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Original Research

Sphingomyelin in Human Breast Milk might be Essential for the Hippocampus Maturation

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Abstract

Background: It has been established that sphingomyelin present human breast milk is useful for the brain maturation and cognitive development. At 10 days of breastfeeding the sphingomyelin content is double that present in cow's milk and its content is independent of the maternal diet. The aim of the study was to analyze the content of sphingomyelin in breast milk at 3 months of breastfeeding and to consider the effect of this molecule on synaptic function and nerve conduction through the probable expansion of myelinated axons. **Methods**: Therefore, to begin to define and assess this, we performed sphingolipidomic analysis in human breast milk. Then, we cultured embryonic hippocampal cells (HN9.10) in the presence of sphingomyelin at a concentration from 0.6% to 31% of human milk, estimated by considering its bioavailability and its passage into the interstitial fluid. To highlight the effect of sphingomyelin in the cells, cell viability and morphology were evaluated. Analyses of neutral sphingomyelinase gene and protein expression was performed. The entry of sphingomyelin into the cell was studied in immunofluorescence; the expression of heavy neurofilament (NF200) was tested with immunocytochemical technique. **Results**: We demonstrated that sphingomyelin is able to enter cell nucleus and overexpress the sphingomyelin phosphodiesterase 4 (*SMPD4*) gene encoding for neutral sphingomyelinase (nSMase), an enzyme useful for its own metabolism. Later, cells displayed changes of the soma and the appearance of neurites supported by NF200 overexpression. **Conclusions**: We speculated that the sphingomyelin present in human breast milk is useful in part to regulate nuclear activity and in part to form myelin sheet to facilitate nerve cell maturation. As brain development occurs at 0–3 years, these data open a new avenue of potential intervention to integrate the infant formulas with SM to obtain a product similar to the maternal milk.

Keywords: human milk; sphingomyelin; embryonic hippocampal cells; cell differentiation; neurites

1. Introduction

Multiple studies show intimate connections between maternal nutrition, among different environmental and maternal factors, and human breast milk (HBM) composition [1]. Thus, several studies have implicated changes in human fatty acid (FA) composition but not in macronutrient composition in relation to maternal dietary intakes [2–5]. This is relevant considering that human milk fat globules (MFGs) contain specific lipids, proteins, non-protein nitrogenous compounds and oligosaccharides [6]. Previous work in our group demonstrated a difference in sphingolipid (Sph) content between human and bovine milk, indicating a specie-specific composition of the milk [7]. Moreover, the Sph component of HBM at 10 days of breastfeeding was independent of maternal nutrition. In fact, the milk from mother fed on Mediterranean diet was very similar to that of mothers fed on a diet rich in vegetables or in proteins (meat, fish and eggs) [7]. We concluded that Sphs of HBM was produced by the metabolism of mammalian gland. Recently, Jiang *et al*. [8] have reviewed studies showing the effect of dietary sphingomyelin (SM) from HBM in impacting as well as gut health also cognitive development. Thus, SM composition in HBM is specifically designed to meet infant nutritional requirements during early life when the brain develops. Therefore, mother's own milk might be considered an optimal nutrition source for the nervous system maturation in infants.

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SM is the cornerstone of the large family of Sphs including interconnected metabolic lipids able to regulate various cellular mechanisms and biologic processes [9]. The biologic function of SM is regulated by tissue distribution and cellular compartmentalization. First, SM is an ubiquitous lipid but it is particularly abundant in the nervous system where has a structural and functional role. Importantly, it is an essential molecule in myelin sheath that surrounds some nerve cell axons, a critical component of both central and peripheral nervous systems, forming a protective barrier against axonal damage and facilitating the movement of nervous impulses [10]. As a consequence, SM in the cerebral spinal fluid was considered a biomarker of demyelination [11]. Second, SM is present in biological membranes where interacts with cholesterol to form lipid rafts [12]. Interestingly, dysregulation of lipid rafts was related with neurodegenerative disease pathogenesis [13]. Moreover, SM is an important regulatory molecule of the nuclear function [14]. Inside the nucleus, SM is preferentially located in perichromatin regions [15] in association with inner nuclear membrane microdomains [14]. Here, SM both anchors active chromatin and is catabolized by neutral sphingomyelinase (nSMase) by regulating duplication and transcription processes and, consequently, controlling the brain health and disease [16], including neurodegenerative disease [17].

Given the connections between the SM in HBM and SM in brain physiopathology, it became important to identify the possible specific changes of SM at different breastfeeding times and to study the bioactive role of SM in the differentiation of the brain embryonic cells. In this study, we analyzed the SM species in the human milk at 10 days, 3 and 12 months from the beginning of breastfeeding. We further studied the effect of SM in embryonic hippocampal cell differentiation.

2. Materials and Methods

2.1 Reagent

Lipids standards for lipidomic analysis were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The chemicals, at analytical grade, were purchased by Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using purified water at a Milli-Q grade (Burlington, MA, USA). Anhydrous sodium sulfate, chloroform, hexane, methanol and potassium hydroxide were purchased from Carlo Erba Reagents (Milan, Italy). Supelco™ 37 component fatty acid methyl esters (FAME) mix, containing the methyl esters of 37 fatty acids was supplied by Supelco (Bellefonte, PA, USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, trypsin, and ethylenediaminetetraacetic acid disodium and tetra-sodium salt (EDTA), fetal bovine serum (FBS) were from Microgem srl (Pozzuoli, NA, Italy). Penicillin–streptomycin, and 6X loading dye were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's phosphate buffer saline (PBS) pH 7.4, and agarose were from Invitrogen srl (Milan, Italy). Dimethylsulfoxide (DMSO), ethanol, hydrochloric acid, sodium chloride, and sodium hydroxide were purchased from Carlo Erba Reagenti srl (Milan, Italy). Fixing solution and cell stain solution were from Cell Biolabs, INC (San Diego, CA, USA). Anti-nSMase, anti-βtubulin, goat anti-rabbit secondary antibodies were from Abcam (Cambridge, UK). SDS-PAGE molecular weight standards were purchased from Nzythech (Lisboa, Portugal). Chemiluminescence kits were purchased from Amersham (Rainham, Essex, UK).

2.2 Population

Ten milk donor women at Banca del Latte Umano Donato (BLUD, Struttura Complessa di Neonatologia e Terapia Intensiva Neonatale– Azienda Ospedaliera Santa Maria della Misericordia - Perugia, Italy) were considered for the study from January 2020 to January 2021. The Bioethics Committee of Perugia University has approved the study (n.2018-05), all procedures were performed accordingly. Women were included on a voluntary basis, signed the informed consent and were anonymized. No sensitive data were collected. The inclusion criteria were mothers who had followed a Mediterranean diet during pregnancy and breastfeeding. The exclusion criteria were a history of pathology, the presence of pathologies transmissible with milk, the use of drug, alcool and smoke, gemellar pregnancies.

2.3 Milk Samples

Milk of ten donor mothers at 10 days of breastfeeding, at 3 months and at 1 year of breastfeeding were considered for the study. The mothers did not undergo any treatment. Immediately after collecting at nine o'clock, the milk samples were submitted to Holder pasteurization to eliminate harmful germs [4]. All samples were stored in a –20 °C freezer before analysis. Milk from the donor bank was used for babies of other mothers who had none or had low milk supply. The quantity administered was dependent on the quantity of milk that the direct mother of the child was able to produce and was calculated on the basis of the pre- and post-breastfeeding infant weight.

2.4 Fatty Acid Analysis

Milk lipid extraction and gas chromatography analysis were performed as previously reported [4]. Of note, the fatty acid methyl esters (FAME) of total lipids were prepared by transmethylation with methanolic KOH and analyzed by high-resolution gas chromatography. A DANI 1000DPC gas-chromatograph (Norwalk, CT, USA), equipped with a split–splitless injector and a flame ionization detector, was used. FAME separation was performed with a CP-Select CB for FAME fused silica capillary column (50 m $\times$ 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy). The injector and detector tempera-

tures were 250 °C. The oven temperature was 60 °C, held for 5 min then raised to 225 °C at 3 °C/min; the final temperature was held for 10 min. The chromatograms were acquired and processed using Clarity integration software (DataApex, Ltd., Prague, Czech Republic).

2.5 Sphingolipidomic Analysis

Sph extraction and LC–MS/MS analysis were performed as previously described [7]. Sphs were extracted from three independent 25 μL aliquots of HBM and were analyzed by LC (Dionex 3000 UltiMate, Thermo Fisher Scientific) coupled to a tandem mass spectrometer (AB Sciex 3200 QTRAP, Sciex). 12:0 SM and 6:0 Cer were used as internal standards (500 ng/mL). The separation was achieved by a reversed-phase analytical column (Acquity BEH C8 100 $\times$ 2.1 mm $\times$ 1.7 μm, Waters) through a linear gradient between eluent A (0.2% formic acid, 2 mM ammonium formate water-solution) and eluent B (0.2% formic acid, 1 mM ammonium formate in methanol). Mass spectrometry was performed in the positive ion mode (ESI+). The ion spray voltage was set at 5.5 kV, and the source temperature was set at 300 °C. Nitrogen was used as a nebulizing gas (GS 1, 45 psi), turbo spray gas (GS 2, 50 psi), and curtain gas (25 psi). Source spectra were recorded in separate experiments in the Enhanced MS (EMS) mode at a scan speed of 1000 Da/s that yielded baseline separation of unit mass peaks in the m/z range 450–1000. The collision-activated dissociation (CAD) MS-MS experiment used nitrogen as collision gas at the low pressure setting (1.2×10^{-5} Torr).

Quantitative analysis was performed interpolating each peak area of analyte/area internal standards with a calibration curve for each sphingolipid. Data represent the mean for the three independent aliquots of HBM.

2.6 Cell Culture and Treatments

Immortalized hippocampal neurons HN9.10e (kind gift of Kieran Breen, Ninewells Hospital, Dundee, UK) were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B [18]. Cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air and 5% CO_2. To study the SM effect on cell differentiation, SM (Sigma Aldrich, St. Louis, Missouri, USA), was dissolved in absolute ethanol as vehicle and increasing concentrations were added to the cell cultures. Stock solution was prepared by dissolving 500 μg/100μL absolute ethanol and by adding PBS to 5 mL final volume. 100 μL absolute ethanol to 5 mL final volume PBS was used as control sample. A control sample without absolute ethanol was added.

2.7 Cell Viability

MTT assay was used to test cellular viability, as previously reported [18]. HN9.10 cells were seeded into 96-well plates (1×10^4 cells/well density) with DMEM complete medium. After 24 h, culture medium was removed and fresh complete medium containing SM at different concentrations (0.5, 1, 5, 10, 15, 20, 25 μM) was added; the cells were incubated for 24 h. Then, MTT reagent was dissolved in PBS 1x and added to the culture at 0.5 mg/mL final concentration. After 3 h incubation at 37 °C, the supernatant was carefully removed, and formazan salt crystals were dissolved in 200 μL DMSO that was added to each well. The absorbance (OD) values were measured spectrophotometrically at 540 nm using an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH, Marburg, Germany). Each experiment was performed two times in triplicate. Cell viability was expressed as a percentage relative to the control cells. A control sample with or without absolute ethanol was added. 1% DMSO and 2% DMSO were used as positive controls.

2.8 Cell Morphology

HN9.10e cells were cultured as above reported for 72 h in absence or presence of increasing concentration of SM (0.5, 1, 5, 10, 15, 20, 25 μM). The observations were performed by using inverted microscopy EUROMEX FE 2935 (ED Amhem, Netherlands) equipped with a CMEX 5000 camera system (40x magnification) and the morphometric analysis was performed by using ImageFocus software (EUROMEX, Arnhem, The Netherlands).

2.9 Immunocytochemistry

HN9.10e were cultured for 48 h for immunocytochemical analysis performed as previously reported [19]. The cells were centrifuged at 1200 g for 15 min. The pellet was fixed with 10% formalin for 24 h and embedded in paraffin. Bond Dewax solution was used to remove paraffin from sections before rehydration and immunostaining on the Bond automated system (Leica Biosystems Newcastle, Ltd., United Kingdom). Immunostaining for neurofilament heavy protein (NF200) detection was performed by using anti-NF200 antibody (NOVOCASTRA Laboratories, Ltd., Newcastle, United Kingdom) and Bond Polymer Refine Detection (Leica Biosystems, Newcastle, Ltd., United Kingdom). The observations were performed by using inverted microscopy EUROMEX FE 2935 (ED Amhem, Netherlands) equipped with a CMEX 5000 camera system (40$\times$ magnification). The intensity of immunostaining was evaluated. The findings were classified as no reactive cells (without labelling), low positive cells, medium positive cells, and strong positive cells. Only the strong positive cells were considered for quantification, as previously reported [19].

2.10 Fluorescence

The SM localization was studied by using the SM probe, enhanced green fluorescent protein-nontoxic-lysenin (EGFP-NT-Lys). The probe was purified from

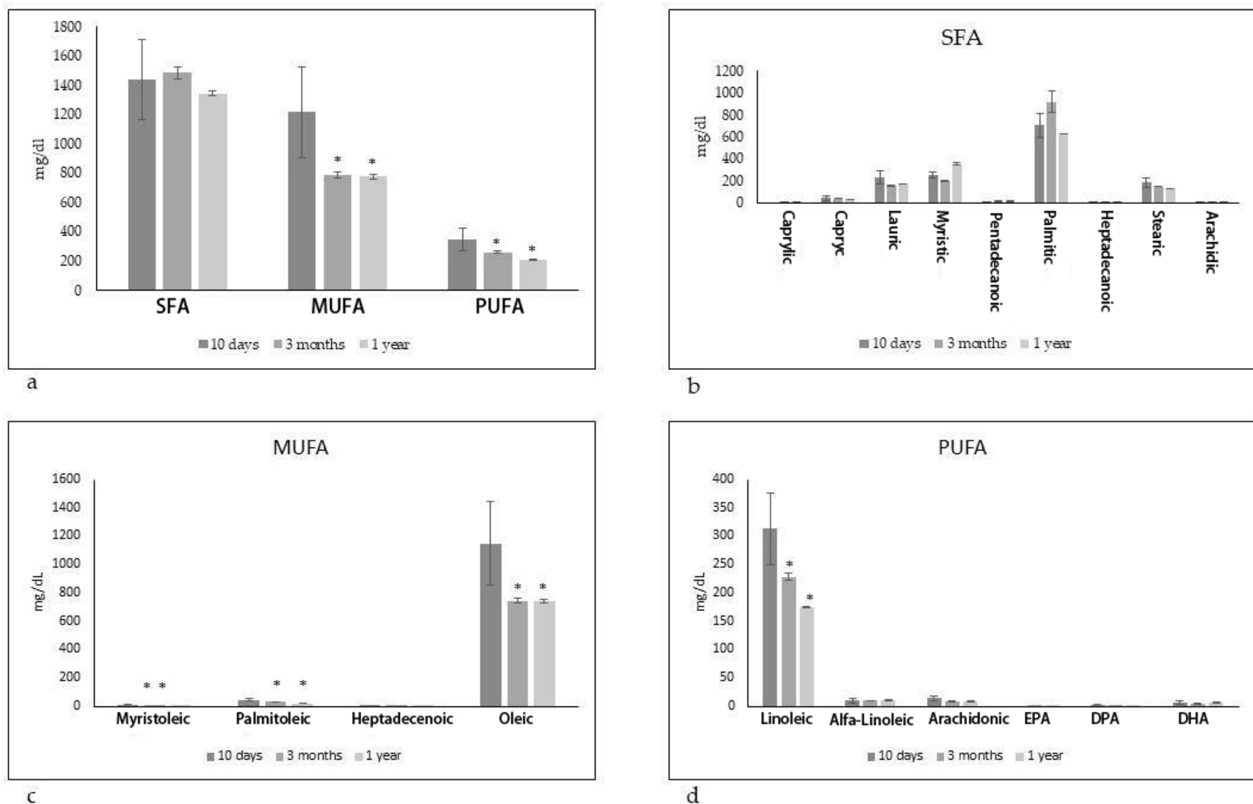

Fig. 1. Fatty acid composition in human breast milk of mothers fed on Mediterranean diet at different times of breastfeeding. SFA, saturated fatty acids, MUFA, monounsaturated fatty acids and PUFA polyunsaturated fatty acids. (a) Total lipids. (b) SFA species. (c) MUFA species. (d) PUFA species. Data are expressed as mean ± SD calculated as reported in "Statistical analysis". Significance of 3 months and 1 year versus 10 days, * $p < 0.01$.

E.coli strain BL21(DE3) harboring pET28/EGFP-NT-Lys according to Tomishige *et al*. [20], with a little modification. In brief, after bacteria culture reaches OD_{600} = approx. 1, the expression of EGFP-NT-Lys was induced at 18 °C for two overnights in the presence of 125 μM IPTG (VWR life science). EGFP-NT-Lys was purified by Hitrap TALON crude column (Cytiva, Munzinger Str. 5, 79111 Freiburg im Breisgau, Germania) from bacteria lysate using its His-tag. Imidazole used in the elution of the protein from the column was removed by dialysis. The dialyzed protein was mixed with glycerol (VWR chemicals) at 50% (v/v) and stored at –20 °C. On the day of the experiment, the medium was removed, cells were washed with DMEM/5% lipid depleted serum (LPDS) and treated with 15 μg/mL EGFP-NT-Lys diluted in DMEM/5% LPDS for 30, 60 and 120 min, Then, cells were fixed with 250 μL of 4% paraformaldehyde (PFA) at room temperature (RT) for 30 min. After washing, the residual PFA was neutralized by 0.1 M NH4Cl at RT for 15 min. Cells were washed three times with 500 μL of PBS and nuclei counterstained with DAPI. Coverslips were mounted and cells viewed in a DMRB Leica epimicroscope equipped with a digital camera.

2.11 Electrophoresis and Western Blot Analysis

Cells were cultured for 24 h in the absence or presence of 1.0 μM SM. The analysis of nSMase protein expression was performed as previously reported [18]. Briefly, sixty micrograms of protein were submitted to SDS–PAGE electrophoresis in 10% polyacrylamide slab gel. For electrophoresis image analysis, the gel was stained with Coomassie blue. The transfer of protein was carried out onto nitrocellulose in 90 min. The membranes were blocked for 60 min with 5% nonfat dry milk in PBS (pH 7.4) and incubated overnight at 4 °C with anti nSMase specific antibody. Anti-βtubulin antibody was used to normalize the data. Anti-nSMase, anti-βtubulin, goat anti-rabbit secondary antibodies were from Abcam (Cambridge, UK). The blots were treated with horseradish-conjugated secondary antibodies for 60 min. Visualization was performed with the enhanced chemiluminescence kit by Chemidoc Imagequant LAS500 – Ge Healthcare-Life Science (Milano, Italy). Densitometric analysis was performed by specific IQ program. Chemiluminescence kits were purchased from Amersham (Rainham, Essex, UK).

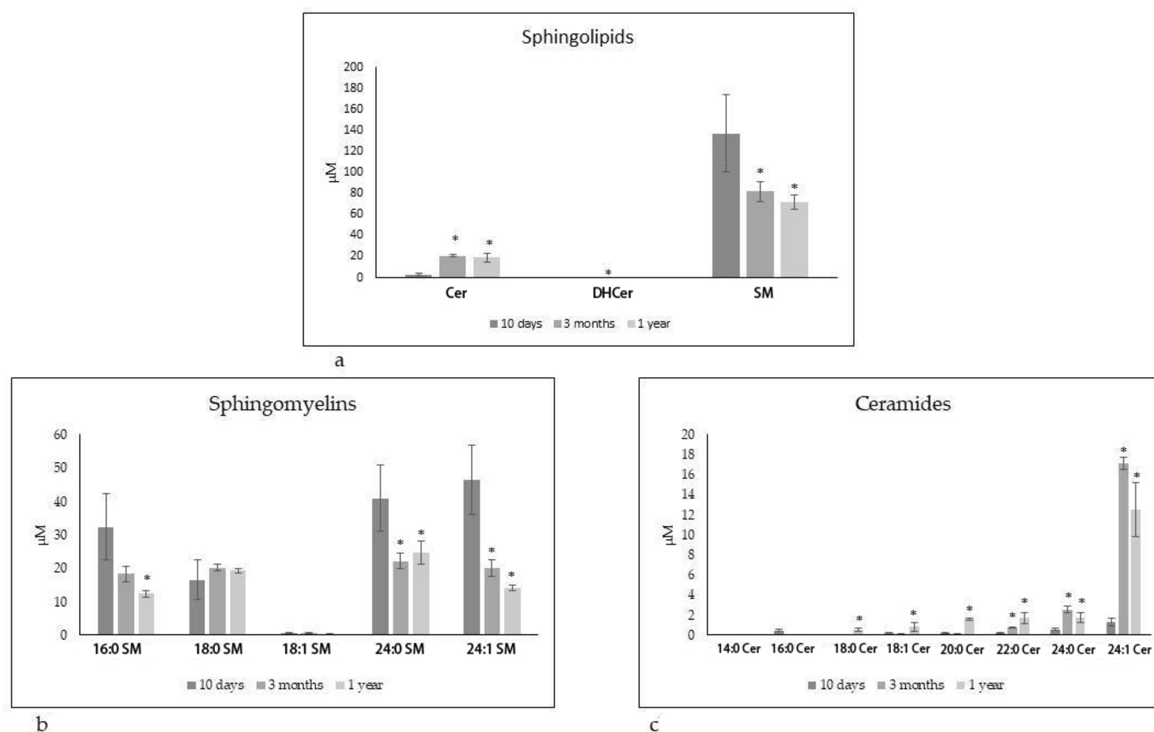

Fig. 2. UFLC MS/MS analysis of human breast milk. Sphingolipid composition at different times of breastfeeding. (a) Total sphingolipid family. (b) Sphingomyelin species. (c) Ceramide species. Data are expressed as μM concentration and represent the mean $\pm$ SD. Cer, ceramide; SM, sphingomyelin; DHCer, dihydroceramide. Significance of 3 months and 1 year versus 10 days, * $p < 0.01$.

2.12 Reverse Transcription Quantitative PCR (RTqPCR)

HN9.10e were cultured for 24 h for RTqPCR analysis. Total RNA was extracted from control and SM-treated cells by using RNAqueous®-4PCR kit (Ambion, Inc., Austin, TX, United States). Samples were treated and RTqPCR was performed to study the gene expression SM phosphodiesterase 4 (*SMPD4*, Hs04187047_g1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1) and 18S rRNA (*S18*, Hs99999901_s1) were used as housekeeping genes. mRNA relative expression levels were calculated as $2^{-\Delta\Delta Ct}$ by comparing the results of SM-treated sample with those of untreated samples as previously reported [19].

2.13 Statistical Analysis

Three experiments in duplicate where performed for each analysis. Graphs were represented as mean $\pm$ SD. Statistical differences were investigated by either unpaired t test or one-way ANOVA coupled with Bonferroni post hoc test, in the case of more than two experimental groups and were set as $*p < 0.01$.

3. Results

3.1 Human Milk Lipids Change during Breastfeeding Time

In previous studies, we showed that the maternal diet changed fatty acid (FA) composition and did not alter Sph composition of HBM at 10 days of breastfeeding [4,7].

Given the undeniable positive effects of the Mediterranean diet for the properties of breast milk, we examined the variations in the composition of fatty acids in women who followed the Mediterranean diet throughout the breastfeeding period. Therefore, milk donor mothers from BLUD of Perugia hospital who had followed a Mediterranean diet were chosen for the study. The criteria of inclusion and exclusion were above reported. Mothers of different ages and with different weights in early pregnancy and with different weight gain during pregnancy were considered. The milk of the same mothers was analyzed at 10 days, 3 months and 1 year of breastfeeding. As shown in Fig. 1a,b, total and each saturated FA (SFA) did not change in time. Differently, both monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) reduced at 3 months and even more a 1 year. Of note, among MUFA myristoleic, palmitoleic and oleic acids and among PUFA oleic acid significantly reduced (Fig. 1c,d).

Given the above results it was interesting to analyze the Sph composition in the same HBM samples. Fig. 2a shows total SM or dihydroceramides (DHCer) or Cer species. It is evident that a longer breastfeeding time induced a lower SM level and a higher Cer level. Furthermore, the value of DHCer was 0.18 + 0.07 μM at 10 days and undetectable at 3 months and 1 year. The reduction was an index of slowing down of SM synthesis in the mammary

gland. Accordingly, when you consider each Sph species, you can see a reduction of SM species (Fig. 2b) and an increase of Cer species (Fig. 2c). If we compared women with weight gain during pregnancy between 9 and 12 kg and those with weight gain greater than 12 kg, the results were very similar. Therefore, milk samples from all women were considered regardless of weight.

3.2 Effects of Sphingomyelin in Embryonic Hippocampal Cell Differentiation

To understand the significance of the results obtained, we hypothesized the relationship between SM and the maturation of nerve cells. To test this hypothesis, we investigated the contribution of SM on the HN9.10 embryonic hippocampal cell differentiation. We first set out to study the dose-dependent effect of SM on HN9.10 cell viability after 24 h of culture by using MTT assay and using 1% and 2% DMSO were used as positive controls. Considering the value of SM in 3 months milk (about 80 μmol/L), its bioavailability and its passage into the interstitial fluid, it was estimated to evaluate a range of concentration from 0.5 μmol/L to 25 μmol/L (from 0.6% to 31% of 3 months milk)

The results demonstrated that SM from 0.5 μmol/L to 25 μmol/L concentration did not induce damage in the cells, indicating that this lipid has no toxic effect at all considered concentrations (Fig. 3).

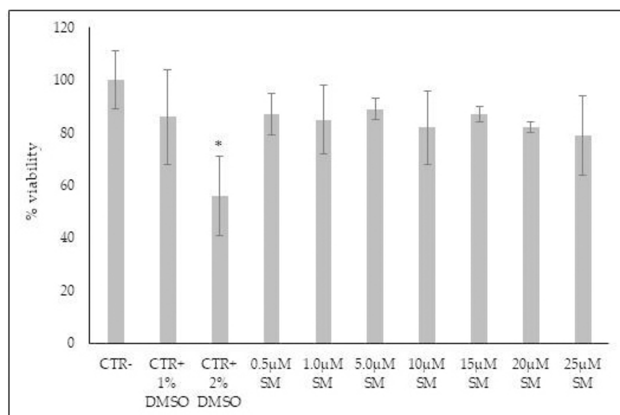

Fig. 3. The effect of sphingomyelin on HN9.10 cell viability. Cells were cultured with increasing doses of SM prepared by using absolute ethanol as vehicle and the viability was measured by MTT assay. Values were reported as percentage viability of the control sample prepared by using only absolute ethanol prepared as reported in 3.6. (CTR-dark gray) or without absolute ethanol (CTR-light gray). 1% DMSO and 2% DMSO were used as positive controls. Data were expressed as mean ± SD. Significance versus the control sample, * $p < 0.01$.

Therefore, we sought to see the effect of each SM concentration in the morphological changes of HN9.10, knowing from our previous studies that the differentiation of these embryonic hippocampal cells was demonstrable with change of the soma characteristics and with the appearance of neurites [19,21]. In order to accomplish this goal, a series of experiments of cell culture for 72 h with increasing doses of SM was performed to determine the minimally sufficient SM dose necessary for HN9.10 differentiation. As shown in Fig. 4a, the control sample was characterized by a very low percentage of cells with neurites that appeared short (Fig. 4c,d). 0.5 μM SM induced a modification of the soma in a large number of cells but the number and length of neurites was very similar to the control. Differently, the number and length of neurites was significantly increased already with 1.0 μM and the values remained high at all used concentration (Fig. 4a,c,d). Numerous cells treated with 1.0 μM had particularly long neurites (Fig. 4b). Thus, 1.0 μM SM was used for all following experiments. Neurite growth requires the expression of neurofilament heavy protein NF200 [19]. To examine whether SM induced expression of this protein, we performed an immunocytochemistry study by using a specific antibody. As demonstrated in Fig. 5, the strong positive cells were 2.2 times more numerous under SM treatment than control cells.

This result led us to hypothesize that SM could enter the cell and reach the nucleus where it regulated gene transcription, supporting our previous studies [15,16]. In order to verify this hypothesis, we used for the first time a specific SM probe (EGFP-NT-Lys) to ascertain the SM localization inside the cells. To monitor the probe incorporation, the cells were counterstained with DAPI (4',6-diamidin-2-fenilindol) to label the nuclei and the samples were analyzed at progressive times. Interestingly, the fluorescent probe was located after 30 min away from the nucleus, then in the perinuclear area and finally inside the nucleus (Fig. 6). To test whether the entrance of SM into the nucleus might be responsible for the regulation of gene expression and consequently of protein synthesis of enzymes responsible for its metabolism, RT-PCR and western blotting analysis were performed. In that contest, SM upregulated *SMPD4* gene encoding for nSMase (Fig. 7a). Accordingly, nSMase protein was overexpressed (Fig. 7b,c).

4. Discussion

Based on previous studies, we showed that lipid composition of HBM at 10 days of breastfeeding was dependent on the maternal diet [4]. Of note, a maternal diet rich in proteins or in vegetables was responsible for the production of a milk with a FA composition different from a milk of mothers who had followed a Mediterranean diet. Based on our biochemical analysis, we showed a reduction of MUFA and PUFA in HBM after 3 months of breastfeeding maintaining constant the Mediterranean diet in mothers. Interestingly, the mothers followed the same type of diet in all the periods considered, including the lipid content. This is relevant considering that generally the maternal intake of carbohy-

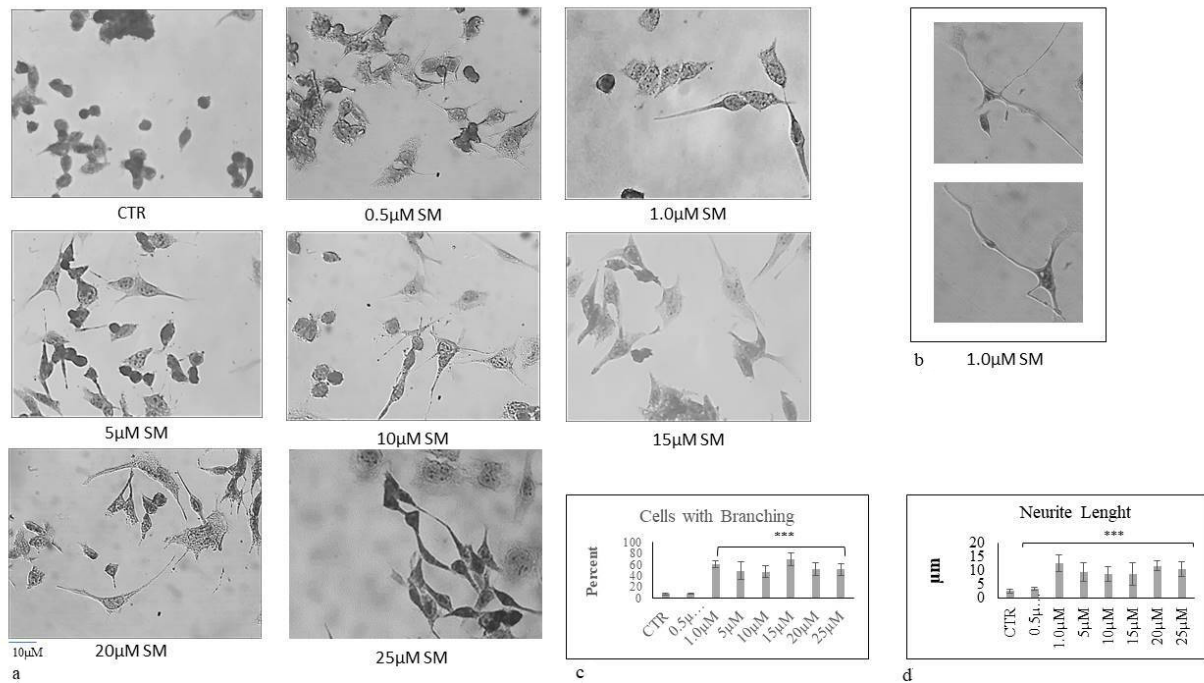

Fig. 4. The effect of sphingomyelin on HN9.10 cell morphology. Cells were cultured with increasing doses of SM and the morphologic analysis was performed as reported in the "Materials and Methods". (a,b) The observations were performed by using inverted microscopy EUROMEX FE 2935 (ED Amhem, Netherlands) equipped with a CMEX 5000 camera system (40× magnification). (c,d) The morphometric analysis was performed by using ImageFocus software. Data were expressed as mean ± SD. Significance versus the control sample, * $p < 0.01$.

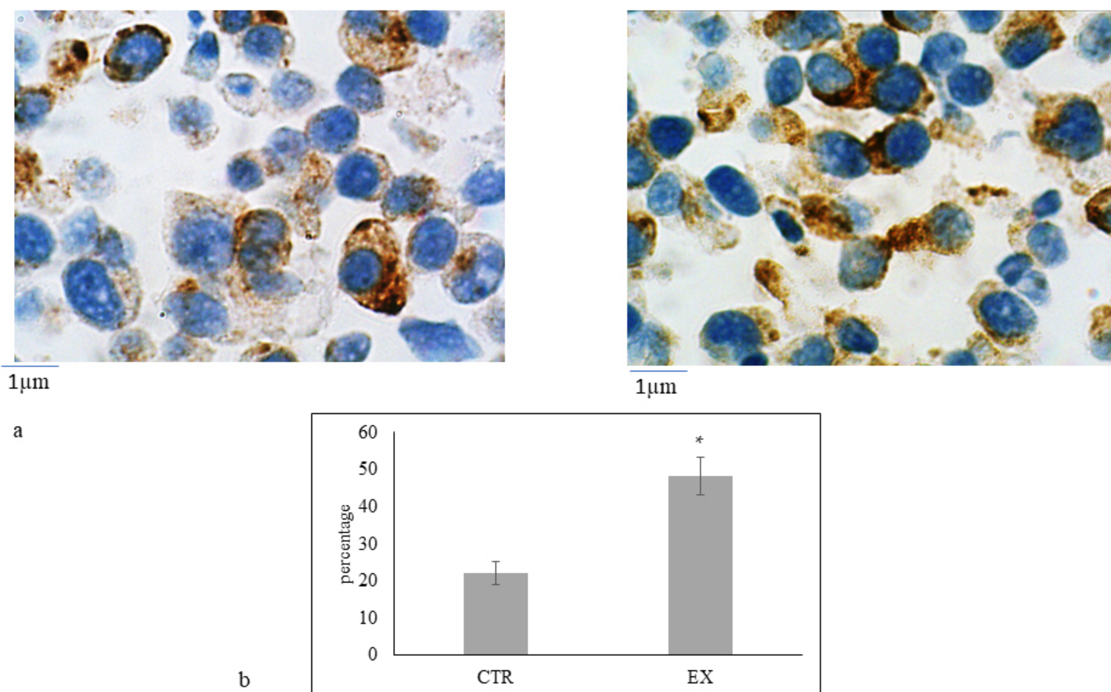

Fig. 5. Immunocytochemical analysis of Neurofilament 200 kDa (NF200). (a) On the left control sample and, on the right experimental sample (HN9.10 treated with 1.0 μM sphingomyelin. (b) Percentage of total cells highly stained (positive cells). Data were expressed as mean ± SD. Significance versus the control sample, * $p < 0.01$.

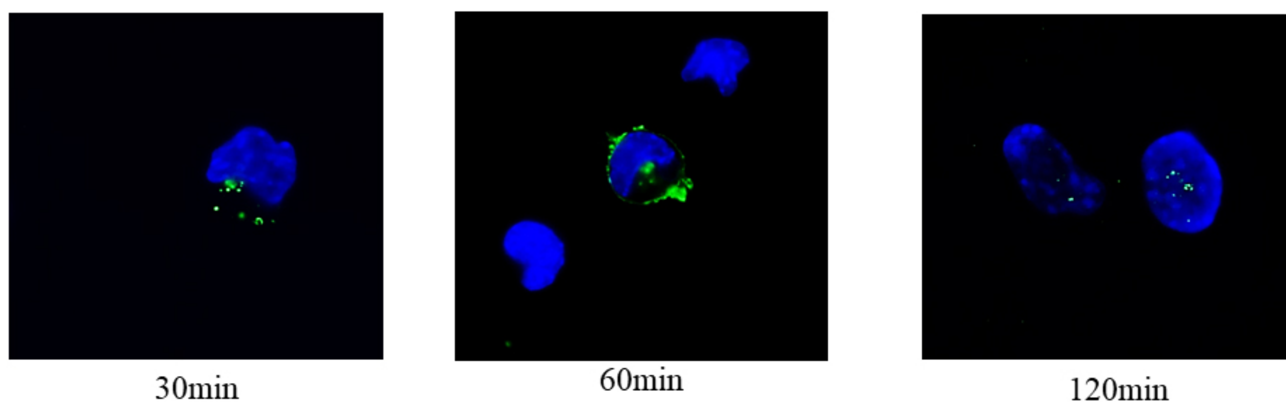

Fig. 6. Localization of sphingomyelin in time with fluorescent probe EGFP-NT-Lys.

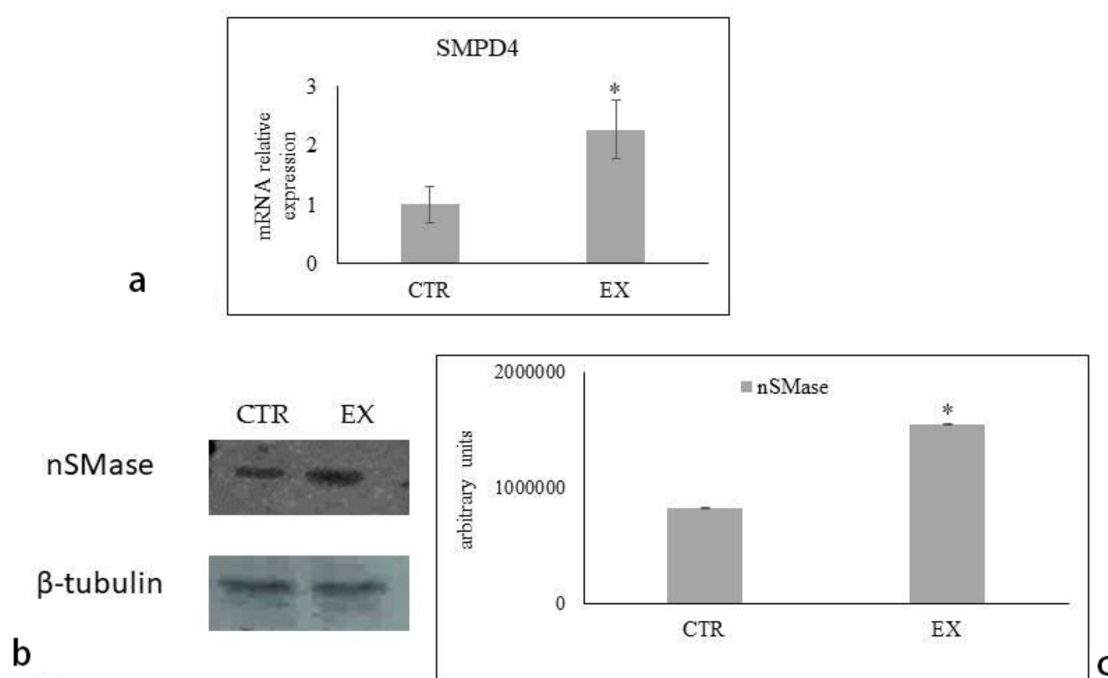

Fig. 7. Effect of sphingomyelin on gene and protein expression. (a) *SMPD4* gene expression analyzed by RT-PCR. (b) Immunoblotting of nSMase protein. (c) Quantification of area density by Chemidoc Imagequant LAS500 by specific IQ programm. Data were expressed as mean ± SD. Significance versus the control sample, * $p < 0.01$.

drates and fatty acids (in quantity and quality) influences the FA composition of HBM. Thus, our data suggest a change of lipid metabolic pathway in mammary gland in time.

This question that consider of mammary gland as a metabolic actor for the production of a milk in which some components are independent of the maternal diet is important. Notably, we have previously demonstrated that a maternal diet rich in proteins or in vegetables or a Mediterranean diet did not influence the Sph composition of the milk [7]. Moreover, the Sph level in HBM was double that found in cow's milk [7].

Taken together, these results raise the possibility that Sphs of HBM was specie-specific and might be dependent on the human mammary gland metabolism. In this regard, considering that Sphs are the most abundant lipids in the brain and that they are essential for brain health [22], Sphs of HBM might have important roles for the newborn development. Recently, implication of SM beneficial effects in the neuronal development in infants was reported [23]. It is know that the maturation process of the brain continues at least until the third year of life [24]. Of note, the hippocampus, which is crucial for mediating spatial memory begins its rapid growth phase at approximately 32 weeks gestation, continuing for at least the first 18 postnatal months [25,26]. In this study, we identified a reduction of SM and an increase of Cer in HBM during the progression of breastfeed-

ing. At present it is difficult to say with certainty that there is an adaptation of the content of HBM SMs to the development of the breastfed infant's brain and the maturation of nerve cells. However, given the findings of this study and published work, the Sph composition in HBM should be always considered thereby providing a possible and plausible hypothesis that high level of SM might be essential at 0–3 year of life for the maturation of nerve cells. Therefore, an integration with SM might be useful during breastfeeding beyond 3 months. Consistent with this theory, we studied the effect of SM in embryonic hippocampal cell differentiation. The results demonstrated that SM treatment induced a change of the soma and the appearance of neurites. To highlight the molecular mechanism of the neurite growth, we demonstrated that SM induced NF200 overexpression.

Importantly, we used a specific probe to investigate whether SM was able to enter the nucleus of HN9.10 cells where it could act regulating gene expression and transcription [15]. Due to its lipophilic nature, it is possible that SM enters the nucleus without a specific transporter as a free molecule or in the form of vesicles but there are no data on this in literature yet. The positive results was not surprising; indeed, there is considerable evidence linking SM to the nuclear activity in nerve cell physiopathology, reviewed by Garcia-Gil and Albi E [16]. However, to our knowledge, this is the first study to describe the entry of SM into the nucleus with a fluorescent probe (EGFP-NT-Lys), notably in HN9.10 cells.

Thus, we hypothesized that SM, reached the nucleus, was able to influence gene expression. Interestingly, we found that SM was able to upregulate *SMPD4* gene encoding for nSMase, an enzyme essential for its own metabolism, supporting previous studies showing how SM metabolism is fine regulated inside the nucleus [14]. Our data suggest that nSMase is regulated by SM, indicating a nutrigenomic effect of SM. Together, these findings have suggested that SM might contribute to HN9.10 cell differentiation. Although the underlying reason for the reduction of SM in HBM in time is unknown, we speculate that nSMase may increase in mammary gland in time. Further studies are underway to study this point.

We can speculate that the high level of SM in HBM in the first period of breastfeeding might be useful in part to enter by 2 h into cell nucleus to stimulate nuclear activity and in part to form myelin sheet after 72 h, when neurite are formed. Further studies will clarify the role of SM in other nerve cells.

5. Conclusions

In summary, this study demonstrates reduction of SM in HBM after 3 months of breastfeeding. *In vitro* system of embryonic hippocampal cells, SM is able first to enter into the nucleus overexpressing the gene for nSMase useful for its own metabolism and then to induce cell differentiation characterized by change of the soma and appearance of neurites. The results implicates the SM as important mediator of neural cell differentiation. As brain development occurs at 0–3 years, these data open a new avenue of potential intervention to integrate the infant formulas with SM to obtain a product similar to the maternal milk.

Abbreviations

Cer, ceramide; nCerase, ceramidase, neutral ceramidase; SM, sphingomyelin; nSMase, neutral sphingomyelinase; RTqPCR, reverse transcription quantitative PCR; SMPD4, sphingomyelin phosphodiesterase 4; SphK2, sphingosine kinase 2.

Author Contributions

The conception and design of the study (SC and EA), experiments (CA, TK, NT, MDC, RP, PS, L Co, ST, CG, MRC, AM) acquisition of data (CA, RP, PS), analysis of data (CA, AM, EA, L Ce), interpretation of data (SC, EA, TB, MGG), drafting the article or revising it critically for important intellectual content (SC, EA), final approval of the version to be submitted (SC, EA and TB). All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The Bioethics Committee of Perugia University has approved the study (n.2018-05), all procedures were performed accordingly. Women were included on a voluntary basis, signed the informed consent and were anonymized.

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Conflict of Interest

The authors declare no conflict of interest. SC is serving as one of the Editorial Board member of this journal. We declare that SC had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to TH.

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