of bone formation and bone growth in the developing offspring as a consequence of altered bone marrow mesenchymal stem cells fate and function are likely to affect bone quality and provide candidate mechanisms for the programming of the skeletal growth trajectory by dietary protein restriction during intrauterine life. Furthermore, whilst bone growth and development is influenced by hormones from other systems, there is evidence that bone itself may control production of these hormones, implying a role for bone in fetal growth.

## Changes to Bone Development *in Utero*

There is now substantial accumulating evidence recording alterations in bone development during *gestation or shortly after birth*. Hastings-Roberts and Zeman (Hastings-Roberts and Zeman, 1977) used a severe protein restriction rat model comprising a diet of 24 % casein (control) or 4 % casein (deficient), both fed *ad libitum* through day 20 of gestation, or 4 % casein fed *ad libitum* until day 16 of gestation followed by 24 % casein *ad libitum* to day 20 (supplemented). Fetuses of protein-deficient dams had the fewest number of ossification centres compared to controls. The number of ossification centres in the fetuses in the supplemented group was also observed to be significantly reduced in comparison to controls. However, protein supplementation resulted in a significant improvement in fetal bone development over the deficient group as evidenced by an increase in the number of visible ossification sites.

Fleeman's group (Fleeman *et al*., 2005) used a different dietary manipulation and studied the effect of total nutrient restriction in pregnant rat dams. There were no skeletal malformations associated with nutrient restriction or fetal weight reduction in gestational day 21 fetuses. There were reductions in ossification levels associated with the lowest nutrient level (62.5 % reduction). Interestingly, although maternal and fetal body weights were significantly reduced in the other feed-restricted groups (25 % and 50 % reduction), there was no evidence of delayed skeletal ossification in these groups. Whereas Cappon *et al*. (Cappon *et al*., 2005) found that using a food restriction of 50 % or more in the rabbit from gestational days 7-19 was associated with reduced ossification of the fetal skeleton at gestational day 29, but there were no skeletal malformations associated with nutrient restriction. This may reflect differences in food utilisation efficiencies between different species.

Using a low-protein model in micro-swine, Lanham *et al*. found that the proximal femur from offspring 2 days before birth showed a reduced level of bone (Lanham *et al*., 2009). In addition, the trabeculae displayed raised density, although there were no significant differences in trabecular thickness or spacing. This structure was shown to support a lower load at failure. In contrast, in vertebra from the same animals, there were no observed differences in trabecular density or thickness, although there was an increase in trabecular spacing. The vertebral bodies were shown to support a lower load at failure. The femoral midshaft in the maternal low protein group showed no differences, whereas the midshaft of the tibia showed altered density and failed at a lower load. Furthermore, the calvaria in this group tended to be thinner, although this did not reach a significant level. Hence, cortical bone may have been less affected than trabecular bone. Interestingly, no differences were found in anthropometric measurements of the femur or vertebra.

Osgerby and colleagues (Osgerby *et al*., 2002), fed pregnant ewes a 30 % total nutritional restriction from day 26 of gestation. Fetal samples were taken at gestational days 45, 90, and 135. The authors reported full-term fetal IGF-I concentrations mirrored maternal levels; fetuses from undernourished mothers appearing to be higher at day 90, but were significantly lower than controls by day 135. Insulin concentrations were not affected by maternal nutrition at day 90, but were significantly lower at day 135 in the undernourished fetuses. IGFBP-2 levels increased significantly between days 90 and 135 in undernourished fetuses, but not in controls. Maternal diet did not significantly affect IGFBP-3 levels. From day 90, maternal under-nutrition resulted in an increase in the length of the fetal femur and metatarsal. The trend for IGF-I to be higher in the underfed mothers in mid-gestation, and in their fetuses on day 90, may have contributed to this increase in bone length. By day 135, the fetal femur and metatarsal were of a similar length in both groups, although the fetal humerus and scapula were shorter in undernourished fetuses, suggesting differential regulation of bone growth by nutrition in a time-dependent manner.

Tatara (Tatara, 2008) studied the neonatal period by investigating the effects of treatment with β-hydroxy-β-methylbutyrate (HMB) during the first 3 weeks of life in male sheep. HMB is a metabolite of the essential amino acid leucine, which appears to increase collagen deposition. Serum concentrations of bone-specific alkaline phosphatase, osteocalcin, GH and IGF-1 where raised at 21 days of age in the HMB treated group, and levels of

the C-terminal telopeptide of type I collagen (CTX) were increased in the same group at 130 days of age. In addition, HMB improved volumetric bone mineral density and bone morphological and mechanical properties of the femur and lumbar spine, as well as increasing femoral length and femoral and vertebral volumes.

Tatara and co-workers (Tatara *et al*., 2007) also examined the effects of HMB treatment on pregnant pig sows during the two last weeks of gestation. HMB increased serum levels of GH, IGF-1 and bone alkaline phosphatase activity in the offspring. Furthermore, HMB also increased volumetric BMD of the trabecular and cortical bone of the femur in the offspring at 6 months of age. Overall, HMB increased the maximum elastic strength and ultimate strength of the femur. The authors suggested that these effects, together with the increased level of GH and IGF-1 in the newborns, indicated involvement of improved somatotrophic axis function in prenatal programming of skeletal development in pigs.

Thus to summarise, using a variety of different animal models and dietary challenges, these results show that maternal dietary restriction during pregnancy and shortly after birth can directly alter bone ossification in the growing fetus apparently by altering fetal serum IGF-1 and IGF binding protein function as indicated by modified expression.

## Long-term Changes to Bone Growth

While there is a wealth of data on the effects of maternal diet on bone development *in utero*, to determine if maternal diet has a permanent effect on bone development, growth, and maintenance, longitudinal, and thus long-term, studies of animals into adulthood and old age are a necessity.

Langley-Evans developed the low-protein rat model over 15 years ago (Langley and Jackson, 1994), yet it still remains one of the most widely used animal models to examine the role of maternal nutritional deficiencies on the development of the offspring. In brief, pregnant rat dams are fed a diet containing 180 g casein/kg (control diet) or 90 g casein/kg (low-protein diet). The low protein diet is balanced in energy content by the addition of carbohydrates. Animals are provided with free access to water and food at all times. On the day of birth, mothers are switched to a standard laboratory chow and all litters reduced to a maximum of 8 pups. The nutritional composition of the low-protein fed mother's milk has been recorded to return to that of control mothers within 24 hours of transfer to the standard laboratory chow. Thus, differences in pup phenotype can be attributed directly the protein insufficiency *in utero*. Using this model, pups have been shown to develop functional changes in adulthood, including hypertension, impaired renal function and immune response, cardiovascular disease, and altered lifespan (Langley-Evans *et al*., 1996; Langley-Evans, 1999; Langley-Evans *et al*., 1999a; Langley-Evans *et al*., 1999b; Aihie *et al*., 2001).

Studies by Mehta and colleagues in 2002 using the Langley-Evans rat model together with dual-energy X-ray absorptiometry (DXA) analysis, demonstrated the mean bone area of the low-protein group adult offspring to be 10 % lower than that of the control offspring (Mehta *et al*., 2002). A similar magnitude in difference was observed for whole body bone mineral content (BMC). However, there were no differences between control and low protein groups in whole body bone mineral density (BMD). There was a significant association between whole body fat mass and whole body BMC. Adjusting for differences in fat mass eliminated the significant effect of maternal diet on the BMC of the offspring. No effect was observed for whole body lean mass. The differences seen in bone area remained after adjusting for both lean and fat mass. These results indicate that a maternal low-protein diet culminate in a reduction in bone area and BMC, but not BMD, among the aged offspring, suggesting from these studies, that bone size, rather than volumetric bone density, is the principle outcome modified by maternal protein restriction. In the same study, the authors (Mehta *et al*., 2002) found that the height of the growth plate (both the cartilaginous and the continuous metaphyseal band of bone) in the proximal tibia at 72 weeks of age was significantly greater in male rat offspring whose mothers were protein undernourished during pregnancy. These findings suggest increased height of the growth plate in the low protein group offspring at the time when longitudinal bone growth had ceased or cessation of growth *at an earlier age* in the protein-undernourished rats. However, a subsequent study has shown that the low protein group male offspring display no significant differences in femur or tibia length from 4 to 75 weeks of age (Lanham *et al*., 2008a), suggesting the former explanation. Increased insulin levels can cause increased tibial growth plate height (Alarid *et al*., 1992) and aged males offspring from low protein fed mothers have been shown to have raised plasma insulin levels at 20 weeks of age (Sugden and Holness, 2002). Hence the increased growth plate height may be a consequence of higher insulin levels.

Oreffo and co-workers (Oreffo *et al*., 2003) using the Langley-Evans model of protein insufficiency examined the cellular mechanisms involved in the programming of bone development. Total CFU-F numbers, indicative of the colony forming efficiency of the mesenchymal stem cells and proliferation potential of these cells, at 8 weeks of age, was found to be reduced by approximately 40 % in cultures from offspring whose mothers were fed a low-protein diet. The authors observed no difference at 12 weeks and a subsequent increase of 111 % at 16 weeks compared to controls cultures. Examination of alkaline phosphatase-positive CFU-F number, indicative of osteogenic potential and differentiation, produced similar results. Furthermore, the authors noted the addition of osteogenic growth factors GH, vitamin D, and IGF-1, under the conditions examined, was insufficient to overcome or reverse the effects of maternal dietary manipulation. In the skeletally mature control offspring (12-16 weeks of age) there was a reduction in CFU-F colony number and alkaline phosphatase specific activity, reflecting reduced osteoblast activity as skeletal growth maturity was reached. In contrast, for low-protein diet offspring, the reduced osteoblast activity/skeletal development at 8 weeks, during peak skeletal growth, and significantly increased

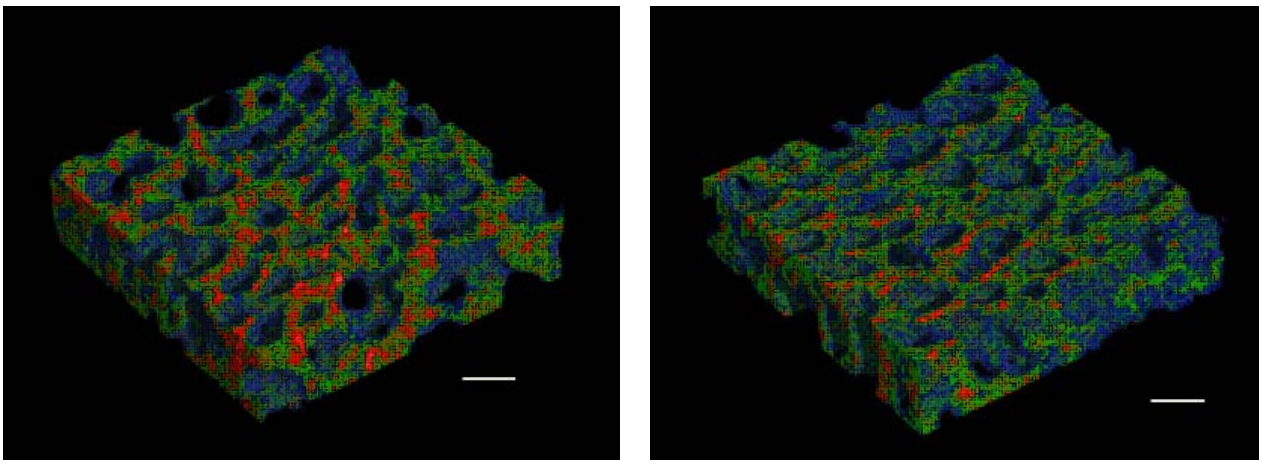

**Fig. 1.** False colour representative section through femoral head for 75-week-old female offspring of rat mothers fed either control protein diet (18 %, left) or low protein (8 %, right) diet during pregnancy. Images show variation in voxel density; low density bone is shown blue, medium density is shown green, and high density bone is shown red. Bar 0.5 mm.

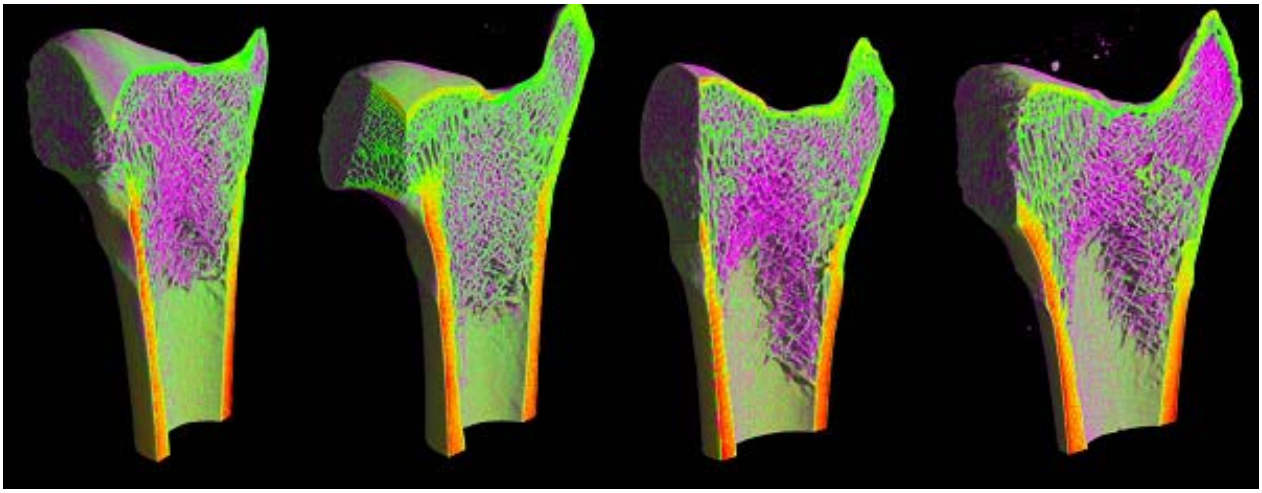

**Fig. 2.** Pregnant ewes were subjected to either control diet (C-) or 50 % total nutrient restriction for the first 30 days of gestation (R-), male offspring were then either fed control diet (-C) or a postnatal nutrient challenge to reduce body weight to 85 % of their target weight from weaning at 12 weeks to 25 weeks postnatal age (-R). Representative images show false colour section through proximal femur of male sheep for CC (left), CR (middle left), RC (middle right), or RR (right) fed offspring. Low density bone is shown magenta, medium density is shown green, and high density bone is shown yellow/orange.

osteoblast activity at 12 and 16 weeks may be attributable to intrauterine programming, with subsequent "catch-up" skeletal growth occurring at 12 and 16 weeks. However, sex differences were not reported, and in a similar study only males displayed catch-up growth (Lanham *et al*., 2008b).

Using the Langley-Evans model of protein insufficiency, Lanham *et al*. (Lanham *et al*., 2008b) studied markers of the osteogenic environment. Serum IGF-1 levels were significantly lower in female low-protein group offspring at 4 weeks of age and serum osteocalcin was significantly higher at 4 weeks of age in male and female offspring from mothers maintained on a low-protein diet, whereas serum 25-OH vitamin D was significantly lower in low-protein group males at 8, 12, and 20 weeks of age. The increased levels of osteocalcin, a specific marker for osteoblast activity (Brown *et al*., 1984), seen in the low-protein diet group at 4 weeks of age suggested increased osteoblast activity and increased bone formation in these animals compared to controls.

Lanham *et al*. (Lanham *et al*., 2008a), used the Langley-Evans model together with micro-CT, to analyse bone structure of 75 week old female offspring from mothers fed a low-protein diet during pregnancy. The femoral heads were found to have thinner, less dense trabeculae (Fig. 1), while the femoral neck bone displayed closer packed trabeculae. Furthermore, the vertebrae had thicker, denser trabeculae, and the midshaft tibiae displayed greater dense cortical bone. Strength testing demonstrated that the femoral heads and midshaft tibiae to be structurally weaker, whereas the femoral necks and vertebrae were found to be structurally stronger. These studies provided

the first direct evidence that maternal nutrition could alter the microscopic bone architecture and structure of the aged offspring. Interestingly, there were site-specific differences in the effects of the maternal nutrition, as the femoral head and midshaft tibia showed alterations characteristic of osteoporosis, whereas the femoral neck and vertebra showed increased bone strength. There were no differences in the anthropometric measurements of the bones. Interestingly, there appeared to be sex-specific differences in the effect of the maternal low-protein diet. Thus, when 75 week old male offspring were tested, no differences were found in structure or density in the femoral head, femoral neck, midshaft femur, midshaft tibia, or vertebra between controls and the low-protein group offspring (Lanham, Cooper, Oreffo unpublished data).

Romano *et al*. (Romano *et al*., 2009) used a bilateral uterine vessel ligation to induce a fetal total nutrient restriction in rat offspring. The authors noted that at 6 months of age, in comparison to controls, the restricted offspring had reduced BMC, reduced anthropometric measurements and reduced bone strength. These parameters could be restored to levels seen in controls if the restricted offspring was cross-fostered onto a control mother. However, this was only observed with female offspring and not male offspring, again suggesting sex differences in the long-term influence of maternal diet. In addition, supplementation with a high calcium diet from adolescence increased adult cortical bone density in low birth weight males and females, and normal weight females. However, calcium supplementation was not sufficient to rescue the bone dimension and strength deficits which were programmed *in utero*, suggesting that the early life environment is critical for bone programming (Romano *et al*., 2010).

Over twenty five years ago Schrader *et al*. (Shrader and Zeman, 1973) examined the male offspring from rat dams fed either a control diet (24 % casein) or a protein-deprived diet (6 % casein) during pregnancy. Offspring were reared in foster litters of 10 (large litters) or four (small litters). Body weight was significantly reduced in the large control litters and both protein-deprived litter sets. However, the protein-deprived small litter set had a similar mass to the large control litter indicating there was a compensatory change in mass from augmentation of the food supply. Only the protein-deficient offspring displayed a delay in ossification that was not altered between the large and small litters suggesting this had been "set" by the maternal diet. From birth to 65 days of age, the rate of bone elongation and weight gain of control male pups exceeded that of deficient male pups. However, from 65 to 90 days, the tibial elongation rate was the same in the two groups while body weight gain decreased at a faster rate in controls than in deficient young.

Using a sheep model, Lanham *et al*. (Lanham *et al*., 2005) used a maternal 50 % total nutrient restriction for the first 30 days of gestation, a postnatal nutrient challenge to reduce body weight to 85 % of their target weight from weaning at 12 weeks to 25 weeks postnatal age, or both. Bone measurements were taken when the male offspring reached 2.5 years of age. Anthropometric measurements of femurs showed no significant differences between the groups, however all the measurements showed a trend compared to controls for the mean to be lower in the postnatal challenge group, higher in the gestation/postnatal challenge group, and similar in the gestation challenge group. When the femur was analysed by pQCT, again, no significant differences were observed between the groups, although the same trends persisted, this time with cortical and trabecular BMD, BMC, cortical thickness and trabecular area. These trends remained after normalising to body mass. Examination of vertebrae using DXA showed BMD in the gestation challenge group was significantly lower than controls, whereas the gestation/postnatal challenge group was similar to controls. No differences were found in BMC. Significance was not observed when results were normalised to mass. No significant differences were found with vertebral BMD or BMC if 50 % maternal nutrient restriction was applied for 30 days before conception.

These various studies clearly demonstrate that a maternal dietary restriction during pregnancy affect bone development with effects on the bone of the growing juvenile and long-term effects in the adult offspring.

**Effects of dietary excess on bone growth**

Nutritional challenge does not necessitate a lack of nutrients, but may also be due to increased nutrients. Given the current obesity epidemic in the western and developing world, lessons from increased nutrient intake on skeletal development may prove informative. Driscoll *et al*. (Driscoll *et al*., 1990) studied the effect of a maternal high protein diet (40 % protein, controls 20 % protein), from day 7 of gestation, on the mandibles of the newborn rat. The authors showed that the alkaline phosphatase and acid phosphatase activity from the high-protein diet group was lower than controls. Protein levels in the mandibles were higher in the high-protein diet groups, although there were no differences in the mass of the mandibles.

Lanham *et al*. (Lanham *et al*., 2010) examined the bone development in a mouse model of high fat intake, during pregnancy and/or for the offspring. Offspring were analysed at 30 weeks of age. Both males and females in the two high fat offspring groups (offspring high fat diet with maternal control or maternal high fat diet) showed increased fat deposition in the distal femur, with the maternal high fat with offspring high fat diet group demonstrating the greatest level of deposition. The male offspring were less affected than the female offspring. Male high fat fed offspring, regardless of maternal diet, showed increased mass and reduced femur length. High fat fed males from high fat fed mothers also showed an increased load at fracture of the midshaft, likely due to the increased cross-sectional area seen at this point in this group. High fat fed female offspring from control mothers displayed increased mass, increased bone volume, and increased cross-sectional area of the midshaft. In contrast, high fat fed female offspring from high fat fed mothers displaying increased mass (although not to the extent of the maternal control diet/offspring high fat diet females), showed a reduced femur length, and increased cross-sectional area of the midshaft (Fig. 3). These data

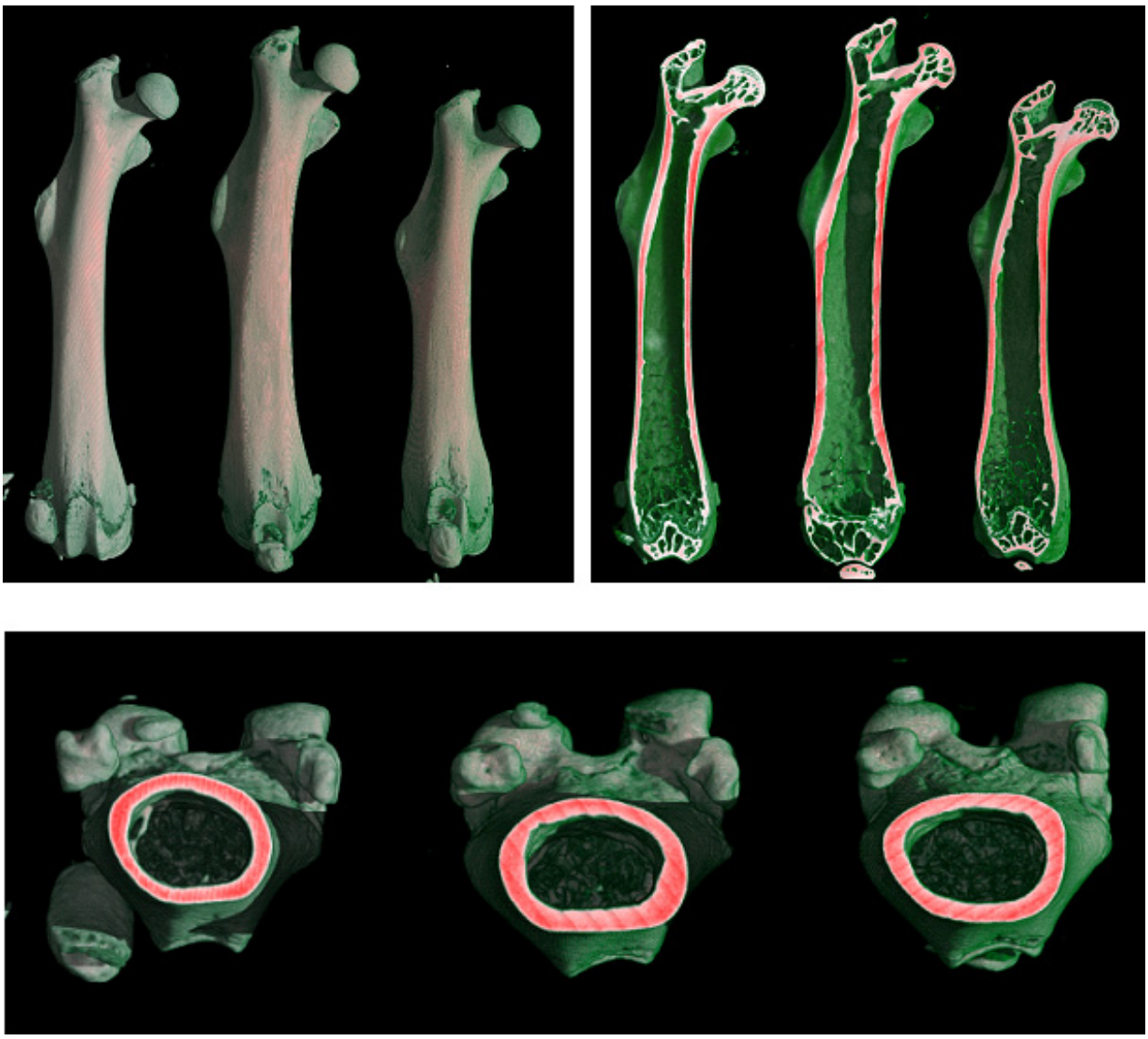

**Fig. 3.** False colour representations of femur from high fat fed female mouse offspring aged 30 weeks. For all images, left femur is control animal, middle is high fat fed offspring from control fed mother (C-HF), and right is high fat fed offspring from high fat fed mother (HF-HF). Top left image shows reduced femur length in HF-HF group compared to control and C-HF groups. Top right image shows internal view of the same samples. Bottom image shows increased cross-sectional area at the midshaft in the C-HF and HF-HF groups compared to controls.

demonstrate that the maternal control diet/offspring high fat diet group increased the cross-sectional area of the femur to withstand the greater mass of the animal. Interestingly, as other bone parameters were maintained (femur length and trabecular thickness), this indicated an increase in the total amount of bone present in the femur. In contrast, the maternal high fat diet/offspring high fat diet females had similar trabecular indices to those seen in controls (Fig. 3). The data indicate a reduction in femur length in order to supply bone material to increase the cross-sectional area of the midshaft, to adapt with the extra mass of the animal. This suggests programming of the total amount of bone, and re-modelling to redistribute the bone to cope with an increase in mass of the animal. However, these maternal high fat diet/offspring high fat diet females failed to increase in mass to a level equal to that of the maternal control diet/offspring high fat diet group as was observed in the male offspring. Thus in summary, it would appear a maternal high fat diet altered the basal metabolic activity of the female offspring.

These data demonstrate that excess nutrients during pregnancy can affect the bones of the offspring. By inducing excess deposition or storage of nutrients, it can lead to permanent alterations of bone development and structure. As detailed below, the key will be to correlate some of these observations with human data and to begin to unravel potential mechanisms.

**Mechanisms of action**

Given that maternal diet appears to be able to programme bone growth in the offspring independently of offspring DNA sequence, an epigenetic mechanism would appear an important area for investigation. Epigenetic modification consists of three distinct, but closely interacting processes;

(i) DNA methylation of carbon 5 of cytosine residues in CpG nucleotides, (ii) histone modifications, in particular acetylation and methylation on specific lysines, and (iii) microRNAs (miRNA) (Jaenisch and Bird, 2003; Iorio *et al*., 2010). Conformationally relaxed chromatin indicates a transcriptionally active region displaying hypomethylated DNA, acetylation of histones H3 and H4, as well as methylation of H3 at lysine 4 (H3 K4). Compact chromatin is transcriptionally silent, hypermethylated, and bound to non-acetylated histones with methylation at H3 K9 and K27 (Feinberg, 2007; Gan *et al*., 2007). Methylation of DNA occurs *via* the addition of methyl groups to 5'-cytosines next to guanines (CpG sites) by DNA methyltransferases (Dnmts) (Pradhan and Esteve, 2003; Klose and Bird, 2006). These methyl groups are primarily supplied from serine. Methylated CpG sites attract methyl-binding proteins, which are associated with histone deacetylases and histone methyl transferases (Fujita *et al*., 2003; Fuks *et al*., 2003), thus there is a reciprocal relationship between DNA methylation and histone modifications. However, DNA methylation is important for maintaining silencing in the long-term (Bird, 2002; Reik, 2007), since DNA methylation patterns are generally stable in somatic cells throughout adult life (Berger, 2007). During DNA replication, the methylation pattern is rapidly reproduced on the new DNA by the methyl transferase DNMT1, which associates with proliferating-cell nuclear antigen and tracks with DNA polymerase (Newell-Price *et al*., 2000; Attwood *et al*., 2002). The histone code can then be re-established after cell division by various mechanisms involving methyl binding domains (MBDs) and DNMTs, which can interact with histone methyltransferases and histone deacetylases. Although a number of studies have concentrated on CpG methylation, there is evidence that non-CpG methylation may also be important (Fuso *et al*., 2010).

Methylation is affected by dietary factors, in particular by levels of methyl-donors such as folic acid (Jhaveri *et al*., 2001; Van den Veyver, 2002; Davis and Uthus, 2004). In addition, the DNA methylation pattern for a specific gene varies between cell types. In general, cells expressing the gene will have hypomethylated promoters, whereas hypermethylation of the promoter silences the gene (Chan *et al*., 2000; Attwood *et al*., 2002; Salozhin *et al*., 2005). The DNA methylation status is heritable, i.e. the cell type-specific gene expression repertoire is stably transmitted to daughter cells. However, if changes occur, for example as a consequence of intrauterine protein deficiency, then the aberrant methylation pattern will also be transmitted to daughter cells, which may result in either abnormal expression of silenced genes or suppression of normally expressed genes. Studies using the agouti mouse (Wolff *et al*., 1998) elegantly demonstrate this principle. Yellow (Avy/a) agouti mice are hypomethylated. However, feeding pregnant black a/a dams methyl-supplemented diets resulted in increased methylation and increased silencing of agouti expression in their offspring, as shown by increased agouti/black mottling in the direction of the pseudoagouti phenotype. The epigenetic phenotypes were maternally heritable and, critically, shown to be modulated by maternal diet, thereby adding evidence to the importance of maternal nutrition on the health and longevity of their offspring.

In our rodent studies (unpublished data), we have cultured bone marrow cells under osteogenic conditions from newborn offspring and searched for genes that were over- or under-expressed in the offspring from low protein fed mothers. We have identified 184 genes (17 over and 167 under-expressed) in males and 483 (213 over and 270 under-expressed) in female offspring. Thus, a maternal protein restriction caused sex-specific alterations in gene expression. Preliminary analyses of the DNA methylation status of genes important in bone development, found over-expression of a candidate gene was associated with decreased methylation of the promoter in 100 % of over-expressing samples, but only 43 % of under-expressing samples ($p < 0.05$). We also found loss of DNA methylation in the 3' UTR region of the gene (100 % of low protein offspring samples versus 0 % of control samples, $p = 0.001$), which is a potential binding region for microRNAs and may also be involved in the epigenetic regulation of genes. These observations provide further evidence of the influence of maternal diet on offspring bone structure and development.

**Animal models – relevance to human biology**

A number of non-communicable diseases of adult life have been identified as having a developmental origin, including, coronary heart disease, stroke, type 2 diabetes mellitus, adiposity, and the metabolic syndrome (reviewed in Gluckman *et al*., 2008). The authors show that currently identified genetic markers only account for a small proportion of the variation in bone mass and risk of fracture of individuals. Data from animal studies are particularly interesting when extrapolated to data from human studies such as the Saskatchewan bone mineral accrual study (Bailey *et al*., 1999) and the Finnish cohort studies (Cooper *et al*., 2001). These studies show that maturational delay may increase the risk of hip fracture through altered accrual of bone density and disproportionate bone growth. In addition, studies have shown that human intrauterine growth retarded babies are capable of demonstrating "catch-up" growth for various anthropometric parameters (Villar *et al*., 1982; Villar *et al*., 1984). Furthermore, infants malnourished *in utero* had a significant retardation in skeletal maturity at birth, when compared to controls (Walther *et al*., 1981). However, at 2.5-3.5 years of age, the average skeletal maturity of the intrauterine malnourished children approached that of controls, demonstrating "catch-up" in skeletal growth during infancy.

Yin *et al*. (Yin *et al*., 2009) studied maternal dietary intake in the third trimester of pregnancy and bone mass in the male and female offspring at age 16. The authors concluded that maternal milk intake was positively associated with offspring bone mineral density at age 16, whereas maternal fat intake showed a negative association. The authors concluded that maternal diet influenced peak bone mass in the offspring possibly through programming bone responses.

A recent meta-analysis (Baird *et al*., 2011) looked at birthweight or weight at 1 year of age to bone mineral content, bone mineral density or osteoporotic fracture in adults aged 18 years and over. From over 4000 abstracts, 14 worldwide studies fitted their inclusion criteria. From

these, the authors concluded there was a consistent positive association between birth weight and adult bone mineral content (BMC) at the lumbar spine and hip; the association being stronger in women. However, birth weight was not a predictor of areal bone mineral density of the lumbar spine and hip. In addition, there was less consistent evidence about the relationship between birth weight and bone mass at the neck of femur, radius or ulna (Baird *et al*., 2010a).

## Summary

A wealth of data is now emerging indicating that maternal diet can greatly affect the size, structure and strength of the bones in the offspring. Abnormalities of skeletal development are initiated in early life, potentially through impaired IGF-1 activity, which would favour down-regulation of bone deposition. Thus, changes in skeletal growth and structure following poor nutrition in pregnancy may lead to reduced bone mass in later life. This could potentially results in osteoporotic fracture in humans. However, as indicated above, caution must be applied before extrapolating animal findings to human populations.

It is clear, animal models of maternal dietary insufficiency and excess provide useful models to study skeletal programming *in utero*. The challenge for the next decade will be to identify the mechanisms of programming of bone growth and to enhance our understanding of the factors influencing bone development, adult bone structure and peak bone mass. Advances in our understanding of the developmental origins of bone and bone diseases will undoubtedly provide a step change in clinical treatment and reduction in the burden of osteoporosis for an increasing ageing population.

## Acknowledgements

Research by the authors is supported by the Biotechnology and Biological Sciences Research Council, Research into Ageing and Medical Research Council.

## Disclosures

The authors state that they have no conflicts of interest.

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## Discussion with Reviewers

**Reviewer II**: Do you think that there may be differences in the mechanism causing the programming of poor adult bone health via maternal protein restriction models and those using bilateral uterine vessel ligation as a model of uteroplacental insufficiency?

**Authors**: Our working hypothesis is that *in utero* males adapt to the nutritional environment to provide maximum growth and size and birth, whereas females adapt for maximum life expectancy. Hence, we believe the pathways involved would be the same in both nutrient situations, however, there outcomes would be expected to be different due to the proportions of each available nutrient (protein, fat, etc).

**Reviewer II**: Considering that maternal undernutrition is mainly now a 3rd world issue, how relevant are maternal protein restriction models to the wider community? In saying this, I would suspect that maternal overnutrition or obestiy in pregnancy may be a more important issue now affecting Western Society.

**Authors**: We believe there are "nutritional adaptive" pathways. In the first phase of our work - some 5 years ago, we utilised both low protein and high fat diets to validate and provide proof of concept data for the skeletal system, for this hypothesis. It has been long recognised in the cardiovascular and hypertension arena the importance of maternal nutrition modulation and the effects on offspring in later life and consequent disease risk.

What is important, and hopefully outlined in the review, is that "abnormal" nutrition either by total excess or reduction of a component (e.g., protein) can produce long term alteration of bone growth and structure – which is now only gradually gaining acceptance. Critically, the challenge facing us all is the growing epidemic of diabetes, metabolic syndrome and obesity in the western world and, critically and sadly still perhaps not fully appreciated in all quarters, the staggering rise in countries such as India and the Asian content.