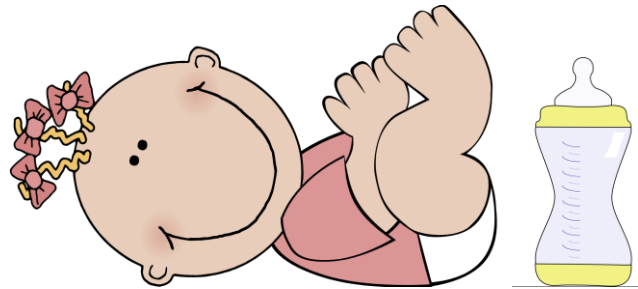# THE SAHLGRENSKA ACADEMY

The role of early nutrition on bone development in children

Degree Project in Medicine

Anneli Landgren

Programme in Medicine

Gothenburg, Sweden 2019

Supervisor: Jovanna Dahlgren

Department of Pediatrics

Table of contents

ABBREVIATIONS

AA – arachidonic acid

ALA – alpha-linolenic acid

BA – bone area

BMAD – bone mineral apparent density

BMC – bone mineral content

BMD – bone mineral density

BMI – body mass index

DEXA – dual-energy x-ray absorptiometry

DHA – docosahexaenoic acid

EFA – essential fatty acids

EPA – eicosapentaenoic acid

FA – fatty acids

IGF-1 – insulin-like growth factor-1

LA – linoleic acid

LCPUFA – long-chain polyunsaturated fatty acids

P1NP – N-terminal propeptides of type 1 collagen

PUFA – polyunsaturated fatty acids

SE – standard error

SFA – saturated fatty acids

ABSTRACT

Title: The role of early nutrition on bone development in children.

Master thesis, Programme in Medicine, Sahlgrenska Academy at the University of Gothenburg, Sweden, 2019.

Authors: Anneli Landgren, medical student, Jovanna Dahlgren MD, Professor in pediatric endocrinology, Department of Paediatrics, Sahlgrenska Academy, Sweden.

Background: Alterations in fetal and postnatal nutrition may have long-term effects on bone status. Omega-3 fatty acids (FA, n-3) are believed to promote bone accrual, while omega-6 FA (n-6) are believed to promote bone resorption. A balanced n-6:n-3 ratio in infancy may reduce the risk of osteoporosis later in life. Bone development can be estimated with dual-energy X-ray absorptiometry (DEXA) measuring mineral density (BMD) and with bone formation markers such as osteocalcin and N-terminal propeptides of type 1 collagen (P1NP).

Aims: To investigate the role of infant nutrition on bone development during infancy and childhood by evaluating the correlation between infant fatty acids, infant bone formation markers and BMD in 8-year-olds, and compare the outcomes between breast-fed and formula-fed infants.

Methods: Between year 2008 and 2009, blood samples were collected from 398 infants at Halmstad hospital, Sweden. Breast-feeding habits during the first year of life were recorded. Lumbar spine BMD was determined in 167 of the children at age 8 years.

Results: Breast-fed had higher serum osteocalcin and n-3 FA, but lower n-6 FA at 4 months of age compared to formula-fed infants. No FA correlated with osteocalcin after adjusting for confounders. BMD did not differ between feeding groups and was not associated with infant FA or bone markers after adjusting for confounders. Girls had significantly higher BMD than boys. Unsurprisingly, weight and gender predicted BMD in the multiple regression analysis.

Conclusions: Breast feeding is important for osteocalcin and FA pattern with high n-3, but does not have long-term effects on BMD.

Key words: early nutrition, bone development, osteoporosis.

INTRODUCTION/BACKGROUND

Early nutrition and later health outcomes

Fetal life and early infancy are periods of rapid growth (1). Intrauterine and early postnatal nutrition is crucial for normal growth and development (2). Alterations in nutrition during fetal life and early infancy is thought to influence later health and disease (1). Epidemiologic studies have shown an association between small birth size and increased risk of chronic disease including type 2 diabetes, coronary heart disease, stroke, hypertension and osteoporosis later in life (3). In parallel with the epidemiological observations, animal studies have demonstrated similar results. Manipulation of nutrition during pregnancy has produced different phenotypes in the offspring, mimicking aspects of human disease (4,5,6). The underlying mechanisms are thought to involve epigenetic modifications in genes, induced by nutrients, which modify gene expression without altering DNA sequences (7). These epigenetic modifications reflect fetal adaptations, in order to meet the demands of the predicted later environment (8). The results are alterations in the expression of genes involved in e.g. endocrine homeostasis and metabolism (9) that may be permanent and later on increase the risk for adult disease.

Not only are the prenatal events of importance for setting an individual on the path of health and disease. Scientists also stress the importance of the postnatal environment during suckling, infancy and early childhood for later health outcomes (10,11). For example, rapid weight gain during the 6 first months of life is a risk factor for later obesity (12). It is known that bottle feeding increases such rapid weight gain (13). This may be due to both higher protein content (14) and higher omega-6 fatty acid content (15) in formula milk compared to breast milk. Moreover, insulin-like growth factor-1 (IGF-1), a major hormonal mediator of perinatal growth, cellular proliferation and differentiation (16), is mainly regulated by nutrient supply during infancy (17). It is known that infants given breast milk have lower serum concentration of IGF-1 than formula-fed infants, which correlates with less weight gain during the first 6 months of life (14).

The lifestyle and diet has changed rapidly during the last century (18). In light of the early origins of health and disease, it is important to investigate how these dramatic changes affect the new-born baby and its later health.

Fatty acids and their balance

Essential fatty acids (EFAs) are fatty acids that cannot be synthesized in the human body but must be obtained by diet (19). EFAs and their products are important components in cell membranes, and influence cell signalling, ion channels, receptors, enzymes and gene expression (20). There are two polyunsaturated fatty acids (PUFA) that are essential for humans, the omega-3 (n-3) alpha-linolenic acid (ALA), and the linoleic acid (LA, n-6 fatty acid) (19). ALA and LA are found abundantly in plant-based oils. Flaxseed, canola and soybean oils are major sources of ALA, while safflower, sunflower and corn oil are major sources of LA (20).

The ALA and LA PUFA compete for the same enzymes for further elongation and desaturation into long-chain polyunsaturated fatty acids (LCPUFA) (Figure 1), thus the balance between them is important (21). Among the most important LCPUFA for biological function are arachidonic acid (AA, 20:4n6), docosahexaenoic acid (DHA, 22:6n3) and eicosapentaenoic acid (EPA, 20:5n3) (22). AA and EPA are precursors to eicosanoids such as prostaglandins and leukotrienes (23). DHA is a structural component in the cerebral cortex, skin and retina (24). EPA and DHA are also naturally found in fish oils and sea food (20).
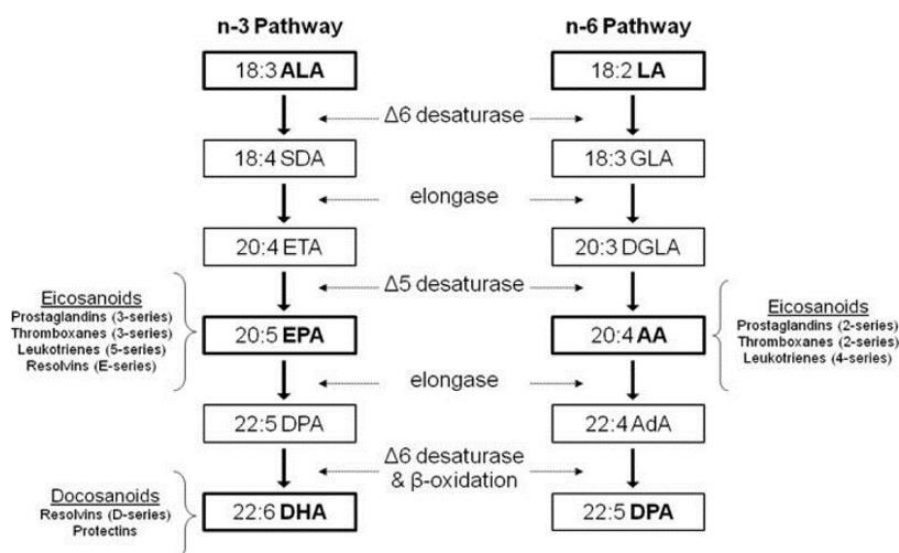
FIGURE 1: Synthetic pathways of n-3 and n-6 polyunsaturated fatty acids (20).

Note: n-3 fatty acids: ALA = alpha-linolenic acid, SDA = stearidonic acid, ETA = eicosatetraenoic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid.

n-6 fatty acids: LA = linoleic acid, GLA = gamma-linolenic acid, DGLA = dihomo-gamma-linolenic acid, AA = arachidonic acid, AdA = adrenic acid.

N-3 and n-6 fatty acids (FA) work together to promote health. Production of eicosanoids from n-3 FA help reduce inflammation while the products of n-6 FA promote inflammation (25). Overconsumption in foods rich in n-6 FA, while neglecting n-3 FA, disrupt the balance of eicosanoids (23). This places an individual in a pro-inflammatory state, associated with the risk of developing disease such as cardiovascular (26), autoimmune and inflammatory disease and cancer (27). Current Western diets typically have excessive amounts of n-6 FA and lower n-3 FA, leading to a much higher ratio than humans historically have lived on (26). This change may be due to the increased intake of fast foods and snacks rich in n-6, and the recent decade recommendations to replace saturated fat with n-6 polyunsaturated fat (e.g. margarine), in order to lower serum cholesterol (26).

Bone development

Bone remodelling is a continuous process of bone formation and destruction (resorption), giving bone its mature structure (28). During childhood growth, bone formation outpaces resorption (28). The peak bone mass occurs in early adulthood, after which formation and resorption maintain an approximate balance (29). Although genetic factors play a key role in attaining peak bone mass, environmental factors such as physical activity and nutrition are important as well (29). Peak bone mass is a major determinant of the risk of osteoporosis later in life, thus optimizing peak bone mass may decrease the prevalence of osteoporosis and problems related to it (30). More research is needed to identify modifiable lifestyle factors in childhood, and how they affect bone health in adulthood (29).

Early nutrition and bone development

Early nutrition, during intrauterine and post-natal life, is important for bone development. In fact, maternal diet in pregnancy has been linked to bone mineral density in children, e.g. magnesium, phosphorus and protein density of the maternal diet (31), and elevated intakes of fruit, vegetables and wholemeal groceries (32). However, the specific nutrients that modulate bone accrual in early life remain to be determined.

Results from animal models suggests a role for LCPUFAs in regulating the metabolism of bones (33). Both n-6 and n-3 FA have been found to be related to bone development in animal

studies (34). For example, mice exposed to high levels of n-3 FA during prenatal and early postnatal period have shown to accelerate bone growth and improve bone quality in terms of trabecular and mechanical structure (35). Possibly, the mechanisms of n-3 FA on bone are by enhancing calcium absorption in the intestine, decreasing urinary excretion of calcium and enhancing synthesis of bone collagen (36).

As for n-6 FA, the eicosanoids derived from AA, especially prostaglandin PGE_2, seems to be of key importance in bone metabolism. At low levels, PGE2 stimulate bone formation (37) but at high levels, PGE2 stimulate osteoclast differentiation and bone resorption (38,37).

Since the n-3 and n-6 PUFA compete for the same enzymes for elongation, increased availability of n-3 LCPUFA (especially EPA), acts to restrain formation of prostaglandins from AA. Low dietary ratios of n-6:n-3 in rats has shown to be associated with higher bone mineral density (BMD) of the vertebrae determined by dual-energy x-ray absorptiometry (DEXA)(39). The findings have been corroborated in human research. In a Swedish study of 85 healthy 8-year-olds (28), serum LA and AA were found to be negatively associated with BMD, measured with DEXA. It also suggested that an overload of n-6 FA was associated with lower BMD. Another cohort study of young Swedish men showed a positive correlation between the n-3 fatty acids DHA and EPA, and peak BMD (40). In terms of early nutrition, one study of LCPUFA status of 727 women in late pregnancy, showed that n-3 LCPUFA were positively associated with a number of DEXA-derived bone indices in the children at age 4 years (41). Arachidonic acid, on the other hand, was inversely related to whole body BMC and BMD.

Human milk is typically the sole and ideal source of nutrition that must supply the infant with appropriate amounts of energy and nutrients. However, surveys in developed countries report that the majority of infants receive some infant formula during the first year of life (42). Studies on long term effect of different feeding habits on bone mass have yielded mixed results (43). Some authors have shown a positive effect of breast feeding on bone mass in childhood and adolescence (44,45). However, other studies have found no association or a negative effect of being breastfed on bone mass (46,47). In addition, a recent Swedish study of 104 infants, where of 56 infants are also included in this study, showed that the n-6:n-3 ratio was significantly lower in breast-fed infants compared to formula-fed infants (15). It

remains to see whether the different fatty acid patterns in infancy impacts bone mineral density at 8 years of age.

Dual-energy x-ray absorptiometry

Dual-energy x-ray absorptiometry (DEXA) is a technique that can be used for evaluating body composition and bone mass (Picture 1)(48). Measurement is based on the differential attenuation of two photon beams as the various tissues of the body absorb them (49). It is an appropriate method for children due to its low radiation exposure, relatively quick scan time, availability of paediatric reference data and its high precision (48). DEXA is a useful tool for studying skeletal maturation and growth development (50). In terms of bone, it can assess

bone mineral content (BMC, g), bone area (cm^2) and bone mineral density (BMD, g/cm^2) of the whole body or specific regions (49).

The technique has limitations due to its two-dimensional nature, meaning that it only measures the cross sectional area of the bone and areal density (BMD, g/cm^2) but cannot detect the depth and the volumetric density (g/cm^3) (51). Therefore, DEXA is vulnerable to size-related artefacts which leads to the risk of underestimating BMD in short/slim children and overestimating BMD in tall/overweight children (52). One way to correct for bone size in growing children is to calculate the bone mineral apparent

PICTURE 1: Dual-energy x-ray absorptiometry (DEXA) was performed at 8 years of age.

density (BMAD, g/cm^3) using the formula $BMC/BA^{1.5}$ (52).

Bone formation markers

Bone formation markers can be measured in serum and reflect the different phases of osteogenesis. Osteoid is formed in the early phase of bone formation and consists mainly of type 1 collagen, which is cleaved to N-terminal (P1NP) and C-terminal propeptides of type 1 collagen and released into the blood (53). Practical advantages of P1NP include its low diurnal variability, its stability at room temperature, and that it does not need to be measured fasting, since circulating levels are not significantly influenced by food intake (53).

Another bone formation marker is osteocalcin, a non-collagenous protein produced by osteoblasts (54). Osteocalcin levels increase when osteoblast differentiation is promoted and is therefore a marker of osteoblast activity and bone formation (55).

HYPOTHESES

The n-3 FA show positive correlations with the bone formation markers (osteocalcin and P1NP) in cord blood/serum in infancy, and BMD_{L1-L4} and $BMAD_{spine}$ at 8 years of age.

High levels of n-6 FA and a high ratio of n-6:n-3 will have negative effects on bone mineralization in terms of bone formation markers (osteocalcin, P1NP) in infants and BMD_{L1-L4} and $BMAD_{spine}$ at 8 years of age.

There is a significant difference in bone formation markers and patterns of FA between infants having received breast milk and infants having received formula milk exclusively at 4 months of age. This difference may have long-lasting effect on bone mass measured as BMD_{L1-L4} and $BMAD_{spine}$ at 8 years of age.

AIMS

General aim

The overall aim of this study is to examine the role of early nutrition on bone development in children.

Specific aims

This thesis originally aimed at investigating the correlation between levels of n-3 and n-6 FA, bone formation markers in infants and BMD_{L1-L4} at age 8 years, then compare breast-fed infants and formula-fed infants in regard to these measures.

The aim was extended during the working process to include all fatty acids derived from the analysis of the cord blood/serum, being saturated fatty acids (SFA), monounsaturated FA and PUFA from the n-3, n-6 and n-9 series. Also, an established method was found later during the research process that can adjust for different bone sizes, i.e. BMAD. Thus, the expanded research questions are:

Is there a correlation between fatty acid patterns (SFA, monounsaturated FA and PUFA from the n-3, n-6, and n-9 series) and the bone formation markers P1NP and osteocalcin at birth and at 4 months of age?

Is there a correlation between fatty acids in cord blood at birth and in serum at 4 months and BMD_{L1-L4} and $BMAD_{spine}$ at 8 years of age?

Is there a significant difference in fatty acids, bone formation markers or BMD_{L1-L4} and $BMAD_{spine}$ between infants having been breast-fed vs formula-fed at 4 months of age?

Is there a correlation between bone formation markers (PINP and osteocalcin) measured at birth and at 4 months of age, and BMD_{L1-L4} and $BMAD_{spine}$ in 8-year-olds?

MATERIAL AND METHODS

Study design

This is a prospective observational study of a population-based Swedish cohort of full-term vaginally delivered infants recruited at birth between year 2008 and 2009 in Halmstad, Sweden.

Participants and data collection

398 healthy full term infants were recruited to the study population at birth after written consent by their parents. Blood samples were collected and directly frozen from the infants' umbilical cord blood at birth and from serum at 4 months of age. Parents completed questionnaires regarding breast-feeding practices including formula supplements and frequency of feeding at recruitment as well as at the first, fourth and twelfth months of life. Weight and height of the mothers were measure and body mass index (BMI) calculated at the first visit for maternal health care. They were also asked questions regarding smoking habits and level of education. A new written consent for DEXA and blood sampling was obtained from 167 parents and children (84 boys, 83 girls) at the age of 8 years. Out of the 167 children performing DEXA, 144 had full series of saturated FA, monounsaturated FA and PUFA at birth and 117 had full series of lipids at 4 months of age.

A drop-out analysis was performed, comparing early demographics of the follow-up study population (n=167) versus dropouts (n=231) (appendix, Table A). The analysis included gestational age, weight and length at birth, weight at 4 months, maternal smoking and BMI, breast feeding habits and maternal educational level (primary school, high school, university, other). The follow-up study population was representative except for the average BMI of the mothers (0.9 kg/m² higher in the follow-up cohort) and the average age at deliverance (1 year older in the follow-up, $p<0.05$ for both. Reasons for drop-out were for example family moving to another region, difficulties for parents to get time off work for the checkups, divorce or that the children did not wish to participate any more.

Anthropometric measurements

Length and weight were measured at delivery, at 4 and 12 months, then at 3, 5, 6 and 8 years of age. Only anthropometric data from birth, 4 months and 8 years is here used. Until two years of age, height was measured with a stadiometer in the supine position. Before DEXA performance, height was measured with a digital wall stadiometer (Picture 2). Until the infants reached a weight of 15 kg, they were weighed on baby scales in the supine position, while older children were weighed on electronic step scales in their underwear.

Maternal BMI was measured at the first visit for maternal health care in the first trimester.

PICTURE 2: Hight measured with a digital wall stadiometer.

Biochemical measures

Cord blood at birth and blood samples at 4 months of age were immediately centrifuged and serum was frozen at -80º C until analyzed at the Göteborg Pediatric-Growth Research Center laboratory. Lipids were extracted from 25 µg of lyophilized serum. They were then separated on a SEP-PAK aminopropyl cartridge and the phospholipid fraction transmethylated (56,57). Finally, the serum FA methyl esters were analyzed using gas chromatography-mass spectrometry as previously described (58).

Osteocalcin in cord blood and serum was measured using the Immunodiagnostic System Ltd (IDS)-iSYS technique and expressed as ng/mL. The intra-assay coefficient of variation (CV) for osteocalcin was 1.4% and the inter-assay CV was 4.0%.

P1NP in cord blood and serum was also measured using Immunodiagnostic System Ltd (IDS)-iSYS technique and expressed as ng/mL. The intra-assay CV for P1NP was 3% and the inter-assay CV was 5.3%.

Bone measurements

DEXA was performed at Halland's Hospital Halmstad at 8 years of age. Height and weight were measured. Bone mineral density (BMD, g/cm^2) of lumbar spine (L1-L4, Picture 3); BMD, bone mineral content (BMC, g) and bone area (BA, cm^2) of the spine were determined using DEXA. In order to correct for different bone sizes, the bone mineral apparent density of the spine ($BMAD_{spine}$, g/cm^3) was later calculated using the formula $BMC/BA^{1.5}$. Unfortunately, the DEXA-measures did not provide values for BMC and BA of the lumbar spine but only for the whole spine. Thus, the BMAD of the spine was calculated and used here. The reference values were obtained from the USA manufacturer and expressed in age- and race-matched Z-scores.
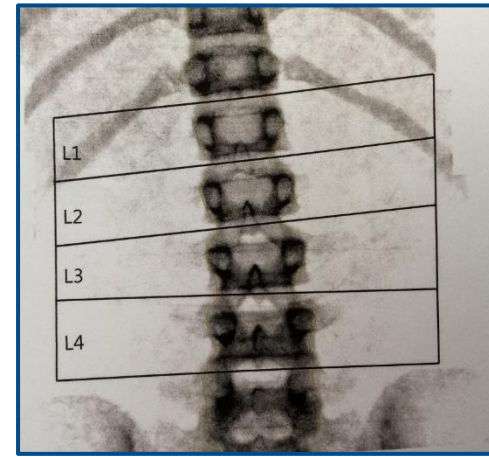
PICTURE 3: Bone mineral density of the lumbar spine was measured with dual-energy x-ray absorptiometry (DEXA).

Statistical methods

Descriptive statistics were performed to describe the study population using frequencies, mean ± standard deviation (SD) for normally distributed data, median and interquartile range (IQR) for skewed data, range and percentage. Data in figures are presented as mean and standard error (SE). The statistical analyses were performed using the Statistical Package for the Social Science (SPSS) version 25 software and Microsoft Excel version 1809. For comparisons between feeding groups, the Mann-Whitney U test was performed for non-normally distributed data. In normally distributed data, the means between groups were compared using the Student's T-test. Spearman rank correlation coefficient was used for non-parametrical correlations between continuous variables and Pearson's correlation coefficient for parametrical test. All tests were 2-tailed. Due to multiple testing of fatty acids, p-values <0.02 were considered statistically significant. However, when fewer testing were needed, e.g. comparisons of bone mineralization between feeding groups, p<0.05 was considered statistically significant. In multiple linear regression analyses, results were adjusted for confounding factors including gender, weight, height and feeding-habits.

Ethics

The study was conducted according to the Helsinki Declaration and was approved by the Regional Ethical Review in Lund (ethics approval number: 44/2008, 2016/442). Clear and thorough information were given to the parents and written informed consent was obtained after deliverance and again before DEXA. The blood samples were carefully collected by experienced nurses. All data was anonymized. Oral informed consent has been obtained from the nurse, mother and child in the pictures included in this thesis.

RESULTS

Descriptive statistics

Demographic data is shown in Table 1 subdivided into girls and boys. The mean gestational age was 281 ± 9 days, where 280 days or 40 weeks represent a full term pregnancy. However, 10 children were born small for gestational age. The mean weight and length were as expected significantly greater in boys at birth and at 4 months of age ($p<0.05$) but not when expressed as SD score. At 8 years of age 23% of the girls were overweight whereof one obese, while 19% of the boys were overweight whereof two obese, according to the International cut off points for BMI for overweight and obesity in children (59).

TABLE 1. Demographic data expressed as mean ± standard deviation or median (interquartile range) in participants and their mothers.

	Boys (n=85)	Girls (n=84)
Gestational age (days)	281 ± 9	282 ± 9
Birth weight (g)	3715 ± 516	3549 ± 507
SDS	-0.11 ± 0.95	-0.18 ± 1.1
Birth length (cm)	51.2 ± 2.3	50.5 ± 2.1
SDS	-0.32 ± 1.13	-0.31 ± 1.25
Weight 4 months (g)	6938 (6320, 7770)	6370 (5970, 6855)
SDS	0.24 (-0.53, 0.94)	-0.14 (-0.71, 0.55)
Length 4 months (cm)	64.2 ± 2.6	62.5 ± 2.0
SDS	0.51 ± 1.13	0.24 ± 0.93
Weight 8 years (kg)	29.0 (26.5, 34)	29.2 (26.0, 33.5)
SDS	0.55 (-0.49, 1.57)	0.33 (-0.60, 1.18)
Height 8 years (cm)	133.3 (130, 138.3)	133.0 (128.8, 136.9)
SDS	0.16 (-0.58, 0.85)	0.05 (-0.64, 0.74)
BMI 8 years (kg/m^2)	16.8 (15.1, 17.8)	16.9 (15.0, 18.3)
SDS	0.33 (-0.64, 1.12)	0.27 (-0.57, 1.20)

Note: SDS = standard deviation score; BMI = body mass index.

The mean maternal age at delivery was 31.4 ± 4.5 years and BMI 23.6 kg/m^2 (21.6, 26.6). Out of the 147 mothers completing the questionnaire at recruitment, 4% were smokers, 29% had no further education after high school and 63% had a university education. At the 4 months visit, 136 mothers completed a questionnaire about feeding habits, including breast-feeding exclusively, mixed (formula and breast milk) and formula-feeding exclusively. The

distribution of feeding habits is shown in Figure 2. In the group of mixed and formula fed infants, 85% received a formula (NAN; Nestlé, Helsingborg, Sweden) supplemented with DHA and AA.

FIGURE 2. Distribution of feeding habits at 4 months of age (n=166).The number (n) and percentage of infants being breast-fed exclusively, mixed-fed (formula and breast-milk) or formula-fed exclusively.
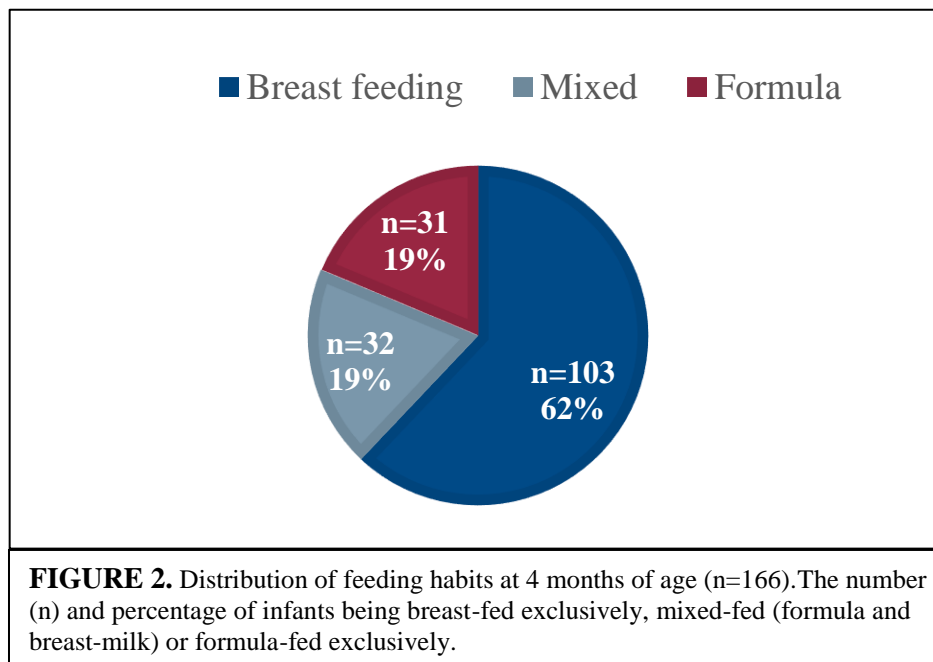
Fatty acids

The data during infancy is shown in Table 2. At birth, the four most predominant fatty acids were, in descending order, 16:0, 18:0, AA and 18:1n9 (oleic acid, OA). At four months of age, the four most predominant FAs were 16:0, LA, 18:0 and OA. The fatty acid patterns changed significantly from birth to 4 months. To summarize, the total saturated FA decreased significantly ($p < 0.001$) and all individual SFA decreased but 23:0 and 12:0. Two monounsaturated FA, OA and 20:1n9, increased, while two monounsaturated FA, 18:1n7 and 22:1n9, decreased. The total amount of monounsaturated FA decreased significantly ($p < 0.001$). The one n-9 LCPUFA (mead acid) decreased significantly. As for n-6 PUFA, 20:3n6, AA, 22:2n6, 22:4n6 and 22:5n6 decreased, while LA increased. The total n-6 increased significantly. Regarding n-3, ALA and 22:5n3 increased ($p < 0.02$) while DHA decreased. The total n-3 decreased significantly. Finally, the n-6:n-3 ratio was significantly higher in serum at 4 months that in cord blood at birth ($p < 0.001$).

TABLE 2. Mean and standard deviation of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in cord blood at birth and serum at 4 months.

Fatty acids	Birth (n=144)	4 months (n=117)
SFA		
12:0	0.00 ± 0.00	0.86 ± 0.57
15:0	0.22 ± 0.06	0.15 ± 0.03 ***
16:0	32.06 ± 3.11	28.43 ± 3.21 ***
17:0	0.67 ± 0.13	0.49 ± 0.07 ***
18:0	18.42 ± 3.62	16.85 ± 2.20 ***
20:0	0.96 ± 0.20	0.67 ± 0.12 ***
22:0	1.54 ± 0.30	1.32 ± 0.23 ***
23:0	0.18 ± 0.05	0.42 ± 0.13 ***
24:0	1.39 ± 0.32	0.93 ± 0.19 ***
Total	55.48 ± 6.02	49.31 ± 4.62***
MUFA		
14:1n5	0.00 ± 0.00	0.00 ± 0.03
16:1n7	0.67 ± 0.23	0.47 ± 2.83
18:1n7	1.46 ± 0.28	1.06 ± 0.16 ***
18:1n9 (OA)	6.66 ± 1.45	9.28 ± 1.22 ***
20:1n9	0.06 ± 0.01	0.14 ± 0.02 ***
22:1n9	0.24 ± 0.12	0.10 ± 0.08 ***
24:1n9	2.85 ± 0.64	2.99 ± 0.64
Total	11.16 ± 1.81	13.47 ± 3.25***
PUFA		
n-9		
20:3n9 (MA)	0.37 ± 0.24	0.05 ± 0.03 ***
n-6		
18:2n6 (LA)	6.13 ± 1.64	20.42 ± 2.57 ***
18:3n6	0.06 ± 0.03	0.20 ± 1.90
20:2n6	0.26 ± 0.05	0.27 ± 0.05
20:3n6	4.86 ± 0.98	2.04 ± 0.61 ***
20:4n6 (AA)	13.72 ± 2.18	8.39 ± 1.57 ***
22:2n6	0.09 ± 0.03	0.03 ± 0.01 ***
22:4n6	0.29 ± 0.20	0.14 ± 0.11 ***
22:5n6	0.34 ± 0.21	0.11 ± 0.28 ***
Total	25.72 ± 2.90	31.69 ± 1.80***
n-3		
18:3n3 (ALA)	0.03 ± 0.04	0.13 ± 0.06 ***
20:4n3	0.04 ± 0.02	0.04 ± 0.03
20:5n3 (EPA)	0.35 ± 0.20	0.55 ± 0.81 *
22:5n3	0.33 ± 0.35	0.49 ± 0.25 **
22:6n3 (DHA)	6.08 ± 1.55	4.37 ± 1.01 ***
Total	6.81 ± 1.77	5.56 ± 1.29***
n-6:n-3 ratio	4.03 ± 1.04	6.15 ± 1.67 ***

Note. Serum concentrations (mol%) are given as mean and standard deviation in all children with complete series of serum fatty acids at birth (n=144) and at 4 months (n=117). OA = oleic acid; MA = mead acid; ALA=alpha linolenic acid; EPA=eicosapentaenoic acid; DHA=docosahexaenoic acid; LA=linoleic acid; AA=arachidonic acid. *p<0.05, **p<0.01, ***p<0.001 where p<0.02 was considered statistically significant.

**Feeding groups and fatty acid patterns**

As can be seen in Table 3, there were several significant differences in saturates, monounsaturated and polyunsaturated FA in serum at 4 months of age between the breast-fed infants and the formula-fed infants. The total SFA, MUFA and n-3 did not differ between the groups. However, total n-6 was significantly higher in formula fed infants. More specifically, breast-fed had higher levels of 15:0, 17:0, 18:0, 20:0, while levels of 22:0, 23:0 and 24:0 were higher in the formula-fed group. Among the monounsaturated FA, 16:1n7 (palmitoleic acid), 18:1n7 and 24:1n9 were higher in the breast-fed infants, while 18:1n9 (OA) was lower.

As for n-6 fatty acids, 20:2n6, 20:3n6 and 20:4n6 (AA) were higher in the breast-fed group, while 18:2n6 (LA) was lower. Regarding n-3 fatty acids, all of them were significantly higher in the breast-fed group, except for 18:3n3 (ALA) which did not differ (figure 3). The n-6:n-3 ratio was significantly higher in the formula-fed group.

TABLE 3. Serum saturated fatty acids (SFA), monosaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in breast-fed vs formula-fed 4 months old infants and p-values.

Fatty acids	Breast fed (n= 135)	Formula-fed (n= 31)	
	Mean ± SD	Mean ± SD	p-value
SFA			
15:0	0.157 ± 0.031	0.117 ± 0.034	**0.004**
16:0	28.18 ± 3.34	29.65 ± 2.20	0.032
17:0	0.505 ± 0.059	0.394 ± 0.075	**<0.001**
18:0	17.08 ± 2.11	15.70 ± 2.31	**<0.001**
20:0	0.677 ± 0.123	0.635 ± 0.104	**<0.001**
22:0	1.27 ± 0.20	1.54 ± 0.26	**<0.001**
23:0	0.381 ± 0.089	0.634 ± 0.113	**<0.001**
24:0	0.895 ± 0.158	1.119 ± 0.229	**<0.001**
Total	49.219 ± 5.016	49.736 ± 1.752	0.651
MUFA			
14:1n5	0.005 ± 0.035	0.000 ± 0.000	0.925
16:1n7	0.533 ± 3.109	0.149 ± 0.048	**<0.001**
18:1n7	1.108 ± 0.140	0.848 ± 0.063	**<0.001**
18:1n9 (OA)	9.11 ± 1.15	10.15 ± 1.20	**<0.001**
20:1n9	0.139 ± 0.023	0.148 ± 0.029	0.358
22:1n9	0.091 ± 0.030	0.136 ± 0.194	0.500
24:1n9	3.12 ± 0.61	2.38 ± 0.45	**<0.001**
Total	13.501 ± 3.544	13.307 ± 1.015	0.809
PUFA			
n-9			
20:3n9	0.055 ± 0.031	0.032 ± 0.024	**<0.001**
n-6			
18:2n6 (LA)	19.84 ± 2.32	23.23 ± 1.72	**<0.001**
18:3n6	0.236 ± 2.086	0.043 ± 0.041	0.066
20:2n6	0.276 ± 0.047	0.227 ± 0.055	**<0.001**
20:3n6	2.14 ± 0.56	1.52 ± 0.58	**<0.001**
20:4n6 (AA)	8.60 ± 1.47	7.33 ± 1.67	**0.001**
22:2n6	0.030 ± 0.005	0.026 ± 0.007	0.046
22:4n6	0.139 ± 0.111	0.131 ± 0.085	0.814
22:5n6	0.116 ± 0.305	0.103 ± 0.049	0.195
Total n-6	31.485 ± 1.823	32.706 ± 1.283	**0.005**
n-3			
18:3n3 (ALA)	0.125 ± 0.062	0.128 ± 0.036	0.314
20:4n3	0.049 ± 0.024	0.022 ± 0.014	**<0.001**
20:5n3 (EPA)	0.624 ± 0.875	0.205 ± 0.096	**<0.001**
22:5n3	0.537 ± 0.246	0.254 ± 0.124	**<0.001**
22:6n3 (DHA)	4.51 ± 0.94	3.72 ± 1.08	**<0.001**
Total n-3	5.821 ± 1.194	4.320 ± 1.059	**<0.001**
Total PUFA	37.243 ± 1.734	36.955 ± 1.454	0.419
n-6:n-3 ratio	5.78 ± 1.16	7.94 ± 2.47	**<0.001**

Note. Serum concentrations (mol%) are given as mean and standard deviation in all children with complete series of serum fatty acids at 4 months (n=117). OA = oleic acid; ALA=alpha linolenic acid; EPA=eicosapentaenoic acid; DHA=docosahexaenoic acid; LA=linoleic acid; AA=arachidonic acid. $p<0.02$ was considered statistically significant.
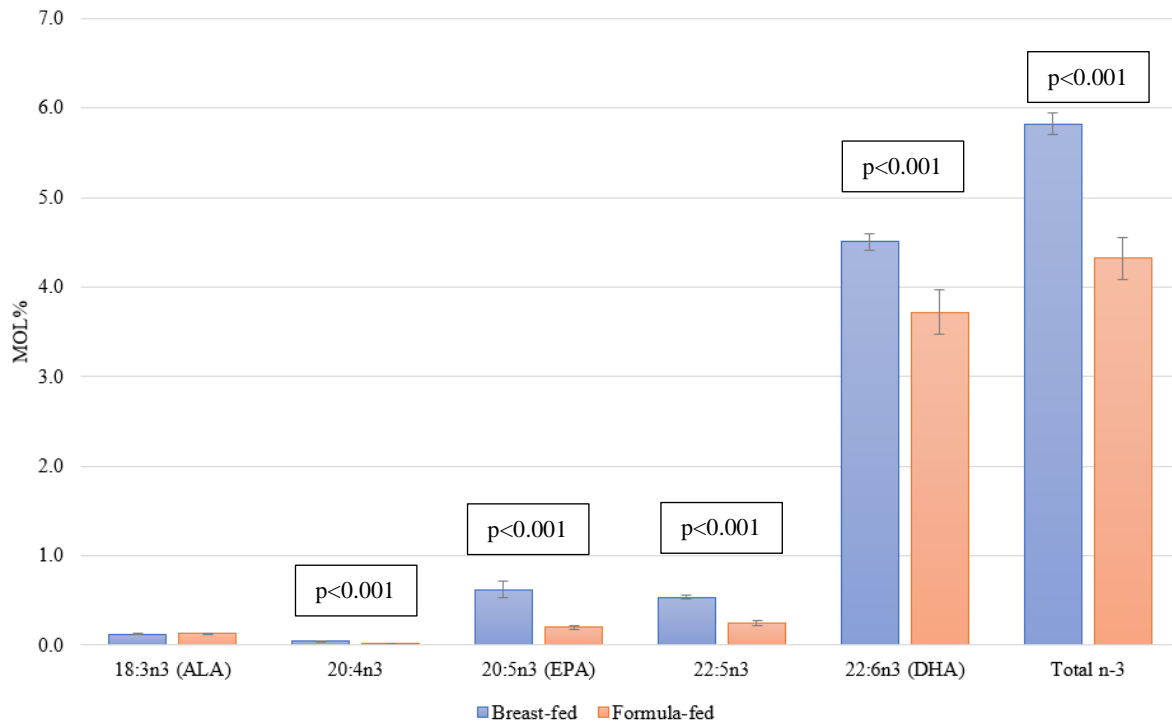
FIGURE 3. Difference in mean levels of n-3 fatty acids between breast-fed and formula-fed infants in serum (mol%) in 4 months old infants. Error bars represent standard error.

Note: ALA = alpha-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

Bone formation markers

Changes over time

In both feeding groups, osteocalcin increased from birth to 4 months from mean and SD 43.2 ± 26.7 ng/mL to 100 ± 36 ng/mL (p<0.001). The same trend was found for P1NP with mean and SD of 1148 ± 501 ng/mL at birth and 1893 ± 458 ng/mL at 4 months (p<0.001).

Feeding groups and bone formation markers

At 4 months of age, breast-fed had significantly higher osteocalcin levels (p<0.001) compared to formula-fed (see Figure 4 for details). The levels of P1NP did not differ between groups, with 1875 ± 456 ng/mL in breast-fed infants compared to 1973 ± 466 ng/mL in formula-fed infants (p=0.22).
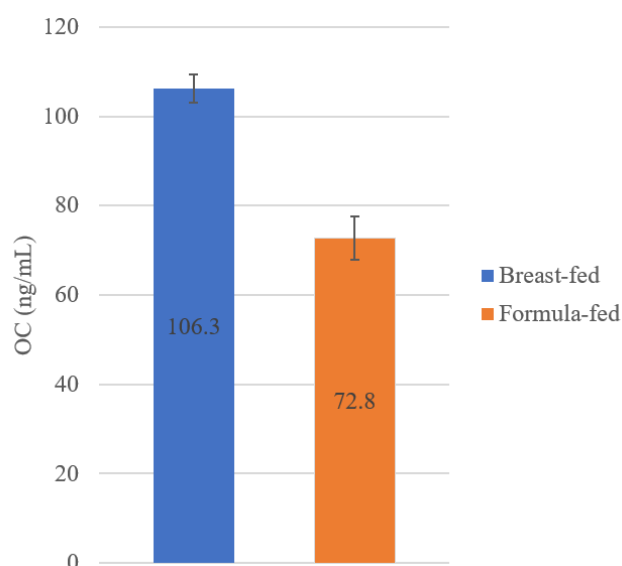
FIGURE 4. Serum osteocalcin (OC) at 4 months of age in breast-fed and formula-fed infants (p<0.001). Error bars represent standard error.

Dual-energy x-ray absorptiometry

In Table 4, the results from DEXA-performance regarding BMD_{L1-L4} and BMD, BMC, BA and BMAD of the spine are presented. Only BMD_{L1-L4} and BMD z-score differed significantly by gender, with girls having higher values (p<0.01 and p<0.001 respectively).

TABLE 4. Bone measurements for boys and girls at age 8 years measured with dual-energy x-ray absorptiometry (DEXA).

Bone measures	Boys (n=84)	Girls (n=83)
BMD_{L1-L4} (g/cm^2)	0.72 ± 0.092	0.76 ±0.078**
Z-score $_{L1-L4}$	0.20 ± 0.93	0.71 ± 0.84***
BMD_{spine}(g/cm^2)	0.64 ± 0.06	0.65 ± 0.06
BMC_{spine} (g)	67.0 ± 13.5	67.1 ± 11.6
BA_{spine} (cm^2)	105.0 ± 13.0	104.0 ± 12.0
$BMAD_{spine}$ (g/cm^3)	0.06 ± 0.01	0.06 ± 0.01
BMD = bone mineral density; BMC = bone mineral content; BA = bone area; BMAD = bone mineral apparent density. **p<0.01, ***p<0.001.		

Feeding-groups and DEXA-results

There was no significant difference between breast-fed or formula-fed infants in BMD_{L1-L4} or $BMAD_{spine}$ at 8 years of age (see Figure 5 for details).
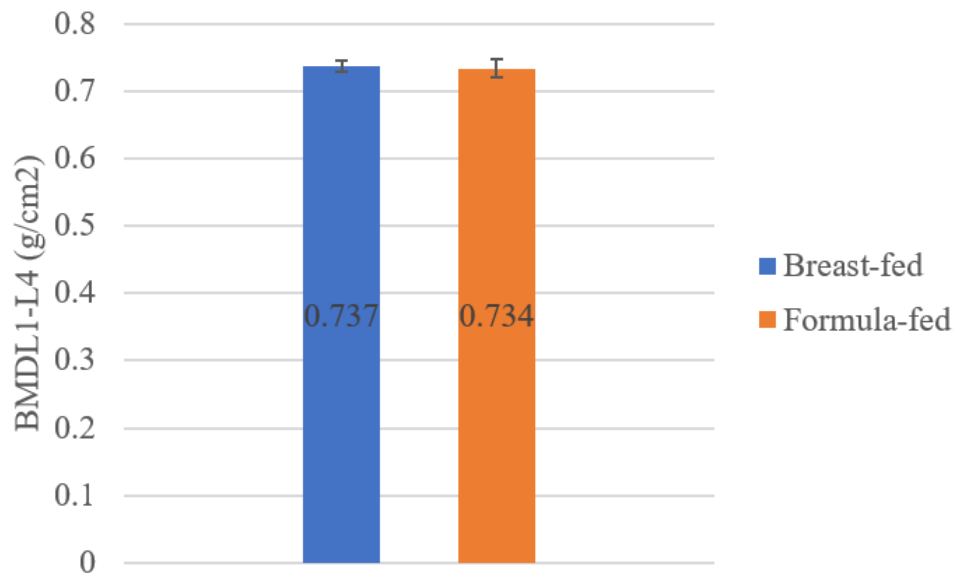
FIGURE 5. Bone mineral density of lumbar spine 1-4 (BMD_{L1-L4}) in breast-fed and formula-fed (non-significant) and error bars with standard error.

Correlations

Fatty acids and bone formation markers

The correlations between bone formation markers and cord blood FA did not reach statistical significance (defined as $p<0.02$) but at 4 months of age, several FA correlated with osteocalcin. Positive correlations with OC were 18:0, 20:3n9, 16:1n7 (palmitoleic acid), 20:3n6, 20:4n6 (AA) and 20:5n3 (EPA) and negative correlations were 22:0, 23:0, 24:0, 18:1n9 and 18:2n6 (LA). The total saturated, monounsaturated and polyunsaturated FA did not correlate with bone formation markers at birth or 4 months of age, but n-6:n-3 ratio almost reached significance ($p=0.03$). See Table 5 for more details.

TABLE 5. Correlation between serum saturated fatty acids (SFA), monosaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) and bone formation markers at birth and 4 months.

Fatty acids	Birth				4 months			
	r P1NP	p P1NP	r OC	p OC	r P1NP	p P1NP	r OC	p OC
SFA								
12:0	0.00	0.000	---	---	-0.10	0.502	0.04	0.789
15:0	0.03	0.757	-0.08	0.481	0.21	0.100	0.12	0.342
16:0	0.01	0.936	-0.15	0.075	0.02	0.860	-0.13	0.157
17:0	-0.06	0.568	-0.13	0.237	0.06	0.661	0.18	0.149
18:0	0.00	0.992	0.08	0.370	0.16	0.085	0.38	**<0.001**
20:0	0.05	0.593	0.13	0.130	-0.17	0.063	0.00	0.971
22:0	0.04	0.869	0.15	0.076	-0.11	0.243	-0.24	**0.010**
23:0	-0.09	0.421	-0.10	0.373	-0.15	0.223	-0.37	**0.003**
24:0	-0.01	0.902	0.05	0.537	-0.13	0.181	-0.22	**0.019**
Total	0.05	0.541	-0.02	0.798	0.12	0.219	0.11	0.168
MUFA								
14:1n5	---	---	---	---	-0.10	0.502	0.04	0.789
16:1n7	0.09	0.309	-0.07	0.394	-0.02	0.827	0.08	0.402
18:1n7	0.14	0.196	-0.05	0.646	-0.09	0.480	0.37	**0.003**
18:1n9	0.07	0.412	-0.09	0.273	-0.03	0.751	-0.23	**0.015**
20:1n9	-0.16	0.139	-0.14	0.201	-0.05	0.695	0.07	0.613
20:3n9	0.04	0.664	-0.07	0.386	0.03	0.791	0.23	**0.015**
22:1n9	-0.10	0.382	0.03	0.762	-0.10	0.442	0.07	0.562
24:1n9	0.07	0.405	0.07	0.430	-0.13	0.171	0.27	**0.004**
Total	0.11	0.197	-0.16	0.062	-0.17	0.070	-0.09	0.339
PUFA								
n-6								
18:2n6 (LA)	0.07	0.379	0.06	0.516	-0.01	0.939	-0.27	**0.003**
18:3n6	0.11	0.185	0.09	0.283	-0.02	0.865	-00.14	0.130
20:2n6	0.02	0.782	-0.01	0.948	0.10	0.277	0.19	0.039
20:3n6	-0.03	0.694	0.16	0.060	0.03	0.753	0.29	**0.001**
20:4n6 (AA)	-0.05	0.581	0.08	0.358	-0.03	0.768	0.22	**0.019**
22:2n6	-0.16	0.134	-0.01	0.952	-0.09	0.477	0.25	0.044
22:4n6	-0.05	0.556	-0.08	0.324	0.07	0.458	0.04	0.698
22:5n6	-0.02	0.788	-0.03	0.775	0.05	0.586	-0.03	0.741
Total	-0.16	0.062	-0.04	0.600	-0.01	0.885	-0.07	0.465
n-3								
18:3n3 (ALA)	-0.07	0.400	-0.00	0.968	-0.00	0.955	-0.02	0.860
20:4n3	0.01	0.938	0.07	0.516	0.08	0.515	0.28	0.025
20:5n3 (EPA)	-0.08	0.324	-0.06	0.505	-0.00	0.993	0.26	**0.006**
22:5n3	-0.18	0.035	-0.10	0.256	0.00	0.979	0.21	0.026
22:6n3 (DHA)	-0.16	0.061	0.01	0.955	0.04	0.652	-0.05	0.604
Total	-0.18	0.037	-0.02	0.815	0.02	0.834	0.09	0.341
n-6:n-3 ratio	0.12	0.161	0.04	0.627	0.01	0.904	0.21	0.032

Note. r = correlation coefficient. p<0.02 was considered statistically significant. P1NP = procollagen type 1 amino-terminal propeptide. OC = osteocalcin. FA = fatty acid. ALA=alpha linolenic acid; EPA=eicosapentaenoic acid; DHA=docosahexaenoic acid; LA=linoleic acid; AA=arachidonic acid.

Fatty acids, BMD$_{L1-L4}$ and BMAD$_{spine}$

The correlations between fatty acids at birth and 4 months, and BMD$_{L1-L4}$ and BMAD$_{spine}$ are shown in the appendix (Table B). Total saturated, monounsaturated and polyunsaturated FA did not correlate with bone measures at 8 years of age. Only one fatty acid, 16:1n7, in serum at 4 months of age showed a significant negative correlation with BMD$_{L1-L4}$ (r= -0.31, p=0.012). Close to significance was EPA with BMAD$_{spine}$ (r=0.20, p=0.029).

Bone formation markers and BMD$_{L1-L4}$ and BMAD$_{spine}$

There were no correlations found between levels of the bone formation markers osteocalcin and P1NP at birth and 4 months of age, and BMD$_{L1-L4}$ and BMAD$_{spine}$ at 8 years of age (appendix, Table C).

Linear regression

To predict BMD$_{L1-L4}$ based on multiple independent variables, a multiple regression analysis was performed. The fatty acids that were included were the ones correlating or nearly correlating with BMD$_{L1-L4}$ (Table 6), being 16:1n7 and EPA at 4 months of age. The other independent variables were gender, weight and height at age 8 years of age. The multiple regression model statistically predicted BMD$_{L1-L4}$ $F(5, 111) = 3.69$, p<0.004, adjusted $R^2 = 0.104$. However, only gender (female, p=0.007), and weight at 8 years of age (p=0.014) added statistically significantly to the prediction. Thus, the influence of fatty acids on BMD$_{L1-L4}$ disappeared when adjusting for confounding factors.

TABLE 6. Summary of multiple regression analysis with bone mineral density (BMD$_{L1-L4}$) as dependent variable.

Variable	B	SE$_B$	β	p-value
Weight 8 years	0.005	0.002	0.310	**0.014**
Height 8 years	-0.001	0.002	-0.075	0.547
Gender	0.043	0.016	0.240	**0.008**
4m 16:1n7	0.001	0.008	0.042	0.871
4m 20:5n3 (EPA)	0.009	0.029	0.083	0.751

Note. B = unstandardized regression coefficient; SE$_B$ = standard error of the coefficient; β = standardized coefficient. 4m = 4 months.

A multiple regression was also carried out including gender, weight and length at 4 months, and all the FA correlating with osteocalcin at 4 months. There was interdependence of residuals, as assessed by a Durbin-Watson statistic of 2.06. The model did not statistically predict osteocalcin at 4 months. However, when including breast-feeding instead of FA, the model statistically predicted osteocalcin at four months (Table 7), where $F(4, 147) = 7.72$, $p<0.001$ and adjusted $R^2 = 0.15$. The independent variables adding statistical significance to the model were weight (p=0.02) and feeding habits (p<0.001). Lower weight and breast-feeding predict higher osteocalcin levels at 4 months of age.

TABLE 7. Summary of multiple regression analysis with osteocalcin as dependent variable.

Variable	B	SE$_B$	β	p-value
Weight 4m	-0.011	0.005	-0.257	**0.020**
Length 4m	1.058	1.567	0.074	0.500
Gender	-5.790	5.708	-0.081	0.312
Feeding-habits	-34.198	6.916	-0.371	**<0.001**

Note. B = unstandardized regression coefficient; SE$_B$ = standard error of the coefficient; β = standardized coefficient. 4m = 4 months.

DISCUSSION

The main hypothesis of infant n-3 and n-6 FA correlating with BMD_{L1-L4} at the age of 8 years was not confirmed. Instead, regression analyses showed that the main influence on BMD_{L1-L4} was actual weight and gender. During infancy, correlations were found between FA and osteocalcin. Nutrition had a significant impact on 4 month osteocalcin with higher values in breast-fed. However, the association with FA disappeared in the regression analysis, which showed that breast-feeding was the greatest predictor of osteocalcin.

This is the first time the association between FA in infancy and bone mineralization at 8 years of age has been studied. One FA, palmitoleic acid, was negatively associated with BMD_{L1-L4}, but not after adjusting for confounding variables. This association could be a result of multiple testing analyses and therefore of minor significance. Moreover, despite differences in osteocalcin between breast-fed and formula-fed during infancy, there was no difference in bone mineralization at 8 years of age between the groups.

Correlations in infancy

In terms of PUFA, total n-6 and n-3 FAs were not associated with bone formation markers at any time. Nevertheless, specific FAs such as 20:3n6, 20:4n6 (AA) and 20:5n3 (EPA) were positively associated with osteocalcin, while 18:2n6 (LA) was negatively associated with osteocalcin. Breast-feeding had a positive influence on osteocalcin. The major differences between the feeding groups were the higher levels of total n-3 FA and the lower n-6 levels in breast-fed. As lower weight and breast-feeding were predictors for higher osteocalcin in the regression analysis, there seem to be other factors than FA in breast milk that have a larger influence on osteocalcin.

Although we did not see effects of FA on early bone development, other studies have. For example, one study of mice receiving diets of different n-3 contents showed a modest increase of osteocalcin at 17 months of age in mice with EPA-enriched diet (60). They suggested an anabolic effect of EPA on bone, which in turn was attributed to high levels of insulin-like growth factor-1 (IGF-1) associated with EPA. IGF-1 is known to be fundamental in achieving a normal longitudinal bone growth and mass during the postnatal period (61). Another study by Harvey et al. showed that maternal EPA and docosapentanoic acid (22:5n3) in late pregnancy were weakly associated with some DEXA-derived bone measures in 4 year old infants (41). Few associations were shown with n-6 FA, but AA percentage of total FA was inversely related to whole body BMC and BMD, highlighting the importance of a balanced n-

6 to n-3 intake for bone development. In contrast to the present study, their findings suggest effects of maternal FA status on fetal bone metabolism that persist into post-natal life.

One major question is why osteocalcin increased with breast-feeding while P1NP did not. It is therefore uncertain whether the rise in osteocalcin represents increased bone formation or if it in fact signifies something else. Osteocalcin is primarily produced by osteoblasts and serves as a biochemical marker for bone formation (62). It is known that osteocalcin exists in two forms: one carboxylated form which has high affinity for hydroxyapatite (the chief structural element of bone) and is mainly embedded in the bone matrix, and one decarboxylated form that has lower affinity for hydroxypatite and is mainly released into the circulation with primarily metabolic effects (62). In this study, we only looked at total osteocalcin. Other studies have shown that carboxylated osteocalcin interacts with hydroxyapatite and modulates its growth, whereas decarboxylated osteocalcin acts as a hormone in glucose homeostasis and fertility (63,64). More specifically, decarboxylated osteocalcin increases insulin secretion and beta-cell proliferation in the pancreas, insulin sensitivity in the muscles and energy expenditure (65). Consequently, studies have shown that total osteocalcin in the blood correlates inversely with body fat percentage and BMI in children (66,67). Likewise was observed in this study, since lower weight at 4 months of age predicted higher osteocalcin levels at the same age in the regression analysis. This is important since weight gain in infancy has shown to correlate with overweight and obesity later in life (68).

Finally, osteocalcin is known to be regulated by the hormone leptin (69) and insulin (70). Ferron et al (70) showed that osteoblasts express insulin receptors and that insulin signalling in osteoblast was a significant determinant of whole-body glucose homeostasis. Insulin achieves this function by favouring osteocalcin decarboxylation, which in turn leads to increased insulin production and sensitivity.

Correlations in childhood

There was no long-term effect of nutrition on DEXA-derived bone measures in this study. As mentioned earlier, Harvey et al. found correlations between maternal LCPUFA in late pregnancy and bone density at 4 years of age in 724 children (41). Since we could not see this in 8-year-olds, it is possible that the effect of LCPUFA on bone diminishes with time and other factors become greater determinants of bone status. In fact, high osteocalcin at 4 months was not associated with higher BMD at 8 years. One study of parents and their children showed that 46-62% of the variance in BMD could be attributed to genetic factors (71).

Another study that investigated the association of weight, height, pubertal stage, calcium intake and physical activity with BMD of the lumbar spine and whole body, showed that pubertal stage was the major independent variable in girls and weight in boys (72).

Furthermore, Eriksson et al. showed that physical activity, especially weight bearing activity, was associated with femoral BMD in 8-years-olds (52). Actual weight was in Eriksson's study the most influential factor, since overweight children had greater BMD and BMC of the total body and lumbar spine than normal weight. However, there was no difference in BMAD, suggesting that the higher BMD in overweight children was due to larger bones and not higher mineralization. Likewise was weight the greatest predictor of BMD_{L1-L4} in our study. Presumably, the influence of weight would decrease if we were able to accurately adjust for different bone sizes through accurate calculation of volumetric bone density of the lumbar spine or through other imaging techniques, which will be further discussed under methodological considerations.

In this study, girls had significantly higher BMD_{L1-L4} than boys and a greater z-score despite pre-puberty. This is in line with the previously mentioned Dutch study showing that girls had higher BMD and BMAD of the lumbar spine at all ages (72). In contrast, no difference in lumbar spine BMD was found between the genders in Eriksson's study of 8-year-olds, but total body and hip BMD were significantly higher in boys (52). One possible explanation for the difference in this study could be that girls had slightly higher median BMI, greater spread and a higher percentage of overweight. Since we could not adjust for different bone sizes, it is difficult to say if there is an actual difference between girls and boys at this age.

Methodological considerations

There are strengths and weaknesses regarding the methods of this study. First, we applied correlation analyses in order to investigate the association between fatty acids and bone development. Correlations do not prove cause and effect relationships. The independent variables, believed to be the serum fatty acids, were not under the control of the researcher. To more fully investigate the cause and effect relationship between the variables, a randomized controlled trial would be preferred, where the infants would be exposed to milk with different ratios of fatty acids. In reality, such a study would be difficult to carry out and the ethical aspects can be questioned.

A second concern regards the DEXA technique and its measures. A strength is that it is a validated method, appropriate to use in children and provides pediatric reference values.

However, DEXA is a two dimensional X-ray method that measures cross sectional area, not volume. Thus, the influence of bone size can lead to an overestimation in tall people and an underestimation in short people. We attempted to adjust for bone size by calculating bone mineral apparent density, BMAD. Unfortunately, we did not manage to successfully calculate BMAD for the lumbar spine but only for the whole spine. Peripheral quantitative computed tomography (pQCT) is a more sophisticated X-ray method, allowing for measures of bone geometry and volumetric bone density (73). However, due to its higher cost, higher radiation and lack of reference values for children, it was not used in this study. Furthermore, we examined BMD of only one site, the lumbar spine. The reason for that was to limit our variables and also because the lumbar spine is one of the most studied bones. In fact, it is one of the bones with the highest content of trabecular bone, which is more metabolically active than trabecular bone (74). Osteoporosis is a metabolic disease that most commonly affects the spine, characterized by low bone mass (74). To investigate how fatty acids affects other sites of the body, future studies should include for example hip, femur and even whole body BMD.

Moreover, there are uncertainties about the relevance of measuring BMD at the age of 8 years. In most skeletal regions the peak bone mass, being the maximum amount of bone accrued, is attained in young adulthood (75). Peak bone mass is known to be associated with the risk of osteoporosis in elderly (76). How well BMD at 8 years of age predicts peak bone mass and osteoporosis in elderly can be questioned. Ideally, DEXA would be measured not only at 8 years of age, but at different ages including young adulthood and later in life. This may, hopefully, be done in the same cohort within some decades.

A strength was that we were able to attain two biochemical markers for bone formation at 4 months of age. Due to the relatively high cost and inconvenience of measuring DEXA in infants, the serum markers are plausible alternatives. However, the congruity between the bone markers at 4 months of age and actual bone status can be questioned. Studies have shown that osteocalcin is an appropriate screening biomarker of osteoporosis in post-menopausal women and a useful tool for monitoring treatment response (77,78). However, it was concluded that osteocalcin cannot be substituted for BMD measurements, but is useful in conjunction with BMD measurements. How well levels of osteocalcin during bone modelling in infants correlate with bone measurements derived from DEXA, have to our knowledge not yet been studied. To more accurately estimate bone status in infants, BMD measurements ought to be in combination with osteocalcin and P1NP measures. The same goes for bone

status at 8 years of age, where we only had BMD measures from DEXA but no biochemical markers.

Furthermore, the infants were divided into two groups depending on feeding habits. The breast-fed group consisted of infants being exclusively breast-fed and mixed-fed (breast milk and formula milk). The reason for allowing mixed-fed to be included in the breast-fed was that they were few and because we showed in a previous study that it is sufficient with a small amount of breast milk to influence the gut microbiota in a beneficial way (79). Preferably, the infants would be divided into at least three groups according to the amount of formula received.

Lastly, there was a substantial number of drop-outs in this study. Fortunately, the drop-out analysis showed that the study group was representative in base-line characteristics (except for a slightly higher maternal BMI and age). Since this study is population-based, the findings may be generalizable to other 8-year-olds. However, we had a relatively small number of participants and even fewer full series of serum fatty acids in infancy. Thus, at this stage, we cannot fully exclude the possibility that the lack of FA correlations with bone status were due to the relatively small number of children.

Further research is needed to investigate what nutrients in breast milk affect what hormones, leading to an increase in osteocalcin. Furthermore, future studies should include a larger number of participants and use bone formation markers in conjunction with DEXA measures when estimating bone status, and then evaluate the utility of bone markers as measures of bone density in infants and children.

CONCLUSIONS

Breast feeding is associated with infant osteocalcin but does not have long-term effects on bone mineral density. Increased levels of n-3 FA and osteocalcin are found in serum of breast-fed infants, but fatty acids did not influence bone formation after adjusting for confounders.

Populärvetenskaplig sammanfattning

I västvärlden idag är benskörhet och dess följder ett växande hälsoproblem. Maximal benmassa uppnås i ung vuxen ålder och denna påverkas bl.a. av genetik, motion och kost. Den maximala benmassan har betydelse för utveckling av benskörhet senare i livet. För att optimera benmassan och motverka benskörhet är det av största vikt att identifiera faktorer tidigt i livet som gynnar bentillväxten.

Faktum är att forskare under de senaste decennierna har intresserat sig mer och mer för hur den tidiga nutritionen påverkar sjukdomsutfall långt senare. Djurstudier har visat att avkommans tillgång på fettsyror under sen graviditet och första levnadsveckor påverkar benkvalitet i vuxenlivet. Detta tillräknas fettsyrors förmåga att modifiera genuttryck som har med metabolism och tillväxt att göra. Exempelvis spelar fleromättade fettsyror såsom omega-3 och omega-6 roll, där omega-3 tros främja nybildning av ben medan omega-6 tros påverka nedbrytning av ben. Eftersom benskörhet är ett resultat av en obalans mellan bennybildning och bennedbrytning är kvoten mellan omega-6 och omega-3-fettsyror av betydelse. Denna studie syftar till att undersöka hur kostens sammansättning av fettsyror i spädbarnsåren påverkar benformationen under samma tidsperiod och även bentäthet senare i barndomen.

Mellan år 2008 och 2009 inkluderades 398 nyfödda barn på Halmstads sjukhus till studien. Blodprover togs vid födelse samt vid 4 månaders ålder och fettsyror och benformations-markörerna osteocalcin och P1NP analyserades. Föräldrarna besvarade frågeformulär angående amningsvanor och barnen delades in i två grupper beroende på om de blivit ammade eller fått mjölkersättning (Nestlé NAN) vid 4 månaders ålder. Vid 8 års ålder genomfördes en mätning av benmineraldensitet (BMD) av ländryggen med dual-energy X-ray absorptiometry (DEXA) på 167 av barnen från ursprungspopulationen. DEXA innebär en lågdos-röntgen och passar sig väl för barn.

Resultaten visade att ammade barn hade högre nivåer av omega-3- och lägre nivåer av omega-6-fettsyror vid 4 månaders ålder jämfört med barn som fått ersättningsmjölk. Ammade hade även högre nivåer av benmarkören osteocalcin men inte P1NP. Ett antal fettsyror korrelerade med osteocalcin men sambanden försvann efter att ha justerat för störande faktorer. Inte heller sågs korrelationer mellan fettsyror, benmarkörer och BMD av ländryggen vid 8 års ålder. Istället var vikt och kvinnligt kön associerat till högre BMD.

Slutsatsen av denna studie är därför att amning, men inte specifikt fettsyror, påverkar benformationsmarkören osteocalcin under spädbarnstiden men har troligen ingen bestående effekt på bentäthet vid 8 års ålder. För att med säkerhet uttala sig i frågan behövs kompletterande studier.

Acknowledgements

I am greatly thankful to the following people:

Jovanna Dahlgren for her outstanding supervision, her wonderful companionship and general wisdom. She introduced me to the world of research, in which I have found great joy.

The nurses Monica and Eivor at Halmstad Hospital for their contagious enthusiasm and diligent job that made this study possible.

Senior researchers Josefine Roswall and Emma Kjellberg for their great support and help in data collection.

Mats Andersson for his kindness and incredible job in the lab, spending many hours analysing fatty acids.

The Seidal family for letting me stay in their guest house and for providing with food and good company during my weeks in Halmstad.

REFERENCES

1.	Barker DJ, Clark PM. Fetal undernutrition and disease in later life. Rev Reprod. 1997 May;2(2):105–12.

2.	Barker DJ. Fetal origins of coronary heart disease. BMJ. 1995 Jul 15;311(6998):171–4.

3.	Godfrey K. The 'developmental origins' hypothesis: Epidemiology. In: Developmental origins of health and disease. New York, NY, US: Cambridge University Press; 2006. p. 6–32.

4.	Woodall SM, Johnston BM, Breier BH, Gluckman PD. Chronic Maternal Undernutrition in the Rat Leads to Delayed Postnatal Growth and Elevated Blood Pressure of Offspring. Pediatr Res. 1996 Sep;40(3):438–43.

5.	Persson E, Jansson T. Low birth weight is associated with elevated adult blood pressure in the chronically catheterized guinea-pig. Acta Physiol Scand. 145(2):195–6.

6.	Korotkova M, Ohlsson C, Gabrielsson B, Hanson LA, Strandvik B. Perinatal essential fatty acid deficiency influences body weight and bone parameters in adult male rats. Biochim Biophys Acta. 2005 Jan 5;1686(3):248–54.

7.	Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic Mechanisms and the Mismatch Concept of the Developmental Origins of Health and Disease. Pediatr Res. 2007 May 1;61(5 Part 2):5R-10R.

8.	Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Carel J-C, et al. Child Health, Developmental Plasticity, and Epigenetic Programming. Endocr Rev. 2011 Apr 1;32(2):159–224.

9.	Zhang X, Ho S-M. Epigenetics meets endocrinology. J Mol Endocrinol. 2011 Feb;46(1):R11–32.

10.	Eriksson JG, Forsén T, Tuomilehto J, Winter PD, Osmond C, Barker DJP. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. BMJ. 1999 Feb 13;318(7181):427–31.

11.	Singhal A, Fewtrell M, Cole TJ, Lucas A. Low nutrient intake and early growth for later insulin resistance in adolescents born preterm. The Lancet. 2003 Mar 29;361(9363):1089–97.

12.	Davidson S, Hod M, Merlob P, Shtaif B. Leptin, insulin, insulin-like growth factors and their binding proteins in cord serum: insight into fetal growth and discordancy. Clin Endocrinol (Oxf). 65(5):586–92.

13.	Karaolis-Danckert N, Buyken AE, Kulig M, Kroke A, Forster J, Kamin W, et al. How pre- and postnatal risk factors modify the effect of rapid weight gain in infancy and early childhood on subsequent fat mass development: results from the Multicenter Allergy Study 90. Am J Clin Nutr. 2008 May 1;87(5):1356–64.

14.	Socha P, Grote V, Gruszfeld D, Janas R, Demmelmair H, Closa-Monasterolo R, et al. Milk protein intake, the metabolic-endocrine response, and growth in infancy: data from a randomized clinical trial. Am J Clin Nutr. 2011 Dec 1;94(suppl_6):1776S-1784S.

15.	Kjellberg E, Roswall J, Bergman S, Strandvik B, Dahlgren J. Serum n-6 and n-9 Fatty Acids Correlate With Serum IGF-1 and Growth Up to 4 Months of Age in Healthy Infants. J Pediatr Gastroenterol Nutr. 2018 Jan;66(1):141–6.

16.   AGROGIANNIS GD, SIFAKIS S, PATSOURIS ES, KONSTANTINIDOU AE. Insulin-like growth factors in embryonic and fetal growth and skeletal development (Review). Mol Med Rep. 2014 Aug;10(2):579–84.

17.   Baird J. Being big or growing fast: systematic review of size and growth in infancy and later obesity. BMJ. 2005;331(7522):929.

18.   Sørensen TI. The changing lifestyle in the world. Body weight and what else? Diabetes Care. 2000 Apr;23 Suppl 2:B1-4.

19.   Berquin IM, Edwards IJ, Chen YQ. Multi-targeted Therapy of Cancer by Omega-3 Fatty Acids. Cancer Lett. 2008 Oct 8;269(2):363–77.

20.   Lau BYY, Cohen DJA, Ward WE, Ma DWL. Investigating the Role of Polyunsaturated Fatty Acids in Bone Development Using Animal Models. Molecules. 2013 Nov 15;18(11):14203–27.

21.   Strandvik Birgitta. Perinatal programming by diets with essential fatty acid deficient/high saturated fatty acids or different n-6/n-3 ratios for diseases in adulthood. Eur J Lipid Sci Technol. 2015 Jul 30;117(10):1513–21.

22.   Hartl J, Herrmann R. Do they always say no? German consumers and second-generation GM foods. Agric Econ. 40(5):551–60.

23.   Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res. 2001 Mar 1;40(1–2):125–48.

24.   Neuringer M, Connor WE. n-3 Fatty acids in the brain and retina: Evidence for their essentiality. Nutr. Rev. 1986.

25.   Pham-Huy LA, He H, Pham-Huy C. Free Radicals, Antioxidants in Disease and Health. Int J Biomed Sci IJBS. 2008 Jun;4(2):89–96.

26.   Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med Maywood NJ. 2008 Jun;233(6):674–88.

27.   Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002 Dec;21(6):495–505.

28.   Eriksson S, Mellström D, Strandvik B. Fatty acid pattern in serum is associated with bone mineralisation in healthy 8-year-old children. Br J Nutr. 2009 Jan;102(3):407–12.

29.   Faulkner RA, Bailey DA. Osteoporosis: A Pediatric Concern? Optim Bone Mass Strength. 2007;51:1–12.

30.   Bonjour J-P, Rizzoli R. Chapter 25 - Bone Acquisition in Adolescence. In: Marcus R, Feldman D, Kelsey J, editors. Osteoporosis (Second Edition) [Internet]. San Diego: Academic Press; 2001 [cited 2018 Jul 6]. p. 621–38. Available from: http://www.sciencedirect.com/science/article/pii/B978012470862450026X

31.   Cole ZA, Gale CR, Javaid MK, Robinson SM, Law C, Boucher BJ, et al. Maternal dietary patterns during pregnancy and childhood bone mass: a longitudinal study. J Bone Miner Res Off J Am Soc Bone Miner Res. 2009 Apr;24(4):663–8.

32.   Jones G, Riley MD, Dwyer T. Maternal diet during pregnancy is associated with bone mineral density in children: a longitudinal study. Eur J Clin Nutr. 2000 Oct;54(10):749–56.

33.   Poulsen RC, Moughan PJ, Kruger MC. Long-Chain Polyunsaturated Fatty Acids and the Regulation of Bone Metabolism. Exp Biol Med. 2007 Nov 1;232(10):1275–88.

34.   Ilich JZ, Kerstetter JE. Nutrition in bone health revisited: a story beyond calcium. J Am Coll Nutr. 2000 Dec;19(6):715–37.

35.   Koren N, Simsa-Maziel S, Shahar R, Schwartz B, Monsonego-Ornan E. Exposure to omega-3 fatty acids at early age accelerate bone growth and improve bone quality. J Nutr Biochem. 2014 Jun 1;25(6):623–33.

36.   Calcium metabolism, osteoporsis and essential fatty acids: A review. Prog Lipid Res. 1997 Sep 1;36(2–3):131–51.

37.   Watkins BA, Li Y, Allen KGD, Hoffmann WE, Seifert MF. 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 Sep 1;130(9):2274–84.

38.   Gao Q, Xu M, Alander CB, Choudhary S, Pilbeam CC, Raisz LG. Effects of prostaglandin E2 on bone in mice in vivo. Prostaglandins Other Lipid Mediat. 2009 Jun 1;89(1):20–5.

39.   Lau BYY, Ward WE, Kang JX, Ma DWL. Vertebrae of developing fat-1 mice have greater strength and lower n-6/n-3 fatty acid ratio. Exp Biol Med Maywood NJ. 2009 Jun;234(6):632–8.

40.   Högström M, Nordström P, Nordström A. n-3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the NO2 Study. Am J Clin Nutr. 2007 Mar;85(3):803–7.

41.   Harvey N, Dhanwal D, Robinson S, Kim M, Inskip H, Godfrey K, et al. Does maternal long chain polyunsaturated fatty acid status in pregnancy influence the bone health of children? Osteoporos Int. 2012 Sep 1;23(9):2359–67.

42.   CDC. 2018 Breastfeeding Report Card [Internet]. Centers for Disease Control and Prevention. 2018 [cited 2018 Dec 2]. Available from: https://www.cdc.gov/breastfeeding/data/reportcard.htm

43.   Muniz LC, Menezes AMB, Buffarini R, Wehrmeister FC, Assunção MCF. Effect of breastfeeding on bone mass from childhood to adulthood: a systematic review of the literature. Int Breastfeed J. 2015;10:31.

44.   Andres A, Casey PH, Cleves MA, Badger TM. Body fat and bone mineral content of infants fed breast milk, cow's milk formula, or soy formula during the first year of life. J Pediatr. 2013 Jul;163(1):49–54.

45.   Foley S, Quinn S, Jones G. Tracking of bone mass from childhood to adolescence and factors that predict deviation from tracking. Bone. 2009 May;44(5):752–7.

46.   Harvey NC, Robinson SM, Crozier SR, Marriott LD, Gale CR, Cole ZA, et al. Breast-feeding and adherence to infant feeding guidelines do not influence bone mass at age 4 years. Br J Nutr. 2009 Sep;102(6):915–20.

47.   Young RJ, Antonson DL, Ferguson PW, Murray ND, Merkel K, Moore TE. Neonatal and infant feeding: effect on bone density at 4 years. J Pediatr Gastroenterol Nutr. 2005 Jul;41(1):88–93.

48.   Crabtree NJ, Leonard MB, Zemel BS. Dual-Energy X-Ray Absorptiometry. In: Bone Densitometry in Growing Patients [Internet]. Humana Press; 2007 [cited 2018 Aug 15]. p. 41–57.

(Current Clinical Practice). Available from: https://link.springer.com/chapter/10.1007/978-1-59745-211-3_3

49.	Pietrobelli A, Peroni DG, Faith MS. Pediatric body composition in clinical studies: which methods in which situations? Acta Diabetol. 2003 Oct 1;40(1):s270–3.

50.	Goran MI. Measurement Issues Related to Studies of Childhood Obesity: Assessment of Body Composition, Body Fat Distribution, Physical Activity, and Food Intake. Pediatrics. 1998 Mar 1;101(Supplement 2):505–18.

51.	Ott SM, O'Hanlan M, Lipkin EW, Newell-Morris L. Evaluation of vertebral volumetric vs. areal bone mineral density during growth. Bone. 1997 Jun;20(6):553–6.

52.	Eriksson S, Mellström D, Strandvik B. Volumetric bone mineral density is an important tool when interpreting bone mineralization in healthy children. Acta Paediatr. 2009 Feb 1;98(2):374–9.

53.	Kučukalić-Selimović E, Valjevac A, Hadžović-Džuvo A. The utility of procollagen type 1 N-terminal propeptide for the bone status assessment in postmenopausal women. Bosn J Basic Med Sci. 2013 Nov;13(4):259–65.

54.	Cox G, Einhorn TA, Tzioupis C, Giannoudis PV. Bone-turnover markers in fracture healing. J Bone Joint Surg Br. 2010 Mar 1;92-B(3):329–34.

55.	Nakamura A, Dohi Y, Akahane M, Ohgushi H, Nakajima H, Funaoka H, et al. Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. Tissue Eng Part C Methods. 2009 Jun;15(2):169–80.

56.	Sabel K-G, Lundqvist-Persson C, Bona E, Petzold M, Strandvik B. Fatty acid patterns early after premature birth, simultaneously analysed in mothers' food, breast milk and serum phospholipids of mothers and infants. Lipids Health Dis. 2009 Jun 10;8:20.

57.	Peng YM, Zhang TY, Wang Q, Zetterström R, Strandvik B. Fatty acid composition in breast milk and serum phospholipids of healthy term Chinese infants during first 6 weeks of life. Acta Paediatr. 2007 Nov 1;96(11):1640–5.

58.	Nilsson AK, Löfqvist C, Najm S, Hellgren G, Sävman K, Andersson MX, et al. Long-chain polyunsaturated fatty acids decline rapidly in milk from mothers delivering extremely preterm indicating the need for supplementation. Acta Paediatr. 2018 Jun 1;107(6):1020–7.

59.	Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000 May 6;320(7244):1240.

60.	Bonnet N, Ferrari SL. Effects of long-term supplementation with omega-3 fatty acids on longitudinal changes in bone mass and microstructure in mice. J Nutr Biochem. 2011 Jul 1;22(7):665–72.

61.	Bouillon R, Prodonova A. Growth Hormone Deficiency and Peak Bone Mass. J Pediatr Endocrinol Metab. 2014;13(Supplement):1327–1342.

62.	Mizokami A, Kawakubo-Yasukochi T, Hirata M. Osteocalcin and its endocrine functions. Biochem Pharmacol. 2017 May 15;132:1–8.

63.	Wei J, Ferron M, Clarke CJ, Hannun YA, Jiang H, Blaner WS, et al. Bone-specific insulin resistance disrupts whole-body glucose homeostasis via decreased osteocalcin activation. J Clin Invest. 2014 Apr;124(4):1–13.

64.   Karsenty G, Oury F. Regulation of male fertility by the bone-derived hormone osteocalcin. Mol Cell Endocrinol. 2014 Jan 25;382(1):521–6.

65.   Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, et al. Endocrine Regulation of Energy Metabolism by the Skeleton. Cell. 2007 Aug 10;130(3):456–69.

66.   Boucher-Berry C, Speiser PW, Carey DE, Shelov SP, Accacha S, Fennoy I, et al. Vitamin D, osteocalcin, and risk for adiposity as comorbidities in middle school children. J Bone Miner Res Off J Am Soc Bone Miner Res. 2012 Feb;27(2):283–93.

67.   Wang J-W, Tang Q-Y, Ruan H-J, Cai W. Relation between serum osteocalcin levels and body composition in obese children. J Pediatr Gastroenterol Nutr. 2014 Jun;58(6):729–32.

68.   Wang G, Johnson S, Gong Y, Polk S, Divall S, Radovick S, et al. Weight Gain in Infancy and Overweight or Obesity in Childhood across the Gestational Spectrum: a Prospective Birth Cohort Study. Sci Rep [Internet]. 2016 Jul 15 [cited 2018 Dec 22];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4945912/

69.   Kalra SP, Dube MG, Iwaniec UT. Leptin increases osteoblast-specific osteocalcin release through a hypothalamic relay. Peptides. 2009 May;30(5):967–73.

70.   Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, et al. Insulin Signaling in Osteoblasts Integrates Bone Remodeling and Energy Metabolism. Cell. 2010 Jul 23;142(2):296–308.

71.   Krall EA, Dawson-Hughes B. Heritable and life-style determinants of bone mineral density. J Bone Miner Res. 1993;8(1):1–9.

72.   Boot AM, de Ridder MAJ, Pols HAP, Krenning EP, de Muinck Keizer-Schrama SMPF. Bone Mineral Density in Children and Adolescents: Relation to Puberty, Calcium Intake, and Physical Activity. J Clin Endocrinol Metab. 1997 Jan 1;82(1):57–62.

73.   Stagi S, Cavalli L, Cavalli T, de Martino M, Brandi ML. Peripheral quantitative computed tomography (pQCT) for the assessment of bone strength in most of bone affecting conditions in developmental age: a review. Ital J Pediatr [Internet]. 2016 Sep 26 [cited 2018 Dec 8];42. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5037897/

74.   Jimenez Avila JM. Vertebral Destruction Syndrome: From Knowledge to Practice. J Spine [Internet]. 2015 [cited 2018 Dec 9];04(04). Available from: http://www.omicsgroup.org/journals/vertebral-destruction-syndrome-from-knowledge-to-practice-2165-7939-1000251.php?aid=59237

75.   Matkovic V, Jelic T, Wardlaw GM, Ilich JZ, Goel PK, Wright JK, et al. Timing of peak bone mass in Caucasian females and its implication for the prevention of osteoporosis. Inference from a cross-sectional model. J Clin Invest. 1994 Feb 1;93(2):799–808.

76.   Newton-John HF, Morgan DB. The loss of bone with age, osteoporosis, and fractures. Clin Orthop. 1970;71:229–52.

77.   Singh S, Kumar D, Lal AK. Serum Osteocalcin as a Diagnostic Biomarker for Primary Osteoporosis in Women. J Clin Diagn Res JCDR. 2015 Aug;9(8):RC04–7.

78.   VS K, K P, Ramesh M, Venkatesan V. The Association of Serum Osteocalcin with the Bone Mineral Density in Post Menopausal Women. J Clin Diagn Res JCDR. 2013 May;7(5):814–6.

79.   Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe. 2015 May 13;17(5):690–703.

Appendix

TABLE A. Drop-put analysis showing early demographic characteristics of the follow-up study population (n=167) versus dropouts (n=231) included at birth but not at DEXA.

	Follow-up cohort	Dropouts	p-value
Gestational age (days)	280±9	279±9	NS
Birth weight (g)	3635±517	3552±497	NS
Birth length (cm)	51.0±4	51±2	NS
Weight 4 months	6737±879	6728±953	NS
Breastfeeding			NS
exclusive	108 (66%)	132 (65%)	
mixed	26 (16%)	37 (18%)	
formula	30 (18%)	35 (17%)	
Maternal BMI (kg/m²)	24.6±4	23.7±4.8	**0.028**
Maternal education			NS
Primary school	4 (3%)	7 (4%)	
High school	43 (29%)	49 (35%)	
University	92 (63%)	143(56%)	
Other	8 (5%)	9 (5%)	
Maternal smoking			NS
Yes	6 (4%)	10 (6%)	
No	141 (96%)	167 (94%)	
Age of mother at delivery (years)	31±4	30±5	**0.007**
Note: BMI = body mass index			

TABLE B. Summary of correlation between monounsaturated fatty acids (MUFA) and polysaturated fatty acids (PUFA), BMD_{L1-L4} and $BMAD_{spine}$.

Fatty acids	Birth				4 months			
	r BMD_{L1-L4}	p BMD_{L1-L4}	r $BMAD_{spine}$	p $BMAD_{spine}$	r BMD_{L1-L4}	p BMD_{L1-L4}	r $BMAD_{spine}$	p $BMAD_{spine}$
MUFA								
14:1n5	-0.038	0.652	-0.034	0.686	0.031	0.739	0.063	0.502
16:1n7	0.006	0.958	-0.049	0.655	-0.308	**0.012**	-0.201	0.109
18:1n7	0.006	0.958	-0.049	0.655	0.191	0.174	0.145	0.306
18:1n9	-0.070	0.521	0.017	0.877	-0.141	0.261	-0.082	0.514
20:1n9	0.057	0.497	-0.031	0.713	0.094	0.315	0.146	0.116
22:1n9	-0.042	0.616	-0.067	0.428	-0.108	0.246	-0.068	0.465
24:1n9	0.006	0.940	-0.070	0.402	-0.011	0.907	-0.024	0.798
Total	0.029	0.728	-0.048	0.566	-0.114	0.222	-0.060	0.520
PUFA								
n-9								
20:3n9	0.013	0.909	-0.098	0.367	-0.242	0.052	-0.103	0.413
n-6								
18:2n6 (LA)	0.061	0.470	-0.006	0.946	0.082	0.381	0.147	0.115
18:3n6	0.053	0.526	-0.025	0.768	-0.039	0.680	-0.001	0.993
20:2n6	0.043	0.609	-0.068	0.418	-0.078	0.402	-0.012	0.899
20:3n6	0.004	0.963	0.022	0.796	-0.152	0.103	-0.138	0.138
20:4n6 (AA)	0.069	0.529	-0.069	0.526	-0.250	0.044	-0.033	0.792
22:2n6	0.070	0.403	0.030	0.718	0.075	0.419	0.147	0.113
22:4n6	0.104	0.213	0.030	0.722	0.147	0.114	.187	0.044
22:5n6	-0.049	0.557	0.000	0.995	0.050	0.591	0.134	0.150
Total	0.088	0.296	0.052	0.539	-0.020	0.833	0.106	0.254
n-3								
18:3n3 (ALA)	-0.176	0.105	-0.200	0.065	-0.013	0.918	0.160	0.202
20:4n3	-0.104	0.215	-0.073	0.387	-0.020	0.833	-0.026	0.779
20:5n3 (EPA)	-0.035	0.679	0.009	0.912	0.101	0.279	.202	0.029
22:5n3	0.001	0.986	0.082	0.329	-0.016	0.863	0.018	0.846
22:6n3 (DHA)	0.044	0.603	-0.036	0.670	-0.016	0.863	0.018	0.846
Total	-0.009	0.913	0.072	0.391	0.027	0.771	0.077	0.409
n-6:n-3 ratio	0.044	0.603	-0.036	0.670	0.044	0.643	-0.030	0.750

Note. $p < 0.02$ was considered statistically significant. r= correlation coefficient. ALA=alpha linolenic acid; EPA=eicosapentaenoic acid; DHA=docosahexaenoic acid; LA=linoleic acid; AA=arachidonic acid. P-values of BMD= bone mineral density and BMAD= bone mineral apparent density.

TABLE C. Summary of correlations between bone formation markers and BMD_{L1-L4} and $BMAD_{spine}$

Bone formation markers	r BMD_{L1-L4}	p BMD_{L1-L4}	r $BMAD_{spine}$	p $BMAD_{spine}$
$P1NP_b$	0.059	0.463	0.007	0.933
OC_b	0.042	0.606	0.029	0.726
$P1NP_{4m}$	-0.014	0.859	-0.049	0.546
OC_{4m}	-0.071	0.387	-0.116	0.155
Note. r= correlation coefficient; $p < 0.05$ was considered statistically significant. P1NP = procollagen type 1 amino-terminal propeptide. OC = osteocalcin. Bone formation markers were measured in cord blood at birth (b) and 4 months of age (4m). BMD= bone mineral density and BMAD= bone mineral apparent density.				