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## Effects of new lipid ingredients during pregnancy and lactation on rat offspring brain gene expression†

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Maternal dietary fat intake during pregnancy and lactation may influence the bioavailability of essential lipophilic nutrients, such as docosahexaenoic acid (DHA), that are important for both the mother and her child's development. This study aimed to evaluate the effects of different maternal fat diets on fat absorption and pup brain development by analyzing gene expression. Rats were fed diets with different lipid matrices during pregnancy and lactation: diet A, mono and diglycerides (MDG) + soy lecithin phospholipids (PL); diet B, MDG + soy lecithin PL + milk-derived PL; and a control diet. All diets contained the same amount of DHA. We determined maternal dietary fat absorption, as well as the offspring fatty acid (FA) profile in both plasma and brain samples at birth and in pups at 14 days post-natal. In addition, microarray analysis was performed to characterize the pup brain gene expression. Maternal dietary fat and DHA apparent absorption was enhanced only with diet B. However, we observed higher plasma DHA and total FA concentrations in lactating pups from the experimental groups A and B compared to the control. Both brain DHA and total FA concentrations were also higher in fetuses and 14-day-old pups from group A with respect to the control, with diet B following the same trend. Offspring brain gene expression was affected by both diets A and B, with changes observed in synaptic and developmental processes in the fetuses, and the detoxification process in 14-day-old pups. Incorporating MDG and PL-rich lipid matrices into maternal diets during pregnancy and lactation may be highly beneficial for ensuring proper neurodevelopment of the fetus and newborn.

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## Introduction

The human brain is a lipid-rich organ<sup>1</sup> that undergoes significant growth from the 12th week of gestation through the first 3 years of life.<sup>2</sup> During this period, polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA, 22 : 6 omega-3), accumulate in the infant's brain.<sup>3</sup> This fatty acid (FA) can be synthesized in the liver and brain, among other tissues, from alpha-linolenic acid (ALA, C18:3 omega-3) through desaturation (desaturases delta-5 and delta-6) and elongation (elongases 2 and 5).<sup>4</sup> Although this conversion is a relevant bio-

chemical process for the organism, it can be limited by various factors, such as hepatic steatosis or oxidative stress.<sup>5</sup> In addition, during the fetal stage, this conversion is not sufficient to ensure adequate fetal DHA accretion, making the maternal contributions during gestation and lactation crucial.<sup>3</sup> Currently, many women in Western countries do not meet the recommended dietary intake of omega-3 FA,<sup>6</sup> requiring nutritional supplements to ensure adequate fetal supply. In this context, any dietary strategy that enhances bioavailability of DHA during pregnancy would be highly beneficial.

It is known that the bioavailability of lipophilic nutrients in the gastrointestinal tract can be modified by their emulsion structure.<sup>7</sup> The addition of specific lipid species to the maternal diet may improve fat absorption in the small intestine due to their emulsifying properties.<sup>7-10</sup> Mono and diglycerides (MDG) are major intermediates in intestinal fat uptake, and together with phospholipids (PL), they are known to promote the formation of smaller fat droplets that are more readily accessible to digestive enzymes.<sup>9,11</sup> Thus, it is interesting to understand their roles in the absorption of compounds that are important for neurodevelopment, such as DHA.

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DHA is the major structural component of the brain and is mostly concentrated at the sn-2 position of PL in cell membranes, playing a key role in neurogenesis and synaptogenesis.<sup>12</sup> High plasma levels of DHA in the mother and breast milk have been associated with better growth and development of the brain and visual system in children.<sup>13</sup> Greater intestinal bioavailability of PL and DHA during pregnancy and lactation could improve the cognitive development of the fetus/newborn. Certain PL, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM), play critical roles in the nervous system.<sup>14–16</sup> Their availability during development is essential for the membrane structure and function of nerve cells.<sup>17,18</sup> In fact, the milk fat globule membrane (MFGM), rich in these PL, has been associated with better cognitive development in children,<sup>19,20</sup> and its inclusion in formula-fed infants is relevant.<sup>21</sup>

The main objective of this study was to determine if adding specific lipids, such as MDG and PL, to the maternal diet may improve fat absorption and DHA content during gestation and lactation. Additionally, microarray analyses were conducted in the offspring brain to identify potential benefits of these lipid matrices on neurodevelopment.

## Materials and methods

### Animals and study design

68 female adult Sprague–Dawley rats (8 weeks of age) were supplied by the Animal Laboratory Service of the University of Murcia. Animals were housed in groups (4 animals per cage) with *ad libitum* access to food and water in a humidity and temperature-controlled ( $22 \pm 1$  °C) room under a 12 h light/dark cycle. Animal weight was recorded every week throughout the experiment. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Murcia (Murcia, Spain) (A13221102, December 14, 2021) and conformed to the ARRIVE guidelines for animal research.<sup>22</sup> Animals received humane treatment in accordance with the European Union guidelines for the care and use of laboratory animals.

Female rats were randomized into three groups that received different experimental diets during the study: a control diet ( $n = 22$ ), without lipid matrices; diet A ( $n = 22$ ), with MDG and soy lecithin PL; and diet B ( $n = 24$ ), with the same composition as diet A plus an extra source of PL (whey protein PL concentrated, WPPC). The compositions of MDG (Radiamuls MG 2152K, Oleon, Belgium) and WPPC (ISO Chill® 6000, Agropur, Minnesota, USA) are included in Table 1. All diets conformed to the standardized rodent AIN-93G diet requirements of vitamins and minerals,<sup>23</sup> and contained the same amount of DHA in the form of triglycerides (Table 1).

After a one-week period of adaptation to the cage and the diets, rats were placed in individual metabolic cages for four days for an apparent fat digestibility assay of the diets. This

**Table 1** Composition of the experimental diets

Diet	Control (g kg <sup>-1</sup> diet)	A (g kg <sup>-1</sup> diet)	B (g kg <sup>-1</sup> diet)
Corn starch	317.97	317.97	317.97
Maltodextrin	100.41	100.01	85.04
Sucrose	91.60	91.57	93.06
Lactose	0.31	0.31	2.96
Glucose	2.79	2.78	2.36
Casein	175.02	175.02	150.71
Soy oil	65.37	62.10	51.66
DHA	1.00	1.00	1.00
MDG	0	2.90	2.90
Soy lecithin	0	0.60	0.60
WPPC	0	0	40
Fiber	45.80	45.80	45.80
Choline	1.27	1.29	1.29
L-Cystine	3.48	3.48	3.92
Mineral mix	32.80	32.80	32.80
Vitamin mix	9.40	9.40	9.40

DHA, docosahexaenoic acid; MDG, mono and diglycerides; PL, phospholipids; WPPC, whey protein phospholipid concentrated. MDG is a commercial oil with 42% monoglycerides, 33% diglycerides, and 25% triglycerides. WPPC composition per gram of product: 22.18 mg of phosphatidylcholine, 18.83 mg of phosphatidylethanolamine, 18.28 mg of sphingomyelin, 8.07 mg of phosphatidylserine, and 3.32 mg of phosphatidylinositol.

allowed measurement of diet intake and fecal collection prior to pregnancy to study dietary fat absorption. Then, male rats were placed into the female cages for mating (1 : 2) and, once mating had taken place (indicated by sperm presence in a vaginal smear under a microscope), they were removed. Female pregnant rats were allocated to appropriate cages (4 animals per cage) and fed their assigned test diets throughout the pregnancy period.

At days 20–21 of gestation, before delivery, some pregnant rats in each group (17 control, 17 group A, and 19 group B) were sacrificed, and samples from both mothers and fetuses were taken. The remainder ( $n = 5$  per group) were allowed to give birth to their pups in individual cages (4 offspring per mother, 2 males and 2 females), where they were maintained until day 14 of life, when all the remaining animals were sacrificed.

### Sample collection

At days 20–21 of gestation, animals were first anesthetized by inhalation of 5% isoflurane, followed by an intramuscular injection of 5 mg ketamine hydrochloride and 0.2 mg xylazine hydrochloride per 100 g animal weight. Maternal blood was extracted by heart puncture using EDTA-coated tubes. After separation of the fetuses, female rats were killed by an intracardiac injection of 20 mg of sodium pentobarbital per 100 g of animal weight. In the case of the fetuses, they were sacrificed by decapitation, and blood and brain samples were collected. The plasma and brain tissues from all fetuses from the same mother were pooled. All sacrifices were made at 10 am after overnight fasting. At day 14 post-partum, lactating animals were fasted for only 4 hours and anesthetized with the above-mentioned protocol, and blood and brain samples were collected.



Blood samples were centrifuged at 1400g for 10 min at 4 °C to obtain the plasma. Brains were frozen in liquid nitrogen and stored at -80 °C until analysis.

### Dietary fat and DHA absorption

Fat apparent absorption was calculated as the ratio between total fat content in feces and total fat intake. The total fat content in feces was determined gravimetrically; 5 g of lyophilized feces was treated with hydrochloric acid 37.5% (v/v) at 100 °C for 1 h and then total fat was extracted with diethyl ether under reflux (Tecator Soxtec System HT 1043, FOSS, Barcelona, Spain), dried, and weighed.<sup>24</sup> Regarding total fat intake, it was calculated based on the dry weight of the diet consumed during the digestibility assay and its fat percentage. The moisture of the diets was determined gravimetrically after 24 h by heating at 105 ± 1 °C until constant weight (JP 200 SELECTA, Barcelona, Spain).

In addition, in the same way as the fat absorption, we estimated dietary DHA apparent absorption considering DHA intake and DHA in the feces (according to the method given by Folch *et al.*,<sup>25</sup> see the section 'Fatty acid analyses' in the 'Materials and methods').

### Fatty acid analyses

Total lipids were extracted from 200 mg of feces, 100 µL of plasma, and 50 mg of brain tissue into chloroform:methanol (2:1 v/v) according to the method given by Folch *et al.*<sup>25</sup> The feces and brain were first homogenized in chloroform:methanol (2:1 v/v) with butylated hydroxytoluene (BHT) using a metal-blade homogenizer. Prior to extraction, 0.05 mg of pentadecanoic acid was added to the samples as the internal standard. FA methyl esters were produced according to Stoffel *et al.*<sup>26</sup> by adding 1 mL of 3 N methanolic HCl (Supelco, Sigma-Aldrich, Massachusetts, USA) and heating at 90 °C for 1 h. The derivatives were extracted into hexane and stored at -20 °C until gas chromatographic analysis.

FA methyl esters were analyzed by gas chromatography using an SP-2560 capillary column (100 m × 0.25 mm i.d. × 20 µm) (Supelco, Sigma-Aldrich, Massachusetts, USA) in a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, Madrid, Spain) equipped with a flame ionization detector.<sup>27</sup> The temperature of the detector and the injector was 240 °C. The oven temperature was programmed at 175 °C for 30 min, increased at 2 °C min<sup>-1</sup> to 230 °C and held at this temperature for 17 min. Helium was used as the carrier gas at a pressure of 45 psi. Peaks were identified by comparison of their retention times with appropriate FA methyl ester standards (Sigma-Aldrich, Massachusetts, USA) and the FA concentrations were determined in relation to the peak area of the internal standard. The FA data are represented as the concentration (g L<sup>-1</sup> or mg g<sup>-1</sup>) and percentage of total FA (g per 100 g of total FA).

### Microarray analysis

RNA extraction was carried out using 30 mg of brain samples (from fetuses and 14-day-old pups) using the RNeasy Mini Kit

(Qiagen, Hilden, Germany). The amount and quality of the RNA were verified by using a Nanodrop 2000 (Thermo Fisher Scientific, Massachusetts, USA) and a Bioanalyzer 2100 (Agilent Technologies, California, USA). Single-stranded cDNAs (ss-cDNAs) were synthesized from 100 ng of the aforementioned RNA, using the Clariom S Assay Rat (Thermo Fisher Scientific, Massachusetts, USA; 902935). According to the protocol supplied by the manufacturer, 2.3 µg of fragmented and biotinylated ss-cDNA were included in the hybridization mix, using the GeneChip Hybridization, Wash, and Stain Kit (Thermo Fisher Scientific, Massachusetts, USA; 900720) and then hybridized to the Clariom S Array Rat (Thermo Fisher Scientific, Massachusetts, USA; 902935). This type of array is designed to provide extensive coverage of all known well-annotated genes including more than 231800 total probes to define the level of expression of more than 22900 genes of the rat transcriptome.

After scanning, microarray data were processed using the Affymetrix Expression Command Console (Affymetrix; Thermo Fisher Scientific, California, USA). Raw data analysis was then performed using the robust multiarray average (RMA) method which allows background correction, log<sub>2</sub> transformation, and quantile normalization to obtain the individual intensity values for each probe set.

### Statistical analysis

Statistical analysis of animal weight, dietary intake, and FA variables was conducted using the SPSS 28.0 software package (IBM Corp., New York, USA). The normal distribution of continuous variables was checked using the Kolmogorov-Smirnov normality test. Differences between the experimental groups were assessed using one-way ANOVA, followed by a *post hoc* Bonferroni test. The results were expressed as mean ± standard error of the mean (SEM), considering statistical significances at  $P \leq 0.05$ .

Regarding the microarray statistical analysis, we used Partek Genomics Suite and Partek Pathways software (Partek Incorporated, Missouri, USA), and performed a one-way ANOVA test with a restrictive threshold at  $P \leq 0.05$  and |fold-change| ≥ 1.5. The molecular interaction, reaction, and relation networks that showed differentially expressed genes were finally analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the Gene Ontology (GO) classification. These pathways and processes were classified according to their respective enrichment scores.

## Results

### Dietary fat digestibility and fatty acid status

Maternal weight was similar among groups at day 20 of pregnancy and day 14 after delivery (Table 2). Placental weight tended to be higher in group B compared to the control group. Fetal weight and the number of fetuses presented no differences among the groups; however, offspring at 14 days of life



Table 2 Animal weight, dietary intake, and fatty acid status of the plasma and brain

Diet	Control	A	B	P
<b>Mothers</b>	<b>n = 17</b>	<b>n = 17</b>	<b>n = 19</b>	
Diet intake during digestibility assay (g day <sup>-1</sup> ) (dry matter)	11.69 ± 0.34 <sup>a</sup>	11.37 ± 0.33 <sup>a</sup>	13.69 ± 0.90 <sup>b</sup>	0.013
Fat intake during digestibility assay (g)	2.45 ± 0.07 <sup>a</sup>	2.39 ± 0.07 <sup>a</sup>	3.07 ± 0.20 <sup>b</sup>	<0.001
Fat in feces during digestibility assay (mg)	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.384
DHA in feces during digestibility assay (μg g <sup>-1</sup> )	16.73 ± 0.88	17.45 ± 0.73	12.63 ± 0.76	0.076
Maternal weight at delivery – d20 (g)	367.37 ± 7.34	351.38 ± 7.96	359.40 ± 8.45	0.388
Maternal plasma DHA at delivery (%)	9.49 ± 0.30	9.14 ± 0.38	9.02 ± 0.39	0.641
Maternal plasma DHA at delivery (g L <sup>-1</sup> )	0.83 ± 0.05	0.71 ± 0.04	0.68 ± 0.06	0.105
Maternal plasma total FA at delivery (g L <sup>-1</sup> )	8.95 ± 0.75	7.95 ± 0.60	7.62 ± 0.66	0.355
Placental weight (g)	0.45 ± 0.01	0.47 ± 0.01	0.50 ± 0.02	0.052
Maternal weight at d14 (g) (n = 5 per group)	265.30 ± 4.95	258.94 ± 7.11	281.76 ± 11.31	0.171
<b>Fetuses</b>	<b>n = 17</b>	<b>n = 17</b>	<b>n = 19</b>	
Number of fetuses	15.29 ± 0.38	13.88 ± 0.83	12.58 ± 1.23	0.118
Fetal weight at delivery – d20 (g)	3.45 ± 0.16	3.66 ± 0.16	3.88 ± 0.17	0.192
Fetal plasma DHA (%)	10.74 ± 0.27	11.08 ± 0.29	10.18 ± 0.53	0.275
Fetal plasma DHA (g L <sup>-1</sup> )	0.21 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.225
Fetal plasma total FA (g L <sup>-1</sup> )	1.96 ± 0.06	1.80 ± 0.04	1.86 ± 0.04	0.081
Fetal brain DHA (%)	14.10 ± 0.29	14.51 ± 0.19	14.18 ± 0.22	0.441
Fetal brain DHA (mg g <sup>-1</sup> )	1.73 ± 0.06 <sup>a</sup>	1.99 ± 0.05 <sup>b</sup>	1.82 ± 0.06 <sup>ab</sup>	0.023
Fetal brain total FA (mg g <sup>-1</sup> )	12.03 ± 0.23 <sup>a</sup>	13.71 ± 0.29 <sup>b</sup>	12.79 ± 0.39 <sup>ab</sup>	0.010
<b>Offspring (14 days old)</b>	<b>n = 20</b>	<b>n = 20</b>	<b>n = 20</b>	
Offspring sex (male/female)	10/10	10/10	10/10	1.000
Offspring weight at d14 (g)	40.46 ± 0.51 <sup>a</sup>	42.10 ± 0.35 <sup>a</sup>	38.09 ± 0.63 <sup>b</sup>	<0.001
Offspring plasma DHA at d14 (%)	13.32 ± 0.21	12.81 ± 0.19	13.35 ± 0.20	0.105
Offspring plasma total FA at d14 (g L <sup>-1</sup> )	4.48 ± 0.12 <sup>a</sup>	5.60 ± 0.14 <sup>b</sup>	5.17 ± 0.12 <sup>b</sup>	<0.001
Offspring brain DHA at d14 (%)	18.38 ± 0.11 <sup>a</sup>	18.81 ± 0.14 <sup>b</sup>	18.45 ± 0.08 <sup>ab</sup>	0.010
Offspring brain total FA at d14 (mg g <sup>-1</sup> )	22.46 ± 0.89 <sup>a</sup>	25.22 ± 0.43 <sup>b</sup>	23.83 ± 0.40 <sup>ab</sup>	0.023

Data are expressed as mean ± SEM. Data not sharing the same superscript letters indicate statistically significant differences between the groups ( $P \leq 0.05$ ). DHA, docosahexaenoic acid; FA, fatty acid.

were smaller in group B compared to the other two groups (Table 2).

During the digestibility assay of diets before gestation, we found that group B had higher diet intake and fat intake compared to the other groups (Table 2). Nevertheless, the fecal fat content was similar among the groups (Table 2), resulting in higher dietary fat apparent absorption in group B compared to group A and the control group (Fig. 1a). In addition, the feces of mothers from group B tended to show a lower DHA concentration (Table 2) and the estimated dietary DHA apparent absorption was significantly higher in these mothers with respect to the other two groups (Fig. 1b).

No significant differences were observed in the plasma content of DHA and total FA, for either mothers or fetuses at delivery (Table 2). However, lactating offspring from groups A and B presented higher plasma DHA (Fig. 1b) and total FA concentrations (Table 2) compared to the control group. Regarding brain analyses, group A fetuses and lactating offspring presented higher total FA and DHA concentrations, as well as DHA percentage compared to the control group (Table 2 and Fig. 1d). Diet B followed the same line. These results could indicate an improvement in the offspring DHA status by the administered maternal dietary lipid matrices.

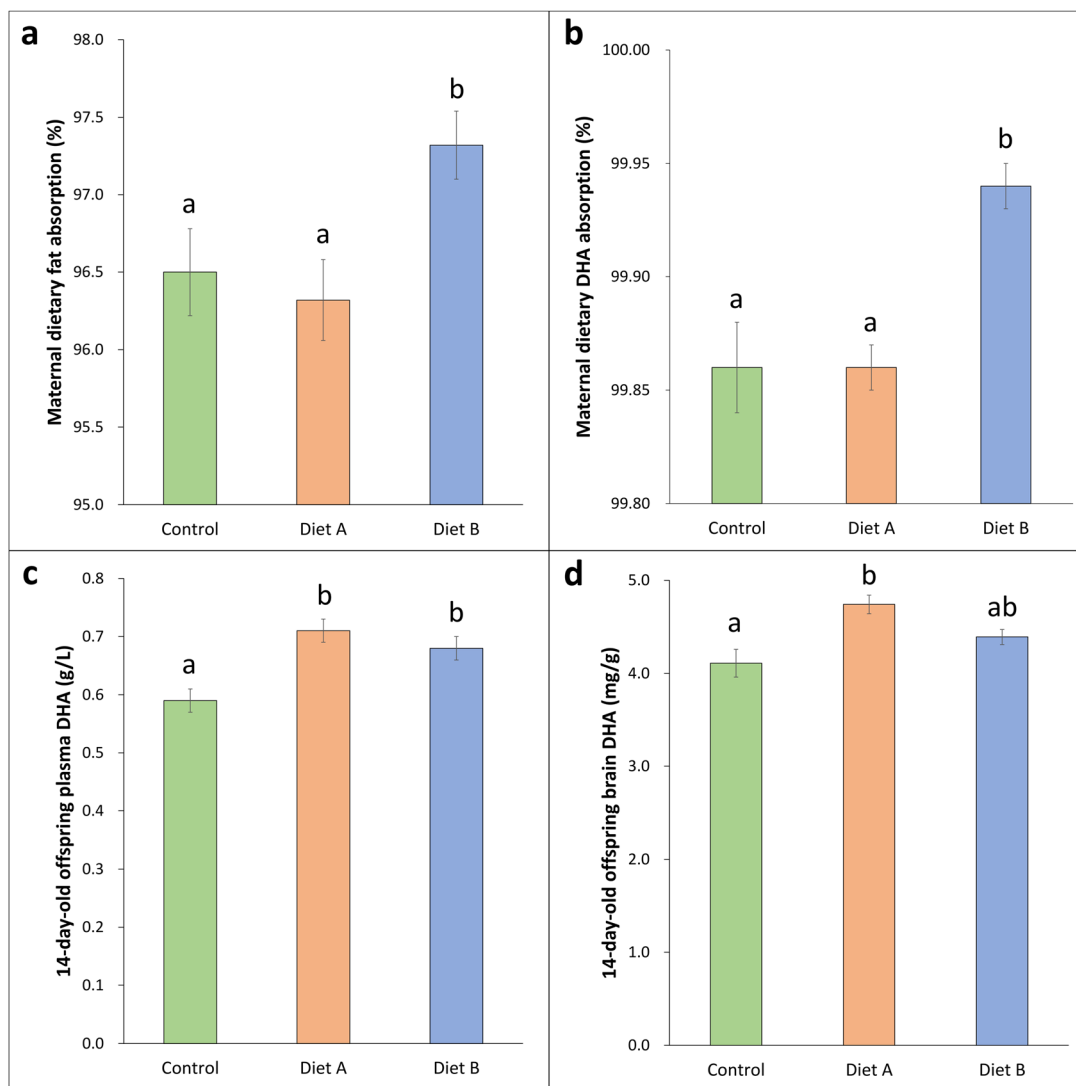
### Gene expression in the fetal brain

Gene expression in the fetal brain was impacted by maternal diet. The shared GO processes altered by both experimental

diets A and B compared to the control were the multicellular organismal process, the developmental process, biological regulation, behavior, and locomotion, among others (Fig. 2a). Diet B had higher enrichment scores than diet A in most of the affected processes (Fig. 2a). All the GO processes impacted in the fetal brain are shown in the ESI (Annex 1†). Concerning KEGG pathways, serotonergic synapse and tryptophan metabolism were the shared pathways affected in groups A and B compared to the control (ESI, Annex 2†).

The fetal brain from group A presented 10 differentially expressed genes compared to the control group: 1 of them was down-regulated (*Dlx6*) and 9 were up-regulated (*Prph*, *Dbh*, *Tph2*, *Hoxb5*, *Hoxc4*, *Lamp5*, *Hoxa2*, *Pax2*, and *Slc6a5*) (Table 3). On the other hand, the fetal brain tissue from group B had 16 differentially expressed genes compared to the control: 12 of them were down-regulated (*Tiam2*, *Satb2*, *Tbr1*, *Neurod6*, *Sla*, *Fezf2*, *Foxg1*, *Neurod2*, *Mpped1*, *Dlx2*, *Chrm1*, and *Zfp238*) and the remaining 4 were up-regulated (*Tph2*, *Pax2*, *Slc6a5*, and *Hoxa2*) (Table 3). These 4 up-regulated genes were the only ones shared by both experimental diets and are related to neurological development: *Tph2* (tryptophan hydroxylase 2) is required for the serotonin biosynthesis, *Pax2* (paired box 2) is involved in the development of the central nervous system, *Slc6a5* (solute carrier family 6, member 5) is related to the maintenance of the presynaptic pool of neurotransmitters, and *Hoxa2* (homeo box A2) is related to the embryonic development of the face.





**Fig. 1** (a) Maternal dietary fat apparent absorption (%); (b) maternal dietary DHA apparent absorption (%); (c) 14-day-old offspring plasma DHA ( $\text{g L}^{-1}$ ); and (d) 14-day-old offspring brain DHA ( $\text{mg g}^{-1}$ ), in animals from the control, diet A, and diet B groups. Data are means  $\pm$  SEM. Different letters indicate statistically significant differences between the experimental groups ( $P \leq 0.05$ ). DHA, docosahexaenoic acid.

### Gene expression in 14-day-old pups' brain samples

At 14 days of age, different sets of genes were impacted by maternal diets compared to what we observed in the fetal brains. The GO processes affected by both maternal diets A and B included detoxification, the multi-organism process, response to stimulus, localization, and the developmental process, among others. Similar to what was observed in the fetal brain, diet B presented a greater effect than diet A on these GO processes, according to higher enrichment scores (Fig. 2b). All the GO processes affected in the 14-day-old pups' brain samples are also shown in the ESI (Annex 3<sup>†</sup>). Regarding KEGG pathways, mineral absorption was the shared pathway affected by both experimental diets A and B compared to the control (ESI, Annex 4<sup>†</sup>).

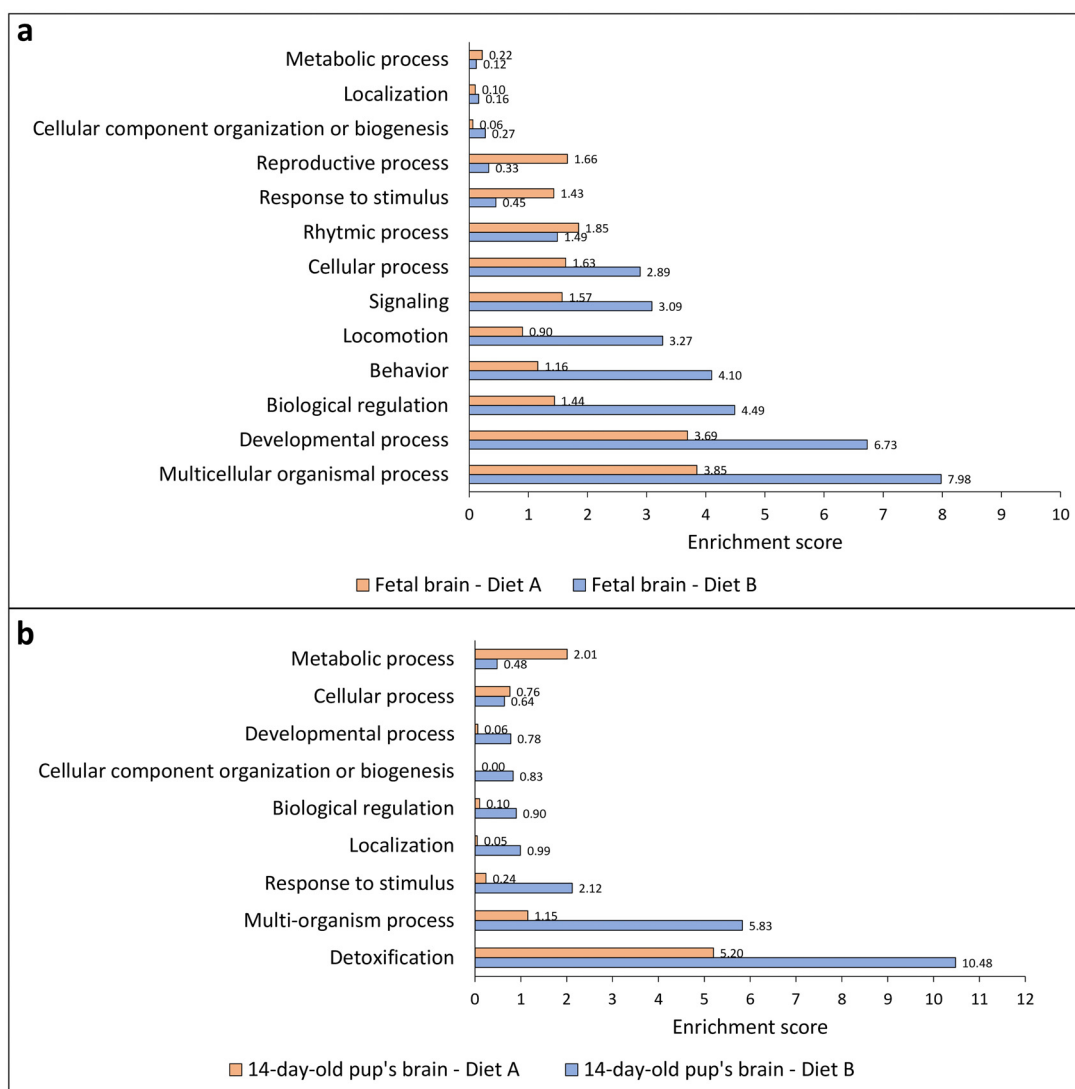
In the pups' brain samples from group A, 13 genes were differentially expressed compared to the control group, all of

them down-regulated (*Mt1M*, *Mt2A*, *Myeov2*, *Slc1a6*, *Ppp1r17*, *Pcp2*, *Rps29*, *Atp5e*, *Nhlh1*, *Cbln1*, *Dbp*, *Mfap4*, and *Ifi27*) (Table 4). In group B, only 2 genes were differentially expressed compared to the control, and both were down-regulated (*Hbaa1* and *Mt2A*) (Table 4). *Mt2A* (metallothionein 2A) was the only gene affected – shared by both diet groups. It is involved in detoxification by regulating the intracellular levels of heavy metals.

### Discussion

In this study, we fed female rats during pregnancy and lactation with diets enriched with different lipid species, all of them containing the same amount of DHA: diet A was fortified with MDG and soy lecithin PL; diet B had the same fortifica-





**Fig. 2** Brain GO processes affected in diets A and B compared to the control, classified by the enrichment score. (a) Fetuses. (b) 14-day-old offspring. GO, gene ontology.

tion as diet A with an extra source of milk PL (WPPC); and the control diet was without these fortifications. Maternal dietary fat and DHA absorption was higher with diet B, although lactating pups from both experimental groups A and B showed higher DHA and total FA concentrations in plasma compared to the control. Regarding the brain results, fetuses and 14-day-old pups from diet A contained higher total fat and DHA content with respect to the control, with diet B following the same trend. Both diets A and B up-regulated the fetal brain expression of genes involved in synaptic and developmental processes – *Tph2*, *Pax2*, *Slc6a5*, and *Hoxa2* – compared to the control. In contrast, *Mt2A*, a detoxification related gene, was down-regulated in both groups compared to the control during lactation. These lipid matrices affected the brain DHA composition in lactating pups, as well as that for its transcriptome.

Dietary fat digestibility was significantly enhanced by diet B compared to diet A and the control diet. The impact of MDG

and PL on intestinal fat absorption has been extensively studied due to their emulsifying capacity.<sup>7–10</sup> In fact, it has been proposed that they could synergistically be employed to improve the intestinal absorption of lipophilic nutrients.<sup>28</sup> In this study, the inclusion of MDG and soy lecithin PL (diet A) was not enough to improve maternal fat apparent absorption. However, the addition of WPPC (similar PL composition to MFGM<sup>29</sup>) as an extra source of PL (diet B) did increase intestinal fat and DHA absorption. Similarly, studies both *in vitro* and *in vivo* have suggested that PL from milk are absorbed more efficiently than those from soybean.<sup>30–32</sup> This is associated with the lower stability of soybean PL liposomes<sup>31</sup> and with their lower production of free FA.<sup>32</sup> All of this suggests that the source of dietary PL is crucial to determine the intestinal absorption efficiency.

Regarding DHA and total FA status in plasma at delivery, no differences were observed in the mothers, and consequently,



**Table 3** Differentially expressed genes in the fetal brains from diets A and B vs. the control

Gene symbol	Encoded protein	Biological function <sup>a</sup>	Gene ID	P	Fold change
<b>Diet A</b>					
<i>Down-regulated</i>					
<i>Dlx6</i>	Distal-less homeo box 6	Forebrain and craniofacial development	ENSRNOT00000014468	0.003	-1.524
<i>Up-regulated</i>					
<i>Prph</i>	Peripherin	Cytoskeletal protein in neurons of the peripheral nervous system	NM_012633	0.032	1.508
<i>Dbh</i>	Dopamine beta-hydroxylase	Conversion of dopamine to norepinephrine	NM_013158	0.004	1.520
<i>Tph2</i>	Tryptophan hydroxylase 2	Biosynthesis of serotonin	NM_173839	0.041	1.576
<i>Hoxb5</i>	Homeo box B5	Developmental regulatory system	NM_001191925	0.007	1.685
<i>Hoxc4</i>	Homeo box C4	Morphogenesis in all multicellular organisms	NM_001109884	0.019	1.829
<i>Lamp5</i>	Lysosomal-associated membrane protein family, member 5	Short-term synaptic plasticity in a subset of GABAergic neurons	NM_001014183	0.004	1.896
<i>Hoxa2</i>	Homeo box A2	Embryonic development	ENSRNOT00000008023	0.005	1.927
<i>Pax2</i>	Paired box 2	Eyes and central nervous system development	NM_001106361	0.018	1.931
<i>Slc6a5</i>	Solute carrier family 6, member 5	Glycinergic synapse	NM_203334	0.018	1.947
<b>Diet B</b>					
<i>Down-regulated</i>					
<i>Tiam2</i>	T-cell lymphoma invasion and metastasis 2	Neural cell development (GDP-GTP exchange activity)	ENSRNOT00000065386	0.012	-1.829
<i>Satb2</i>	SATB homeo box 2	Transcription regulation and chromatin remodeling	NM_001109306	0.029	-1.803
<i>Tbr1</i>	T-box, brain, 1	Cortical development	NM_001191070	0.021	-1.773
<i>Neurod6</i>	Neuronal differentiation 6	Nervous system development and differentiation	NM_001109237	0.014	-1.644
<i>Sla</i>	Src-like adaptor	Cell differentiation	NM_178097	0.024	-1.638
<i>Fezf2</i>	Fez family zinc finger 2	Nervous system development and neuron differentiation	NM_001107251	0.031	-1.602
<i>Foxg1</i>	Forkhead box G1	Establishment of regional subdivision of the developing brain	NM_012560	0.021	-1.586
<i>Neurod2</i>	Neuronal differentiation 2	Neuronal differentiation	NM_019326	0.017	-1.575
<i>Mpped1</i>	Metallophosphoesterase domain containing 1	Predicted to enable hydrolase activity	NM_001130569	0.046	-1.541
<i>Dlx2</i>	Distal-less homeo box 2	Forebrain and craniofacial development	NM_001191746	0.009	-1.533
<i>Chrm1</i>	Cholinergic receptor muscarinic 1	Influences the effects of acetylcholine on the central and peripheral nervous systems	NM_080773	0.048	-1.516
<i>Zfp238</i>	Zinc finger protein 238	Neuronal development	NM_022678	0.016	-1.508
<i>Up-regulated</i>					
<i>Tph2</i>	Tryptophan hydroxylase 2	Biosynthesis of serotonin	NM_173839	0.040	1.534
<i>Pax2</i>	Paired box 2	Eyes and central nervous system development	NM_001106361	0.045	1.686
<i>Slc6a5</i>	Solute carrier family 6, member 5	Glycinergic synapse	NM_203334	0.048	1.716
<i>Hoxa2</i>	Homeo box A2	Embryonic development	NM_012581	0.046	1.790

Only genes with  $P \leq 0.05$  and  $|\text{fold-change}| \geq 1.5$  are listed. <sup>a</sup> Biological functions extracted from GeneCards: the human gene database.

not in the fetuses either. The DHA statuses of the mother and the newborn are highly correlated at the time of birth.<sup>13</sup> Nevertheless, in 14-day-old offspring from groups A and B, we observed a higher concentration of DHA and total FA in the plasma compared to the control. Valenzuela *et al.*, in a study in rat with different sources of DHA supplementation during pregnancy, reported higher maternal tissue DHA accretion and higher milk DHA content after its dietary supplementation as PL to the mother, despite no differences being observed in maternal plasma DHA levels.<sup>33</sup> In fact, DHA is primarily transported in blood lipoproteins in the form of PC.<sup>34</sup> Whether a higher availability of PL structures in maternal tissues might lead to increased DHA secretion and/or milk fat digestibility for the pups needs further investigation. Nevertheless, the higher plasma DHA content observed in 14-day-old pups from

groups A and B in the present study would be highly beneficial, since it has been reported that newborns with a high DHA status exhibit more mature encephalogram patterns and better attention capacity.<sup>35</sup>

The brain samples from both fetuses and pups showed higher fat content and DHA concentration and percentage. These differences were statistically significant in terms of diet A with respect to the control, with diet B following the same line, and this could have affected the brain transcriptome. In fact, in terms of the gene expression in the fetal brain, both diets A and B up-regulated 4 genes related to synaptic and developmental processes (*Tph2*, *Pax2*, *Slc6a5*, and *Hoxa2*) compared to the control. The *Tph2* gene encodes tryptophan hydroxylase 2, which catalyzes the initial and rate limiting steps in serotonin biosynthesis,<sup>36</sup> and its deletion in mice has



**Table 4** Differentially expressed genes in the 14-day-old pup brain samples from diets A and B vs. the control

Gene symbol	Encoded protein	Biological function <sup>a</sup>	Gene ID	P	Fold change
<b>Diet A</b>					
<i>Down-regulated</i>					
<i>Mt1M</i>	Metallothionein 1M	Detoxification	ENSRNOT00000047663	0.001	-1.746
<i>Mt2A</i>	Metallothionein 2A	Detoxification	NM_001137564	0.002	-1.705
<i>Myeov2</i>	Myeloma overexpressed 2	Pseudogene	NM_001109044	0.015	-1.688
<i>Slc1a6</i>	Solute carrier family 1, member 6	Glutamate uptake	NM_032065	0.021	-1.670
<i>Ppp1r17</i>	Protein phosphatase 1, regulatory subunit 17	Protein phosphatase inhibitor in cerebellar Purkinje cells	NM_153467	0.037	-1.669
<i>Pcp2</i>	Purkinje cell protein 2	Catalytic activity in neuronal cell body	NM_001107116	0.009	-1.603
<i>Rps29</i>	Ribosomal protein S29	Component of the small ribosomal subunit	NM_012876	0.017	-1.569
<i>Atp5e</i>	ATP synthase subunit epsilon, mitochondrial	Subunit of mitochondrial ATP synthase	NM_139099	0.021	-1.561
<i>Nhlh1</i>	Nescient helix loop helix 1	Cell-type determination in the developing nervous system	NM_001105970	0.015	-1.553
<i>Cbln1</i>	Cerebellin 1 precursor	Synapse integrity and synaptic plasticity	NM_001109127	0.049	-1.529
<i>Dbp</i>	D site of albumin promoter (albumin D-box) binding protein	Circadian period and sleep regulation	NM_001289982	0.011	-1.517
<i>Mfap4</i>	Microfibrillar-associated protein 4	Extracellular matrix protein	NM_001034124_2	0.028	-1.514
<i>Iffi27</i>	Interferon alpha-inducible protein 27	Cellular protein metabolic process	NM_203410	0.001	-1.514
<b>Diet B</b>					
<i>Down-regulated</i>					
<i>Hba-a1</i>	Hemoglobin alpha adult chain 1	Oxygen transport	NM_001013853	0.010	-1.593
<i>Mt2A</i>	Metallothionein 2A	Detoxification	NM_001137564	0.027	-1.517

Only genes with  $P \leq 0.05$  and  $|\text{fold-change}| \geq 1.5$  are listed. <sup>a</sup> Biological functions extracted from GeneCards: the human gene database.

been linked to delayed growth and persistent thinness.<sup>37</sup> In fact, low levels of serotonin have been associated with autism.<sup>38</sup> The *Pax2* gene encodes a highly conserved transcription factor with an important role in the development of the central nervous system.<sup>39</sup> It is involved in the differentiation of GABA precursor neurons<sup>40</sup> and its deletion in mice has resulted in altered GABA levels and impaired synaptic processes, constituting a potential cause of anxiety-like behaviors.<sup>41,42</sup> The *Slc6a5* gene encodes a Na/Cl-dependent glycine transporter (GlyT2), responsible for glycine reuptake at presynaptic terminals.<sup>43</sup> The activity of GlyT2 is essential for proper motor function, and its alterations have been associated with neuromotor deficiencies such as human hyperekplexia.<sup>44,45</sup> Finally, *Hoxa2* is a highly conserved transcription factor that plays a critical role in the developing brain by regulating the formation and differentiation of neural structures.<sup>46</sup> Taking all this together, our results indicate changes in synaptic and developmental processes in the fetal brains of groups A and B compared to the control. The shared source of PL in both experimental diets was soy lecithin, which mainly contains PC and represents a source of choline. This could favor the synthesis of acetylcholine in the brain,<sup>47</sup> essential for the growing nervous system.<sup>48</sup> In addition, PC is important for neuronal membrane fluidity and interneuronal communication, as it is one of the most abundant PL in neuronal membranes.<sup>17</sup>

The related GO processes modified by diets A and B in the fetal brain compared to the control were development, behavior, and locomotion, among others. However, according to the higher enrichment scores, diet B affected these functions to a greater extent. Diet B contained WPPC as an additional source

of PL like PC, PE, PS, and SM, including others. Early supplementation of these PL has been reported to enhance cognitive development in neonatal piglets.<sup>14</sup> In particular, PE and PS are abundant PL in nerve cell membranes and usually contain DHA esterified in the sn-2 position.<sup>12,49</sup> PS has been reported to support cognitive functions, including memory, learning, concentration, communication, and locomotion.<sup>15</sup> In fact, a decline of PL in the neuronal membrane, particularly PS, has been associated with memory impairment and deficits in mental cognitive abilities.<sup>15</sup> On the other hand, SM plays an important role in myelin integrity and function, as well as in axonal maturation.<sup>50</sup> SM constitutes a relevant lipid during brain development from mid-gestation to the end of the first postnatal year, when central nervous system myelin dramatically increases.<sup>16</sup> In infant formulas, PL prepared from milk are considered to be closer to human milk than soy lecithin because they contain SM.<sup>30</sup> Thus, the supplementation of women's diet during pregnancy and lactation with these lipid matrices may improve both maternal fat and DHA bioavailability, which might influence neurodevelopment and growth during the perinatal period.

Regarding the 14-day-old pup brain gene expression, both experimental diets down-regulated the *Mt2A* gene which encodes metallothionein 2A, a metal-binding protein generally classified as a stress responder.<sup>51</sup> Exposure to heavy metals or oxidative stress causes elevated gene expression of metallothioneins<sup>52</sup> and this could be relevant in the brain since it presents a high level of oxygen consumption and is highly susceptible to oxidative stress.<sup>51</sup> The addition of WPPC in diet B again affected GO processes to a greater extent, particularly detoxification, showing higher enrichment scores than diet



A. Considering all this, *Mt2A* gene down-regulation in the pup brain samples of groups A and B could indicate a lower oxidative stress status in these animals. This is in line with the higher plasma and brain concentrations of DHA observed in the lactating pups of groups A and B, since omega-3 FA, especially eicosapentaenoic acid and DHA, exert antioxidant properties.<sup>53</sup> In addition, despite the limited literature available, some studies in animal models and human neurons cultures suggest a possible antioxidant effect of PC and PS, promoting significant reductions of reactive oxygen species production.<sup>54,55</sup> However, further studies are needed to better understand the possible antioxidant effect of MDG/PL lipid matrices in fetuses/newborns.

The main limitation of our study is that we analyzed the whole brain rather than specific regions. In addition, due to the complexity of the brain's transcriptome and its boundless molecular networks, it is challenging to determine the ultimate biological effect resulting from diets with lipid matrices. However, our study also has some strengths. We evaluated for the first time the whole-genome gene expression in the brains of fetuses and lactating pups after supplementation with different lipid matrices to the mothers.

## Conclusions

In conclusion, maternal supplementation with lipid matrices containing MDG and PL affected offspring brain gene expression, related to processes like synaptic function, development, and detoxification. Increased plasma DHA and total FA concentrations were observed during lactation in 14-day-old pups in the experimental groups receiving MDG/PL supplements. The brain DHA content was also higher in both fetuses and 14-day-old pups. Thus, the inclusion of MDG/PL-rich lipid matrices in maternal supplements during pregnancy and lactation could be of great interest to ensure proper neurodevelopment of fetuses/newborns.

## Author contributions

VO: methodology, formal analysis, and writing – original draft. AG: methodology, formal analysis, and writing – review and editing. MJLP: methodology and writing – review and editing. PBV: dietary preparation and writing – review and editing. MV, JMLP, and BJL: writing – review and editing. MK and JPC: conceptualization and writing – review and editing. EL: conceptualization, resources, funding acquisition, supervision, project administration, and writing – review and editing.

## Data availability

The data that support the findings of this study are not publicly available due to funding agreements. However, the data can be provided under reasonable request.

## Conflicts of interest

PBV, MV, JMLP, BJL, MK, and JPC are employees at Abbott Nutrition S.L. The remaining authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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