Special Article

Bone metabolism in very preterm infants receiving total parenteral nutrition: do intravenous fat emulsions have an impact?

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Very preterm infants (<32 weeks' gestation) are at high risk for impaired skeletal development because of factors that limit the provision of extrauterine nutrients. Cumulative net deficiencies of calcium, phosphorus, docosahexaenoic acid (DHA), and arachidonic acid (ARA) are evident in these infants after prolonged administration of total parenteral nutrition (TPN). This is significant because minerals as well as metabolites of DHA and ARA are important modulators of bone cell differentiation, lengthening of bone, and bone matrix deposition. Furthermore, diets containing only precursors of DHA and ARA result in suboptimal skeletal growth. With the emergence of new intravenous lipid emulsions, it is important to understand the impact of fatty acids on bone metabolism in the third trimester in order to optimize the provision of TPN in very preterm infants. The purpose of this review is to evaluate current evidence regarding intravenous lipid emulsions and bone metabolism in very preterm infants receiving prolonged TPN and to identify areas of research needed.

INTRODUCTION

Very preterm infants (<32 weeks' gestation) are at high risk for impaired skeletal development because the period of greatest nutrient accretion in utero is missed.[1] Although the exact incidence of metabolic bone disease remains unknown, in part due to a lack of consensus on the definition of metabolic bone disease, it has been estimated to occur in 23% of very low-birth-weight (<1500 g) infants and in 55%–60% of extremely low-birth-weight (<1000 g) infants.[2] The risk of metabolic bone disease is positively associated with duration of total parenteral nutrition (TPN).[3,4] The capability to mimic the high placental transfer rate of nutrients such as calcium and phosphorus via TPN is limited, which undoubtedly contributes to the higher incidence of metabolic bone disease in this population.[5] Little emphasis has been placed on the adequacy of other nutrients that contribute to TPN-related metabolic bone disease, such as polyunsaturated fatty acids (PUFAs). Contrary to early beliefs, the impact of PUFAs in health and disease is dependent on the total fatty acid profile rather than on a single fatty acid. The n-6 and n-3 PUFAs have complex metabolic interactions and compete for a number of enzymatic pathways.[6,7] Evidence from studies on neonatal animal species, effects of maternal diet on fetal growth, and enteral lipid sources used in preterm infant nutrition support suggestions that changes

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in both the amount and the type of fatty acid provided can impact bone development.[8–10] Given the higher risk of metabolic bone disease in very preterm infants receiving TPN, it is important to consider the potential impact of lipids that regulate bone tissue formation in congruence with strategies to optimize bone mineralization and microarchitecture.[5] This review evaluates the effect of intravenous fat emulsions (IVFEs), both currently available as well as those in development, on bone metabolism in very preterm infants.

Since few studies on this topic have been conducted in humans, animal studies addressing the effect of PUFAs and the n-6:n-3 PUFA ratio on bone metabolism are reviewed for a more comprehensive understanding of the mechanisms involved.

BONE GROWTH AND METABOLISM IN VERY PRETERM INFANTS

Preterm birth is associated with development and subsequent treatment of medical problems arising from developmental immaturity. Some treatments for these medical problems, such as steroids, diuretics, TPN, etc., may affect bone metabolism.[5] Bone mineral density (BMD) or bone mineral content (BMC) increases either by deposition of minerals into the existing bone matrix or through an increased rate of bone tissue formation.[5,11] Metabolic bone disease is a term often used to collectively define and summarize skeletal development disorders seen in preterm infants.[5] Rickets or osteomalacia in preterm infants results primarily from insufficient mineralization of bone; on the other hand, osteopenia of prematurity results from either insufficient bone tissue formation or an increased rate of bone resorption.[2,11]

Several systemic hormones and localized growth factors coordinate bone formation and resorption to support bone growth in early life.[12] Important factors produced in the bone microenvironment that impact skeletal biology include prostaglandins, cytokines, and insulin-like growth factors (IGFs).[12] Bone formation requires protein and energy for collagen matrix synthesis as well as adequate intakes of calcium, phosphorus, magnesium, and zinc for mineralization.[11,13] The high demand for nutrients during rapid skeletal growth and mineral accretion in the third trimester is evidenced by increased placental transfer of calcium (90–120 mg/kg/d) and phosphorus (60–75 mg/kg/d).[14] Additionally, the detrimental effect of limited parenteral calcium and phosphorus infusion on bone metabolism has been well documented.[15,16]

Other nutrients that exert biological effects on the skeletal tissues are the n-3 long-chain PUFAs (LC-PUFAs) eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) and the n-6 LC-PUFA arachidonic acid (ARA, 20:4 n-6).[12] These specific n-3 and n-6 LC-PUFAs are substrates for prostanoids that influence the differentiation and activity of bone cells and cartilage tissues.[12] High placental transfer of ARA (212 mg/kg/d) and DHA (43 mg/kg/d), providing an ARA:DHA ratio of 5:1, is evident during the third trimester.[17] The inability to simulate placental transfer of these nutrients (i.e., minerals and lipids) via TPN in very preterm infants inevitably results in deficiencies.[18] Therefore, optimal skeletal development requires not only adequate nutrient supply for bone mineralization but also adequate precursors and stimulation for the formation of new bone matrix for longitudinal and cross-sectional growth.

MECHANISMS OF THE EFFECTS OF POLYUNSATURATED FATTY ACIDS ON BONE METABOLISM

Variation in the linoleic acid (LA, 18:2 n-6) to α-linolenic acid (ALA, 18:3 n-3) ratio influences endogenous synthesis of both n-6 LC-PUFAs and n-3 LC-PUFAs because of competition for the same substrates and, in turn, synthesis of products. The same Δ-6 desaturase enzyme catalyzes the first step in the synthesis of EPA and DHA from ALA and of ARA from LA. LC-PUFAs compete for the sn-2 position in cell membrane phospholipids; therefore, supplementation of EPA or DHA increases n-3 LC-PUFA tissue concentrations at the expense of ARA.[7] Similarly, high concentrations of ARA influence tissue incorporation of EPA and DHA. DHA supplementation decreases intermediary metabolites of both LA and ALA in plasma lipid profiles.[19] Dietary intake of the LC-PUFA precursors, LA and ALA, alone prevents essential fatty acid deficiency but may still lead to inadequate tissue levels of LC-PUFAs due to limited endogenous synthesis via elongation and desaturation rates.[7,20] Current practices of feeding high levels of LA postnatally without a source of preformed LC-PUFAs failed to prevent decline in plasma phospholipid levels of either ARA or DHA.[21,22]

Dietary n-3 and n-6 LC-PUFA intake alters prostanoid formation, cell-to-cell signaling processes, and transcription factors in vivo. For this reason, these biologically active LC-PUFAs may be considered nutraceutical fatty acids.[12] ARA, the main n-6 LC-PUFA, plays a role in normal bone development.[23] Membrane-bound ARA is a substrate of cyclooxygenase 2, which is required for the production of prostaglandin E_2 (PGE_2). At physiological levels, PGE_2 stimulates normal bone growth.[8,23,24] At excessive levels, PGE_2 stimulates osteoblasts to express the protein receptor activator for nuclear-factor κβ ligand (RANKL). RANKL binds to its

receptor (RANK) on osteoclast precursors and stimulates the differentiation and maturation of these precursors into activated osteoclasts.[8] Imbalance between the rate of osteoclast and osteoblast activity influences bone metabolism. Osteoblasts produce osteoprotegerin, which inhibits maturation of osteoclasts by acting as a decoy receptor for RANKL. Exposing cultured osteoblast-like cells to ARA treatment inhibited osteoprotegerin production.[8,25]

The n-3 and n-6 LC-PUFAs have opposing effects on osteoclastogenesis. The n-6 PUFAs favor osteoclastic activity by downregulating the ratio of osteoprotegerin to *RANKL* gene expression in osteoblasts.[26] Figure 1 summarizes the effects of different fatty acids on mesenchymal stem cell differentiation. The n-6 PUFAs also promote bone marrow–derived pluripotent human mesenchymal stem cell differentiation into adipocytes rather than osteoblasts.[26] Increasing the intake of the n-3 LC-PUFAs, DHA and EPA, relative to the n-6 LC-PUFA, ARA, provides protection against bone mass loss.[27] The extent of this protective effect on bone mass is dependent on both the amount as well as the specific n-3 PUFA provided. Greater incorporation of n-3 LC-PUFAs, particularly EPA, into cell membranes displaces ARA from membrane phospholipids.[8] High amounts of EPA relative to ARA inhibit osteoclast differentiation and activity without stimulating osteoblast activity by decreasing ARA, a substrate for PGE_2 synthesis.[27,28] In contrast, DHA exerts an inhibitory effect on osteoclasts by downregulating *RANKL* gene expression while also shifting differentiation of bone marrow human mesenchymal stem cells toward osteoblasts rather than adipocytes.[27,28] Additionally, n-3 LC-PUFAs may reduce production of proinflammatory cytokines, increase production of IGF-1, and improve calcium accretion in bone.[28,29]

In the absence of adequate n-3 LC-PUFA intake, proinflammatory processes counteract the modulatory effects of PGE_2 on bone formation.[27,28] Li et al.[30] reported that the dietary n-6:n-3 PUFA ratio modulated bone PGE_2 production ex vivo and the concentration of IGF-1 in bone tissues, leading to altered rates of bone formation in growing rats and chicks. Therefore, modulating osteoclastogenesis and osteoblastogenesis through a reduced dietary n-6:n-3 LC-PUFA ratio may promote bone formation in early life.[8]

PRECLINICAL STUDIES ON THE EFFECT OF INTAKE OF VARIOUS RATIOS OF POLYUNSATURATED FATTY ACIDS ON BONE

In the absence of large human clinical trials evaluating the impact of IVFEs on bone metabolism in very preterm infants, evidence derived from animal studies may help guide recommendations and the design of future studies. The fatty acid profiles of currently available formulations of IVFEs are limited. Animal studies provide

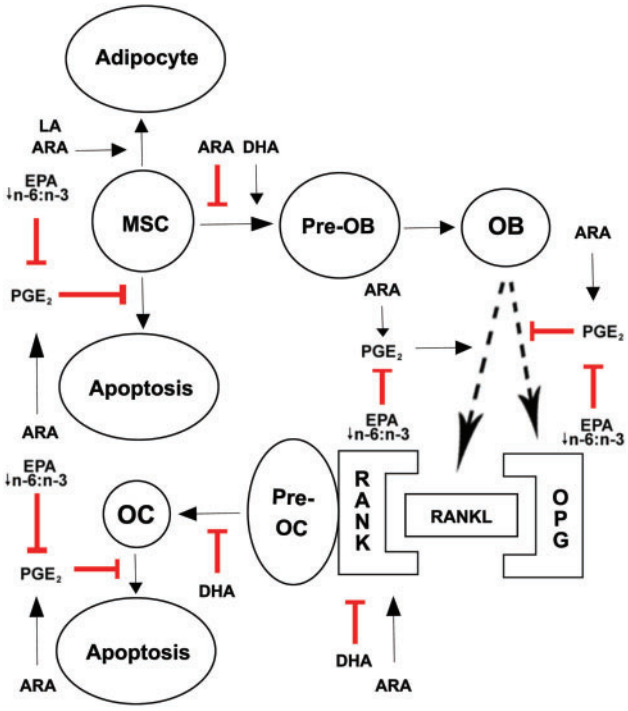

Figure 1 **Pathway for differentiation of mesenchymal stem cells.** *Abbreviations*: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MSC, mesenchymal stem cell; OB, osteoblast; OC, osteoclast; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; RANK, receptor activator of nuclear factor-kappa β; RANKL, receptor activator of nuclear factor-kappa β ligand.

insight on a much wider variation in the total n-6:n-3 PUFA ratio as well as the specific effects of individual LC-PUFAs provided in IVFEs. Since the mechanism of action in which individual LC-PUFAs impact bone metabolism are differential, this data is essential to optimize the fatty acid profiles of future IVFE products intended for preterm infants.

Li et al.[31] randomized female rats to an ALA-deficient diet (medium-chain triglyceride-rich coconut oil + LA-rich safflower oil), an ALA-deficient but high-LA diet (safflower oil), an ALA-adequate diet (coconut, safflower, and ALA-rich flaxseed oils), or an ALA-adequate plus DHA diet (coconut, safflower, flaxseed, and DHA single-cell oils) and mated these rats with chow-fed male rats. The female rats were maintained on their assigned treatment diet through pregnancy and lactation. Pups were weaned (postnatal day 21) and fed the same diet as their dams for 12 weeks.[31] Tibia and femur cortical bone and bone marrow were evaluated for fatty acid composition.[31] Only rats exposed to the ALA+DHA-supplemented diet had significantly reduced ARA content in cortical bone; rats fed this diet also had the greatest ($P = 0.0001$) bone marrow DHA content.[31] However, the effects of fatty acid composition changes on bone formation, mineral content, microarchitecture, and strength were not determined.

Watkins et al.[23] investigated the effect of different n-6:n-3 PUFA ratios on bone formation in weanling rats using various blends of EPA+DHA-rich menhaden oil and LA-rich safflower oil, resulting in total n-6:n-3 ratios of 23.8:1, 9.8:1, 2.6:1, and 1.2:1. These diets consisted of EPA:DHA ratios ranging from 2.35 to 2.7:1 and trace amounts of ARA.[23] As expected, rats fed diets with the higher EPA:DHA ratios had greater bone EPA content relative to DHA.[23] There was a positive correlation ($R^2 = 0.51$; $P = 0.003$) between the ARA:EPA ratio and PGE_2 in bone. Furthermore, a significant negative correlation was found between the bone formation rate and the ARA:EPA ratio ($R^2 = 0.34$; $P = 0.01$) and between bone formation and PGE_2 ($R^2 = 0.22$; $P = 0.05$).[23] Serum activity of alkaline phosphatase isoenzymes increased as the n-6:n-3 dietary ratio decreased, but there was no significant effect on serum osteocalcin in this study.[23] Despite its lack of specificity, serum alkaline phosphatase is a biomarker commonly used in clinical practice to evaluate neonatal bone metabolism.[32] These results collectively suggest that insufficient EPA intake may compromise modulation of bone formation because of the resultant increase in both ARA and PGE_2.

Lukas et al.[33] compared the effect of 5 different lipid sources that had different fatty acid profiles and different n-6:n-3 ratios on bone mass and microarchitecture of growing rats. Weanling rats fed a high-fat diet (33% kcal) as tuna oil (n-6:n-3 ratio of 0.09:1; EPA:DHA ratio of 0.5:1) for 8 weeks exhibited higher tibia BMD and BMC compared with rats fed corn oil (n-6:n-3 ratio of 73:1).[33] Other oil sources used in the study were high in EPA, such as krill, menhaden, and salmon oils (EPA:DHA ratios of 3–5:1), but had no effect on bone mass.[33] Although EPA may be beneficial for bone formation, these findings suggest that a lower EPA:DHA ratio (i.e., <3:1) may be indicated to optimize bone mineralization. Furthermore, the authors reported that oils high in ALA, such as flaxseed oil, improved bone microarchitecture.[33] However, none of the oils studied improved both bone formation and microarchitecture; therefore, all 3 types of n-3 PUFAs may be required to enhance bone health.[33]

Previous studies have provided n-3 PUFAs to rats after the postnatal period at weaning. Rodent models are advantageous due to ease of experimental manipulation, cost-effectiveness, and general acceptance as an experimental animal model for preliminary studies.[34] Along with the short gestation of rodents, the extensive body of knowledge on rodent bone metabolism and periods in which different organs develop is an additional advantage of rodent models in preterm bone research.[35] Before clinical trials are conducted, data from larger animals are often required in addition to data from rodent studies.[34] Miniature pigs are one of the few animals in which spontaneous fracture has been reported; other notable advantages of piglets over other larger animal species as an animal model of nutrition in preterm infants include their omnivorous diet and their similarity to humans in gastrointestinal physiology.[34,36] Thus, neonatal piglet models have been used to evaluate infant formulas because of similarities between piglets and human infants in the metabolism of and requirements for nutrients.[37] Mollard et al.[37] evaluated the effects of a 5:1 ARA:DHA ratio consisting of various concentrations of ARA and DHA on bone mass in neonatal piglets. Concentrations of ARA:DHA used in this study were 0.5:0.1 g per 100 g fat, 1.0:0.2 g per 100 g fat, and 2.0:0.4 g per 100 g fat, added as ARA and DHA single-cell oils. The authors used a constant concentration and an LA:ALA ratio of 9:1 for all diets, but they omitted EPA from all diets. The control diet was devoid of the LC-PUFAs, EPA, DHA, and ARA.[37] Dietary supplementation with an ARA:DHA ratio of 5:1 was demonstrated to enhance BMD in the lumbar spine and whole body; however, increasing the total dietary intake of ARA and DHA above 0.5:0.1 g per 100 g fat did not appear to have any further benefit.[37]

No significant differences were found upon evaluation of ex vivo PGE_2 release from bone or plasma IGF-1 compared with findings in the control group; the

authors attributed this to similar n-6:n-3 PUFA ratios in all diets.[37] Plasma osteocalcin was measured as a marker of osteoblast activity, and urinary cross-linked N-telopeptide of type 1 collagen, corrected for urinary creatinine, was measured as a marker of osteoclast activity. Osteoclast activity was significantly reduced in piglets fed a ratio of ARA:DHA at 1.0:0.2 g per 100 g fat, but no significant difference in osteocalcin levels was demonstrated.[37] The authors concluded that the highest dietary concentrations of ARA:DHA used in this study may be limited in inhibiting bone resorption.[37] The decline in liver tissue EPA levels and the concomitant increase in the ratio of tissue ARA:EPA seen with dietary ARA:DHA concentrations above 0.5:0.1 g per 100 g fat suggest that the addition of a small amount of dietary EPA may be essential to blunt this effect.[37] Since LC-PUFA levels in very preterm infants can be measured only in erythrocytes and not in bone tissue,[38] further studies using animal models to evaluate the potential correlation of LC-PUFA levels in erythrocytes and liver tissue with BMD and trabecular and cortical bone microarchitecture may be beneficial.

It has also been demonstrated that feeding rats DHA increases basal calcium absorption by increasing the activity of calcium-ATPase, the rate-limiting enzyme in calcium uptake.[39] Higher calcium retention and BMD in femur and tibia bones was shown in growing rats fed DHA-rich tuna oil.[33] Since the benefits of DHA for bone metabolism may be partially attributed to the effect of DHA on calcium bioavailability, it is possible that the optimal proportion of EPA:DHA may differ for parenteral and enteral formulations. Therefore, the type of n-3 PUFA included in these formulations should be taken into account, as different n-3 PUFAS may have different effects on bone formation and resorption.[33]

To summarize, ALA appears to be essential for modulation of bone microarchitecture, and supplementation of DHA alone does not appear to increase liver tissue EPA content. This is important because PGE_2 levels are influenced by the ARA:EPA ratio in tissues rather than the ARA:DHA ratio, and PGE_2 plays an important role in modulation of osteoclastogenesis and osteoclast activity. On the basis of current evidence, it would be of interest to evaluate various treatment diets with PUFA composition within the following ranges: an LA:ALA ratio of 5–9:1, an EPA:DHA ratio of 0.3–0.5:1, and an ARA:DHA ratio of 2:1. Rodent studies with PUFA intervention between the perinatal period and the immediate postnatal period favor beneficial effects of DHA enrichment on bone composition.[40–44] However, a study that compared bone composition, density, and quality in animal models most commonly used in bone research (rats, pig, dog, chicken, sheep, and cow) reported that rat bone composition differs the most from human bone.[45] Further studies using larger animal models such as piglets, which are more similar to humans in bone composition and nutrient requirements, are needed to more fully understand the extent to which individual n-3 PUFAs and n-6 PUFAs modulate bone development and influence bone mass, bone microarchitecture, and bone strength.[8]

CLINICAL STUDIES ON INTAKE OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND BONE GROWTH

Human trials evaluating the effect of LC-PUFAs on bone development are limited and have reported inconsistent effects. Providing supplements containing n-3 LC-PUFAs in combination with n-6 PUFAs to adolescent boys was reported to have a nonsignificant effect on bone accumulation.[46] A study conducted in preterm infants born at an average of 30 weeks' gestation found that formulas fortified with ARA plus DHA had little effect on BMD, as measured by dual energy X-ray absorptiometry (DEXA), after 1 year.[47] However, patients who received formulas fortified with LC-PUFA ratios of ARA:DHA at 2.7:1 and 1.6:1, with n-6:n-3 ratios ranging from 6.7:1 to 7.7:1, displayed improved circulating levels of DHA and increased lean body mass compared with controls.[47] The authors concluded that dietary provision of LC-PUFAs at these levels promoted body growth but did not enhance or impair bone mineral accretion.

The effects of LC-PUFAs on bone may not be detectable by DEXA at either term or 1 year of age in this study population because of the absence of other risk factors for metabolic bone disease, such as the lack of significant medical problems or the short duration of TPN. Other biochemical markers of bone metabolism,[32] such as serum alkaline phosphatase and serum phosphate, were not reported in this study. DEXA has been reported to lack sensitivity to detect short-term changes in bone mass.[9] Newer approaches that combine the use of quantitative ultrasound with DEXA may also improve the evaluation of skeletal development in this population.[5,48,49]

Brantsaeter et al.[50] assessed a food frequency questionnaire completed by a cohort of Norwegian women at midpregnancy (≈22 weeks' gestation) and found that intake of fish such as tuna or cod was positively associated with birth length, suggesting greater longitudinal bone growth. However, this association was not found with intake of shellfish, other fatty fish, or n-3 PUFA supplementation.[50] This is likely related to the different fatty acid profiles of various fish and n-3 PUFA supplements. Tuna and cod both contain EPA:DHA ratios

between 0.3:1 and 0.6:1[51]; this is consistent with reports from animal studies suggesting that EPA may have a less beneficial effect on bone when provided in a high dietary EPA:DHA ratio.[33] There may be a window during the perinatal period in which differential fatty acid profiles have a greater effect on fetal programming of mesenchymal stem cell differentiation into osteoblasts rather than adipocytes (Figure 1).[41]

Screening and monitoring bone health in very preterm infants

The availability of an early indicator of metabolic bone disease may enable the prevention of long-term effects, although there is no gold standard for diagnosis at an early stage.[32] Biochemical markers and imaging techniques have been used to assess bone health in preterm infants, with routine biochemical markers being the most commonly used criteria.[52] Table 1 outlines the most common screening and monitoring markers of metabolic bone disease in preterm infants.

A systematic review on serum and/or urinary markers of metabolic bone disease in preterm infants was recently published.[32] These markers, including biochemical markers of bone formation – such as serum phosphate and serum alkaline phosphatase – and biochemical markers of bone resorption – such as urinary calcium-to-creatinine and phosphate-to-creatinine ratios and percent tubular reabsorption of phosphate – have been compared, with imaging techniques used as the reference.[2]

While some studies found no correlation between low serum phosphate and low BMD as measured by imaging techniques, 2 studies demonstrated significant correlations.[32] However, correlation appears dependent on the chosen cutoff value for serum phosphate. Serum phosphate <1.8 mmol/L (5.6 mg/dL) has been found to have specificity of 96% and sensitivity of 50%,[53] whereas serum phosphate <1.2 mmol/L (3.7 mg/dL) has reported specificity of 100%, sensitivity of 33%, positive predictive value of 100%, and negative predictive value of 57%.[54] On the other hand, serum calcium is a poor biomarker of metabolic bone disease; no significant correlation has been found between serum calcium concentrations and DEXA or X-ray assessments.[32]

Table 1 **Most common markers used in screening for and monitoring metabolic bone disease in preterm infants**

Marker	Level of interest	Key points
Biochemical marker		
Serum phosphate	Moderate biochemical marker of bone formation	Low levels correlate with MBD
Serum ALP (total)	Moderate biochemical marker of bone formation	High values associated with MBD. Better correlation of serum ALP bone isoenzyme with bone mineral accretion
Combination of serum ALP and serum phosphate	Good biochemical marker of bone formation	Serum ALP ≥900 U/L *plus* serum phosphate ≤1.8 mmol/L (5.6 mg/dL): sensitivity 100% and specificity 70% for MBD
Serum calcium	Poor biochemical marker of bone formation	No significant correlation with DEXA or X-ray assessments
Parathyroid hormone	Promising early marker of bone formation	PTH level >180 mg/dL *plus* serum phosphate <1.5 mmol/L (4.6 mg/dL): sensitivity 100% and specificity 94% for MBD
$TRP = 1-(U_{phos}/S_{phos} \times S_{cr}/U_{Cr})$	Reasonable indicator of phosphate reabsorption	TRP >95% indicates inadequate supplementation
Urine Ca:Cr and P:Cr (spot)	Poor indicator of phosphate reabsorption	Very dependent on type of feed and some medications (loop diuretics and methylxanthines)
Imaging techniques		
X-ray	Good but late marker of MBD	Bone mineral density needs to be decreased by ≥20%–40% for radiographic changes to be visible
DEXA	Current standard for measurement of bone mineral content	Accurately measures bone mass (bone mineral content and bone mineral density). Fast scan time and low radiation exposure
QUS	Promising method for bone strength assessment	Measures bone strength, which includes bone mineralization, cortical thickness, elasticity, and microarchitecture. Reliable measurements, but insufficient evidence to support its other clinimetric properties

Data from.[2,32,52–58,60–65] *Abbreviations*: ALP, alkaline phosphatase; Ca:Cr, calcium to creatinine; DEXA, Dual energy X-ray absorptiometry; MBD, metabolic bone disease; P:Cr, phosphorus to creatinine; S_{cr}, serum creatinine; PTH, parathyroid hormone; S_{phos}, serum phosphate; TRP, tubular reabsorption of phosphate; U_{Cr}, urinary creatinine; U_{phos}, urinary phosphorus; QUS, quantitative ultrasound.

Total serum alkaline phosphatase is the sum of 3 isoforms (liver, intestine, and bone), of which the bone isoform contributes about 90%. In several studies, no clear correlation was demonstrated between total serum alkaline phosphatase and BMD, despite significant negative correlation between the bone isoenzyme of serum alkaline phosphatase and the rate of bone mineral accretion.[55] Hypophosphatemia, the principal nutritional deficiency in metabolic bone disease, is a key factor involved in accelerating the turnover of matrix vesicles and, hence, increasing plasma alkaline phosphatase activity.[52] The combination of serum alkaline phosphatase >900 U/L and serum phosphate <1.8 mmol/L (5.6 mg/dL) is very sensitive (100%) and specific (70%) for diagnosing inadequate intake and low BMD.[32,53] Secondary hyperparathyroidism, reflected by elevation of parathyroid hormone levels, is associated with undermineralized bone in premature infants.[56] In this context, parathyroid hormone seems to be an early serum biomarker with better sensitivity than alkaline phosphatase in screening for metabolic bone disease. A recent study reported that a parathyroid hormone level >180 mg/dL *plus* a phosphate level <1.5 mmol/L (4.6 mg/dL) yields a sensitivity of 100% and a specificity of 94% for severe metabolic bone disease.[56]

Urinary calcium-to-creatinine and phosphorus-to-creatinine ratios (spot urine evaluation) are other biomarkers evaluated for assessment of bone health. While the 95th percentile for urinary calcium to creatinine is 3.8:1 (mmol/mmol), decreasing with increasing postnatal age, the 95th percentile for urinary phosphorus to creatinine is 26.7:1 (mmol/mmol), remaining stable with increasing postnatal age. These measurements are very dependent on type of feed and on medications such as loop diuretics and methylxanthines, and it is still not proven that they are reliable markers of BMC.[57] A high percentage of tubular reabsorption of phosphate suggests low urinary phosphate wasting and vice versa.[58] Tubular reabsorption of phosphate is a good guide to adequacy of phosphate supplementation, with a value >95% indicating inadequate supplementation.[57] Type 1 procollagen C-terminal peptide and osteocalcin are biochemical markers of bone formation not routinely used as metabolic bone disease screening tools in preterm infants because of the influence of circadian variations and limited references for this population.[52,59] Additionally, osteocalcin is difficult to measure accurately due to instability, and type 1 procollagen C-terminal peptide is not specific to bone.[59]

The most common imaging techniques currently used to diagnose metabolic bone disease are X-ray, DEXA, and quantitative ultrasound. "Thin" and "washed-out" bones, healing fractures, subperiosteal new bone formation, and enlargement of epiphysis are radiologic characteristics of metabolic bone disease in preterm infants.[2] The radiographic signs are late markers, since BMD needs to be decreased by at least 20%–40% for changes to be visible radiographically.[2,52]

DEXA measures the mineral content of the bones, expressed as grams of hydroxyapatite per centimeter squared.[3] This is a validated technique that has high precision and accuracy for measuring BMC and BMD and is considered the standard tool for assessing bone mass in small subjects.[2,60] DEXA is convenient in infants because radiation exposure is low, scan time is fast, and the BMC results are independent of anthropometric variables and gestational age.[2,3,60] Unfortunately, the clinical availability of DEXA is limited.[2] Due to the difficulty in obtaining a scan for whole-body measurements, regional sites may have more clinical utility.[61] The trabecular bone is preferred for measurements, and the lumbar region is generally used in neonates, although modern portable equipment can analyze the forearm and the calcaneus.[3] Normative longitudinal data for bone mass in healthy term infants from birth to 1 year of age are available, as are percentile charts for areal and volumetric BMD in lumbar spine and femur.[61]

Quantitative ultrasound, which measures the speed of sound in bone, is a nonionizing, portable, and relatively low-cost method for assessing bone status. This method measures determinants of bone strength, including bone mineralization and cortical thickness, elasticity, and microarchitecture.[62,63] Although quantitative ultrasound has been standardized in preterm infants,[64] no correlation was found between DEXA and speed of sound measurements.[49] It has been suggested that both methods may be used to complement each other in the assessment of bone health.[49] Quantitative ultrasound has the ability to generate reliable measurements, but evidence to support its other clinimetric properties is insufficient.[65] Another imaging technique with limited clinical use in infants is the quantitative computed tomography scan, which can assess BMC but involves exposure of infants to high doses of radiation.[2]

Clinical consequences of metabolic bone disease. Limitations in both the specificity and the sensitivity of clinical markers and inconsistent diagnostic criteria for TPN-related metabolic bone disease make it difficult to quantify the impact of IVFEs on long-term outcomes in very preterm infants.[32] Most studies assessing the effects of prematurity on late BMD have used imaging methods, including DEXA, quantitative ultrasound, and computerized tomography.[66] Some follow-up studies have found no difference in bone mineralization between term and ex-preterm infants in late childhood[67];

contrarily, others have reported that preterm infants became shorter, lighter, and had lower BMC than controls through childhood and possibly until puberty.[68–70] These results are difficult to interpret because of the confounding effects of puberty and the interaction with bone size and later BMD.[66] Moreover, studies suggesting that adults who were born preterm are shorter than their term-born counterparts have not made appropriate adjustments for current size, making it difficult to determine whether BMD is appropriate for current size or not.[66,71]

More immediate complications of metabolic bone disease, such as fractures, poor growth, and increased hospital length of stay, are not typically studied. However, preterm infants with biochemical evidence of metabolic bone disease during the neonatal period are at high risk of bone fractures and long-term stunting effect, with lower lumbar spinal BMC and density compared with children born at term.[67,72–74] Appropriate intakes of calcium, phosphorus, and vitamin D during childhood may improve their prognosis.[21] Nevertheless, interventions early in life, such as adequate diet, supplements, or passive exercise with gentle joint compressions, can improve the quality of the preterm infant's bones and play a role in the prevention and treatment of metabolic bone disease.[75]

FURTHER RESEARCH: PARENTERAL LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND BONE GROWTH

Very preterm infants need a sufficient supply of n-3 and n-6 LC-PUFAs because they miss out on the last trimester of pregnancy, the period of greatest accretion of these nutrients.[76] Most of these infants cannot tolerate full enteral feedings within the first days or weeks after birth, and therefore nutrients need to be delivered by TPN.[77] The soybean oil IVFE currently available in the United States provide 6.8:1 n-6:n-3 ratio of LA to ALA but lack preformed LC-PUFAs.[16] The biologically active n-3 and n-6 LC-PUFAs, EPA, DHA, and ARA, can be synthesized from the essential fatty acids ALA and LA; however, metabolic conversion is inefficient in adults and even less efficient in very preterm infants.[7,78] This is not surprising, since placental transfer of maternal preformed LC-PUFAs is the primary means of acquisition for the fetus.[78] For this reason, provision of LA and ALA without LC-PUFAs may have negative implications for skeletal development.[23] The LC-PUFA profile of soybean oil IVFE is subphysiological, and administration of soybean oil IVFE has been shown to result in a net deficiency of DHA accretion in very preterm infants.[79]

The ideal fatty acid profile of IVFE should contain a variety of fatty acids in ratios optimal for tissue incorporation, appropriate inflammatory response, and support of metabolic processes that contribute to optimal growth. Recommendations for intakes of enterally administered PUFAs for preterm infants have been updated to support such outcomes; however, the optimal fatty acid profile of IVFE for preterm infants has yet to be defined.[80,81] Lapillonne et al.[81] recommended an enteral ARA intake of 18–45 mg/kg/day, an enteral DHA intake of 12–60 mg/kg/day, and an enteral EPA intake of $\leq$20 mg/kg/day. These guidelines also take enteral bioavailability of nutrients ($\approx$80%) into consideration to compensate for potential malabsorption.[81] In order to establish guidelines for total intake of LC-PUFAs via IVFE that better reflect in utero accretion, estimating the rate of placental LC-PUFA transfer may be more relevant. The translation of recommendations for total intake of LC-PUFAs via IVFE may require careful consideration with regard to potential differences in bioavailability and metabolism.

Recently, new IVFEs for clinical use containing n-3 LC-PUFAs have been commercialized,[82] representing a significant advance in TPN formulation, especially for the reduction of TPN-associated liver disease.[83] Other advantages of these formulations, related to n-3 PUFA content, include reduction of bronchopulmonary dysplasia and prevention of severe retinopathy.[84,85] Conditions such as bronchopulmonary dysplasia and necrotizing enterocolitis not only affect the organ involved but also limit the nutrient intake; thus, these conditions also affect other systems and tissues, including bone.[2,3,52] Studies using new IVFE in very preterm infants are promising in terms of reducing inflammation, improving LC-PUFA status, and attenuating the aforementioned diseases that have been positively associated with metabolic bone disease. Previous in vitro and animal studies have demonstrated beneficial effects of LC-PUFAs on bone cells and bone health. As far as can be determined, no studies have addressed the potential impact of new IVFE formulations on neonatal bone development and the subsequent long-term effects on bone health.

The American Academy of Pediatrics Committee on Nutrition[86] currently recommends intake of LA:ALA at a ratio of 5–15:1, with LA constituting 8.5%–27% of total fat for TPN in stable preterm infants weighing <1500 g; however, TPN recommendations for ARA, EPA, and DHA were not specified. Ratios for LA:ALA of 5–15:1 and for ARA:DHA of 1–2:1 have been previously recommended by expert consensus panels for enteral nutrition in very preterm infants.[87–89] However, Lapillonne et al.[81] suggested that future recommendations for enteral feedings in very preterm

Table 2 **Fatty acid profiles of intravenous lipid emulsions compared with recommendations for enterally administered LC-PUFAs**

Fatty acid	Notation	Type of emulsion						Suggested ranges for skeletal development	Recommended enteral ranges
		S 100[a]	SM 50:50[a]	SO 20:80[a]	F 100[a]	SMF 40:50:10[a]	SMOF 30:30:25:15[a]	(% total lipids)	(% total lipids)
LA	C18:2 n-6	53.2	27	17.2	4	21.9	18.6	8–25	8–25
ALA	C18:3 n-3	7.9	3.5	2.4	2	2.9	2.4	0.9–5	≥0.9
GLA	C18:3 n-6	ND	0.4	ND	NA	0.2	ND	NA	NA
ARA	C20:4 n-6	ND	0.2	0.6	2	0.4	0.5	0.5–1.0	0.3–0.7
EPA	C20:5 n-3	ND	0.1	ND	19	3.3	2	0.06–0.25[b]	0.06–0.15[b]
DPA	C22:5 n-3	ND	0.1	ND	NA	0.5	0.4	NA	NA
DHA	C22:6 n-3	ND	0.1	0.6	12	2.5	2.2	0.2–0.5	0.2–0.5
LA:ALA		6.8:1	7.7:1	7.2:1	2:1	7.6:1	7.8:1	5–9:1	5–15:1
ARA:DHA		NA	2:1	1:1	0.17:1	0.16:1	0.23:1	2–5:1	1–2:1
EPA:DHA		NA	1:1	NA	1.6:1	1.3:1	1.1:1	0.3–0.5:1	<0.33:1
n-6:n-3		6.8:1	7.3:1	7.8:1	0.18:1	2.4:1	2.6:1	NA	NA

Data from.[81,86–90] *Abbreviations*: ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; F, fish; GLA, γ-linoleic acid; LA, linoleic acid; NA, not available; ND, not detectable; S, soybean oil; SM, soybean oil + medium-chain triglyceride oil; SMF, soybean oil + medium-chain triglyceride oil + fish oil; SMOF, soybean oil + medium-chain triglyceride oil + olive oil + fish oil; SO, soybean oil + olive oil.
[a]Percent oil by weight.
[b]Estimated on the basis of percentage of total DHA recommended.

infants focus on total LC-PUFA intake rather than ratios because of the wide variability of ARA:DHA ratios in human milk. Since many benefits of fish oil have been attributed to the modulation of inflammatory response, neither the n-6:n-3 ratio nor the ARA:DHA ratio should be overlooked when developing recommendations for the lipid profile of IVFE. Data on accretion of ARA and DHA in the fetus suggest in utero placental transfusion rates of ARA:DHA of 2–5:1, while current recommendations for enteral intake of ARA:DHA suggest a ratio of 1–2:1.[78,86] Suggested PUFA profiles of IVFE for the study of skeletal development in very preterm infants are outlined in Table 2; these suggestions may help guide the development of IVFE products and the design of future studies of IVFE in this population.

As shown in Table 1, the fatty acid profiles of newer IVFE have variable n-6:n-3 ratios (0.18–7.8:1) compared with soybean oil IVFE (6.8:1). While soybean oil IVFE contains no ARA, DHA, or EPA[90], newer IVFE contain EPA:DHA ratios of 1–1.6:1 and ARA:DHA ratios of 0.16–2:1 (Table 2). Of note, current guidelines for enteral nutrition support of very preterm infants suggest that ARA intake should slightly exceed DHA intake to support normal metabolic processes.[81] New IVFE contain either appropriate ratios of ARA:DHA (1–2:1) or suboptimal ratios of ARA:DHA (0.17–0.25:1), but lack EPA or exceed the upper recommendations for the EPA:DHA ratio (Table 2). Therefore, on the basis of current enteral recommendations, LC-PUFA ratios in many of these IVFE may be suboptimal for TPN support in very preterm infants.[81]

Table 3 summarizes clinical studies of IVFE in preterm infants in which bone was assessed, mostly as a secondary outcome. Vasudevan et al.[91] performed a pilot study to evaluate the effect of either a soybean oil–based IVFE or an olive oil–based IVFE (80% olive oil + 20% soybean oil) on pulmonary hemodynamics and urinary eicosanoid metabolites. This study was performed on very low-birth-weight preterm infants <30 weeks' gestation (n = 15).[91] Infants who received the olive oil IVFE demonstrated a decline in the urinary proinflammatory thromboxane B_2 to 6-keto-prostaglandin-$F_{1\alpha}$ ratio from baseline to day 3 compared with the minimal change from baseline seen in infants who received a soybean oil IVFE.[91] The dose of olive oil IVFE administered on day 3 of this study provided ARA at approximately 180 mg/kg and DHA at approximately 180 mg/kg, meeting approximately 85% of the estimated daily placental transfer of ARA and approximately 400% of the estimated daily placental transfer of DHA. While the olive oil IVFE provides most PUFAs within recommended ranges for PUFA ratios and percent total fatty acids (Table 2), the ratio of ARA:DHA and the absence of EPA may be suboptimal for bone metabolism, based on information derived from animal studies. However, this study was important as it suggests potential short-term benefits for eicosanoid metabolism with even small amounts of ARA and DHA. Another study also compared soybean oil IVFE with olive oil IVFE in preterm infants, but the authors did not evaluate any outcomes potentially related to bone metabolism.[92]

Other studies have evaluated the metabolic effects of various IVFE in preterm infants. Skouroliakou et al.[84] evaluated the effects of an IVFE containing a blend of soybean oil, medium-chain triglyceride oil, olive oil, and fish oil (SMOF) (n = 54) vs an soybean oil IVFE (n = 75) in very low-birth-weight preterm infants

Table 3 **Studies of the effects of various intravenous lipid emulsions on bone metabolism in preterm infants**

Reference	Study design	Characteristics and no. of study subjects	Study duration	IVFE source	Outcomes relevant to bone metabolism
Vasudevan et al. (2013)[91]	RCT	Median GA 26–28 wk BW 500–1249 g n = 15 (n = 5–10 per group)	4 d; measurements at baseline and 24 h after maximum lipid intake	Control: S Treatment: SO	Decreased ratio of urinary thromboxane B_2 to 6-keto-$PGF_{1\alpha}$ with SO compared with S at endpoint
Skouroliakou et al. (2012)[84]	Prospective observational	GA <34 wk BW <1500 g n = 129 (n = 54–75 per group)	≈25 d of IVFE; data collected through time of discharge	Control: S Treatment: SMOF	Decreased total serum ALP and increased serum P, independent of confounding variables, in SMOF group compared with S group at endpoint
Tomsits et al. (2010)[93]	RCT	GA <34 wk BW 1000–2500 g n = 60 (n = 30 per group)	7–14 d PN; measurements at baseline, day 8, and day 15	Control: S Treatment: SMOF	No significant differences in serum ALP or P. RBC n-6:n-3 ratio significantly lower in SMOF group compared with S group at endpoint. Significant difference in change in RBC n-6:n-3 ratio from baseline between groups
D'Ascenzo et al. (2014)[94]	RCT	BW 500–1249 g n = 80 (n = 20 per group)	7–18 d PN; measurements at baseline, day 7, and day 14	Control: S Treatment: SMOF (stratified to 2 lipid doses)	No significant differences in ALP levels between groups. Decreased RBC LA and ARA and increased EPA from baseline at days 7 and 14 in the SMOF group
Savini et al. (2013)[97]	Single-center RCT	BW 500–1249 g n = 144 (n = 27–30 per group)	3 wk; <5 d of exclusive PN	Control: S Treatment: MS, MSF, SO, SMOF	No differences in total serum ALP between groups at 6 wk postnatal age; length NR
Vlaardingerbroek et al. (2014)[96]	Single-center, double-blind RCT	BW <1500 g n = 96 (n = 48 per group)	≈11–12 d of IVFE; measurements at baseline, day 6, and day 14	Control: S Treatment: SMOF	RBC and TG n-6:n-3 ratio significantly lower and EPA and DHA increased at days 6 and 14 in SMOF group compared with control group. No difference in lower leg length between groups at day 28
Rayyan et al. (2012)[95]	Double-blind RCT	GA <34 wk BW 500–2000 g n = 53 (n = 26–27 per group)	At least 7 d of PN; measurements at baseline, day 8, and day 15 or day of treatment DC	Control: S Treatment: SMOF	RBC n-6:n-3 ratio at day 8 significantly lower and closer to baseline value in SMOF group compared with S group. No significant difference in change of length from baseline between groups
Fallon et al. (2014)[98]	Single-center, retrospective review	GA <37 wk PN dependence ≥4 wk n = 181 (n = 131, F group; n = 50, S group)	Radiographs as needed until treatment DC or 4 mo after lipid initiation	Control: S Treatment: F	Fractures identified in 12% of S group compared with 5.3% of F group. Recurrence of fracture identified in 67% of S group compared with 29% of F group

Abbreviations: ALP, alkaline phosphatase; ARA, arachidonic acid; BW, birth weight; DC, discontinuation; DHA, docosahexaenoic acid; F, fish oil; GA, gestational age; IVFE, intravenous fat emulsion; LA, linoleic acid; MS, medium-chain triglyceride oil + soybean oil; MSF, medium-chain triglycerides + soybean + fish oil; NR, not reported; P, phosphate; PGF, prostaglandin F; PN, parenteral nutrition; RBC, red blood cells; RCT, randomized controlled trial; S, soybean oil; SMOF, soybean oil + medium-chain triglycerides + olive oil + fish oil; SO, soybean oil + olive oil; TG, triglycerides.

<34 weeks' gestation. The authors found that infants who received the SMOF IVFE had significantly lower serum alkaline phosphatase concentrations at day 45 of life ($P < 0.001$) and at the time of discharge from the hospital ($P = 0.01$) compared with infants who received the soybean oil IVFE.[84] Furthermore, infants who received the SMOF IVFE had significantly higher ($P = 0.02$) serum phosphate at the time of discharge from the hospital.[84] Since elevated serum alkaline phosphatase with decreased serum phosphate is positively associated with the incidence of metabolic bone disease,[32] these results indicate the need for additional studies on the source of oils in IVFE relative to specific outcomes on bone metabolism in preterm infants.

Multiple regression analysis revealed that the SMOF IVFE was independently associated with a reduction in the development of bronchopulmonary dysplasia and in serum alkaline phosphatase.[84] Cholestasis was not a significant factor associated with elevated serum alkaline phosphatase, and indicators of liver function, serum gamma-glutamyl transpeptidase and direct bilirubin levels, did not differ between groups.[84] Although the alkaline phosphatase bone isoenzyme was not measured, the authors attributed changes in total serum alkaline phosphatase to metabolic bone disease rather than to cholestasis.[84] While the SMOF IVFE contained a suboptimal ARA:DHA ratio (0.23:1), it contained adequate percent total lipid ARA and exceeded the recommended percent total lipids for EPA and DHA (Table 2). Evidence from animal studies suggest that addition of LC-PUFAs, even at suboptimal levels, may have a beneficial effect on bone.[31,37,43]

Tomsits et al.[93] evaluated the metabolic effects of SMOF IVFE compared with soybean oil IVFE in preterm infants (n = 60) <34 weeks' gestation with birth weight <2500 g. In contrast to Skouroliakou et al.,[84] they found no significant differences in final serum alkaline phosphatase or phosphate or in change serum alkaline phosphatase or phosphate from baseline between groups at study termination (≈15 days).[93] Infants in this study had average birth weights of approximately 1670 g and received IVFE for a short duration of 7–14 days.[93] D'Ascenzo et al.[94] performed a similar study in preterm infants (n = 80) with birth weights ranging 500–1249 g. They also reported no differences in serum alkaline phosphatase concentrations between or within groups in samples collected on days 7 and 14 of TPN.[94] In both studies, TPN was tapered after day 7, while enteral feedings were increased.[93,94] It is possible that the effects of IVFE on serum alkaline phosphatase and serum phosphate may not be reflected during early TPN.

Other short-term studies[95–97]comparing SMOF IVFE with soybean oil IVFE in preterm infants evaluated few parameters specific or potentially related to bone metabolism (Table 3). Rayyan et al.[95] investigated the metabolic effects of soybean oil IVFE vs SMOF IVFE in preterm infants <34 weeks' gestation (n = 53) with birth weights ranging 500–2000 g who were expected to receive at least 7 days of TPN. However, infants with conditions that would predispose them to TPN dependence and metabolic bone disease, such as liver disease, extreme immaturity, or severe congenital malformations, were not included because of the potential for therapeutic interventions to interfere with the treatment. The authors did not report serum phosphate or serum alkaline phosphatase. There were no significant differences in change in length from baseline to day 15 or post treatment between groups. This study provided no further insight into the effect of soybean oil IVFE vs SMOF IVFE on skeletal development because of its short duration, the exclusion of infants at highest risk for metabolic bone disease, and the lack of measured biochemical markers directly related to metabolic bone disease.

Vlaardingerbroek et al.[96] evaluated the growth and fatty acid profiles of preterm infants with birth weight <1500 g (n = 96) receiving either soybean oil IVFE or SMOF IVFE. The authors did not measure any biochemical indices specific to bone. A significant improvement in *z* scores for change from baseline to discharge head circumference and weight was observed in infants receiving SMOF IVFE compared with those receiving soybean oil IVFE; however, no difference between groups was seen in change from baseline at 28 days in *z* scores for either measurement.[96] Linear growth was measured by average lower length gain in millimeters per day rather than change in *z* score for the first 28 days, but not at discharge; no significant difference in linear growth was seen between groups at day 28.[96] Since *z* scores may help capture change specific to age and no *z* scores were used to evaluate linear growth, these results are likely of limited value for evaluation of bone health in this study.

Savini et al.[97] studied the effects of 5 different IVFE on preterm infants (n = 144) with birth weights ranging 500–1249 g. Infants were randomized to receive soybean oil IVFE, olive oil IVFE, SMOF IVFE, SM IVFE (50% soybean oil + 50% medium-chain triglyceride oil), or SMF IVFE (40% soybean oil + 50% medium-chain triglyceride oil + 10% fish oil) until full enteral feedings were reached (up to 21 days).[97] Liver function tests were measured at 6 weeks postnatal age (≈3 weeks after treatment discontinuation), and the authors found no significant differences in total serum alkaline phosphatase between groups at this time. Serum phosphate was not measured. Change in length or length *z* scores from

baseline were not reported. The authors found no differences in the incidence of patent ductus arteriosus, necrotizing enterocolitis, bronchopulmonary dysplasia, or sepsis between groups.[97] Again, the short study duration and the nonspecific measurements of skeletal growth used in this study provide insufficient information to evaluate bone health.

Metabolic bone disease in very preterm infants has been reported to be positively correlated with the duration of TPN.[3] Most recently, Fallon et al.[98] observed an apparent decrease in fracture incidence in TPN-dependent (TPN > 4 wks) neonates (n = 181) receiving 100% fish oil–based IVFE (n = 131) compared with those receiving soybean oil IVFE (n = 50). The authors thus performed a retrospective review of prospectively collected data; fractures were identified in 5.3% of infants who received fish oil IVFE compared with 12% of infants who received soybean oil IVFE. Furthermore, 67% of neonates in the soybean oil IVFE fracture group had extremity fractures compared with 16.7% of those in the fish oil IVFE, who exhibited mostly rib fractures.[98] Sixty-seven percent of neonates in the soybean oil IVFE fracture group also had recurrent fractures compared with 29% of neonates in the fish oil IVFE fracture group.[98] Since radiographic changes such as fractures are considered late markers of metabolic bone disease, future studies should evaluate longer durations of IVFE administration. It would be ideal to include earlier biomarkers of metabolic bone disease, such as the combination of serum alkaline phosphatase and serum phosphate or serum parathyroid hormone, as routine measurements of bone health in studies of very preterm infants receiving IVFEs. Additionally, long-term follow-up studies measuring later bone health in these infants should be performed to determine the benefits of early intervention.

The type of IVFE should also be evaluated in future studies. Based on the review of the evidence, none of the fatty acid profiles of the current IVFE appear sufficient to meet the needs of very preterm infants relative to typical in utero accretion of LC-PUFAs. Likewise, enteral formulas designed for very preterm infants utilize a combination of dietary oil sources in order to customize the fatty acid profile of these products.[86] Common dietary oils used in IVFE such as plant oil and, recently, fish oil alone are insufficient to meet ARA requirements because preformed dietary ARA is typically found in animal fats. Currently, infant formulas utilize single-cell-organism biosynthesized sources of preformed ARA, EPA, and DHA as additives to meet recommendations.[99] These single-cell sources of LC-PUFAs may need to be evaluated with an aim toward optimizing the lipid profiles of future IVFEs for use in very preterm infants.

CONCLUSION

Early studies evaluating the effect of supplementation with increased DHA and ARA on neonatal bone were unable to replicate outcomes projected by studies using rodent and piglet models. Many of these studies failed to account for the effect of manipulating single fatty acids on both the total n-6:n-3 ratio and the ARA:DHA ratio. Animal studies initiated at weaning age may not account for potential critical windows for neonatal programming in utero. Collectively, these factors may, in part, explain the inconsistent effects of LC-PUFA intake on bone observed in early animal studies and in human neonatal studies. Thus, it cannot be ruled out that the lack of significance was related to suboptimal fatty acid ratios rather than to an increased intake of LC-PUFAs, such as DHA or ARA. To determine the effects of nutritional programming on bone health, future studies should administer LC-PUFAs during gestation and lactation. It is also important to consider the use of preterm rather than term rodent and piglet models because of differences in organ maturation at birth.

Research of IVFEs continues to advance. Development of new IVFEs should focus on optimization of fatty acid profiles and the potential use of single-cell oils. It would be of interest to evaluate the intake of different PUFA ratios within ranges thought to promote skeletal development. Studies specific to very preterm infants, using metabolic bone disease as the primary outcome, are also needed. Since methods for measurement of bone mass, mineralization, microarchitecture, and growth in very preterm infants vary in sensitivity and specificity, it may be beneficial to assess a combination of measurements. Early biomarkers of metabolic bone disease, such as the combination of serum alkaline phosphatase or parathyroid hormone and serum phosphate, along with long-term follow-up measurements, such as change over time using both quantitative ultrasound and DEXA, are needed to help determine the impact of IVFEs on bone health later in life.

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REFERENCES

1. Lyon AJ, McIntosh N, Wheeler K, et al. Radiological rickets in extremely low birthweight infants. Pediatr Radiol. 1987;17:56–58.
2. Vachharajani AJ, Mathur AM, Rao R. Metabolic bone disease of prematurity. Neoreviews. 2009;10:e402–e411.
3. Figueras-Aloy J, Alvarez-Domínguez E, Pérez-Fernández JM, et al. Metabolic bone disease and bone mineral density in very preterm infants. J Pediatr. 2014;164:499–504.
4. Viswanathan S, Khasawneh W, McNelis K, et al. Metabolic bone disease: a continued challenge in extremely low birth weight infants. JPEN J Parenter Enter Nutr. 2014;38:982–990.
5. Harrison C, Gibson A. Osteopenia in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2013;98:F272–F275.
6. Bridges KM, Gigliotti JC, Altman S, et al. Determination of digestibility, tissue deposition, and metabolism of the omega-3 fatty acid content of krill protein concentrate in growing rats. J Agric Food Chem. 2010;58:2830–2837.
7. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. Am J Clin Nutr. 2006;83(suppl 6):1467S–1476S.
8. Lau BYY, Cohen DJ, Ward WE, et al. Investigating the role of polyunsaturated fatty acids in bone development using animal models. Molecules. 2013;18:14203–14227.
9. Harvey N, Dhanwal D, Robinson S, et al. Does maternal long chain polyunsaturated fatty acid status in pregnancy influence the bone health of children? The Southampton Women's Survey. Osteoporos Int. 2012;23:2359–2367.
10. Devlin MJ, Grasemann C, Cloutier AM, et al. Maternal perinatal diet induces developmental programming of bone architecture. J Endocrinol. 2013;217:69–81.
11. Rauch F, Schoenau E. Skeletal development in premature infants: a review of bone physiology beyond nutritional aspects. Arch Dis Child Fetal Neonatal Ed. 2002;86:F82–F85.
12. Watkins BA, Li Y, Seifert MF. Nutraceutical fatty acids as biochemical and molecular modulators of skeletal biology. J Am Coll Nutr. 2001;20(suppl 6):410S–416S.
13. Rigo J, Mohamed M, De Curtis M. Disorders of calcium, phosphorus and magnesium metabolism. In: R Martin, A Fanaroff, M Walsh, eds. Fanaroff & Martin's Neonatal Perinatal Medicine: Diseases of the Fetus and Infant. St Louis, Missouri: Elsevier Mosby; 2011:1523–1526.
14. Ziegler EE, O'Donnell AM, Nelson SE, et al. Body composition of the reference fetus. Growth. 1976;40:329–341.
15. Nehra D, Carlson SJ, Fallon EM, et al. A.S.P.E.N. clinical guidelines: nutrition support of neonatal patients at risk for metabolic bone disease. JPEN J Parenter Enter Nutr. 2013;37:570–598.
16. Boullata JI, Gilbert K, Sacks G, et al. A.S.P.E.N. clinical guidelines: parenteral nutrition ordering, order review, compounding, labeling, and dispensing. JPEN J Parenter Enteral Nutr. 2014;38:334–377.
17. Lapillonne A, Jensen CL. Reevaluation of the DHA requirement for the premature infant. Prostaglandins Leukot Essent Fat Acids. 2009;81:143–150.
18. Pereira-da-Silva L, Macedo I, Rosa M, et al. Calcium and phosphorus intake by parenteral nutrition in preterm infants. In: R Rajendram, V Patel, V Preedy, eds. Diet and Nutrition in Critical Care. New York, New York: Springer; 2015:1–16.
19. Emken EA, Adlof RO, Duval SM, et al. Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. Lipids. 1999;34:785–791.
20. Benatti P, Peluso G, Nicolai R, et al. Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties. J Am Coll Nutr. 2004;23:281–302.
21. Robinson DT, Carlson SE, Murthy K, et al. Docosahexaenoic and arachidonic acid levels in extremely low birth weight infants with prolonged exposure to intravenous lipids. J Pediatr. 2013;162:56–61.
22. Bernhard W, Raith M, Koch V, et al. Plasma phospholipids indicate impaired fatty acid homeostasis in preterm infants. Eur J Nutr. 2014;53:1533–1577.
23. Watkins BA, Li Y, Allen KG, et al. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. J Nutr. 2000;130:2274–2284.
24. Gao Q, Xu M, Alander CB, et al. Effects of prostaglandin E_2 on bone in mice *in vivo*. Prostaglandins Other Lipid Mediat. 2009;89:20–25.
25. Coetzee M, Haag M, Kruger MC. Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E_2 and parathyroid hormone on osteoprotegerin and RANKL secretion by MC3T3-E1 osteoblast-like cells. J Nutr Biochem. 2007;18:54–63.
26. Casado-Díaz A, Santiago-Mora R, Dorado G, et al. The omega-6 arachidonic fatty acid, but not the omega-3 fatty acids, inhibits osteoblastogenesis and induces adipogenesis of human mesenchymal stem cells: potential implication in osteoporosis. Osteoporos Int. 2013;24:1647–1661.
27. Poulsen RC, Moughan PJ, Kruger MC. Long-chain polyunsaturated fatty acids and the regulation of bone metabolism. Exp Biol Med. 2007;232:1275–1288.
28. Kruger MC, Coetzee M, Haag M, et al. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. Prog Lipid Res. 2010;49:438–449.
29. Kajarabille N, Díaz-Castro J, Hijano S, et al. A new insight to bone turnover: role of ω-3 polyunsaturated fatty acids. Sci World J. 2013;2013:589641. doi:10.1155/2013/589641.
30. Li Y, Seifert MF, Ney DM, et al. Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein concentrations and reduce bone formation in rats fed (*n*-6) or (*n*-3) fatty acids. J Bone Miner Res. 1999;14:1153–1162.
31. Li Y, Greiner RS, Salem N, et al. Impact of dietary n-3 FA deficiency on rat bone tissue FA composition. Lipids. 2003;38:683–686.
32. Visser F, Sprij AJ, Brus F. The validity of biochemical markers in metabolic bone disease in preterm infants: a systematic review. Acta Paediatr. 2012;101:562–568.
33. Lukas R, Gigliotti JC, Smith BJ, et al. Consumption of different sources of omega-3 polyunsaturated fatty acids by growing female rats affects long bone mass and microarchitecture. Bone. 2011;49:455–462.
34. Turner A. Animal models of osteoporosis – necessity and limitations. Eur Cell Mater. 2001;1:66–81.
35. Andersen HS, Gambling L, Holtrop G, et al. Maternal iron deficiency identifies critical windows for growth and cardiovascular development in the rat postimplantation embryo. J Nutr. 2006;136:1171–1177.
36. Sangild PT. Gut responses to enteral nutrition in preterm infants and animals. Exp Biol Med. 2006;231:1695–1711.
37. Mollard RC, Kovacs HR, Fitzpatrick-Wong SC, et al. Low levels of dietary arachidonic and docosahexaenoic acids improve bone mass in neonatal piglets, but higher levels provide no benefit. J Nutr. 2005;135:505–512.
38. Shoji H, Hisata K, Suzuki M, et al. Effects of parenteral soybean oil lipid emulsion on the long-chain polyunsaturated fatty acid profile in very-low-birth-weight infants. Acta Paediatr. 2011;100:972–976.
39. Haag M, Magada ON, Claassen N, et al. Omega-3 fatty acids modulate ATPases involved in duodenal Ca absorption. Prostaglandins Leukot Essent Fat Acids. 2003;68:423–429.
40. Green KH, Wong SCF, Weiler HA. The effect of dietary n-3 long-chain polyunsaturated fatty acids on femur mineral density and biomarkers of bone metabolism in healthy, diabetic and dietary-restricted growing rats. Prostaglandins Leukot Essent Fat Acids. 2004;71:121–130.
41. Korotkova M, Ohlsson C, Hanson LA, et al. Dietary *n*-6:*n*-3 fatty acid ratio in the perinatal period affects bone parameters in adult female rats. Br J Nutr. 2004;92:643–648.
42. Lau BYY, Ward WE, Kang JX, et al. *Fat-1* gene modulates the fatty acid composition of femoral and vertebral phospholipids. Appl Physiol Nutr Metab. 2010;35:447–455.
43. Li Y, Seifert MF, Lim SY, et al. Bone mineral content is positively correlated to *n*-3 fatty acids in the femur of growing rats. Br J Nutr. 2010;104:674–685.
44. Nielson F, Stoecker B. Boron and fish oil have different beneficial effects on strength and trabecular microarchitecture of bone. J Trace Elem Med Biol. 2009;23:195–203.
45. Aerssens J, Boonen S, Lowet G, et al. Interspecies differences in bone composition, density, and quality: potential implications for *in vivo* bone research. Endocrinology. 1998;139:663–670.
46. Damsgaard CT, Mølgaard C, Matthiessen J, et al. The effects of n-3 long-chain polyunsaturated fatty acids on bone formation and growth factors in adolescent boys. Pediatr Res. 2012;71:713–719.
47. Groh-Wargo S, Jacobs J, Auestad N, et al. Body composition in preterm infants who are fed long-chain polyunsaturated fatty acids: a prospective, randomized, controlled trial. Pediatr Res. 2005;57:712–718.
48. Pereira-da-Silva L, Costa A, Pereira L, et al. Early high calcium and phosphorus intake by parenteral nutrition prevents short-term bone strength decline in preterm infants. J Pediatr Gastroenterol Nutr. 2011;52:203–209.
49. Gianni ML, Mora S, Roggero P, et al. Quantitative ultrasound and dual-energy x ray absorptiometry in bone status assessment of ex-preterm infants. Arch Dis Child Fetal Neonatal Ed. 2008;93:F146–F147.
50. Brantsæter AL, Birgisdottir BE, Meltzer HM, et al. Maternal seafood consumption and infant birth weight, length and head circumference in the Norwegian Mother and Child Cohort Study. Br J Nutr. 2012;107:436–444.
51. Vlieg P, Body D. Lipid contents and fatty acid composition of some New Zealand freshwater finfish and marine finfish, shellfish, and roes. New Zeal J Mar Fresh Res. 1988;22:151–162.
52. Lothe A, Sinn J, Stone M. Metabolic bone disease of prematurity and secondary hyperparathyroidism. J Paediatr Child Health. 2011;47:550–553.
53. Backström MC, Kouri T, Kuusela AL, et al. Bone isoenzyme of serum alkaline phosphatase and serum inorganic phosphate in metabolic bone disease of prematurity. Acta Paediatr. 2000;89:867–873.
54. Ashmeade T, Pereda L, Chen M, et al. Longitudinal measurements of bone status in preterm infants. J Pediatr Endocrinol Metab. 2007;20:415–424.
55. Crofton PM, Shrivastava A, Wade JC, et al. Bone and collagen markers in preterm infants: relationship with growth and bone mineral content over the first 10 weeks of life. Pediatr Res. 1999;46:581–587.
56. Moreira A, Swischuk L, Malloy M, et al. Parathyroid hormone as a marker for metabolic bone disease of prematurity. J Perinatol. 2014;34:787–791.

57. Harrison CM, Johnson K, McKechnie E. Osteopenia of prematurity: a national survey and review of practice. Acta Paediatr Int J Paediatr. 2008;97:407–413.
58. Rustico SE, Calabria AC, Garber SJ. Metabolic bone disease of prematurity. J Clin Transl Endocrinol. 2014;1:85–91.
59. Yang L, Grey V. Pediatric reference intervals for bone markers. Clin Biochem. 2006;39:561–568.
60. Hammami M, Koo W, Hockman EM. Body composition of neonates from fan beam dual energy X-ray absorptiometry measurement. JPEN J Parenter Enter Nutr. 2003;27:423–426.
61. Gallo S, Vanstone C, Weiler H. Normative data for bone mass in healthy term infants from birth to 1 year of age. J Osteoporos. 2012;672403. doi:10.1155/2012/672403.
62. Teitelbaum JE, Rodriguez RJ, Ashmeade TL, et al. Quantitative ultrasound in the evaluation of bone status in premature and full-term infants. J Clin Densitom. 2006;9:358–362.
63. McDevitt H, Ahmed SF. Quantitative ultrasound assessment of bone health in the neonate. Neonatology. 2007;91:2–11.
64. Littner Y, Mandel D, Mimouni FB, et al. Bone ultrasound velocity curves of newly born term and preterm infants. J Pediatr Endocrinol Metab. 2003;16:43–47.
65. Wang KC, Wang KC, Amirabadi A, et al. Evidence-based outcomes on diagnostic accuracy of quantitative ultrasound for assessment of pediatric osteoporosis – a systematic review. Pediatr Radiol. 2014;44:1573–1587.
66. Wood C, Wood A, Harker C, et al. Bone mineral density and osteoporosis after preterm birth: the role of early life factors and nutrition. Int J Endrocrinology. 2013;902513:1–7.
67. Fewtrell MS, Prentice A, Jones SC, et al. Bone mineralization and turnover in preterm infants at 8–12 years of age: the effect of early diet. J Bone Miner Res. 1999;14:810–820.
68. Bowden L, Jones C, Ryan S. Bone mineralisation in ex-preterm infants aged 8 years. Eur J Pediatr. 1999;158:658–661.
69. Fewtrell M, Prentice A, Cole T, et al. Effects of growth during infancy and childhood on bone mineralization and turnover in preterm children aged 8–12 years. Acta Paediatr. 2000;89:148–153.
70. Abou Samra H, Stevens D, Binkley T, et al. Determinants of bone mass and size in 7-year-old former term, late-preterm, and preterm boys. Osteoporos Int. 2009;20:1903–1910.
71. Hovi P, Andersson S, Järvenpää A, et al. Decreased bone mineral density in adults born with very low birth weight: a cohort study. PLoS One. 2009:e1000135. doi:10.1371/journal.pmed.1000135.
72. Carroll DM, Doria AS, Paul BS. Clinical-radiological features of fractures in premature infants – a review. J Perinat Med. 2007;35:366–375.
73. Chan GM, Armstrong C, Moyer-Mileur L, et al. Growth and bone mineralization in children born prematurely. J Perinatol. 2008;28:619–623.
74. Lucas-Herald A, Butler S, Mactier H, et al. Prevalence and characteristics of rib fractures in ex-preterm infants. Pediatrics. 2012;130:1116–1119.
75. Vignochi CM, Silveira RC, Miura E, et al. Physical therapy reduces bone resorption and increases bone formation in preterm infants. Am J Perinatol. 2012;29:573–578.
76. Haggarty P. Meeting the fetal requirement for polyunsaturated fatty acids in pregnancy. Curr Opin Clin Nutr Metab Care. 2014;17:151–155.
77. Valentine CJ, Puthoff TD. Enhancing parenteral nutrition therapy for the neonate. Nutr Clin Pract. 2007;22:183–193.
78. Crawford M. Placental delivery of arachidonic and docosahexaenoic acids: implications for the lipid nutrition of preterm infants. Am J Clin Nutr. 2000;71:275–284.
79. Lapillonne A, Eleni Dit Trolli S, Kermorvant-Duchemin E. Postnatal docosahexaenoic acid deficiency is an inevitable consequence of current recommendations and practice in preterm infants. Neonatology. 2010;98:397–403.
80. Kapoor V, Glover R, Malviya MN. Soy oil based versus alternative lipid emulsions for parenterally fed preterm infants. Cochrane Database Syst Rev. 2011;(6):CD009172. doi:10.1002/14651858.CD009172.
81. Lapillonne A, Groh-Wargo S, Lozano Gonzalez CH, et al. Lipid needs of preterm infants: updated recommendations. J Pediatr. 2013;162(suppl 3):S37–S47.
82. Waitzberg D, Torrhinas R, Jacintho T. New parenteral lipid emulsions for clinical use. JPEN J Parenter Enter Nutr. 2006;30:351–357.
83. Muhammed R, Bremner R, Protheroe S, et al. Resolution of parenteral nutrition-associated jaundice on changing from a soybean oil emulsion to a complex mixed-lipid emulsion. J Pediatr Gastroenterol Nutr. 2012:797–802.
84. Skouroliakou M, Konstantinou D, Agakidis C, et al. Cholestasis, bronchopulmonary dysplasia, and lipid profile in preterm infants receiving MCT/ω-3-PUFA-containing or soybean-based lipid emulsions. Nutr Clin Pract. 2012;27:817–824.
85. Pawlik D, Lauterbach R, Turyk E. Fish-oil fat emulsion supplementation may reduce the risk of severe retinopathy in VLBW infants. Pediatrics. 2011;127:223–228.
86. American Academy of Pediatrics Committee on Nutrition. Nutritional needs of the preterm infant. In: Kleinman R, Greer F, eds. Pediatric Nutrition. 7th ed. Elk Grove City, Illinois: American Academy of Pediatrics; 2014:83–121.
87. Klein CJ. Nutrient requirements for preterm infant formulas. J Nutr. 2002;132(6 suppl 1):1395S–1577S.
88. Agostoni C, Buonocore G, Carnielli VP, et al. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. J Pediatr Gastroenterol Nutr. 2010;50:85–91.
89. Koletzko B, Innis SM. Lipids. In: R Tsang, R Uauy, B Koletzko, et al., eds. Nutrition of the Preterm Infant: Scientific Basis and Practical Guidelines. 2nd ed. Cinncinnati, Ohio: Digital Education Publishing; 2005:97–140.
90. Driscoll DF, Bistrian BR, Demmelmair H, et al. Pharmaceutical and clinical aspects of parenteral lipid emulsions in neonatology. Clin Nutr. 2008;27:497–503.
91. Vasudevan C, Johnson K, Miall LS, et al. The effect of parenteral lipid emulsions on pulmonary hemodynamics and eicosanoid metabolites in preterm infants: a pilot study. Nutr Clin Pract. 2013;28:753–757.
92. Demirel G, Oguz SS, Celik IH, et al. The metabolic effects of two different lipid emulsions used in parenterally fed premature infants – a randomized comparative study. Early Hum Dev. 2012;88:499–501.
93. Tomsits E, Pataki M, Tolgyesi A, et al. Safety and efficacy of a lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil: a randomised, double-blind clinical trial in premature infants requiring parenteral nutrition. J Pediatr Gastroenterol Nutr. 2010;51:514–521.
94. D'Ascenzo R, Savini S, Biagetti C, et al. Higher docosahexaenoic acid, lower arachidonic acid and reduced lipid tolerance with high doses of a lipid emulsion containing 15% fish oil: a randomized clinical trial. Clin Nutr. 2014;33:1002–1009.
95. Rayyan M, Devlieger H, Jochum F, et al. Short-term use of parenteral nutrition with a lipid emulsion containing a mixture of soybean oil, olive, oil, medium-chain triglycerides, and fish oil: a randomized double-blind study in preterm infants. JPEN J Parenter Enter Nutr. 2012;36(1 suppl):81S–94S.
96. Vlaardingerbroek H, Vermeulen MJ, Carnielli VP, et al. Growth and fatty acid profiles of VLBW infants receiving a multicomponent lipid emulsion from birth. J Pediatr Gastroenterol Nutr. 2014;58:417–427.
97. Savini S, D'Ascenzo R, Biagetti C, et al. The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. Am J Clin Nutr. 2013;98:312–318.
98. Fallon EM, Nazarian A, Nehra D, et al. The effect of docosahexaenoic acid on bone microstructure in young mice and bone fracture in neonates. J Surg Res. 2014;191:148–155.
99. Ratledge C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie. 2004;86:807–815.