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Maternal Western diet alters milk leptin levels and early postnatal leptin handling in rat offspring

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Author contributions:

- **Pere Bibiloni:** Formal analysis, Investigation, Data curation, Writing - Original Draft, Writing – Review & Editing, Visualization
- **Rita de Matteis:** Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – Review & Editing, Supervision, Project administration
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Abstract

Objective - Lactation is a critical developmental window during which maternal conditions influence breast milk composition, thereby affecting infant growth and long-term health. Leptin, a key bioactive component of breast milk involved in metabolic programming, may have its beneficial effects attenuated under maternal obesogenic diets. This study evaluated whether a maternal Western-style diet (WD) is associated with changes in early postnatal absorption of milk-derived leptin and leptin-related miRNAs, and their relationship with gastric content as an indirect indicator of nutrient intake in the offspring. **Methods -** Female rats were fed a control diet (C-Dams) or a WD (WD-Dams) starting one month before gestation and throughout gestation and lactation. Dams and pups were euthanized at lactation/postnatal day 4 (LD4/PND4). **Results -** WD-Dams displayed higher leptinemia (3.8 ± 1.5 vs. 2.1 ± 0.67 ng/mL, $p = 0.026$) and milk leptin levels (1.6 ± 0.46 vs. 1.0 ± 0.33 ng/mL, $p = 0.012$). Despite increased milk leptin supply and stronger gastric leptin signal detected by immunohistochemistry, WD-pups did not show a quantitative increase in gastric leptin content or circulating leptin levels. Notably, they presented a tendency towards greater gastric content (0.24 ± 0.12 vs. 0.12 ± 0.11 g, $p = 0.089$). No differences were found in leptin-related miRNAs absorption; however, miR-17 levels were higher in WD-milk (increment of 70.7%) and correlated with duodenal levels only in WD-pups. **Conclusions:** Maternal WD consumption during the perinatal period alters milk leptin levels and early leptin exposure and is associated with slight differences in gastric content in the offspring, suggesting early alterations in regulatory processes.

Introduction

Early-life development represents a critical window during which environmental conditions can shape physiological processes and influence disease risk later in life (1). While adverse metabolic programming was initially attributed to maternal undernutrition during gestation

(2), accumulating evidence has also linked it to maternal overnutrition throughout the perinatal period. Specifically, maternal exposure to an obesogenic diet and/or maternal obesity during pregnancy and lactation increases offspring susceptibility to obesity, diabetes, cardiovascular disease, and other metabolic disorders (3–5). Given the high prevalence of obesity and unhealthy dietary patterns among women of reproductive age in industrialized countries (6), a deeper understanding of their effects on offspring health and the underlying mechanisms is needed.

Lactation represents a critical window for the metabolic programming of health and disease (7). This period may also offer important opportunities for the implementation of preventive or corrective interventions in the context of maternal obesity. In this regard, breast milk is considered the gold standard for infant nutrition during the initial six months of life (8), not only due to its macro- and micronutrient composition, but also because of its rich profile of bioactive compounds essential for proper development and growth (9). However, the composition of milk is influenced by maternal factors, such as dietary intake and metabolic status (7, 10, 11), which may have implications for infant health in both the short and long term (7).

Among the bioactive compounds present in milk, leptin plays a prominent role in metabolic programming (12). Rodent studies have demonstrated that, during the early postnatal period, orally ingested leptin is absorbed by the immature stomach and may exert biological effects, potentially influencing short-term food intake (13). Furthermore, daily supplementation with physiological doses of leptin during the suckling period has been associated with protective effects against obesity and related disorders in later life (14–17). However, this beneficial role appears to be mitigated when dams are fed a Western-style diet (WD) (18). In humans, indirect evidence also supports a role for breast milk leptin (19), as several independent studies have reported an inverse association between milk leptin concentrations and infant body weight, particularly among infants of normal-weight mothers (20–22). However, this relationship is less evident in women with overweight/obesity (12). While breast-milk leptin levels are

positively correlated with maternal adiposity (12, 23), there is a consistent association between maternal overweight and obesity and an increased risk of obesity in offspring (3). This suggests that the potential benefits of increased milk leptin supply may be counterbalanced or diminished under these maternal conditions. Although other contributing factors cannot be ruled out, disruptions in leptin absorption or signaling in the offspring, stemming from an unbalanced maternal diet or an altered metabolic state, may underlie this impaired protective effect. Alterations in the supply or absorption of miRNAs related to leptin signaling may also contribute to these effects.

MicroRNAs (miRNAs), short non-coding RNAs that regulate gene expression post-transcriptionally, represent another group of milk-derived bioactives that are the focus of increasing research interest (24). Although the extent of their absorption remains a matter of debate, accumulating evidence suggests that milk-derived miRNAs can be absorbed by the neonatal gut, where they may act locally or enter the circulation to influence target tissues (9). Notably, it has been demonstrated that maternal diet can modify the composition of milk miRNAs, which may have implications for the offspring metabolism (25–27). In this context, our research focuses on milk-derived miRNAs that may target the leptin signaling pathway. However, the impact of maternal dietary intake on the absorption of such miRNAs remains to be elucidated.

Therefore, this study aimed to determine whether maternal consumption of an unbalanced diet (WD) in rats alters early postnatal milk leptin levels and its early postnatal handling in offspring, as well as leptin-related miRNA profiles, and to assess their association with early postnatal growth parameters.

Methods

Experimental design and animal procedures

The animal protocols implemented in this study were approved by the Bioethical Committee of the University of the Balearic Islands (Exp. 2023/16/AEXP, September 1, 2023). All

procedures adhered to the University guidelines for the use and care of laboratory animals. An overview of the experimental design is shown in **Figure 1.**

Fifteen virgin female Wistar rats (Envigo-Inotiv, Barcelona, Spain) were housed in the University facilities under controlled conditions (temperature of 22 ºC, 12-hour light/dark cycle) with free access to food and water. At two months of age, the animals were caged in pairs and allocated to one of two diet groups: standard chow diet (Control dams group, C-dams, n=6) providing 3.31 kcal/g, with 8.7% of calories from fat, 72.9% from carbohydrate, and 18.4% from protein (SAFE® A40, Safe, Augy, France) or a high-fat, high-sucrose diet (Western diet, WD dams group, WD-dams, n=9) providing 4.7 kcal/g, with 40.0% of calories from fat, 43.0% from carbohydrate and 17.0% from protein (D12079B, Research Diets, New Brunswick, NJ, USA). Dams were assigned to experimental groups using stratified randomization. To ensure group homogeneity, rats were balanced based on baseline body weight (measured twice prior to diet onset) and litter of origin, with siblings distributed across different groups to minimize potential litter-specific biases. All dams were of the same age. They were maintained on their assigned diet throughout the experiment. At three months of age, females were bred with male rats (previously fed a standard chow diet) and subsequently housed individually. All 15 female rats contributed offspring to the study. At postnatal day (PND) 1, litters were standardized to 10 pups per dam, aiming for a balanced sex ratio (5 males and 5 females per litter) and body weight homogeneity. Extreme weight outliers were excluded to minimize within-group variability. In cases where a 1:1 sex ratio was not naturally available, pups were selected to maintain an overall sex balance across the entire experimental cohort.

Throughout the experimental procedure, maternal body weight and food intake were monitored three times per week. Food intake was calculated by subtracting the weight of the remaining pellets from the initial amount provided. Measurements were recorded per cage, and the average intake per dam was calculated accordingly. To ensure accuracy, bedding was

inspected during each weighing session; any significant fragments of food were recovered and weighed together with the remaining pellets to account for potential spillage.

Pups were weighed at PND1 and immediately prior to sacrifice. At PND4, a pair of pups (male and female) or a single pup per litter were selected, ensuring overall group sex balance and weight homogeneity. In total, 11 C-pups (6 females and 5 males) and 16 WD-pups (8 females and 8 males) were included; the remaining pups were assigned to other experimental procedures. Animals were sacrificed by decapitation without anesthesia within the first 2 hours of the light cycle. Blood samples were collected in EDTA-treated tubes and centrifuged (1000 x g, 4 ºC, 10 min) to collect the plasma. The stomach was immediately removed and weighed. Subsequently, it was opened, rinsed with saline buffer, and reweighed to calculate the gastric content by weight difference. One longitudinal half was frozen in liquid nitrogen and stored at -70 ºC until analysis, while the other half was fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) overnight at 4 ºC for immunohistochemistry. A 5-cm segment of the duodenum was also collected, cut transversely into two halves, and preserved using the same methods.

Dams were euthanized by decapitation without anesthesia on lactation day (LD) 4, immediately after their respective litters. Five minutes prior to sacrifice, oxytocin (4 IU kg^{-1} of body weight, Facilpart, Laboratory Syva S.A.U., León, Spain) was administered intraperitoneally to facilitate post-mortem milk extraction, which was performed manually from all teats. Trunk blood was collected and processed as described above, and mammary gland samples were obtained and stored at -70 ºC until analysis.

Quantification of leptin and glucose levels

Pup stomach samples were homogenized in phosphate-buffered saline (1/4 w/v) at 4 ºC. Homogenates were then centrifuged at 7500 x g for 2 min at 4 ºC. Leptin concentrations in the resulting supernatants, milk samples, and plasma from both pups and dams were

determined using a mouse/rat leptin ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Blood glucose levels were measured in pups and dams immediately after decapitation using an Accu-Chek glucometer (Roche Diagnostics, Barcelona, Spain).

Immunohistochemistry

Stomach and duodenum samples were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4) overnight. After washing with PB, specimens were dehydrated through a graded ethanol series and embedded in paraffin. Sections of 3 µm were processed for immunostaining using the ABC method with diaminobenzidine as the chromogen, as previously described (28). Dewaxed sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase, followed by a 20-min incubation in normal goat serum (1:75 in PBS) to reduce nonspecific background staining. Samples were then incubated with a rabbit polyclonal anti-leptin antibody (Santa Cruz, CA) overnight at 4 ºC. This was followed by incubation with a biotinylated goat anti-rabbit immunoglobulin G secondary antibody (1:200) for 30 min. Subsequently, sections were treated with an avidin-biotin complex (Vectastain ABC kit, Vector) for 30 min. Peroxidase activity was visualized using SIGMA*FAST* 3,3-diaminobenzidine tablets (Sigma, St. Louis, MO) dissolved in water and applied for 5 min in a dark room. Sections were counterstained with hematoxylin, dehydrated in serial ethanol dilutions, cleared in xylene, and mounted with Eukitt. Negative controls, involving the omission of the primary antibody, were processed in parallel.

Total RNA extraction and mRNA levels quantification

Total RNA was extracted from mammary gland samples according to EZNA Total RNA Kit I guidelines (Omega Bio-TekInc., Georgia, USA). Isolated RNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Llc, Wilmington, DE, USA). Purity was confirmed by optimal A260/280 and A260/230 ratios, and integrity was assured via 1% agarose gel electrophoresis.

For reverse transcription (RT), 250 ng of total RNA were first denatured at 65 ºC for 10 min using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain). Reactions were conducted in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Madrid, Spain) at 25 ºC for 10 min, 37 ºC for 50 min, 70 ºC for 15 min, and a final holding step at 4 ºC. Quantitative polymerase chain reaction (qPCR) was performed using a 1/5 dilution of the complementary DNA (cDNA) product with Power SYBER Green PCR Master Mix (Life Technologies, Madrid, Spain) and forward and reverse primers at 20 µM each (Sigma Aldrich Química SA, Madrid, Spain). Primers for guanosine diphosphate dissociation inhibitor (*Gdi*) were CCGCACAAGGCAAATACATC/ GACTCTCTGAACCGTCATCAA, while for leptin (*Lep*) were AGGAAAATGTGCTGGAGACC/ATACCGACTGCGTGTGTGAA (forward/reverse, respectively). The reaction was carried out in an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Madrid, Spain) with an initial cycle of 95 ºC for 10 min, 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min, and a final melting curve stage (60 – 95 ºC). Threshold cycle (Ct) values were obtained by StepOne Software v2.3. Relative mRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method, using *Gdi* as the housekeeping gene. Results were expressed as percentages, with the C group mean set at 10 %.

miRNA extraction and miRNA levels quantification

Total RNA enriched in small RNAs was extracted from maternal milk, pup duodenum, and pup plasma using the mirVana™ miRNA Isolation Kit (Life Technologies Corporation, Waltham, MA, USA). Isolated miRNAs were quantified using a Qubit 4 Fluorometer with the Qubit microRNA assay kit (Life Technologies Corporation, Waltham, MA, USA).

From 10 ng of enriched RNA, cDNA was synthesized using the miRCURY LNA RT kit (QIAGEN, Madrid, Spain) following the manufacturer's protocol. A 1/15 dilution of the product was used as substrate for the miRCURY LNA SYBR Green PCR Kit (QIAGEN, Madrid, Spain) to amplify U6 snRNA (v2) and several miRNAs (miR-7a-1-3p, -16-5p, -17-5p, -26a-5p, -27a-3p, -27b-5p, -29a-5p, -103-3p, -191-5p, -200a-3p, and -222-3p; GeneGlobe IDs in **Table S1**). Selected miRNAs included those predicted to target the leptin signaling pathway (miR-7a-1, miR-27b, and miR-

29a, according to miRDB (29)) or previously reported in breast milk to vary with maternal diet or metabolic status (miR-16, miR-17, miR-26a, miR-27a, miR-27b, miR-29a, miR-103, miR-200a, miR-222) (26, 30–33). PCR cycling conditions were set to an initial activation cycle at 95 ºC for 2 min, 40 cycles of two steps (95 ºC for 10 s and 56 ºC for 60 s), and a final melting curve analysis from 60 ºC to 95 ºC. Additionally, RT-qPCR was performed on milk samples, as described above, to amplify and quantify 18S rRNA expression using the primers CGCGGTTCTATTTTGTTGGT and AGTCGGCATCGTTTATGGTC.

Data were obtained and analyzed as described previously. For duodenum and plasma, U6 snRNA served as the housekeeping gene. For milk, the mean of miR-191-5p and 18S rRNA was used as the reference Ct, as it showed the most stable expression across groups.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). Sample sizes (n, referring to biological replicates) are detailed in each figure caption. For maternal analyses, the experimental unit was the dam. For offspring analyses, individual pups were analyzed while accounting for litter as a random effect. The significance threshold was set at 0.05.

Maternal weight trajectories were analyzed using Area Under the Curve (AUC) and two-way repeated measures ANOVA (RM ANOVA), followed by Tukey’s post-hoc test. Other maternal variables were compared using the Mann-Whitney U test. The magnitude of change was assessed by Cohen’s D. Correlations between variables of interest were determined via Spearman's rank correlation coefficient.

Regarding offspring analyses, linear mixed-effects (LME) models were used to account for potential inter-litter variability, with experimental group (C-pups or WD-pups) and offspring sex (male or female) included as fixed effects, and litter included as a random intercept. When appropriate, significant interactions were further explored through sex-stratified Mann–Whitney U tests.

To assess associations between variables of interest while accounting for litter influence, additional LME models were fitted with the same fixed and random effects structure. Spearman correlations were additionally performed within experimental subgroups to further explore patterns suggested by the LME models.

miRNA functional analysis identified predicted target genes using *miRDB* (target score > 80, (29)) and *TargetScan* (score > 0.1, (34)). Gene Ontology enrichment analysis was performed using the *Clusterprofiler* R package (35), with the top 10 biological processes selected based on adjusted p-values.

R version 4.5.0 (36) was used to perform all statistical procedures described. All graphs shown were constructed with the *ggplot2* package (37).

During the preparation of this manuscript, the authors used ChatGPT-4.0 (OpenAI) to assist with code development for data analysis and to improve the clarity and readability of the text. All content was subsequently reviewed and edited by the authors, who take full responsibility for the final version of the manuscript.

Results

Western diet increases energy intake and alters maternal growth trajectory prior to mating

To evaluate the impact of WD on maternal energy balance, body weight and calorie intake were monitored from the start of the dietary intervention (two months of age) until mating, approximately one month later. As shown in **Figure 2,** WD-dams exhibited a significantly higher cumulative energy intake compared to their counterparts (32% increment, **Figure 2A**). This was primarily driven by the higher energy density of the WD (4.7 kcal/g) and its elevated fat-to-carbohydrate ratio (40% vs. 8.7% kcal from fat). Although the difference in absolute body weight before mating did not reach statistical significance ($p = 0.194$, **Figure 2B**), WD-fed dams showed a more pronounced weight gain over time, reaching values up to 8% than the C group by the end of the pre-mating period. While the total accumulated growth did not differ significantly between groups (AUC, $p = 0.328$), the trajectory of body weight gain was

significantly altered by the diet, as revealed by a robust diet-by-time interaction (two-way RM ANOVA, $p < 0.001$). Regarding individual timepoints, post-hoc analysis showed that the weight difference reached statistical significance at day 24 after the start of the dietary intervention ($p < 0.05$; **Figure 2C**). The Western diet also influenced body weight gain during gestation. Despite comparable litter sizes between groups, WD-dams showed a strong trend toward reduced gestational weight gain compared to C-dams ($p = 0.068$, **Table 1**).

WD-dams show increased systemic and milk leptin levels independently of mammary gland synthesis

To characterize the metabolic status of dams during lactation, glycemia, leptinemia, and milk leptin levels were determined at LD4. No significant differences in maternal glycemia were observed between groups. However, WD intake resulted in a significant increase in both circulating and milk leptin concentrations (**Table 1**). A strong correlation was found between these two parameters (**Figure 3A**), suggesting that elevated milk leptin levels primarily reflected systemic leptin. In contrast, milk leptin concentrations were not correlated with leptin mRNA levels in the mammary gland (**Figure 3B**). Moreover, mammary gland leptin gene expression remained unaffected by WD intake (**Figure 3C**), suggesting that local leptin synthesis contributed minimally to the increased milk leptin content in WD-dams.

Male WD-pups display lower birth weight but increased gastric content

Biometric and leptin-related parameters of the offspring are summarized in **Figure 4** and in **Table S2**. Weight parameters showed an interactive effect of the experimental diet and sex, restricting the effects of maternal WD consumption to males. At PND1, male WD-pups exhibited a significantly lower body weight compared to male C-pups (**Figure 4A**). Weight gain from PND1 to PND4 was similar between groups and thus the same male-specific difference in body weight at PND4 was observed (**Figure 4B**).

Notably, despite this lower body weight in males at PND4 (10.3 ± 0.69 for C-pups vs 8.86 ± 1.19 for WD-pups), WD-pups displayed a trend toward significantly greater gastric content

than controls (**Figure 4C**). No significant differences were observed between groups in circulating glucose levels (**Figure 4D**), leptin levels (**Figure 4E**) or gastric leptin content (**Figure 4F**).

Gastric immunolocalization of leptin suggests enhanced absorption of milk-derived leptin in WD-pups

Leptin was immunolocalized within the gastric mucosa to further explore its absorption from milk (**Figure 5**). Immunoreactivity was primarily localized to the apical region of the gastric mucosa, spanning from the apex to the base of superficial epithelial cells, which suggests the internalization of milk-derived leptin from the lumen. A less intense leptin-positive signal was also detected along the depth of the gastric glands, specifically in chief cells, reflecting endogenous leptin production. Notably, WD-pups exhibited a more robust and continuous immunoreactive signal across the gastric mucosa (including both mucoid and chief cells) (**Figure 5A, C**), whereas C-pups displayed a fragmented and less intense staining pattern (**Figure 5B, D**).

No leptin immunostaining was detected in the duodenal tissue of either group (**Figure 5E, F**), confirming that, at PND4, leptin production and absorption are restricted to the stomach.

Associations between maternal and neonatal leptin levels with offspring growth

To further characterize leptin absorption and its potential impact on the pups, correlation analyses were conducted across the previously described parameters. Despite the absence of differences in body weight gain between C-pups and WD-pups, milk leptin levels were negatively associated with pup weight gain from PND1 to PND4 (**Figure 6A**), supporting a regulatory role for milk-derived leptin in early energy balance. A sex-specific pattern was observed, with a trend towards stronger associations in male offspring, consistent with the sex-dependent trends observed in body weight parameters.

No significant association was observed between milk leptin and gastric leptin content (**Figure 6B**).

In contrast, milk leptin levels were negatively associated with circulating leptin levels in pups (**Figure 6C**). Stratified analyses indicated that this association was more pronounced in WD-pups than in C-pups.

Finally, circulating leptin levels in pups were positively associated with body weight at PND4 (**Figure 6D**), with a stronger association observed within the WD-pups group.

Maternal Western diet selectively increases milk miR-17 levels and its accumulation in the offspring duodenum

Levels of selected milk-derived miRNAs – either targeting leptin-signaling genes or previously linked to maternal diet and metabolic status – were measured in milk, duodenum, and plasma to assess the impact of maternal WD intake on miRNA transfer and absorption (**Figure 7**).

Overall, no significant differences were observed between groups for the majority of miRNAs across the biological sources analyzed. The sole exception was miR-17, which exhibited a marked and statistically significant increase in the milk of WD-dams compared to controls.

Focusing on miR-17, a positive association was found between its levels in milk and the duodenum, although the association did not reach statistical significance. Stratified analysis suggested a significant positive association specifically within the WD-pups group (**Figure 8A**). However, this relationship was not observed between duodenum and plasma levels (**Figure 8B**), suggesting that while miR-17 may be absorbed by the duodenal mucosa, it does not necessarily reach systemic circulation in detectable quantities.

Aiming to explore the potential functional implications of increased miR-17 levels, an enrichment analysis of its predicted target genes was performed. The primary biological processes associated with miR-17 targets included the regulation of cell growth during development, neurodevelopmental pathways, mechanisms of autophagy, and protein lipidation (**Figure 8C**).

Regarding sex effects, miR-27a and miR-27b levels differed between male and female pups (**Table S2**), with higher levels observed in males. However, no significant interaction between sex and maternal diet was detected in the LME models, suggesting that the effect of maternal diet was consistent across sexes. Consequently, sex stratification was not applied, and miRNA analyses were conducted in the combined offspring cohort.

Discussion

Early-life exposures are known to shape offspring health, either conferring protection against disease or increasing susceptibility later in life (3). We have previously demonstrated in rats that the detrimental effects of maternal WD consumption during the perinatal period may be partially mediated by alterations in breast milk composition, including changes in metabolic hormones (38), metabolites (39), lipid profiles (40), and miRNAs (26, 27, 33). Moreover, it is plausible that the beneficial effects of milk-derived bioactives are compromised under adverse maternal dietary or metabolic conditions. This appears to be the case for leptin, as the beneficial long-term effects of leptin supplementation during suckling in rats are attenuated in offspring of WD-fed dams (18). Similarly, in humans, the higher milk leptin levels in women with obesity do not consistently translate into metabolic benefits for their infants (12). Notably, although variations in milk bioactive components are well-documented, their early absorption by the neonate and how maternal diet modulates this process have received comparatively little attention. In this study, we provide novel evidence that, although maternal WD intake increases milk leptin supply and its subsequent absorption by the immature stomach, this does not result in a quantitative proportional increase in leptin levels. Moreover, while milk-derived leptin may be associated with some physiological parameters, as suggested by its negative association with body weight gain, other expected relationships were not observed. In particular, no evidence of reduced food intake was found, as reflected by the slightly increased gastric content in WD-pups. Overall, these findings suggest early alterations in leptin-related regulatory processes associated with maternal WD exposure.

As reported in previous studies with similar protocols (26, 27, 33, 38–40), maternal consumption of an obesogenic diet initiated one month prior to mating and maintained through gestation and lactation exerted notable effects on both dams and their offspring. In dams, although their body weight did not significantly exceed that of C-dams, the growth trajectory was significantly altered. This change likely reflects a greater accumulation of fat mass, as previously reported in studies using comparable dietary interventions (38). Consistent with this, WD-dams exhibited elevated circulating leptin levels, which are directly proportional to adipose tissue depots (41), suggesting that these animals were in the initial stages of developing an obesity-related phenotype. Major changes in other metabolic hormones were not yet expected at this stage; indeed, in this model, it has been previously shown that insulin and adiponectin levels remain unaltered during early lactation (LD5), while by the end of lactation, dams exhibited elevated circulating insulin levels as well as increased NEFA concentrations, consistent with an altered metabolic state (38). Therefore, the observed effects on the offspring may stem from the direct nutritional impact of the unbalanced maternal diet, an early-stage metabolic shift in the dam, or a combination of both factors.

Leptin levels in breast milk are dynamic and influenced by both the stage of lactation and maternal conditions. Herein, milk leptin concentrations were elevated in WD-dams at the early lactation stage (LD4) compared to C-dams. Interestingly, previous studies using a similar dietary model have reported the opposite trend at later stages (38). Specifically, while leptin levels in the milk of C-dams increased progressively over time, they remained relatively stable in WD-dams, resulting in lower levels by the end of lactation in comparison to those of C-dams (38). These findings suggest that an unbalanced maternal diet exerts a differential and sustained influence on milk leptin dynamics throughout lactation. Both mammary tissue and circulating leptin contribute to the total leptin content of milk (42). Notably, at LD4, mammary leptin gene expression was not significantly altered by WD intake. Thus, the rise in milk leptin levels in WD-dams at this stage appears to be driven primarily by elevated circulating leptin rather than increased local synthesis. This is further supported by the positive correlation

between serum and milk leptin levels, an observation consistent with human data (43). This pattern points to distinct regulatory mechanisms for leptin expression in adipose tissue versus the mammary gland, which may explain why the divergent effects of WD on these two sources, a phenomenon also reported in similar dietary models (38) and under moderate maternal caloric restriction (44).

Regardless of its origin, WD-pups were exposed to higher leptin levels through maternal milk. At PND4, orally ingested leptin is known to be absorbed by the gastric mucosa (13). This stomach-localized uptake was observed in the present study, along with limited endogenous leptin synthesis in chief cells, consistent with previous reports (28). Although duodenal absorption of gastric-derived leptin has been reported in young adult rats (45), no such uptake was detected here, suggesting that leptin absorption remains anatomically restricted to the stomach at PND4.

Our findings indicate that the increased milk leptin content of WD-dams led to greater gastric leptin exposure in their offspring, as evidenced by the robust immunoreactivity in the gastric mucosa. However, this did not translate into a significant increase in gastric leptin content or systemic leptinemia. This discrepancy may suggest that differences in leptin absorption were too subtle to be detected quantitatively yet remained discernible via immunohistochemistry. Alternatively, elevated exogenous leptin intake may be linked to a negative modulation of endogenous leptin synthesis, thereby maintaining similar overall content between groups. In line with this hypothesis, an inverse association between milk leptin and circulating leptin levels was identified, with stratified analyses indicating a stronger relationship in the WD-pups group. While correlation does not imply causality, these findings could be compatible with a negative feedback mechanism, as previously proposed for leptin autoregulation (46, 47). Notably, circulating leptin in pups was significantly correlated with body weight at PND4, potentially indicating that the leptin production is already responsive to adiposity at this early stage. Consistent with this possibility, previous studies with the same dietary intervention

have shown that WD-pups accumulate more fat mass than controls as early as PND6-PND8 (38).

Early postnatal leptin exposure influences both neonatal development and long-term metabolic outcomes (12). In the short term, oral leptin has been suggested to contribute to the regulation of food intake by acting as a satiating signal, either through activation of afferent vagal nerves in the gastric lumen or centrally after entering the systemic circulation (13). However, its role as a hypothalamic signal is likely limited at this developmental stage (48). Notably, we observed that despite receiving larger amounts of milk-derived leptin, WD-pups exhibited a trend towards increased gastric content. Gastric content measurement is a practical, indirect approach to estimating neonatal intake and has been successfully used in previous studies examining leptin-related effects on neonatal feeding (13). Thus, the apparent increase in gastric content despite higher leptin exposure suggests a decoupling between leptin supply and expected satiety-related effects during early life. Additionally, male WD-pups exhibited lower birth weight and reduced body weight at PND4, in agreement with previous studies showing that high-fat maternal diets can restrict fetal growth in rats (49). This reduction may have contributed to the somewhat lower gestational weight gain observed in WD-dams. Together with the slightly increased gastric content observed in this study, these findings may be consistent with an accelerated postnatal growth trajectory described in similar WD models (38), a phenomenon associated with adverse metabolic programming later in life (50). Nonetheless, other factors that may contribute to increased gastric content should be considered, including potential alterations in gastric emptying, a process in which leptin has been implicated (51), as well as intra-litter variability in feeding or differences in milk composition, including palatability.

Notably, our results showed that while body weight gain from PND1 to PND4 did not differ significantly between groups, milk leptin levels were negatively associated with body weight gain during this early postnatal period, particularly in males. This association, which has also been reported in human studies (22), suggests a potential relationship between milk-derived

leptin and early growth trajectories. While these correlations should be interpreted with caution, as they do not establish causality, they suggest that increased leptin supply from WD-dams could still be associated with early body weight gain. Potential mechanisms underlying this association may involve processes related to energy expenditure, such as thermogenesis in brown adipose tissue (52). Supporting this, leptin treatment has been reported to enhance cold-defense capacity in thermoneutrality-reared rat pups (53, 54). Thus, in contrast to adult organisms, where leptin is primarily known for its anorexigenic effects, its role during early life may involve a combination of modest or transient effects on feeding together with changes in energy expenditure (52). However, these mechanisms were not directly assessed in the present study and warrant further investigation. Nevertheless, these potential effects may not be sufficient to counterbalance the adverse effects associated with the maternal metabolic exposome. This interpretation aligns with our previous observations in rats, where the long-term metabolic benefits of leptin supplementation during the suckling period were reduced in the progeny of WD–fed dams (18). These findings may also have implications for humans; specifically, they could help explain why some studies involving women with obesity have found no clear association between milk leptin concentrations and infant weight gain (55), and why the delivery of higher milk-derived leptin levels to the offspring of women with obesity does not necessarily translate into the expected metabolic advantages for their infants (12), particularly when compared to infants of mothers with normal-weight.

Integrating these findings, the lower birth weight observed in WD offspring at PND1 in males suggests an initial state of intrauterine adaptation to maternal nutritional imbalance. This metabolic starting point may prime the neonate for a compensatory postnatal response. In this context, the somewhat greater gastric content should not be viewed as an isolated event, but as part of a potentially altered regulatory framework. Specifically, it might represent an early attempt to modulate energy balance. However, the apparent lack of intake inhibition despite the higher leptin supply and increased leptin signal in the gastric mucosa, points

toward the possibility that these adaptations to a suboptimal perinatal environment might bypass or desynchronize some postnatal regulatory processes. This would result in a biased response to higher leptin exposure, where only some expected physiological outcomes are observed, such as a negative regulation of early weight gain. Altogether, this highlights a complex interplay between prenatal programming and early milk-mediated signaling.

Adding further complexity, the effects of maternal diet on the leptin system may be modulated by changes in other milk bioactives. Our group has previously shown that an unbalanced maternal diet during the perinatal period alters the miRNA composition of milk (26, 33, 38). Here, we focused on early diet-modulated changes in miRNA that could influence leptin signaling. To this end, we analyzed the levels of selected miRNAs with predicted target genes within the leptin signaling pathway (miR-7a-1, miR-27b, miR-29a, as checked in miRDB (29)), as well as others present in milk and previously associated with maternal diet or metabolic status (26, 30–32). Their levels were semi-quantified in milk, duodenum, and plasma to explore potential indirect evidence of their absorption. Overall, maternal WD intake did not significantly affect the levels of miRNAs related to leptin signaling across the analyzed sources, suggesting they are unlikely to be inhibiting leptin responsiveness. The sole exception among the other miRNAs was miR-17, which was significantly elevated in the milk of WD-dams. Notably, only within the WD group did milk miR-17 levels correlate with those in the duodenum, suggesting a potential selective uptake of this regulatory molecule. Even though this was not mirrored in plasma, it cannot be ruled out that sustained intake throughout the suckling period could eventually lead to systemic detection, consistent with previous observations showing that miRNA milk variations and transfer to pups become more evident at later stages of lactation (26, 27). Additionally, potential local effects of milk-derived miR-17 at the intestinal level should not be disregarded. Given its involvement in development processes, including the immune system (56), neurogenesis, and neuronal function (57), changes in its supply may contribute to alterations in the maturation of the digestive and enteric nervous systems, both of which are critical processes during the postnatal period.

However, further research, including gene expression analysis of miR-17 targets in specific tissues, is needed to explore this possibility.

The inclusion of sex as a biological variable is crucial in metabolic programming studies (58). In the present study, no consistent sex-specific differences were detected across the leptin-related parameters evaluated. However, a sex-dependent effect of maternal diet was observed on neonatal weight and weight at PND4, with lower body weights in male WD-pups, whereas no significant effects were detected in females. Previous studies using similar models have reported that sex-specific differences in response to maternal WD intake, particularly in biometric parameters, emerge from PND6 onwards in males and from PND8 onwards in females (38). It is, therefore, plausible that at the very early developmental stages analyzed here, sexual dimorphism may already exert a significant influence on these outcomes. Moreover, duodenum levels of miR-27a and miR-27b showed sex-specific divergence, with significantly higher expression in males. To the best of our knowledge, sexual dimorphism in these miRNAs during the postnatal period has not been previously reported. Such developmental variations in miRNA expression may contribute to the establishment of sexual dimorphism and may carry physiological implications for postnatal development that warrant further investigation.

This study has several strengths, including the use of a well-characterized dietary model that combines high fat and sugar content to closely reflect contemporary human obesogenic diets. In addition, by focusing on leptin naturally present in breast milk and spontaneously ingested by the offspring, and by integrating analyses of leptin in milk and plasma with immunohistochemical approaches and physiological measurements, this work provides a physiologically relevant assessment of how maternal WD influences early milk leptin levels and its absorption during the neonatal period. However, some limitations must be acknowledged. Firstly, factors beyond changes in milk leptin levels or function may have contributed to the observed increase in gastric content in the offspring, as previously discussed, including prenatal effects that may not be fully disentangled within the current

study design. Secondly, maternal diet affects the overall bioactive composition of breast milk, as mentioned above. Thus, although this study focused on leptin and miRNAs – both recognized as key mediators of metabolic programming – the potential contribution of other milk-derived components should not be disregarded. Thirdly, even though the chow diet herein applied represents a reference condition to support normal physiological and reproductive function in laboratory rodents, it should be considered that the use of a purified control diet could have provided a more controlled nutritional comparison. Lastly, it should be acknowledged that our findings are primarily associative. While they provide a basis for generating novel hypotheses regarding milk leptin supply, neonatal absorption, and early-life effects, they do not establish causality and should be interpreted with caution until further mechanistic studies using causal experimental approaches are conducted.

Conclusion

In summary, our findings show that maternal WD consumption during the perinatal period affects milk leptin supply and is associated with differences in its early handling in neonatal rats. Although WD-dams produced milk with higher leptin levels during early lactation, and immunohistochemical analyses suggested increased gastric absorption in their offspring, this was not accompanied by higher gastric leptin content or circulating leptin levels. This discrepancy suggests the presence of compensatory mechanisms that may mask the expected anorexigenic effects typically associated with higher leptin exposure. These outcomes do not seem to be directly attributed to changes in the absorption of miRNAs targeting leptin-related pathways. However, the observed increase and potential uptake of miR-17 suggest that other milk-derived regulatory molecules may also vary in response to maternal WD at the gastrointestinal level. Overall, these results emphasize the importance of maternal nutritional status in shaping both the composition and neonatal uptake of breast milk bioactives. Further functional studies are needed to clarify the mechanisms underlying these observations, and longitudinal approaches will be required to determine their potential implications for metabolic programming in the offspring.

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Figure 1

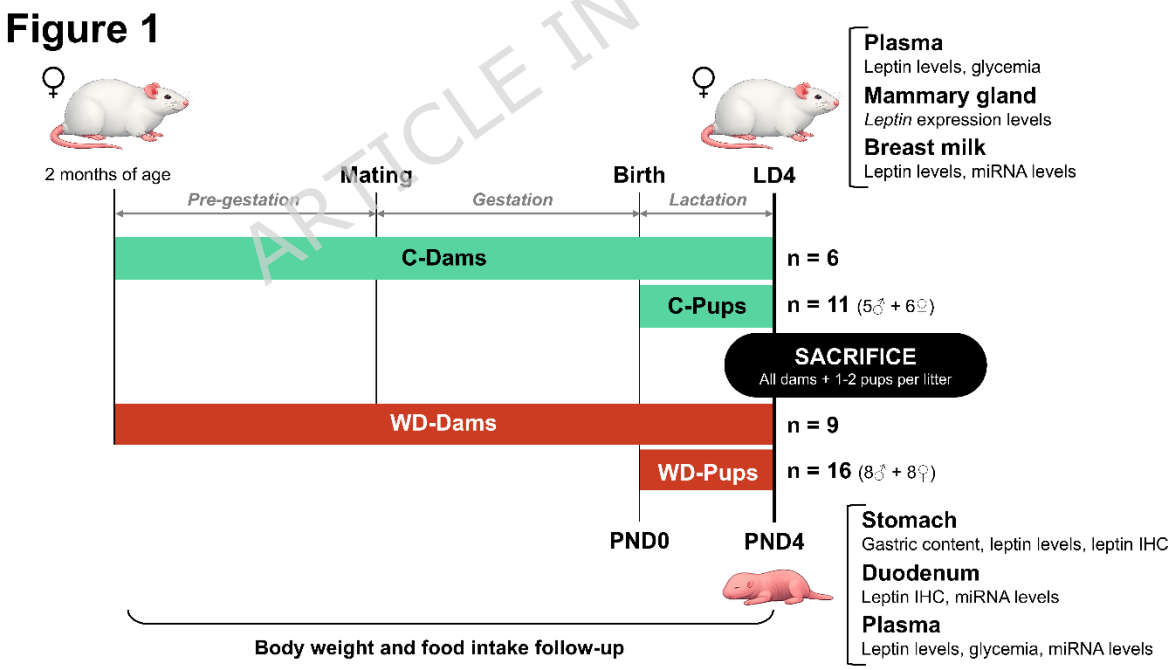

Figure 1

Schematic overview of the experimental design and analyses performed.

Figure 2

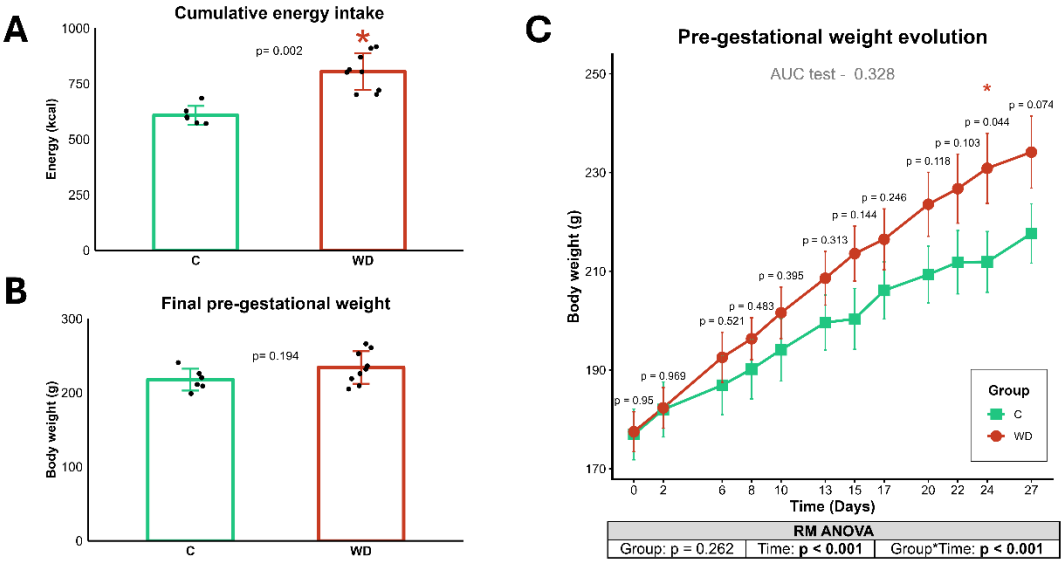

Figure 2

Maternal energy balance trajectory during the pregestational period. **A**) Cumulative energy intake of dams from 2 to ~3 months of age (at the time of mating). Bars represent group means with SD as error bars; individual data points are displayed in black (C, n = 6; WD, n = 9). Group comparison was conducted using the Mann-Whitney U test. **B**) Final pre-gestational weight (day 27 after diet group allocation). Bars represent group means with SD as error bars; individual data points are displayed in black (C, n = 6; WD, n = 9). Group comparison conducted using the Mann-Whitney U test. **C**) Body weight progression of dams over the same period. Data points represent group means (green: C, n = 6; red: WD, n = 9), with error bars indicating standard deviation (SD). The overall weight trajectories were compared by the area under the curve (AUC) analysis using the Mann-Whitney U-test. Weight progression was analyzed using repeated measures ANOVA (RM ANOVA) test, with Group and Time as factors. Exact p-values are indicated in the table below the graph. Post-hoc comparisons at each time point were performed using Tukey's multiple comparisons test. Exact p-values for post-hoc comparisons are indicated above each time point.

Figure 3

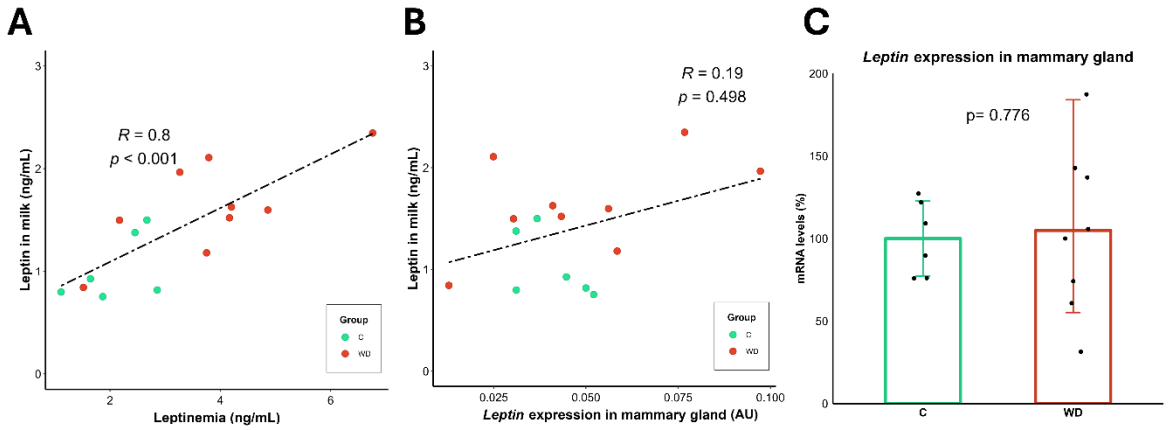

Figure 3

Correlation between leptin levels in milk and (**A**) plasma leptin in dams or (**B**) leptin expression in the mammary gland. Each point represents an individual dam from the C (green, n = 6) or WD (red, n = 9) group. Spearman's rank correlation coefficient (R) and corresponding p-value for both groups combined are indicated. **C**) Leptin expression in the mammary gland across both experimental groups. Bars represent group means, with SD shown as error bars; individual data points are shown in black (C, n = 6; WD, n = 8). Statistical comparison between groups was performed using the Mann-Whitney U test.

Figure 4

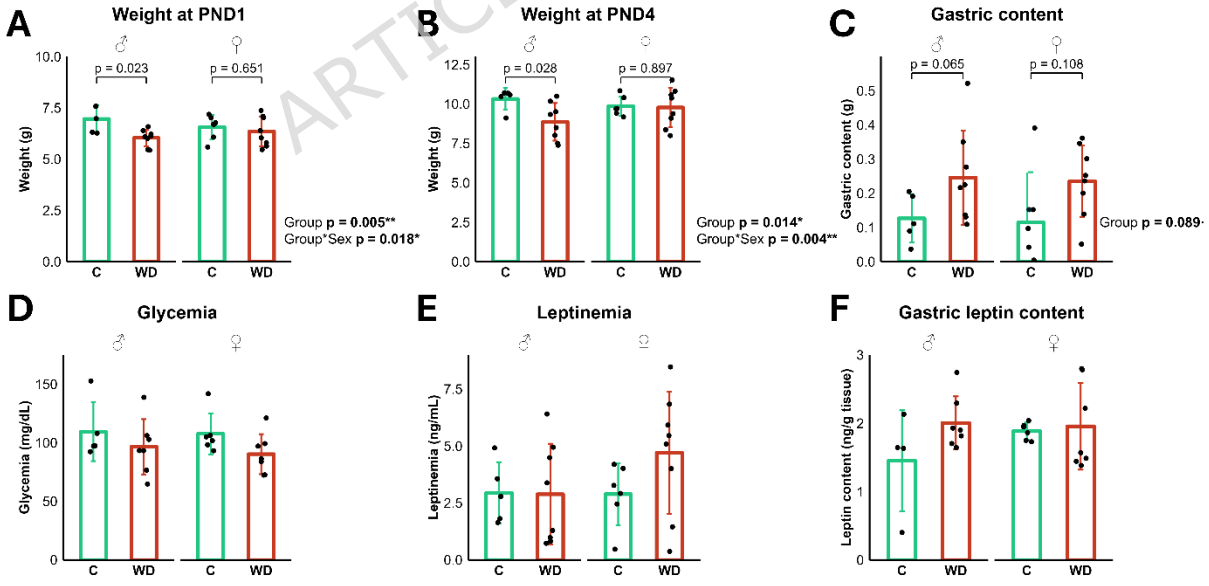

Figure 4

Biometric and leptin-related parameters in the offspring. A) Weight at PND1. B) Weight at PND4. C) Gastric content. D) Glycemia. E) Leptinemia. F) Gastric leptin content. In all cases,

bars represent group means, with SD shown as error bars; individual data points are shown in black (C♂, n = 5; C♀, n = 6; WD♂, n = 8; WD♀, n = 8; animals from the same litter are considered as different subjects). Statistical comparison was performed using a linear mixed-effects model, with Group and Sex as fixed effects and dam as random effect. Pairwise comparisons were applied using Mann-Whitney U test.

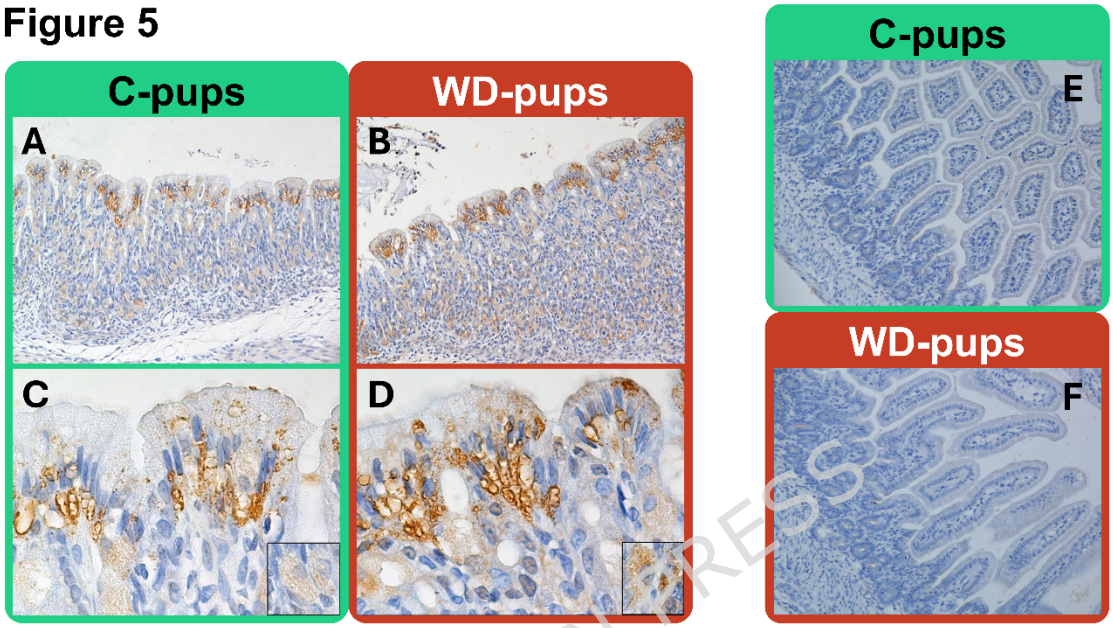

Figure 5

Left panel - Immunostaining for leptin in the gastric mucosa. Samples from WD-pups (B, D) displayed a stronger signal in the apical region – reflecting absorbed leptin – and in the chief cells – reflecting synthesized leptin – compared to C-pups (A, C). Light microscopy: A and B, x20, showing representative longitudinal sections of the gastric mucosa; C and D, x100, showing the apical portion of the mucosa and a representative chief cell in the lower right inset. Immunolocalization was visualized using diaminobenzidine (brown) and nuclei were counterstained with hematoxylin (blue).

Right panel - Immunostaining for leptin in the duodenal mucosa. Samples from both C-pups (E) and WD-pups (F) showed no detectable leptin signal. Light microscopy: x20. Immunolocalization was visualized using diaminobenzidine (brown) and nuclei were counterstained with hematoxylin (blue).

Figure 6

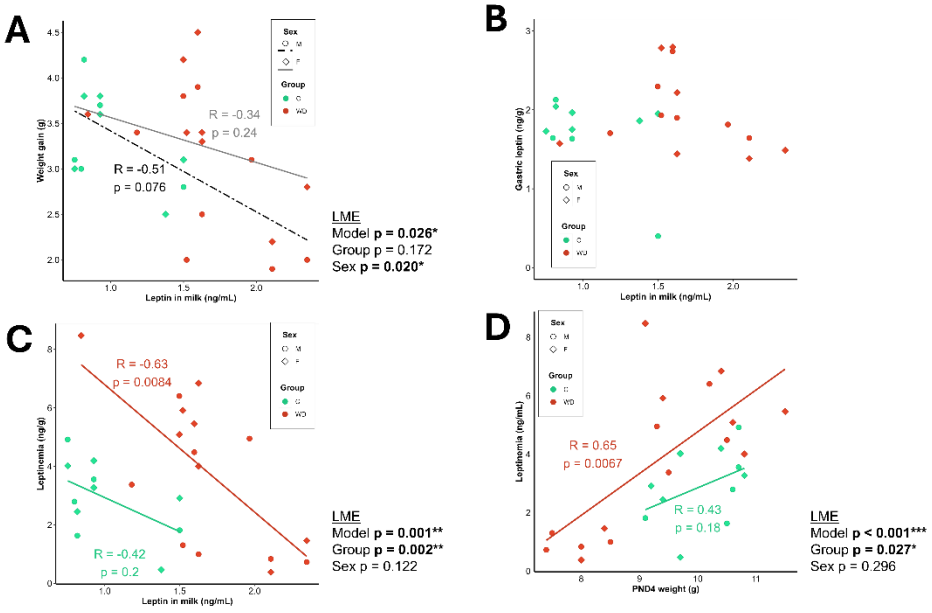

Figure 6

Associations between leptin-related measures and biometric parameters in pups. **A**) Leptin in milk vs. weight gain (from PND1 to PDN4) (C, n = 11, WD, n = 16), (**B**) leptin in milk vs. gastric leptin content (C, n = 10, WD, n = 14), (**C**) leptin in milk vs. plasma leptin (C, n = 11, WD, n = 16), (**D**) PDN4 weight vs. plasma leptin (C, n = 11, WD, n = 16). Each point represents an individual pup from the C (green) or WD (red) group, including males (circles) and females (diamonds). Animals from the same litter are considered as different subjects. Linear mixed-effects (LME) model parameters are shown. Models included Group and Sex as fixed effects and dam as a random effect. p-values for the overall model and individual terms are indicated for those significant models. Spearman's rank correlation coefficient (R) and corresponding p-value for subgroups correlations are shown. Trend lines are represented by line type for sex (black dot-dashed for males and grey solid for females) and by color for experimental group

(red for WD-pups and green for C-pups). ·, p < 0.1; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 7

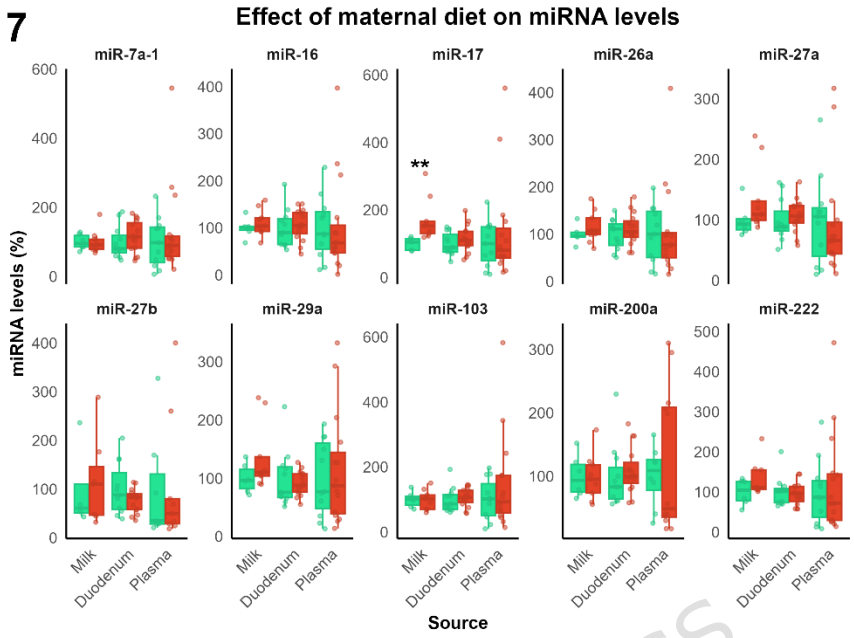

Figure 7

Effect of maternal diet on miRNA levels across different biological sources. Boxplots show the median and interquartile range of selected miRNAs in dams' milk (C, n = 6, WD, n = 9), pups' duodenum (C, n = 11, WD, n = 16), and pups' plasma (C, n = 11, WD, n = 16) for C (green) and WD (red) groups. Each point represents an individual pup from the C (green) or WD (red) group (animals from the same litter are considered as different subjects). Statistical comparisons between experimental groups in milk were performed using the Mann-Whitney U test. **, p < 0.01. In duodenum and plasma, linear mixed-effects (LME) models were constructed with Group and Sex as fixed effects and dam as random effect.

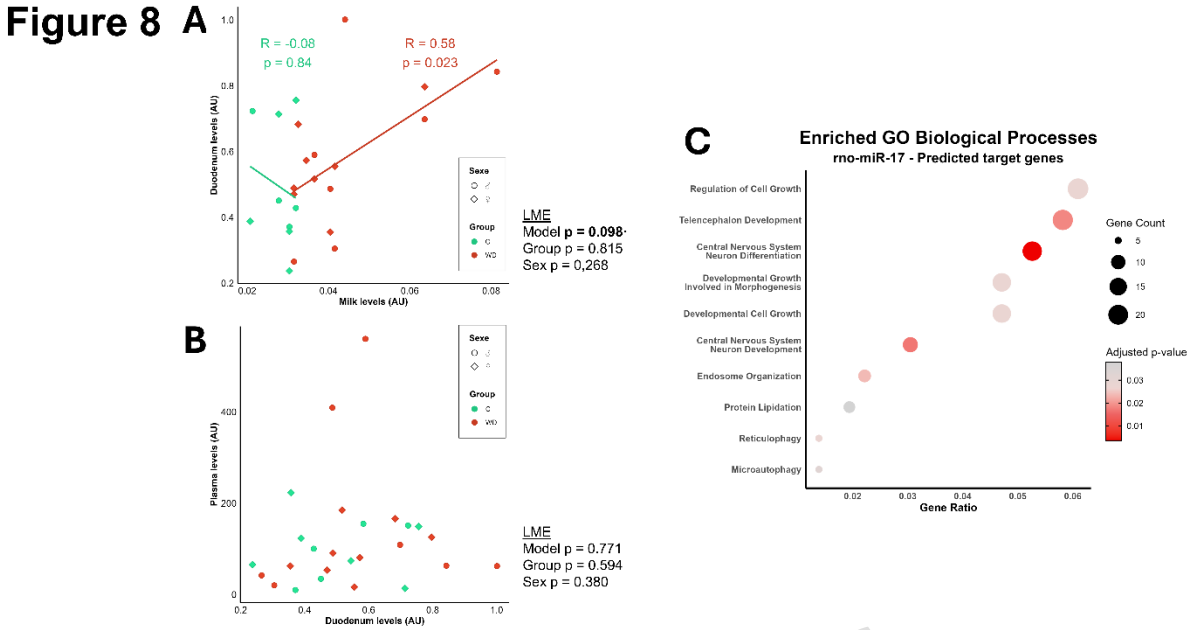

Figure 8

Correlations for rno-miR-17 levels in dams' milk vs. pups' duodenum (C, n = 9, WD, n = 15; **A**) or pups' duodenum vs pups' plasma (C, n = 11, WD, n = 15, **B**). In both cases, each point represents an individual pup from either the C (green) or WD (red) group, including males (circles) and females (diamonds). Linear mixed-effects (LME) model parameters are shown. Models included Group and Sex as fixed effects and dam as a random effect. p-values for the overall model and individual terms are indicated for those significant models. Spearman's rank correlation coefficient (R) and corresponding p-value for subgroup correlations are shown. Trend lines are represented by color according to experimental group (red for WD-pups and green for C-pups).**C**) Enrichment analysis of rno-miR-17 predicted target genes. Dot plot depicts the top 10 enriched pathways according to adjusted p-value.

Table 1. Dams' parameters.

	C-dams	WD-dams	p-value	Cohen's D
n	6	9		
Relative gestational weight gain (%)	51 ± 5.1	44 ± 6.2	0.068	-1.3
Number of pups	11 ± 1.6	12 ± 2.4	0.77	0.26
Glycemia (mg/dL)	136 ± 9.2	148 ± 33	0.39	0.47
Leptinemia (ng/mL)	2.1 ± 0.67	3.8 ± 1.5	**0.026**	1.4
Milk leptin (ng/mL)	1.0 ± 0.33	1.6 ± 0.46	**0.012**	1.5

Parameters of control (C-dams) and Western-Diet fed dams (WD-dams). Serum and milk determinations were performed post-mortem at lactation day 4 (LD4). Data are presented as mean ± SD. Differences between groups were analyzed using the Mann–Whitney U test; p-values < 0.05 (indicating statistical significance) are shown in bold. Effect sizes were calculated using Cohen's D, with the control group as reference.

Table S1. GeneGlobe ID of the miRNAs amplified and quantified by miRCURY LNA miRNA PCR assay.

miRNA	GeneGlobe ID
U6 snRNA (v2)	YP02119464
miR-7a-1-3p	YP00205573
miR-16-5p	YP00205702
miR-17-5p	YP02119304
miR-26a-5p	YP00206023
miR-27a-3p	YP00206038
miR-27b-5p	YP02112954
miR-29a-5p	YP00204430
miR-103-3p	YP00204063
miR-191-5p	YP00204306
miR-200a-3p	YP00204707
miR-222-3p	YP00204551

Table S2. Parameters from linear mixed-effects models for offspring variables. For the fixed effects (Group and Sex) and their interaction, beta coefficients and p-values are reported.

Variable	Group		Sex		Interaction	
	β	p-value	β	p-value	β	p-value
Weight at PND1 (g)	**-1.08**	**0.005**	-0.44	0.07	**0.78**	**0.018**
Weight at PND4 (g)	**-1.51**	**0.014**	-0.5	0.156	**1.55**	**0.004**
Weight gain (g)	-0.41	0.287	-0.05	0.738	**0.77**	**0.003**
Relative weight gain (%)	1.1	0.856	2.78	0.239	6.24	0.055
Gastric content (g)	0.12	0.089	-0.01	0.882	0.02	0.795
Glycemia (mg/dL)	-12.24	0.336	-1.62	0.77	5.51	0.465
Leptinemia (ng/mL)	402.07	0.741	68.95	0.944	1687.1	0.204
Gastric leptin (ng/g tissue)	529.91	0.103	452.15	0.136	-459.2	0.238
Duodenum miR-7a-1-3p	$4.1 \cdot 10^{-03}$	0.331	$3.90 \cdot 10^{-03}$	0.224	$-4.17 \cdot 10^{-03}$	0.323
Duodenum miR-16-5p	0.03	0.952	0.30	0.386	0.16	0.723
Duodenum miR-17-5p	0.11	0.363	0.06	0.495	-0.03	0.768
Duodenum miR-26a-5p	0.06	0.639	0.01	0.947	$-2.98 \cdot 10^{-03}$	0.985
Duodenum miR-27a-3p	0.18	0.309	**0.28**	**0.038**	-0.29	0.090
Duodenum miR-27b-5p	$-7.8 \cdot 10^{-05}$	0.616	**$3.23 \cdot 10^{-04}$**	**0.016**	$-2.19 \cdot 10^{-04}$	0.178
Duodenum miR-29a-5p	$-5.6 \cdot 10^{-04}$	0.480	$2.98 \cdot 10^{-04}$	0.554	$-8.88 \cdot 10^{-05}$	0.894
Duodenum miR-103-3p	-0.02	0.893	-0.04	0.797	0.13	0.557
Duodenum miR-200a-3p	-6.0·10-05	0.981	$7.43 \cdot 10^{-04}$	0.618	1.5310-03	0.446
Duodenum miR-222-3p	$-7.4 \cdot 10^{-04}$	0.657	$5.42 \cdot 10^{-04}$	0.630	$-7.67 \cdot 10^{-06}$	0.996
Plasma miR-7a-1-3p	0.02	0.392	-0.02	0.340	-0.01	0.544
Plasma miR-16-5p	1.46	0.261	0.74	0.539	-1.93	0.233
Plasma miR-17-5p	396.74	0.400	230.65	0.550	-768.18	0.147
Plasma miR-26a-5p	92.01	0.219	20.01	0.773	-108.55	0.247
Plasma miR-27a-3p	9.72	0.598	0.02	0.999	-23.21	0.286
Plasma miR-27b-5p	14.05	0.531	8.22	0.641	-30.23	0.210
Plasma miR-29a-5p	0.02	0.585	0.02	0.605	-0.01	0.814

Plasma miR-103-3p	28.85	0.284	-0.52	0.982	-29.63	0.326
Plasma miR-200a-3p	0.06	0.346	0.02	0.657	-0.18	0.202
Plasma miR-222-3p	0.28	0.291	0.08	0.713	-0.39	0.165