Docosahexaenoic acid supplementation during pregnancy as phospholipids or triglycerides produces different placental uptake but similar fetal brain accretion in neonatal piglets

Antonio Gázquez[a], María Ruíz-Palacios[a] and Elvira Larqué[a*]

[a]Department of Physiology, Faculty of Biology, University of Murcia, Murcia, Spain.

*Corresponding author:

Dr. Elvira Larqué.

Department of Physiology. Faculty of Biology, University of Murcia.

Campus de Espinardo, 30100 Murcia, Spain.

E-mail: elvirada@um.es. Phone: +34-868-884239. Fax: +34-868-883963.

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Abstract

The great variety of n-3 long chain polyunsaturated fatty acid sources raises the question of the most adequate for using as a docosahexaenoic acid (DHA) supplement during pregnancy. Placental and fetal availability of different DHA sources remains unclear. We investigated DHA availability in maternal lipoproteins, placenta and fetal tissues in pregnant sows fed DHA as phospholipid (PL) or triglyceride (TG) to identify the best DHA source during this period. Pregnant Iberian sows were fed with diets containing 0.8% DHA of total fatty acids as PL from egg yolk or TG from algae oil during the last third of gestation (40 days). Maternal tissues, placentas and fetal tissues were obtained at delivery and DHA quantified by gas-chromatography. MFSD2a carrier expression was analysed in both placenta and fetal brain by western blotting. Sows fed DHA-PL diet showed higher DHA incorporation in plasma LDL but not in plasma total lipids. No differences were found in DHA content between groups in maternal liver, adipose tissue or brain. Placental tissue incorporated more DHA in both total lipids and PL fraction in sows fed DHA-PL. However, this did not lead to an enhanced DHA accretion either in fetal plasma, fetal liver or fetal brain. MFSD2a expression was similar between both experimental groups. Maternal DHA supplementation during pregnancy in sow either as PL or TG produces similar DHA accretion in fetal tissues but not in placenta. Both fat sources are equally available for fetal brain.

Abbreviations: AA, arachidonic acid; DHA, docosahexanoic acid; FA, fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; Lyso-PC, lysophosphatidylcholine; Lyso-PL, lyso-phospholipid; PL, phospholipid; PUFA, polyunsaturated fatty acid; TG, triglyceride.

Introduction

Fatty acid (FA) concentration in maternal plasma increases during pregnancy as a consequence of physiological hyperlipidemia[1]. Phospholipids (PLs) are the major source of polyunsaturated FAs (PUFAs) in serum[2]. The increase in docosahexaenoic acid (DHA, 22:6 n-3) concentration in both maternal plasma PLs[1, 3] and erythrocyte PLs[4] is higher than for others PUFAs. However, despite the increase in absolute concentrations, there is a steady decline from the first trimester until delivery in the percentage of DHA in plasma PLs and erythrocytes PLs[1, 5], as well as in total plasma[5-6], with respect to the total FA content. The high prenatal accumulation of DHA in brain during the third trimester of pregnancy[7-8], the selective and preferential maternal-fetal transfer of long-chain PUFAs (LC-PUFAs) respect to other fatty acids across the placenta[9-11] and the low desaturase activity in both placenta[12] and fetal liver[13] could explain this decline of LC-PUFAs percentage in the maternal compartment. In recent years, great attention has turned to consider whether low DHA intakes in pregnant women may achieve an optimal central nervous system and visual development of the fetus[14]. Moreover, DHA supplementation might slightly reduce the risk of preterm birth, especially before 34 weeks of gestation[15-16], which highlights the potential role of DHA supplementation during pregnancy.

Several health organizations recommend an average dietary intake of at least 200mg DHA/d for pregnant women; intakes of up to 1g/day DHA or 2.7g/day n-3 LC-PUFA have been used in randomized clinical trials without significant adverse effects[17-18]. Several fat sources are currently available in the market for DHA supplements such as egg yolk extracts, single-cell microalgae oils, krill oils and fish oils. These sources contain DHA under different chemical forms, mainly PLs or triglycerides (TGs), resulting in different gut digestion and absorption[19-20]. Moreover, the intake of DHA as PL or TG may influence their distribution in plasma lipoproteins, as reported in piglets fed artificial formulas enriched with the same amount of DHA but from different fat sources (PL *vs.* TG)[21]. Valenzuela et al. compared the bioavailability of natural PL or TG DHA sources in pregnant rats but data from placentas and fetal tissues were not reported[22]. Pigs are the animal model more suitable to study lipid metabolism respect to humans[23], but no studies are available on the effects of DHA supplementation in the form of PL or TG during gestation on lipoprotein metabolism, even when they are crucial for placental FAs uptake and transfer.

In the present study, we examined the effects of DHA supplementation, from egg yolk (PL) or algae oil (TG) during the last period of gestation in sows, on DHA availability in

maternal plasma lipoproteins, placental distribution and accretion in fetal tissues, especially regarding to fetal brain incorporation.

Methods

Animals and diets

The protocol of this study was approved by the Animal Care Committee of the Murcia University (Murcia, Spain) and conforms to the ARRIVE guidelines for animal research. Animals received humane treatment in accordance with the European Union guidelines for the care and use of laboratory animals. In the present study pigs were used as they are considered one of the best models to study nutrition issues in human[23]. Iberian sows of twelve months of age were supplied by Escamez S.L. (Murcia, Spain). Sows were randomly assigned to two groups of six animals per group. Experimental groups were blinded for the staff responsible for the care of animals. Animals consumed water and their assigned experimental diet *ad libitum* during the last third of gestation (40 days), from day 65 to day 105 of gestation. In the present study, we tested two different fat sources of preformed DHA: egg yolk extract as DHA-PL source (OVOLIFE, Belovo, Belgium) and algae oil as DHA-TG source (DHASCO, Martek, MD, EEUU). OVOLIFE contained 88.1% of PL (64-79% phosphatidylcholine, 12-18% phosphatidylethanolamine and <3% lyso-phospholipids, analyzed by the manufacturer using 31^{P}-NMR phospholipid analysis); 43.1% saturated FAs, 23.3% monounsaturated FAs, 33.5% PUFA, 2.2% AA and 18.1% DHA (99.2% in PL form). DHASCO only contained TG and was composed by 22.7% saturated FAs, 15.6% monounsaturated FAs, 61.6% PUFAs, 0.5% AA and 59.0% DHA (97.9% in TG form). Concentrated fungal oil (ARASCO, Martek, MD, EEUU) was used as source of AA in DHA-TG diet in order to equilibrate AA content with respect to DHA-PL diet.

Standard diet for pregnant sows did not contain DHA (Alimer S.Coop., Murcia, Spain). The standard diet was enriched with a fat blend (10g/kg) to obtain diets with 0.8% DHA of total FAs either as PL or TG. In the DHA-PL diet, the total PL content was 3.68% of total fat while virtually 0% in the DHA-TG diet. Diets were finally composed (w/w) by 13.4% protein, 3.4% fat, 6.1% ashes and 88.4% dry matter. The total DHA intake for pregnant pigs was around 330mg DHA/day. Ingredients and FA composition of experimental diets are summarized in Table 1 and 2 respectively.

Collection of samples

On day 105 of gestation, animals were stunned through high CO_2 atmosphere exposition. The maternal and fetal blood was extracted by jugular vein puncture in heparinised tubes and centrifuged at 1400g 10min 4ºC to obtain plasma. Maternal liver, adipose tissue, brain, placentas, fetal liver and fetal brains (2 fetus and placentas were pooled per pig) were frozen in liquid nitrogen and stored at –80ºC until analysis.

Isolation of plasma lipoproteins

Lipoproteins were isolated from 1mL of fresh maternal plasma by ultracentrifugation using a discontinuous NaCl/KBr density-gradient[24] in an Optima L-100 XP ultracentifuge equipped with 100Ti rotor (Beckman Coulter, CA, EEUU). The rest of plasma was frozen in liquid nitrogen and stored at –80ºC until analysis.

Fatty acids analyses

Total lipids were extracted from experimental diets (500mg), 250µL plasma, the whole band of lipoproteins isolated from 1mL plasma, and tissues (30-50mg liver, 15-20mg adipose tissue, 30-50mg brain and 100mg placenta) according to Folch et al. method[25]. Previous to the extraction, an internal standard was added to the samples: 0.05mg pentadecanoic acid for total FA analyses, and 0.01mg FA each of pentadecanoic acid, tripentadecanoin, phosphatidylcholine dipentadecanoyl, and cholesteryl pentadecanoate for lipid fraction analyses. Samples with internal standard were extracted into chloroform:methanol (2:1 v/v). The lipid extract was evaporated to dryness under nitrogen flux. The residue was taken up in 400µL chloroform:methanol (1:1 v/v) for analysis of lipid fractions. It was applied on silica gel plates (Merck, Darmstadt, Germany), and the PL, TG, cholesterol esters and non-esterified FA were isolated by development of the plates in n-heptane/diisopropylether/glacial acetic acid (60:40:3 v/v) [26]. Bands from different lipid fractions were detected with 0.2% 2′,7′-dichlorofluorescein in ethanol (w/v) under UV light and scrapped. The bands of the thin layer chromatography as well as the dried residue for total FA analyses were both methylated according to Stoffel et al.[27] by adding 1mL of 3N methanolic HCl (Supelco, Sigma-Aldrich, MO, EEUU) and heating at 90°C for 1 hour. The derivatives were extracted into hexane and stored at -20ºC until gas chromatographic analysis.

FA methyl esters were analyzed by gas chromatography using a SP-2560 capillary column (100m x 0.25mm x 20µm) (Supelco, Sigma-Aldrich, MO, EEUU) in a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, CA, EEUU) equipped with a flame

ionization detector[28]. The temperature of the detector and the injector was 240ºC. The oven temperature was programmed at 175ºC 30min and increased at 5ºC/min to 230ºC and held at this temperature for 17min. Helium was used as the carrier gas at a pressure of 45psi. Peaks were identified by comparison of their retention times with appropriate FA methyl esters standards (Sigma-Aldrich, MO, EEUU) and FA concentrations determined in relation to peak area of internal standard.

Protein extracts for western blotting

30mg of placental tissue and fetal brain were homogenized in 0.3mL ice-cold lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 5mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1µg/mL leupeptin) from Cell Signaling Technology (MA, EEUU). Phenylmethanesulfonyl fluoride solution 1mM was added to lysis buffer before homogenization[29]. Samples were homogenized using a Tissue Lyser LT device (Qiagen Iberia SL, Madrid, Spain). Protein lysates were obtained from the supernatant after 15min centrifugation at 10.000g 4ºC. Protein was quantified by Bradford assay[30] and samples stored at -80ºC until Western blot analysis.

Western blot analysis

The primary antibodies used were rabbit polyclonal antibody against the orphan transporter called "Major Facilitator Superfamily Domain Containing 2A" (MFSD2a, Abcam, Cambridge, United Kingdom) and mouse monoclonal anti-beta-actin (Sigma-Aldrich, MO, EEUU). Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (TX, EEUU). Protein extracts (15µg protein) diluted in sample buffer were resolved on 10% polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Membranes were then blocked in phosphate saline buffer with 0.05% Tween-20 (PBS-T) containing 2% bovine serum albumin for 1h at room temperature. Thereafter, membranes were incubated with primary antibodies overnight at 4ºC. Blots were then washed with PBS-T and probed for 1h at room temperature with the correspondent secondary antibodies conjugated with horseradish peroxidase. Finally, membranes were stripped with Tris/HCl buffer pH 2.3 containing beta-mercaptoethanol 0.1M and re-probed with anti-beta-actin to perform loading controls. Proteins were detected using a chemioluminescence kit according to the manufacture's instruction (Pierce ECL 2 Western Blotting Substrate; Thermo Fisher Scientific, MA, EEUU)[31]. Density of all bands was determined by densitometry using

Image Quant LAS 500 software (GE Healthcare, CA, EEUU). Relative protein expression data were normalized for beta-actin expression.

Statistical analysis

Sample size was estimated based on DHA percentages in plasma PLs of piglets published by Alessandri et al.[32]. Type I error was set at α=0.05 and type II error β=0.2 (power 80%), obtaining a minimum sample size of 3 animals per group. The software used for this estimation was nQuery 7.0 (Statsols HQ, Cork, Ireland).

The SPSS 15.0 software (SPSS, Inc., IL, EEUU) was used for statistical analyses of data obtained. To evaluate the effects of DHA supplementation as PL *vs*. TG on FA profiles of maternal and fetal tissues, a t-test was performed. Differences between plasma lipoproteins composition were assessed by one way ANOVA followed by *post hoc* of Bonferroni. Statistical significant differences were establish at $P<0.05$. Data are expressed as means ± SEM.

Results

DHA supplementation as PL or TG during the last third of gestation did not affect DHA percentage in total lipids of maternal plasma (Fig 1A). In both experimental groups, DHA was preferentially incorporated in PL-rich lipoproteins (HDL and LDL), being DHA percentage significantly higher in LDL of animals fed DHA-PL with respect to DHA-TG diet (Fig 1B). In fact, when we analyzed plasma lipid fractions, maternal plasma showed a slight trend towards higher DHA percentage in PL fraction (P=0.13) of animals fed the DHA-PL diet compared to those fed DHA-TG diet (Table 3). No differences were observed for the rest of plasma lipid fractions. Total DHA concentration in plasma was also similar between groups (DHA-PL group: 4.00 ± 0.99 mg/dL *vs.* DHA-TG group: 3.44 ± 0.79 mg/dL, P=0.64). Therefore, the administration of 0.8% DHA in the form of PL or TG produced different lipoprotein incorporation in the mother although the enrichment in maternal serum PL was moderated.

Concerning the others maternal tissues, we found statistically significant differences in DHA composition in total lipids between both experimental groups neither in liver, adipose tissue nor maternal brain (Fig 1A). However, placental tissue showed significantly higher DHA percentage (Fig 2A) and also higher DHA concentration in total lipids of the DHA-PL group (DHA-PL group: 0.07 ± 0.01 mg/g *vs.* DHA-TG group: 0.05 ± 0.01 mg/g, P=0.03).

This DHA increase in placenta was exclusively due to the incorporation of DHA in PL fraction (Table 3). Placenta is a tissue with a high amount of PL in its structure, and this could be related with the fact that only placenta, and not other maternal tissues, was comparatively more enriched in DHA after feeding the animals with DHA-PL diet compared to DHA-TG fed group. Despite enhanced DHA accumulation in placentas of DHA-PL fed animals, MFSD2a protein expression, which carries up lyso-phospholipids (lyso-PLs), was not higher in placenta of this group (DHA-PL: 1.54 ± 0.53 *vs.* DHA-TG: 1.75 ± 0.27 arbitrary units, p=0.72). Concerning AA percentage, no differences were found in lipid fractions of maternal plasma. However, DHA-TG group presented lower AA percentage in total lipids of placenta (DHA-PL: 10.75 ± 0.67% vs. DHA-TG: 8.28 ± 0.78%, p=0.025) and also in PL fraction (Table 3).

Despite the higher incorporation of DHA in placenta, maternal DHA-PL supplementation did not enhance DHA accretion in fetal structures, either in fetal plasma, liver or brain respect to fetus from mothers fed DHA-TG diet (Fig 2B). Fetus re-distributes again DHA in plasma lipids and no differences were found in PL fraction between groups (Table 4). In fact, only DHA percentage in plasma cholesterol esters was higher in the offspring of DHA-PL group (Table 4), which could be due to the link between PL and cholesterol esters formation. More importantly, no differences were found in DHA percentage either in total lipids or PL fraction of fetal brain (Fig 2, Table 4) or DHA content in the whole brain (DHA-PL group: 2.23 ± 0.30 mg/g *vs.* DHA-TG group: 2.41 ± 0.19 mg/g, P=0.59). Fetus from DHA-TG group presented higher values of AA in total lipids of plasma than in the DHA-PL fed group (DHA-PL: 12.55 ± 0.75% *vs.* DHA-TG: 15.32 ± 0.62%, p=0.011), and this was also observed in fetal plasma PL fraction (Table 4), in contrast to placental tissue of these animals. The DHA-PL group tended to higher MFSD2a expression in fetal brain (DHA-PL: 1.38 ± 0.15 *vs.* DHA-TG: 0.96 ± 0.25 arbitrary units, p=0.14). MFSD2a bands pattern in fetal brain was completely different to that observed in placenta, which only a predominant band of ~100kDa detected (Fig 3), that corresponded to the expected size of such protein.

Therefore, maternal lipid supplementation with DHA in the form of PL during the last third of gestation positively affected DHA accumulation in placenta, but it did not enhance fetal DHA accretion compared to the administration of DHA in TG form. Fetal brain DHA accretion seems to be strongly regulated.

Discussion

It is uncertain whether DHA consumption during pregnancy in PL form could be a source with higher placental bioavailability and fetal brain accretion than DHA in the form of TG. In the present study, sows fed a diet supplemented with 0.8% DHA in the form of PL or TG during the last third of gestation (40 days) showed similar DHA accumulation in total lipids of maternal plasma. However, there was a slight trend towards higher DHA incorporation in maternal plasma PL which indicates that maternal metabolism modulates in certain degree the effect of dietary DHA, modifying its incorporation in maternal serum lipid fractions. Some reports in piglets, full-term infants and children also indicated that the plasma lipid fraction in which DHA is incorporated after gut absorption is not always related with the chemical form of DHA consumed[33-35]. Jiménez et al.[36] showed higher DHA incorporation in plasma PL of newborn piglets fed a formula supplemented with LC-PUFA PLs from pig brain extract than control group fed sow milk or control formula without DHA, while no differences were observe in brain composition of piglets. In case of pig brain concentrates, other lipid components apart from PL, e.g. cerebrosides, gansliosides, sphingolipids and lysophosphatidylcholine can be present and might alter the absorptive process and later metabolism of FAs.

In non-pregnant humans, Ramprasath et al.[37] reported an increase of n-3 index in healthy individuals receiving for 4-weeks n-3 FAs supplements from krill oil *vs.* fish oil. However, the magnitude of DHA change in plasma was minimal and they introduced an inappropriate variable into their study due to fish oil capsules contained over 32% linoleic acid (18:2 n-6) of total FAs, which is an antagonist of n-3 incorporation in mammalian tissues[38]. Other authors did not report differences in humans after PL or TG DHA supplementation[34-35, 39].

There is only one study conducted in pregnant animals but developed in rats, in which animals were fed during gestation with DHA as PL or TG, resulting in similar DHA levels in total lipids of maternal plasma[22]; these authors reported only higher levels of DHA in erythrocytes PLs when using the PL source. Respect to maternal tissues, liver PLs and adipose tissue had higher DHA in the PL-DHA group before mating than in the DHA TG group, but these differences were not significant at the time of delivery[22]. These results are in agreement with our data, and there is no evidence of enhancement DHA content in maternal tissues with DHA supplementation as PL *vs.* TG at the same dose during pregnancy. Maybe it

is more efficient to increase the DHA dosage administered to pregnant mothers than change the lipid source used to improve DHA availability.

Despite similar DHA incorporation in total lipids of maternal plasma, we found higher incorporation of DHA in PL-rich lipoproteins (HDL and LDL), probably linked to the observed trend towards higher DHA percentage in plasma PLs. This higher DHA incorporation in LDL of DHA-PL group could have been promoted the higher placental uptake detected in our study. Amate et al.[21] also reported different DHA incorporation in serum LDL PLs and HDL PLs of piglets when using PL *vs.* TG sources while not differences were reported in DHA percentage in plasma PLs fraction. We did not analyse FAs composition of lipoproteins per lipid fractions.

Placenta displays HDL-receptors and LDL-receptors in its membranes as well as lipases to release FAs from plasma lipoproteins. Endothelial lipase mainly releases the FA from the *sn-1* position of the PL, producing a lyso-PL[9]. Nevertheless, with the time this enzyme releases also the FA esterified in the *sn-2* position of the Lyso-PL[40]. These lyso-PL could be an additional source of FAs for the placenta and other tissues[41-42]. In the present study we describe for the first time a higher incorporation of DHA in placentas from animals fed DHA from the PL source. Since placenta is a tissue with more than 85% of PL in its structure[43], this could facilitate a higher DHA uptake from PL rich lipoproteins. This finding also support, at least in part, the hypothesis that endothelial lipase could release DHA from maternal circulating PL, especially in DHA-PL group, which might have been preferentially uptake by placental tissue.

DHA lyso-phosphatidylcholine (lyso-PC) has been proposed as a preferred physiological carrier of DHA to the brain[41-42], probably via MFSD2a transporter[44]. Recently, we have demonstrated that lower MFSD2a expression was related to disturbed DHA placental transfer in the offspring of gestational diabetes mothers[31]. Inactivation of MFSD2a protein has been also linked to severe outcomes such as microcephaly syndrome[45-46]. However, in the present study placental MFSD2a did not change among groups while fetal brain expression tended to higher values in the DHA-PL group.

Human placenta is a discoidal endotheliochoreal placenta, which means that the distance between maternal blood and the fetal capillaries is minimal (sometimes just a single layer of throphoblast cells), while pigs have a diffuse epitheliochoreal placenta with intact layers of epithelial cells between both blood circulations. These histological differences in placenta of humans and pigs could affect or modulate the FAs uptake and transport across the tissue in a different way. Moreover, a complex pattern of bands appeared for MFSD2a by

329 western blot analysis in sow placentas and fetal brain. Placenta expressed MFSD2a with three
330 different molecular weights: ~100kDa, ~65kDa and ~50kDa while fetal brain only presented a
331 clear band at ~100kDa. Previous studies in mice and several human cell lines have shown
332 different levels of protein glycosilation[45, 47-48]. Berger et al.[47] also reported different
333 MFSD2a glycosilations in liver and brown adipose tissue in mice. Different glycosilation
334 patterns or others post-translational changes of MFSD2a between different tissues of the same
335 animal, or the same tissue between different species, could imply different functions. A
336 regulatory effect of fasting/refeeding on liver MFSD2a expression and lipid metabolism has
337 been even described in mice by the same authors[47]. More studies are needed to fully
338 understand the role of MFSD2a in body growth, lipid metabolism and brain integrity.
339 Surprisingly, the major accumulation of DHA in placenta of these animals did not lead
340 to a higher DHA status in the offspring. Valenzuela et al.[49] showed increased DHA levels in
341 PL fraction of cerebellum and hippocampus of pups at two months of age after maternal
342 supplementation during pregnancy with DHA as lyso-PC in rats. In our study, the level of
343 lyso-PC was minimal in the diets since Ovolife product contained < 3% of total fat as lyso-
344 PL, being phosphatidylcholine the major PLs source (64-79% of total PLs). The DHA-TG
345 diet was not supplemented with free choline to compensate the choline provided by
346 phosphatidylcholine in the DHA PL-diet, which should be mentioned. We did not find
347 statistically significant differences in DHA content in total FAs analysis or lipid fractions of
348 fetal brain between both experimental groups. Placenta releases FAs as non-esterified fatty
349 acids[50] and the re-esterification by the fetal liver limits the selective accretion of DHA in PL
350 of fetal plasma and hence tissue accretion.
351 One of the most remarkable strength of this study is the using of sows as experimental
352 model, which is difficult to handle, being one of the species with a lipid metabolism closest to
353 the humans and allowing us to collect very important tissues as maternal or fetal brain.
354 Among the limitations of this study, to mention that pigs have a diffuse epitheliochoreal
355 placenta while humans have a discoidal endotheliochoreal placenta where maternal blood is in
356 contact with trophoblast cells. It is not known whether these structural differences of
357 placentas might imply differences in lipid placental transfer. Only total phospholipids were
358 considered and it would be interesting to measure LysoPC even when they were administrated
359 in low doses in this study.
360 In conclusion, this study provides evidence that DHA as PL from egg yolk extract and
361 DHA as TG from microalgae oil have similar availability for fetal tissues. The maternal
362 supplementation during the last third of gestation with DHA as PL *vs.* TG resulted in small

differences in maternal plasma, with higher incorporation in maternal LDL that could explain higher placental DHA uptake. Nevertheless, this higher placental uptake was not linked to higher DHA levels in fetal brain that seems to be well regulated.

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Authors' contributions: A.G. and E.L. designed research. A.G. and M.R-P. conducted research and samples determinations. A.G. performed statistical analysis. A.G. and E.L. wrote the manuscript. All authors have read and approved the final manuscript.

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Legend of figures

Fig. 1. Docosahexaenoic acid (DHA) percentage at delivery in maternal plasma, liver, adipose tissue, brain (**A**) and lipoproteins (**B**) of pigs fed during the last third of gestation with DHA (0.8% of total fatty acids) as phospholipid (DHA-PL) or triglyceride (DHA-TG). Values are means ± SEM (n=6/group). *Indicates significant differences between PL and TG groups ($P < 0.05$). †Indicates significant differences between HDL and VLDL lipoproteins within the same PL or TG group ($P < 0.05$). §Indicates significant differences between LDL and VLDL lipoproteins within the same PL or TG group ($P < 0.05$).

Fig. 2. Docosahexaenoic acid (DHA) percentage at delivery in placenta (**A**) and fetal plasma, fetal liver and fetal brain (**B**) after maternal DHA supplementation (0.8% of total fatty acids) as phospholipid (DHA-PL) or triglyceride (DHA-TG) during the last third of gestation. Values are means ± SEM (n=6/group). *Indicates significant differences between PL and TG groups ($P < 0.05$).

Fig. 3. Western blotting membranes of MFSD2a in placenta (**A**) and fetal brain (**B**) of pigs after maternal docosahexaenoic acid supplementation (0.8% of total fatty acids) as phospholipid (DHA-PL) or triglyceride (DHA-TG) during the last third of gestation. Three different bands are present in placental tissue (~100kDa, ~65kDa and ~50kDa), which might correspond with different glycosylation pattern, while only a predominant band (~100kDa) appeared in fetal brain.

Table 1. Composition of experimental diets.

Ingredients (g/kg)	DIETS	
	DHA-TG	DHA-PL
Sunflower pellets	25	25
Barley	380	380
Wheat bran	180	180
Corn	258.5	258.5
Rapeseed flour	100	100
Sugar cane molasses	15	15
Experimental fat		
DHASCO	0.45	-
OVOLIFE	-	2.9
ARASCO	0.15	-
Olive oil	3.3	3.2
Soybean oil	4.5	3.9
Palm oil	1.6	-
Lysine	1.8	1.8
Sepiolitic clay	5	5
Antioxidants and preservatives	2.39	2.39
Minerals and vitamins*		
DHA	0.0017	0.0017

ARASCO: fungal oil rich in AA (20:4n-6) provided by Martek. DHASCO: algae oil rich in DHA (22:6n-3) in the form of TGs provided by Martek. OVOLIFE: egg yolk extract rich in DHA in the form of PLs provided by Belovo.

DHA-TG, diet supplemented with DHASCO in which DHA is in TGs form. DHA-PL, diet supplemented with OVOLIFE in which DHA is in PLs form.

*Minerals (per kg of diet): NaCl 4g, $CaCO_3$ 12.9g, $Ca_3(PO_4)_2$ 5.2g, $MnSO_4$ 35mg, $FeCO_3$ 50mg, $CuSO_4 \cdot 5H_20$ 15mg, ZnO 90mg, KI 0.5mg, $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$ 0.45mg and Na_2SeO_3 0.06mg. Vitamins (per kg of diet): vitamin A 2.7mg, vitamin D_3 0.04mg and vitamin E 30mg.

Table 2. Fatty acids profile of experimental diets.

Fatty acid	DIETS	
	DHA-TG	DHA-PL
	g/100g total fatty acids	
14	0.2	0.3
16	15.9	16.0
18	3.4	2.4
18:1 n-9	30.2	29.6
18:1 n-7	1.4	1.2
18:2 n-6	42.9	45.0
18:3 n-3	3.1	2.9
20:4 n-6	0.1	0.2
22:6 n-3	0.8	0.8
SFA	20.5	20.0
MUFA	32.3	31.3
PUFA	47.2	49.2
PUFA n-6	43.2	45.5
PUFA n-3	4.0	3.7

DHA-TG, diet supplemented with algae oil in which DHA is in TGs form. DHA-PL, diet supplemented with egg yolk extract in which DHA is in PLs form. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. Percentage of docosahexaenoic acid (DHA) and arachidonic acid (AA) in lipid fractions of maternal plasma, maternal liver and placenta of sows after DHA supplementation (0.8% of total fatty acids) as phospholipid (DHA-PL) or triglyceride (DHA-TG) during the last third of gestation.

DHA (g/100g total fatty acids)	DIETS				
	DHA-PL		DHA-TG		
	Mean	SEM	Mean	SEM	P
Maternal plasma					
Phospholipids	3.44	0.50	2.80	0.80	0.13
Triglycerides	0.44	0.12	0.40	0.26	0.89
Free fatty acids	0.87	1.09	0.99	2.06	0.91
Cholesterol esters	0.27	0.10	0.47	0.24	0.42
Maternal liver					
Phospholipids	5.75	1.06	4.98	0.84	0.20
Triglycerides	0.23	0.06	0.25	0.06	0.78
Free fatty acids	1.15	0.33	1.03	0.14	0.43
Cholesterol esters	0.83	0.58	1.69	0.47	0.24
Placenta					
Phospholipids	1.28	0.10	1.03	0.19*	**0.02**
Triglycerides	0.71	0.30	0.71	0.30	0.99
Free fatty acids	1.16	0.37	1.52	0.50	0.19
Cholesterol esters		n.d.		n.d.	
AA %					
Maternal plasma					
Phospholipids	13.27	1.00	14.33	2.60	0.37
Triglycerides	0.96	0.24	1.22	0.13	0.32
Free fatty acids	0.55	0.41	0.35	0.29	0.37
Cholesterol esters	6.71	0.30	7.15	0.70	0.54
Maternal liver					
Phospholipids	17.80	0.73	18.55	1.64	0.33
Triglycerides	2.14	0.23	2.67	0.52	0.35

Free fatty acids	5.94	0.71	7.15	0.81*	**0.02**
Cholesterol esters	2.75	0.23	2.55	0.21	0.52
Placenta					
Phospholipids	11.70	2.08	7.07	2.60*	**0.01**
Triglycerides	4.36	0.67	3.46	0.74	0.35
Free fatty acids	6.62	2.01	6.64	2.42	0.99
Cholesterol esters	3.68	0.31	2.94	0.80	0.37

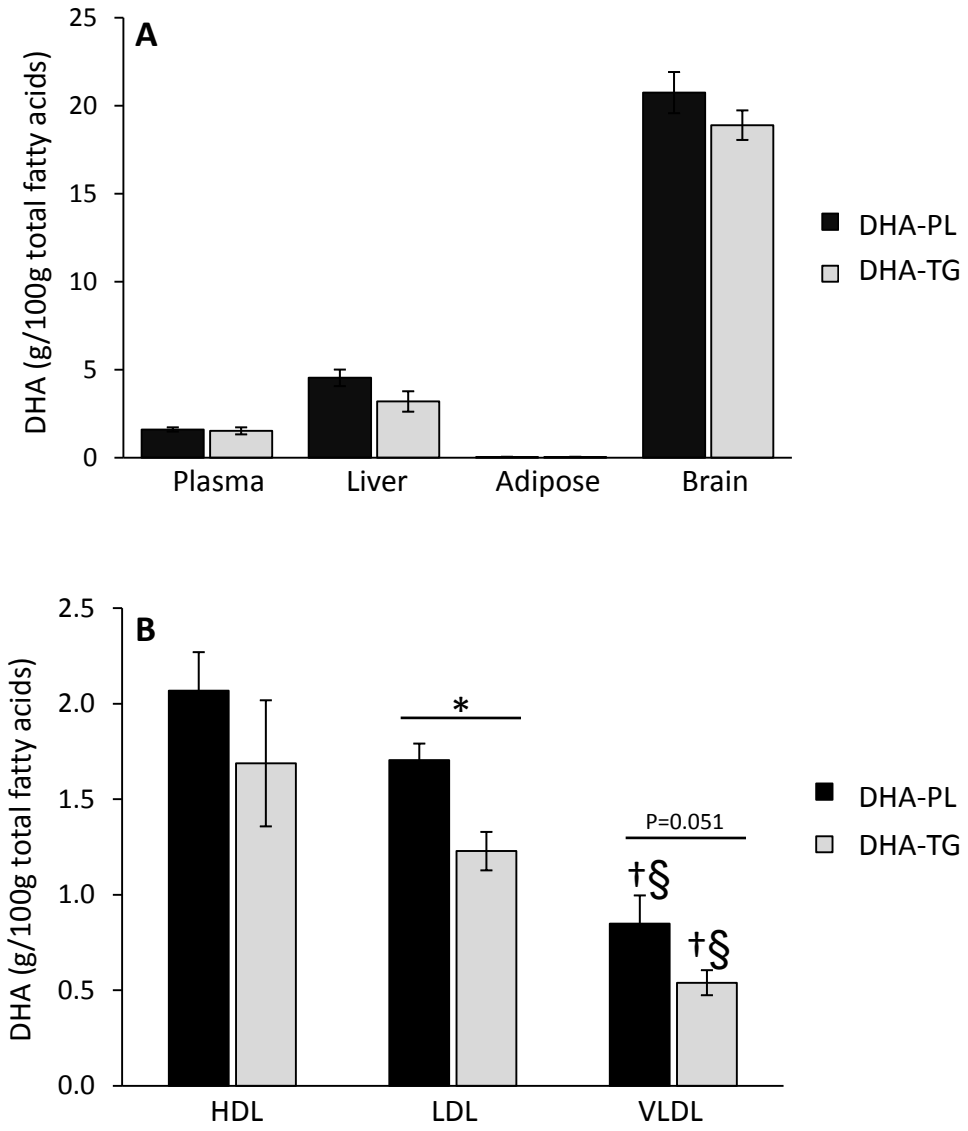
Values are means ± SEM (n=6/group). Bold face was used when P<0.05. *Indicates significant differences between PL and TG groups (P <0.05). N.d., non detectable.

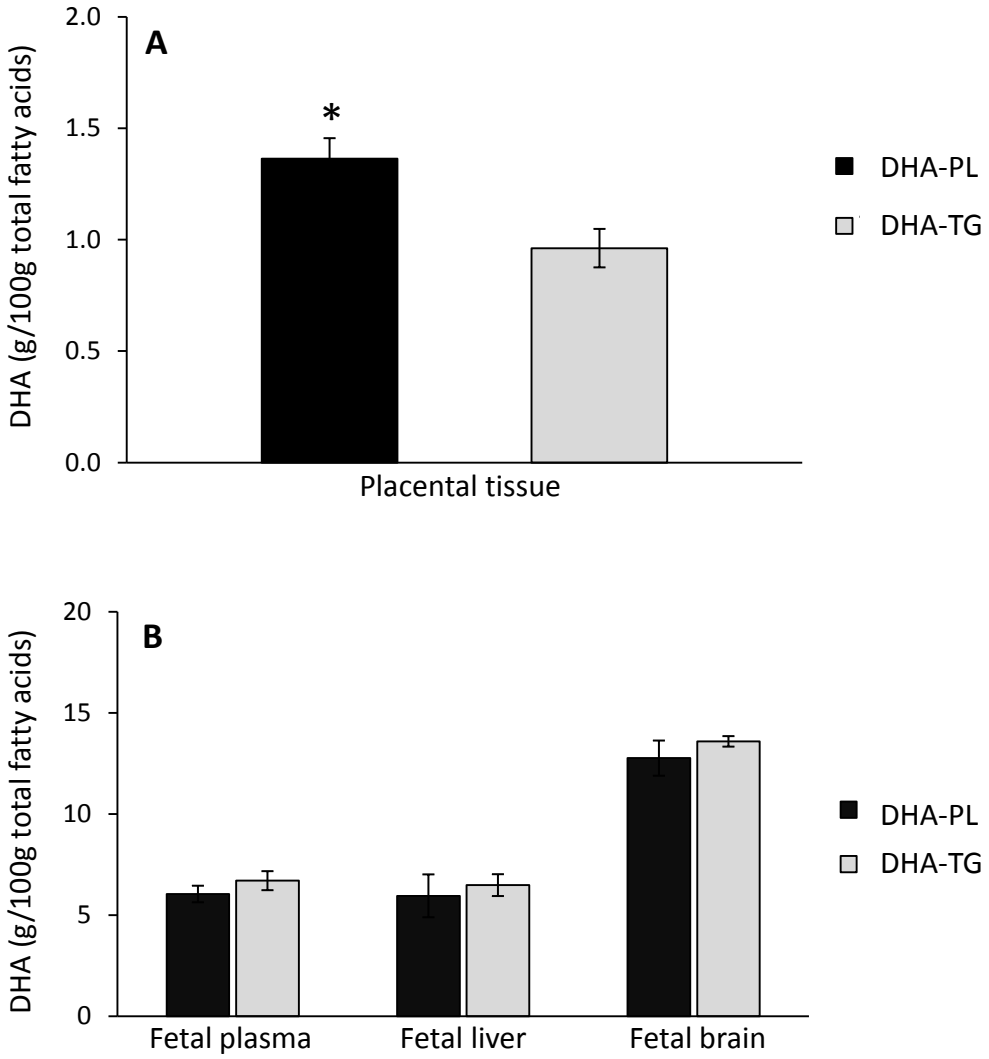
Table 4. Percentage of docosahexaenoic acid (DHA) and arachidonic acid (AA) in lipid fractions of fetal plasma, fetal liver and fetal brain after maternal DHA supplementation (0.8% of total fatty acids) as phospholipid (DHA-PL) or triglyceride (DHA-TG) during the last third of gestation.

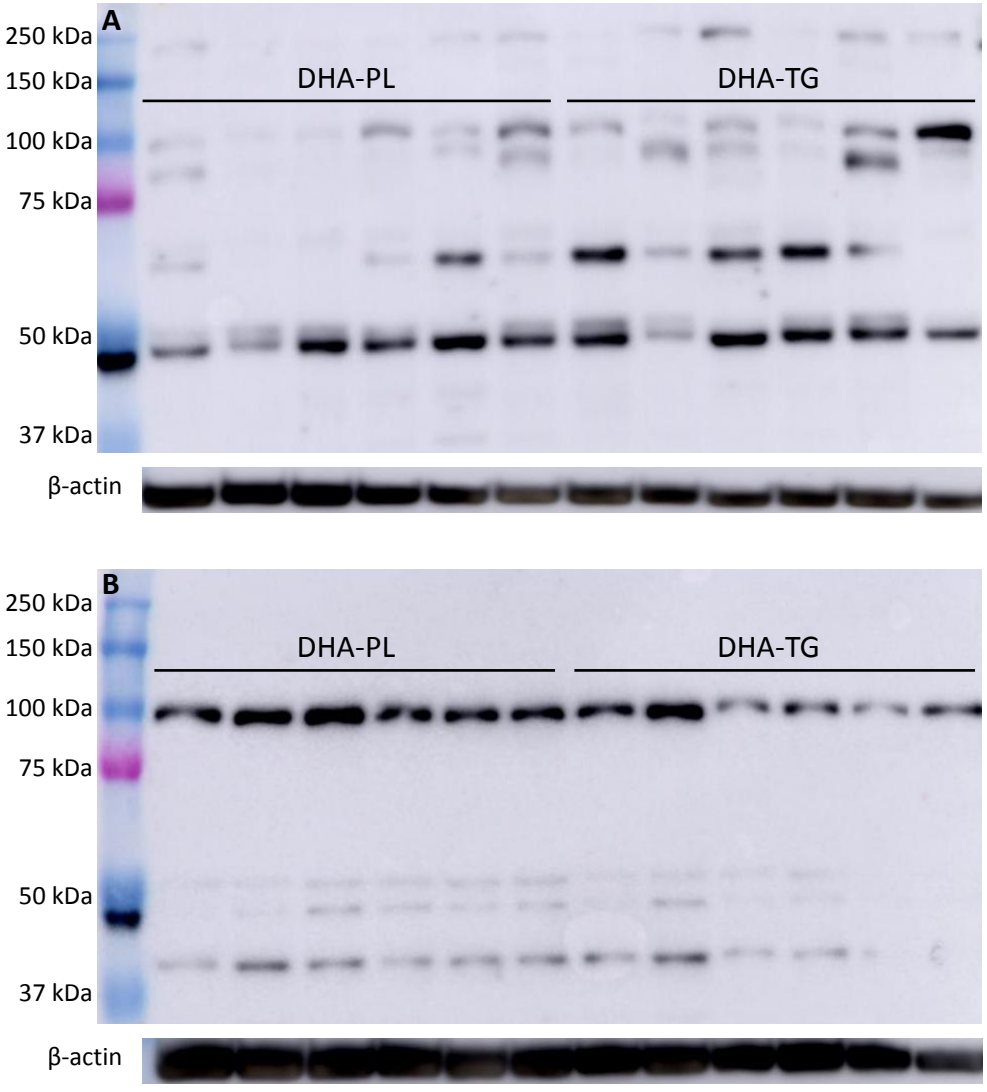
DHA (g/100g total fatty acids)	DIETS				
	DHA-PL		DHA-TG		
	Mean	SEM	Mean	SEM	P
Fetal plasma					
Phospholipids	8.44	2.06	8.81	0.73	0.69
Triglycerides	3.98	0.56	2.56	0.91	0.18
Free fatty acids	3.45	1.82	2.77	0.81	0.42
Cholesterol esters	1.41	0.18	0.79	0.12*	**0.01**
Fetal liver					
Phospholipids	9.75	1.15	9.76	1.47	0.97
Triglycerides	0.82	0.18	1.45	0.41	0.17
Free fatty acids	2.08	0.22	4.26	2.15	0.06
Cholesterol esters	0.56	0.28	0.62	0.68	0.93
Fetal brain					
Phospholipids	13.68	0.71	13.59	0.62	0.82
Triglycerides	0.01	0.01	1.43	1.28	0.28
Free fatty acids	5.06	1.49	4.83	1.08	0.77
Cholesterol esters	0.01	0.01	0.69	0.75	0.36
AA %					
Fetal plasma					
Phospholipids	18.27	1.35	19.71	0.56*	**0.04**
Triglycerides	4.23	0.51	5.51	1.41	0.38
Free fatty acids	2.89	1.00	2.98	1.01	0.88
Cholesterol esters	9.04	0.39	7.98	0.71	0.18
Fetal liver					
Phospholipids	21.00	2.25	21.92	1.10	0.39
Triglycerides	2.02	0.35	4.58	1.29	0.08

Free fatty acids	7.10	1.21	12.24	4.57*	**0.04**
Cholesterol esters	2.10	0.41	3.92	0.79	0.06
Fetal brain					
Phospholipids	12.15	0.63	11.79	0.48	0.29
Triglycerides	8.45	1.94	9.15	1.87	0.78
Free fatty acids	16.25	3.66	19.02	4.63	0.28
Cholesterol esters	0.75	0.82	2.45	1.87	0.38

Values are means ± SEM (n=6/group). Bold face was used when $p<0.05$. *Indicates significant differences between PL and TG groups ($P<0.05$).


A
DHA (g/100g total fatty acids)
25
20
15
10
5
0
Plasma
Liver
Adipose
Brain
DHA-PL
DHA-TG
B
DHA (g/100g total fatty acids)
2.5
2.0
1.5
1.0
0.5
0.0
*
P=0.051
†§
†§
HDL
LDL
VLDL
DHA-PL
DHA-TG


A
2.0
1.5
1.0
0.5
0.0
DHA (g/100g total fatty acids)
*
Placental tissue
DHA-PL
DHA-TG
B
20
15
10
5
0
DHA (g/100g total fatty acids)
Fetal plasma
Fetal liver
Fetal brain
DHA-PL
DHA-TG


A
250 kDa
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100 kDa
75 kDa
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37 kDa
DHA-PL
DHA-TG
β-actin
B
250 kDa
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75 kDa
50 kDa
37 kDa
DHA-PL
DHA-TG
β-actin