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Perinatal maternal dietary supplementation of omega-3 fatty acids transiently affects bone marrow microenvironment, osteoblast and osteoclast formation and bone mass in male offspring

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Abstract

It is increasingly evident that micronutrient environment experienced before birth and in infancy is important for achieving optimal bone mass by adolescence and maintaining bone health. This study determined whether maternal supplementation with omega-3 polyunsaturated fatty acids (n3FA) improved offspring bone growth and adult bone mass. Female rats were fed a diet containing 0.1% (Control, n=10) or 1% (n3FA, n=11) docosahexanoic acid (DHA) during pregnancy and lactation. Offspring were weaned onto a control diet. Tibial growth plate and metaphysis structure, osteoblast/osteoclast density and differentiation, and gene expression were assessed in offspring at 3 weeks (weaning), 6 weeks (adolescent) and 3 months (adult). Maternal n3FA supplementation elevated offspring plasma n3FA levels at 3- and 6-weeks. While total growth plate heights were unaffected at any age, the resting zone thickness was increased in both male and female offspring at 3 weeks. In n3FA males, but not females, bone trabecular number and thickness were increased at 3 weeks but not other ages. The week-3 n3FA males also exhibited an increased bone volume, an increased osteoblast but decreased osteoclast density, and higher expression of osteoclastogenesis inhibitor OPG but lower expression of osteoclastogenic cytokines RANKL, TNF-α and IL-6. No effects were seen at 6 weeks or 3 months in either sex. Thus, perinatal n3FA supplementation is associated with increased bone formation, decreased resorption and a higher bone mass in males, but not in females, at weaning; these effects do not persist into adolescence and adulthood and are unlikely to produce lasting improvements in bone health.

INTRODUCTION

Osteoporosis has long been recognised as a problem of older adults, however poor bone health is now being seen increasingly in children and adolescents (1). For example, low bone mass and/or more frequent fractures are commonly seen in children or adolescents suffering from poor nutrition or micronutrient deficiency (2). Whilst seventy-five to eighty percent of peak bone mass attained by an individual is attributed to genetics, 20-25% is related to life style and environmental factors, in particular nutrition (3). In addition to the role of nutrition in determining adult bone health, previous

research has also shown that the nutrient supply before birth and into early infancy is important in determining the health outcomes of offspring into adulthood (4, 5), including bone mass (6). In addition, it is well documented that malnutrition or under-nutrition during critical growth periods in childhood can cause growth retardation and decreased bone formation. This, in turn, reduces the peak bone mass attained in adolescence (7-10), which is known to be an important determinant of bone mass/strength throughout later life (11). Therefore it is evident that nutritional strategies that can be applied during critical periods of bone growth during early infancy, childhood and adolescence are important in order to achieve optimal bone growth and peak bone mass and ensure good bone health and prevention of osteoporosis in adult life. Thus, devising effective strategies for optimising bone health during early life is important not only for achieving optimal childhood bone health, but for minimising/delaying onset of ageing-related osteoporosis and fractures (12).

In addition to an adequete supply of overall energy, optimal growth and development of the human skeleton and maintenance of bone health also requires an adequate supply of specific macronutrients, micronutrirents as well as bioactive ingredients, including protein, inorganic minerals (e.g., calcium, magnesium, phosphorus), vitamins (vitamins A, D, E, K, C), and long chain omega-3 polyunsaturated fatty acids (n3FAs) (13). Although not classically associated with bone development, there is accumulating evidence that the supply n3FAs during critical periods of bone growth can also play an important role in optimizing bone health and bone mass.

A number of studies have demonstrated that, in addition to their well-established effects on cardiac health and anti-inflammatory properties, n3FAs also have positive effects on bone development and metabolism. Dietary supplementation with n3FAs increased bone density and bone strength in adult rats and piglets (14-16). In growing rats, n3FAs increased osteoblast formation and activity resulting in an increased rate of bone formation, suggesting that n3FAs may be linked to an alteration in osteoblast formation and/or function (17, 18). Apart from promoting bone formation, n3FAs also inhibit bone resorption in adult rats by suppressing expression of inflammatory cytokines and NF-κB

activation and thereby reducing osteoclast formation and activity (19, 20). There is also evidence that n3FA supplementation is beneficial for bone health in humans, and n3FA supplementation has been shown to promote bone health by suppressing bone resorption (21) and improving bone density in older adults and postmenopausal women (21-24). Together these findings indicate that n3FAs exert a positive effect on bone formation and metabolism, which could represent a potential nutrition supplementary strategy that could be explored for improving bone growth and increasing bone mass.

Due to compliance and delivery issues encountered with children, delivery to the mother, rather than the child would represent a more practical means of delivering n3FAs to the fetus and breast-fed infacts to improve offspring bone development and growth. A number of large scale clinical trials have confirmed that maternal n3FA supplementation is safe for both the mother and the infant, and may have benefiical effects on infant cognitive development (25, 26). However, there are no studies to date which have determined whether exposure to an increased supply of n3FA during the perinatal period, i.e. a critical period for bone growth and development, is associated with long-lasting improvements in bone health. In the current study, we have therefore used a rat model of maternal n3FA supplementation to determine the effects of maternal n3FA dietary supplementation during pregnancy and lactation on offspring endochondral bone formation, bone structure and volume, bone resorption, bone marrow osteogenic and osteoclastogenic progenitor cell pools of young and adult offspring.

METHODS AND MATERIALS

Animals and Diets

Twenty-one female and eight male Albino Wistar rats, weighing between 200-250g, were individually caged under a 12 hour light/ 12 hour dark cycle at a room temperature of 25°C. Female rats were acclimatised to a diet designed with a fatty acid composition comparable to a *typical* Australian

(Western) diet (40% saturates, 40% monounsaturates, 20% polyunsaturates) (27) (Specialty Feeds, Glen Forrest, Western Australia, Australia) for 3 weeks prior to mating to ensure that the fatty acid profile of all animals was comparable prior to the dietary intervention. Following this dietary acclimatisation, females were mated and allocated into one of two dietary groups: receiving either the pre-mating basal diet (5% fat, 0.1% DHA, Control, n=10), or a high n3FA diet (5% fat, 1% DHA, n3FA, n=11) throughout pregnancy and lactation. Both diets were isocaloric (20% protein, 59.4% carbohydrate and 5% fat by weight). Detailed information on the fatty acid composition of this diet has been published previously (28). Food intake was determined 2-3 times weekly during pregnancy and lactation and fresh food supplied. Dams were weighed weekly throughout the experimental period.

Dams had a natural birth and pups were allowed to suckle from their mothers until weaning. At 21 days of age (weaning), pups were separated from their mothers. Male and female from the same litter were separated and pups of the same sex in the same litter housed together for the remainder of the experiment. All offspring were then fed standard rodent chow ad libitum (20% protein, 59.4% carbohydrate and 4.8% fat by weight) (Rat and Mouse cubes, Specialty Feeds, Glen Forrest, Western Australia, Australia) without additional n3FAs added to this standard diet. From birth until weaning, pups were weighed every 2 days; after weaning, the offspring was weighed every week. All animals were euthanized by CO_2 overdose as stipulated in the Australian Code of Practice for the Care and Use of Animals and all procedures were approved by the Animal Ethics Committee of Institute of Medical and Veterinary Science, South Australia.

Tissue Collection, Processing, and Measurements of Bone Lengths and Weights

For assessing effects of maternal n3FA supplementation on offspring's bone growth, bone mass accrual, and adult bone health outcomes, offspring (n=1 from each dam) were euthanized at *3 separate time-points*: 3 weeks (immediately after treatment, newly weaned, childhood), 6 weeks (towards the end of rapid growth period or adolescence) and 3 months (12 weeks) (skeletally mature adulthood). At the designated time points rats were euthanased with an overdose of CO_2 and bloods

collected via cardiac puncture. Body length (nose to base of tail) and abdominal circumference were also measured. All organs were dissected and weighed, with special attention paid when collecting the long bones: femurs, tibiae and humeri.

The left proximal tibia (1cm length) was dissected, fixed in 10% formalin for 24 hours, decalcified in EDTA for 7 days at 4^{o}C, routinely processed, paraffin embedded, sectioned at 4µm and stained with haematoxylin and eosin (H&E). The right tibia was used for collecting fresh growth plate cartilage (by scraping off using a fine scalpel blade), which was then snap frozen in liquid nitrogen and stored at -80^{o}C. In addition, the proximal metaphysis of the right tibia was also collected and stored frozen at -80^{o}C for subsequent RNA extraction. All the remaining bone and the humeri were used to collect bone marrow samples for cell culture using established methods (29).

Fatty Acid Analysis

Blood collected via cardiac puncture was centrifuged for obtaining plasma and the remaining red blood cell mass was then prepared for fatty acid analysis of red blood cell phospholipids. Lipid extraction, fatty acid methylation and gas chromatographic analysis were performed as previously described (28, 30).

Histomorphometric Analysis of Growth Plate and Metaphysis

On the left tibial sections stained with haematoxylin and eosin (H&E), morphometric measurements were conducted as previously described (31) for average growth plate zonal thickness (of the resting, proliferative, and hypertrophic zones), height of the primary spongiosa bone, trabecular number and thickness within the metaphysis. Trabecular bone volume BV/TV% was calculated by dividing the total trabecular bone area by the total tissue field area.

Metaphysis Osteoblast/Osteoclast Surface Density

On H&E-stained tibial sections, osteoblasts on the trabecular surface in the secondary spongiosa of the metaphysis were counted and were expressed as number of cells per unit length of trabecular bone

perimeter (cells/mm) as previously described (31). To identify osteoclasts, sections were stained for tartrate-resistant acidic phosphatase (TRAP) using established methods (32) (Sigma, NSW, Australia) and counterstained with haematoxylin. Multinucleated (>3 nuclei) and TRAP+ cells were counted by light microscopy and expressed as number of TRAP+ cells/mm on the trabecular surface in the secondary spongiosa.

CFU-f ALP and Mineralisation Assays

Effects of n3FA supplementation on the osteoprogenitor cell pool in the bone marrow were examined ex vivo using bone marrow specimens from the offspring in a Colony Forming Unit- Fibroblast (CFU-f) assay as previously described (29, 33). Briefly, bone marrow cells were plated in duplicate at 2 x 10^6 mononuclear cells/well in 6 well plates in complete medium (α-MEM supplemented with 10% foetal bovine serum, HEPES, 100μm Lascorbate, 2mM L-glutamine, 100μ/ml penicillin and 100μg/ml streptomycin). Cells were cultured for 14 days and media changed every 3 days. After 14 days colonies were fixed with 4% paraformaldehyde and stained for osteoblast differentiation marker alkaline phosphatase. (ALP^+ stained colonies (aggregates of >50 cells) were counted and expressed as ALP^+ colonies/well.

In addition, to assess treatment effects on osteogenic potential of bone marrow stromal cells, cells were cultured in osteogenic medium and were assessed for their osteogenesis potential as previously described (29). Briefly, cells were cultured in 24 well plates at a density of 4.75 x 10^4 cells/well with 6 wells per animal. The first 3 wells were fed "mineralisation" media (basal media supplemented with 10nM dexamethasone and 10mM β-glycerol-2-phosphate) with the remaining cells cultured in unsupplemented basal media. Cells were re-fed with the respective culture media twice weekly and maintained under these conditions for 4 weeks. Following fixation with formaldehyde, cells were washed 3 times in H_20 and incubated on a shaker for 20 minutes with 200μL/well 40nM alizarin red dye. After washing off unincorporated dye, the plate was stored at -20°C. For quantification, the incorporated dye was extracted on a shaker with 160 μL of 10% (v/v) acetic acid, and after being

neutralised with 40µL of 10% (v/v) ammonium hydroxide, was analysed colorimetrically at 405nm.

Quantitative RT-PCR gene expression analysis

Real-time RT-PCR was used to assess the relative mRNA expression of major stimulator of osteoclast formation and activity (receptor activator of nuclear factor-κB ligand or RANKL) and osteoclast formation inhibitor (osteoprotegerin or OPG) (34) as well as tumour necrosis factor (TNF-a) and interleukin-6 (IL-6) (two inflammatory cytokines known to promote osteoclastogenesis). In addition, expression of osteogenesis-related gene osterix and bone matrix protein, osteocalcin mRNA were also determined. Total RNA from frozen metaphyseal bone samples were isolated using TRIZOL solution (Sigma, NSW, Australia). Two micrograms of metaphyseal RNA were then reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA) according to the manufacturer's instructions. This cDNA was used to determine the mRNA expression for the genes of interest by quantitative real-time PCR as previously described (35) using gene specific primers detailed in Table 2 (primers designed using Primer Express® software v2.0, Applied Biosystems, CA) and cyclophilin-A (CycA) as the housekeeping control loading gene. SYBR green PCR assays for each target molecule and internal reference CycA were performed in duplicate on these cDNA samples in a 10 µl reaction using the Applied Biosystems 7500 FAST 96-well PCR machine. From the amplification curves, relative expression was calculated using the comparative Ct ($2^{-\triangle Ct}$) method, with Cyclophilin A serving as the endogenous control.

Statistical Analysis

Data are presented as means ± SEM. The effects of maternal n3FA supplementation, during pregnancy and lactation, on offspring erythrocyte fatty acid composition and RT-PCR expression data were determined by Student's unpaired t-test. Effects of supplementation on maternal weight change during pregnancy and lactation, and postnatal weight gain in male and female offspring was determined using an ANOVA with repeated measures. When significant levels ($p<0.05$) were achieved, a Tukey's Post Hoc test was performed. In tables and figures, *, **, and *** represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

RESULTS

Effects of maternal n3FA supplementation on maternal parameters

Before mating, the weights of the mothers were comparable between the two dietary groups. Prior to commencement of supplementation, there were no differences in n3FA content in maternal erythrocytes between the dams fed on the two diets (Basal, 6.01 ± 0.09%; n3FA, 6.40 ± 0.33%). At weaning, the levels of DHA (Basal, 2.30 ± 0.11%; n3FA, 3.68 ± 0.22%) and total n3FAs (Basal, 3.81 ± 0.04%; n3FA, 5.42 ± 0.26%) were significantly higher in the n3FA supplemented dams than in the basal control ($P<0.001$). There was no effect of maternal n3FA supplementation on maternal weight gain during pregnancy or weight loss during lactation, and there were no differences in maternal body weight at the end of lactation between the basal and n3FA groups as previously reported (28). Maternal omega-3 intake had no effects on maternal food intake during either pregnancy or lactation (28).

Effects of maternal n3FA supplementation on pregnancy outcomes and offspring fatty acid status

Maternal supplementation with n3FA during pregnancy and lactation resulted in increased offspring erythrocyte DHA ($p<0.001$) and total n3FA levels ($p<0.001$) at 3-weeks of age (Figure 1A, B). Despite n3FA supplementation being ceased at weaning, increased DHA and total n3FA levels persisted until 6-weeks of age ($p<0.001$), but had returned to control levels by 3-months of age. Interestingly, 3-week old females offspring from n3FA supplemented dams exhibited a marked increase in erythrocyte levels of arachidonic acid (AA) (Basal: 18.90 ± 0.45 vs n3FA: 20.86 ± 0.60; $P=0.044$) and linoleic acid (LA) (Basal:18.90 ± 0.45 vs n3FA: 20.86 ± 0.60; $P=0.044$) when compared to basal females and n3FA supplemented males (n3FA female: 20.86 ± 0.60 vs n3FA male 17.55 ± 0.69; $P=0.028$). Levels of AA and LA returned to basal levels by 6-weeks of age (data not shown).

As reported previously (28), maternal n3FA supplementation did not alter body length or body weight of the offspring at the ends of critical growth periods (3 week, 6 week and 3 months of age). n3FA

supplementation also did not affect the length of gestation, litter size or mean pup birth weight (28). Male/females offspring ratios were also comparable (28).

Effect of maternal n3FA supplementation on offspring growth plate structures

Histological analysis of proximal tibial bones from basal (Figure 2A) and n3FA supplemented offspring (Figure 2B) revealed that while n3FA supplementation did not affect overall thickness of the growth plate at any time point (Figure 2C), n3FA supplementation significantly increased the height of the resting zone in both male ($p<0.02$) and female ($p<0.01$) offspring at 3-weeks of age (Figure 2D). There was no effect of n3FA supplementation on the thickness of the resting zone at either 6 weeks or 3 months of age. There was also no effect of maternal n3FA supplementation on the morphometric features of the proliferative zone or hypertrophic zone irrespective of age or sex (data not shown).

Effect of maternal n3FA supplementation on structural changes in the metaphysis of offspring

Examination of H&E stained sections of the proximal tibia showed that the primary spongiosa height in both male and female offspring was unaffected by maternal dietary n3FA supplementation (results not shown). Further histological analysis of the trabeculae in the secondary spongiosa showed little variability across the diets or sexes; however, when compared to the basal group, 3-week old male, but not female, offspring of dams supplemented with n3FA exhibited an increased bone volume/tissue volume ratio (BV/TV%) (Figure 3A, B, C). The increase in BV/TV% was attributed to an increase in both the average trabecular thickness ($p<0.05$; Figure 3D) and an increase in trabecular number in the n3FA 3-weekold male offspring compared to Controls ($p<0.001$; Figure 3E).

Changes in osteoclast density and expression of osteoclastogenesis regulatory genes in the metaphysis

Consistent with the increased BV/TV%, 3-week old male offspring of n3FA dams exhibited a decreased number of osteoclasts per millimetre of trabecular bone surface ($p<0.05$; Figure 4A, B, C). Furthermore, RT-PCR gene expression analysis revealed a significant decrease in mRNA expression

of the critical osteoclast formation gene, RANKL ($P<0.01$; Figure 4D) but no change in osteoclastogenesis inhibitor, OPG ($P>0.05$; Figure 4E) in these offspring. Thus, the RANKL/OPG ratio was significantly decreased in 3-week old n3FA supplemented male offspring compared to controls ($P<0.05$; results not shown). mRNA expression of pro-inflammatory cytokines TNF-a ($P<0.05$; Figure 4F) and IL-6 ($P<0.05$; Figure 4G) were also decreased in 3-week old male offspring of n3FA supplemented dams, indicating that maternal supplementation with n3FA decreases expression of signals important for osteoclast formation and activity. There was no effect of maternal n3FA supplementation on any of these measures in 3-week female offspring or in 6-week or 3-month old offspring of either sex.

Changes in osteoblast density in the metaphysis

Previously n3FA supplementation has not only been shown to affect osteoclast number and activity, but also the number of bone forming osteoblasts (36). To examine whether the increase in BV/TV% in the 3-week old male offspring of n3FA-supplemented dams were attributed to alterations in osteoblast formation and density, osteoblast numbers on bone trabecular surfaces of the 3-week old male offspring were also analysed. Analysis of H&E stained sections of secondary spongiosa showed that n3FA-supplemented offspring had an increased number of osteoblasts lining the bony trabeculae ($p<0.05$) compared to controls (Fig. 5A, B, C). Consistent with the higher osteoblast density, offspring in the n3FA group exhibited increased mRNA expression of osterix, a transcription factor essential for osteoblast differentiation and maturation ($P<0.05$; Figure 5D). There was no effect of maternal n3FA supplementation on mRNA expression of the bone matrix protein osteocalcin in the offspring at any time point (Figure 5E).

Effects of maternal n3FA supplementation on ex vivo osteogenic potential

In order to examine the effects of n3FA supplementation on osteoblast differentiation from bone marrow stromal progenitor cells, an ex vivo CFU-F assay plus ALP staining was performed with bone marrow stromal cells isolated from the offspring. The number of ALP^+ colonies (or number of osteoprogenitor cells) was significantly increased in 3-week old male offspring of n3FA

supplemented dams compared to the controls ($P<0.01$; Figure 5F). In addition, as a measure of the treatment effects on late stage osteogenic potential of the bone marrow stromal cells, a mineralisation assay with the bone marrow cells showed that maternal n3FA supplementation resulted in an increase in the mineralising potential of bone marrow stromal cells in the 3-week old male offspring compared to the basal diet control (Figure 5G).

DISCUSSION

Recently it has become increasingly apparent that nutrient supply before birth and in early infancy is important for achieving optimal bone growth and peak bone mass during childhood/adolescence. Although n3FA has been previously shown to improve bone deposition and bone mass in growing animals and in adult humans, no previous studies have assessed whether supplementing the maternal diet with n3FA improves bone health in the offspring. Here we have shown that maternal n3FA supplementation during pregnancy and lactation leads to elevated n3FA RBC concentrations in the offspring. This increase resulted in several positive outcomes within the metaphysis of long bones in 3-week old male offspring. Interestingly, the beneficial effects in the bone were found to be quite short lived, suggesting that whilst there may be short-term benefits of increased perinatal exposure to n3FA on bone growth and development in males, the effects are unlikely to be maintained into adolescence and adulthood when these offspring are weaned onto a standard (low n3FA) chow diet.

Maternal n3FA supplementation increases omega-3 index in the offspring

It is known that eicosapentaenoic acid (EPA) and DHA are never completely absent from breast milk, and the level is largely determined by the mother's diet (37, 38). This was apparent in the current study as erythrocyte EPA, DHA and total omega-3 LCPUFA concentrations in n3FA supplemented pups were significantly higher than in the control group. It is important to note that these levels remained elevated up to 6 weeks of age, even though both control and n3FA groups had been consuming standard rat chow since weaning. This therefore suggests that fatty acids incorporated into erythrocyte phospholipids before and during the suckling period can be retained after weaning and these have the potential to have prolonged biological effects (28). As it has been previously shown

that it takes 2-3 weeks for dietary fat composition changes to alter erythrocyte fatty acid composition, this result was not unexpected (39).

Can early exposure to n3FAs promote bone development?

Whilst previous studies have reported beneficial effects of n-3 LCPUFA supplementation on bone strength in adolescent and adult animals, our current study showed that n3FA supplementation did not appear to alter the structure of the region of the bone responsible for determining the future length and shape of the mature bone – the epiphyseal plate/growth plate. While there is very little data concerning the effects of n3FAs on specific regions of growth plate, studies investigating the effects of n3FAs on chick epiphyseal growth plate cartilage have indicated that dietary lipids are able to affect cartilage metabolism (40). In this study, treating chick chondrocytes with the omega-6 (n6) PUFA, linoleic acid (LA), resulted in decreased collagen synthesis, whereas treatment with n3FAs increased collagen synthesis (40). The epiphyseal plate of the growing chick has also been reported to be able to selectively incorporate certain dietary fatty acids (41). In the current study, maternal supplementation with n3FAs did not affect the overall height of the growth plate of the offspring. Interestingly, the size of the resting zone, the region comprising of undifferentiated or resting cartilage cells, was increased in both male and female 3-week old offspring of n3FA supplemented dams. The functional significance of this change is unclear, however it is unlikely to have translated into any long term effects on bone health, as all parameters of the growth plate were again comparable by 6-weeks of age.

The n3FA have long been implicated in bone homeostasis (20, 24, 42-44) with increasing evidence that lack of n-3FA can induce bone loss (22, 45), while dietary n3FA supplementation may promote bone formation (20, 22). In the current study we have shown that maternal supplementation with n3FAs during pregnancy and lactation did not result in any increases in the height of primary spongiosa bone, the region containing woven trabecular bone that is newly converted from the calcified hypertrophic growth plate cartilage (46). However, 3-week old male offspring of n3FA supplemented dams had an increased number and thickness of trabeculae within the secondary

spongiosa resulting in an overall increase in the ratio of bone volume to total volume (BV/TV%), accompanied by a significant decrease in the number of resorptive osteoclasts. This result is consistent with existing studies which have demonstrated the ability of n3FA to inhibit osteoclast number and function both in vitro (47) and in vivo (19, 21, 48), and suggests that increasing the supply of n3FA during the early period of bone development has the potential to promote bone formation.

There are currently two known mechanisms by which n3FA inhibit osteoclastogenesis. Firstly, these FA can act indirectly by increasing osteoblast number and RANKL expression and secondly, via a more direct method, PUFA metabolites (ie prostaglandins or PGs) acting to inhibit osteoclast progenitors and osteoclast formation (47). The results of the present study, consistent with the literature, have shown that supplementation with n3FA during pregnancy and lactation decreases the number of osteoclasts present on the bone surface in the 3-week male offspring. This appeared to be due to an increased mRNA expression of OPG, which has an important role in inhibiting osteoclastogenesis (49, 50) and suppressing osteoclast survival (51), coupled with a simultaneous downregulation of the expression of RANKL mRNA, which is critical for osteoclast formation and differentiation, in the metaphysis of the growing offspring. Consistent with previous studies in adult rats which showed that n3FA supplementation inhibits osteoclast formation/activation and bone resorption by suppressing the expression of proinflammatory cytokines and osteoclastogenic factors (19), the current study showed that maternal n3FA supplementation had also decreased the expression of pro-inflammatory cytokines, TNF-a and IL-6, in the 3-week old male offspring.

Not only has n3FA supplementation been shown to suppress formation of osteoclasts, but it has also been associated with enhanced activity of bone forming osteoblasts (19, 43), such that the increase in BV/TV% in the present study could be due not only to a reduction in osteoclastogenesis, but also enhanced osteogenesis. In support of the relationship between dietary PUFAs and bone metabolism, it has previously been reported that feeding post-weaning male Fisher rats DHA substantially increased bone marrow cell number (52). As the bone marrow consists of precursor cells including

mesenchymal stem cells, it is possible that the higher number of marrow cells could translate into an increase in the osteoblastogenic potential (53). Runx2 or cbf-α1, an essential transcription factor for the commitment of mesenchymal cells to the osteogenic lineage, has previously been reported to be increased in fetal rat calvarial cells when stimulated with n3FA (EPA) (43). In the present study, we showed that maternal n3FA supplementation could enhance the differentiation of pre-osteoblast like cells into mature mineralizing osteoblasts in the offspring with the ability to form mineralized extracellular matrix.

In the literature there is increasing interest in the concept of programming, the idea that "a stimulus or insult during a critical or sensitive period of development can have long-term or lifetime effects" (4). There are numerous studies investigating early nutrition “programming” in animals but now randomized intervention trials with long-term follow-up are appearing, showing nutrition in early life has a major impact on health into early adulthood, including cardiovascular disease risk and bone health (54, 55). The ability of particular treatments to induce long-term effects is dependent on both the duration and timing during development when it is applied. For example, Jones et al (55) found a positive correlation between duration of breastfeeding and increased bone mineral density at 8 years of age, and concluded that breastfeeding for less than 3 months was not associated with increased bone mass at any site in the children. This suggests, therefore, that the lack of a sustained effect of maternal n3FA supplementation on structure and function of bone in the offspring, may have been because the duration of supplementation in the current study was insufficient to maintain the positive bone changes, the timing of supplementation was not right, or a combination of both these factors. It is also likely that the children in the few studies concerning early nutrition and later bone mass received sufficient amounts of n3FAs from their diets post breastfeeding. In contrast, the rats in the current study were all weaned onto a standard rat chow which contained very low levels of EPA and DHA, and n3FA levels in the offspring of n3FA supplemented dams had returned to the levels seen in control offspring by 3 months of age. This implies that any n3FA that were stored in offspring tissues at the end of the intervention (weaning) were likely depleted as dietary intake was no longer able to

meet requirements for n3FAs throughout the rest of the critical growth period, and hence lasting effects were not observed. It will be important in future studies to determine whether these positive effects are maintained if the offspring are maintained on a high n3FA diet post-weaning.

Offspring gender difference in the bone benefit effects of maternal n3FA supplementation

This study has shown that there is a significant difference between the effects of n3FA maternal supplementation between male and female offspring. The slightly higher levels of DHA (P=0.055) and increased AA levels (P=0.028) in the female supplemented offspring, when compared to their male counterparts, could be due to gender differences in sex hormone status and therefore different hormonal regulation of PUFA metabolism. This is supported by clinical data, which shows that estrogens, but not androgens, can increase concentrations of AA and DHA in the blood lipids of patients treated with sex hormone steroids (56, 57). Other studies have also shown that female rats exhibit higher DHA levels in their livers (58) and replete their DHA status more readily than males (59). The molecular, cellular and bone volume differences seen between our 3-week old supplemented male and female offspring are possibly due to the elevated levels of AA present in the female offspring. AA and its metabolite PG-E_2 are proinflammatory and several studies have established the significance of PG-E_2 in osteoclast formation and bone resorption (60-62) . However, DHA is able to inhibit PG-E_2 synthesis (63) perhaps by replacing AA in the cell membrane and therefore limiting the amount of AA available for PG-E_2 production (64). It is possible that the increased levels of AA in the female offspring provide increased competition with the n3FAs and hence could be dampening the positive effects that were seen in the n3FA supplemented male offspring.

Summary

We have therefore reported that maternal n3FA supplementation during pregnancy and lactation translates into elevated DHA, EPA and total LC-PUFA RBC concentrations in all offspring at 3- and 6-weeks of age, and is associated with an increased number of osteoblasts and a decreased osteoclast density, and consequently a higher volume of trabecular bone only in 3-week old male, but not

female, offspring and that these effects are not maintained at 6-weeks or 3 months of age. These results, as in some previous reports, have established that there are sex-dependent differences in the beneficial effects of n3FA and indicate that further research is needed in order to derive sex-specific nutritional/supplement recommendations. In addition, this study has demonstrated that although n3FA supplementation throughout pregnancy and lactation can exert positive effects on early bone development, at least in males, the positive effects diminish once the supplementation has ceased and n3FA tissue stores have returned to control levels. Overall, the results of this study suggest that increasing the supply of dietary n3FA during the prenatal and early postnatal period has no lasting benefits for bone health in the offspring, and that the maintenance of a high intake of n3FA post-weaning is likely to be required in order to achieve lasting effects.

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Figure legends:

Figure 1: **Effects of maternal dietary n3FA supplementation during pregnancy and lactation on erythrocyte n3FA and LC-PUFA concentrations of offspring**. n3FA supplementation increased red blood cell concentrations of DHA **(A)** and total LC-PUFAs **(B)** in 3-week and 6-week old offspring ($p<0.001$) which returned to control levels by 3-months of age.

Figure 2: **Effects of maternal dietary n3FA supplementation during pregnancy and lactation on the total thickness and individual zonal heights of growth plate in proximal tibia** of offspring. H&E section of control **(A)** and n3FA supplemented (**B)** offspring rat at weaning (21d). The dashed lines in A and B arbitrarily separate the three individual growth plate zones (Resting, Rz; Proliferative, Pz; Hypertrophic, Hz) (Bar = 200μm), and the sum of individual zonal heights amounts to the total growth plate thickness **(C)**. No differences in zonal heights were detected, with exception to the resting zone which was increased in both male and female offspring of n3FA-supplemented dams ($P<0.001$) **(D).**

Figure 3: **Effects of maternal dietary n3FA supplementation during pregnancy and lactation on the trabecular bone volume and architecture in the proximal tibial metaphysis** of the offspring. H&E stained sections of control metaphysis **(A)** and n3FA supplemented metaphysis **(B)** at weaning (Bar = 200μm). Histological analysis shows an increase in bone volume/tissue volume (BV/TV%) in male but not female offspring **(C)**, which was associated with increases in trabecular thickness **(D)** and trabecular number **(E)**.

Figure 4: **Osteoclast density on trabecular bone surface and mRNA expression of important regulatory genes of osteoclastogenesis in the metaphysis** of control and n3FA supplemented offspring. TRAP- stained sections of control metaphysis **(A)** and n3FA supplemented metaphysis **(B)** (Bar = 100μm). Osteoclasts are TRAP-positively stained cells

adhering to the trabecular surface, and numbers counted and expressed as TRAP+ cells/mm trabecular perimeter surface **(C**). Relative expression was calculated using the comparative Ct ($2^{-\triangle Ct}$) method, with Cyclophilin A serving as the endogenous control: RANKL **(D)**, OPG **(E),** TNF-alpha **(F)**, Interleukin-6 **(G)**.

Figure 5: **Osteoblast density, mRNA expression of important osteogenic genes and in vitro analysis of osteogenic potential in the metaphysis** of 3-week old control and n3FA supplemented male offspring. Osteoblasts were identified and counted via routine H&E staining in sections of control **(A)** and n3FA supplemented metaphysis **(B)** (Bar = 100μm). Osteoblasts were identified as cuboidal cells lining that metaphyseal surface and expressed as cells/mm trabecular perimeter surface **(C)**. Relative real-time RT-PCR analysis of mRNA expression of important osteogenic genes osterix **(D)** and osteocalcin **(E)** expression was calculated using the comparative Ct ($2^{-\triangle Ct}$) method, with Cyclophilin A serving as the endogenous control:. In vitro analysis of osteogenic potential was carried out via colony forming unit -fibroblast assay, expressed as ALP+ colonies per well **(F)** and colormetric analysis of Alizarin Red staining after stimulation with osteogenic media **(G)**.

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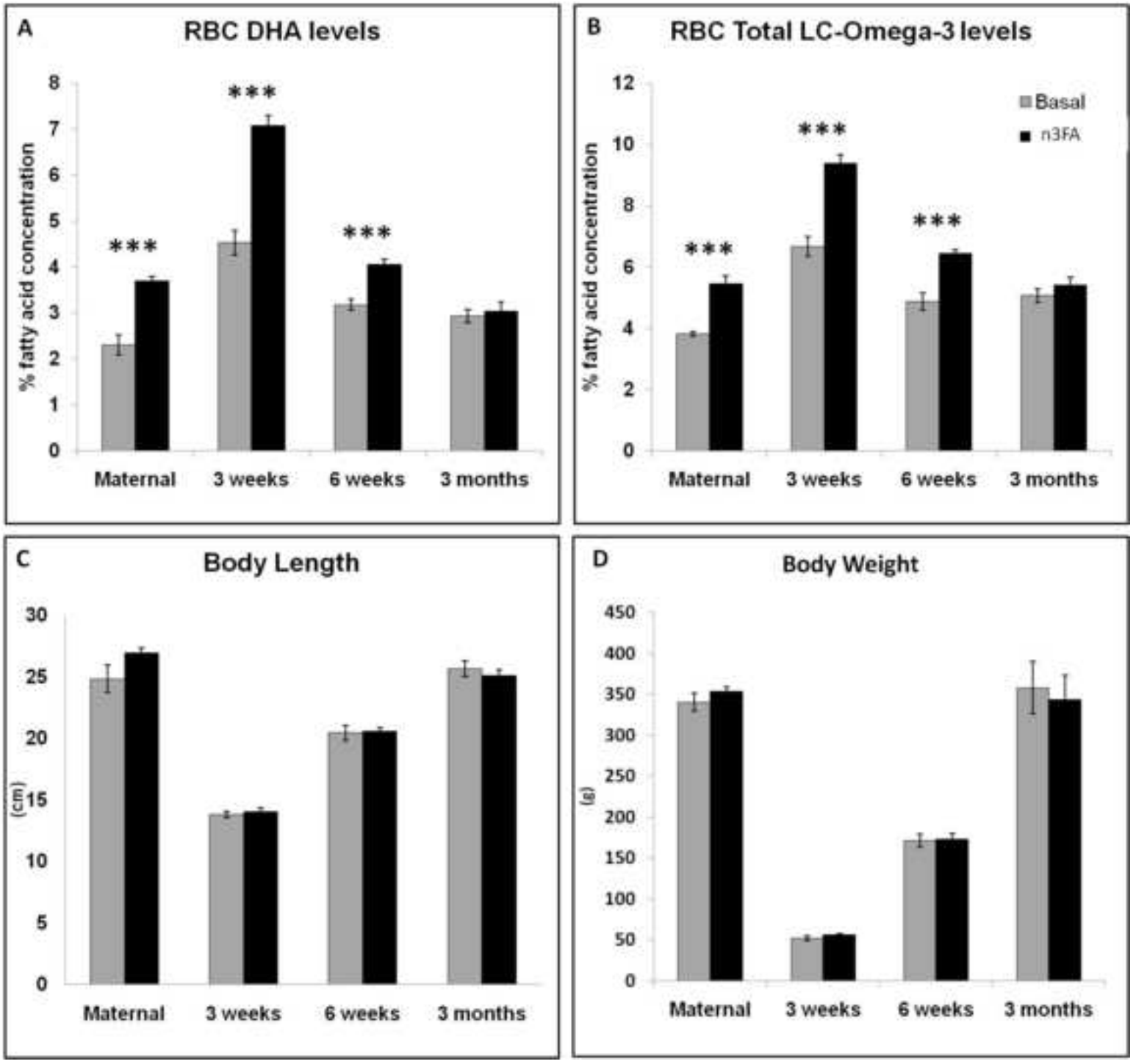

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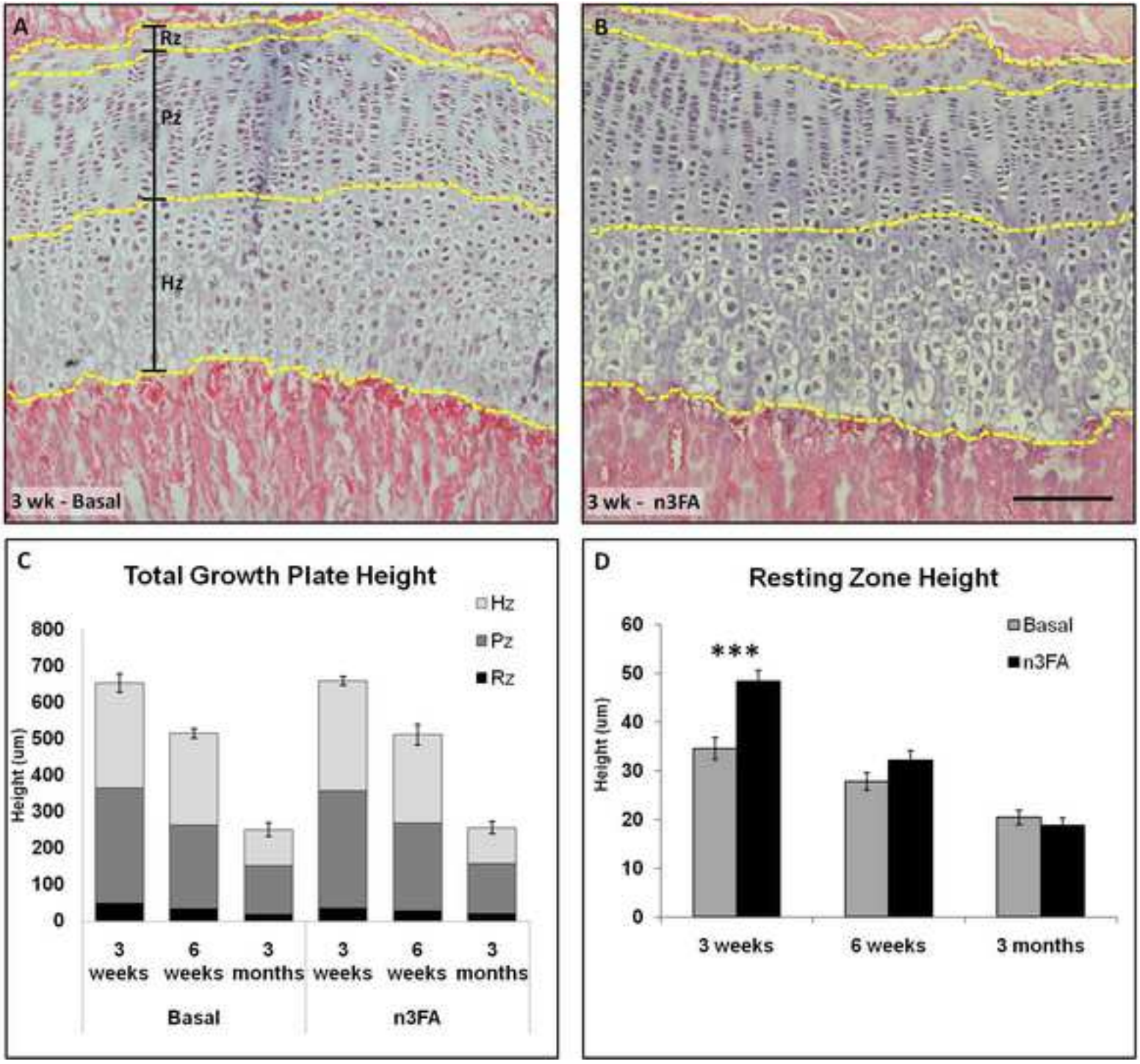

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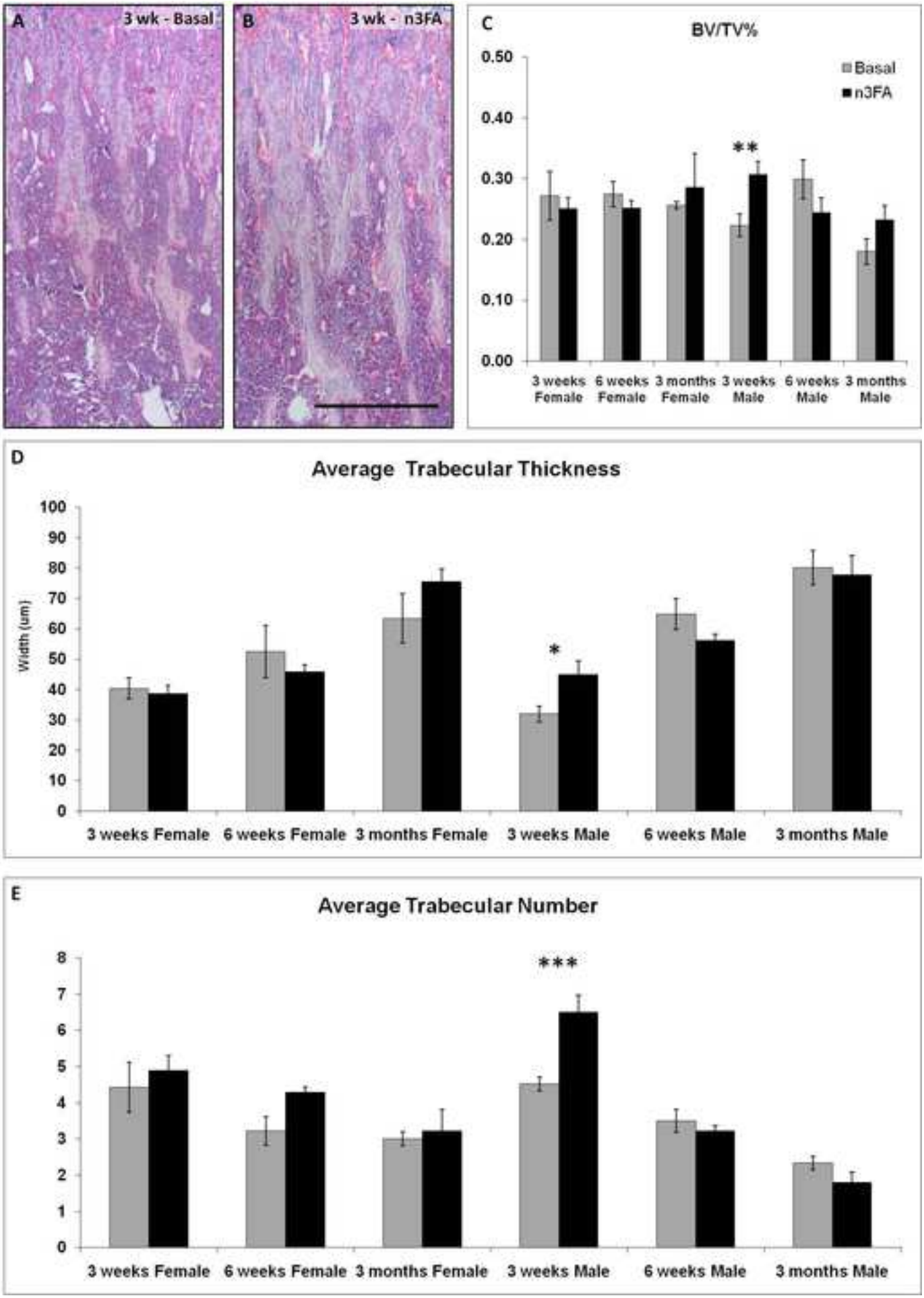

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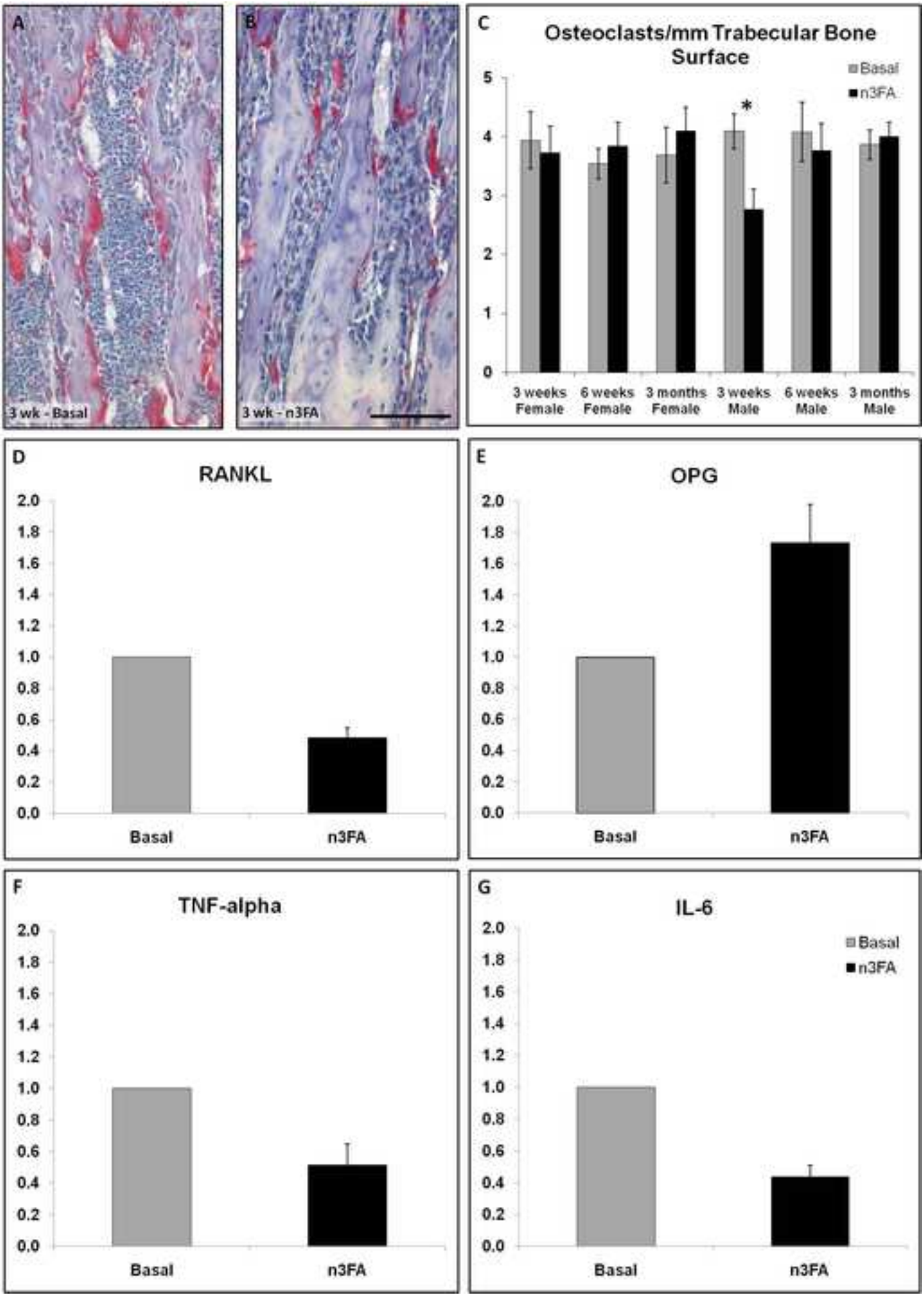

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