Journal of Parenteral and Enteral Nutrition
Volume 00 Number 0
xxx 2019 1–9

DOI: 10.1002/jpen.1533
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Early Postnatal Changes of Bone Turnover Biomarkers in Very Low-Birth-Weight Neonates—The Effect of Two Parenteral Lipid Emulsions with Different Polyunsaturated Fatty Acid Content: A Randomized Double-Blind Study

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Abstract

Background: ω-3 polyunsaturated fatty acids (n-3 PUFAs) are reported to have beneficial effect on bone mineral density. This study aimed to evaluate early changes of bone turnover biomarkers in very low-birth-weight (VLBW) neonates and the effect of 2 parenteral lipid emulsions (PLEs) with different PUFA composition. *Methods:* This is a randomized double-blind study with parallel design. VLBW neonates (n = 66) receiving parenteral nutrition (PN)>70% of daily energy requirements for >14 days were assigned into 2 groups that were prescribed soybean oil–based (n = 35) and n-3–enriched PLE (n = 31), respectively. Osteoprotegerin (OPG), soluble receptor activator of nuclear factor-kB ligand (sRANKL), osteocalcin (OC), interleukin-6 (enzyme-linked immunoblot assay kits), Ca, and P plasma levels were assessed before PLE implementation (T1) and on day 20 of life (T2). *Results:* In the total population, sRANKL and OC significantly increased, whereas OPG and the OPG/sRANKL ratio decreased from T1 to T2. Within each group, T1-to-T2 changes of OC were significant in both groups, whereas those of OPG/sRANKL were significant only in the soybean-based group. Multiple regressions showed an independent effect of group allocation on OPG change. Significant associations were observed between PN duration and sRANKL change (negatively), n-6/n-3 and OC changes (positively), and OPG and sRANKL changes (positively). *Conclusions:* A high bone-turnover rate in VLBW neonates with predominance of bone resorption is confirmed. The lower rate of OPG/sRANKL reduction in the n-3–enriched PLE group indicates that n-3 PUFA–enriched PLEs may help to attenuate early bone loss in VLBW neonates. (*JPEN J Parenter Enteral Nutr.* 2019;00:1–9)

Keywords

bone health; fatty acids; lipids; neonates; parenteral nutrition

Clinical Relevancy Statement

The study provides evidence confirming a high bone-turnover rate in very low-birth-weight (VLBW) neonates with predominance of bone resorption. The observed lower rate of osteoprotegerin/soluble receptor activator nuclear factor-kB ligand ratio reduction in the Smoflipid group indicates that ω-3 polyunsaturated fatty acid

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Financial disclosure: None declared.

Conflicts of interest: None declared.

Received for publication November 16, 2018; accepted for publication February 20, 2019.

This article originally appeared online on xxxx 0, 2019.

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(n-3 PUFA)–enriched parenteral lipid emulsions (PLEs) might help reduce early bone loss in VLBW neonates, suggesting that, in clinical practice, PLEs enriched in n-3 PUFAs may be the preferable source of parenteral fat for them. Moreover, plasma bone biomarker levels can be useful in following up the bone health status of VLBW neonates in clinical practice, since they can be measured in every lab using accessible techniques, requiring minimal amounts of blood.

Introduction

Metabolic bone disease of prematurity is a common complication of prematurity, mainly affecting very low-birthweight (VLBW) neonates. VLBW neonates are deprived of the last trimester of pregnancy when the highest calcium (Ca) and phosphorus (P) accretion occurs. At birth, transplacental active-mineral transfer is interrupted, and the preterm neonate becomes dependent on mineral supplies via nutrition. On the other hand, physiological adaptation of bones to extrauterine life leads to increased bone resorption.[1,2] These factors contribute to significant decline in bone strength during the first weeks of extrauterine life, which is followed by a catch-up to the third or the sixth month of life.[3,4]

Among the plasma biomarkers proposed for the evaluation of bone metabolic disease, blood levels of osteoprotegerin (OPG), soluble receptor activator of nuclear factor-kB ligand (sRANKL), and osteocalcin (OC) have been extensively studied.[5-10] The sRANKL binds to receptor RANK on the surface of osteoclast progenitor cells and promotes osteoclast differentiation, activity, and survival, eventually leading to bone resorption.[5-7] OPG binds to the sRANKL, thereby preventing its attachment to RANK receptor and the subsequent bone resorption.[8] On the other hand, the OC, produced by the osteoblasts, reflects its activity and has been used as a marker of new bone formation (Figure 1).[9,10]

Fat intake is included among the nutrition factors involved in bone metabolism.[2] Experimental and adult studies associated fat quality with development of osteoporosis, which is equivalent to osteopenia of prematurity.[11,12] The effect of fatty acids on bone turnover is attributed mainly to the inflammatory properties of the ω-6 (n-6) polyunsaturated fatty acids (PUFAs) and the anti-inflammatory properties of the n-3 PUFAs.[13] The balance between n-6 and n-3 PUFAs plays an important role in cytokine production and bone turnover.[14] The ultimate result on bone metabolism depends on the PUFA profile, with eicosapentanoic acid (EPA) acting as inducer and docosahexanoic acid (DHA) and arachidonic acid (AA) acting as inhibitors of bone formation, whereas prostaglandin E2, a byproduct of AA metabolism, exerts a differential effect on bone turnover (Figure 1).[11,15-17]

Most VLBW neonates depend on parenteral nutrition (PN) for several days or weeks. Composition of the fatty acids delivered by PN differs depending on the type of parenteral lipid emulsion (PLE) used. Formulations of PLEs most widely used in neonates include soybean oil–based PLE and n-3 PUFA/medium-chain triglyceride (MCT)–enriched PLE. The soybean-based PLE is rich in n-6 PUFAs, whereas the n-3 PUFA–enriched PLE contains increased n-3 PUFAs (EPA and DHA) derived from fish oil. Previous studies by our research team demonstrated that preterm neonates receiving n-3 PUFA–enriched PLEs had significantly lower plasma levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8 on day 30 of life and oxidative stress compared with those receiving soybean-based PLEs.[18,19]

We hypothesized that a PN formulation containing PLE enriched in n-3 PUFAs could attenuate the early postnatal decline of bone mineral density in VLBW neonates. The objective of this study was to evaluate the changes of 3 bone turnover biomarkers, the OPG, sRANKL, and OC, in VLBW neonates during the first 3 postnatal weeks and the potential effect of administration of 2 PLEs with different PUFA composition on bone turnover biomarkers.

Study Population and Methods

Study Design and Population

This was a randomized, double-blind clinical trial with parallel design (2 treatment groups) and an allocation ratio 1:1. Preterm neonates with gestational age < 32 weeks and birth weight < 1500 g that were admitted to a tertiary neonatal intensive care unit between February 2017 and February 2018 within 12 hours after birth were assessed for eligibility. Exclusion criteria were anticipated needs for PN at >70% of total daily energy (hereafter referred to as PN duration) for <10 days, evidence of intrauterine infection, perinatal asphyxia, major congenital anomalies, and refusal of parental consent. Eligible neonates were randomly assigned into 2 groups: soybean oil–based PLE (Intralipid) and n-3/MCT-enriched PLE (Smoflipid). Randomization was based on a computer-generated randomization list, and the PN regimens were automatically produced as described previously.[20] Cluster randomization was performed based on birth weight (<1000 g and 1000–1500 g). Postrandomization exclusion criteria were PN duration < 15 days, diagnosis of inherited disorders of metabolism, withdrawal of parental consent, and transportation to other hospital or early death within the first 14 days of life. The included neonates were followed up to the 20th day of life (study period). Of the 112 VLBW neonates assessed for eligibility, 75 were randomized in the 2 study groups. Sixty-six of them finished the study, 35 in the soybean-based group and 31

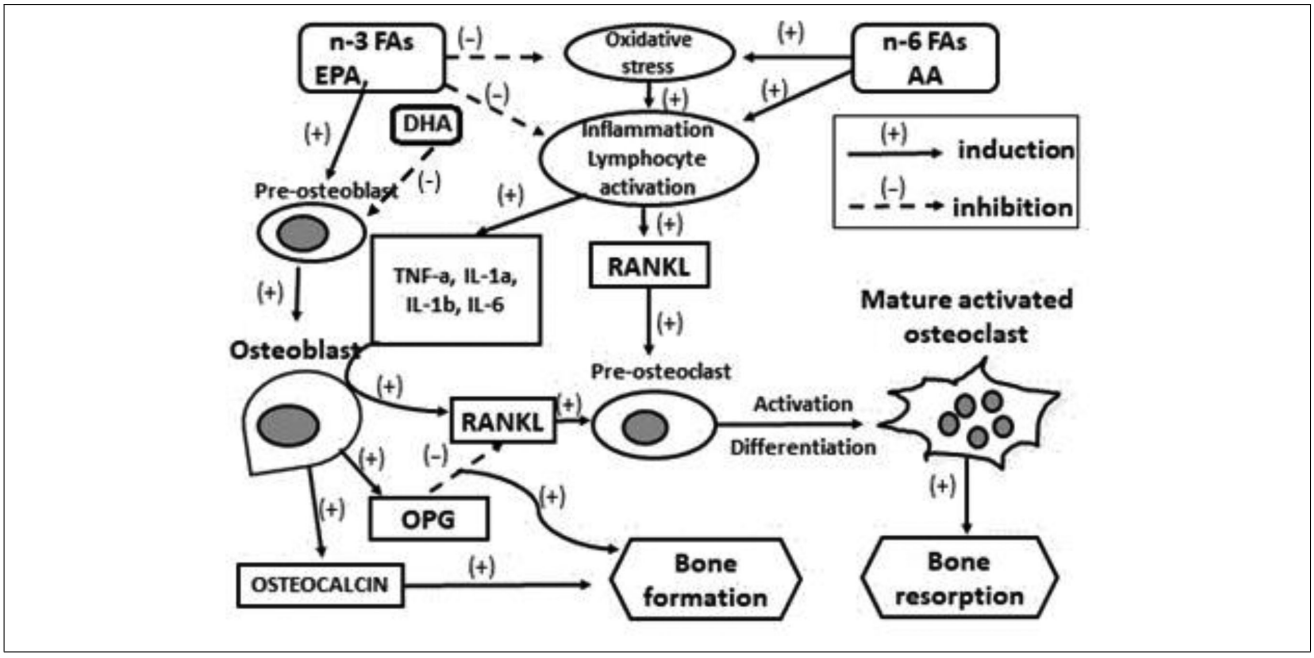

Figure 1. Pathophysiology mechanisms of bone turnover and association with polyunsaturated FAs. Schematic presentation of the main pathways involved in bone turnover and the role of n-3 and n-6 FAs. AA, arachidonic acid; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; FA, fatty acids; IL, interleukin; OPG, osteoprotegerin; PUFA, polyunsaturated fatty acid; RANKL, receptor activator nuclear factor-kB ligand; TNF-a, tumor necrosis factor-a.

in the n-3–enriched group, as shown in the flow diagram (Figure 2).

Bioethics

The study protocol was approved by the Scientific and Ethical Committee of the Hospital, and written consent was obtained from all parents before enrollment. This work is registered in ClinicalTrials.gov (Protocol Record 29042015) and was written according to the Consolidated Standards of Reporting Trials (CONSORT) statement (http://www.consort-statement.org).

PN Protocols

PLE was added on the first or second day of life at a dose of 1 g/kg/d, which increased daily by 1 g/kg/d up to a maximum of 3 g/kg/d. Protein was initiated on the first day of life at 2 g/kg/d, reaching 3.5–4.5 g/kg/d by day 3–4, depending on neonate's body weight, in accordance with the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommendations.[21] PLEs were infused over 20–24 hours. Amino acids were derived from Vamin Infant (Fresenius Kabi HELLAS, Athens, Greece). The PLE provided to the Smoflipid group was the n-3–enriched PLE Smoflipid (Fresenius Kabi HELLAS, Athens, Greece), containing fish oil (3 g%), soybean oil (6 g%), olive oil (5 g%), MCTs (6 g%), egg yolk phospholipids (1.2 g%), glycerin (2.5 g%), and α-tocopherol (200 mg/L). The Intralipid group received the soybean oil–based PLE Intralipid 20% (Fresenius Kabi HELLAS, Athens, Greece) containing soybean oil (20 g%), egg yolk phospholipids (1.2 g%), glycerin (2.25 g%), and α-tocopherol (38 mg/L). The pharmacist who prepared the 2 PN formulations and assigned neonates in 1 of the 2 groups was not involved in the neonates' care. All neonates received 160 IU/kg/d of ergocalciferol (vitamin D2) through PN (Vitalipid Infant, Fresenius Kabi, Athens, Greece) from day 1 to 2. Enteral feeding was implemented as soon as possible with either maternal milk or preterm formula. All personnel of the neonatal unit and participants were unaware of the assignment.

Clinical and Laboratory Data

Recorded data included gestational age, birth weight, perinatal history, neonatal problems, treatment, and outcome. Nutrient and energy intake via PN and enteral feeding were recorded daily. Serum levels of Ca and P were assessed every 3 days. Measurement of plasma levels of OPG, sRANKL, OC, and IL-6 was performed within 24 hours after birth (time 1 [T1]), before PLE implementation, and on day 20 of life or when the PN-derived energy decreased to $\leq$70% of total energy (time 2 [T2]), whichever was earlier, albeit not before the 15th day of life. The age of 15–20 days for final evaluation was chosen in order to assess biomarker levels

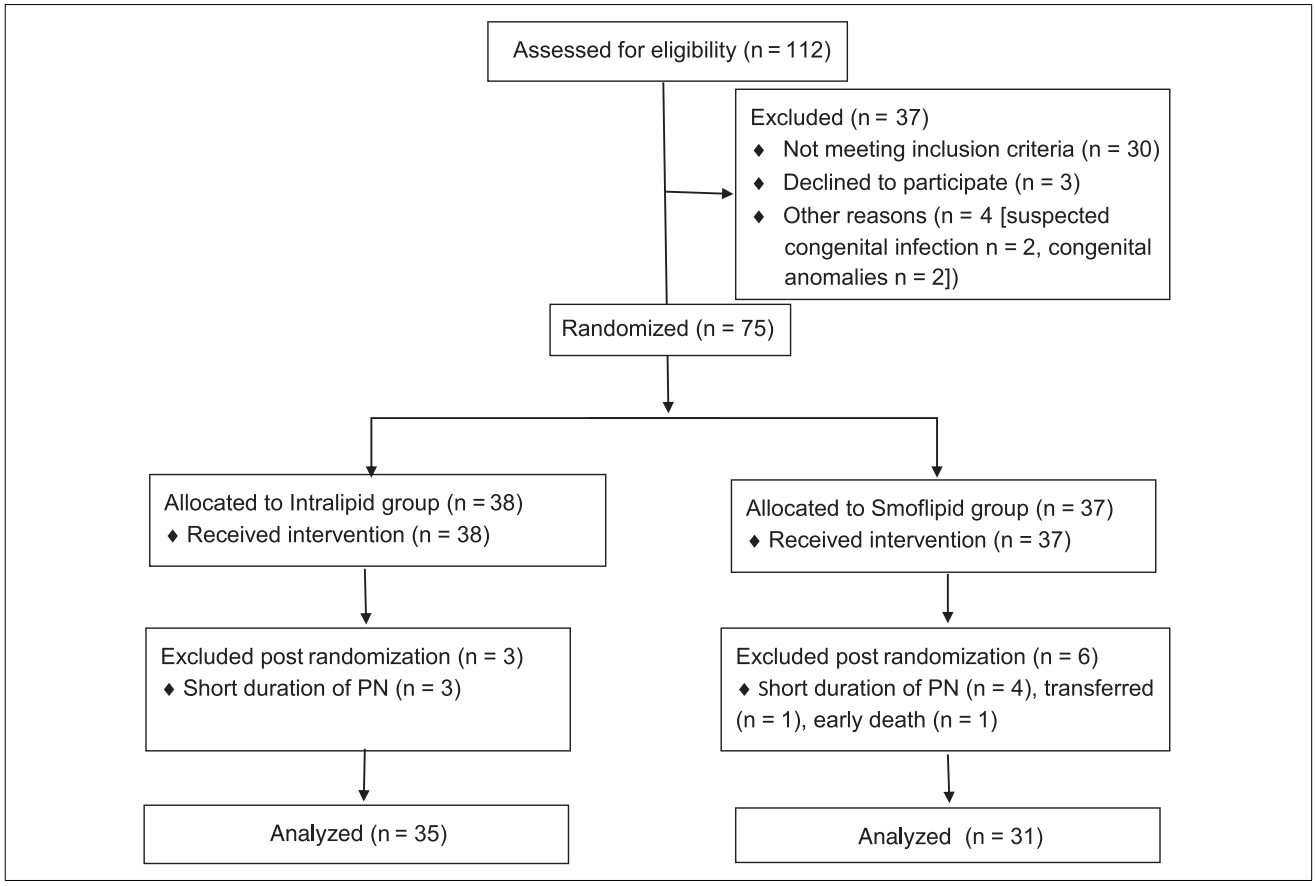

Figure 2. Flow diagram of the study design. Early postnatal changes of bone turnover biomarkers in very low-birth-weight neonates. The effect of 2 parenteral lipid emulsions with different polyunsaturated fatty acid content. A randomized double-blind study. PN, parenteral nutrition.

during the period of high PN requirements and before the development of complications of prematurity (ie, chronic lung disease) which can affect bone turnover. T2 samples were obtained in the morning, following overnight interruption of the PLE and before feedings. For measurement of the study parameters, 2 mL of blood was obtained during sampling for routine laboratory tests.

High-sensitivity enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) were used to measure OPG (Human Osteoprotegerin /TNFRSF11B, DuoSet ELISA development kit, 15 plates), sRANKL (Mouse TRANCE/RANK Ligand/TNFSF11 Quantikine ELISA Kit 96 tests, sensitivity 5 pg/mL, assay range 31.2–2000 pg/mL), OC (Human Osteocalcin Quantikine ELISA Kit 96 tests, sensitivity 0.898 ng/mL, assay range 2.0–64 ng/mL), and IL-6 (Human IL-6 Quantikine ELISA Kit, sensitivity to 0.110 pg/mL and intra-assay coefficient of variability < 7%). Plasma FAs were assessed by gas liquid chromatography in the form of their methyl esters and were expressed as percentages wt/wt of total fatty acids measured as previously described.[22]

Outcome Measures

Primary outcome measures were changes of bone turnover biomarkers in VLBW neonates during the first 2–3 postnatal weeks and differences in biomarkers levels between the 2 study groups. Secondary outcome measures were the differences in plasma n-3 and n-6 PUFA between the 2 groups at the end of the study.

Statistical Analysis

Continuous variables were presented as means and standard deviations and categorical variables as counts and percentages. The between-group differences were assessed by the Mann-Whitney *U* test and Fisher exact test, as appropriate. The paired *t*-test was used for assessing the changes from T1 to T2. Multivariate linear regression models, separate for each biomarker, were constructed to assess the factors independently associated with the adjusted percentage changes of the biomarker plasma levels. The percentage changes between baseline and the end of the study period were calculated for all variables to be used in the regression

Table 1. Clinical Characteristics of the 2 Study Groups.

	Intralipid Group	Smoflipid Group	*P**	OR (95% CI)
n	35	31		
Birth weight (g, mean ± SD)	1232 ± 263	1240 ± 260	0.947	N.A.
Gestational age (weeks, mean ± SD)	29.3 ± 1.7	29.2 ± 1.9	0.975	N.A.
Male gender, n (%)	22 (63)	18 (58)	0.802	1.2 (0.45, 3.87)
Prenatal steroids, n (%)	28 (80.0)	24 (77.4)	1.0	1.17 (0.36, 3.80)
Intrauterine growth restriction, n (%)	1 (2.9)	2 (6.5)	0.597	0.43 (0.037. 4.95)
Respiratory distress syndrome, n (%)	17 (48.6)	18 (58.1)	0.469	0.68 (0.26, 1.81)
Sepsis during study period, n (%)	2 (5.7)	1 (3.2)	1.0	1.82 (0.16, 21.1)
Patent ductus arteriosus, n (%)	10 (29)	4 (13)	0.143	2.70 (0.75, 9.72)
Chronic lung disease, n (%)	7 (20.0)	4 (12.9)	0.521	1.67 (0.44, 6.43)
Intraventricular hemorrhage grades II–IV, n (%)	4 (11.4)	4 (12.9)	1.0	0.87 (0.20, 3.82)
Retinopathy of prematurity (n, %)	2 (5.7)	0 (0)	0.494	N.A.
Days on parenteral nutrition at proportion > 70% (mean ± SD)	17.3 ± 1.4	16.7 ± 2.0	0.047	N.A.
Age at T2 (days, mean ± SD)	19.5 ± 2.0	18.7 ± 1.6	0.052	N.A.
Days in hospital (mean ± SD)	64 ± 24	57 ± 24	0.074	N.A.

N.S., nonsignificant; N.A., nonapplicable, OR, odds ratio; T2, time 2.
*P**, Mann-Whitney test or Fisher exact test.

models. Nonparametric variables were transformed using natural logarithms for use in the multiple regression models. The threshold for significance was set at $P < 0.05$. Statistical analysis was performed using IBM SPSS software, version 21 (IBM, Chicago, IL, USA).

Results

Clinical Data

The 2 study groups were comparable in regard to the gestational age, birth weight, perinatal/neonatal morbidity, medications, and days in hospital (Table 1). The PN duration was significantly higher in the Intralipid group, but a difference in mean values of 0.6 days between the 2 groups is not considered clinically significant. All infants received parenteral ergocalciferol (D2, 160 IU/kg/d) and caffeine from the first day of life, and none received postnatal steroids during the study period. Regarding furosemide administration, only 3/66 neonates received 1 to 2 doses during the whole study period. The mean daily fluid, energy, macronutrient, and mineral intake did not differ between the 2 groups (Table 2). The percentage of energy intake via enteral nutrition and PN during the first 15 days of the study is shown in Table 3.

Levels of Bone Turnover Biomarkers, IL-6, Ca, and P

In the total population, comparison between T1 and T2 plasma levels showed significant increase in sRANKL and OC and decrease in the OPG/sRANKL ratio, whereas OPG showed a nonsignificant decrease. Paired comparisons between T1 and T2 levels within each study group showed a nonsignificant increase in sRANKL and decrease of OPG and significant increase of OC in both groups, whereas the OPG/sRANKL ratio decreased significantly only in the Intralipid group. Significant changes were also observed in levels of IL-6 (decrease), Ca, and P (increase) in the total population as well as within each study group (Table 4).

The between-group comparisons showed no significant difference in values of the OPG, sRANKL, OPG/sRANKL, OC, IL-6, Ca, and P at either T1 or T2.

Levels of PUFAs

Comparisons between groups regarding the plasma PUFA levels at T2 showed that the Smoflipid group had significantly higher plasma values of n-3 (2.8 ± 1.1 vs 2.2 ± 0.8, $P = 0.008$) and EPA (0.64 ± 0.44 vs 0.39 ± 0.30, $P = 0.009$) and lower values of n-6 (25.3 ± 3.9 vs 28.9 ± 3.9. $P = 0.001$), n-6/n-3 ratio (10.4 ± 4.7 vs 14.5 ± 4.7, $P < 0.001$), and AA (4.6 ± 1.4 vs 6.1 ± 2.2, $P = 0.001$) than the Intralipid group, whereas the DHA values were comparable in the 2 groups.

Multiple Regression Analysis

Multiple linear regression models were built with the percentage change of each bone turnover biomarker as dependent variable and birth weight, group allocation representing the type of PLE, PN duration, and changes in n-6/n-3 PUFA ratio, IL-6, and Ca as independent variables. The model constructed for the OPG included the changes of sRANKL among the independent variables to evaluate the association between these 2 biomarkers.

Table 2. Mean (± SD) Energy, Nutrient, and Mineral Intake During the Intervention Period Via Parenteral and Enteral Routes.

	Intralipid Group	Smoflipid Group	*P* (Mann-Whitney)
n	35	31	
Fluid volume, mL/kg/d	151.3 ± 5.9	148.4 ± 34.2	0.181
Energy, kcal/kg/d	85.5 ± 8.5	84.7 ± 17.2	0.448
Proteins, g/kg/d	3.2 ± 0.32	3.1 ± 0.63	0.862
Glucose, g/kg/d	13.3 ± 1.4	13.0 ± 2.6	0.496
Fat, g/kg/d	3.0 ± 0.41	3.0 ± 0.70	0.949
Calcium, mEq/kg/d	2.1 ± 0.5	2.0 ± 0.6	0.057
Phosphate, mmol/kg/d	1.3 ± 0.3	1.2 ± 0.2	0.1512
Magnesium, mEq/kg/d	0.77 ± 0.1	0.76 ± 0.1	0.676
Sodium, mEq/kg/d	2.9 ± 1.3	2.8 ± 1.3	0.129
Potassium, mEq/kg/d	2.0 ± 0.4	2.0 ± 0.3	0.316

Table 3. Total Daily Energy Intake and Percentage of Energy Intake Via PN and Enteral Nutrition During the Study Period.

	Intralipid Group			Smoflipid Group		
Day of Life	Total Energy Intake, kcal/kg/d	Percent of Energy from PN	Percent of Energy from Milk	Total Energy Intake, kcal/kg/d	Percent of Energy From PN	Percent of Energy From Milk
2nd	51.8 ± 6.2	100 ± 0	0	67.2 ± 14.7	100 ± 0	0
5th	88.7 ± 11.7	99.2 ± 2.7	0.8 ± 2.7	91.0 ± 12.5	94.7 ± 9.5	5.3 ± 9.5
10th	89.5 ± 12.8	94.3 ± 10.5	5.61 ± 10.5	88.4 ± 16.4	96.8 ± 8.0	3.2 ± 8.0
15th	77.2 ± 23.3	95.5 ± 8.6	4.5 ± 8.6	85.9 ± 23.7	96.9 ± 8.1	3.1 ± 8.1

PN, parenteral nutrition.

Group allocation was a significant independent predictor of OPG, whereas the sRANKL was significantly positively associated with the OPG. The PN duration and birth weight were significantly positively and negatively, respectively, associated with sRANKL after adjustment for confounders. The change of total n-6/n-3 ratio was independently positively associated with the OC adjusted changes. The change in serum Ca was negatively associated with the OPG/sRANKL and positively with the OC changes after adjustment for confounders (Table 5). Additional regression models including the incidence of respiratory distress syndrome and sepsis as confounders did not show any significant independent effect of these complications of prematurity on biomarker changes (data not shown).

Discussion

This is the first study to investigate the effect of PLEs with different PUFA composition on bone turnover biomarkers during the early postnatal weeks. The changes in biomarker levels indicated a prevalence of bone resorption over bone formation in the total study population as well as within each study group. The greater reduction of OPG/sRANKL ratio in the Intralipid group indicates greater bone resorption in this group. Multiple regression models revealed that the type of PLE and the PN duration had a significant independent effect on the change of OPG and sRANKL, respectively, whereas the change in n-6/n-3 ratio was a significant independent predictor positively associated with the OC change.

OPG, sRANKL, OPG/sRANKL, and OC Changes During the Study Period in the Total Population

For evaluation of bone turnover, the use of 2 biomarkers, 1 for bone formation and 1 for bone resorption, have been recommended.[23] In the current study, we have used 2 biomarkers of bone formation, the OPG and OC, and 1 of bone resorption, the sRANKL[9], and we observed significant changes in sRANKL and OPG/sRANKL ratio but not OPG during the study period. These changes are consistent with increased bone turnover with predominant osteoclastic activity. Our results are in line with previous studies in neonates that used quantitative ultrasound, which reported a progressive decrease of bone strength during the first few weeks or months of life, especially in preterm neonates.[3,4]

In order to clarify the independent association between OPG and sRANKL after controlling for potential

Table 4. Mean (± SD) of Serum/Plasma Levels of Biochemistry Parameters at Baseline (T1) and the End of the Study Period (T2).

	Total Population			Intralipid Group			Smoflipid Group		
	T1	T2	*P**	T1	T2	*P**	T1	T2	*P**
n	66	63		35	33		31	30	
OPG, pg/mL	19.7 ± 18.9	16.5 ± 12.6	0.143	20.1 ± 21.2	18.5 ± 15.7	0.646	19.2 ± 16.9	14.2 ± 7.5	0.114
sRANKL, pmol/L	7.7 ± 12.9	13.5 ± 16.1	0.015	7.1 ± 13.7	12.8 ± 15.8	0.067	8.2 ± 12.2	14.2 ± 16.6	0.115
OPG/sRANKL ratio	4.1 ± 5.0	2.6 ± 4.3	0.002	5.3 ± 3.7	2.6 ± 2.6	<0.001	5.6 ± 6.2	2.6 ± 5.7	0.113
Osteocalcin, ng/mL	173.2 ± 249	761.2 ± 527	<0.001	264.5 ± 277	706.9 ± 553	<0.002	281.5 ± 225	788.9 ± 507	<0.001
IL-6, pg/mL	8.6 ± 1.7	6.1 ± 3.5	<0.001	8.5 ± 1.7	6.3 ± 3.3	0.031	8.6 ± 1.7	6.0 ± 3.7	0.003
Calcium, mmol/L	2.12 ± 0.2	2.49 ± 0.1	<0.001	2.12 ± 0.2	2.40 ± 0.1	<0.001	2.15 ± 0.2	2.4 ± 0.1	<0.001
Phosphorus, mmol/L	1.81 ± 0.4	2.13 ± 0.4	<0.001	1.87 ± 0.4	2.13 ± 0.4	0.012	1.81 ± 0.42	2.13 ± 0.3	0.002

Comparison between the 2 study groups (Mann-Whitney *U* test) showed no significant difference at T1 and T2.
IL, interleukin; OPG, osteoprotegerin; sRANKL, soluble receptor activator nuclear factor-kB ligand; T1, time 1; T2, time 2.
*P**, paired *t*-test.

Table 5. Multivariate Linear Regression Models With the Dependent Variable the Mean Percentage Changes of Serum/Plasma Levels of Bone Metabolism Biomarkers During the Study Period.

Dependent Variable	Group and Significant Independent Variables	β	*P*	Exp (B)	Confidence Intervals 5%	Confidence Intervals 95%	Nonsignificant Variables
Percent OPG change	Group	59.173	0.034	4.992E+25	85.538	2.92E+49	Percent n-6/n-3 change, percent Ca change, percent IL-6 change, BW
	Percent change RANKL	0.136	0.013	1.145	1.029	1.274	
Percent sRANKL change	PN duration	48.979	0.033	5.352E-22	1.468E-41	0.020	Group, percent change n-6/n-3, percent change Ca, percent change IL-6
	BW	−0.442	0.024	0.642	0.438	0.943	
Percent OPG/sRANKL change	Percent change Ca	−2.30	0.006	0.100	0.020	0.511	Group, PN duration, percent change n-6/n-3, percent change IL-6, BW
Percent OC change	Percent change n-6/n-3	3.774	0.009	43.575	2.569	738.965	Group, PN duration, percent change IL-6, percent change Ca, BW

BW, birth weight; Group, allocation group according to the type of LE; IL, interleukin; n-3, ω-3; n-6, ω-6; OC, osteocalcin; OPG, osteoprotegerin; sRANKL, soluble receptor activator nuclear factor-kB ligand; PN, parenteral nutrition.

confounders, we included these 2 biomarkers in the same regression model. In the context of their opposite effects on bone turnover, an inverse correlation would be expected between OPG and sRANKL. However, the analysis revealed a significant positive association of the adjusted OPG changes with sRANKL, which may reflect preservation of OPG production as a compensatory mechanism against the RANK/RANKL-associated bone resorption. On the other, the OC levels highly increased in our study population, suggesting increased bone formation, in agreement with previous reports in term neonates.[24] Overall, the significant increase of OC, combined with changes in OPNPG, sRANKL, and OPG/sRANKL ratio, indicates an ongoing bone remodeling in VLBW infants during early postnatal life.

Data on OPG, sRANKL, and OC levels in human neonates are limited. Early studies in term neonates showed that blood levels of OC increase during the first few days after birth,[24] whereas breastfeeding is associated with higher OPG levels compared with formula fed infants because of the OPG contained in human milk.[25] Studies in term neonates with intrauterine growth restriction reported that

levels of OC, OPG, and sRANKL remained stable[26,27], whereas other studies demonstrated a significant increase of OPG/sRANKL ratio[28] in early postnatal life.

In agreement with our results, previous studies in preterm neonates reported increasing OC levels during the first 3 weeks of life.[29] In addition, prenatal exposure to steroids has been associated with increased OPG, whereas postnatal administration was associated with decreased levels of OC.[30,31] Thus far, no study has explored the effect of early nutrition on early bone-turnover biomarkers.

Effect of the Type of PLE on PUFA Profile and Bone Turnover Biomarker Plasma Levels

The infusion of PLEs with different PUFA composition resulted in significant differences of plasma PUFA profile between the 2 study groups. At the end of study period, the Smoflipid group had significantly lower total n-6 PUFA, n-6/n-3 PUFA ratio, and AA and higher n-3 PUFA and EPA levels than the Intralipid group had. Evaluation of bone turnover biomarkers in relation to the PLE infused showed that their plasma levels at the study's end did not differ significantly between the 2 groups. This can be attributed to the short PN duration that could not have any significant impact on plasma biomarkers. However, the OPG/sRANKL ratio decreased significantly from T1 to T2 in the Intralipid but not Smoflipid group. It has been reported that the OPG/sRANKL ratio is a more reliable marker of bone turnover than the OPG and sRANKL.[27,32] In this respect, the changes in OPG/sRANKL ratio found in the current study are compatible with the higher rate of bone loss in the Intralipid group. Studies in adults showed that the n-3 PUFA intake is positively associated with OPG and OPG/sRANKL ratio as well as with bone mineral density.[33,34] Combined, these data suggest that the lower rate of OPG/sRANKL ratio decrease in the Smoflipid group may indicate that the n-3–enriched PLEs could help reduce the rate of postnatal bone loss in VLBW infants.

Multiple Regression Analysis

In addition to the composition of the fatty acid administered, bone turnover in preterm infants can be influenced by many factors, such as Ca intake, chronic inflammation, and medications.[2,35,36] Therefore, further analysis was performed to evaluate the potential independent effect of the type of PLE, PN duration, and changes of n-6/n-3 PUFA ratio on biomarker changes after controlling for potential confounders. We found that the group allocation, mirroring the type of PLE, had a significant independent effect on OPG change, whereas the PN duration had a negative independent effect on sRANKL levels. The latter association indicates that longer exposure to n-3 PUFA–enriched PLE mitigates the bone resorption observed during early postnatal weeks in VLBW neonates. This is in line with findings of experimental studies demonstrating a significant association of long-term intake of n-3 PUFA, specifically the EPA, with improvement of cortical bone volume and thickness.[37] The positive association of the n-6/n-3 ratio change with the OC changes, indicating that levels of OC increase with increasing n-6/n-3 ratio, was an unexpected finding. This association may represent a compensatory increase of OC aiming at limiting the detrimental effects of increased n-6/n-3 ratio on bone metabolism.

Regression analysis revealed that Ca change was significant independent factor associated inversely with the OPG/sRANKL ratio. A possible interpretation of this finding is that the increased bone resorption, as evidenced by the decrease of OPG/sRANKL ratio during the study, leads to increased Ca delivery from bones to the circulation.

In our study, complications of prematurity could not possibly have any significant effect on the differences in bone biomarkers between the 2 groups, because the incidence of respiratory distress syndrome was comparable in the 2 groups, the incidence of sepsis during the study was very low, and chronic lung disease develops beyond the 28th day of life. Also the proportion of neonates that had received prenatal steroids was comparable between the 2 groups, whereas no neonate was treated with steroids during the study period, furosemide was given extremely rarely, and caffeine was administered to all neonates. Therefore, any medication-associated contribution to the observed differences in biomarker levels between the 2 study groups seems rather unlikely.

Strengths and Weaknesses

The strength of our study is the fact that it is the first to explore the effect of the PLE on early levels of bone turnover biomarkers in VLBW infants. A limitation of our study is the wide range of the biomarker values, which combined with the relatively low numbers of the study population underpowered our results. Nevertheless, multiple regression models revealed important associations between the type and duration of PLE administration with changes in biomarkers during the study period.

In conclusion, overall the changes of bone turnover biomarkers found in our study population of VLBW neonates suggest a high bone-turnover rate in the first 3 weeks of life, with predominance of bone resorption over bone formation. The lower decrease of the OPG/sRANKL ratio in the Smoflipid group from baseline to the study's end indicates a lower rate of bone loss in this group. The latter finding together with the reported beneficial effect of n-3 PUFAs on bone metabolism suggests that the use of PLE enriched in n-3 PUFAs may restrain bone loss in VLBW neonates during the early postnatal period.

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