RESEARCH ARTICLE

Maternal supplementation with an excess of different fat sources during pregnancy and lactation differentially affects feeding behavior in offspring: Putative role of the leptin system

Juana Sánchez, Teresa Priego, Ana Paula García, Marina Llopis, Mariona Palou, Catalina Picó and Andreu Palou

Molecular Biology, Nutrition and Biotechnology (Nutrigenomics), University of the Balearic Islands (UIB), and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Spain

Received: April 13, 2012
Revised: August 7, 2012
Accepted: August 14, 2012

Scope: This study investigates the lasting effects of maternal supplementation with different fat sources during pregnancy and lactation on feeding behavior and energy homeostasis of their offspring, and its relation to hypothetical effects in the development of main central structures involved in leptin signaling.

Methods and results: Offspring of dams supplemented with olive oil, butter, or margarine during late pregnancy and lactation were fed with normal fat (NF) diet until 4-month-old, and then with NF or high fat (HF) diet until 6-month-old. Results showed that 21-day-old margarine group pups presented a higher cell number in the arcuate nucleus (ARC) (females) and higher hypothalamic ObRb/SOCS3 mRNA ratio (males). In adulthood, and under HF diet, they displayed a lower body weight (both genders) and body fat (males) than the butter group, a lower preference for fat food (both genders), and lower leptin levels than the olive oil (both genders) and butter (males) groups.

Conclusions: Maternal supplementation with different fat sources during the perinatal period may affect the development of hypothalamic structures and hence predisposition to obesity. Margarine, compared with other fats, may program the offspring for increased leptin sensitivity and a lower preference for fat food, thus providing relative protection against body weight gain in adulthood, particularly under an obesogenic environment.

Keywords:
Butter / Margarine / Metabolic programming / Obesity / Olive oil

1 Introduction

Human epidemiologicaland experimental animal studies have shown that maternal nutrition during the perinatal period, and particularly both undernutrition and overnutrition, influences the susceptibility to diet-related chronic diseases in adulthood, including cardiovascular diseases, type 2 diabetes, obesity, and cancer [1–3]. In this sense, maternal prenatal undernutrition has been described to have long-term consequences on offspring metabolic energy regulatory systems increasing the propensity to develop obesity [4–8]. In contrast, moderate caloric restriction during lactation in rat dams has been associated in the offspring with a moderate protection against obesity development in adult life and obesity-related metabolic alterations, particularly dyslipidemia, insulin resistance, and hyperleptinemia, associated with high fat (HF) diet feeding [9, 10].

In addition to the amount of energy, differences in the macronutrient composition of maternal diet could also elicit different outcomes in the offspring. For instance, offspring of dams exposed to a low protein diet during pregnancy are more prone to exhibit hypertension, altered glucose handling,

Correspondence: Prof. Catalina Picó, Universitat de les Illes Balears, Biología Molecular, Nutrición y Biotecnología (Nutrigenómica), Edifici Mateu Orfila. Carretera de Valldemossa Km 7.5, 07122-Palma de Mallorca, Spain
E-mail: cati.pico@uib.es
Fax: +34 971173426

Abbreviations: **αMSH**, melanocyte-stimulating hormones alpha; **AgRP**, agouti-related protein; **ARC**, arcuate nucleus; **CHO**, carbohydrate; **HF**, high fat; **NF**, normal fat; **NPY**, neuropeptide Y; **ObRb**, leptin receptor; **POMC**, pro-opiomelanocortin; **SOCS3**, the suppressor of cytokine signaling 3

higher abdominal fat [11], and present enhanced preferences for fat in adulthood [12]. Similarly, prenatal high dietary protein exposure also results in lower energy expenditure and higher adiposity at juvenile age in the offspring [13]. Other studies have also shown that offspring of dams fed a HF diet or overfeeding a varied, high fat and highly palatable "cafeteria diet" during pregnancy and/or lactation present greater adiposity and body weight [14–17]. Thus, it can be generally recognized that an early exposure to an excess of fat may have negative effects in the late metabolic health of the offspring, especially during fetal life and infancy, which are periods showing the highest adaptability and vulnerability to external factors. However, in addition to the amount of fat, the type of fat eaten during this period may also be determinant and, to this regard, changes in the n3/n6 ratio in breast milk have been proposed to contribute to the increasing prevalence of childhood overweight and obesity [18–21]. Apart from these studies, a comparison of the programming effects of the intake of an excess of different types of fat during gestation and lactation on later energy homeostasis has not been performed.

The hypothalamus plays a critical role in energy homeostasis, integrating leptin and other metabolic signals. Key targets of leptin action are the orexigenic neurons within the hypothalamic arcuate nucleus (ARC) that coexpress neuropeptide Y (NPY) and agouti-related protein (AgRP), which are inhibited by leptin, and anorexigenic pro-opiomelanocortin (POMC) neurons, which, by contrast, are activated by leptin [22]. Knowing the importance of the hypothalamus and of leptin signaling in the central regulation of energy balance, it seems reasonable to think that alterations of these structures and systems occurring during early life may result in permanent changes that could be relevant for the control of energy balance, and some data support such hypothesis [7, 23]. In this sense, a decrease in the number of cells in the hypothalamus occurring in the offspring of rat dams exposed to caloric restriction during gestation, has been proposed as a mechanism that predisposes to later insulin and leptin resistance and is responsible for higher propensity to overweight gain [5, 7]. NPY release in the hypothalamic paraventricular nucleus, food intake, and dietary preferences have been shown to be affected by the type of diet (rich in carbohydrates or fat) of the mother during pregnancy and lactation [23].

Here, we aimed to assess whether supplementation of maternal diet with an excess of different fat sources during the perinatal period may have different consequences on feeding behavior and energy homeostasis in the offspring and whether these different outcomes may be related with the development/maturation of the main central structures involved in leptin signaling. The selected three fat sources are those commonly consumed in the European diets, which are rich in different fatty acid types: butter (rich in saturated fatty acids), olive oil (rich in monounsaturated fatty acids, MUFA), and margarine (rich in polyunsaturated fatty acids, PUFA).

Table 1. Fatty acid composition of fat sources used in the experimental design. Data are mean of three different measurements expressed as a percentage of total fat

Fatty acids	Olive oil	Margarine	Butter
C10:0	nd	nd	2.06
C12:0	nd	4.56	6.44
C14:0	0.63	1.54	15.7
C16:0	12.7	13.9	36.9
C18:0	0.91	1.28	10.7
C20:0	nd	nd	0.77
C23:0	nd	nd	0.86
SFA	14.3	21.2	73.5
C16:1n7	1.05	nd	1.53
C18:1n7	1.87	1.04	0.42
C18:1n9c	70.6	20.9	16.5
C24:1	nd	1.72	0.61
MUFA	74.0	23.8	19.8
C18:2n6	7.70	41.9	1.17
C18:3n6	nd	nd	0.64
C18:3n3	1.02	5.52	0.75
CLA 9,11	nd	nd	0.79
C20:3n6	nd	nd	0.12
C22:5n3	nd	1.48	0.52
PUFA	8.72	49.0	3.99
$n-6$	7.70	42.0	1.93
$n-3$	1.02	7.01	1.27
$n-6/n-3$	7.53	5.99	1.51

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; nd, nondetectable levels.

2 Material and methods

2.1 Animals and experimental design

Virgin female Wistar rats (Charles River Laboratories Spain, SA, Barcelona, Spain), housed at 22°C with a period of light/dark of 12 h (lights on from 08:00 to 20:00 h) and with free access to food and water, were mated with male rats. Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and then female rats were single caged. Pregnant rats were supplemented with 30% of the normal caloric intake of a group of dams fed ad libitum in the form of different fat sources (olive oil, margarine, or butter) from day 14 of pregnancy to day 21 of lactation. According to the labeling, butter used in the study contained 0.5% carbohydrates and 0.8% protein (w/w), in addition to fat. The fatty acid profile of the different fat sources was determined as described bellow and is shown in Table 1. The offspring of these animals are referred to as olive oil, margarine, and butter groups, respectively. The offspring of dams fed ad libitum (control group) were also followed. Supplementation of the different fat sources was carried out by gavage once a day. At day 1 after delivery, excess pups in each litter were removed to keep ten pups per dam (five males and five females, when possible). At day 6 and 12 of lactation, blood samples (from the saphenous vein) and milk samples (as previously described, [24]) were obtained

from dams. At day 12 of lactation, blood samples (from the end of the tail) were also collected from pups and they were pooled by sex per litter.

At weaning (21 days of life), some pups from the control, olive oil, margarine, and butter groups were killed by decapitation during the first 2 h of the beginning of the light cycle. After killing the animals, the hypothalamus was rapidly removed by using the following landmarks, i.e., frontal edge of the optical chiasm, lateral sulci, caudal edge of the mammary bodies, and a depth of 2 mm. Samples were immediately frozen in liquid nitrogen and stored at −70°C until RNA analysis. Blood was also collected and centrifuged at 1000×g for 10 min to collect the serum, which was stored at −20°C until analysis.

A second set of animals was kept alive and fed with standard diet until the age of 4 months; then they were divided into two groups: one group continued under normal fat (NF) diet (standard diet containing 3.2 Kcal/g, with 2.9% calories from fat) and the other group was exposed to a high fat (HF) diet (containing 4.7 Kcal/g, with 45% calories from fat, Research Diets, Inc., NJ, USA). The HF diet contained 5.5% calories from soybean oil and 39.5% from lard.

Body weight and food intake of dams during gestation and lactation and the offspring until the age of 6 months were followed. In addition, body fat content (by EchoMRI-700™, Echo Medical Systems, LLC, TX, USA) of dams at weaning and of the offspring at different ages was measured.

Blood samples were collected from the offspring at different ages (4 and 6 months) under feeding conditions. Plasma was obtained by centrifugation of blood at 1000×g for 10 min, and stored at −20°C until analysis.

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University, and national guidelines for the use and care of laboratory animals of our university were followed.

2.2 Two-bottle preference test

Food preferences were assessed in adult animals (when they were 3 months old) by the two-bottle preference test, as previously described [25]. Briefly, the rats had to choose between two bottles containing either a carbohydrate (CHO)-rich liquid diet or a fat-rich liquid diet. The two diets had identical caloric density (2.31 kcal/g) and the following ingredients: for the CHO-rich diet, 10 g/100 mL skimmed milk, 40 g/100 mL sucrose, 4 g/100 mL olive oil, and 0.35 g/100 mL xanthan gum (Sigma, Madrid, Spain); and for the fat-rich diet, 10 g/100 mL skimmed milk, 10 g/100 mL sucrose, 17.3 g/100 mL olive oil, and 0.35 g/100 mL xanthan gum. Before the test started, and during a period of 8 days, the rats were habituated to each bottle given individually on alternate days for 1 h, without withdrawing the standard chow diet. The test was started 2 days after the adaptation period. Solid food was withdrawn at the beginning of the light phase. Two bottles containing preweighed quantities of either the CHO- or fat-rich diet were placed side-by-side 4 h after the beginning of the light cycle for 1 h. The bottles were then reweighed, and the intake of each diet was determined and corrected for spillage. Spillage was estimated by weighing small collection plates placed underneath the spout of the bottles.

2.3 Fatty acid analysis of the different fat sources

Total lipid was simultaneously saponified and methylated according to the method of Bondia-Pons et al. [26]. Ten microliters of olive oil, margarine, or butter diluted with hexane were transesterified in tubes containing 2.5 μg of the internal standard (tridecanoic acid from Sigma, St. Louis, MO, USA), by adding 0.25 mL sodium methylate reagent (0.5 M) (Flucka, Barcelona, Spain) and heated to 80°C for 10 min after vigorous shaking. After cooling down to 25°C, samples were esterified with 0.25 mL of boron trifluoride–methanol reagent (Sigma) also at 80°C for 3 min. Once the tubes were cooled, fatty acid methyl esters (FAMEs) were isolated by adding 150 μL of n-hexane (Merck, Darmstadt, Germany). After shaking for 1 min, 200 μL of a saturated sodium chloride solution was added. Afterwards, the tubes were centrifuged for 10 min at 4000 rpm. After drying with anhydrous sodium sulphate (Flucka), a 90 μL aliquot of the clear n-hexane top layer was transferred into an automatic injector vial equipped with a volume adapter of 300 μL and used for GC.

GC analyses were performed on HP-5890 Series GC System (Hewlett-Packard, Waldbronn, Germany) equipped with a flame ionization detector and a HP-5890 Series Injector. Separation of FAMEs was carried out in a capillary column (40 m × 0.18 mm id, 0.10 μm) coated with Rtx-2330 nonbonded stationary phase (poly 90% biscyanopropyl-10% cyanopropylphenyl) siloxane from Thames Restek UK (Saunderton, UK). Operating conditions were as follows: the split–splitless injector was used in splitless mode. The injection volume of the sample was 1 μL. The injector and detector temperatures were kept at 250 and 270°C, respectively. The temperature program was as follows: initial temperature 140°C, increased at 20°C/min to 170°C, and held at this temperature for 20 min and increased at 10°C/min to 230°C and held at this temperature for 5 min (total run time: 31 min). Helium was used as the carrier gas, with a head pressure of 300 kPa that referred to a linear velocity of 27.5 cm/s at 140°C. Detector gas flows: H_2, 40 mL/min; make-up gas (N_2), 40 mL/min; air, 450 mL/min. Data acquisition and processing were performed with HP-Chemstation software for GC systems. FAMEs were compared with purified standards (Supelco 37 component FAME mix from Sigma). Individual fatty acids are expressed as a percentage of total fatty acids in the sample.

2.4 RNA extraction and RT-qPCR analysis

Total RNA was extracted from the hypothalamus by Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA

was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Wilmintog, Delaware USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time PCR was used to measure mRNA expression levels of ObRb, SOCS3, NPY, and POMC in hypothalamus of the offspring of control, olive oil, butter, and margarine dams at the age of 21 days. The ratio between the relative expressions levels of ObRb and SOCS3 was also calculated.

A total of 0.25 μg of total RNA (in a final volume of 5 μL) was denatured at 65°C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in a Applied Biosystems 2720 Thermal Cycler (Applied Biosystem). Each PCR was performed from diluted (1/20) cDNA template, forward and reverse primers (1 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers, obtained from Sigma, for the different genes are described in [27]. Real-time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated as previously described with the 18 rRNA as reference gene [28].

2.5 Analyses of blood parameters

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) following standard procedures. Plasma and milk leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.6 Morphometric and immunohistochemical analysis in the hypothalamus

In the fixed brains, a coronal block containing the hypothalamus was cut, dehydrated in graded series of ethanol, cleared in xylene, and embedded in paraffin. Anatomically matched coronal sections (5 μm thick) from the hypothalamus (−2.3–−3.3 mm posterior to bregma)—according to published coordinates [29] with the help of hematoxylin/eosin-stained sections—were cut with a microtome and mounted in SuperFrost/Plus slides.

Immunohistochemical demonstration of NPY and αMSH was performed with the avidin-biotinperoxidase (ABC) method [30]. Sections were incubated sequentially at room temperature in the following solutions: 0.3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase; EDTA-based solution (pH = 8.2) in microwave oven for 30 min and 20 min on ice for antigen retrieval; 2% goat normal serum in Tris 0.1% Triton X-100 (Tris) for 20 min to reduce nonspecific background staining prior to incubation with primary antibody (polyclonal anti-NPY antibody produced in rabbit, N9528 Sigma-Aldrich Co., 1:200 in Tris with 1% BSA for 1 h and 15 min at 37°C; and rabbit anti-αMSH antibody, AB946 Chemicon (Millipore Corporation, Billerica, MA, USA), 1:800 in PBS 0.1% Triton X-100 with 1% BSA overnight at 4°C); biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) 1:200 in Tris 1% BSA for 1 h at room temperature; peroxidase-labeled ABC reagent (Vectastain ABC kit, Vector) in Tris for 30 min at room temperature and Fast 3,3′-diaminobezidine tablet, DAB (Sigma) in Tris for 3 min in dark room for enzymatic development of peroxidise. Subsequently, slides were washed with deionized water, dehydrated with increasing concentrations of ethanol and xylene, mounted with Eukitt (Panreac Quimica SA, Barcelona, Spain), and cover-slipped. Negative controls were performed by omission of primary antibody.

ARC images from light microscopy (3/animal) were digitalized. The boundaries of the ARC were drawn interactively in each cresil violet-stained section and the area and number of cresil violet-stained cells were measured using AxioVision40V 4.6.3.0. software (Carl Zeiss, Imaging Solutions GmbH, Germany). The number of immunoreactive neurons in this nucleus was also measured. Image analysis from all groups was examined in a blind fashion.

2.7 Statistical analysis

All data are expressed as the mean ± SEM.

Multiple comparisons were assessed by repeated measures ANOVA and two-way ANOVA to determine the effects of different factors (maternal type of fat supplementation, sex, and type of diet). Differences between groups were assessed by one-way ANOVA followed by least significances difference (LSD) post-hoc comparison. As we aimed to study the specific effect of different fat sources rather than the effect of high fat supplementation in itself, we did not include the offspring of control dams in the ANOVA analysis. Single comparisons were assessed by Student's *t*-test or pair *t*-test analysis. Analyses were performed with SPSS for Windows (SPSS, Chicago, IL, USA). Threshold of significance was defined at $p < 0.05$.

3 Results

3.1 Results in dams

Dams supplemented with olive oil, margarine, or butter presented a similar body weight to control dams, both at the

Table 2. Body weight (at the end of gestation and at the end of lactation), cumulative caloric intake (from day 14 to 20 of gestation and during the whole lactation period), body fat content at the end of lactation, and serum and milk leptin levels on days 6 and 12 of lactation of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine,or butter) during late pregnancy and lactation

Gestation		Control	Olive oil	Margarine	Butter
Body weight (end of gestation) (g)		329 ± 6	329 ± 8	334 ± 11	337 ± 6
Cumulative food intake (from day 14 to day 20) (Kcal)		477 ± 20	531 ± 16	534 ± 24	570 ± 14 ↑
Lactation		**Control**	**Olive oil**	**Margarine**	**Butter**
Body weight (end of lactation) (g)		280 ± 4	271 ± 7	271 ± 12	281 ± 8
Body fat content (end of lactation) (%)		9.56 ± 0.67	9.67 ± 0.25	10.97 ± 0.88	10.98 ± 0.66
Cumulative food intake (from day 1 to day 20) (Kcal)		3509 ± 52	3215 ± 135	3353 ± 127	3417 ± 102
Serum leptin (pg/mL)	Day 6	2073 ± 415	1801 ± 225	2053 ± 301	1910 ± 278
	Day 12	1947 ± 259	1744 ± 199	2065 ± 295	1867 ± 213
Milk leptin (pg/mL)	Day 6	325 ± 44	410 ± 44	439 ± 41	378 ± 47
	Day 12	394 ± 51	382 ± 28 (a)	535 ± 55 (b)	433 ± 45 (ab)

ANOVA analysis was assessed without the control group. Data are mean ± SE.M. (n = 5–6). Statistics: a≠b, LSD post-hoc analysis. ↑, different versus control ($p < 0.05$, Student's t-test).

end of gestation and lactation (Table 2). Body fat content measured at the end of lactation was also similar between the different groups. Notably, both olive oil- and margarine-supplemented dams decreased the intake of chow diet to compensate the daily fat supplementation, while the group of dams supplemented with butter, presented a greater accumulated food intake from day 14 to day 20 of gestation (Table 2). At day 12 of lactation, no differences were observed in serum leptin concentration in nursing dams, but margarine-supplemented dams presented a higher milk leptin concentration compared to olive oil-supplemented dams (Table 2).

3.2 Results in offspring

3.2.1 Body weight and blood parameters

The olive oil group (both males and females) exhibited lower body weight at birth than the margarine and butter groups ($p < 0.05$, one-way ANOVA), and, in the case of females, they also showed lower body weight in comparison with controls ($p < 0.05$, Student's t-test) (Table 3). Females of the margarine group showed the highest body weight at birth, but this difference with respect to controls disappeared during lactation. At day 12 of life, all fat-supplemented groups showed lower body weight that controls ($p < 0.05$, Student's t-test). Animals in the olive oil group also showed lower body weight than the margarine and butter groups ($p < 0.05$, one-way ANOVA). At this age, no differences in serum leptin concentration were observed between the different groups (Table 3). The lower body weight of the olive oil group was maintained during the whole suckling period, and could be related with lower food intake during this period, as deduced from the lower gastric contents measured on day 21 (Table 3). No significant differences were found in the margarine and butter groups with respect to controls regarding body weight at weaning.

Circulating glucose, insulin, and leptin levels of pups at weaning are also shown in Table 3. No differences were observed between groups in glucose levels either in males or in females. Maternal intake of different fat sources had different effects on circulating insulin and leptin levels in male and female offspring (sex by treatment interaction effects, $p = 0.060$ and $p = 0.015$, respectively, two-way ANOVA). Females of the margarine group displayed lower circulating insulin levels compared to the olive oil group. No changes in circulating insulin levels were observed in males. In contrast, olive oil group males displayed lower circulating leptin levels compared to the margarine and butter group ($p < 0.05$, one-way ANOVA); in addition, compared to controls, leptin levels were lower in olive oil group males and higher in margarine group males ($p < 0.05$, Student's t-test).

Body weight and food intake of pups were followed until the age of 6 months (Fig. 1). The table in this figure shows body weight, fat content, and lean mass of animals at the ages of 4 months (when all animals were fed with NF diet) and 6 months (with half of the animals moved from the last 2 months to a HF diet). Evolution of body weight and cumulative food intake from 4 to 6 months of age are also represented in the figure. At the age of 4 months, margarine group males

Table 3. Body weight (at birth and at weaning), gastric contents and circulating glucose, insulin, and leptin in 21-day-old male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation

Birth		Control	Olive oil	Margarine	Butter	ANOVA
Body weight (g)	Males	6.58 ± 0.11	6.43 ± 0.07 (a)	6.87 ± 0.17 (b)	6.84 ± 0.09 (b)	S
	Females	6.32 ± 0.11	6.01 ± 0.09 (a) ↓	6.84 ± 0.16 (c) ↑	6.43 ± 0.09 (b)	T
Day 12		Control	Olive oil	Margarine	Butter	ANOVA
Body weight (g)	Males	24.6 ± 0.3	21.9 ± 0.3 (a) ↓	23.4 ± 0.5 (b) ↓	23.2 ± 0.3 (b) ↓	S
	Females	24.1 ± 0.3	21.1 ± 0.3 (a) ↓	22.8 ± 0.4 (b) ↓	23.0 ± 0.4 (b) ↓	
Leptin (pg/mL)	Males	962 ± 135	996 ± 115	941 ± 136	902 ± 92	
	Females	867 ± 139	1037 ± 138	866 ± 144	886 ± 82	
Weaning		Control	Olive oil	Margarine	Butter	ANOVA
Body weight (g)	Males	45.4 ± 0.6	38.8 ± 1.0 (a) ↓	41.5 ± 2.4 (ab)	43.5 ± 0.9 (b)	T
	Females	43.3 ± 0.8	37.2 ± 0.7 (a) ↓	41.1 ± 1.6 (b)	42.4 ± 0.8 (b)	
Gastric contents (g)	Males	1.175 ± 0.112	1.086 ± 0.105 (a)	1.580 ± 0.256 (b)	1.468 ± 0.105 (b)	S
	Females	1.426 ± 0.101	0.929 ± 0.068 ↓	1.111 ± 0.177	1.338 ± 0.111	T
Glucose (mg/dL)	Males	129 ± 4	123 ± 4	127 ± 3	129 ± 3	
	Females	136 ± 3	127 ± 3	126 ± 3	132 ± 3	
Insulin (μg/L)	Males	0.058 ± 0.008	0.045 ± 0.006	0.072 ± 0.015	0.057 ± 0.012	S×T
	Females	0.076 ± 0.011	0.091 ± 0.019 (a)	0.045 ± 0.010 (b)	0.078 ± 0.009 (ab)	
Leptin (pg/mL)	Males	1634 ± 62	1329 ± 106 (a) ↓	2169 ± 197 (b) ↑	1819 ± 83 (b)	S×T
	Females	1947 ± 255	2009 ± 157	1894 ± 178	1894 ± 147	

ANOVA analysis was assessed without the control group. Data are mean ± SEM. ($n = 5$–6). Statistics: S, effect of sex; T, effect of maternal supplementation; S×T, interaction between sex and maternal supplementation, ($p < 0.05$, two-way ANOVA). a≠b≠c, LSD post-hoc analysis. ↑/↓, different versus control ($p < 0.05$, Student's *t*-test).

showed lower body weight compared to the butter group ($p < 0.05$, one-way ANOVA), but no significant differences were found in their body fat content or lean mass. Female animals showed no significant differences between groups concerning body weight, fat content, or lean mass. Neither were any differences either found concerning cumulative food intake between the different groups of animals during this period, either in males or in females (data not shown).

At the age of 6 months, no significant differences were found regarding body weight of the different groups of animals; however, it is interesting to highlight that within those animals exposed to HF diet, only the olive oil group (both males and females) showed higher body weight compared to their NF-fed counterparts ($p < 0.05$ Student's *t*-test). Nevertheless, male animals exposed to HF diet showed a higher percentage of body fat and a lower percentage of lean mass compared to those animals maintained under NF diet ($p < 0.05$, NF versus HF, Student's *t*-test), with the exception of animals in the margarine group (Fig. 1). These animals also showed a lower body fat percentage compared to the butter group. Female animals in the margarine group presented a higher lean mass content than the olive oil (under both NF and HF diets) and butter (under NF diet) groups ($p < 0.05$, one-way ANOVA). Neither were significant differences found concerning cumulative food intake between the different groups of animals during this 2 months period, but, interestingly, all HF diet-fed groups presented a higher cumulative food intake compared to their respective NF diet-fed counterparts, with the exception of margarine group females that maintained a similar intake of calories.

Figure 2 shows plasma leptin concentration of the different groups of animals at the ages of 4 and 6 months. At the age of 4 months, leptin levels were not significantly different between groups of male animals, but margarine group females showed lower leptin levels than the olive oil group and also lower than control animals. This tendency was maintained at the age of 6 months in the animals maintained under NF diet, but differences were not significant. At the age of 6 months, circulating leptin levels of male animals exposed to HF diet were higher than those of animals exposed to NF diet, with the exception of the margarine group. These animals also displayed lower leptin levels than the olive oil and butter groups. Female animals of the olive oil group showed higher circulating leptin levels compared to the margarine and butter groups. These animals were the only group within females that showed higher leptin levels under HF diet in comparison with those under NF diet.

No significant differences were found concerning circulating glucose levels at the ages of 4 and 6 months (results not shown).

Males			Control	Olive-oil	Margarine	Butter	ANOVA
4-month	Body weight (g)		408 ± 7	399 ± 7 (ab)	381 ± 8 (b)	405 ± 7 (a)	
	Total fat content (%)		13.1 ± 0.8	12.8 ± 0.4	13.0 ± 0.8	14.0 ± 0.6	
	Lean mass (%)		73.2 ± 0.8	73.5 ± 0.4	74.5 ± 0.9	72.2 ± 0.5	
6-month	Body weight (g)	NF	450 ± 12	442 ± 10	439 ± 9	462 ± 14	D
		HF	485 ± 19	491 ± 16 #	462 ± 13	486 ± 13	
	Total fat content (%)	NF	15.8 ± 1.3	17.0 ± 0.8	17.1 ± 1.6	17.6 ± 1.5	D
		HF	22.9 ± 1.7 #	23.0 ± 1.2 (ab) #	21.1 ± 1.6 (b)	25.9 ± 1.0 (a) #	
	Lean mass (%)	NF	70.9 ± 1.4	69.9 ± 0.8	70.6 ± 1.2	69.1 ± 1.3	D
		HF	65.4 ± 1.6 #	65.4 ± 0.9 #	67.8 ± 1.0	62.8 ± 0.9 #	
Females							
4-month	Body weight (g)		248 ± 5	243 ± 4	239 ± 4	241 ± 4	
	Total fat content (%)		14.3 ± 0.6	14.4 ± 0.6	13.44 ± 0.6	13.8 ± 0.5	
	Lean mass (%)		71.5 ± 0.5	71.5 ± 0.5	72.6 ± 0.5	71.7 ± 0.4	
6-month	Body weight (g)	NF	267 ± 6	254 ± 4	255 ± 6	256 ± 8	D
		HF	276 ± 6	284 ± 10 #	265 ± 10	272 ± 9	
	Total fat content (%)	NF	15.3 ± 1.4	14.9 ± 0.9	13.1 ± 1.0	14.1 ± 0.6	D
		HF	19.2 ± 1.0 #	21.6 ± 2.2 #	18.1 ± 1.5 #	19.6 ± 1.7 #	
	Lean mass (%)	NF	70.5 ± 1.3	71.4 ± 0.8 (a)	73.4 ± 0.7 (b)	71.2 ± 0.5 (a)	D
		HF	68.0 ± 0.9	65.9 ± 1.8 (a) #	70.2 ± 0.8 (b)	67.1 ± 1.5(ab) #	T

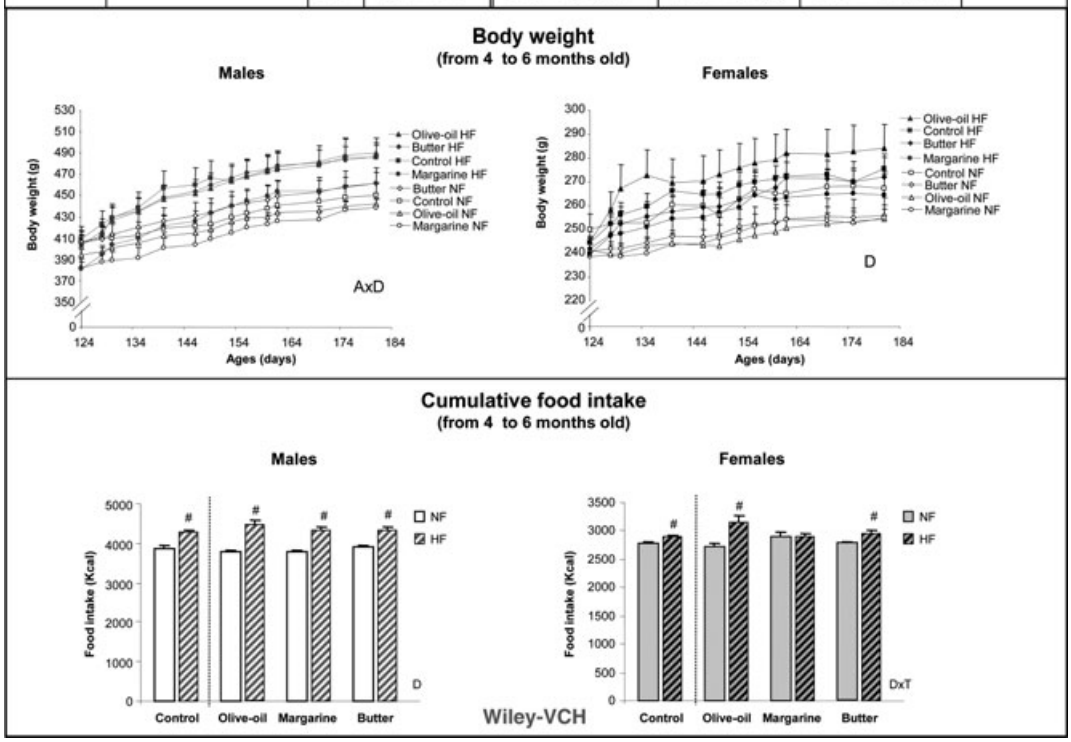

Figure 1. Body weight and fat content, at 4 and 6 months of age, of male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation. Animals were fed with standard NF diet until the age of 4 months and then with a NF or HF diet until the age of 6 months. The insert figures represent body weight over time and the cumulative food intake in kcal during the last 2 months when half of the animals were fed a NF diet or HF diet. The double line (Table) or the dotted line (Figure) indicates that the ANOVA analysis was assessed without the control group. Data are mean ± SEM. ($n = 7$–12). Statistics: D, effect of diet (NF or HF); D×T, interaction between diet and maternal supplementation, ($p < 0.05$, two-way ANOVA). a≠b, LSD post-hoc analysis. #, different from their respective NF-fed group ($p < 0.05$, Student's *t*-test). ↓, different versus control ($p < 0.05$, Student's *t*-test). In the body weight figure: D, effect of diet; A×D, interaction between age and diet; ($p < 0.05$, repeated measures ANOVA).

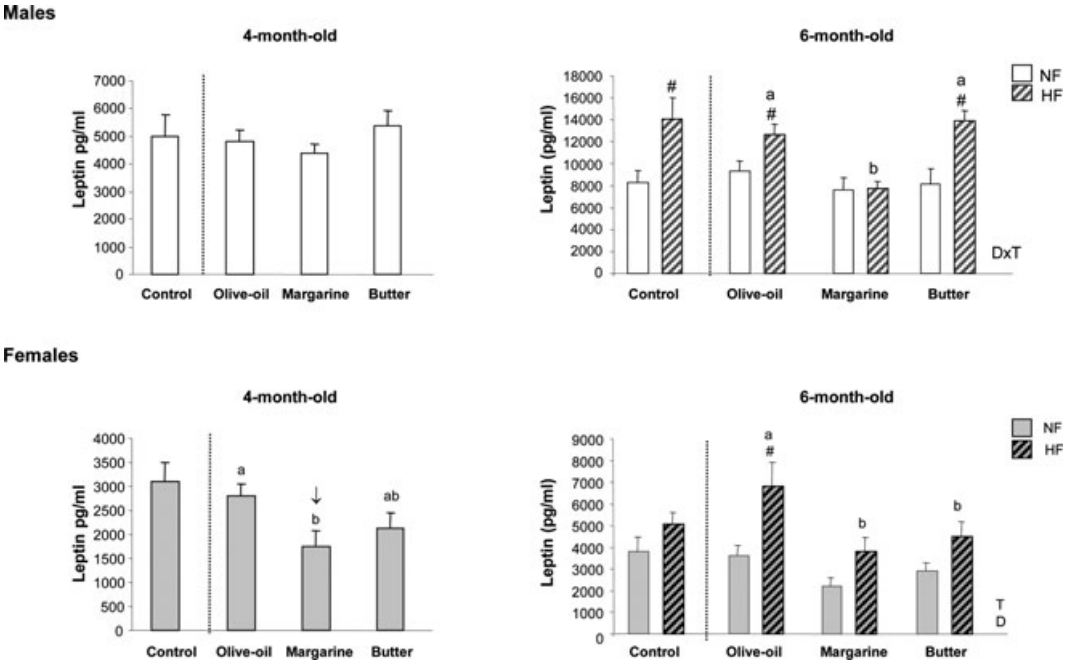

Figure 2. Circulating leptin in male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation, at the ages of 4 months (when all animals were fed a NF diet) and 6 months (with half of the animals moved from the last 2 months to a HF diet). The dotted line indicates that the ANOVA analysis was assessed without the control group. Data are mean ± SEM. ($n = 8$–11). Statistics: T, effect of maternal supplementation; D, effect of diet; D×T, interaction between maternal supplementation and diet ($p < 0.05$, two-way ANOVA). a≠b, LSD post-hoc analysis. *, different from their respective fed group ($p < 0.05$, Paired *t*-test). #, different from their respective NF-fed group ($p < 0.05$, Student's *t*-test). ↑/↓, different versus control ($p < 0.05$, Student's *t*-test).

3.2.2 Two-bottle food preference test

Food preferences were measured at the age of 3 months with the two-bottle preference test (Fig. 3). Male animals from all groups showed a significant preference for CHO-rich food versus fat-rich food ($p < 0.05$, pair *t*-test), and this difference was exacerbated in the margarine group. These animals showed a lower preference for fat-rich food compared to the other groups of animals ($p < 0.05$, LSD post-hoc analysis). Female animals showed a similar preference for CHO- and

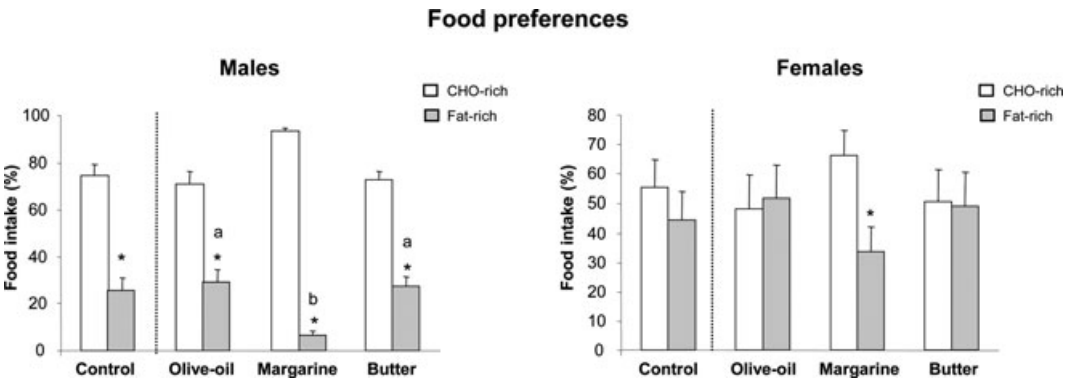

Figure 3. Dietary preferences measured by the two-bottle choice test of 3-month-old male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation. All animals were under a normal fat diet. Bars represent the percentage of the amount of CHO-rich food and fat-rich food with respect to the total food ingested in the choice test. The two diets had identical caloric density (2.31 kcal/g). The dotted line indicates that the ANOVA analysis was assessed without the control group. Data are mean ± SEM. ($n = 8$–11). Statistics: Within each rich diet a≠b ($p < 0.05$, one-way ANOVA and LSD post-hoc test). *, CHO- versus Fat-rich diet ($p < 0.05$, pair sample *t*-test).

fat-rich food, with the exception of the margarine group that showed a higher preference for CHO-rich food than for fat-rich food.

3.2.3 Morphometry, immunohistochemistry, and gene expression in hypothalamus of weaned pups

To ascertain whether different effects of the type of maternal fat supplementation during perinatal life may by related to different programming effects on the development of hypothalamic structures involved in body weight control, we performed morphometric and immunohistochemical studies in the ARC of the hypothalamus. Expression of selected genes involved in leptin signaling in the hypothalamus was also measured.

Results of morphometric analysis of cresil violet-stained cells from the ARC in weaned rats are shown in Table 4, and representative brain sections immunostained for NPY and αMSH in the hyphothalamc ARC nucleus are shown in Fig. 4. In the ARC, the number of cresil violet-stained cells and numerical density were significantly higher in margarine group females compared to the olive oil and butter groups ($p < 0.05$, one-way ANOVA). This increase was not observed in male animals, but compared to controls, margarine group males showed a higher numerical density ($p < 0.05$, Student's *t*-test).

Immunostaining for NPY in the ARC revealed that the number of NPY^+ cells was differently affected by the type of maternal fat supplementation in a sex-dependent manner (sex by treatment interaction, $p < 0.05$, two-way ANOVA). Male animals in the butter group showed a higher number of NPY^+ cells compared to controls, but margarine group females showed a higher number of NPY^+ cells compared to those of the butter group ($p < 0.05$, one-way ANOVA) and to controls ($p < 0.05$, Student's *t*-test). No differences in the number of αMSH^+ cells, or in the ratio of $NPY^+/\alpha MSH^+$ cells were observed between groups in the immunostained sections of the ARC.

Expression levels of selected genes in hypothalamus at weaning (day 21) are shown in Fig. 5. ObRb mRNA levels were affected by the type of maternal fat supplementation ($p < 0.05$, two-way ANOVA). The olive oil group of animals showed lower ObRb mRNA levels compared to the margarine and butter groups ($p < 0.05$ LSD post-hoc analysis), as well as compared to controls ($p < 0.05$, Student's *t*-test). In addition, margarine group males presented higher ObRb mRNA expression levels compared to controls ($p < 0.05$, Student's *t*-test). No significant differences were observed in SOCS3 mRNA levels between the different groups of animals. Yet, interestingly, concerning the ObRb/SOCS3 mRNA ratio, which could considered, among other parameters, as a marker of leptin sensitivity, an interactive effect between sex and treatment ($p < 0.05$, two-way ANOVA) was found, with male animals of the margarine group showing a higher value compared to those in the olive oil and butter groups. POMC and NPY expression levels were higher in female pups compared to males, but no differences depending on the type of maternal fat supplementation were observed; however, compared to controls, male animals in the margarine and butter groups displayed higher POMC mRNA levels ($p < 0.05$, Student's *t*-test).

4 Discussion

The intake of an excess of fat during pregnancy and lactation may have negative consequences on the metabolic health of offspring. Here, we have characterized the lasting effects of maternal supplementation with different fat sources during late pregnancy and lactation on body weight, food intake, and food preferences of the offspring, and assessed whether different outcomes could be attributed to different programming effects on central structures involved in the regulation of energy balance, particularly affecting leptin signaling. The fat sources were selected because they are commonly consumed in the European diets and are rich in different fatty acid types: butter (rich in saturated fatty acids), olive oil (rich in MUFA), and margarine (rich in PUFA). However, it has to be pointed out that these fat sources contain not only fatty acids, but also other ingredients, which may contribute or even be responsible of some of the effects of these fat types.

Detrimental effects of maternal HF diet on obesity and other related metabolic diseases in offspring have been reported [14–16, 31–33], but studies were based mainly on saturated fat sources. We describe here that maternal intake of an excess of fat during the perinatal period has different outcomes in the offspring depending on the type of fat, and the different effects are more evident when animals are exposed in adulthood to a HF diet. Maternal olive oil supplementation results in a lower body weight of offspring at birth that remains lower until weaning, but differences compared to the other treated groups disappears in adulthood; however, under the challenge of a HF diet, these animals show a greater increase in body weight and fat content. Similar lasting effects to those of olive oil are observed with maternal supplementation with butter, with regards to body weight, although with a greater increase in body fat content when male animals are exposed to a HF diet. In contrast, the offspring of dams supplemented with margarine during the perinatal period show a greater body weight at birth and at weaning, compared to the offspring of dams supplemented with olive oil, but differences disappear when the animals grow. Moreover, these animals seem to be protected, at least to some extent, from further development of overweight when exposed to HF diet. These protective effects are seen when comparing with the offspring of dams supplemented with the other fat sources, and even with those fed a nonsupplemented control diet.

Different effects of maternal supplementation with different fat sources during the perinatal period on offspring response to a HF diet in adulthood could be related, in part,

Table 4. Morphometry and immunohistochemical analysis of the ARC nucleus. Number and numerical density of hematoxylin/eosin-stained neurons, and number of NPY, αMSH, and the ratio $NPY^+/\alpha MSH^+$ immunoreactive neurons (NPY^+, αMSH^+) within ARC nucleus of 21-day-old male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation. Numerical density is expressed as number of cells per square millimetre

ARC		Control	Olive oil	Margarine	Butter	ANOVA
Number of cells (n)	Males	430 ± 23	493 ± 47	502 ± 37	467 ± 41	S
	Females	409 ± 14	376 ± 18 (a)	465 ± 21 (b)	375 ± 15 (a)	
Numerical density (n/mm^2)	Males	1791 ± 82	2177 ± 204	2264 ± 120 ↑	1821 ± 167	S
	Females	1726 ± 99	1575 ± 145 (a)	1893 ± 100 (b)	1582 ± 36 (a)	T
Number of NPY^+ neurons	Males	100 ± 5	113 ± 8	118 ± 10	132 ± 4 ↑	S×T
	Females	107 ± 3	111 ± 9 (ab)	128 ± 2 (b) ↑	>98.4 ± 6 (a)	
Number of αMSH^+ neurons	Males	55.7 ± 10.5	58.0 ± 11.8	74.4 ± 9.9	87.9 ± 16.7	
	Females	72.3 ± 15.9	62.8 ± 15.2	70.3 ± 13.0	59.2 ± 8.1	
Ratio $NPY^+/\alpha MSH^+$ neurons	Males	2.06 ± 0.49	2.12 ± 0.32	1.63 ± 0.13	1.70 ± 0.25	
	Females	1.97 ± 0.75	2.11 ± 0.50	2.14 ± 0.22	1.77 ± 0.18	

ANOVA analysis was assessed without the control group. Data are mean ± SEM ($n = 5$). Statistics: S, effect of sex; T, effect of maternal supplementation; S×T, interaction between sex and maternal supplementation, ($p < 0.05$, two-way ANOVA). a≠b, LSD post-hoc analysis. ↑, different versus control ($p < 0.05$, Student's *t*-test).

to different programming effects on the establishment of food preferences. As previously described, we observed that control adult male rats have a higher preference for CHO-rich food compared to fat-rich food [9, 12]. This preference is exacerbated in the offspring of margarine-supplemented dams during the perinatal period. Adult female rats show a rather similar preference for both types of macronutrients, but again those in the margarine group show a higher preference for CHO-rich food compared to fat-rich food. These programming effects on food preferences occurring in the margarine group could be responsible, in the case of female offspring, for the lack of increase in caloric intake when exposed to a HF diet, with respect to the intake under a NF diet. Thus, maternal intake of different fat sources during pregnancy and lactation might play a role influencing the long-term appetite of the offspring.

In addition to differences in food preferences, rats from the margarine group compared to the olive oil and butter groups, seem to have a better response when exposed to a HF diet. Specifically, male animals do not show a significant increase in body fat content in comparison with their controls under NF diet, and females appear to have a better control of food intake. Interestingly, 6-month-old male animals in the margarine group under HF diet display lower circulating leptin levels compared to the other treated groups and to controls suggesting higher sensitivity to leptin. In the case of females, differences are only significant when comparing with the olive oil group, which shows higher leptinemia than controls. However, females in the margarine group already show lower levels than the olive oil group and lower than controls at the age of 4 months, when all animals are under NF diet. These results suggest that the margarine group seem to be better prepared to face HF diet conditions, in comparison with the other groups from dams supplemented with other fat sources, and the leptin system could be tentatively involved in this adaptation.

In rats, it is known that the differentiation of the neuronal systems regulating energy homeostasis begins during gestation and continues until weaning [34, 35]. Increasing evidence supports that nutritional environment during early development can influence the development of the hypothalamus, exerting changes in the expression of genes critically involved in the regulation of energy intake and expenditure that can be permanent and related with the predisposition for adult-onset metabolic disorders [7, 36–38]. In this sense, morphometric analysis of the hypothalamus in weaned pups reveals that females in the margarine group exhibit a significant increase in the number of cells and cell density in the ARC compared to the other treated groups. No significant changes are found in males between the treated groups, although male pups in the margarine group show higher cell density in the ARC than the control group. Modification in the structure of the hypothalamus, particularly a reduction in the number of cells in the ARC has been found in obesity-prone models, particularly in offspring from dams fed a low protein diet or under energy restriction during gestation, and has been related with the long lasting detrimental effects observed in these animals [7, 39]. Therefore, these results suggest that the type of fat during the perinatal period may lead to structural changes in hypothalamic centers involved in food intake and energy expenditure in the offspring, thus providing an anatomic basis for a different capacity to regulate food intake and body weight. However, despite it is known that gestation and lactation are critical periods in the differentiation of the neuronal systems regulating energy homeostasis [34, 35], it must be pointed out that changes described in the present study were found at weaning and it is not known whether they are maintained in adulthood.

In addition to changes in the total number of cells in the ARC, female pups in the margarine group also show a greater amount of NPY^+ neurons than those in the butter group and in controls, with no significant changes in the amount of

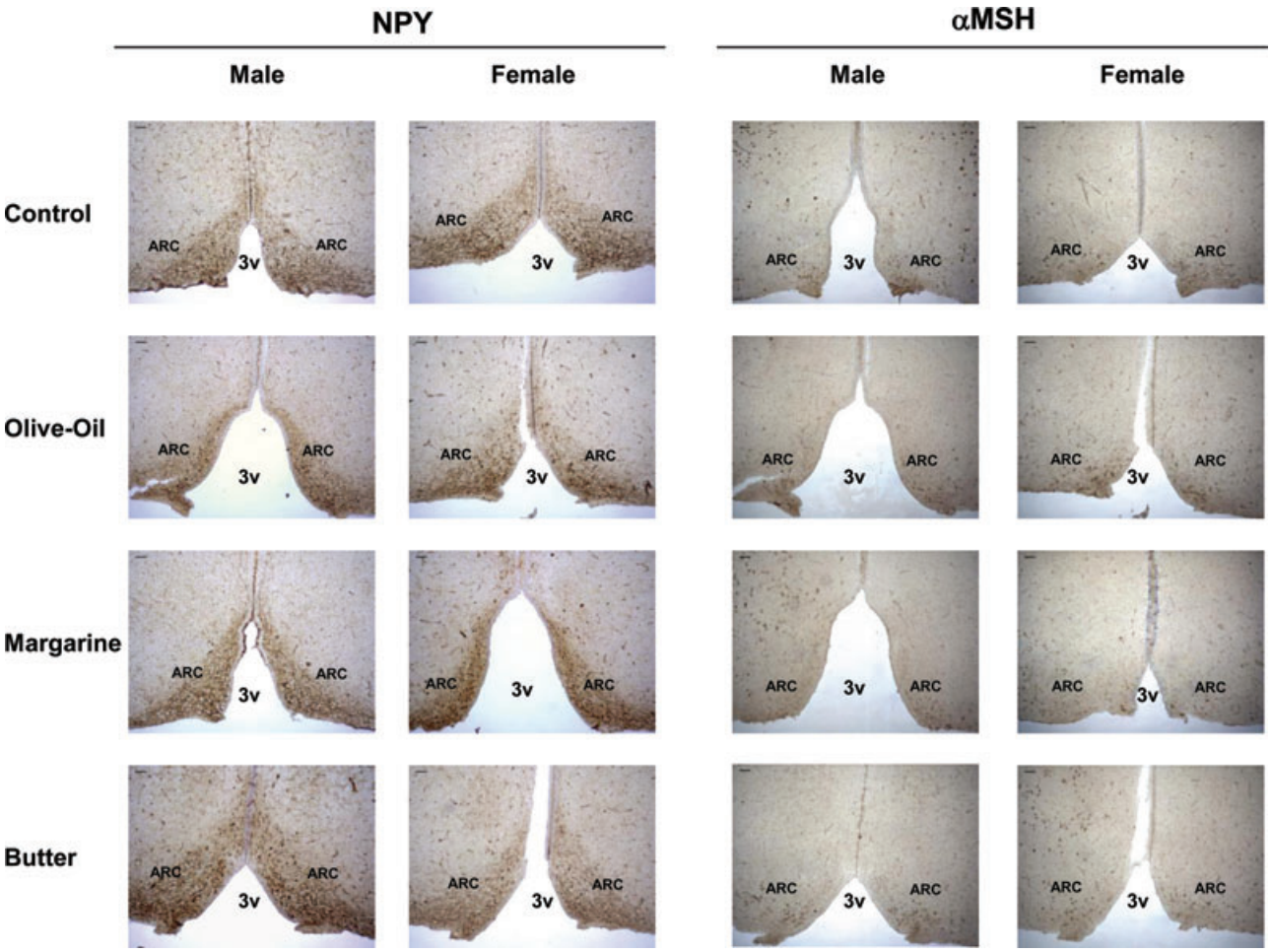

Figure 4. Representative brain sections immunostained for NPY and αMSH in the hypothalamic ARC nucleus of 21-day-old male and female offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter). ARC, arcuate nucleus; 3v, third ventricle. Scale bar: 50 μm.

αMSH^+ neurons or in the $NPY^+/\alpha MSH^+$ ratio in any group. Male pups in the butter group also show a higher amount of NPY^+ neurons compared with controls. HF diet feeding during pregnancy has been previously described to increase the proliferation of hypothalamic orexigenic peptide-producing neurons [40]. Conversely, Kozak et al. [23] showed that a maternal HF diet decreased NPY expression in the ventromedial nucleus of their offspring. We show here that the effect of maternal HF diet during the perinatal period on neuroendocrine system development may be dependent on the fat source, and that an increase in the number of orexigenic peptide-producing neurons does not necessarily imply negative consequences on the capacity to regulate energy balance, promoting energy intake. In fact, a decrease in the number of NPY^+ neurons has been described in offspring from moderate caloric-restricted dams during the first part of gestation [7] and these animals display in adulthood an impaired regulation of food intake and energy expenditure [7,8]. Here, despite some changes in the number of NPY^+ neurons, we do not observe any change in NPY mRNA expression levels in the hypothalamus of these animals. However, since we have studied gene expression levels only under ad libitum feeding conditions, changes in NPY mRNA levels under different nutritional conditions, e.g. fasting, cannot be ruled out. Interestingly, margarine and butter group females show higher mRNA expression levels of POMC compared to controls. Margarine and butter group animals also show greater hypothalamic ObRb mRNA expression levels compared to the olive oil group, but only margarine group males show a greater ObRb/SOCS3 ratio compared to the other treated groups. These results support the fact that the type of fat eaten during perinatal life may exert different programming effects on mechanisms involved in central leptin sensitivity, with margarine appearing to have, in these particular conditions, some positive effects in comparison with other fat sources.

Which particular components of margarine may be involved in these early programming effects and through which

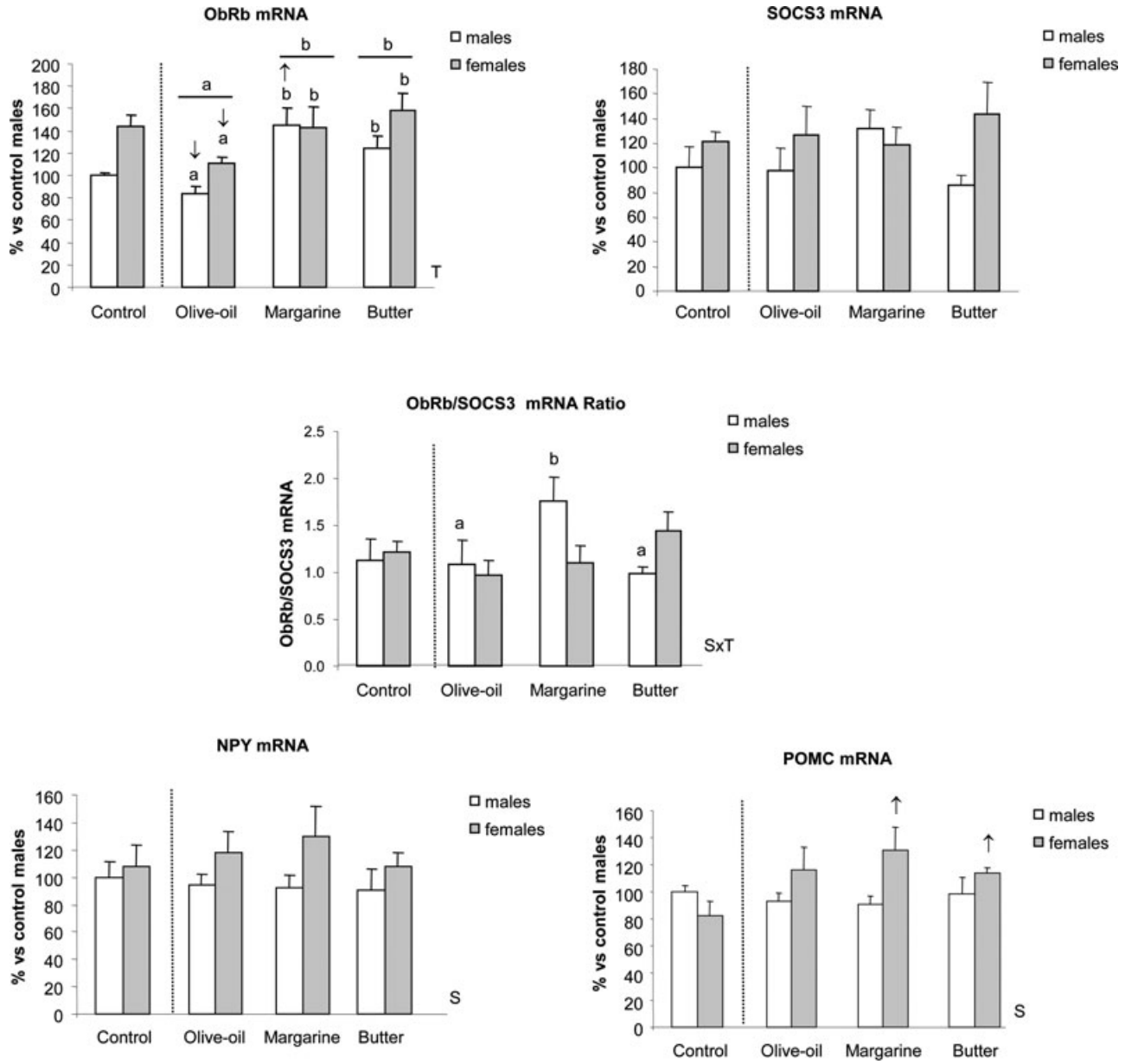

Figure 5. Leptin receptor (ObRb), the suppressor of cytokine signaling 3 (SOCS3), ObRb/SOCS3, Neuropetide Y (NPY), and Pro-opiomelacortine (POMC) mRNA expression levels in hypothalamus of 21-day-old offspring of dams supplemented with 30% of the normal caloric intake with different fat sources (olive oil, margarine, or butter) during late pregnancy and lactation. Data are expressed as a percentage of the mean value of male offspring of control dams. Data are mean ± SEM of 5–7 animals per group. The dotted line indicates that the ANOVA analysis was assessed without the control group. Statistics: S, effect of sex; T, effect of maternal supplementation; ($p < 0.05$, two-way ANOVA). a≠b, LSD post-hoc analysis. ↑/↓, different versus control ($p < 0.05$, Student's *t*-test).

mechanisms they may be acting are not known yet. Leptin has been proposed as an important neurotrophic factor during postnatal development. A lack of leptin during early life in mice has been shown to compromise the neuronal organization of hypothalamic nuclei involved in food intake control [41], thus potentially affecting sensitivity to this hormone in adulthood. Moreover, leptin supplementation with physiological doses during lactation in rat pups has been described to prevent overweight in adulthood and other features of the metabolic syndrome, to improve leptin sensitivity, and to determine a lower preference for fat-rich food [25, 28, 42]. Interestingly, a postnatal leptin surge around day 10 has been described in mice [43] and in rats [44], and alterations in this leptin surge by changes in perinatal nutrition have been related with long-lasting effects on metabolism including an increased susceptibility to the detrimental effects of a

postnatal obesogenic diet [44–46]. In this sense, margarine-supplemented dams presented higher milk leptin concentration compared to olive oil dams at day 12 of lactation, coinciding with the period of the natural occurring leptin surge. These higher milk leptin levels do not seem to be related with changes in maternal fat mass, suggesting a specific regulation of some component of the margarine in the milk-borne leptin more than the amount of maternal fat per se. Notably, males pups of the margarine and butter group displayed higher circulating leptin levels at weaning than those in the olive oil group, and those in the margarine group were also higher than those in the control group. These changes in leptin levels are not directly reflecting changes in body weight of the pups, but might reflect an increased maternal milk-borne leptin. Thus, it could be speculated that the higher supply of milk-borne leptin, and/or the moderately higher levels of plasma leptin as well as its better signaling (increased hypothalamic ObRb expression and greater ObRb/SOCS3 ratio) found in margarine group males at the end of the suckling period may indicate that these animals present a neuroendocrine milieu that promotes a better establishment of the hypothalamic homeostasis circuitry, which can be relevant to reduce the risk of suffering obesity in adulthood. However, this latter explanation does not apply to females, and the reasons for the sex-dependent differences need to be further studied.

In conclusion, maternal supplementation with an excess of fat during late pregnancy and lactation may differently program the offspring for later predisposition to or prevention of obesity and leptin resistance depending on the source of fat. Supplementation with margarine, compared with other fat sources, may program the offspring for increased leptin sensitivity in adulthood and a lower preference for fat food, and hence provide relative protection against body weight gain in adulthood, particularly under an obesogenic environment. Differential effects depending on the type of fat on the development of hypothalamic structures involved in food intake control could be one of the underlying molecular mechanisms responsible for the different outcomes.

This work was supported by the Spanish Government (AGL2009-11277) and European Union (BIOCLAIMS FP7-244995) and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERobn. Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n° FP6-506360).

The authors have declared no conflict of interest.

5 References

[1] Barker, D. J., Eriksson, J. G., Forsen, T., Osmond, C., Fetal origins of adult disease: strength of effects and biological basis. *Int. J. Epidemiol.* 2002, *31*(6), 1235–1239.

[2] Langley-Evans, S. C., Developmental programming of health and disease. *Proc. Nutr. Soc.* 2006, *65*(1), 97–105.

[3] Martorell, R., Stein, A. D., Schroeder, D. G., Early nutrition and later adiposity. *J. Nutr.* 2001, *131*(3), 874S–880S.

[4] Jones, A. P., Friedman, M. I., Obesity and adipocyte abnormalities in offspring of rats undernourished during pregnancy. *Science* 1982, *215*(4539), 1518–1519.

[5] Palou, M., Priego, T., Sanchez, J., Palou, A. et al., Sexual dimorphism in the lasting effects of moderate caloric restriction during gestation on energy homeostasis in rats is related with fetal programming of insulin and leptin resistance. *Nutr. Metab.* 2010, *7*, 69–79.

[6] Ravelli, G. P., Stein, Z. A., Susser, M. W., Obesity in young men after famine exposure in utero and early infancy. *N. Engl. J. Med.* 1976, *295*(7), 349–353.

[7] Garcia, A. P., Palou, M., Priego, T., Sanchez, J. et al., Moderate caloric restriction during gestation results in lower arcuate nucleus, N. P.Y- and alphaMSH-neurons and impairs hypothalamic response to fed/fasting conditions in weaned rats. *Diabetes Obes. Metab.* 2010, *12*(5), 403–413.

[8] Garcia, A. P., Palou, M., Sanchez, J., Priego, T. et al., Moderate caloric restriction during gestation in rats alters adipose tissue sympathetic innervation and later adiposity in offspring. *PLoS One* 2011, *6*(2), e17313.

[9] Palou, M., Priego, T., Sanchez, J., Torrens, J. M. et al., Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life. *Endocrinology* 2010, *151*,(3), 1030–1041.

[10] Palou, M., Torrens, J. M., Priego, T., Sanchez, J. et al., Moderate caloric restriction in lactating rats programs their offspring for a better response to HF diet feeding in a sex-dependent manner. *J. Nutr. Biochem.* 2011, *22*(6), 574–584.

[11] Langley-Evans, S. C., Bellinger, L., McMullen, S., Animal models of programming: early life influences on appetite and feeding behaviour. *Matern. Child Nutr.* 2005, *1*(3), 142–148.

[12] Bellinger, L., Lilley, C., Langley-Evans, S. C., Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. *Br. J. Nutr.* 2004, *92*(3), 513–520.

[13] Daenzer, M., Ortmann, S., Klaus, S., Metges, C. C., Prenatal high protein exposure decreases energy expenditure and increases adiposity in young rats. *J. Nutr.* 2002, *132*(2), 142–144.

[14] Bayol, S. A., Simbi, B. H., Fowkes, R. C., Stickland, N. C., A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic fatty liver disease in rat offspring. *Endocrinology* 2010, *151*(4), 1451–1461.

[15] Bayol, S. A., Simbi, B. H., Stickland, N. C., A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J. Physiol.* 2005, *567*(Pt 3), 951–961.

[16] Buckley, A. J., Keseru, B., Briody, J., Thompson, M. et al., Altered body composition and metabolism in the male offspring of high fat-fed rats. *Metabolism* 2005, *54*(4), 500–507.

[17] Guo, F., Jen, K. L. High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. *Physiol. Behav.* 1995, *57*(4), 681–686.

[18] Ailhaud, G., Guesnet P., Fatty acid composition of fats is an early determinant of childhood obesity: a short review and an opinion. *Obes Rev.* 2004,*5*(1), 21–26.

[19] Ailhaud, G., Massiera, F., Alessandri, J. M., Guesnet, P., Fatty acid composition as an early determinant of childhood obesity. *Genes Nutr.* 2007, *2*(1), 39–40.

[20] Innis, S. M., Polyunsaturated fatty acids in human milk: an essential role in infant development. *Adv. Exp. Med. Biol.* 2004, *554*, 27–43.

[21] Innis, S. M., Human milk: maternal dietary lipids and infant development. *Proc. Nutr. Soc.* 2007, *66*(3), 397–404.

[22] Schwartz, M., Woods, S., Porte, D. J., Seeley, R. et al., Central nervous system control of food intake. *Nature* 2000, *404*(6778), 661–671.

[23] Kozak, R., Richy, S., Beck, B., Persistent alterations in neuropeptide Y release in the paraventricular nucleus of rats subjected to dietary manipulation during early life. *Eur. J. Neurosci.* 2005, *21*(10), 2887–2892.

[24] Sanchez, J., Oliver, P., Miralles, O., Ceresi, E. et al., Leptin orally supplied to neonate rats is directly uptaken by the immature stomach and may regulate short-term feeding. *Endocrinology* 2005, *146*(6), 2575–2582.

[25] Sanchez, J., Priego, T., Palou, M., Tobaruela, A. et al., Oral supplementation with physiological doses of leptin during lactation in rats improves insulin sensitivity and affects food preferences later in life. *Endocrinology* 2008, *149*(2), 733–740.

[26] Bondia-Pons, I., Molto-Puigmarti, C., Castellote, A. I., Lopez-Sabater, M. C., Determination of conjugated linoleic acid in human plasma by fast gas chromatography. *J. Chromatogr. A* 2007, *1157*(1–2), 422–429.

[27] Palou, M., Sanchez, J., Rodriguez, A. M., Priego, T. et al., Induction of NPY/AgRP orexigenic peptide expression in rat hypothalamus is an early event in fasting: relationship with circulating leptin, insulin and glucose. *Cell Physiol. Biochem.* 2009, *23*(1–3), 115–124.

[28] Pico, C., Oliver, P., Sanchez, J., Miralles, O. et al., The intake of physiological doses of leptin during lactation in rats prevents obesity in later life. *Int. J. Obes.* 2007, *31*(8), 1199–1209.

[29] Paxinos, G., Watson, C., *The Rat Brain in Stereotaxic Coordinates*, 4th edn. Academic Press, San Diego, CA 1998, pp. 27–33.

[30] Hsu, S. M., Raine, L., Fanger, H., Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 1981, *29*(4), 577–580.

[31] Bayol, S. A., Farrington, S. J., Stickland, N. C., A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *Br. J. Nutr.* 2007, *98*(4), 843–851.

[32] Bayol, S. A., Macharia, R., Farrington, S. J., Simbi, B. H. et al., Evidence that a maternal "junk food" diet during pregnancy and lactation can reduce muscle force in offspring. *Eur. J. Nutr.* 2009, *48*(1), 62–65.

[33] Bayol, S. A., Simbi, B. H., Bertrand, J. A., Stickland, N. C., Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. *J. Physiol.* 2008, *586*(13), 3219–3230.

[34] Bouret, S. G., Simerly, R. B., Development of leptin-sensitive circuits. *J. Neuroendocrinol.* 2007, *19*(8), 575–582.

[35] Grove, K. L., Grayson, B. E., Glavas, M. M., Xiao, X. Q. et al., Development of metabolic systems. *Physiol. Behav.* 2005, *86*(5), 646–660.

[36] Chen, H., Simar, D., Morris, M. J., Hypothalamic neuroendocrine circuitry is programmed by maternal obesity: interaction with postnatal nutritional environment. *PLoS ONE* 2009, *4*(7), e6259.

[37] Ikenasio-Thorpe, B. A., Breier, B. H., Vickers, M. H., Fraser, M., Prenatal influences on susceptibility to diet-induced obesity are mediated by altered neuroendocrine gene expression. *J. Endocrinol.* 2007, *193*(1), 31–37.

[38] Page, K. C., Malik, R. E., Ripple, J. A., Anday, E. K., Maternal and postweaning diet interaction alters hypothalamic gene expression and modulates response to a high-fat diet in male offspring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2009, *297*(4), R1049–R1057.

[39] Plagemann, A., Harder, T., Rake, A., Melchior, K. et al., Hypothalamic nuclei are malformed in weanling offspring of low protein malnourished rat dams. *J. Nutr.* 2000, *130*(10), 2582–2589.

[40] Chang, G. Q., Gaysinskaya, V., Karatayev, O., Leibowitz, S. F., Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. *J. Neurosci.* 2008, *28*(46), 12107–12119.

[41] Bouret, S. G., Draper, S. J., Simerly, R. B. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science* 2004, *304*(5667), 108–110.

[42] Priego, T., Sanchez, J., Palou, A., Pico, C., Leptin intake during the suckling period improves the metabolic response of adipose tissue to a high-fat diet. *Int. J. Obes.* 2010, *34*(5), 809–819.

[43] Ahima, R., Prabakaran, D., Flier, J., Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implication for energy homeostasis and neuroendocrine function. *J. Clin. Invest.* 1998, *101*(5), 1020–1027.

[44] Delahaye, F., Breton, C., Risold, P. Y., Enache, M. et al., Maternal perinatal undernutrition drastically reduces postnatal leptin surge and affects the development of arcuate nucleus proopiomelanocortin neurons in neonatal male rat pups. *Endocrinology* 2008, *149*(2), 470–475.

[45] Palou, M., Konieczna, J., Torrens, J. M., Sanchez, J. et al., Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed. *J. Nutr. Biochem.* 2012. Available from http://www.sciencedirect.com/science/help/doi.htm

[46] Pico, C., Jilkova, Z. M., Kus, V., Palou, A. et al., Perinatal programming of body weight control by leptin: putative roles of AMP kinase and muscle thermogenesis. *Am. J. Clin. Nutr.* 2011, *94*(6), 1830S–1837S.

