Female rats consuming an iron and omega-3 fatty acid deficient diet preconception require combined iron and omega-3 fatty acid supplementation for the prevention of bone impairments in offspring

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Abstract

We previously showed in rats that pre- and postnatal deficiencies in iron and omega-3 (n-3) fatty acids can impair bone development, with additive and potentially irreversible effects when combined. This study aimed to investigate, in female rats consuming a combined iron and n-3 fatty acid deficient (ID + n-3 FAD) diet preconception, whether supplementation with iron and docosahexaenoic/eicosapentaenoic acid (DHA/EPA), alone and in combination, can prevent bone impairments in offspring. Using a 2 × 2 factorial design, female Wistar rats consuming an ID + n-3 FAD diet preconception were randomised to receive an: 1) iron supplemented (Fe + n-3 FAD), 2) DHA/EPA supplemented (ID + DHA/EPA), 3) Fe + DHA/EPA, or 4) ID + n-3 FAD diet from gestational day 10 throughout pregnancy and lactation. Post-weaning offspring (n = 24/group; male/female = 1:1) remained on the respective experimental diets for three weeks until postnatal day 42–45. Offspring born to female rats consuming a control diet preconception and an Fe + DHA/EPA diet throughout pregnancy and lactation served as non-deficient reference group (Control + Fe + DHA/EPA). Bone mineral density (BMD) was measured using dual-energy X-ray absorptiometry and bone strength using three-point bending tests. Only offspring in the Fe + DHA/EPA group had significantly higher spine and femur BMD, and higher femur stiffness than offspring in the ID + n-3 FAD group, and had similar spine BMD and femur stiffness as the Control + Fe + DHA/EPA group. Offspring in the Fe + DHA/EPA group further had significantly higher femur strength (ultimate load) than the other experimental groups, and a similar femur strength as the Control + Fe + DHA/EPA group. This study shows that only combined iron and DHA/EPA supplementation can prevent bone impairments in offspring of female rats consuming an iron and n-3 FA deficient diet preconception.

Introduction

Almost 20% of the global population is suffering from osteoporosis.1,2 Peak bone mineral density (BMD) is a crucial factor in the development of osteoporosis, as a 10% increase in peak BMD is predicted to delay the onset of osteoporosis by 13 years.3 A 10% increase in peak bone mass is also estimated to reduce osteoporotic fracture risk in postmenopausal women by 50%.4 Osteoporosis has even been labelled as a “paediatric disease with geriatric consequences”.5 As it is evident that the prevention of osteoporosis begins with optimal bone development during early life. While some factors such as genetic risk are non-modifiable, lifestyle factors such as nutrition can influence 20%–40% of peak bone mass.6 The International Osteoporosis Foundation’s 4th priority, therefore, states that “expectant mothers should be well nourished to support an infant’s development in utero” and “children and adolescents (should) achieve genetic potential for peak bone mass through a nutritious diet with adequate calcium intake and regular physical activity”.7 However, besides calcium, there are other nutrients that may play an important role in bone development.

Iron intake and status have been associated with osteoporosis or BMD in adult men and women.8-12 Animal studies have shown that iron deficiency (ID) can lower bone strength indicators.13-18 ID is the most common micronutrient deficiency, especially in pregnant women and young children.19 Prenatal ID may lead to maternal anaemia and other pregnancy complications, as well as detrimental health outcomes in the offspring, likely including effects on bone development.10,20-22 The World Health Organisation, therefore, recommends daily oral iron and folic acid supplementation in pregnant women.23 The effects of antenatal iron supplementation on bone health and development are less known. A study in young ID women

Keywords:
Iron; omega-3 fatty acids; prenatal supplementation; bone development

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found no effect of iron supplementation on bone turnover markers. Premenopausal ID women who recovered from anaemia with pharmacological treatment, however, showed improvements in bone turnover markers.

Another nutrient essential for healthy pregnancies and optimal fetal growth and development is omega-3 (n-3) fatty acids (FA). Intakes of n-3 FA, especially the functionally important n-3 long-chain polyunsaturated FAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are often insufficient in pregnant and lactating women in developing countries. An increase in the intake of omega-6 (n-6) FAs in developed countries has also resulted in an increased n-6/n-3 ratio. A higher maternal n-6/n-3 ratio during pregnancy may lead to unfavourable outcomes in offspring, such as low birth weight, obesity, and impairments in neurocognitive and immune function. Evidence shows that n-3 FA supplementation during pregnancy can reduce the risk for preterm delivery and might be beneficial for offspring health outcomes like birthweight, neurological outcomes, and allergy risk, but the evidence is not conclusive enough yet to recommend routine n-3 FA supplementation during pregnancy.

Effects of n-3 FA supplementation during pregnancy on bone outcomes in offspring are less known. Animal studies have shown that offspring bone development can be influenced by maternal FA status, and different effects have been observed between male and female offspring. To our knowledge, no studies have determined associations of maternal n-3 FA status with bone development in offspring in humans. Human studies have, however, shown positive associations between n-3 FA status and bone outcomes in adults. One study in children further found higher n-3 FA intake to be associated with higher lumbar spine BMD. In contrast, another in boys found no association between serum total n-3 phospholipid FAs and femur, lumbar spine, or total body BMD. In older adults, n-3 FA supplementation was associated with higher BMD. The effects of n-3 FA supplementation on bone might depend on the type of FA, with stronger evidence for a beneficial effect of DHA and EPA than alpha-linolenic acid (ALA).

A lower n-6/n-3 FA ratio in bone compartments was further shown to increase bone modelling and bone strength in rats.

Data on the interactions between iron and n-3 FAs during bone development are scarce. In a previous study, we found that ID during early life has a negative effect on bone development, with potential compounding effects when combined with n-3 FA deficiency. The effects of ID alone were reversible when offspring received a sufficient diet post-weaning, however, a combined iron and n-3 FA deficiency resulted in irreversible deficits in measured bone outcomes. Further, both iron and n-3 FA status have previously been associated with alterations in insulin-like growth factor-1 (IGF-1), a hormone essential for normal growth and bone development.

The aims of this study were, firstly, to investigate in female rats consuming an ID þ n-3 fatty acid deficient (FAD) diet preconception, whether pre- and postnatal iron and DHA/EPA supplementation, alone and in combination, can prevent impairments in bone development in offspring and, secondly, to determine whether effects are sex-specific. We hypothesised that possible bone impairments in offspring from female rats that consumed a combined ID and n-3 FAD diet preconception cannot be prevented with pre- and postnatal iron or DHA/EPA supplementation alone but can be prevented with combined iron and DHA/EPA supplementation.

### Materials and methods

#### Experimental design

Forty female Wistar rats at 21 ± 3 days of age (postnatal day [PND] 21) and a minimum weight of 25 g were housed in pairs and placed on the control diet for a two-week period of preconditioning as illustrated in Fig. 1. At the end of the preconditioning phase (at PND 35), n = 8 rats were randomly selected (using Excel’s computerised random number generator) to remain on the control diet, while the rest were placed on an n-3 FA deficient diet for seven weeks before mating, to imitate a chronic n-3 FAD diet. Three weeks before mating, at PND 63, these female rats were switched to a combined ID þ n-3 FAD diet.

At PND 84, the female rats were placed in individual cages and mated with ~PND 84 male breeders of the same strain (maintained on standard laboratory chow) using monogamous breeding (one male bred with one female). Males were accustomed to purified control diets a few days prior to adding them to the females' cages. Conception was confirmed by the presence of a vaginal plug (gestational day [GD] 0). Ten days after conception (GD 10), the pregnant dams were randomly allocated to one of three supplementation diet groups: 1) iron [Fe] + n-3 FAD (n = 8); 2) ID + DHA/EPA (n = 8); 3) Fe + DHA/EPA (n = 8), or remaining on the ID + n-3 FAD (n = 8) diet (deficient reference group). The dams were maintained on this diet throughout pregnancy. The female rats allocated to the control diet at PND 35 were also switched to a combined iron and n-3 supplemented diet [Control + Fe + DHA/EPA (n = 8)] from GD 10 throughout their pregnancy (non-deficient reference group). Maternal body weights and food intake were recorded to the nearest 0.1 g every second day during the gestational period. During the mating period, however, the rats were minimally handled and only during the light phase, as mating takes place during the dark phase.

Dams were allowed to deliver spontaneously at approximately GD 22 (PND 0). Dam weights after parturition, litter size and litter weights at birth were recorded. Within three to five days after birth, the litters were culled to eight pups (to maintain nutritional adequacy), with ideally four males and four females per litter [eight pups/litter; minimum of seven litters/group].

The dams stayed with their pups and were maintained on their respective experimental diets throughout lactation. At the start of the post-weaning period (PND 21) the dams and pups that were no longer used for the experiment were euthanised. Thereby, we aimed for a sample size of 24 pups per experimental group, with a male:female ratio of 1:1, and representing a minimum of eight litters (dams) per experimental group. The pups were maintained on their respective experimental diets for three weeks until PND 42–45. During this period, the littermates were housed in pairs by sex. All offspring were weighed three times per week. At PND 42–45, the offspring were euthanised and samples were collected.

Sample sizes were determined by power calculations appropriate for animal model studies in nutrition research as described by Ricci et al.

#### Animals and diets

The rats in this experiment were bred and housed in a temperature and humidity-controlled (22 ± 2 °C and 55 ± 15% relative humidity) animal facility (Vivarium of the Preclinical Drug Development Platform of the North-West University [NWU] in Potchefstroom, South Africa) with a reversed 12-h dark-light cycle (lights off at 06:00 a.m.).
Rats were pair-housed in standard individually ventilated solid floor cages (Techniplast GR900 SealSafe R Plus; 300 mm × 300 mm × 189 mm) with low trace element (<2.00 ppm iron) alpha-cellulose bedding (Alpha-Dri R; LBS Serving Biotechnology Ltd, Horley, UK). All rats had ad libitum access to purified diets and demineralised water (18 MΩ).

Purified experimental diets were obtained commercially from Dyets Inc. (Bethlehem, USA) and were based on the American Institute of Nutrition (AIN) 93G purified diets for laboratory rodents for growth, pregnancy, and lactation.69 All diets were isocaloric and contained 10% fat. The diets were modified in their iron content and FA composition (Table 1). The basal AIN-93G formulation (control diet) contained 40 mg/kg iron, while the ID and iron supplemented diets contained 15–18 mg/kg iron and 80 mg/kg iron, respectively. The control diet contained ca. 5 g/kg total n-3 FA, mainly in the form of ALA, the n-3 FAD diet 0.1 g/kg total n-3 FA,70-72 and the DHA/EPA supplemented diets contained 6 g/kg total n-3 FA in the form of ALA, EPA, and DHA. The analysed FA and iron contents of the diets are presented in Supplemental Table 1.

**Sample collection, storage, and analyses**

After euthanasia by decapitation, trunk blood was collected and stored as previously described by Strydom et al.18 Total red blood cell (RBC) phospholipid FA analysis was performed as described by Baumgartner et al.,73 as a modification of the method by Folch et al.76

Haemoglobin concentrations were measured in whole blood using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden). Liver tissue was collected, aliquoted, snap frozen, and stored for liver iron analysis as described previously.18

Analysis of BMD was done ex vivo in the lumbar spines and right femurs18 using dual-energy X-ray absorptiometry (Hologic Discovery W DXA scanner with APEX system software version 2.3.1). Left femurs were used to measure the femur weight (g), length (mm), and cross-sectional area (mm²). The left femurs were also used to perform three-point bending tests with a servo-hydraulic machine (Instron model 1026).18 The bottom supports (3 mm wide with rounded points) were placed 8 mm apart, with a...
perpendicular force (load) exerting pressure at the midpoint in the anterior-posterior direction. Force deformation curves were used to determine mechanical properties, such as ultimate load (a complete break, measured in newton [N]), ultimate deflection (deflection at the point of ultimate load, measured in mm), ultimate stress (ultimate load over cross-sectional area, measured in megapascal [MPa; N/mm²]), and ultimate stress (ultimate load over cross-sectional area, measured in megapascal [MPa; N/mm²]). During elastic deformation no damage is incurred on the bone, therefore the bone will return to its original state and internal strength if the load is removed during this stage. At the point of transition from the elastic to the plastic region (after which the bone is damaged permanently due to structural changes but not necessarily completely broken yet), the load, deformation, and stress were also determined. The bone stiffness was determined by the gradient of the linear line (elastic region) of the load over deformation, where a higher stiffness is evident of a more brittle bone.

Body weight, haemoglobin, liver iron, FA markers, and BMD were measured in all pups within the different diet groups. Some measurements, however, were unsuccessful and the total usable numbers are reported in the Results section.

Ferritin and IGF-1 were analysed in a sub-sample (n = 12 per group) of plasma using rat ferritin and IGF-1 enzyme-linked immunosorbent assay (ELISA) kits (Sandwich-ELISA principle) provided by Elabscience (Wuhan, China) as per ELISA kit manual.

Researchers were blinded for diet groups during weight measurements, sample collections, and laboratory analyses.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics software (version 27) and Excel 2016. Data were examined for normality of distribution (using q-q plots, histograms, and the Shapiro–Wilk test) and the presence of outliers (using box plots and standard deviation). Homogeneity of variance were examined by the Levene’s test.

The effects of pre- and postnatal iron and DHA/EPA supplementation, as well as their interactions, on BMD and indices of bone strength at PND 42–45 were determined using 2×2×2 ANCOVA (iron [sufficient vs. deficient] × n-3 FA [sufficient vs. deficient] × sex [male vs female]). When no significant interaction with sex was obtained, sex was included in the models as a covariate. Even though many main effects of sex were observed, only diet × sex interactions are discussed. As body weight can influence bone development, and indices of bone strength at PND 42–45 were determined using one-way ANCOVA followed by Bonferroni’s post hoc test and adjusted for sex and body weight. Cross-sectional area of the femur was also included as a covariate in femur load and displacement as the size of a bone can influence the strength thereof. No adjustment for cross-sectional area is made when ultimate or transition stress is considered, however, as the area is used to calculate the stress.

Dunnett’s post hoc test was used to compare each of the four experimental diet groups (ID + n-3 FAD, Fe + n-3 FAD, ID + DHA/EPA and Fe + DHA/EPA) to the non-deficient reference group (Control + Fe + DHA/EPA).

Data are expressed as means ± standard error of means (SEM), and the significance of all tests was set at P < 0.05.

Results

Growth, iron, and n-3 fatty acid status of the offspring

Table 2 provides results on body weight, iron, and n-3 FA status for offspring at PND 42–45, adjusted for sex.

There were iron × sex interactions on body weight at PND 42–45 (p < 0.001) and weight gain from PND 21 (p < 0.001) in the offspring. In male offspring, both iron and DHA/EPA supplementation resulted in higher body weight at PND 42–45 (iron, p < 0.001; sex, p < 0.05).
Table 2. Offspring body weight, iron, and n-3 fatty acid status at postnatal day 42–45

<table>
<thead>
<tr>
<th></th>
<th>Control + Fe + DHA/EPA</th>
<th>n</th>
<th>ID + n-3 FAD</th>
<th>n</th>
<th>Fe + n-3 FAD</th>
<th>n</th>
<th>ID + DHA/EPA</th>
<th>n</th>
<th>Fe + DHA/EPA</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Means ± SEM for different groups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Male body weight (g)</strong></td>
<td>255 ± 9.1</td>
<td>10</td>
<td>191 ± 4.3‡**</td>
<td>12</td>
<td>214 ± 3.8‡‡*</td>
<td>10</td>
<td>207 ± 8.4‡‡*</td>
<td>12</td>
<td>235 ± 5.3‡*</td>
<td>13</td>
<td>&lt;0.001  0.003  0.616</td>
</tr>
<tr>
<td><strong>Female body weight (g)</strong></td>
<td>186 ± 5.9</td>
<td>12</td>
<td>165 ± 4.3‡‡*</td>
<td>12</td>
<td>162 ± 2.1‡‡*</td>
<td>13</td>
<td>169 ± 4.8‡‡*</td>
<td>12</td>
<td>177 ± 2.7‡*</td>
<td>13</td>
<td>0.561  0.011  0.160</td>
</tr>
<tr>
<td><strong>Male weight gain (g)</strong></td>
<td>184 ± 6.8</td>
<td>10</td>
<td>138 ± 3.2‡‡*</td>
<td>12</td>
<td>157 ± 2.7‡‡*</td>
<td>10</td>
<td>143 ± 6.6‡‡*</td>
<td>12</td>
<td>165 ± 3.8‡*</td>
<td>13</td>
<td>&lt;0.001  0.166  0.845</td>
</tr>
<tr>
<td><strong>Female weight gain (g)</strong></td>
<td>118 ± 3.6</td>
<td>12</td>
<td>108 ± 2.3‡*</td>
<td>12</td>
<td>106 ± 1.9‡*</td>
<td>13</td>
<td>109 ± 3.3‡*</td>
<td>12</td>
<td>112 ± 2.0*</td>
<td>13</td>
<td>0.918  0.174  0.263</td>
</tr>
<tr>
<td><strong>Haemoglobin (g/dL)</strong></td>
<td>12.9 ± 0.3</td>
<td>22</td>
<td>9.1 ± 0.2‡‡*</td>
<td>24</td>
<td>13.8 ± 0.3‡*</td>
<td>23</td>
<td>8.9 ± 0.3‡‡*</td>
<td>24</td>
<td>13.6 ± 0.2‡*</td>
<td>25</td>
<td>&lt;0.001  0.560  0.849</td>
</tr>
<tr>
<td><strong>Male liver iron (μg/L)</strong></td>
<td>247 ± 53</td>
<td>10</td>
<td>28 ± 4§‡*</td>
<td>11</td>
<td>190 ± 37‡*</td>
<td>10</td>
<td>30 ± 4§‡*</td>
<td>11</td>
<td>179 ± 28‡*</td>
<td>13</td>
<td>&lt;0.001  0.827  0.775</td>
</tr>
<tr>
<td><strong>Female liver iron (μg/L)</strong></td>
<td>570 ± 113</td>
<td>12</td>
<td>31 ± 3‡*</td>
<td>12</td>
<td>499 ± 86†</td>
<td>12</td>
<td>30 ± 1†</td>
<td>12</td>
<td>310 ± 45†*</td>
<td>13</td>
<td>&lt;0.001  0.058  0.060</td>
</tr>
<tr>
<td><strong>Plasma ferritin (ng/mL)</strong></td>
<td>48 ± 8</td>
<td>11</td>
<td>58 ± 8</td>
<td>10</td>
<td>188 ± 86</td>
<td>9</td>
<td>41 ± 3</td>
<td>10</td>
<td>40 ± 4</td>
<td>10</td>
<td>0.122  0.050  0.112</td>
</tr>
<tr>
<td><strong>RBC total n-3 FA (% total FA)</strong></td>
<td>9.8 ± 0.1</td>
<td>21</td>
<td>0.9 ± 0.1‡‡*</td>
<td>21</td>
<td>1.0 ± 0.1‡‡*</td>
<td>22</td>
<td>9.1 ± 0.3‡‡*</td>
<td>23</td>
<td>9.8 ± 0.1‡</td>
<td>25</td>
<td>0.013  &lt;0.001  0.040</td>
</tr>
<tr>
<td><strong>RBC total n-6 FA (% total FA)</strong></td>
<td>37.2 ± 0.2</td>
<td>20</td>
<td>40.2 ± 2.1‡*</td>
<td>23</td>
<td>45.3 ± 0.6‡*</td>
<td>22</td>
<td>36.5 ± 0.6‡*</td>
<td>22</td>
<td>38.0 ± 0.1†*</td>
<td>25</td>
<td>0.005  &lt;0.001  0.119</td>
</tr>
<tr>
<td><strong>RBC n-6/n-3 FA (% total FA)</strong></td>
<td>3.8 ± 0.1</td>
<td>20</td>
<td>45.7 ± 2.3‡*</td>
<td>21</td>
<td>44.5 ± 1.0‡*</td>
<td>21</td>
<td>4.0 ± 0.1‡</td>
<td>21</td>
<td>3.9 ± 0.1‡</td>
<td>25</td>
<td>0.555  &lt;0.001  0.630</td>
</tr>
<tr>
<td><strong>RBC DHA (% total FA)</strong></td>
<td>6.23 ± 0.16</td>
<td>21</td>
<td>0.80 ± 0.17‡‡*</td>
<td>22</td>
<td>0.65 ± 0.17‡‡*</td>
<td>22</td>
<td>5.29 ± 0.16‡‡*</td>
<td>24</td>
<td>6.21 ± 0.16‡‡*</td>
<td>26</td>
<td>0.021  &lt;0.001  0.001</td>
</tr>
<tr>
<td><strong>RBC EPA (% total FA)</strong></td>
<td>0.70 ± 0.02</td>
<td>22</td>
<td>0.04 ± 0.02‡‡*</td>
<td>21</td>
<td>0.03 ± 0.02‡‡*</td>
<td>22</td>
<td>0.79 ± 0.18‡‡*</td>
<td>24</td>
<td>0.68 ± 0.18§‡*</td>
<td>26</td>
<td>0.002  &lt;0.001  0.014</td>
</tr>
</tbody>
</table>

Two-way ANCOVA was used to test effects of iron, DHA/EPA, and iron × DHA/EPA supplementation interactions, adjusted for sex. Where a diet × sex interaction is present, results are split between males and females. Between-group differences were determined using one-way ANCOVA followed by Bonferroni’s post hoc test (adjusted for sex if no sex interaction is present). Dunnett’s post hoc test was used to determine the differences between the Control + Fe + DHA/EPA (non-deficient reference) group and each of the other groups (two-sided). Means in a row with different superscripts without a common letter differ (p < 0.05). * Differs to the Control + Fe + DHA/EPA (non-deficient reference) group. Values are means ± SEM.

DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, Fe: iron, ID: iron deficiency, n: number, n-3 FAD: omega-3 fatty acid deficiency, FA: fatty acids; n-6 FA: omega 6 fatty acids; PND: postnatal day; RBC: red blood cell.
DHA/EPA, \( p = 0.003 \)), with only the Fe + DHA/EPA group having a higher body weight compared to the ID + n-3 FAD group. Mean body weights of the ID + n-3 FAD, Fe + n-3 FAD, and ID + DHA/EPA groups differed from the non-deficient reference group (Control + Fe + DHA/EPA; \( p < 0.001 \) for all) while there was no difference between the Fe + DHA/EPA group and the Control + Fe + DHA/EPA group (\( p = 0.114 \)). Iron supplementation resulted in greater weight gain in male offspring from PND 21 (\( p < 0.001 \)), with both iron supplemented groups having a greater weight gain than the ID + n-3 FAD group. All experimental groups, however, had lower weight gain compared to the Control + Fe + DHA/EPA group (ID + n-3 FAD; \( p < 0.001 \); ID + DHA/EPA, \( p < 0.001 \); Fe + n-3 FAD, \( p = 0.003 \); Fe + DHA/EPA, \( p = 0.024 \)). In female offspring, DHA/EPA supplementation resulted in higher body weight (\( p = 0.011 \)). The weights in the ID + n-3 FAD, Fe + n-3 FAD, and ID + DHA/EPA groups differed from the Control + Fe + DHA/EPA group (\( p = 0.004, p < 0.001, \) and \( p = 0.028, \) respectively) while there was no difference between the Fe + DHA/EPA group and the non-deficient reference group (Control + Fe + DHA/EPA; \( p = 0.342 \)). No significant effects were found on female weight gain.

No diet \( \times \) sex interaction was found on haemoglobin. Iron supplementation increased haemoglobin (\( p < 0.001 \)) and both iron supplemented groups (Fe + n-3 FAD and Fe + DHA/EPA) had higher haemoglobin concentrations compared to the ID diet groups (ID + n-3 FAD and ID + DHA/EPA; \( p = 0.312 \)), and similar haemoglobin concentrations to the Control + Fe + DHA/EPA group (\( p = 0.544 \)). An iron \( \times \) sex interaction was found on offspring liver iron concentrations at PND 42–45 (\( p < 0.001 \)). Iron supplementation resulted in higher liver iron concentrations in male and female offspring (\( p < 0.001, \) in both), with significantly higher liver iron concentrations in the iron supplemented groups compared to the ID diet groups. In female offspring, there was a tendency towards an iron \( \times \) DHA/EPA interaction on liver iron (\( p = 0.060 \)), with the Fe + n-3 FAD group having higher liver iron concentrations compared to the Fe + DHA/EPA group. In males, the iron supplemented diet groups reached liver iron concentrations similar to the Control + Fe + DHA/EPA group (Fe + n-3 FAD, \( p = 0.515 \); Fe + DHA/EPA, \( p = 0.301 \)), whereas the ID groups had lower concentrations than the Control + Fe + DHA/EPA group (\( p < 0.001, \) in both). In females, only the Fe + n-3 FAD group reached iron concentrations similar to the Control + Fe + DHA/EPA group (\( p = 0.864 \)), whereas the ID groups (\( p < 0.001 \)) and the Fe + DHA/EPA group (\( p = 0.024 \)) had lower concentrations.

No diet \( \times \) sex interactions were found on ferritin concentrations. Supplementation with DHA/EPA tended to lower ferritin (\( p = 0.050 \)), but there were no differences between the DHA/EPA supplemented groups and non-supplemented groups.

No diet \( \times \) sex interactions were found on any RBC FA markers. Iron \( \times \) DHA/EPA interactions were observed on RBC total n-3 FA (\( p = 0.040 \)), DHA (\( p = 0.001 \)), and EPA (\( p = 0.014 \)). Both DHA/EPA supplemented groups had significantly higher RBC total n-3 FA, DHA, and EPA than the n-3 FAD diet groups, but RBC total n-3 FA and DHA was even higher in the Fe + DHA/EPA group compared to the ID + DHA/EPA group. In contrast, RBC EPA was significantly higher in the ID + DHA/EPA group compared to the Fe + DHA/EPA group. Only the Fe + DHA/EPA group had similar RBC total n-3 FA, DHA, and EPA compared to the Control + Fe + DHA/EPA group (total n-3 FA, \( p = 1.00 \); DHA, \( p = 1.00 \); EPA, \( p = 0.91 \)), while all other experimental groups had lower RBC total n-3 FA, DHA, and EPA, except for the ID + DHA/EPA group, which had higher RBC EPA than the Control + Fe + DHA/EPA group (\( p = 0.004 \)). Iron supplementation led to higher RBC n-6 FA (\( p = 0.005 \)) while DHA/EPA supplementation lowered n-6 FA (\( p < 0.001 \)). This is also shown by the Fe + n-3 FAD group having higher n-6 FA levels compared to all other diet groups and the Control + Fe + DHA/EPA group (\( p < 0.001 \)). Supplementation with DHA/EPA lowered the RBC n-6/n-3 FA ratio (\( p < 0.001 \)), with both DHA/EPA supplemented groups having lower RBC n-6/n-3 FA ratios compared to the n-3 FAD groups. Both DHA/EPA supplementation groups had RBC n-6/n-3 FA ratios similar to the Control + Fe + DHA/EPA group (\( p = 1.000, \) in both), while the n-3 FAD groups had higher n-6/n-3 FA ratios than the Control + Fe + DHA/EPA group (\( p < 0.001, \) in both).

### Bone mineral density

Iron and DHA/EPA supplementation elevated lumbar spine (iron, \( p < 0.001 \); DHA/EPA, \( p < 0.001 \)) and right femur BMD (iron, \( p = 0.008 \); DHA/EPA, \( p = 0.030 \)) in the offspring at PND 42–45, with the Fe + DHA/EPA group having higher spine and femur BMD (spine, \( 0.140 \pm 0.002 \); femur, \( 0.145 \pm 0.002 \)) than the ID + n-3 FAD group (Fig. 2; spine, \( 0.121 \pm 0.002 \); femur, \( 0.137 \pm 0.001 \)). The Fe + DHA/EPA group had similar spine BMD (\( p = 1.000 \)), but lower femur BMD (\( p = 0.005 \)), compared to the Control + Fe + DHA/EPA group (spine, \( 0.139 \pm 0.003 \); femur, \( 0.154 \pm 0.002 \)).

A tendency towards an iron \( \times \) DHA/EPA interaction on spine BMD (\( p = 0.058 \)) was observed, but this tendency did not remain after adjusting for body weight in addition to sex adjustment (\( p = 0.140 \)). When adjusting for body weight, iron supplementation elevated spine BMD (\( p = 0.016 \)), but not DHA/EPA supplementation (\( p = 0.080 \)). Only the combined Fe + DHA/EPA supplementation group resulted in higher spine BMD compared to the ID + n-3 FAD group and all other experimental diet groups differed from the Control + Fe + DHA/EPA group (ID + n-3 FAD, \( p < 0.001 \); Fe + n-3 FAD, \( p = 0.008 \); ID + DHA/EPA, \( p = 0.002 \)). No effects remained in the right femur BMD after adjusting for bodyweight.

### Femur size and bone strength indicators

Table 3 indicates the femur size and strength indicators in offspring at PND 42–45.

No diet \( \times \) sex interactions were observed on femur weight or femur area. Iron \( \times \) DHA/EPA interactions were observed on femur weight (\( p = 0.008 \)) and cross-sectional area (\( p = 0.012 \)), with the Fe + DHA/EPA group having higher femur weight compared to the Fe + n-3 FAD group and higher cross-sectional area compared to both n-3 FAD diet groups. The DHA/EPA groups had similar weight and area compared to the Control + Fe + DHA/EPA group (ID + DHA/EPA, \( p = 0.148 \) and \( p = 0.097 \), Fe + DHA/EPA, \( p = 1.000 \) in both), while the n-3 FAD groups had lower femur weight and area compared to the Control + Fe + DHA/EPA group (ID + n-3 FAD, \( p = 0.015 \) and \( p = 0.030 \), Fe + n-3 FAD, \( p < 0.001 \) in weight and area). After adjusting for sex and bodyweight, the iron \( \times \) DHA/EPA interactions remained; the Fe + n-3 FAD group had lower femur weight compared to the other three experimental diet groups and the Fe + DHA/EPA group had higher area compared to the Fe + n-3 FAD group. None of the experimental groups differed in weight compared to the Control + Fe + DHA/EPA group, but the Fe + n-3 FAD group had lower area compared to the Control + Fe + DHA/EPA group.

An iron \( \times \) sex interaction was observed on femur length (\( p = 0.025 \)). In male offspring, iron supplementation increased femur length (\( p = 0.008 \)), but there were no differences between the
experimental groups. No effects on femur length were observed in female offspring.

Neither femur stiffness, ultimate load, ultimate displacement, ultimate stress, nor transition stress showed diet \( \times \) sex interactions. Iron and DHA/EPA supplementation resulted in higher bone stiffness (iron, \( p = 0.022 \); DHA/EPA, \( p = 0.013 \)), with the combined Fe + DHA/EPA supplementation group having higher bone stiffness compared to the ID + n-3 FAD group. Only the ID + n-3 FAD group had lower bone stiffness compared to the Control + Fe + DHA/EPA group (\( p = 0.025 \)). Effects remained the same when adjusting for sex and body weight, as well as for sex, body weight, and femur area.

An iron \( \times \) DHA/EPA interaction was observed on ultimate load (\( p = 0.015 \)), with the combined Fe + DHA/EPA supplemented group having significantly higher ultimate load compared to the ID + n-3 FAD, Fe + n-3 FAD, and ID + DHA/EPA groups. The combined supplementation group also had a similar ultimate load compared to the Control + Fe + DHA/EPA group (\( p = 1.000 \)), but none of the other experimental groups (\( p < 0.001 \) for all). The results did not change when adjusting for body weight and femur area in addition to sex.

A DHA/EPA \( \times \) sex interaction was found on transition load (\( p = 0.015 \)). In male offspring, DHA/EPA supplementation resulted in higher transition load (\( p = 0.001 \)), and iron supplementation showed a tendency towards a higher transition load (\( p = 0.061 \)). The Fe + DHA/EPA group had a higher transition load compared to the ID + n-3 FAD and Fe + n-3 FAD groups. The combined supplementation group reached a similar transition load compared to the Control + Fe + DHA/EPA group (\( p = 0.950 \)), but none of the other experimental groups (ID + n-3 FAD, \( p = 0.001 \); Fe + n-3 FAD, \( p = 0.004 \); ID + DHA/EPA, \( p = 0.047 \)). After adjusting for body weight, DHA/EPA supplementation (\( p = 0.008 \)), but not iron supplementation (\( p = 0.387 \)), resulted in higher transition load. No significant effects were observed on transition load in female offspring, but iron supplementation tended to result in higher transition load without adjustments (\( p = 0.065 \)) and after adjusting for body weight (\( p = 0.050 \)).

No effects were observed on ultimate or transition displacement. There were no diet \( \times \) sex interactions observed on ultimate or transition stress. Iron supplementation resulted in higher ultimate stress (\( p = 0.018 \) when adjusting for sex only; \( p = 0.055 \) when adjusting for sex and body weight), but there were no differences between different diet groups or with the Control + Fe + DHA/EPA group. No effects were observed on transition stress.

**Insulin-like growth factor-1**

DHA/EPA supplementation lowered plasma IGF-1 in the offspring (\( p = 0.046 \)) (Fig. 3). There were, however, no between-group differences for any of the experimental diets or with the Control + Fe + DHA/EPA group.

**Discussion**

We previously showed that combined ID and n-3 FAD during the pre- and postnatal period leads to greater impairments in bone development than a single deficiency, and that the negative effects of the combined deficiency may not be reversible after introducing a sufficient diet post-weaning. In this study, we aimed to investigate whether impairments in offspring bone development can be prevented when female rats consuming an ID and n-3 FAD diet preconception are supplemented with iron and DHA/EPA, alone or in combination, during gestation and lactation. The results of this study show that pre- and postnatal iron and DHA/EPA supplementation can prevent the detrimental effects of combined ID and n-3 FAD on lumbar spine and femur BMD, as well as femur weight, area, stiffness, and ultimate load in offspring during early adolescence in an additive or synergistic way. Only combined supplementation with iron and DHA/EPA resulted in spine BMD and femur bone strength outcomes similar to offspring from female rats that received iron and DHA/EPA supplementation but were never deficient (Control + Fe + DHA/EPA group). However, femur BMD remained significantly lower. The results from this study further indicate that some effects of pre- and postnatal deficiency and supplementation might be sex-dependent.

The rat model was designed to mimic the situation where women of reproductive age consume diets low in iron and n-3 FAs and thus start a pregnancy with a combined iron and n-3 FA deficiency. Due to the high prevalence of ID anaemia in women

Figure 2. Offspring lumbar spine (A) and right femur (B) bone mineral density at postnatal day 42–45. Two-way ANCOVA was used to test effects of iron, DHA/EPA, and iron \( \times \) DHA/EPA supplementation interactions, adjusted for sex. Between-group differences were determined using one-way ANCOVA followed by Bonferroni’s post hoc test (adjusted for sex). Dunnett’s post hoc test was used to determine differences between the Control + Fe + DHA/EPA (non-deficient reference) group and each of the other groups (two-sided). Means in a row with different superscripts without a common letter differ (\( p < 0.05 \)). *Diffs to the Control + Fe + DHA/EPA (non-deficient reference) group. Values are means \( \pm \) SEM. BMD: bone mineral density, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, Fe: iron, ID: iron deficiency, n-3 FAD: omega-3 fatty acid deficiency.
<table>
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<td>ID + n-3 FAD n</td>
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<tr>
<td>Femur weight (g)</td>
<td>0.56 ± 0.02 21</td>
<td>0.50 ± 0.01^ab*</td>
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<td>Male femur length (mm)</td>
<td>17.7 ± 0.1 9</td>
<td>16.9 ± 0.2^*</td>
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<tr>
<td>Female femur length (mm)</td>
<td>17.5 ± 0.1 12</td>
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<td>Femur area (mm²)</td>
<td>3.9 ± 0.1 21</td>
<td>3.3 ± 0.1^*</td>
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<tr>
<td>Stiffness (N/mm)</td>
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<td>102 ± 6.7^*</td>
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<td>Ultimate load (N)</td>
<td>49 ± 2.1 17</td>
<td>37 ± 1.2^ab*</td>
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<td>Male transition load (N)</td>
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Two-way ANCOVA was used to test the effects of iron, DHA/EPA, and iron x DHA/EPA supplementation interactions, adjusted for sex. Where a diet x sex interaction is present, results are split between males and females. Between-group differences were determined using one-way ANCOVA followed by Bonferroni’s post hoc test (adjusted for sex if no sex interaction). Dunnett’s post hoc test was used to determine differences between the Control + Fe + DHA/EPA (non-deficient reference) group and each of the other groups (two-sided). Means in a row with different superscripts without a common letter differ (p < 0.05). * Differs to the Control + Fe + DHA/EPA (non-deficient reference) group. Values are means ± SEM.

DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, Fe: iron, ID: iron deficiency, n: number, n-3 FAD: omega-3 fatty acid deficiency.
of reproductive age, especially in low- and middle-income countries, and the increased iron requirements during pregnancy for placental and fetal development as well as maternal blood volume expansion, iron supplementation is provided routinely as part of antenatal care in many settings. Furthermore, several scientific and public health bodies are recommending a higher intake of n-3 long-chain PUFA, especially DHA, during pregnancy for optimal fetal development. However, DHA/EPA supplementation is currently not provided routinely as part of pre- and postnatal care in most countries. To our knowledge, this is the first study to investigate the effects of pre- and postnatal iron and DHA/EPA supplementation, alone and in combination, on bone development in offspring from female rats consuming a diet deficient in iron and n-3 FA preconception. Thereby, the model was designed for supplementation to start at the beginning of the second trimester, which corresponds to GD 10 in rats, to mimic the time when women in developing countries would typically seek antenatal care and start supplementation.

The amount of iron in the iron-deficient experimental diets (15-18 mg/kg diet vs. 40 mg/kg diet in AIN-93G diet) induced moderate anaemia (Hb 8-10 g/dL) in the offspring, similar to what can be expected in the human context. Iron supplementation was able to prevent anaemia and significantly increased liver iron concentrations (indicative of iron stores) in both male and female offspring. Based on the higher (non-significant) plasma ferritin concentrations observed in the Fe + n-3 FAD offspring, which is not only a biomarker of iron stores but also an acute-phase protein, we speculate that iron supplementation in the presence of an n-3 FAD may have induced inflammation leading to the sequestration of iron in storage sites, including hepatocytes. This may also explain the higher liver iron concentrations observed in female offspring in the Fe + n-3 FAD group compared to female offspring in the Fe + DHA/EPA group. However, this finding will need further investigation.

The DHA/EPA supplemented diets prevented a decrease in RBC total n-3 FA, DHA, and EPA. Only combined iron and DHA/EPA supplementation resulted in RBC total n-3 FA, DHA, and EPA similar to the offspring born to female rats that received iron and DHA/EPA supplementation and were never deficient (Control + Fe + DHA/EPA). Iron is a co-factor for the FA desaturase and elongase enzymes responsible for the conversion of ALA to EPA and further to DHA. The DHA/EPA supplemented diets also contained ALA. Thus, iron may have improved RBC n-3 LC-PUFA status by increasing the conversion of ALA into EPA and DHA. This may also explain why RBC EPA was higher in offspring in the ID + DHA/EPA group compared to the Fe + DHA/EPA group, resulting in even higher RBC EPA than in the non-deficient reference offspring.

Figure 3. Offspring insulin-like growth factor-1 at postnatal day 42-45. Two-way ANCOVA was used to test effects of iron, DHA/EPA, and iron × DHA/EPA supplementation interactions, adjusted for sex. Between-group differences were determined using one-way ANCOVA followed by Bonferroni’s post hoc test (adjusted for sex). Dunnett’s post hoc test was used to determine differences between the Control + Fe + DHA/EPA (non-deficient reference) group and each of the other groups (two-sided). Values are means ± SEM. DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, Fe: iron, ID: iron deficiency, IGF-1: insulin-like growth factor-1, n-3 FAD: omega-3 fatty acid deficiency.
determined the effects of n-3 FA supplementation in non-n-3 FAD animals.

The mechanisms by which iron and n-3 FA influence bone development appear to be complex and multifactorial. Iron has been shown to play a role in collagen bone matrix synthesis and the conversion of vitamin D to its active form, which is necessary for calcium absorption. Hypoxia, caused by ID anaemia, can also stimulate bone resorption or acidosis, which induces osteoclast activity and therefore bone loss. The possible mechanisms by which n-3 FA may affect bone include their influence on calcium absorption, the differentiation of mesenchymal stem cells into osteoblasts, changes in membrane function, and in the regulation of inflammatory cytokines. The additive effects of iron and DHA/EPA on BMD and stiffness indicate that iron and DHA/EPA supplementation may affect these bone outcomes via independent mechanisms, while the synergistic effects observed on femur weight, area, and ultimate load may indicate potential shared mechanisms. Nonetheless, the results of this study indicate that adequate intake of both nutrients is necessary during early life to ensure optimal bone development, since supplementation of the one nutrient in the presence of a deficiency in the other nutrient will not be able to prevent alterations in bone outcomes.

In this study, pre- and postnatal DHA/EPA supplementation lowered circulating IGF-1 in offspring, even though there were no significant between-group differences. This seems unexpected, as summarised in a review Yakar et al., IGF-1 is an important polypeptide for growth and development and has been shown to affect bone growth and BMD. Decreased serum IGF-1 has been strongly associated with an increased risk of osteoporotic fractures independently of BMD in healthy postmenopausal women. In a study investigating the effects of intrauterine growth restriction in rats, a low protein diet during pregnancy resulted in lower IGF-1 in offspring at PNDs 7 and 21. The mechanism behind the IGF-1 findings in this study is unclear. However, in our previous study, there was a trend of pre- and postnatal n-3 FAD to increase IGF-1. Thus, pre- and postnatal DHA/EPA supplemented may have lowered IGF-1 to the level of the iron and DHA/EPA supplemented non-deficient control offspring. It is further possible that effects of IGF-1 on bone differ according to developmental stages.

Body weight can influence bone development, and we previously showed that both pre- and postnatal ID and n-3 FAD alone result in lower body weight and body weight gain in the offspring, with an additive reduction in the combined ID + n-3 FAD offspring. In the current study, the effects of iron and DHA/EPA supplementation on offspring body weight and body weight gain were sex-dependent. However, in both males and females, only the offspring supplemented with combined iron and DHA/EPA achieved a body weight similar to the offspring born to iron and DHA/EPA supplemented female rats that were never deficient. Nonetheless, most effects of iron and DHA/EPA supplementation in this study remained after adjusting for sex and body weight. Therefore, the effects of supplementation are likely independent of their effect on body weight.

Strengths of this study include the 2 × 2 factorial design investigating the effects of pre- and postnatal iron and n-3 FA supplementation, alone and combined, on bone development and the possible translation to the human context. The amount of iron provided induced a moderate ID and was an amount of supplementation similar to what can be expected in humans. Using a rat model, three-point bending tests could be used to determine bone strength, which is not possible in a human context. Also, it would not be ethical to purposely initiate nutrient deficiencies in pregnant women or women of reproductive age. The human and rat skeleton have enough similarities in pathophysiologic responses to make the rat a valuable model for research on bone health.

Our main limitation was the inability to follow offspring up to late adulthood to determine whether effects remain and affect bone later in life, due to it being part of a larger study. Furthermore, we did not account in our analysis that the experimental groups represented more than one offspring per dam/litter. We did not measure food intake and did not pair-feed groups. However, potential differences in food intake were at least partially controlled for by adding body weight as a covariate. Iron and DHA/EPA breast milk concentrations were not measured as part of this study and therefore the extent to which maternal supplementation during lactation influenced nutrient transfer to the offspring via breast milk is uncertain. Furthermore, in the human context, iron supplementation in individuals with sufficient iron stores might have negative effects on growth and other health outcomes. Iron overload can have detrimental effects on bone health in healthy individuals. Therefore, it can be speculated that the offspring in the supplemented control group (Control + Fe + DHA/EPA) might not be the best representation of ideal bone development.

In conclusion, improving bone growth and development during early life will decrease osteoporosis risk later in life and may also have current benefits to children and adolescents. This study shows that only combined iron and DHA/EPA supplementation can prevent impairments in bone development measured during adolescence in the offspring of female rats that consumed a combined iron and n-3 FA deficient diet preconception. Therefore, pregnant women with a co-existing iron and n-3 FA deficiency might need both iron and DHA/EPA supplementation, from early pregnancy throughout lactation, to ensure their children achieve their full bone developmental potential.

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Author contribution. E.V., C.M.S., H.K., and J.B. conceptualised and designed the study. E.V., L.Z., E.K., and J.B. executed the study and collected data. E.V., L.Z., P.V., and J.B. performed biochemical analyses. E.V. and J.B. performed the statistical analyses and wrote the first draft of the manuscript, and have primary responsibility for its final content. All authors reviewed and approved the final manuscript.

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Competing interests. None.

Ethics standard. The experiments in this study have been conducted following the ARRIVE guidelines for animal research. Ethical approval has been obtained from the AnimCare Ethics Committee (National Health Research Ethics Council reg. number AREC-130913-015) of the Faculty of Health Sciences of the NWU (NWU-00566-19-A5).


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