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# The effect of docosahexaenoic acid on bone microstructure in young mice and bone fracture in neonates

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ABSTRACT

*Background:* As low bone mineral density is a risk factor for fracture in childhood, optimizing age appropriate bone mass is recommended and might lower the impact of bone loss related to age. Consumption of omega-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic and docosahexaenoic (DHA) acids have been shown to beneficially modulate bone metabolism. The objective of this study was to determine the incidence of fracture in neonates receiving a fish compared with soybean oil–based intravenous lipid emulsion and evaluate the effect of varying dietary omega-3 PUFA consumption on growing bone in young mice.

*Materials and methods:* Eligibility criteria for the clinical study included gestational age ≤37 wk and parenteral nutrition–dependence for ≥4 wk. Radiographs were reviewed after lipid initiation to identify radiologic bone fracture. The animal study evaluated female C57/Bl6 mice randomized into one of five groups from age 3–12 wk, at which time femurs were harvested for micro–computed tomography and light microscopy analysis.

*Results:* A lower incidence of bone fracture was found in neonates maintained on fish compared with soybean oil. In the animal study, findings suggest the DHA diet provides the best protection against trabecular bone loss as evidenced by increased bone volume fraction, increased trabecular number, and decreased trabecular separation on micro–computed tomography. These protective effects appeared to affect the bone microstructure alone.

*Conclusions:* The lower fracture risk observed in fish oil fed neonates in combination with the protective effects of DHA observed in the femurs of young C57/BL6 mice suggest an important role for omega-3 PUFAs on bone growth.



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## 1. Introduction

At least 90% of peak bone mass is obtained by the age of 18 y [1]. As low bone mineral density is a risk factor for fracture in childhood, optimizing age appropriate bone mass is recommended and might lower the impact of bone loss related to age. Consumption of omega-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are important biomediators and have been shown to beneficially modulate bone metabolism. Murine studies have shown reduced bone loss after dietary omega-3s in ovariectomized and aged animals [2–4] and beneficial effects on bone development and metabolism in young growing animals [5–11]. In human studies, it has been shown that dietary fat modulates the formation of bone and remodeling during development, especially over the first year of life. Growth failure and metabolic bone disease are commonly seen in parenteral nutrition (PN)-dependent neonates and those born prematurely, as the greatest mineral accumulation occurs during the third trimester. In caring for PN-dependent neonates at our institution, the administration of intravenous fish oil appeared to lessen the frequency of bone fracture and fragility compared with soybean oil. This was purely an observation regarding the type of lipid administration and warranted further investigation. Therefore, we hypothesize that omega-3 PUFA fortified diets provide the best protection against trabecular bone loss. To that end, the objective of this study was two-fold: to determine the incidence of fracture in neonates receiving a fish oil compared with a soybean oil–based intravenous lipid emulsion and subsequently to evaluate the effect of varying dietary omega-3 PUFA consumption on growing bone in young C57/BL6 mice.

## 2. Materials and methods

### 2.1. Clinical neonatal review

A research study protocol (No IRB-P00004009) was approved by the Institutional Review Board at Boston Children's Hospital to conduct a single-center retrospective review of prospectively collected data to evaluate the fracture risk of PN-dependent neonates on fish *versus* soybean oil–based lipid emulsions. Neonates with cholestasis (direct bilirubin ≥2 mg/dL) due to congenital or acquired gastrointestinal disease who were enrolled in an open-label treatment protocol with a fish oil–based lipid emulsion at 1 g/kg/d (Omegaven; Fresenius Kabi AG, Bad Homburg v.d.H., Germany) from 2005–2012, were compared with a historical cohort of PN-dependent cholestatic neonates who received a soybean oil–based lipid emulsion at 1–4 g/kg/d (Intralipid; Fresenius Kabi, Uppsala, Sweden) from 1999–2007 [12,13]. The comparison cohort preceded the introduction of fish oil and received the standard of care dose and type of lipid emulsion at the time. Neonates that were included in the control cohort had two consecutive direct bilirubin values ≥2 mg/dL while on PN that could not be attributed to another cause of hepatic disease. Eligibility criteria for the present study included a gestational age ≤37 wk and PN-dependence for ≥4 wk. There were 131 neonates on fish oil and 50 neonates on soybean oil who met study criteria. Radiographs were taken as clinically indicated and reviewed from lipid start until stop date or 4 mo after lipid initiation to identify radiologic bone fracture. Radiological reports reviewed by an attending radiologist were used to confirm bone fracture. The incidence and type of fracture was evaluated between the fish and soybean oil cohorts.

### 2.2. Murine dietary protocol

Female mice (aged 21 d) were obtained from Jackson Laboratories (Bar Harbor, ME). These animals were fed standard rodent chow (AIN-93M Purified Rodent Diet No. 110900; Dyets Inc, Bethlehem, PA) and were randomized and housed in groups of five in regular vented cages within a barrier room with a 12-h light cycle. Male mice were not used as preliminary studies showed no difference in the outcomes evaluated. For the present study, mice were randomized into one of five dietary groups ($n = 10$ per group), which varied by the lipid type. Group 1 (Soy) was fed standard rodent chow (AIN-93M; Dyets No. 110900) with soybean oil, group 2 (HCO) was fed hydrogenated coconut oil (HCO), an essential fatty acid free diet (Dyets No. 102328), and groups 3–5 were fed modified AIN-93M diets. Group 3 (Menhaden) was fed menhaden oil as the sole source of fat (Dyets No. 102332), group 4 (20:1 DHA: arachidonic acid [AA]) was fed a 20:1 ratio of DHA:AA consisting of 2.0% calories from DHA, 0.1% from AA, and 7.9% from HCO (Dyets No. 102536), and group 5 (DHA) was fed a DHA-rich diet consisting of 2.1% calories from DHA and 7.9% from HCO (Dyets No. 102681). The selection and composition of these diets were based on previous research in our laboratory [14–17]. All diets contained equal caloric and food weight components with total fat calories at 5%. HCO and AA (98% grade) were purchased from Cayman Chemical (Ann Arbor, MI). Esterified DHA (87.4% DHA, 12.6% sterols) was provided by Martek (Columbia, MD). HCO, AA, and DHA were stored at −60°C.

After randomization into respective groups, animals were earmarked and fed their assigned diets for nine consecutive weeks (aged 3–12 wk), a period of rapid growth. Animals had full and uninhibited access to food and water. Animals were individually weighed every third day, and growth and appearance were closely monitored and documented. Food was checked daily and refreshed every 2–3 d.

The animal protocol (No. 10-03-1620) complied with the National Institutes of Health Animal Research Advisory Committee guidelines and was approved by the Boston Children's Hospital Animal Care and Use Committee.

### 2.3. Murine femur harvest

At 12 wk, mice were euthanized, and femurs were harvested for micro–computed tomography (μCT) and descriptive histomorphometric analyses using light microscopy. For imaging analysis, cleaned femurs were placed in normal saline and frozen at −20°C until imaging was performed. For histology, femurs were placed in 10% formalin for 24 h followed by Bouin solution for 24 h, then 70% ethanol in phosphate buffered saline. Samples were embedded in paraffin and were cut