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RESEARCH PAPER

Implementation of a healthy diet to lactating rats attenuates the early detrimental programming effects in the offspring born to obese dams. Putative relationship with milk hormone levels

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Abstract

Lactation is a critical period of development and alterations in milk composition due to maternal diet or status may affect infant growth. We aimed to evaluate in rats whether improving maternal nutrition during lactation attenuates early imprinted adverse metabolic effects in the offspring born to obese dams. Three groups were studied: Control (C) dams, fed with standard diet; Western diet (WD) dams, fed with WD 1 month prior to gestation and during gestation and lactation; and Reversion (Rev) dams, fed as WD-dams, but moved to a standard diet during lactation. Macronutrient content, insulin, leptin and adiponectin levels were determined in milk. Phenotypic traits and circulating parameters in dams and their offspring were determined throughout lactation. Results showed that, at weaning, WD-dams displayed lower body weight and greater plasma insulin and non-esterified fatty acids levels than C-dams, and signs of hepatic steatosis. Milk from WD-dams showed lower protein content and insulin, leptin, and adiponectin levels during the entire or the late lactation. Rev-dams retained excess body fat content, but milk composition and most circulating parameters were not different from controls at late lactation and showed higher leptin mRNA levels in mammary gland than WD-dams. The offspring of WD-dams, but not that of Rev-dams, displayed higher body weight, adiposity, and circulating leptin and glucose levels than controls at weaning. In conclusion, dietary improvement during lactation prevents early adverse effects in offspring associated with maternal intake of an obesogenic diet, that may be related with the normalization of milk hormone levels.

Keywords: Lactation period; Metabolic programming; Milk composition; Maternal obesity; Maternal diet.

1. Introduction

Fetal and early postnatal life represent critical windows of development in which environmental conditions may have a profound influence on physiological functions and risk of diseases in adult life, such as obesity, type 2 diabetes, and cardiovascular diseases [1,2]. Besides the known adverse effects of undernutrition during fetal life, which initially supported the concept of developmental origins of health and disease [3,4], cumulative evidence, mainly from animal studies, also show that overnutrition during both gestation and lactation periods increases the prevalence of obesity and related alterations in adulthood [5–8].

Lactation is a critical period of major relevance in the programming of metabolic-related diseases. In humans, a rapid weight gain during this period has been shown to predispose to a distorted metabolic phenotype in later life, with increased risk of overweight, central adiposity and insulin resistance [9,10]. Studies conducted in animal models have also shown that maternal obesity and/or exposure to an obesogenic diet during lactation may affect postnatal development of the offspring, predisposing them to metabolic syndrome and related alterations [11–13]. Conversely, improved nutrition during this period may mitigate some of the adverse effects caused by an obesogenic environment during gestation on food preferences and susceptibility to diet-induced obesity

Abbreviations: C, Control; WD, Western diet; Rev, Reversion; LD, Lactation day; PND, Postnatal day; O-C, Offspring of C-dams; O-WD, Offspring of WD-dams; O-Rev, Offspring of Rev-dams.

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[14,15]. However, studies that discriminate between the adverse effects of maternal overnutrition and maternal obesity are scarce [15]. Considering that both conditions are usually associated, but not always, it is of interest to discern their programming effects, as well as to find out whether the effects induced by maternal obesity and/or an unbalanced diet during gestation could be reversed or attenuated through an improvement in the maternal diet during lactation. This could be of relevance to improve the health of future generations.

Breast milk is largely recognized as the best source of nutrition for infants during lactation, as it contains, besides macro and micronutrients, a large range of bioactive components that may influence infant growth and development [16]. However, the composition of breast milk is not uniform, and many factors, such as maternal metabolic state and dietary inputs may affect its composition and compromise infant health [15,17,18]. We have previously described that maternal consumption of a cafeteria diet during lactation produces lasting effects in the metabolic health of their offspring, which are associated with changes in milk macronutrient composition [12]. Furthermore, using another model of cafeteria diet-induced obesity, in which cafeteria diet was removed during gestation and lactation (post-cafeteria model), maternal diet, rather than maternal obesity itself, was shown to have a main influence on milk triacylglycerol (TG) profile [19]. Maternal diet and/or maternal status may also entail changes in endogenous synthesized metabolic hormones and these alterations may have an impact on infant development [20]. Among these hormones, there is evidence of the role of leptin ingested during lactation in metabolic programming, as deduced from intervention studies in animal models [21–23] and observational studies in humans [23–25], but the role of other milk hormones, such as insulin and adiponectin has been, so far, less explored. The study of the impact of maternal obesity and maternal exposure to an unbalanced diet on the content of these hormones in milk, as well as the possible contribution of alterations in their levels to the adverse effects on the development of the offspring, could shed light on its potential role in breast milk.

Therefore, in the present study, we aim to evaluate the effects of dietary improvement during lactation in diet-induced obese rats on milk macronutrient composition and the concentration of selected metabolic hormones present in milk, and the effects in the offspring development and growth during lactation.

2. Material and methods

2.1. Animals and experimental design

The animal protocol carried out in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Exp. 2018/13/AEXP, January 23, 2019) and measures for the use and care of laboratory animals of the University were followed.

The study was done using 27 litters as described below. Virgin female Wistar rats housed at controlled temperature (22°C), with a 12-hour light–dark period and with free access to food and water, were fed with a standard chow diet (SD; 3.3 kcal•g^{-1}, with 8.4% calories from fat, 72.4% from carbohydrates and 19.3% from protein; Safe, Augy, France) (control-dams, C-dams) or a high-fat and high-sucrose diet (Western diet, WD; 4.7 kcal•g^{-1}, with 40.0% calories from fat, 43.0% from carbohydrates and 17.0% from proteins; Research Diets, New Brunswick, NJ, USA) for 1 month prior to being bred with male rats (this period was referred as pre-gestation). The nutritional composition of both commercial diets is provided in Supplementary Table 1. Pregnant dams were housed individually and continued with their assigned diets during gestation period. At postnatal day (PND) 1, litters were equated to 10 pups per dam, five males and five females when possible. Throughout lactation, C-dams continued with SD (n=8), but dams fed with WD either continued with WD diet (WD-dams, n=9) or were exposed to SD during this period (Reversion dams, Rev-dams; n=10). A scheme of the experimental design is represented in Supplementary Figure 1.

Body weight of dams and of their offspring was recorded daily during the suckling period. At three time points of lactation (lactation day (LD) 5, LD10 and LD15), milk and plasma samples from dams were collected. For this, dams were separated from their pups for 2–3 hours. Then, blood samples were collected from the saphenous vein and plasma was obtained by centrifugation (1000 g). For milk extraction, 4 IU kg^{-1} of body weight of oxitocin (Facilpart, Laboratory syva s.a.u, León, España) was administered intraperitoneally to dams for milk extraction and dams were anesthetized using isoflurane (IsoFlo, Abbott Laboratories Ltd., North Chicago, IL, USA), and subsequently were kept anesthetized. Milk was extracted manually from all teats. At PND10 and 15, and at weaning (PND21), a blood sample was collected in a capillary from the end of the tail of the pups. Plasma was obtained as above described, and samples of the same litter and sex were pooled. Plasma and milk samples were stored at −80°C until further analysis.

Dams were killed by decapitation at LD21 (at weaning) during the first 2-hour at the beginning of the light cycle. Maternal tissues were rapidly removed and frozen in liquid nitrogen and stored at -80°C until analysis.

2.2. Maternal liver histological analysis

Maternal liver samples were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C. Then, they were washed in phosphate buffer, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin blocks. Five-micrometer-thick sections of tissues were cut with a microtome and mounted on slides. For histological analysis, liver sections were classified into four grades depending on fat accumulation following Burnt et al. classification [26]: grade 0 was assigned when there was no fat accumulation; grade 1 when fat vacuoles were observed in less than 33% of hepatocytes; grade 2 when 33–66% of hepatocytes contained fat vacuoles; and grade 3 when they were found in more than 66% of hepatocytes.

2.3. Analysis of blood parameters

Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain) was used to measure blood glucose concentration. Enzyme-linked immunosorbent assay kits were used for the quantification of insulin (Mercodia AB, Uppsala, Sweden), leptin and adiponectin (R&D Systems, Minneapolis, MN, USA). Enzymatic colorimetric kits were used for the quantification of plasma non esterified (or free) fatty acids (NEFA) (Wako Chemicals GmbH, Neuss, Germany) and TG (Química Clínica Aplicada, Tarragona, Spain) levels.

2.4. Analysis of milk composition

Milk samples were analyzed for lactose, total protein, TG, leptin, adiponectin and insulin concentration. Lactose concentration was determined by a commercial colorimetric method (Biovision, Milpitas, CA, USA). Total milk protein concentration was measured by the Pierce BCA Protein Assay Kit (Thermhofisher Scientific, Madrid, Spain). For the determination of milk TG levels, lipids were first extracted as previously described [27] and then TG levels were quantified using a commercial kit (Química Clínica Aplicada, Tarragona, Spain). Enzyme-linked immunosorbent assay kits were used as described above for the quantification of insulin, leptin, and adiponectin.

2.5. RNA Extraction and real-time quantitative RT-PCR analysis

Real-time polymerase chain reaction was used to measure leptin mRNA expression levels in maternal mammary gland and retroperitoneal white adipose tissue (rWAT). Total RNA was extracted using the 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 Wilmington, DE, USA). Its integrity was confirmed using agarose gel electrophoresis.

Real-time polymerase chain reaction was used to measure mRNA expression levels of leptin. Total RNA (0.25 μg, in a final volume of 5μL) was denatured at 65°C for 10 minutes and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 minutes, 42°C for 30 minutes, with a final step of 5 minutes at 95°C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (5 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Real-time PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 minutes at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 minute at 60°C). 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 v2.3., Applied Biosystem) and the relative expression of each mRNA was calculated as previously described [28]. Guanosine diphosphate dissociation inhibitor was used as reference gene. All primers were obtained from Sigma (Sigma Aldrich Co., LLC, Madrid, Spain).

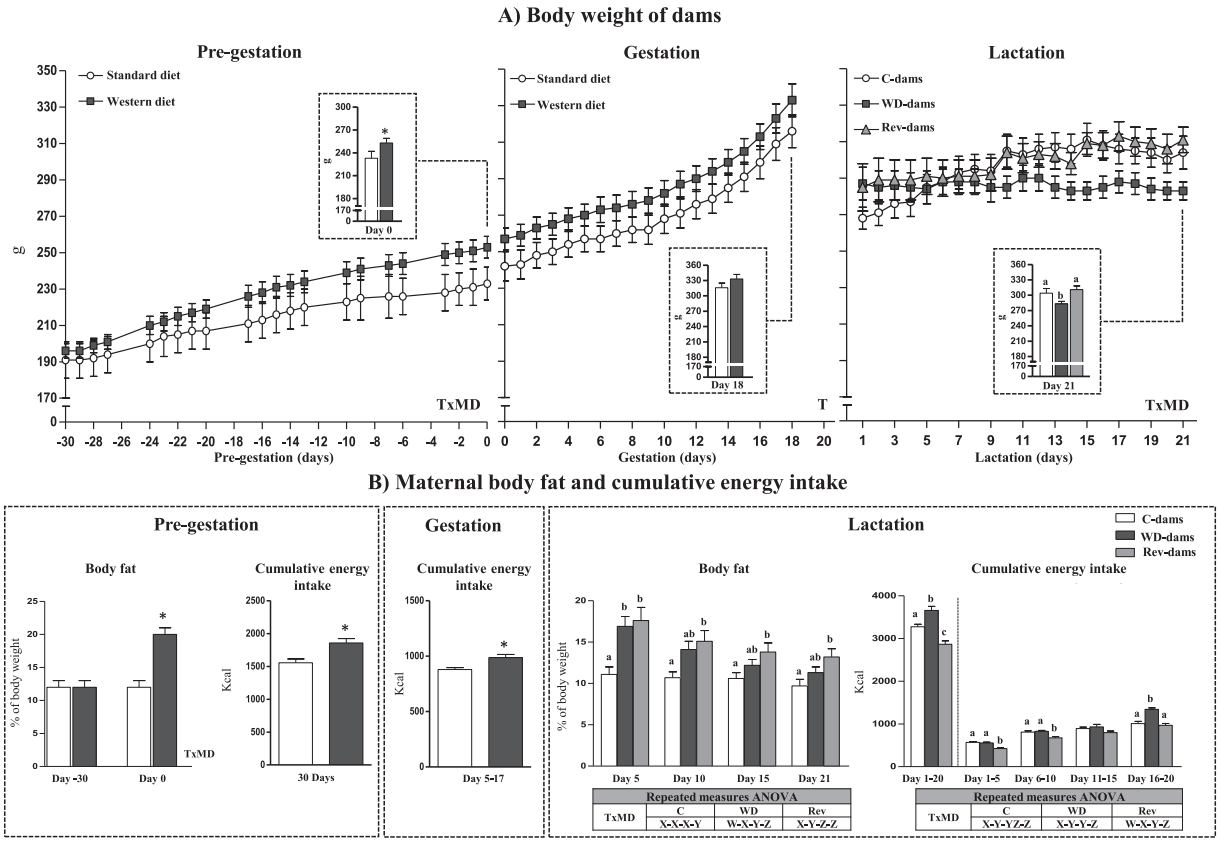


Fig. 1. Body weight (A) and body fat and cumulative energy intake (B) of female rats over the pre-gestation, gestation and lactation periods of control (C-dams), western-diet (WD-dams) and reversion dams (Rev-dams). Data are expressed as the mean±s.e.m. of eight to ten animals per group. Statistics: MD, effect of maternal diet; T, effect of time; TxMD, interactive effect between time and maternal diet (repeated measures ANOVA). LSD *post hoc* test, W≠X≠Y≠Z (Effect of time repeated measures ANOVA) and a≠b≠c (Effect of maternal diet one-way ANOVA). *, Western-diet *versus* control diet (Mann–Whitney U test). Statistic details of LSD *post hoc* test of body weight during lactation: LD14 (a-b-ab); LD15, LD16, LD21 (a-b-a); LD17 and LD19 (ab-a-b).

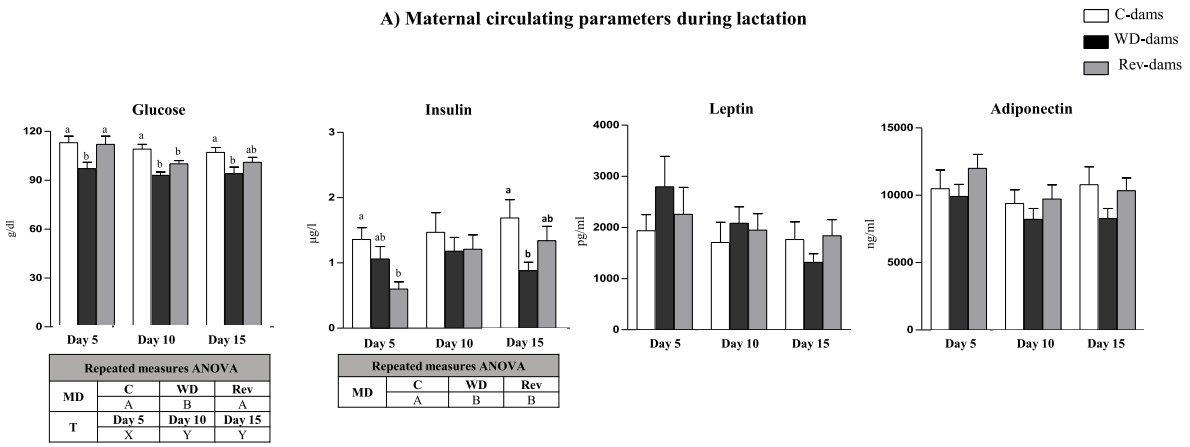
2.6. Statistical analysis

Data are expressed as the mean±SEM (n=8–10). Data were checked for normality using Shapiro-Wilks normality test and Levene's test was performed to assess homogeneity of variances between groups. Logarithmic transformation was applied when required before analysis. During pre-gestation and gestation, comparisons between two groups were assessed by the non-parametric Mann–Whitney U test. During lactation, differences among groups were assessed by one-way ANOVA followed by least significances difference (LSD) *post hoc* comparison. In those parameters measured at different time points, repeated measures ANOVA followed by LSD *post hoc* tests were used to compare the mean differences between groups and/or between the time-points of the lactation period. In addition, relation between two variables were assessed using the Person's correlation coefficient. The test used for each comparison is indicated in the footnote of the figures. Threshold of significance was defined at $P<.05$. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL, USA).

3. Results

3.1. Maternal phenotypic traits during pre-gestation, gestation and lactation

Body weight of female rats over the 30 days prior to gestation (pre-gestation) and during the gestation and lactation periods are shown in Fig. 1A. Body fat content and cumulative food intake is shown in Fig. 1B. During pre-gestation period, dams exposed to WD consumed more calories compared to their controls exposed to SD (19% greater, 1555±58 *vs.* 1857±68 Kcal) (Mann-Whitney U test). They also gained more weight and fat mass than controls (interactive effect between diet and time, repeated measures ANOVA). At the time of mating, body weight and fat mass of WD fed rats were, respectively, 8% and 66% greater than controls (Mann-Whitney U test). During gestation, dams exposed to WD continued consuming more calories than their controls (Mann-Whitney U test) and showed a trend to a higher body weight. However, during lactation, body weight of WD-dams remained practically unaltered, whereas body weight of C- and of Rev-dams (WD-dams that returned to SD after delivery) increased progressively during lactation (interactive effect between diet and time, repeated measures ANOVA). In fact, WD-dams displayed a body weight significantly lower than C-dams between LD14 and LD16 and at the end of lactation (LD21) (one-way ANOVA, *post hoc* analysis; statistic details of LSD *post hoc* test are included in the figure legend). They also displayed a lower body weight than Rev-dams from LD15 onwards (one-way ANOVA, *post hoc* analysis). No significant differences were observed in body weight between Rev- and C-dams during this period. Regarding body fat content, both WD- and Rev-dams displayed a greater body fat percentage than C-dams on LD5, but from LD10 onwards the difference with respect to controls was only maintained for the Rev-dams (one-way ANOVA, *post hoc* analysis). Of note, body fat percentage decreased throughout lactation in WD- and Rev-dams, but it remained more stable in C-dams, with the exception of LD21 in which it underwent a significant decrease (interactive effect between diet and time, repeated measures ANOVA). Despite the lower body weight of WD-dams at the end of lactation compared to C-dams, their cumulative calorie intake throughout lactation was higher than that of C-dams (12%), although the difference was mainly found in the late period, between LD16 and LD20 (33%). In contrast, Rev-dams maintained a

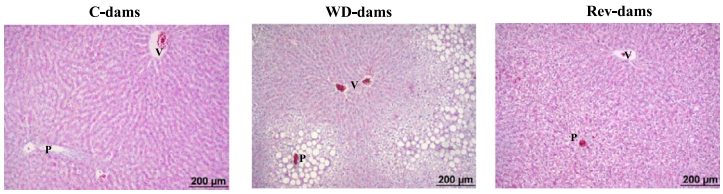


Repeated measures ANOVA			
MD	C	WD	Rev
	A	B	A
T	Day 5	Day 10	Day 15
	X	Y	Y

Repeated measures ANOVA			
MD	C	WD	Rev
	A	B	B

B) Maternal Phenotypic traits and liver histological analysis at weaning (LD21)

	C-dams	WD-dams	Rev-dams	ANOVA
Mammary Gland (g)	6.51 ± 0.53	7.82 ± 0.49	7.58 ± 0.35	-
Liver (g)	12.8 ± 0.67	13.4 ± 0.61	13.0 ± 0.31	-
rWAT (g)	2.35 ± 0.26a	2.98 ± 0.24ab	3.73 ± 0.49b	MD
Glucose (g/dl)	112 ± 5	104 ± 5	109 ± 4	-
Insulin (µg/l)	1.39 ± 0.25a	2.20 ± 0.30b	1.38 ± 0.21a	MD
Leptin (pg/ml)	1130 ± 215a	1645 ± 200ab	1956 ± 219b	MD
Adiponectin (ng/ml)	5440 ± 1656	6091 ± 1310	7571 ± 1254	-
Triglycerides (mg/ml)	1.23 ± 0.10	2.02 ± 0.34	1.42 ± 0.21	-
NEFA (nmol/µl)	0.25 ± 0.05a	0.40 ± 0.05b	0.27 ± 0.03a	MD



Degree of steatosis	C- dams (n=6)	WD- dams (n=10)	Rev- dams (n=9)
Grade 0	6	1	8
Grade 1	-	4	1
Grade 2	-	-	-
Grade 3	-	5	-

V = central vein
P = portal space

Fig. 2. Maternal circulating parameters during lactation (A), and maternal phenotypic traits and liver histological analysis at weaning (LD21) (B). Data are expressed as the mean±s.e.m. of eight to ten animals per group. Statistics: MD, effect of maternal diet; T, effect of time; TxMD, interactive effect between time and maternal diet (repeated measures ANOVA or one-way ANOVA). LSD *post hoc* test, A≠B (Effect of maternal diet repeated measures ANOVA); X≠Y (Effect of time repeated measures ANOVA) and a≠b (Effect of maternal diet one-way ANOVA).

greater adiposity than C-dams during the whole lactation period, despite their reduced calorie intake (12.6%). The decrease in energy intake in Rev-dams was more marked in the first half of lactation, what could be explained by the change from WD to SD. All groups of dams increased their calorie intake throughout lactation. Cumulative intake during the entire period studied (pre-gestation, gestation and lactation) was significantly greater in WD-dams compared with C-dams and Rev-dams, and no significant differences were observed between C-dams and Rev-dams (C-dams: 5731±98 Kcal; WD-dams: 6615±138 Kcal; Rev-dams: 5739±116 Kcal) (a,b,a; one-way ANOVA, *post hoc* analysis).

Maternal circulating parameters at different time-points of lactation are shown in Fig. 2A, and phenotypic traits and liver histological analysis at weaning (LD21) are shown in Fig. 2B. WD-dams exhibited lower glucose levels than C- and Rev-dams (repeated measures ANOVA, *post hoc* analysis). Rev-dams also displayed lower circulating glucose levels than controls at LD10 (one-way ANOVA, *post hoc* analysis). Both WD- and Rev-dams displayed lower insulin levels than C-dams (repeated measures ANOVA, *post hoc* analysis). The differences compared to C-dams were more pronounced at LD5 for Rev-dams (one-way ANOVA, *post hoc* analysis) and at LD15 for WD-dams (one-way ANOVA, *post hoc* analysis). A different pattern was observed at the end of lactation (LD21), since WD-dams displayed greater insulin levels than C- and Rev-dams (one-way ANOVA, *post hoc* analysis). No significant differences were observed in maternal leptin and adiponectin circulating levels during lactation, but at LD21 Rev-dams displayed greater circulating leptin levels than C-dams (one-way ANOVA, *post hoc* analysis).

As shown in Fig. 2B, Rev-dams had a greater weight of the rWAT than C-dams (one-way ANOVA, *post hoc* analysis), but no differences were observed in the weight of the mammary gland and liver between groups (one-way ANOVA, *post hoc* analysis). WD-dams displayed greater NEFA levels than C- and Rev-dams (one-way ANOVA, *post hoc* analysis) and a tendency to greater circulating levels of TG than C-dams (P=.08, one-way ANOVA, *post hoc* analysis LSD). Liver histological analysis revealed that nine of the ten WD-dams presented different degrees of hepatic steatosis, between grade 1 and grade 3, but no signs of steatosis were observed in C-dams. In the case of Rev-dams, one of the nine animals presented grade 1 steatosis.

3.2. Offspring phenotypic traits during the suckling period

Body weight evolution during the suckling period was different between the offspring of C-dams (O-C), the offspring of WD-dams (O-WD), and the offspring of Rev-dams (O-Rev), both males and females (interactive effect between diet and time, repeated measures ANOVA) (Fig. 3A). O-WD pups displayed a greater body weight from PND6 (males) and PND8 (females) onwards compared with O-C and O-Rev pups (one-way ANOVA, *post hoc* analysis). In turn, O-Rev pups displayed a lower body weight than O-C pups from PND1 to PND7 and from PN12 onwards (males) or from PN16 onwards (females) (one-way ANOVA, *post hoc* analysis). Body composition analysis at weaning revealed that O-WD pups, both males and females, displayed greater body fat mass and fat percentage compared to O-C and O-Rev pups (one-way ANOVA, *post hoc* analysis). In addition, O-Rev pups displayed a lower body fat mass than

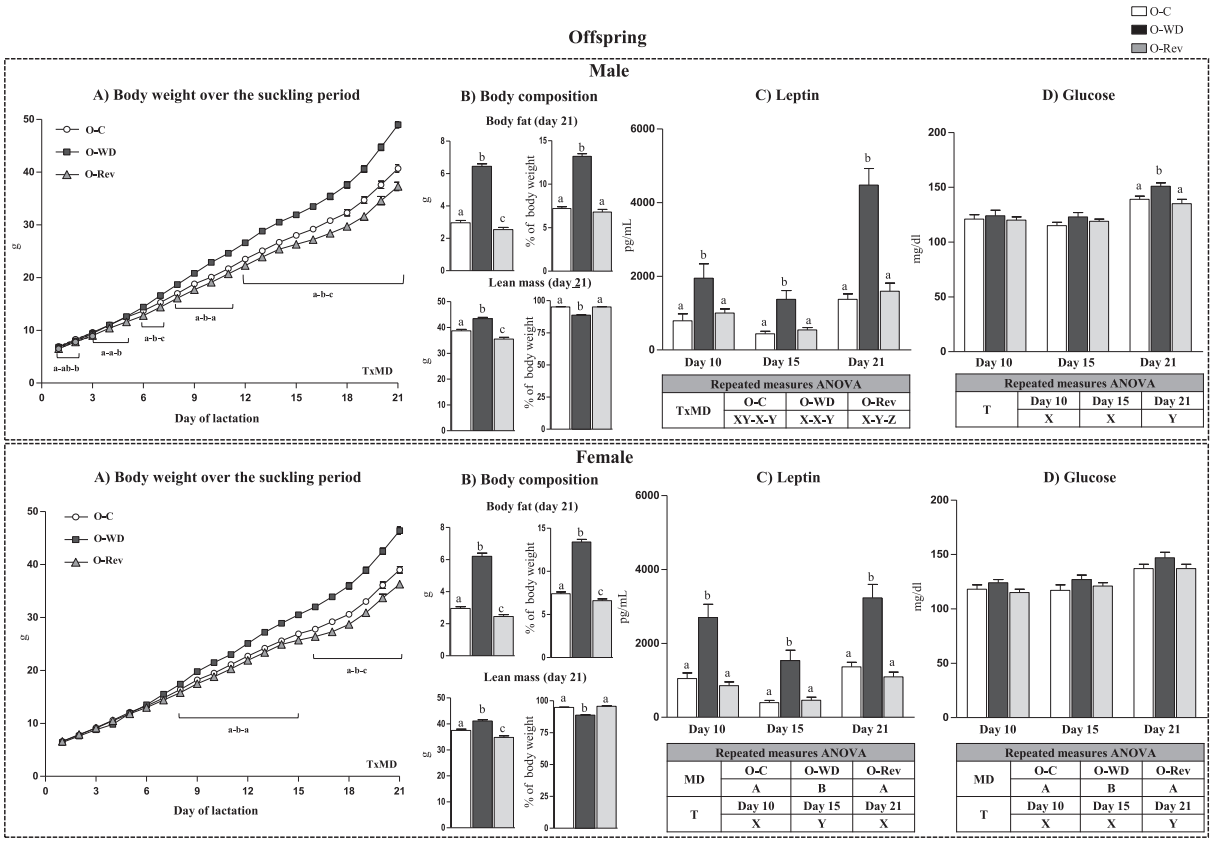


Fig. 3. Body weight over suckling period of male and female offspring of control (O-C), western-diet (O-WD) and reversion dams (O-Rev) (A). Body fat and lean mass of male and female O-C, O-WD and O-Rev at PN21 (B). Leptin and glucose levels of male and female O-C, O-WD and O-Rev at PN10, PN15 and PN21. Data are expressed as the mean±s.e.m. of eight to ten animals per group. Statistics: MD, effect of maternal diet; T, effect of time; TxMD, interactive effect between time and maternal diet (repeated measures ANOVA). LSD *post hoc* test, A≠B (Effect of maternal diet repeated measures ANOVA); X≠Y≠Z (Effect of time repeated measures ANOVA) and a≠b≠c (Effect of maternal diet one-way ANOVA).

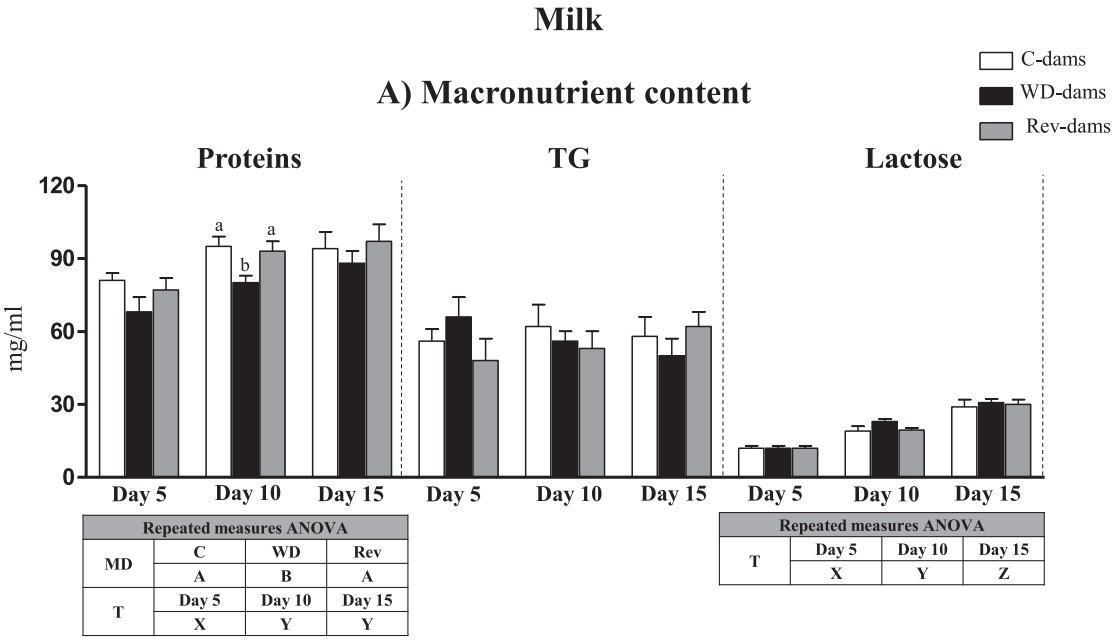
O-C pups, and O-Rev females, but not males, also displayed a lower fat percentage than O-C (one-way ANOVA, *post hoc* analysis). Notably, male and female O-WD pups also displayed a higher lean mass content than O-C and O-Rev pups (one-way ANOVA, *post hoc* analysis). In turn, O-Rev rats displayed lower lean mass than O-C rats. However, when considering lean mass percentage, O-WD pups showed lower lean mass than O-C and O-Rev pups (one-way ANOVA, *post hoc* analysis), and no differences were found between O-C and O-Rev groups. Thus, the differences between groups regarding body weight can be attributed mainly to differences in body fat content, but also in lean mass content.

Circulating leptin and glucose levels were measured in these animals under ad libitum feeding conditions at PN10, PN15 and PN21 (Fig. 3C). In accordance with their higher adipose mass, O-WD pups displayed greater circulating leptin levels than O-C and O-Rev pups (one-way ANOVA, *post hoc* analysis), whereas no significant differences were observed between O-Rev and O-C pups. In fact, a positive correlation was found between plasma leptin levels and body fat percentage at PND21 (r=0.886; $P<.001$ (males), and r=0.815, $P<.001$ (females), Pearson's correlation). In addition, in males there was an interactive effect between circulating leptin levels and the day of lactation (interactive effect between diet and time, repeated measures ANOVA). Circulating levels of leptin at PND15 were significantly lower than at PND10 only in O-Rev males (repeated measures ANOVA, *post hoc* analysis). However, in females, all groups showed a decrease in their circulating levels at PND15 compared to PND10 (repeated measures ANOVA, *post hoc* analysis). Circulating glucose levels in PND21 were greater compared to the other days studied (PND10 and 15) in both males and females (repeated measures ANOVA, *post hoc* analysis). In addition, O-WD females displayed higher glucose levels during the suckling period compared to O-C and O-Rev pups (repeated measures ANOVA, *post hoc* analysis). O-WD males also showed higher glucose levels than O-C and O-Rev pups at the end of the suckling period (PND21) (one-way ANOVA, *post hoc* analysis).

3.3. Milk composition

The concentration of proteins, TG and lactose was measured in milk at three time points during lactation (Fig. 4A). Protein content on LD10 and LD15 of lactation was higher compared to the content on LD5 (repeated measures ANOVA, *post hoc* analysis). Milk from WD-dams presented a lower protein content than that of C- and Rev-dams throughout the lactation period (repeated measures ANOVA, *post hoc* analysis), but specially at LD10 (one-way ANOVA, *post hoc* analysis). No differences were observed in the TG content either due to maternal conditions or the course of lactation. Lactose concentration progressively increased from LD5 to LD15 (repeated measures ANOVA, *post hoc* analysis), but no differences were observed between groups.

The energy content of milk, estimated from the concentration of the macronutrients above mentioned, increased throughout the lactation period (repeated measures ANOVA, *post hoc* analysis), but no significant differences were found between groups (Fig. 4B). The macronutrient distribution of milk is shown in Fig. 4C. The contribution of lactose to the energy content increased throughout lac-

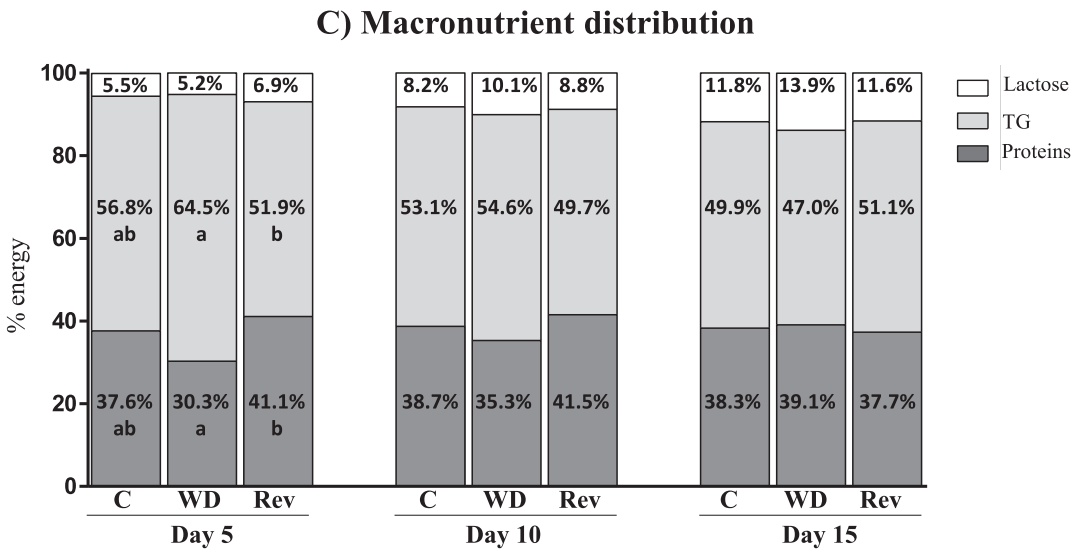


Repeated measures ANOVA			
MD	C	WD	Rev
	A	B	A
T	Day 5	Day 10	Day 15
	X	Y	Y

Repeated measures ANOVA			
T	Day 5	Day 10	Day 15
	X	Y	Z

B) Energy content (Kcal/100ml)

	Day 5		Day 10		Day 15		Repeated measures ANOVA
C-dams	87 ± 4		101 ± 8		101 ± 7		
WD-dams	91 ± 7	X	91 ± 4	XY	93 ± 7	Y	T
Rev-dams	79 ± 9		93 ± 6		107 ± 7		



Repeated measures ANOVA				
Lactose	T	Day 5	Day 10	Day 15
		X	Y	Z
TG	T	Day 5	Day 10	Day 15
		X	XY	Y

Fig. 4. Macronutrient content (A), energy content (B) and macronutrient distribution (C) in milk at day 5, 10 and 15 of lactation of control, western-diet and reversion dams (A). Data are expressed as the mean±s.e.m. of eight to ten animals per group. Statistics: MD, effect of maternal diet; T, effect of time, (repeated measures ANOVA). LSD *post hoc* test, A≠B (Effect of maternal diet repeated measures ANOVA); X≠Y≠Z (Effect of time repeated measures ANOVA) and a≠b (Effect of maternal diet one-way ANOVA).

tation (repeated measures ANOVA, *post hoc* analysis), whereas the contribution of TGs decreased (repeated measures ANOVA, *post hoc* analysis). Milk from WD-dams presented a greater percentage of the total energy from TGs and lower from proteins compared to Rev-dams at LD5 (one-way ANOVA, *post hoc* analysis). No differences were observed in the distribution of macronutrient when comparing WD- and Rev-dams with C-dams.

The levels of candidate hormones (insulin, leptin and adiponectin) were also measured in milk at different time points (Fig. 5A). Insulin levels, unlike leptin and adiponectin, were higher in milk than in maternal plasma. Insulin levels in milk at LD10 and 15 were higher than at LD5 (repeated measures ANOVA, *post hoc* analysis) and were influenced by maternal group (repeated measures ANOVA, *post hoc* analysis). Milk of WD-dams displayed lower insulin levels than C-dams, but no differences were observed between Rev- and C-dams (repeated measures ANOVA, *post hoc* analysis). However, Rev-dams also displayed lower insulin levels than C-dams on LD5 (one-way ANOVA, *post hoc* analysis), but levels were normalized to those of C-dams with the course of lactation. Regarding milk leptin, the pattern at different time points was different between groups (interactive effect between diet and time, repeated measures ANOVA). In C-dams, leptin increased progressively during lactation (repeated measures ANOVA, *post hoc* analysis). At day 15 of lactation, WD-dams, but not Rev-dams, displayed lower milk leptin levels than C-dams (Mann-Whitney U test). No differences were observed between groups concerning adiponectin levels considering the whole lactation period, but WD-dams at LD15 displayed lower levels than C- and Rev-dams (one-way ANOVA, *post hoc* analysis).

Correlations between milk and maternal plasma hormone levels during lactation were done segmented by maternal conditions (Fig. 5B). There was a significant positive correlation for insulin and adiponectin in C-dams (r=0.535, $P<.01$; r=0.753, $P<.001$, respectively, Pearson's correlation) and in Rev-dams (r=0.422, $P<.05$; r=0.503, $P<.01$, respectively), but no correlations were found for these hormones in WD-dams. Regarding leptin, a positive correlation between milk and plasma levels was found only in the WD-dams (r=0.505, $P<.01$).

3.4. Maternal leptin expression by mammary gland and the retroperitoneal white adipose tissue

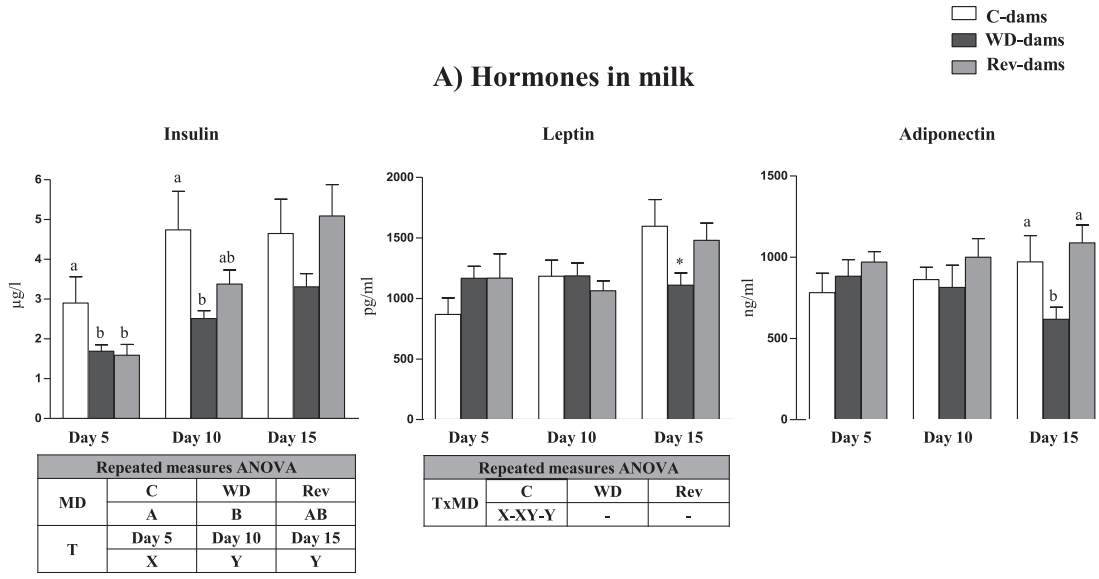
Besides the adipose tissue, the mammary gland also produces leptin, contributing to their levels in milk. Given the lack of correlation between leptin levels in plasma and milk in C- and Rev-dams, we analyzed leptin mRNA levels in the mammary gland and the rWAT at LD21 (at weaning), to determine if they follow the same profile (Fig. 6A). A differential pattern was observed between both tissues. In the rWAT, WD-dams exhibit higher leptin mRNA levels than C-dams (Mann-Whitney U test), and similar to Rev-dams. However, in the mammary gland, Rev-dams showed greater leptin mRNA levels than WD-dams (one-way ANOVA, *post hoc* analysis), whereas no significant differences were found between WD-dams or Rev-dams and C-dams. Of note, leptin mRNA expression levels in the rWAT were positively correlated with both body fat percentage and the weight of this fat depot, considering all groups of rats (r=0.838, $P<.001$; r=0.748, $P<.001$, respectively, Pearson's correlation), whereas no correlation was found concerning leptin expression in the mammary gland and body fat percentage or the weight of the mammary gland (Fig. 6B).

4. Discussion

Exclusively breastfeeding is universally recommended due to its health benefits for mothers and infants. Among others, breastfeeding affords a reduction in childhood obesity risk and type 2 diabetes [29–31]. However, the protective effects of breastfeeding may differ depending on maternal phenotype. An increasing number of children are born to and breast fed by mothers with obesity, as consequence that many women in western countries consume poor-quality diets that contain excessive levels of sugars and fat [32,33]. This consumption has been simulated in animal models exposed to a high-fat and/or high-sucrose diet during gestation and/or lactation, providing important insights on the harmful metabolic programming effects in the offspring [5–8]. Nevertheless, studies allowing to discern the differential metabolic effects of maternal obesity per se from the direct effects of the intake of unbalanced diets are scarce. Here, we show, in rats, that maternal intake of an obesogenic diet during lactation, rather than maternal excess of adiposity (in the absence of dietary alterations during lactation), appears as the main contributor to alterations in milk composition and the accelerated postnatal growth of the offspring. Moreover, dietary improvement during lactation after the intake of an unbalanced diet prior and during gestation prevents, at least in part the early detrimental effects in the offspring.

Lactation is a physiological state that represents important challenges for maternal energy homeostasis to be adapted to this demanding condition. Changes in circulating levels of metabolic hormones, such as leptin and insulin, among others, as well as in the responsiveness to these signals contribute to the modifications in energy balance pathways during this period [34]. The need of energy for milk production supposes a negative energy balance for dams, despite the presence of hyperphagia, what influences their body weight and metabolic profile [35]. Here, we show that such negative energy balance seems to be more marked in WD-dams. They consumed more calories during the lactation period than C-dams, while showing a progressive reduction of their body fat percentage from 17% (LD5) to 11% (LD21), so that it was not different from that of C-dams from LD10. In turn, their offspring (O-WD) displayed a greater body weight from mid lactation than their controls. This was accompanied by the presence of hyperleptinemia, already evident at PND10, higher circulating glucose levels, greater fat mass, and lower lean mass percentage. In this sense, a direct effect of the WD consumed directly by the offspring must be considered, since they begin to eat solid food, in addition to milk, in late lactation. This fact may also contribute to the higher calorie intake attributed to their dams.

The reversion group is of interest to discriminate the effects of maternal overweight from those of overeating an unbalanced diet during lactation, since the intrauterine environment was the same as that of WD-dams. Of note, different outcomes were observed in Rev-dams and their offspring (O-Rev) in comparison to WD-dams and O-WD. As a consequence of the dietary normalization after delivery, Rev-dams displayed a voluntary calorie restriction (12.6% and 21.7% compared to C-dams and WD-dams, respectively, considering the whole lactation period), but maintained excess body fat compared to C-dams throughout lactation. We have previously reported that a forced energy restriction of 20% in normal-weight rats during lactation produced notable effects on body weight in dams and their offspring (24% and 34% reduction *vs.* controls, respectively) [36]. In the present study, the effects of voluntary energy restriction during lactation were not observed in body weight of dams and were more modest in the offspring (8.4% and 6.9% reduction in males and females, respectively, compared to O-C at weaning). The lower body weight of O-Rev animals in comparison to O-C animals may be attributed, in part, to a lower lean mass, although the percentage of lean mass was similar. Very interestingly, O-Rev pups displayed circulating levels of leptin and glucose during lactation similar to those of O-C pups, as well as similar (males), or even lower (females) body fat percentage at weaning,



Repeated measures ANOVA			
MD	C	WD	Rev
	A	B	AB
T	Day 5	Day 10	Day 15
	X	Y	Y

Repeated measures ANOVA			
TxMD	C	WD	Rev
	X-XY-Y	-	-

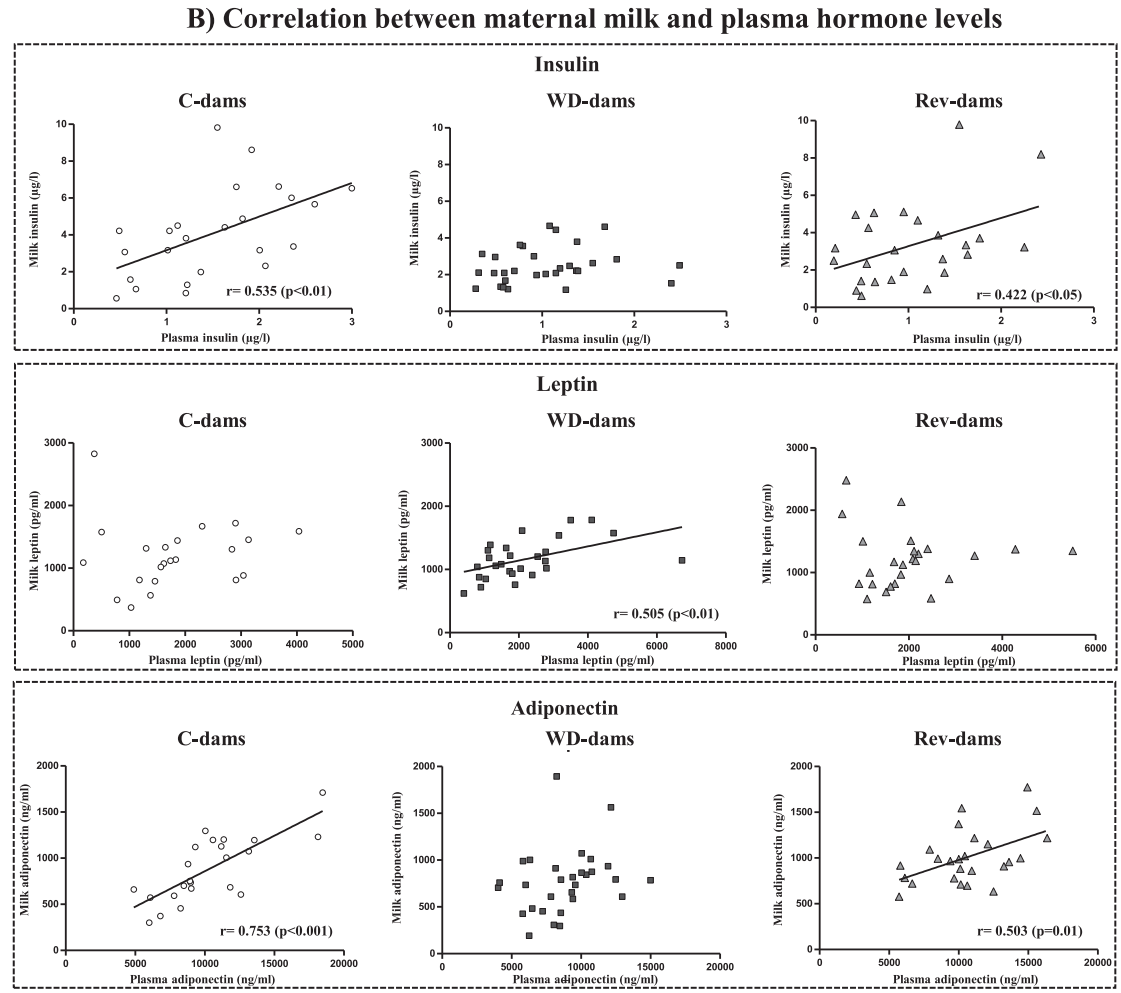


Fig. 5. Concentration of insulin, leptin, and adiponectin levels in milk at day 5, 10 and 15 of lactation of control, western-diet, and reversion dams (A). Correlation between milk and maternal plasma levels in control, western-diet, and reversion dams (B). Data are expressed as the mean±s.e.m. of eight to ten animals per group. Statistics: MD, effect of maternal diet; T, effect of time; TxMD, interactive effect between time and maternal diet (repeated measures ANOVA). LSD *post hoc* test, A≠B (Effect of maternal diet repeated measures ANOVA); X≠Y (Effect of time repeated measures ANOVA) and a≠b (Effect of maternal diet one-way ANOVA). *, Western-diet *versus* control diet (Mann–Whitney U test). The correlation was done by Pearson correlation (two-tailed).

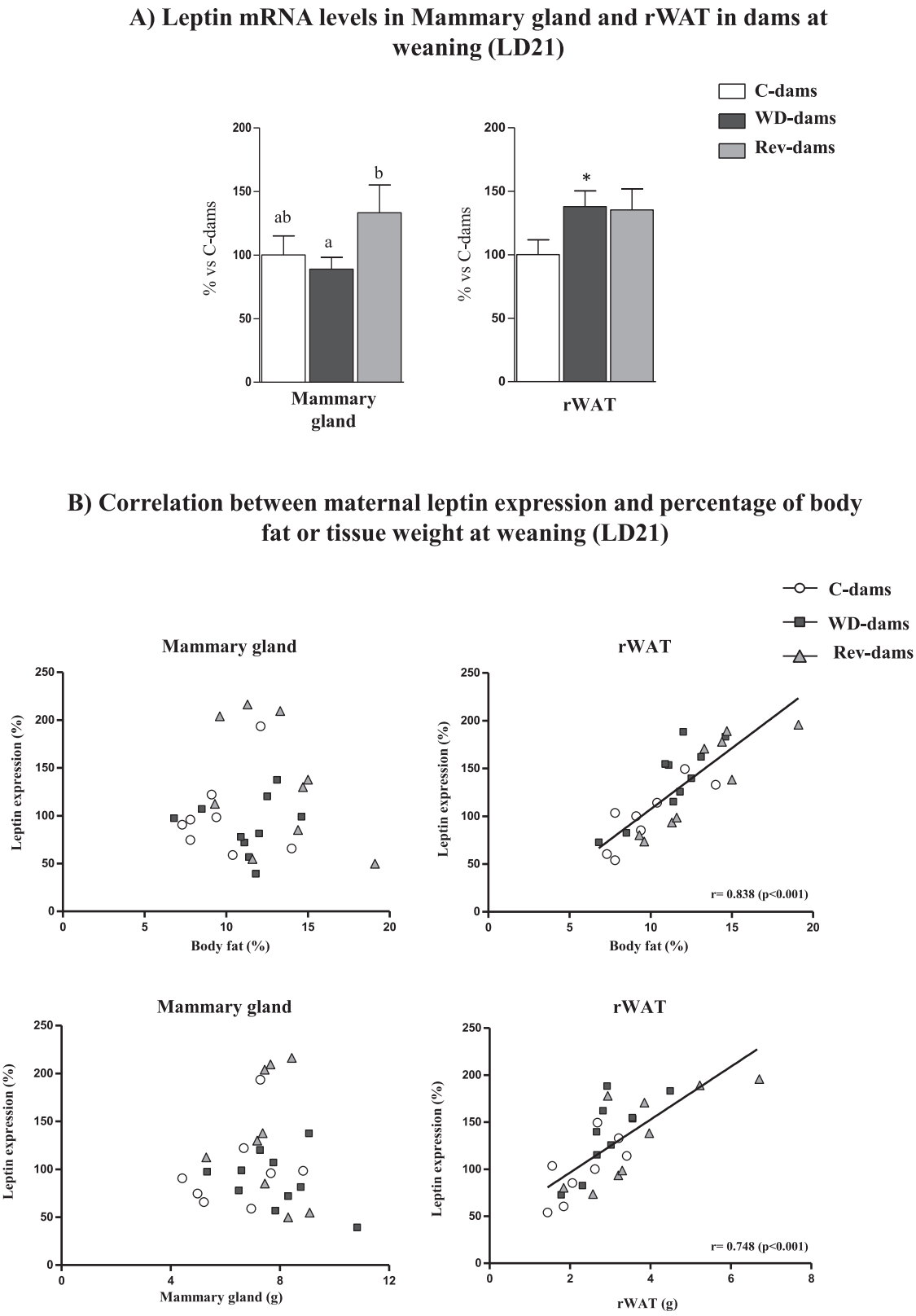


Fig. 6. Leptin mRNA levels in mammary gland and retroperitoneal white adipose tissue (rWAT) of control, western-diet and reversion dams at weaning (LN21) (A). Correlation between maternal leptin expression and percentage of body fat or tissue weight at weaning (LD21) (B). One-way ANOVA followed by LSD *post hoc* test, a≠b. *, Western-diet *versus* control diet (Mann–Whitney U test). The correlation was done by Pearson correlation (two-tailed).

despite having been exposed to an obesogenic environment during gestation. An accelerated growth during lactation has been proposed as an important risk factor for type 2 diabetes and obesity [9]. Thus, here, the improvement of maternal diet during lactation seems to avoid or attenuate the harmful effects in offspring associated to maternal intake of an unbalanced diet.

Circulating parameters in WD-dams during lactation reflected their altered metabolic state. Under feeding conditions, they displayed lower glucose levels compared to C-dams, accompanied by lower insulin levels, particularly at LD15. A decrease in insulin levels has also been described in rat dams exposed to a cafeteria diet during lactation [19]. This may reflect the situation of negative energy balance, that may be accompanied by changes in the sensitivity to this hormone. Concretely, during lactation, maternal tissues, such as the adipose tissue, display a selective insulin resistance, whereas the mammary gland remains insulin sensitive ensuring that metabolic fuels are channeled to this site for milk production [37,38]. It must be considered that TG present in milk can derive from different sources, including dietary lipids, fat reserves, and de novo synthesis within the mammary gland [39]. Despite NEFA were only determined at the end of lactation, the greater circulating levels of NEFA in WD-dams may be in agreement with the increased fat mobilization in response to fuel demands for milk production [40,41]. Nevertheless, the presence of insulin resistance in the mammary gland of WD-dams, impairing de novo fatty acids synthesis, cannot be ruled out, as has been suggested in rats exposed to cafeteria diet during lactation [19]. This would explain the need for a greater TG mobilization from their own fat stores. However, unlike the pattern during lactation, at weaning, WD-dams showed higher insulin levels compared to controls along with the normalization of glucose levels, coincident with the completion of lactation and the end of the increased energetic demands for milk production. The presence of higher insulin levels was expected due to the WD consumption. Moreover, histological analysis revealed that WD-dams also displayed a greater fat accumulation in liver and signs of hepatic steatosis. Interestingly, Rev-dams showed lower insulin and glucose levels than C-dams during the first half of lactation, but levels were similar to that of C-dams at the end of lactation, suggesting that the glucose-insulin homeostasis was normalized due to dietary improvement. No alterations in NEFA levels or in hepatic morphology and lipid accumulation were either observed in Rev-dams. Instead, and reflecting their greater fat mass, Rev-dams also presented greater circulating leptin levels than C-dams and a greater rWAT mass at the end of lactation.

Milk composition is of relevance to infant growth and development [15,42]. The accelerated postnatal growth-up in O-WD pups, but not in O-Rev, may be associated with a higher milk energy content and/or to specific changes in their macronutrient composition due to maternal diet during lactation. Studies conducted in animal models suggest that an obesogenic dietary pattern may impact the milk macronutrient composition [12,15,43]. Here, we observed no changes in the energy content of milk between groups. Total TG and lactose levels were unaffected by maternal diet/conditions during the lactation period. In fact, the concentration of lactose in milk has been described to be generally stable and less sensitive to maternal diet [15,18]. Results regarding TG are also in line with other animal studies showing that total fat remains unchanged in milk after consuming a high-fat diet [44], although other studies have found a greater lipid content in high-fat diet [45,46] or cafeteria-diet [12] fed rats. Total milk protein content was decreased in WD-dams compared to C-dams, whereas the reversion of the diet during lactation normalized the protein content in milk. This suggest that the decrease in the milk protein content in WD-dams is related to the diet rather than to maternal adiposity. Macronutrient distribution was different between WD-dams and Rev-dams at early lactation, since milk from Rev-dams presented a lower percentage of the total energy from lipids and greater from the protein fraction compared to that of WD-dams. These changes in the macronutrient distribution and particularly the reduction in the total protein content could contribute to the differences in infant growth, as previously described in the offspring of rats exposed to a cafeteria diet during lactation [12].

Besides macronutrient composition, potential alterations in bioactive hormones present in milk, particularly, endogenously synthesized peptide hormones and cytokines with a potential role in infant growth and development may also be considered. In fact, the concentration of specific bioactive peptides has been described to be influenced by maternal diet during lactation [47]. Among them, the one that shows clearer evidence as a potential programming factor during lactation is leptin [25], but other hormones such as insulin and adiponectin, which have an important role in energy metabolism may also be of interest, although their roles as milk components have been less explored [20].

Regarding insulin, despite its important and well-known role in energy and glucose homeostasis [48], its function as a component of breast milk is poorly understood. Of interest, milk insulin levels were greater (about double) that those found at the same time-point in maternal circulation. These results are in line with human studies [49], suggesting that insulin could be actively transported from maternal circulation into milk, although the transporter involved has not yet been identified [50]. Additionally, human studies have reported that insulin concentrations in breast milk are higher in overweight and women with obesity compared to their normal-weight counterparts [49,51–53], and levels were correlated with maternal insulin concentration, HOMA-IR and body mass index [49]. Here, we found that WD-dams displayed lower insulin levels in milk than C-dams during the whole lactation period. Rev-dams also showed lower insulin levels than C-dams at the beginning of lactation, but levels were no longer different from controls from LD10, again pointing out the importance of maternal diet. Young et al. have also shown that maternal plasma concentration and insulin sensitivity may impact breast milk insulin concentration more than maternal BMI [49]. Notably, in the present study, insulin levels in milk and plasma were positively correlated in C- and Rev-dams, but not in WD-dams. Considering that insulin does not seem to diffuse into milk via the paracellular pathway, but rather by regulated transport through the mammary gland epithelium, and there is also no evidence that this hormone can be synthesized in the mammary gland [50], current results suggest the existence of alterations in the active transfer of circulating insulin into milk associated to WD consumption. However, possible mechanisms involved and how they are affected by maternal conditions need to be further elucidated.

Therefore, it must be deduced that O-WD pups were chronically exposed to relatively lower doses of oral insulin during lactation than controls, whereas this alteration was normalized in O-Rev pups. The functions of milk insulin and the possible impact of alterations in its concentration are not really known. Some studies suggest that milk insulin may act locally in the enterocytes or be absorbed into circulation and mediate offspring growth effects [54–56]. It has also been described that oral insulin may have an effect on gut [57] and pancreas [58] maturation, and exert favorable effects on blood glucose and lipid profile [59]. Insulin in human milk has also been shown to influence the microbiome in the gastrointestinal tract [60]. Regarding the possible impact of milk insulin on infant body weight, Fields et al. found a negative correlation between milk insulin levels and body weight, weight-for-length z-score, BMI-for-age z-score, and total lean mass in infants born from normal weight and mothers with obesity at 1 month

of age, proposing that milk insulin may have a function in the buildup of body fat and body lean mass in infants [61]. Interestingly, Chan et al. have been described a U-shaped association between breast milk insulin at 4 months and the weight-for-length (WFL) and BMI at 4 months and 1 year, with intermediate concentrations of breast milk insulin predicting the lowest infant WFL and BMI z-score. Thus, intermediate concentrations of breast milk insulin may optimally support infant metabolism as the immature pancreas develops its capacity to produce insulin, whereas insufficient or excessive insulin in breast milk may impair this process [53]. Of note, pup nursed by WD-dams had greater glucose levels during the whole lactation (females) or at the end of lactation (males) as compared with pups nursed by C- and Rev-dams, which could be tentatively associated with an insufficient insulin supply from milk.

Another metabolism-regulating hormone found in effective quantities in breast milk is adiponectin. Circulating adiponectin exhibits insulin sensitizing and anti-inflammatory effects and their levels are inversely related to fat mass and insulin resistance [62,63]. However, adiponectin levels in milk do not seem to follow the same association as in circulation, and its role as a milk component looks controversial. Some human studies have described a positive relationship between maternal adiposity and adiponectin levels in breast milk [64,65]. In turn, higher milk adiponectin has been associated with lower infant weight [66] or WFL z-score [67] during the first 6 months of age; however, this higher supply of adiponectin was subsequently associated with greater weight gain and adiposity in the second year of life in the same children [66,68]. In another study, higher levels of adiponectin in breast milk have also been associated with a greater risk of being overweight at 2 years of age in breastfed infants [51]. Thus, milk adiponectin might influence infant growth and development, but no direct relationship has been shown. Animal studies have not shed much light on the possible role of adiponectin in milk, nor on the relationship between its levels in milk and the mother's dietary or metabolic conditions. For example, milk adiponectin levels have been described to be increased in nursing rats exposed to a mild calorie restriction during lactation [36], a condition associated with a certain protection in the offspring against the development of metabolic alterations [69–71]. However, rats fed a cafeteria diet during lactation [47] and rats made obese by cafeteria diet feeding and moved to a standard diet before mating [72] also displayed greater milk adiponectin levels. These maternal conditions were associated with adverse or neutral effects in the offspring, respectively [12,72]. Interestingly, both maternal overexpression of adiponectin in transgenic mice and maternal adiponectin deficiency in knockout mice have been shown to lead to a systemic inflammatory response in the pups [73]. Therefore, the presence of lower milk adiponectin levels in the final step of lactation in WD-dams compared to controls, but not in Rev-dams, could be tentatively related with the early hallmarks of metabolic syndrome that show O-WD pups, such as greater body weight and adiposity, hyperleptinemia and higher circulating glucose levels compared to O-C and O-Rev pups. However, a cause-effect relationship cannot be established, and the effects observed in the offspring may be the result of the combination of a myriad of milk components. On the other hand, it should be noted that in the present study, and similarly to what observed for insulin, adiponectin levels in milk were correlated with maternal plasma levels of adiponectin in C- and Rev-dams, but not in WD-dams. This suggests the existence of alterations in the transfer from circulation to milk is this group of dams. Moreover, since adiponectin is expressed in the mammary gland [73], alterations in its production associated to WD consumption may also account for these differences. These results in control dams are in accordance with a previous study in rats showing the existence of a positive correlation between milk and maternal plasma levels of adiponectin [74], but the effects of maternal diet and/or maternal conditions have not been specifically addressed.

Among the hormones present in milk, probably the most relevant and for which there is clear evidence of its physiological importance during lactation in metabolic programming is leptin [25]. Leptin levels in breast milk have been reported to be positively correlated with circulating leptin levels and with maternal BMI/adiposity [24,75], so that mothers with overweight/obesity generally have greater amounts of leptin in breast milk than normal-weight mothers [25,61]. Interestingly, observational studies have shown the existence of a negative correlation between breast milk leptin levels and infant body weight gain and BMI [24,25,76,77]. Animal studies have provided direct demonstration of the key role of leptin intake during the suckling period both in neonatal development and long-lasting metabolic programming [3,25]. In rats, leptin oral supplementation at physiological doses during the suckling period has been shown to exert beneficial effects in the offspring, since it prevents against the development of overweight and excess adiposity in adulthood and improves leptin and insulin sensitivity and the general metabolic state, both under standard and HF-diets [21,78,79]. In the present study, leptin levels increased in C-dams throughout the lactation period, as previously described [80], but this increase was not observed in WD-dams and Rev-dams, so that WD-dams, but not Rev-dams, displayed lower milk leptin levels than C-dams on LD15. Notably, unlike what described in humans, no significant correlation was found between milk leptin levels and maternal plasma levels in C-dams and Rev-dams, but a positive correlation was shown in WD-dams. The discrepancy between groups could be tentatively attributed to differences in leptin production by the mammary gland. Interestingly, at weaning, Rev-dams showed higher leptin mRNA expression levels than WD-dams in mammary gland. This pattern was tissue-specific, since leptin expression in the rWAT of WD-dams was similar to that of Rev-dams and greater than controls. Moreover, as expected, leptin mRNA levels in the rWAT were positively correlated with maternal adiposity and with the weight of this fat depot, but no correlation was found regarding leptin expression in the mammary gland. The mechanisms and factors involved in the specific regulation of leptin production by the mammary gland remain unknown, but they deserve to be studied. A different pattern of leptin expression between the mammary gland and the rWAT was also described in rats exposed to a moderate calorie restriction during lactation [71]. These rats showed greater leptin expression in the mammary gland compared to their controls, with no changes in the WAT. Notably, the offspring of these dams were found to be more protected against diet-induced obesity and related metabolic alterations in adulthood [69,71]. In another model consisting of rats made obese by cafeteria diet feeding, removal of this diet 1 month before mating (post-cafeteria rats) was shown to prevent the potential adverse effects associated with maternal obesity in the offspring [72]. These dams exhibited higher leptin concentration in milk in comparison to their controls, and this was tentatively related with the protection of their offspring against excessive fat accumulation in adulthood [72]. Therefore, the presence of higher leptin expression in the mammary gland of Rev-dams and the maintenance of milk leptin levels similar to controls might contribute to the improvement in the growth rate and adiposity of their offspring.

Leptin orally administered during the suckling period has been described to exert its biological functions acting on receptors present in the gastrointestinal tract [25], but it is also absorbed by the immature stomach of neonate rats and transferred into circulation, probably exerting additional systemic effects [80]. The

contribution of oral leptin during the suckling period to circulating levels seems to be lower compared to that produced by the adipose tissue. In fact, the lower milk leptin supply to the offspring of WD-dams in the late lactation period was not associated to lower circulating leptin levels. Instead, these animals displayed greater leptin levels than O-C and O-Rev pups, according to their greater body fat percentage, and a positive correlation was found between both parameters at PND21. Of note, despite the beneficial effects described with the administration of physiological doses of leptin orally [21], the hyperleptinemia present in the offspring of WD-dams during the suckling period is not expected to have long-term beneficial effects. In fact, different outcomes were obtained in animal studies in which leptin was injected, instead of orally administered, into pups during the suckling period, resulting in increased plasma levels of leptin, as they were associated with greater body weight gain [81] or with increased diet-induced body weight gain and with related metabolic alterations [82].

Epigenetic modifications have been proposed as mechanisms by which suboptimal nutrition during critical stages of life, including fetal and early postnatal periods, could contribute to developmental plasticity and affect the long-term health of the individual. In fact, some studies carried out in animal models show that maternal overnutrition leads to alterations in the gene methylation profile in key metabolic tissues such as the liver [83,84] or the hypothalamus [85] leading to long-term epigenetic modifications in the offspring. Therefore, although in the present study we have not analyzed epigenetic changes, it is plausible that they could contribute to some of the effects of maternal conditions on the offspring development. Moreover, besides main programming effects in the offspring are related to maternal conditions, paternal environmental factors can also modulate epigenetics in the offspring, through imprinting processes in the sperm [86]. In fact, paternal obesity, diabetes mellitus and nutritional habits are associated with adverse effects on the metabolic and cardiovascular health of their offspring [86]. In the present study, all reproductive males used for mating the female dams within the three experimental groups had the same age and similar body weight. They were also kept under the same stabling conditions and fed a standard diet, except for the mating days with the dams fed the Western diet (up to 5 days). Therefore, the putative influence on the offspring of paternal exposure to this diet is expected to be small compared to maternal influence but it can't be ruled out, notwithstanding the short period of paternal exposure to this diet.

In conclusion, we show that normalization of the diet during lactation in nursing rats after the intake of an obesogenic diet before and during gestation attenuates the rapid postnatal growth-up and greater adiposity, as well as the hyperleptinemia and higher circulating glucose levels observed in the offspring of dams that were maintained with the obesogenic diet during lactation. These effects may be at least in part attributed to the normalization of milk composition, particularly total protein content and/or levels of metabolic hormones, particularly insulin, adiponectin and leptin, which were reduced in those fed an obesogenic diet during lactation. In addition, the role of the mammary gland in leptin production and its different regulation with respect to that of the adipose tissue is highlighted. All in all, these results are of relevance if they could be extrapolated to humans, since they show the importance of the implementation of a healthy diet during lactation to limit detrimental programming effects in the offspring, particularly in women with overweight or obesity.

Declaration of competing interests

The authors declare that they have no conflict of interest.

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Supplementary materials

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CRediT authorship contribution statement

Catalina A. Pomar: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Pedro Castillo:** Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Mariona Palou:** Methodology, Conceptualization, Investigation, Writing – review & editing, Supervision. **Andreu Palou:** Methodology, Conceptualization, Writing – review & editing, Project administration, Funding acquisition. **Catalina Picó:** Methodology, Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

References

[1] Langley-Evans SC. Developmental programming of health and disease. Proc Nutr Soc 2006;65:97–105. doi:10.1079/pns2005478.
[2] Suzuki K. The developing world of DOHaD. J Dev Orig Health Dis 2018;9:266–9. doi:10.1017/S2040174417000691.
[3] Picó C, Palou M, Priego T, Sánchez J, Palou A. Metabolic programming of obesity by energy restriction during the perinatal period: different outcomes depending on gender and period, type and severity of restriction. Front Physiol 2012;3:436–50. doi:10.3389/fphys.2012.00436.
[4] Barker DJ. Fetal origins of coronary heart disease. BMJ 1995;311:171–4. doi:10.1136/bmj.311.6998.171.
[5] Ainge H, Thompson C, Ozanne SE, Rooney KB. A systematic review on animal models of maternal high fat feeding and offspring glycaemic control. Int J Obes (Lond) 2011;35:325–35. doi:10.1038/ijo.2010.149.
[6] Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. J Physiol 2005;565:3–8. doi:10.1113/jphysiol.2004.079756.
[7] Zambrano E, Ibáñez C, Martínez-Samayoa PM, Lomas-Soria C, Durand-Carbajal M, Rodríguez-González GL. Maternal obesity: lifelong metabolic outcomes for offspring from poor developmental trajectories during the perinatal period. Arch Med Res 2016;47:1–12. doi:10.1016/j.arcmed.2016.01.004.
[8] Kislal S, Shook LL, Edlow AG. Perinatal exposure to maternal obesity: lasting cardiometabolic impact on offspring. Prenat Diagn 2020;40:1109–25. doi:10.1002/pd.5784.
[9] Ong KK, Loos RJ. Rapid infancy weight gain and subsequent obesity: systematic reviews and hopeful suggestions. Acta Paediatr 2006;95:904–8. doi:10.1080/08035250600719754.
[10] Leunissen RW, Kerkhof GF, Stijnen T, Hokken-Koelega A. Timing and tempo of first-year rapid growth in relation to cardiovascular and metabolic risk profile in early adulthood. JAMA 2009;301:2234–42. doi:10.1001/jama.2009.761.
[11] Gorski JN, Dunn-Meynell AA, Hartman TG, Levin BE. Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. Am J Physiol Regul Integr Comp Physiol 2006;291:R768–78. doi:10.1152/ajpregu.00138.2006.
[12] Pomar CA, van Nes R, Sanchez J, Pico C, Keijer J, Palou A. Maternal consumption of a cafeteria diet during lactation in rats leads the offspring to a thin-outside-fat-inside phenotype. Int J Obes (Lond) 2017;41:1279–87. doi:10.1038/ijo.2017.42.
[13] Gomes RM, Bueno FG, Schamber CR, de Mello JCP, de Oliveira JC, Francisco FA, et al. Maternal diet-induced obesity during suckling period programs offspring obese phenotype and hypothalamic leptin/insulin resistance. J Nutr Biochem 2018;61:24–32. doi:10.1016/j.jnutbio.2018.07.006.

[14] Gugusheff JR, Vithayathil M, Ong ZY, Muhlhausler BS. The effects of prenatal exposure to a 'junk food' diet on offspring food preferences and fat deposition can be mitigated by improved nutrition during lactation. J Dev Orig Health Dis 2013;4:348–57. doi:10.1017/S2040174413000330.

[15] Picó C, Reis F, Egas C, Mathias P, Matafome P. Lactation as a programming window for metabolic syndrome. Eur J Clin Invest 2021;51:e13482. doi:10.1111/eci.13482.

[16] Lönnerdal B. Breast milk: a truly functional food. Nutrition 2000;16:509–11. doi:10.1016/s0899-9007(00)00363-4.

[17] Bzikowska-Jura A, Czerwonogrodzka-Senczyna A, Olędzka G, Szostak-Węgierek D, Weker H, Wesołowska A. Maternal nutrition and body composition during breastfeeding: association with human milk composition. Nutrients 2018;10:1379–94. doi:10.3390/nu10101379.

[18] Lönnerdal B. Effects of maternal dietary intake on human milk composition. J Nutr 1986;116:499–513. doi:10.1093/jn/116.4.499.

[19] Pomar CA, Kuda O, Kopecky J, Rombaldova M, Castro H, Picó C, et al. Maternal diet, rather than obesity itself, has a main influence on milk triacylglycerol profile in dietary obese rats. Biochim Biophys Acta Mol Cell Biol Lipids 2020;1865:158556. doi:10.1016/j.bbalip.2019.158556.

[20] Picó C, Palou M, Pomar CA, Palou A. Benefits of breastfeeding in infant health: a role for milk signaling peptides. In: Sanchez MVPC, editor. Molecular Nutrition: Mother and Infant, Academic Press; 2021. ISBN 978-0-12-813862-5. Cambridge, Massachusetts, Estados Unidos p. 29–56.

[21] Picó C, Oliver P, Sánchez J, Miralles O, Caimari A, Priego T, et al. The intake of physiological doses of leptin during lactation in rats prevents obesity in later life. Int J Obes (Lond) 2007;31:1199–209. doi:10.1038/sj.ijo.0803585.

[22] Szostaczuk N, Priego T, Palou M, Palou A, Picó C. Oral leptin supplementation throughout lactation in rats prevents later metabolic alterations caused by gestational calorie restriction. Int J Obes (Lond) 2017;41:360–71. doi:10.1038/ijo.2016.241.

[23] Picó C, Palou M, Pomar CA, Rodríguez AM, Palou A. Leptin as a key regulator of the adipose organ. Rev Endocr Metab Disord 2021 Online ahead of print. doi:10.1007/s11154-021-09687-5.

[24] Miralles O, Sanchez J, Palou A, Pico C. A physiological role of breast milk leptin in body weight control in developing infants. Obesity (Silver Spring) 2006;14:1371–7. doi:10.1038/oby.2006.155.

[25] Palou M, Picó C, Palou A. Leptin as a breast milk component for the prevention of obesity. Nutr Rev 2018;76:875–92. doi:10.1093/nutrit/nuy046.

[26] Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467–74. doi:10.1111/j.1572-0241.1999.01377.x.

[27] Priego T, Sánchez J, García AP, Palou A, Picó C. Maternal dietary fat affects milk fatty acid profile and impacts on weight gain and thermogenic capacity of suckling rats. Lipids 2013;48:481–95. doi:10.1007/s11745-013-3764-8.

[28] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45. doi:10.1093/nar/29.9.e45.

[29] Horta BL, Loret de Mola C, Victora CG. Long-term consequences of breastfeeding on cholesterol, obesity, systolic blood pressure and type 2 diabetes: a systematic review and meta-analysis. Acta Paediatr 2015;104:30–7. doi:10.1111/apa.13133.

[30] Arenz S, Rückerl R, Koletzko B, von Kries R. Breast-feeding and childhood obesity–a systematic review. Int J Obes Relat Metab Disord 2004;28:1247–56. doi:10.1038/sj.ijo.0802758.

[31] Owen CG, Martin RM, Whincup PH, Smith GD, Cook DG. Does breastfeeding influence risk of type 2 diabetes in later life? a quantitative analysis of published evidence. Am J Clin Nutr 2006;84:1043–54. doi:10.1093/ajcn/84.5.1043.

[32] Huseinovic E, Winkvist A, Bertz F, Hellebö Johansson E, Brekke HK. Dietary assessment among women with overweight and obesity in early postpartum. J Hum Nutr Diet 2016;29:411–17. doi:10.1111/jhn.12350.

[33] Blumfield ML, Hure AJ, Macdonald-Wicks LK, Patterson AJ, Smith R, Collins CE. Disparities exist between National food group recommendations and the dietary intakes of women. BMC Womens Health 2011;11:37–46. doi:10.1186/1472-6874-11-37.

[34] Woodside B, Budin R, Wellman MK, Abizaid A. Many mouths to feed: the control of food intake during lactation. Front Neuroendocrinol 2012;33:301–14. doi:10.1016/j.yfrne.2012.09.002.

[35] Smith MS, Grove KL. Integration of the regulation of reproductive function and energy balance: lactation as a model. Front Neuroendocrinol 2002;23:225–56. doi:10.1016/s0091-3022(02)00002-x.

[36] Palou M, Torrens JM, Castillo P, Sánchez J, Palou A, Picó C. Metabolomic approach in milk from calorie-restricted rats during lactation: a potential link to the programming of a healthy phenotype in offspring. Eur J Nutr 2020;59:1191–204. doi:10.1007/s00394-019-01979-6.

[37] Vernon RG, Pond CM. Adaptations of maternal adipose tissue to lactation. J Mammary Gland Biol Neoplasia 1997;2:231–41. doi:10.1023/a:1026380220364.

[38] Vernon RG. Lipid metabolism during lactation: a review of adipose tissue-liver interactions and the development of fatty liver. J Dairy Res 2005;72:460–9. doi:10.1017/S0022029905001299.

[39] Neville MC, Picciano MF. Regulation of milk lipid secretion and composition. Annu Rev Nutr 1997;17:159–83. doi:10.1146/annurev.nutr.17.1.159.

[40] Smith RW, Walsh A. Effect of lactation on lipolysis in rat adipose tissue. Lipids 1976;11:418–20. doi:10.1007/BF02532850.

[41] Dunshea FR, Bell AW, Trigg TE. Relations between plasma non-esterified fatty acid metabolism and body fat mobilization in primiparous lactating goats. Br J Nutr 1989;62:51–61. doi:10.1079/bjn19890007.

[42] Eriksen KG, Christensen SH, Lind MV, Michaelsen KF. Human milk composition and infant growth. Curr Opin Clin Nutr Metab Care 2018;21:200–6. doi:10.1097/MCO.0000000000000466.

[43] Rolls BA, Gurr MI, van Duijvenvoorde PM, Rolls BJ, Rowe EA. Lactation in lean and obese rats: effect of cafeteria feeding and of dietary obesity on milk composition. Physiol Behav 1986;38:185–90. doi:10.1016/0031-9384(86)90153-8.

[44] Alexandre-Gouabau MC, David-Sochard A, Royer AL, Parnet P, Paillé V. Moderate high caloric maternal diet impacts dam breast milk metabotype and offspring lipidome in a sex-specific manner. Int J Mol Sci 2020;21:5428–43. doi:10.3390/ijms21155428.

[45] Cerf ME, Louw J, Herrera E. High fat diet exposure during fetal life enhances plasma and hepatic omega-6 fatty acid profiles in fetal wistar rats. Nutrients 2015;7:7231–41. doi:10.3390/nu7095337.

[46] Franco JG, Fernandes TP, Rocha CP, Calviño C, Pazos-Moura CC, Lisboa PC, et al. Maternal high-fat diet induces obesity and adrenal and thyroid dysfunction in male rat offspring at weaning. J Physiol 2012;590:5503–18. doi:10.1113/jphysiol.2012.240655.

[47] Pomar CA, Sánchez J, Palou A. The intake of a cafeteria diet in nursing rats alters the breast milk concentration of proteins important for the development of offspring. Nutrients 2020;12:2470–83. doi:10.3390/nu12082470.

[48] Scherer T, Sakamoto K, Buettner C. Brain insulin signalling in metabolic homeostasis and disease. Nat Rev Endocrinol 2021;17:468–83. doi:10.1038/s41574-021-00498-x.

[49] Young BE, Patinkin Z, Palmer C, de la Houssaye B, Barbour LA, Hernandez T, et al. Human milk insulin is related to maternal plasma insulin and BMI: but other components of human milk do not differ by BMI. Eur J Clin Nutr 2017;71:1094–100. doi:10.1038/ejcn.2017.75.

[50] Whitmore TJ, Trengove NJ, Graham DF, Hartmann PE. Analysis of insulin in human breast milk in mothers with type 1 and type 2 diabetes mellitus. Int J Endocrinol 2012;2012:296368. doi:10.1155/2012/296368.

[51] Weyermann M, Brenner H, Rothenbacher D. Adipokines in human milk and risk of overweight in early childhood: a prospective cohort study. Epidemiology 2007;18:722–9. doi:10.1097/ede.0b013e3181567ed4.

[52] Ley SH, Hanley AJ, Sermer M, Zinman B, O'Connor DL. Associations of prenatal metabolic abnormalities with insulin and adiponectin concentrations in human milk. Am J Clin Nutr 2012;95:867–74. doi:10.3945/ajcn.111.028431.

[53] Chan D, Goruk S, Becker AB, Subbarao P, Mandhane PJ, Turvey SE, et al. Adiponectin, leptin and insulin in breast milk: associations with maternal characteristics and infant body composition in the first year of life. Int J Obes (Lond) 2018;42:36–43. doi:10.1038/ijo.2017.189.

[54] Fernández-Moreno MD, Fernández-González MA, Diaz-Juárez JL, López-Luna MP, Prieto JC. Interaction of insulin with small intestinal epithelial cells from developing rats. Biol Neonate 1988;54:289–93. doi:10.1159/000242865.

[55] Sodoyez-Goffaux F, Sodoyez JC, De Vos CJ. Insulin receptors in the gastrointestinal tract of the rat fetus: quantitative autoradiographic studies. Diabetologia 1985;28:45–50. doi:10.1007/BF00276999.

[56] Shehadeh N, Sukhotnik I, Shamir R. Gastrointestinal tract as a target organ for orally administered insulin. J Pediatr Gastroenterol Nutr 2006;43:276–81. doi:10.1097/01.mpg.0000226377.03247.fb.

[57] Shamir R, Muslach M, Sukhotnik I, Perlman R, Diamond E, Mogilner J, et al. Intestinal and systemic effects of oral insulin supplementation in rats after weaning. Dig Dis Sci 2005;50:1239–44. doi:10.1007/s10620-005-2766-7.

[58] Kinouchi T, Koizumi K, Kuwata T, Yajima T. Milk-borne insulin with trypsin inhibitor in milk induces pancreatic amylase development at the onset of weaning in rats. J Pediatr Gastroenterol Nutr 2000;30:515–21. doi:10.1097/00005176-200005000-00010.

[59] Shehadeh N, Wies R, Eishach O, Berant M, Etzioni A, Shamir R. Influence of oral insulin supplementation on carbohydrate, lipid and protein metabolism in weaned Balb/c mice. J Pediatr Endocrinol Metab 2003;16:431–7. doi:10.1515/jpem.2003.16.3.431.

[60] Lemas DJ, Young BE, Baker PR, Tomczik AC, Soderborg TK, Hernandez TL, et al. Alterations in human milk leptin and insulin are associated with early changes in the infant intestinal microbiome. Am J Clin Nutr 2016;103:1291–300. doi:10.3945/ajcn.115.126375.

[61] Fields DA, George B, Williams M, Whitaker K, Allison DB, Teague A, et al. Associations between human breast milk hormones and adipocytokines and infant growth and body composition in the first 6 months of life. Pediatr Obes 2017;12:78–85. doi:10.1111/ijpo.12182.

[62] Schöndorf T, Maiworm A, Emmison N, Forst T, Pfützner A. Biological background and role of adiponectin as marker for insulin resistance and cardiovascular risk. Clin Lab 2005;51:489–94.

[63] Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, et al. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 2003;46:459–69. doi:10.1007/s00125-003-1074-z.

[64] Yu X, Rong SS, Sun X, Ding G, Wan W, Zou L, et al. Associations of breast milk adiponectin, leptin, insulin and ghrelin with maternal characteristics and early infant growth: a longitudinal study. Br J Nutr 2018;120:1380–7. doi:10.1017/S0007114518002933.

[65] Martin LJ, Woo JG, Geraghty SR, Altaye M, Davidson BS, Banach W, et al. Adiponectin is present in human milk and is associated with maternal factors. Am J Clin Nutr 2006;83:1106–11. doi:10.1093/ajcn/83.5.1106.

[66] Brunner S, Schmid D, Zang K, Much D, Knoeferl B, Kratzsch J, et al. Breast milk leptin and adiponectin in relation to infant body composition up to 2 years. Pediatr Obes 2015;10:67–73. doi:10.1111/j.2047-6310.2014.222.x.
[67] Woo JG, Guerrero ML, Altaye M, Ruiz-Palacios GM, Martin LJ, Dubert-Ferrandon A, et al. Human milk adiponectin is associated with infant growth in two independent cohorts. Breastfeed Med 2009;4:101–9. doi:10.1089/bfm.2008.0137.
[68] Woo JG, Guerrero ML, Guo F, Martin LJ, Davidson BS, Ortega H, et al. Human milk adiponectin affects infant weight trajectory during the second year of life. J Pediatr Gastroenterol Nutr 2012;54:532–9. doi:10.1097/MPG.0b013e31823fde04.
[69] Palou M, Torrens JM, Priego T, Sánchez J, Palou A, Picó C. 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;22:574–84. doi:10.1016/j.jnutbio.2010.05.001.
[70] Torrens JM, Konieczna J, Palou M, Sánchez J, Picó C, Palou A. Early biomarkers identified in a rat model of a healthier phenotype based on early postnatal dietary intervention may predict the response to an obesogenic environment in adulthood. J Nutr Biochem 2014;25:208–18. doi:10.1016/j.jnutbio.2013.10.009.
[71] Palou M, Priego T, Sánchez J, Torrens JM, Palou A, Picó C. Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life. Endocrinology 2010;151:1030–41. doi:10.1210/en.2009-0934.
[72] Castro H, Pomar CA, Palou A, Picó C, Sánchez J. Offspring predisposition to obesity due to maternal-diet-induced obesity in rats is preventable by dietary normalization before mating. Mol Nutr Food Res 2017;61:1600513. doi:10.1002/mnfr.201600513.
[73] Jin Z, Du Y, Schwaid AG, Asterholm IW, Scherer PE, Saghatelian A, Wan Y. Maternal adiponectin controls milk composition to prevent neonatal inflammation. Endocrinology 2015;156:1504–13. doi:10.1210/en.2014-1738.
[74] Nozhenko Y, Asnani-Kishnani M, Rodríguez AM, Palou A. Milk leptin surge and biological rhythms of leptin and other regulatory proteins in breastmilk. PLoS One 2015;10:e0145376. doi:10.1371/journal.pone.0145376.
[75] Uysal FK, Onal EE, Aral YZ, Adam B, Dilmen U, Ardiçolu Y. Breast milk leptin: its relationship to maternal and infant adiposity. Clin Nutr 2002;21:157–60. doi:10.1054/clnu.2001.0525.
[76] Doneray H, Orbak Z, Yildiz L. The relationship between breast milk leptin and neonatal weight gain. Acta Paediatr 2009;98:643–7. doi:10.1111/j.1651-2227.2008.01192.x.
[77] Schuster S, Hechler C, Gebauer C, Kiess W, Kratzsch J. Leptin in maternal serum and breast milk: association with infants' body weight gain in a longitudinal study over 6 months of lactation. Pediatr Res 2011;70:633–7. doi:10.1203/PDR.0b013e31823214ea.
[78] Sánchez J, Priego T, Palou M, Tobaruela A, Palou A, Picó C. Oral supplementation with physiological doses of leptin during lactation in rats improves insulin sensitivity and affects food preferences later in life. Endocrinology 2008;149:733–40. doi:10.1210/en.2007-0630.
[79] Priego T, Sánchez J, Palou A, Picó C. Leptin intake during the suckling period improves the metabolic response of adipose tissue to a high-fat diet. Int J Obes (Lond) 2010;34:809–19. doi:10.1038/ijo.2010.18.
[80] Sánchez J, Oliver P, Miralles O, Ceresi E, Picó C, Palou A. Leptin orally supplied to neonate rats is directly uptaken by the immature stomach and may regulate short-term feeding. Endocrinology 2005;146:2575–82. doi:10.1210/en.2005-0112.
[81] de Oliveira Cravo C, Teixeira CV, Passos MC, Dutra SC, de Moura EG, Ramos C. Leptin treatment during the neonatal period is associated with higher food intake and adult body weight in rats. Horm Metab Res 2002;34:400–5. doi:10.1055/s-2002-33473.
[82] Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, et al. The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on maternal nutritional status during pregnancy. Endocrinology 2008;149:1906–13. doi:10.1210/en.2007-0981.
[83] Zhang Q, Xiao X, Zheng J, Li M, Yu M, Ping F, et al. A maternal high-fat diet induces DNA methylation changes that contribute to glucose intolerance in offspring. Front Endocrinol (Lausanne) 2019;10:871–84. doi:10.3389/fendo.2019.00871.
[84] Kawakubo-Yasukochi T, Yano E, Kimura S, Nishinakagawa T, Mizokami A, Hayashi Y, et al. Hepatic glycogenolysis is determined by maternal high-calorie diet via methylation of Pygl and it is modified by oteocalcin administration in mice. Mol Metab 2021;54:101360. doi:10.1016/j.molmet.2021.101360.
[85] Gali Ramamoorthy T, Allen TJ, Davies A, Harno E, Sefton C, Murgatroyd C, et al. Maternal overnutrition programs epigenetic changes in the regulatory regions of hypothalamic Pomc in the offspring of rats. Int J Obes (Lond) 2018;42:1431–44. doi:10.1038/s41366-018-0094-1.
[86] Eberle C, Kirchner MF, Herden R, Stichling S. Paternal metabolic and cardiovascular programming of their offspring: a systematic scoping review. PLoS One 2020;15:e0244826. doi:10.1371/journal.pone.0244826.