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Fatty acid and lipid metabolism in liver of pregnant mice and their offspring is influenced by unbalanced folates/vitamin B12 diets

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ABSTRACT

Micronutrients (folates and vitamin B12) and long chain polyunsaturated fatty acids (LC-PUFAs) are linked through the one carbon cycle. We studied the effects of pre and postnatal high FA/low B12 diets (HFLB12) on hepatic fatty acid metabolism. Pregnant C57BL/6 mice were divided in two groups: control (2 mg folic acid: FA/25 μg vitamin B12/Kg food) and HFLB12 diets (8 mg FA/5 μg vitamin B12/Kg food). Offspring continued on the same diets until 60 days old. We determined hepatic fatty acid profile in dams and offspring and the expression of *PPARα, Cpt-1, Acox-1* and *Fas* and the enzymatic activity of desaturases, all involved in lipid metabolism. In liver of dams, the HFHB12 diet decreased total fatty acids and desaturase activities; in offspring, effects were opposite, being more noticeable in females. Prenatal and postnatal unbalanced folic acid/B12 diets play a crucial role in regulating genes and enzymes involved in lipid metabolism in liver of dams and their offspring in adulthood.

1. Introduction

Micronutrients belonging to the family of hydrosoluble vitamins of the complex B, as folates and vitamin B12, along with omega-6 and omega-3 long chain polyunsaturated fatty acid (LC-PUFAs), such as docosahexaenoic acid (C22:6n-3, DHA) are crucial molecules during life cycle, involved in control and regulation of several relevant metabolic pathways [1–3]. Folates and vitamin B12 participate in one-carbon and amino acid metabolism, as well as in cellular growth and division along with DNA synthesis.

Also, Vitamin B12 regulates the energetic balance through its action as a co-factor of the enzyme methyl malonyl CoA mutase in the mitochondria [4–8]. Both vitamins are key molecules for the S-adenosylmethionine (SAM) synthesis, the principal methyl donor involved in the methylation reactions of different molecules including DNA, protein and lipids [5]. Phosphatidylethanolamine-N-methyltransferase (PEMT) catalyzes the sequential methylation of phosphatidylethanolamine to phosphatidylcholine and preferentially utilizes phosphatidylethanolamine that contains docosahexaenoic acid (DHA) generating phosphatidylcholine containing DHA, a key component of cell membranes. The methylation of phosphatidylethanolamine to phosphatidylcholine by PEMT is important in mobilizing LC-PUFAs like DHA from liver to the plasma and other tissues [9–11]. In addition, plasma and hepatic DHA concentrations depend on the efficiency of several enzymatic reactions acting on its precursor, α-linolenic acid. These enzymes (Δ−5 and Δ−6 desaturases) cause elongation and desaturation in the cytoplasm, and thus may also generate substrates for peroxisomal β-oxidation by the acyl-coenzyme A oxidase (Acox-1) [12]. Moreover, DHA regulates lipid metabolism, interacting as a ligand of PPARα to induce CPT-1 and thus stimulating β-oxidation, or as a ligand of SREBP-1c, leading to decreased fatty acid synthesis through the downregulation of fatty acid synthase (FAS) [13–15], (**Fig. 1**).

With all these antecedents, it is not difficult to establish the importance of folic acid, vitamin B12 and DHA during pregnancy and fetal development. For instance, folates and vitamin B12 prevent fetal malformations such as neural tube defects [16–18], and DHA is involved in fetal brain and retina development. Then, any alteration in the maternal one carbon cycle will directly affect the DNA methylation patterns possibly via changes in the expression of genes involved in placental development and function. This condition may result in adverse pregnancy outcomes thereby predisposing the offspring to unhealthy long-term consequences during adulthood [18,19,20]. This circumstance may be influenced by consuming diets with unbalanced folates/vitamin B12 ratio such as those being deficient in folates, or alternatively, diets with elevated folate and low vitamin B12 levels. Consequently, it probably affects populations

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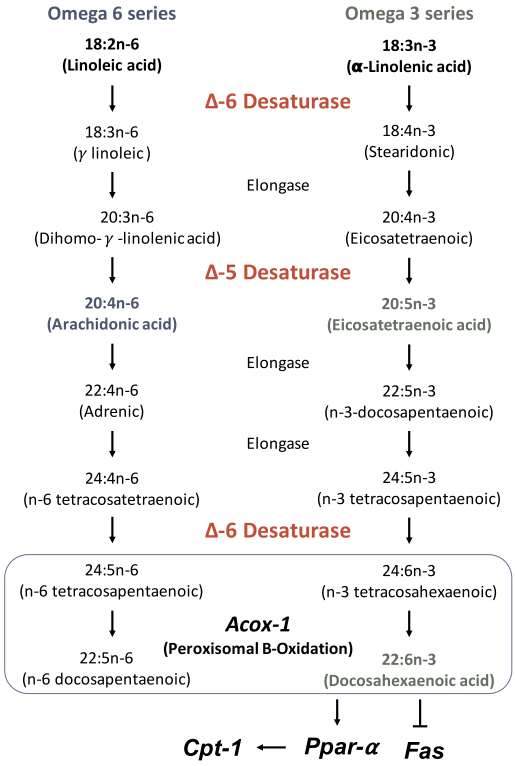

Fig. 1. Scheme of the metabolic pathway for the intracellular synthesis of long chain polyunsaturated fatty acids (LC-PUFA). LC-PUFAs synthesis from n6 and n3 use the same enzymes desaturases and elongases to synthesize arachidonic acid (AA), eicosapentainoic acid (EPA) and docosahexaenoic acid (DHA), like main products. DHA is a ligand of PPARα which increases the transcription of CPT-1 and therefore, DHA modulates beta oxidation. DHA also acts as a ligand for other transcription factors (SREBP-1), decreasing the transcription of enzymes related to the synthesis of fatty acids.

consuming high levels of fortified foods with folic acid and poor amounts of animal origin foods, the main source of vitamin B12 [21]. Therefore, as already stated, the one carbon metabolic pathway will be altered, decreasing the production of S-adenosyl-methionine, thus leading to low or inefficient methylation reactions [8] and increasing levels of circulating homocysteine [22]. It has been also demonstrated that concurrently varying the dietary intake of folic acid, vitamin B12, and vitamin B6 can influence plasma concentration of DHA [23].

Accordingly, the important interaction among these three essential nutrients (folates, vitamin B12 and lipids) for crucial metabolic pathways during life cycle, lead to study, in a mouse model, whether a dietary imbalance between folates and vitamin B12 given pre and postnatally, could affect the fatty acid metabolism in liver of dams and their offspring.

We hypothesize that diets containing high levels of folic acid (FA) and low levels of vitamin B12 (HFLB12) modify the fatty acid levels in liver of dams and offspring by disrupting the action of transcription factors and enzymes involved in the desaturation reactions (Δ- 5 and Δ−6 desaturases) and mitochondrial (PPARα and Cpt-1) and peroxisomal (Acox-1) β-oxidation.

We found that unbalanced folate/vitamin B12 diets given to pregnant mice and their offspring until adulthood, affects lipid metabolism in both, dams and offspring, with higher incidence in female offspring, indicating a sexual dimorphism due to this altered nutritional condition.

2. Materials and methods

2.1. Study design

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the "Guide for the care and use of laboratory animals, Eight Ed. 2010, National Research Council, National Academies Press, Washington, D.C." and the "AVMA Guidelines for the euthanasia of animals: 2013 Edition" [24]. The protocol was approved by the local Committee of Animal Bioethics at the Institute of Nutrition and Food Technology, (INTA), University of Chile.

C57BL/6 female mice (6 - –8 weeks) were acquired in the Institute of Public Health of Chile and then maintained in the bioterium of our institution under normal conditions of humidity and temperature (22–24 °C) and 12:12 h light-dark cycle. They were divided in two dietary groups: control (NFNB12, $n = 10$), which received a diet containing normal FA concentrations (2 mg/kg diet), the synthetic folate, and normal vitamin B12 concentrations (25 μg/kg diet) (Research Diet INC, USA), and treatment group (HFLB12, $n = 10$), fed a diet containing high FA (8 mg/kg diet) and low vitamin B12 levels (5 μg/kg diet). Composition of growth and adult control and HFLB12 diets are shown in **Table 1**. To adapt and synchronize reproductive cycles, females were housed in individual cages and exposed to these diets during two weeks before mating. Food and purified tap water were at libitum. After the adaptation period, females were mated, and conception was confirmed by observation of sperms under microscope in vaginal samples. Half of the pregnant females ($n = 5$ per group) were scheduled for a caesarean (day 18th) and livers and plasma were extracted. See **Fig. 2** for an experimental design scheme.

The rest of pregnant females ($n = 5$ per group) continued with the same diets (NFNB12 or HFLB12) until delivery and during nursing. After weaning (21 days), females were sacrificed according to the guidelines for rodent euthanasia provided by the American Medical Veterinary Association [24] and offspring's were separated by sex (one female and one male per litter) and continued with the same diet as their mothers (NFNB12 or HFLB12, $n = 5$ per sex and group) until 60 days of age. During this period, body weight and food intake were evaluated. At 60 days old, offspring were sacrificed as described before, and blood and livers were extracted. All offspring samples were from different litters (**Fig. 2**).

2.2. Diet composition

The diet was acquired in Research Diet INC (USA) which was elaborated according to AIN-93 G/M for rodents. To obtain a diet high in FA, we chose 8 mg/kg FA because, it is an approximation of the FA intake by Chilean pregnant women. According to the Chilean National Food Consumption survey of 2010 (ENCA 2010) folate intake in women (15–64 years) is ~450 μg of folates [25] which in pregnant women is increased with the intake of FA supplement (1000 μg) reaching 4 times the recommended intake (400 μg/day) [26]. The preparation of diets with low vitamin B12 levels (5 μg/kg) was founded in the percentage of adequacy of nutrient intake in humans [27]. For mice, we chose a 20% of the intake recommendation as a deficiency criterion [28]. The AIN-93 G diet is recommended for growth, pregnancy and lactation phases; AIN-93 M diet has a lower protein and fat content and is recommended for adult and mature animals [28].

2.3. Determination of food intake

The amount of food consumption was recorded every week in

Table 1
Composition of the diets.

	NFNB12		HFLB12	
	G	M	G	M
Calories (Kcal/g)	4.00	3.85	3.96	3.81
Carbohydrates (%)	63.9	75.9	63.9	75.9
Fats (%)	15.8	9.4	15.8	9.4
Proteins (%)	20.3	14.7	20.3	14.7
Folic Acid (mg/kg diet)	**2.0**	**2.0**	**8.0**	**8.0**
Vitamin B12 (μg/kg diet)	**25.0**	**25.0**	**5.0**	**5.0**

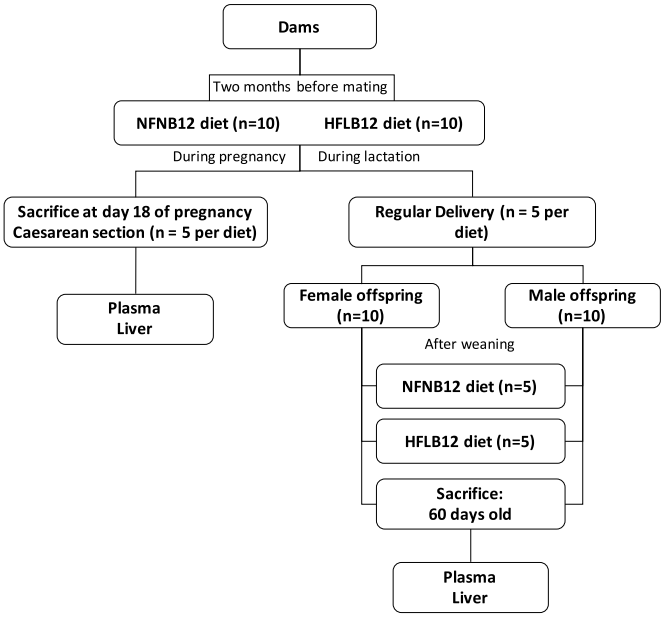

Fig. 2. Schematic representation of the experimental design and the feeding protocol for different groups.

offspring from weaning until 60 days old. Food lost was calculated subtracting spilled out amount from the original amount put in the recipient.

2.4. Liver and blood samples

Livers of mothers and offspring at 60 days old were extracted and weighed immediately after euthanasia. Then, livers were immediately frozen in liquid nitrogen and finally stored at −80 °C until determinations. Plasma was prepared from blood samples collected in EDTA as anticoagulant and stored frozen at −80 °C until processed.

2.5. Determination of total homocysteine

In order to confirm the effect of the HFLB12 diet, homocysteine levels were measured in the plasma of mothers and their offspring, by chemiluminescence in Barnafi Krause certified laboratory, Santiago, Chile (http://bklab.cl/test/homocisteina/).

2.6. Fatty acids analysis

The extraction of total hepatic lipids was carried out according to the method described by Bligh & Dyer [29]; pulverized livers (~200 mg per sample) were homogenized with a chloroform/methanol mixture (2:1 v/v) in a cold environment. Bidistilled water and 0.5 N $MgCl_2$ were added to the homogenized product and centrifuged at 0–4 °C (3000 rpm × 10 min) to collect the chloroform phase. Samples were then dried under a stream of nitrogen and fatty acid methyl esters (FAMEs) were prepared according to the technique described by Morrison and Smith [30]. The saponifiable lipids obtained from liver tissue were prepared with boron trifluoride (BF3, 12% methanolic solution) followed by treatment with NaOH saturated in methanol (0.5 N). Finally, fatty acids were extracted and collected to be quantified by gas-liquid chromatography (GC) in an Agilent Hewlett-Packard equipment (model 7890A, CA, USA). The fatty acids were identified by comparing their retention times, and using C23:0 fatty acid as an internal standard (Nu-Chek Prep Inc., Elysian MN, USA). Results were expressed as mg of fatty acids /g liver.

2.7. RNA extraction, cDNA synthesis and real-time PCR

Total RNA from dams and offspring livers was extracted with Trizol (Invitrogen, Carlsbad, CA) and chloroform according to manufacturer's instructions, and frozen in aliquots at −80 °C until cDNA synthesis. The RNA integrity of each sample was evaluated by a 260/280 ratio of 1.7 ± 1.9 and by electrophoresis in agarose gels. One microgram of total mRNA was used for the synthesis of cDNA with the M-MLV reverse transcriptase kit (RT, Promega, Wisconsin, USA) following manufacturer's instructions. For mRNA quantification of *PPARα, Cpt-1, Acox-1, Fas, Fasd-1 - Δ−6* and *Fasd-2 Δ−5 desaturases*, we used the Eco qPCR System (Illumina San Diego, CA, U.S.A) and sequence of primers described in **Table 2.** Results were analyzed by Eco Real-time PCR System Software v4.1 (Illumina) and calculated by the $-2^{\Delta\Delta CT}$ method [31]31 following the MIQE Guidelines [32]. Results were expressed in relation to the geometric mean expression of three of the most stable housekeeping genes (*Gapdh, Ywhaz* and *β-actin*), using the group fed the NFNB12 diet as control.

2.8. Determination of liver Δ−5 and Δ−6 desaturase activities

Liver samples frozen in liquid nitrogen were homogenized (500 mg) in a buffer solution pH7.9 containing 10 mmol/l HEPES, 1 mmol/l EDTA, 0.6% NonidetP-40, 150 mmol/l NaCl, and protease inhibitors (1 mmol/l phenyl methyl sulfonyl fluoride,1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mmol/l orthovanadate). Liver homogenates were centrifuged three times at 4 °C, first at 2000 rpm for 30 s, followed by centrifugation of the supernatants at 5000 rpm for 5 min, and finally at 100,000 rpm for 60 min, to obtain the extracts for desaturase activity measurements. The activity of Δ−6 desaturase was assayed by measuring the amount of 18:3, omega-6 produced from 18:2, omega-6, whereas that of Δ−5 desaturase was determined by the amount of 20:3, omega-6 converted to 20:4, omega-6, using albumin-bound fatty acid precursors (18:2, omega-6 and 20:3, omega-6) [33]. Desaturase activities were assayed in an incubation medium containing 4 mmol ATP, 0.1 mmol coenzyme-A, 1.28 mmol NADH, 2.42 mmol N-acetylcysteine, 0.5 mmol nicotinamide, 5 mmol $MgCl_2$, 62.5 mmol NaF, and 62.5 mmol phosphate buffer pH7, supplemented with 100 nmol albumin-bound FA

Table 2
Sequence of primers for real time PCR.

Gen	*Ta (°C)	Forward primer	Reverse primer
Pparα	60 °C	5′– AATGTGGCCAGGACATTGGA – 3′	5′–AACAAGTGCCAGCCAGGTTT – 3′
Cpt-1a	60 °C	5′– CCTCACCGCCAATTCCAAAA – 3′	5′ –AAGTCAAACGGTGGAGCCTA – 3′
Acox1	58 °C	5′– AAGCTACGTGCAGCCAGATT – 3′	5′–TTGCACGGCTCTGTCTTGAA – 3′
Fas	58 °C	5′ – GGAGGTGGTGATAGCCGGTAT – 3′	5′–TGGGTAATCCATAGAGCCCAG – 3′
Fasd1 (Δ5 desaturase)	60 °C	5′ – GCCTTCAACAACTGGTTCAGTG	5′ – CGTACTTGGCGCACAGGGATTG – 3′
Fasd2 (Δ6 desaturase)	60 °C	5′ – CCTTTGTCCTCGCTACCTCTCA	5′ – ATAGACAGAAAGGTGGCCATAGTCA – 3′
Actin	58 °C	5′ – AAGAAAGGGTGTAAAACGCA – 3′	5′ – CCGTAAAGACCTCTATGCCA – 3′
Ywhaz	57 °C	5′ – TTGATCCCCAATGCTTCGC – 3′	5′ – CAGCAACCTCGGCCAAGTAA – 3′
Gapdh	65 °C	5′ – AGGTCGGTGTGAACGGATTTG – 3′	5′ – TGTAGACCATGTAGTTGAGGTCA – 3′

* Ta: Annealing temperature.

precursor and 1 mg protein of cytosolic extract in a total volume of 1 ml, incubated at 37 °C for 30 min in a shaking bath. Δ−6 desaturase and Δ−5 desaturase assays were conducted simultaneously. The reaction was stopped by adding 6 ml of a cold methanol/chloroform mixture (2/1 v/v). Freshly prepared heptadecanoic acid (17:0; >99% purity) was added (20 mg) as an internal standard.

To determine the changes in the levels of products or precursors achieved after incubation, lipids were extracted and derivatized to fatty acid methyl esters (FAME), which were analyzed by gas–liquid chromatographic analysis, as described previously [34]. The individual fatty acid methyl esters (FAMEs) peaks were identified by comparing the retention times of the individual fatty acids from an authentic standard mixture and converted to concentration using the 17:0 internal standard. Δ−6 desaturase (net increase in 18:3, omega-6) and Δ−5 desaturase (net decrease in 20:3, omega-6) activities were calculated from GC-FID results as differences between baseline values and those obtained after 30 min incubation, and results were expressed as nmol/mg protein/min.

2.9. Data analysis

Data are expressed as median and 25–75th percentile. Mann–Whitney test was used to know differences between groups, control and treatment, and within sex between both groups. Statistical significance was considered at $p < 0.05$. The data was analyzed using Stata/IC 15.

3. Results

3.1. Determinations in dams

3.1.1. Blood homocysteine levels

This metabolite was determined to verify the well-known effect of HFLB12 diets on homocysteine circulating levels. As expected, dams treated with HFLB12 diets before and during pregnancy showed increased plasma Hcy levels (median ± IQR: 9.8 ± 3.0 μ mol/L) compared to NFNB12 diet (5.5 ± 5.4 μ mol/L, $p = 0.033$).

3.1.2. Hepatic fatty acid levels (mg/g liver)

There was no difference in the liver weights obtained from the NFNB12 group dams compared to the HFLB12-treated group (1.8 ± 0.3 vs 1.5 ± 0.4 g). The liver of dams fed HFLB12 registered lower fatty acid levels; mainly: total SFA, total MUFA, total PUFAS, total omega-3 and total omega-6 fatty acids compared to NFNB12. In addition, the DHA/linolenic acid and AA/linoleic acid ratios were higher and the omega-6/omega-3 ratio was lower in HFLB12 compared to the NFNB12 group (**Table 3**).

3.1.3. Expression of genes and enzymatic activities involved in lipid metabolism

In the HFLB12 group, the mRNA expressions of *PPARα, Cpt-1* and *Fasd1* were higher **(Fig. 3)**, and the enzymatic activities of both, *Δ−5 and Δ−6 desaturases* were lower **(Fig. 4)** compared to NFNB12.

3.2. Determinations in adult offspring (60 days old)

3.2.1. Blood homocysteine levels

Offspring fed a HFLB12 diet during gestation until 60 days old showed no changes in homocysteine levels compared to the NFNB12 group: the concentrations of Hcy in female offspring were 4.1 ± 3.1 μmol/L and 5.4 ± 1.7 μmol/L for the HFLB12 and NFNB12 respectively. In the male offspring, there were no significantly differences in circulating levels of Hcy (2.7 ± 1.6 μmol/L and 3.0 ± 2.2 μmol/L for the HFLB12 and control group respectively).

3.2.2. Hepatic fatty acid levels (mg/g liver)

Offspring from both groups showed no differences in their liver weights (female offspring: 0.9 ± 0.3 vs 1.0 ± 0.2 g for NFNB12 and HFLB12 respectively; and male offspring: 1.4 ± 0.3 vs 1.1 ± 0.4 g for NFNB12 and HFLB12 respectively). In general, offspring of the HFLB12 group had higher hepatic fatty acids levels compared to the control group and to their mothers. The effects observed in female offspring were more remarkable than in males. Female offspring of the HFLB12 group showed higher total SFA, MUFA, PUFAs, LC-PUFAs and DHA, AA, omega 3 and omega 6 fatty acids than the NFNB12 group (**Table 3**). In opposition to their mothers and to the NFNB12 control group, the HFLB12 group showed a lower DHA/linolenic acid and AA/linoleic acid ratios and a higher omega 6/omega 3 ratio. Male offspring of the HFLB12 group, showed higher total SFA, LC-PUFAs, AA and omega 6/omega 3 ratio than the NFNB12 group (**Table 3**).

3.2.3. Expression of genes and enzymatic activities involved in lipid metabolism

Female offspring from the HFLB12 group showed no changes in the mRNA expression of all genes tested **(Fig. 5A)**. In male offspring from the HFLB12 group, no effect was observed in the mRNA expression of all genes. However, *Fas*, has a tendency to be higher *($p = 0.0556$),* and the mRNA expression of *Fasd1* (Δ−5 desaturase) showed a tendency to be lower compared to the expression of the same gene in the liver of the NFNB12 group *($p = 0.0556$,* **Fig. 5B)**. In female offspring of the HFLB12 group**,** we found that the enzymatic activity of both, Δ−5 desaturase and Δ−6 desaturase, were higher compared to NFNB12 **(Fig. 6A)**. Interestingly, in male offspring´s livers, no changes in the enzymatic activities of both desaturases were observed (**Fig. 6B**).

A summary of the effect of the HFLB12 diet on fatty acid profile in liver of dams and offspring is shown in **Table 4**. Also, a summary of the effects of the HFLB12 diet on the expression (mRNA) of genes and enzymatic activities related to lipid metabolism in liver of dams and offspring is shown in **Table 5**.

4. Discussion

In this study we have found that dietary imbalance between folates and vitamin B12 (HFLB12) from gestation to adulthood modifies, hepatic metabolism of lipids. The effects of such unbalanced diet were observed in both, dams and offspring at adulthood, with a higher incidence in females than in males. Interestingly, the effects of HFLB12 diets in female offspring were opposite to those found in dams. In male offspring, minor effects of this HFLB12 diet were observed, thus suggesting a sexual dimorphism response to these unbalanced diets.

Diets supplemented with adequate levels of folic acid, vitamin B12 and DHA in dams during pregnancy have positive effects on body composition and glucose metabolism in offspring [35]35, in addition to normal expression of hepatic transcription factors like PPARα, SREBP-1 and RXRα [36] and methylation patterns [37]37. On the contrary, consumption of low folate and vitamin B12 levels, or a high folate/low vitamin B12 ratio in the diet - also reflected in circulating levels during pregnancy- have been related to adverse effects in the offspring [22,38,22,38,39].

In the present study, when compared to a control diet, the HFLB12 diet given to pregnant females reduced their hepatic levels of total SFA, MUFA, PUFA (mainly EPA), total n3 and total n6. However, the conversion to DHA and AA from their precursors, indicated by the ratios DHA/ALA and AA/LA respectively, was higher. Conversely, sixty days old female offspring fed with the HFLB12 (from the womb to adulthood), showed an increase in total SFA, MUFA, PUFA and LC-PUFAs (predominantly EPA and AA), including total n3 and n6. However, the ratios DHA/ALA and AA/LA, were lower. These opposite effects between mother and offspring suggest that the higher levels of total fatty acids found in the female offspring liver may be due to a fetal programming mechanism leading to increased synthesis, except for DHA

Table 3
Fatty Acid profile in liver of dams and offspring (female and male) (mg /g liver).

Fatty acid	Dams NFNB12 Diet	Dams HFLB12 Diet	*p*value	Female offspring NFNB12 Diet	Female offspring HFLB12 Diet	P Value	Male offspring NFNB12 Diet	Male offspring HFLB12 Diet	*p*value
C10:0 Decanoic acid	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–
C12:0 Dodecanoic Acid	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–
C14:0 Tetradecanoic acid	0.41 ± 0.21	0.08 ± 0.05	**0.0281**	0.059 ± 0.03	0.0245 ± 0.09	**0.0026**	0.16 ± 0.0 9	0.195 ± 0.076	0.1573
C16:0 Palmitic Acid	20.59 ± 4.36	4.804 ± 1.57	**0.0281**	4.023 ± 1.83	1.102 ± 3.71	**0.0007**	7.718 ± 1.77	9.467 ± 3.43	**0.0451**
C18:0 Stearic Acid	4.98 ± 0.43	2.132 ± 1.41	**0.0281**	2.323 ± 0.68	3.948 ± 0.95	**0.0055**	2.645 ± 0.16	3.48 ± 0.43	**0.0022**
C20:0 Eicosanoic Acid	0.017 ± 0.02	0 ± 0	0.1526	0 ± 0	0.017 ± 0.015	**0.0024**	0.084 ± 0.007	0.06 ± 0.027	0.4094
C22:0 Docosanoic acid	0.0.7 ± 0.002	0 ± 0	**0.0109**	0 ± 0	0.028 ± 0.034	**0.0053**	0.048 ± 0.006	0.0.9 ± 0.0 15	0.5557
C24:0 Tetracosanoic Acid	0.031 ± 0.007	0 ± 0	**0.037**	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–
Total Saturated Fatty Acids	26.058 ± 5.03	7.259 ± 2.704	**0.0281**	6.406 ± 2.405	14.696 ± 4.54	**0.0012**	10.636 ± 1.5	13.306 ± 4.58	**0.0251**
C14:1 Tetradecenoic acid	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–
C16:1 Palmitoleic Acid	2.13 ± 1	0.46 ± 0.11	**0.0281**	0.466 ± 0. 13	2.137 ± 0.84	**0.0007**	1.478 ± 0.57	1.365 ± 0.34	1.0000
C18:1n9 Oleic Acid	24.644 ± 1.19	3.057 ± 0.96	**0.0281**	3.865 ± 0.7	15.275 ± 6.33	**0.0012**	8.52 ± 3.79	10.556 ± 6.37	0.0771
C20:1n9 Eicosaenoic Acid	0.264 ± 0.11	0 ± 0	**0.017**	0.042 ± 0.017	0.0172 ± 0.08	**0.0307**	0.188 ± 0.10	0.243 ± 0.07	0.2386
C22:1n9 Erucic acid	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–	0.021 ± 0.0	0.016 ± 0.0	0.4094
C24:1 Tetrakosenoic Acid	0.035 ± 0.0	0 ± 0	**0.0068**	0.065 ± 0.03	0.056 ± 0.03	0.2794	0.033 ± 0.008	0.038 ± 0.0 0	0.0990
Total Monounsaturated Fatty Acid	27.524 ± 1.44	3.505 ± 1.01	**0.0281**	4.423 ± 0.77	17.663 ± 7.20	**0.0012**	10.261 ± 4.45	12.211 ± 6.41	0.1573
C18:2n6 Linoleic Acid (LA)	10.803 ± 1.03	2.139 ± 1.16	**0.0281**	3.925 ± 1.39	9.30 ± 6.5	**0.002**	5.882 ± 1.91	7.61 ± 3.03	0.0990
C18:3n6 Gamma Linolenic Acid (GLA)	0.536 ± 0.2	0.119 ± 0.08	**0.0281**	0.044 ± 0.037	0.138 ± 0.19	**0.0087**	0.075 ± 0.0 02	0.081 ± 0.033	0.4795
C18:3n3 Linolenic Acid (ALA)	0.0411 ± 0.0 9	0.046 ± 0.05	**0.019**	0.127 ± 0.05	0.478 ± 0.46	**0.0016**	0.256 ± 0.15	0.312 ± 0.21	0.4094
C20:2n6 Eicosadienoic acid	0.0124 ± 0.01	0.044 ± 0.0 66	0.1054	0.083 ± 0.033	0.017 ± 0.052	**0.0055**	0.0119 ± 0.0 25	0.0129 ± 0.012	0.5557
C20:3n6 Dihomo-gamma-linolenic acid (DGLA)	0.026 ± 0.03	0.069 ± 0.06	**0.0192**	0.229 ± 0.11	0.422 ± 0.086	**0.0016**	0.44. ± 0.0 5	0.478 ± 0.16	0.4094
C20:3n3 Eicosatrienoic acid	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–	0 ± 0	0 ± 0	–
C20:4n6 (AA)	4.128 ± 0. 63	3.06 ± 1.43	0.0790	3.041 ± 0–88	4.07 ± 1.19	**0.0168**	3.071 ± 0.333	3.542 ± 0.19	**0.0133**
C20:5n3 EPA	0.064 ± 0.05	0 ± 0	**0.0160**	0.035 ± 0.07	0.117 ± 0.07	**0.004**	0.129 ± 0.02	0.118 ± 0.04	0.6374
C22:5n3 DPA n-3	0.19 ± 0.02	0.086 ± 0.07	**0.0192**	0.061 ± 0.04	0.117 ± 0.07	**0.0034**	0.0134 ± 0.03	0.155 ± 0.06	0.0771
C22:6n3 DHA	4.063 ± 0.51	2.213 ± 0.98	**0.0570**	2.151 ± 0. 52	2.625 ± 0.85	**0.0538**	2.189 ± 0.35	2.052 ± 0.254	0.9062
Total Polyunsaturated Fatty Acid	20.80 ± 2.36	7.51 ± 3.44	**0.0281**	9.711 ± 3.35	16.594 ± 8.69	**0.0026**	12.336 ± 2.16	14.62 ± 4.77	0.0990
Total LC-PUFAs	9.007 ± 1.1	5.325 ± 2.34	**0.0570**	5.644 ± 1.78	7.657 ± 2.0	**0.0136**	6.249 ± 0.28	6.6.5 ± 0.39	**0.0184**
Total n3	4.762 ± 0.6	2.379 ± 1.06	**0.0281**	2.369 ± 0.77	3.254 ± 1.34	**0.0308**	2.736 ± 0.49	2.673 ± 0.76	0.6374
Total n6	16.039 ± 1.77	5.161 ± 2.41	**0.0281**	7.343 ± 2.43	13.543 ± 7.22	**0.0020**	9.557 ± 1.75	11.947 ± 4.11	0.0593
EPA + DHA	4.18 ± 0.51	2.224 ± 0.99	**0.0404**	2.188 ± 0.55	2.713 ± 0.87	0.0538	2.318 ± 0.37	2.194 ± 0.25	0.9062
DHA/Linolenic Acid Ratio	0.101 ± 0.0 1	0.46 ± 0.12	**0.0446**	0.157 ± 0.03	0.049 ± 0.05	**0.0009**	0.077 ± 0.03	0.078 ± 0.0 4	0.5557
EPA/Linolenic Acid Ratio	0.002 ± 0	0.001 ± 0.01	0.4601	0.001 ± 000	0.002 ± 00	0.5863	0.005 ± 0.002	0.004 ± 0.003	0.7237
DHA + EPA/Linolenic Acid Ratio	0.102 ± 0.01	0.465 ± 0.12	**0.0446**	0.159 ± 0.03	0.051 ± 4.9	**0.0009**	0.08. ± 0.03	0.08. ± 0.04	0.5557
AA/Linoleic Acid Ratio	0.004 ± 0	0.014 ± 000.4	**0.0192**	000.8 ± 0.001	0.004 ± 000.2	**0.0016**	0.005 ± 0.001	0.006 ± 0.002	1.0000
n3/n6 Ratio	0 ± 0	0 ± 0	**0.0281**	0 ± 0	0.1 ± 0	**0.0026**	0 ± 0	0 ± 0	**0.0451**

Data are presented as median and interquartile range.
bold digits are results with statistical significance, with a p lower than 0.05.

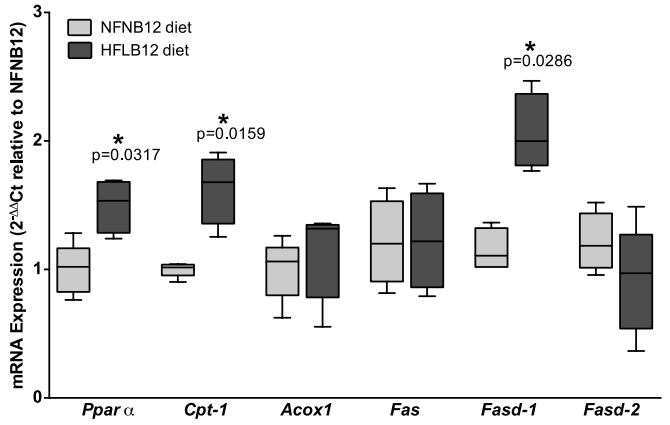

Fig. 3. mRNA expression of *PPARα, Cpt-1, Acox, Fas, Fasd1* (Δ−5 desaturase) and *Fasd2* (Δ−6 desaturase) in liver of dams (n = 5 per group). Results are expressed as box and whisker; the box represent the 25th percentile, 50th percentile and 75th percentile and the whisker represent minimum and maximum values. *$p < 0.05$; HFLB12 compared to NFNB12; Mann–Whitney Test.

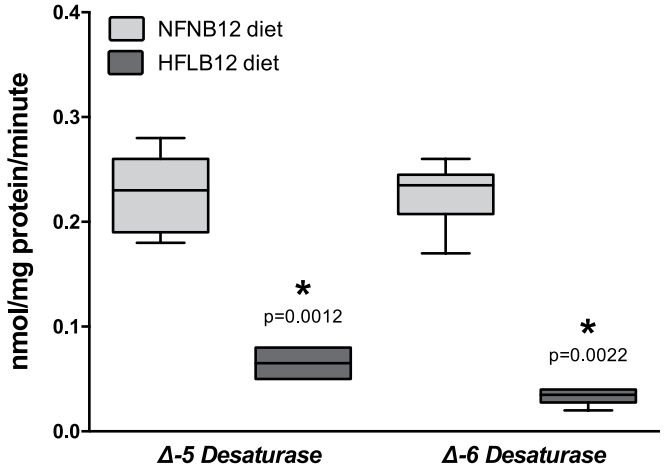

Fig. 4. Δ−5 and Δ−6 desaturase activities in dams. Results are expressed as box and whisker, the box represent the 25th percentile, 50th percentile and 75th percentile and the whisker represent minimum and maximum values. *$p < 0.05$; HFLB12 compared to NFNB12; Mann–Whitney Test.

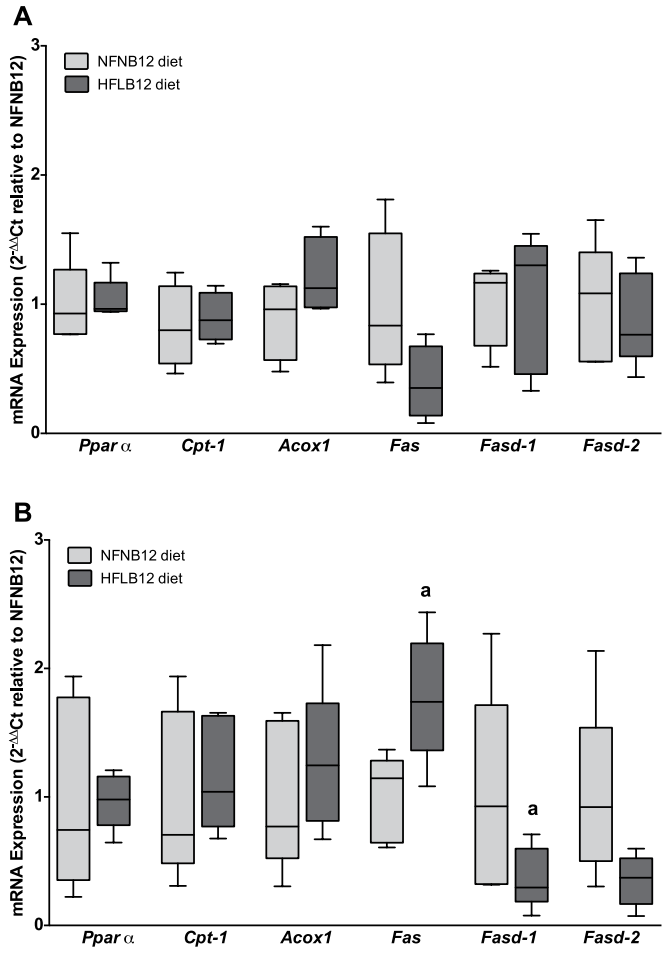

Fig. 5. (A) mRNA expression of *PPARα, Cpt-1, Acox, Fas, Fasd1* (Δ−5desaturase) and *Fasd2* (Δ−6 desaturase) in liver of female offspring. Results are expressed as box and whisker, the box represent 25th percentile, 50th percentile and 75th percentile and the whisker represent minimum and maximum values. Mann–Whitney Test. (B) mRNA expression of *PPARα, Cpt-1, Acox, Fas, Fasd1* (Δ-5 desaturase) and *Fasd2* (Δ-6 desaturase) in liver of male offspring. Results are expressed as box and whisker, the box represent 25th percentile, 50th percentile and 75th percentile and the whisker represent minimum and maximum values. [a]p = 0.0556; HFLB12 compared to NFNB12; Mann Whitney Test.

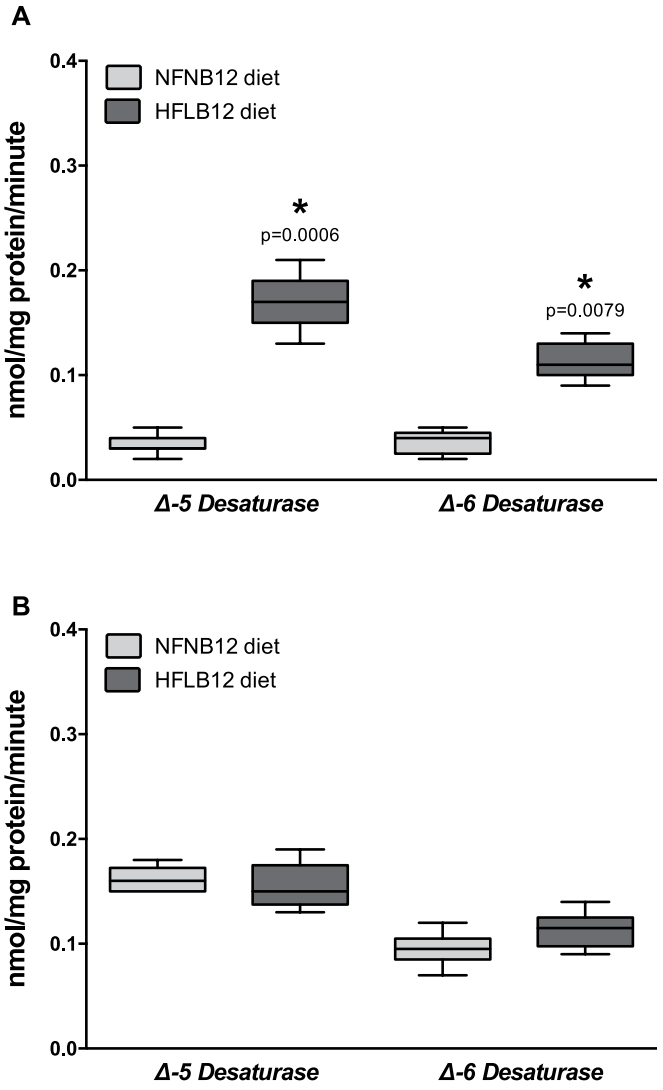

Fig. 6. (A) Δ−5 and Δ−6 desaturase activities in liver of female offspring. Results are expressed as box and whisker, the box represent 25th percentile, 50th percentile and 75th percentile and the whisker represent minimum and maximum values. *$p = 0.0006$ and *$p = 0.0079$ for Δ−5 and Δ−6 desaturase activities respectively in HFLB12 compared to NFNB12; Mann–Whitney Test. (B) Δ−5 and Δ−6 desaturase activities in liver of male offspring. Results are expressed as box and whisker, the box represent 25th percentile, 50th percentile and 75th pe rcentile and the whisker represent minimum and maximum values. Mann–Whitney Test.

and AA or, alternatively, to an enhanced capability of lipids transport from the circulation to the liver, or both.

As expected, direct measurements of hepatic Δ−5 and Δ−6 desaturases activities were decreased in mothers and elevated in female offspring, a finding concordant with the respective amount of fatty acids found in liver of mothers and female offspring. It is worth to mention that the effects observed in female offspring were not completely replicated in male offspring, with the exemption of SFA, AA and total LC-PUFA. In addition, changes in the enzymatic activities of hepatic Δ−5 and Δ−6 desaturases in male offspring were not observed. These results indicate that exposure to a HFLB12 diet from early gestation to adulthood affects in a different manner the lipid metabolism in liver of males and females, indicating sexual dimorphism in response to this diet.

In a previous study, Wadhwani et al. [40], found that hepatic tissue from pregnant Wistar rats (at the 20th day of pregnancy) fed with a diet containing high FA levels (8 mg/kg diet) and without vitamin B12 (0 μg/kg diet), showed higher total MUFA but lower DHA levels than rats fed with control diets. These results are different to our results in mice fed with diets containing the same FA concentrations but different concentrations of vitamin B12 (0 vs 5 μg vitamin B12 /kg diet). However, in that study [40], the activity of Δ−5 desaturase (expressed as 20:4n6/20:3n6 ratio) in offspring was higher, in agreement with the results obtained in this study. Here, it is worth to note that we carried out direct determinations of desaturases activities, and thus, strengthening the validity of the present results.

In other study, same authors analyzed the expression of Δ−5 desaturase and the fatty acid transporter proteins (*Fatp1* and *Fatp4*) in placenta of females fed with the same unbalanced diet, and found decreased levels of both molecules compared to the control group [41]41. Those results indicate that the placenta also synthetizes LC-PUFAs, but the imbalance of FA/vitamin B12 decreased both, the synthesis and transport of fatty acids to the fetus. Unfortunately, we could not be able to analyze the fatty acid content in placentas, which can be considered as a weakness of our study.

Additionally, in the present study, the product/precursor ratio of DHA and AA was higher in dams fed the HFLB12 diet, suggesting that during pregnancy, their livers increase the LC-PUFAs to ensure the

Table 4
Summary of the effect of HFLB12 related to NFNB12 diets on fatty acid profile in dams and offspring.

Fatty acids (mg/ g liver)	Dams	Female offspring	Male offspring
Total Saturated Fatty Acids	↓	↑	↑
Total Monounsaturated Fatty Acid	↓	↑	=
Total Polyunsaturated Fatty Acid	↓	↑	=
Total LC-PUFAs	=[a]	↑	↑
Total n3	↓	↑	=
Total n6	↓	↑	=[b]
C22:6n3 DHA	=[c]	=[d]	=
C20:4n6 (AA)	=	↑	↓
DHA/Linolenic Acid Ratio	↑	↓	=
AA/Linoleic Acid Ratio	↑	↓	=
n6/n3 Ratio	↓	↑	↑

[a] $p = 0.057$.
[b] $= p = 0.059$.
[c] $= 0.057$.
[d] $= 0.0538$.

Table 5
Summary of the effect of HFLB12 related to NFNB12 diets on the enzymatic activities and expression (mRNA) of hepatic Δ−5 and Δ−6 desaturases and fatty acid ratios in dams and offspring.

Lipid metabolism in liver		Dams	Female offspring	Male offspring
mRNA expression	*Pparα*	↑	=	=
	Cpt-1	↑	=	=
	Acox1	=	=	=
	Fas	=	=	=[a]
	Fasd1	↑	=	=[b]
	Fasd2	=	=	=
Enzymatic Activity	Δ−5 desaturase	↓	↑	=
	Δ−6 desaturase	↓	↑	=
Fatty acids ratio of desaturases	Δ−5 desaturase: AA/DGLA Ratio	↑	↓	=
	Δ−6 desaturase: DGLA/LA Ratio	=	=	=

[a] $\uparrow p = 0.0556$.
[b] $\downarrow p = 0.0556$.

transport of adequate levels of omega-3 to the fetus through the placenta, as a compensatory mechanism of being exposed to a HFLB12 diet. This statement is justified by the lower levels of LC-PUFAs in the HFLB12 group of dams (5.325 vs 9.007 mg/g liver in NFNB12) meaning that more LC-PUFAs are being transferred to the fetus in this group. In addition, these results are in agreement with a higher expression of *Fasd1* observed in the liver of HFLB12 dams. No changes in the expression of *Fasd2* were observed, and the enzymatic activities of both hepatic desaturases were lower compared to those activities found in liver from animals fed a NFNB12 diet. This last result may be explained as a feed-back regulation by products (AA and DHA), and/or, alternatively by posttranscriptional modifications of *Fasd1*.

Meher et al. [36], found that in liver of 21 days old rat pups from dams fed a diet deficient in both, FA and vitamin B12 that AA, DHA and total SFA were lower compared to pups from dams fed with a control diet [36]. Other authors [36,36,42], have also found a lower expression of PPARα, PPARγ and CPT-1 in liver of rat offspring (21 days and 12 weeks old) exposed to vitamin B12 deficient diets before and during pregnancy. Those results however, does not give information related to the sex of the animals. Female and male offspring could display a different response *in-utero* with respect to timing, onset and severity of outcomes, while their mothers are exposed to diets containing unbalanced levels of folic acid and vitamin B12 during pregnancy.

Previous studies have reported other biological responses to FA supplementation with or without vitamin B12 to pregnant mice, such as a higher adiposity and altered vascularity in male offspring [43]. Those authors also reported that adult female offspring from dams fed with diets containing elevated FA and without vitamin B12, presented fasting hyperglycemia and glucose intolerance [44].

The relevance of this study for humans is related to a public policy present in some countries in relation to food fortification with folic acid. This policy implemented to prevent fetal NTD, has raised the levels of FA consumption in the whole population, but especially in pregnant mothers, in which levels are even higher due to the additional intake of FA supplements (almost 1000 μg/day, the upper limit). In the last years, this policy has been questioned since it has been related to health impairment in newborns and elderly [45,46]. Arias et al., found that in childbearing age women supplemented with the FA concentration currently recommended by the international health organisms (400 μg/day), circulating vitamin B12 levels decreased [47], suggesting that high FA levels may affect the vitamin B12 metabolism[22,38]. Also it has been reported that 6 years old children born to mothers with high erythrocyte folate (RBC > 1100 μmol/L) and low plasma vitamin B12 (>114 pmol/L) show higher susceptibility to insulin resistance (IR-HOMA > 0.7) compared to children born to mothers with normal RBC folate and normal plasma vitamin B12 [39]. Moreover, we have previously reported that serum cord blood from preterm newborns had higher folate levels (66 μmol/L, 25–219 μmol/L) and low vitamin B12 (219 pmol/L, 97–868 pmol/L), resulting in a significantly higher ($p = 0.002$) serum folate/vitamin B12 ratio (305, 181–428) compared to term newborns (141, 86–216) [48]. Thus, food fortification with high FA levels as in Chile (1.8–2.6 mg/kg product), could be related to premature births [49]. At present, however, we do not know whether these children have changes in the concentration of LC-PUFAs, or if they have an altered hepatic lipid metabolism as a consequence of in-utero exposure to an environment in some way conditioned by the imbalance in those vitamins. We do not know either about long term sequels which may be presumably involved in any unhealthy condition during adulthood.

In summary, this study confirms previous reports suggesting a metabolic interconnection between folates and vitamin B12 with total and omega-3 fatty acids. Our results reveal that diets with unbalanced levels of folates/vitamin B12 during pregnancy modify the lipid metabolism in the liver of pregnant females and in their offspring if this diet is maintained until adulthood. These changes observed in the pregnant females may be triggered by induction of mechanisms aimed to compensate maternal levels of essential fatty acids, which are altered by the dietary folates/B12 imbalance. In this way, it could be ensured that adequate levels of lipids are transferred to the fetus through the placenta, thus limiting dietary deficiencies during fetal development and potential epigenetic alterations; and hence avoiding abnormal fetal programming as a consequence of excess/deficiency of specific vitamin nutrients [50,50,51]. Therefore, finding of specific biomarkers of lipid metabolism in mother's blood and placenta, as a close representative of fetal tissue, is warranted. These determinations will permit to infer whether these effects could also occur in humans and explore associations to potential undesirable long-term consequences.

5. Conclusion

Taken together these results demonstrate that unbalanced diets in folates/vitamin B12 (high folates and low vitamin B12) given during gestation modifies hepatic lipid metabolism of dams and adult offspring when this diet is maintained. The effects observed in dams include decreased levels of all hepatic fatty acids SFA; MUFA, PUFA and LC-PUFA; PUFA and LC-PUFA decrease may be explained by a mechanism involving a decreased activity of both desaturase enzymes (Δ−5 and Δ−6). An opposite effect was observed in the offspring, with special emphasis on females, where hepatic SFA, MUFA, PUFA and LC-PUFA increased with the dietary treatment, as did the Δ−5 and Δ−6 desaturase activities

CRediT authorship contribution statement

Erika Castaño-Moreno: Conceptualization, Methodology, Software, Writing - original draft. **Valeska Castillo:** Software, Methodology, Validation. **Reyna Peñailillo:** Visualization, Investigation. **Miguel N. Llanos:** Investigation, Writing - original draft, Writing - review & editing. **Rodrigo Valenzuela:** Conceptualization, Methodology, Validation, Funding acquisition. **Ana María Ronco:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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