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Fatty Acid Percentage Distribution in Complex Lipids of Breast Milk from Mothers on a Low Docosahexaenoic Acid Diet

--Manuscript Draft--

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Abstract:	The aim of this study was to assess the fatty acid percentage distribution in complex lipids of breast milk from mothers on a low docosahexaenoic acid (DHA) diet. We performed a descriptive, cross-sectional study of milk samples (n=14) collected 90 days after delivery and analyzed them using gas chromatography, thin-layer chromatography and the Fiske-Subbarow method. Complex lipid distribution was 40.70 ± 5.11% sphingomyelin (SM), 26.03 ± 5.98% phosphatidylethanolamine (PE), 21.12 ± 2.32% phosphatidylcholine, 7.94 ± 1.96% phosphatidylserine and 4.22 ± 1.25% phosphatidylinositol. Median DHA and arachidonic acid values were 0.13% (0.12; 0.18) and 0.42% (0.33; 0.53), respectively. Mean fatty acid percentage in SM and PE was as follows: palmitic acid, 34.45 ± 1.94% and 5.38 ± 0.94%; oleic acid, 16.50 ± 4.07% and 9.43 ± 4.05%; linoleic acid, 5.91 ± 4.69% and 9.05 ± 4.5%. DHA was not detectable in SM, but it was found in PE (55.33 ± 14.46). In conclusion, breast milk of mothers on a low DHA diet contained 55% DHA in PE, but no DHA in SM.
Additional Information:	
Question	**Response**
Have any of the data and/or the subjects in the submitted manuscript been previously reported in another publication?	No

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Reviewer Comments:

Reviewer 1: The authors addressed the critiques and made important modifications. The manuscript is easier to read, however, the entire manuscript needs considerable proof reading. Multiple sentences are challenging to understand and have incorrect phrasing.

These a few examples, not an exhaustive list:

In the intro, the paragraph with "Some nutrients keep a constant concentration in maternal milk, regardless of their own nutritional condition" would be more clear as "some nutrients maintain a constant concentration in breast milk, regardless of maternal diet"

In the methods, some abbreviations are not spelled out: IDIP, SD

In results, the sentence that states "the higher percent of FA corresponds to" is not clear - higher compared to what? Or did you mean these are just the FA highest % overall? "The FA with the highest percentages are oleic acid, palmitic acid, etc…"

In the discussion, "Our results showed that predominated FA of SM species" should be "the predominant species of FA in SM were"

"In Japanese women, PE was the most abundant PL, with fairly high percentages (59%) in total FAs, especially ARA (12.7%) and DHA (5.6%). I don't understand the second half of the sentence.

"DHA primarily incorporates into PE and then into PC. It has been shown that a newly arrived DHA molecule has 5.7 higher affinity for PE than PC, indicating that PE would be the main DHA reservoir in membranes."

Consider "DHA is preferentially incorporated in to PE before PC"

When reporting standard deviations, consider adding the +/- symbol before the numbers in parentheses, to make it clear these are standard deviations (especially in the abstract before this is spelled out). The symbol is used in the discussion, but the not the rest of the manuscript.

The authors greatly appreciate all the comments and suggestions made by the reviewer and have accordingly included them in this version of the manuscript. We have further done an English Language review of the entire manuscript, as suggested by the Editor.

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The authors report no conflicts of interests. This study received funding from IDIP.

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Authorship

Horacio F. González: conception of study design, drafting and revision of the final version of the work.

Agustina Malpeli: conception of study design and carrying out nutritional questionnaires.

Victoria Fasano: conception of study design and biostatistical analysis of data.

L.G. Pescio: conception of study design, acquisition and analysis of samples, drafting and revision of the final version of the work.

Norma B. Sterin de Speziale: conception of study design, drafting and revision of the final version of the work.

Silvana Visentin: conception of study design, acquisition and analysis of samples, drafting and revision of the final version of the work.

All authors approved the final version of the work to be published.

ABSTRACT

The aim of this study was to assess the fatty acid percentage distribution in complex lipids of breast milk from mothers on a low docosahexaenoic acid (DHA) diet. We performed a descriptive, cross-sectional study of milk samples (n=14) collected 90 days after delivery and analyzed them using gas chromatography, thin-layer chromatography and the Fiske-Subbarow method. Complex lipid distribution was 40.70 ± 5.11% sphingomyelin (SM), 26.03 ± 5.98% phosphatidylethanolamine (PE), 21.12 ± 2.32% phosphatidylcholine, 7.94 ± 1.96% phosphatidylserine and 4.22 ± 1.25% phosphatidylinositol. Median DHA and arachidonic acid values were 0.13% (0.12; 0.18) and 0.42% (0.33; 0.53), respectively. Mean fatty acid percentage in SM and PE was as follows: palmitic acid, 34.45 ± 1.94% and 5.38 ± 0.94%; oleic acid, 16.50 ± 4.07% and 9.43 ± 4.05%; linoleic acid, 5.91 ± 4.69% and 9.05 ± 4.5%. DHA was not detectable in SM, but it was found in PE (55.33 ± 14.46). In conclusion, breast milk of mothers on a low DHA diet contained 55% DHA in PE, but no DHA in SM.

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What Is Known

- The amount of long-chain polyunsaturated fatty acids in breast milk greatly depends on maternal dietary intake.
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What Is New

- In mature milk samples, SM was the complex lipid with the highest percentage.
- In mothers on a low DHA diet, DHA was incorporated only into PE.

INTRODUCTION

At birth, the human brain weighs approximately 400 g, reaching about 1200 g between two and three years of age. During this period, complex lipids are essential components of brain lipid deposition (1).

Human milk contains 3 to 5% lipids, including 98% triacylglycerols, 0.8% phospholipids (PL) and 0.5% cholesterol, among others. Lipids are present as fat globules surrounded by a three-layered membrane structure composed of specific proteins (mainly glycoproteins) as well as polar (PL and glycosphingolipids) and apolar (cholesterol and cerebrosides) lipids (2). Phospholipids include sphingomyelin (SM) and glycerophospholipids (GPL). Sphingomyelin is the major sphingolipid found in milk and a main component of the myelin sheath that covers neuronal axons. Glycerophospholipids comprise phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylinositol (PI) (3).

In SM, the primary hydroxyl group of the sphingoid base is linked to phosphoryl-choline. Since PL are a source of choline, they regulate the transduction signal and serve as a source of methyl groups in intermediate metabolism, becoming essential for optimum brain development (4).

The chemical structure of SM and PL determine their structural functions in the cell. It has been postulated that the degree of unsaturation of neuronal PL fatty acids (FA) can affect neurological functions since they modulate membrane activities. In this sense, while polyunsaturated FA (PUFA) are essential components for membrane permeability and fluidity (5), PL are involved in cell membrane integrity, cell signaling, proliferation and inflammatory response (6).

Breast milk GPL are a source of PUFA, which are present in large amounts in the brain and the central nervous system. Phosphatidylethanolamine is a highly unsaturated PL containing high proportions of docosahexaenoic and arachidonic acids (DHA and ARA,

respectively). These PUFA are abundant in the central nervous system as well as components of membrane PL, thus favoring the brain development of breastfed infants (7).

Some nutrients maintain a constant concentration in breast milk, regardless of maternal diet. In other cases, their concentration may vary significantly, depending on the nutritional condition of the mother or on nutrient intake. In this sense, long chain PUFA (LCPUFA) are a clear example of the variations observed in maternal milk in response to cultural and dietary habit differences (8). In a previous study carried out by our group in breastfeeding mothers receiving care at the public health system, breast milk DHA content and intake were found to be low with reference to international recommendations (9,10).

Considering that slight changes in lipid composition may greatly affect myelin function (11), the aim of this study was to assess PUFA percentage distribution in SM and PE of breast milk from women on a low DHA diet.

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Breast milk lipids (0.8 ml) were extracted using the Bligh and Dyer protocol (12) and dried under nitrogen atmosphere. Aliquots of the lipid extracts were dissolved in chloroform, spotted onto thin layer chromatography plates (TLC) and developed in chloroform:methanol:acetic acid:water (40:10:10:1, v/v/v/v). The plates were then exposed to iodine vapors and the bands corresponding to SM, PC, PS, PI and PE were scraped off the plates according to the mobility of the lipid standards. Quantitation of each PL was carried out by measuring total lipid phosphate according to the Fiske-Subbarow method (13). Gas chromatography was used for FA quantification.

Maternal Dietary Intake

The consumption of DHA precursors (dried fruits, supplements with alpha-linolenic acid, type of oil, flax and pumpkin seeds) and sources (oily fish such as salmon, tuna, herring and

trout) was surveyed with a food frequency questionnaire (single selection), taking into account the local consumption habits and previous studies performed by our group in lactating mothers (14,15). Data were analyzed using the SARA food chemical composition tables and software (16).

Statistical Analysis

Data were processed with the statistical program R version 3.5.1. The normality of each variable was evaluated with the Shapiro–Wilk test. Student's t-test was used for continuous variables with normal distribution and Mann-Whitney test for those which did not fit a normal distribution. The distribution of FA and complex lipids is presented as a percentage of the total FA analyzed. Variables with a normal distribution are presented as means and standard deviations (SD) and non-parametric variables as medians (interquartile range, IQR). In all cases, results with a significance level of $p < 0.05$ were considered statistically significant.

RESULTS

Fourteen milk samples from exclusively breastfeeding mothers were analyzed. The median age of mothers was 25 years (IQR= 20.25; 30).

The assessment of known DHA precursors and sources showed that none of the mothers reported to have consumed any of the food items surveyed or received supplements during the previous month. The only seed oil consumed was sunflower oil, which is very high in omega-6.

The distribution of complex lipids in milk was as follows: SM, 40.70 ± 5.11%; PE, 26.03 ± 5.98%; PC, 21.12 ± 2.32%; PS, 7.94 ± 1.96%; and PI, 4.22 ± 1.25%.

The FA with the highest percentages were oleic acid (31.77 ± 2.59%), palmitic acid (21.73 ± 1.92%) and linoleic acid (18.86 ± 5.72%). Median DHA and ARA values in milk were 0.13% (0.12; 0.18) and 0.42% (0.33; 0.53), respectively.

The percentage distribution of FA in SM and PE from maternal milk is shown in Table 1.

DISCUSSION

In this study, total FA in maternal breast milk had a low percentage of DHA. Both SM and PE were the most abundant PL classes identified in human milk. Additionally, DHA was not found in SM, but it accounted for 55% of total FA in PE.

In a previous study by our group, the DHA percentage in breast milk of mothers assisted in the Public Health System was low, amounting to less than 0.2% (9). In a report of a meta-analysis of 65 studies of 2474 women (10), mean DHA concentration in breast milk was 0.32 ± 0.22%. The highest DHA concentrations were reported in coastal populations and associated with the consumption of marine food (17). In the present study, the mothers evaluated did not consume either omega-3 or DHA precursors, sources or supplements, corroborating previous findings of our group analyzing maternal dietary intakes in a similar population from the same region (9).

Our results showed that the predominant species of FA in SM of breast milk were saturated FA, namely, palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), behenic acid (C22:0) and lignoceric acid (C24:0). The most common unsaturated FAs were palmitoleic acid (C161ω9:), oleic acid (18:1ω9) and the essential linoleic acid (18:2ω6). Total saturated FAs in SM were higher than those reported by Wang et al. (79.38 ± 2.66% vs. 59.23 ± 4.10%, respectively) (5). Differences were probably due to the high levels of DHA (1.11 ±0.50%) and LCPUFA in the Japanese samples of the mentioned study and the absence of DHA in SM of our study.

The complex mixture of lipids in human milk has been widely studied (6,17). Reports on lipid variations during the first weeks of lactation have shown that they are stabilized after the third week (17). The PL profile in human breast milk fluctuates during the entire lactation period in order to suit the growing needs of newborns. The average value of total PL in human milk significantly decreases from colostrum to transitional and mature milk. The temporal change of PL class distribution through lactation might be connected to their physicochemical properties and the functional requirements of breast milk at different stages (6).

In our study, SM was the most abundant PL in human breast milk. In agreement with Sala-Vila et al. (18), the percentage of SM was the highest, followed by PE in our study and by PC in theirs. Such difference could be due to the fact that we analyzed mature milk samples three months postpartum and the mentioned authors studied one-month postpartum milk. In this sense, Holmes et al. emphasized that PE increased and PC decreased as lactation proceeded (19).

In Japanese women, PE was the most abundant PL, containing a high percentage of unsaturated FA (59%), especially ARA (12.7%) and DHA (5.6%), which represented 0.99 and 1.0% of total lipid content, respectively (5). In our study, DHA corresponded to 55% of PE, but we did not find ARA in PE. DHA is preferentially incorporated into PE before PC. It has been shown that a newly arrived DHA molecule has 5.7 higher affinity for PE than PC, indicating that PE would be the main DHA reservoir in membranes (20).

We also found a greater percentage of essential linoleic acid (18:2ω6) but no ARA in PE. Our results agree with those of Bitman et al. (17), who found that while linoleic acid increased as milk matured, ARA decreased.

One of the limitations of our study was the low number of samples and the low variability in the diet of the mothers surveyed. The analysis of a group of mothers with a higher

consumption of omega 3 would allow assessing changes in the distribution of esterified FAs in complex lipid molecules. Another limitation was that the distribution of FA molecules in PC, PI and PS could not be analyzed. Nevertheless, the main contribution of this study was that lactating women consuming food items low in DHA had a low percentage of DHA in their milk. Accordingly, considering the relevance of DHA for auditory, visual and cognitive development, supplementation of lactating mothers with DHA following international recommendations would be an important public health measure.

CONCLUSION

The evaluation of a limited number of breast milk samples from mothers receiving public health care showed that they did not consume DHA precursors, sources or supplements. The highest percentages of complex lipids in human milk corresponded to SM and PE. We found that PE contained 55% DHA, but we did not find either DHA in SM or ARA in SM and PE.

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TABLE 1. Fatty acid percentage distribution in sphingomyelin and phosphatidylethanolamine of human milk 90 days after delivery.

Fatty acid	Complex lipids	
	SM	PE
C16:0	34.64 (1.94)	5.51 (1.00)
C16:1n9	12.48 (1.18)	-
C18:0	22.16 (3.32)	14.50 (3.21)
C18:1n9	17.10 (3.69)	9.43 (4.07)
C18:2n6	8.93 (1.97)	9.08 (4.55)
C20:0	7.87 (1.56)	7.49 (3.30)
C20:4n6 (ARA)	-	-
C22:0	9.46 (4.72)	6.02 (0.92)
C24:0	5.25 (1.77)	-
C22:6n3 (DHA)	-	55.34 (14.46)

Data are means (SD). SM = sphingomyelin; PE = phosphatidylethanolamine; ARA = arachidonic acid; DHA = docosahexaenoic acid.

ABSTRACT

The aim of this study was to assess the fatty acid percentage distribution in complex lipids of breast milk from mothers on a low docosahexaenoic acid (DHA) diet. We performed a descriptive, cross-sectional study of milk samples (n=14) collected 90 days after delivery and analyzed them using gas chromatography, thin-layer chromatography and the Fiske-Subbarow method. Complex lipid distribution was 40.70 ± 5.11% sphingomyelin (SM), 26.03 ± 5.98% phosphatidylethanolamine (PE), 21.12 ± 2.32% phosphatidylcholine, 7.94 ± 1.96% phosphatidylserine and 4.22 ± 1.25% phosphatidylinositol. Median DHA and arachidonic acid values were 0.13% (0.12; 0.18) and 0.42% (0.33; 0.53), respectively. Mean fatty acid percentage in SM and PE was as follows: palmitic acid, 34.45 ± 1.94% and 5.38 ± 0.94%; oleic acid, 16.50 ± 4.07% and 9.43 ± 4.05%; linoleic acid, 5.91 ± 4.69% and 9.05 ± 4.5%. DHA was not detectable in SM, but it was found in PE (55.33 ± 14.46). In conclusion, breast milk of mothers on a low DHA diet contained 55% DHA in PE, but no DHA in SM.

Key Words: sphingomyelin, phosphatidylethanolamine, breast milk, polyunsaturated fatty acids

What Is Known

- The amount of long-chain polyunsaturated fatty acids in breast milk greatly depends on maternal dietary intake.
- When docosahexaenoic acid (DHA) intake is normal or high, DHA is incorporated into both phosphatidylethanolamine (PE) and sphingomyelin (SM).

What Is New

- In mature milk samples, SM was the complex lipid with the highest percentage.
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INTRODUCTION

At birth, the human brain weighs approximately 400 g, reaching about 1200 g between two and three years of age. During this period, complex lipids are essential components of brain lipid deposition (1).

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respectively). These PUFA are abundant in the central nervous system as well as components of membrane PL, thus favoring the brain development of breastfed infants (7).

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RESULTS

Fourteen milk samples from exclusively breastfeeding mothers were analyzed. The median age of mothers was 25 years (IQR= 20.25; 30).

The assessment of known DHA precursors and sources showed that none of the mothers reported to have consumed any of the food items surveyed or received supplements during the previous month. The only seed oil consumed was sunflower oil, which is very high in omega-6.

The distribution of complex lipids in milk was as follows: SM, 40.70 ± 5.11%; PE, 26.03 ± 5.98%; PC, 21.12 ± 2.32%; PS, 7.94 ± 1.96%; and PI, 4.22 ± 1.25%.

The FA with the highest percentages were oleic acid (31.77 ± 2.59%), palmitic acid (21.73 ± 1.92%) and linoleic acid (18.86 ± 5.72%). Median DHA and ARA values in milk were 0.13% (0.12; 0.18) and 0.42% (0.33; 0.53), respectively.

The percentage distribution of FA in SM and PE from maternal milk is shown in Table 1.

DISCUSSION

In this study, total FA in maternal breast milk had a low percentage of DHA. Both SM and PE were the most abundant PL classes identified in human milk. Additionally, DHA was not found in SM, but it accounted for 55% of total FA in PE.

In a previous study by our group, the DHA percentage in breast milk of mothers assisted in the Public Health System was low, amounting to less than 0.2% (9). In a report of a meta-analysis of 65 studies of 2474 women (10), mean DHA concentration in breast milk was 0.32 ± 0.22%. The highest DHA concentrations were reported in coastal populations and associated with the consumption of marine food (17). In the present study, the mothers evaluated did not consume either omega-3 or DHA precursors, sources or supplements, corroborating previous findings of our group analyzing maternal dietary intakes in a similar population from the same region (9).

Our results showed that the predominant species of FA in SM of breast milk were saturated FA, namely, palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), behenic acid (C22:0) and lignoceric acid (C24:0). The most common unsaturated FAs were palmitoleic acid (C161ω9:), oleic acid (18:1ω9) and the essential linoleic acid (18:2ω6). Total saturated FAs in SM were higher than those reported by Wang et al. (79.38 ± 2.66% vs. 59.23 ± 4.10%, respectively) (5). Differences were probably due to the high levels of DHA (1.11 ±0.50%) and LCPUFA in the Japanese samples of the mentioned study and the absence of DHA in SM of our study.

The complex mixture of lipids in human milk has been widely studied (6,17). Reports on lipid variations during the first weeks of lactation have shown that they are stabilized after the third week (17). The PL profile in human breast milk fluctuates during the entire lactation period in order to suit the growing needs of newborns. The average value of total PL in human milk significantly decreases from colostrum to transitional and mature milk. The temporal change of PL class distribution through lactation might be connected to their physicochemical properties and the functional requirements of breast milk at different stages (6).

In our study, SM was the most abundant PL in human breast milk. In agreement with Sala-Vila et al. (18), the percentage of SM was the highest, followed by PE in our study and by PC in theirs. Such difference could be due to the fact that we analyzed mature milk samples three months postpartum and the mentioned authors studied one-month postpartum milk. In this sense, Holmes et al. emphasized that PE increased and PC decreased as lactation proceeded (19).

In Japanese women, PE was the most abundant PL, containing a high percentage of unsaturated FA (59%), especially ARA (12.7%) and DHA (5.6%), which represented 0.99 and 1.0% of total lipid content, respectively (5). In our study, DHA corresponded to 55% of PE, but we did not find ARA in PE. DHA is preferentially incorporated into PE before PC. It has been shown that a newly arrived DHA molecule has 5.7 higher affinity for PE than PC, indicating that PE would be the main DHA reservoir in membranes (20).

We also found a greater percentage of essential linoleic acid (18:2ω6) but no ARA in PE. Our results agree with those of Bitman et al. (17), who found that while linoleic acid increased as milk matured, ARA decreased.

One of the limitations of our study was the low number of samples and the low variability in the diet of the mothers surveyed. The analysis of a group of mothers with a higher

consumption of omega 3 would allow assessing changes in the distribution of esterified FAs in complex lipid molecules. Another limitation was that the distribution of FA molecules in PC, PI and PS could not be analyzed. Nevertheless, the main contribution of this study was that lactating women consuming food items low in DHA had a low percentage of DHA in their milk. Accordingly, considering the relevance of DHA for auditory, visual and cognitive development, supplementation of lactating mothers with DHA following international recommendations would be an important public health measure.

CONCLUSION

The evaluation of a limited number of breast milk samples from mothers receiving public health care showed that they did not consume DHA precursors, sources or supplements. The highest percentages of complex lipids in human milk corresponded to SM and PE. We found that PE contained 55% DHA, but we did not find either DHA in SM or ARA in SM and PE.

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TABLE 1. Fatty acid percentage distribution in sphingomyelin and phosphatidylethanolamine of human milk 90 days after delivery.

Fatty acid	Complex lipids	
	SM	PE
C16:0	34.64 (1.94)	5.51 (1.00)
C16:1n9	12.48 (1.18)	-
C18:0	22.16 (3.32)	14.50 (3.21)
C18:1n9	17.10 (3.69)	9.43 (4.07)
C18:2n6	8.93 (1.97)	9.08 (4.55)
C20:0	7.87 (1.56)	7.49 (3.30)
C20:4n6 (ARA)	-	-
C22:0	9.46 (4.72)	6.02 (0.92)
C24:0	5.25 (1.77)	-
C22:6n3 (DHA)	-	55.34 (14.46)

Data are means (SD). SM = sphingomyelin; PE = phosphatidylethanolamine; ARA = arachidonic acid; DHA = docosahexaenoic acid.