Received: 1 October 2024 | Revised: 10 December 2024 | Accepted: 2 January 2025

DOI: 10.1002/oby.24246

ORIGINAL ARTICLE

Obesity Biology and Integrated Physiology

Obesity A Research Journal | THE OBESITY SOCIETY | WILEY

Impact of Western diet on milk miRNAs and target genes in offspring adipose tissue: modulation by betaine during suckling

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Funding information
Spanish Ministry of Science, Innovation, and Universities, Grant/Award Numbers: PGC2018-097436-B-I00, PID2022-138140NBI00

Abstract

Objective: We investigated how a maternal Western diet (WD) affects milk microRNA (miRNA) profile and associates with metabolic programming in adipose tissues in pups. We also explored the impact of betaine supplementation during suckling, as betaine levels are reported to be reduced in WD-fed dams' milk.

Methods: A microarray analysis was performed to profile miRNA expression in dams' milk. Betaine levels were measured in the milk of dams and the plasma of their offspring. We also analyzed the expression of miRNA target genes in white and brown adipose tissues through gene expression analysis.

Results: Our findings confirm decreased betaine levels in the milk of WD-fed dams and the plasma of their offspring. The miRNA screening identified 37 deregulated miRNAs (36 downregulated), with the following 6 as the most relevant: miR-223-3p; miR-32-5p; let-7i-5p; miR-140-5p; miR-29a-3p; and miR-29c-3p (downregulated). Some of their target genes were upregulated in brown and white adipose tissues, particularly those related to thermogenesis and browning. Betaine supplementation in pups demonstrated a slight protective effect in females by enhancing thermogenic capacity.

Conclusions: Our results underscore the profound impact of a maternal WD on milk miRNA composition, potentially influencing gene expression, thermogenesis, and adiposity in the offspring, with sex-related differences.

INTRODUCTION

Nutritional challenges during pregnancy and lactation play a crucial role in the metabolic health and development of the infant [1]. Suboptimal maternal dietary conditions have been shown to result in detrimental metabolic programming, which can lead to an increased risk of obesity and other metabolic disorders later in life [2, 3]. Animal studies have suggested that exposure to a Western diet (WD) during critical developmental windows alters gene expression and induces epigenetic modifications in offspring [4, 5]. Despite extensive research, the mechanisms underlying maternal diet (MD)-induced programming remain unclear. Breast milk is the gold standard for infant feeding and nutrition [6], with a plethora of well-established benefits, including reducing infant mortality rates and protecting against the development of obesity, diabetes, and other metabolic disorders [6–9]. Milk composition varies throughout lactation, on a diurnal basis, and based on maternal metabolic status and nutritional behavior [10, 11]. Alterations in specific breast milk metabolites may underlie

the epigenetic and gene expression changes in offspring of obese dams [12]. In this regard, we have previously described that maternal intake of an obesogenic diet during the lactation window induces substantial changes in the milk lipid and metabolomic profiles, as well as in metabolic hormones [13–15].

Compounds in milk influenced by maternal metabolic status, particularly those acting as epigenetic factors, have garnered special attention. Milk represents a significant reservoir of microRNAs (miRNAs), which are small, noncoding RNA molecules that regulate gene expression post-transcriptionally [16]. These molecules play essential roles in various biological processes, including development, metabolism, and disease [16]. Studies have indicated that dietary patterns can disturb the levels of specific milk miRNAs such as miR-222, which is notably upregulated by cafeteria MD or WD [17, 18], potentially leading to greater fat accumulation and altered diet-induced thermogenesis in the offspring [17, 19]. This suggests that milk miRNAs may exert a crossover effect between maternal milk and adipose tissue programming, controlling critical steps in adipocyte differentiation and browning [19]. Animal studies carried out in our laboratory have shown that supplementation with milk bioactive compounds can improve metabolic programming and attenuate the detrimental programming effects caused by inadequate maternal nutrition during gestation [20]. For example, physiological leptin supplementation during suckling improves eating behavior and food preferences as well as leptin and insulin sensitivity and protects against obesity development in adulthood [21, 22]. Furthermore, it reverses many alterations caused by moderate gestational calorie restriction, resulting in a healthier adulthood phenotype [23]. Additionally, physiological myoinositol supplementation during the suckling period improves metabolic health in male rats and prevents insulin resistance and hypertriglyceridemia associated with inadequate fetal nutrition and a diabetogenic diet in adulthood [24].

We have previously identified a notable disparity in rat milk betaine levels between WD-fed dams and controls, with significantly lower concentrations observed in WD-fed dams' milk [15]. Betaine is an important nutrient during development because it is a source of methyl groups in the one-carbon metabolism [25]. In humans, lower maternal betaine levels during the third trimester of pregnancy are associated with increased infant birth weight and adiposity [26]. Moreover, an inverse association between milk betaine content and infant growth has been reported in different human cohorts [27]. In mice, maternal betaine supplementation during lactation decreases weight gain and adiposity in offspring by modulating gut *Akkermansia* abundance [27].

The molecular mechanisms by which milk components such as miRNAs or betaine influence offspring metabolism remain largely unknown. We used a rat model to explore how a maternal obesogenic WD affects miRNA content in breast milk and the connection with metabolic programming in adipose tissue, focusing on energy metabolism and body weight regulation. Additionally, we examined whether betaine supplementation during suckling could mitigate the effects of a detrimental metabolic programming caused by a maternal WD.

Study Importance

What is already known?

- Maternal Western diet (WD) affects milk composition and has detrimental effects on metabolic programming in offspring, influencing their adipose tissue function and propensity to obesity.
- Betaine levels have been found to be reduced in the milk of rats exposed to a WD, and betaine content in milk has been inversely associated with infant growth in different human cohorts.

What does this study add?

- Maternal exposure to a WD impacts the milk microRNA (miRNA) profile, potentially affecting target gene expression in offspring adipose tissue. In particular, decreased let-7i-5p and miR-29a levels may explain increased expression of thermogenic genes in white and brown adipose tissues and leptin in white adipose tissue.
- Betaine supplementation during suckling shows limited effects but may enhance the response to a maternal WD, particularly in the induction of thermogenic capacity in female offspring.

How might these results change the direction of research or the focus of clinical practice?

- The results highlight the need to further explore miRNA profiles in maternal milk depending on maternal diet and their effects on offspring metabolic programming and metabolism.
- The findings guide future research on nutritional interventions for both mothers during pregnancy and lactation and for pups during suckling, highlighting the need for sex-adjusted strategies.

METHODS

Animals and experimental design

The animal protocol was approved by the Bioethical Committee of the University of Balearic Islands (exp. 2018/13/AEXP, January 23, 2019) and followed institutional guidelines.

Virgin female Wistar rats were housed under controlled conditions and divided into two groups: one group was fed a standard chow diet (control diet [CD], Altromin Spezialfutter GmbH & Co. KG; $n = 10$), and the other group was fed a high-fat/high-sucrose diet (WD; Research Diets, Inc.; $n = 9$). Diets were administered 1 month before mating and were continued through gestation and lactation. At postnatal day (PND) 1, litters

were adjusted to 10 pups per dam (5 females and 5 males when possible).

Male and female pups were supplemented with an oral solution of betaine (Sigma Aldrich) or the corresponding vehicle (i.e., water) throughout the suckling period (i.e., PND 1 to PND 20) using a pipette. The dose of betaine was equivalent to twice the average amount of betaine taken by normal breastfeeding. After weaning, dams (day 21) and pups (day 22) were euthanized, and blood, retroperitoneal white adipose tissue (rWAT), and brown adipose tissue (BAT) samples were collected.

Body weight and food intake were followed throughout the experiment. Body composition (i.e., fat and lean mass) was analyzed with EchoMRI-700 (Echo Medical Systems LLC) on day 22 for pups. Glucose levels were measured in fresh blood by Accu-Check Glucometer (Roche Diagnostics).

Nutritional information on diet and betaine daily doses are detailed in the online Supporting Information.

Milk sample collection

Milk samples were collected from dams on days 10, 15, and 21 of lactation, as previously described [14].

miRNA isolation and quantification in milk

Total miRNA levels in milk were analyzed from 50 µL of whole milk using a mirVana miRNA isolation kit (Life Technologies Corporation) according to the manufacturer's protocol. RNA quantification was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Thermo Fisher Scientific). For miRNA expression analysis, RNA was reverse transcribed using miRCURY LNA RT Kit (QIAGEN). The screening of miRNA expression in milk was carried out with a miRCURY LNA miRNA Focus PCR 96-well Panel Rat miFinder Focus YARN-201Z (QIAGEN). The panel allows the analysis of 84 miRNAs, and rno-miR-191-5p was used as a housekeeping miRNA. Detailed procedures are described in online Supporting Information.

Betaine determination

Sample and standard preparation

Milk and plasma samples (5 µL) were deproteinized with acetonitrile/ammonium formate solution, whereas food samples were treated with methanol/water. Betaine-(trimethyl-D_9) hydrochloride was added to all samples. Following vortexing, incubation, and centrifugation, supernatants were diluted for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Milled chow and WD samples underwent specific dilutions before analysis. Detailed protocol and calibration curves for betaine and deuterated betaine standards are explained in online Supporting Information.

LC–MS/MS analysis

The analysis was performed using the UltiMate 3000 HPLC system (Thermo Fisher Scientific) coupled to mass spectrometry (Q Exactive, Thermo Fisher Scientific) in positive mode, equipped with an Ascentis Express HILIC column and precolumn (SUPELCO, Sigma Aldrich). The mobile phase consisted of 10 mM of ammonium formate in water (phase A) and acetonitrile (30:70; phase B) at a flow rate of 400 µL/min. Mass spectrometry settings, including ionization, temperature, and gas adjustments, are detailed in online Supporting Information.

Gene expression analysis

mRNA levels of validated target genes from miRTarBase [28] were analyzed in rWAT and BAT of offspring at weaning by real-time reverse transcriptase-polymerase chain reaction (RT-qPCR). Total RNA was extracted using Tripure Reagent (Roche Diagnostics) and quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Thermo Fisher Scientific). As a housekeeping gene, guanosine dissociation inhibitor (*Gdi*) was used.

Western blot analysis

BAT uncoupling protein 1 (UCP1) protein levels were determined by Western blotting using β-actin (ACTB) as loading and transfer control. The detailed protocol and reagents are given in online Supporting information.

Statistical analysis

Data are expressed as mean (SE) ($n = 8$–13). Statistical analyses included three-way ANOVA (factors of sex, MD, and betaine), with two-way ANOVA applied after sex stratification. Normality was assessed via the Levene test. Mann–Whitney *U* tests were used for single comparisons. Analyses were performed using SPSS Statistics (IBM Corp.), with a significance threshold set at $p < 0.05$. miRNA expression in dams' milk was analyzed using Metaboanalyst [29], including principal component analysis (PCA), variable importance in projection (VIP) values, and a volcano plot to visualize differentially expressed miRNAs.

RESULTS

Phenotypic characteristics of dams and their offspring

Data on dams are summarized in Table 1. After 1 month on the WD, WD-fed dams showed increased total and percentage body fat, although body weight remained unchanged. Cumulative energy intake was higher in WD-fed dams before gestation. No weight or fat

differences were observed during lactation. At weaning, WD dams had higher circulating insulin, lower leptin, unchanged glucose levels, and increased mammary gland weight.

Phenotypic traits at PND 21 and circulating parameters at PND 22 are shown in Table 2. WD-fed males and females exhibited higher body weight, total fat mass, fat mass percentage, rWAT and BAT weights, and circulating leptin levels than their controls. Notably, betaine supplementation during suckling led to a reduction in rWAT weight in the female offspring of dams exposed to a CD. Although three-way ANOVA showed an interactive effect of sex with either MD or betaine treatment on circulating glucose, no significant changes were observed when comparing the different conditions. For circulating insulin, males had higher levels than females.

Betaine levels in maternal milk, CD and WD, and offspring plasma

Betaine levels were determined in milk on days 10, 15, and 21 of lactation (Figure 1A). Milk betaine levels increased throughout lactation in both CD- and WD-fed dams when considered together. Notably, maternal exposure to a WD during the pregestation, gestation, and lactation stages resulted in significantly reduced levels of betaine in milk throughout lactation. Analysis of MD revealed markedly lower betaine levels in the WD compared with the CD (Figure 1B), explaining the decreased milk betaine in WD-fed dams. The reduction of milk betaine concentration under a WD was reflected in the plasma of pups on all days studied (days 10, 15, and 21 of suckling) across all groups, without any effects from betaine supplementation (Figure 1C).

TABLE 1 Phenotypic traits and circulating parameters of dams ($n = 9$–10) over the pregestation period during which WD-fed dams were exposed to a WD (31 days) and over the lactation period (21 days)

Dams			CD	WD
Pregestation period	Day -31	Weight, g	156 ± 6	155 ± 5
		Fat mass, g	15.5 ± 1.7	16.5 ± 1.2
		% Body fat	9.7 ± 0.8	10.6 ± 0.7
	Day 0	Weight, g	207 ± 6	215 ± 8
		Fat mass, g	22.4 ± 2.6	34.5 ± 3.6*
		% Body fat	10.6 ± 1.0	15.9 ± 1.3*
		Cumulative energy intake, kcal	1388 ± 56	1790 ± 66*
Lactation period	Day 10	Weight, g	268 ± 5	261 ± 7
		Fat mass, g	29.7 ± 2.3	32.4 ± 2.2
		% Body fat	11.1 ± 0.7	12.4 ± 0.8
	At weaning	Weight, g	263 ± 6	255 ± 6
		Fat mass, g	26.7 ± 2.1	24.8 ± 1.9
		% Body fat	10.1 ± 0.6	9.7 ± 0.7
		Glucose, mg/dL	93.1 ± 4.0	96.2 ± 5.7
		Insulin, µg/L	0.4 ± 0.1	1.5 ± 0.4*
		Leptin, pg/mL	1602 ± 163	1060 ± 225*
		Mammary gland, g	6.5 ± 0.3	7.9 ± 0.4*

Note: Data are mean ± SEM. Mann–Whitney *U* test was performed to analyze differences between groups.
Abbreviations: CD, control diet; WD, Western diet.
*Statistically significant difference compared with CD-fed dams, $p < 0.05$.

Analysis of milk-derived miRNA expression profiles

A microarray analysis capable of detecting 84 probes for milk-derived miRNAs was conducted in CD- and WD-fed dams ($n = 8$) using milk from day 21 of lactation. There were important changes in the levels of milk miRNAs between both groups, as illustrated by the partial least-squares-discriminant analysis (PLS-DA) shown in Figure 2. The results of the PLS-DA showed that the first two components accounted for 24.5% and 8.8% of the variation between the two groups of dams (Figure 2A). Figure 2B displays the miRNAs that demonstrated the greatest significance in differentiating between CD- and WD-fed groups, as determined by their VIP scores. A total of 37 miRNAs were significantly different between both groups (fold change threshold of 1 and $p < 0.05$; Table S1). Of these, 36 were downregulated, and only 1 was upregulated in the milk of WD-fed dams compared with CD-fed dams. Based on these findings, we selected the miRNAs that exhibit the most significant changes using a fold change threshold of 1.5, which indicates miRNAs with a fold change lower or larger than 1.5, and $p < 0.01$. This led to the final selection of the following six miRNAs: miR-223-3p; miR-32-5p; let-7i-5p; miR-140-5p; miR-29c-3p; and miR-29a-3p (Figure 2C), all of which were downregulated in the milk of WD-fed dams (Figure 2D).

Targeted gene expression of miRNAs in rWAT

In order to explore the downstream effects of the six most significantly altered miRNAs in breast milk, we performed a gene expression analysis of validated target genes in the offspring. We initially focused on rWAT, given the role of the target genes in regulating fat depots.

Figure 3 shows the expression levels of key target genes of the miRNAs in rWAT at day 22 of life. For the targets of miR-223-3p, maternal WD feeding led to a general downregulation of glucose transporter type 4 (also known as solute carrier family 2 member 4, *Slc2a4*; in both sexes) and signal transducer and activator of transcription 3 (*Stat3*) expression (particularly in females), with no changes observed in toll-like receptor 4 (*Tlr4*) expression. No differences between groups were found for phosphatase and tensin homolog (*Pten*) expression, a target of miR-32-5p. In contrast, maternal WD feeding caused a general upregulation of adrenoceptor β 3 (*Adrb3*) and *Ucp1* mRNA levels (the latter especially in females), both of which are targets of let-7i-5p. Additionally, the expression of platelet-derived growth factor receptor α (*Pdgfra*), a target of miR-140-5p, was downregulated with the maternal WD, especially in males. Regarding the targets of miR-29a-3p/29c-3p, leptin (*Lep*) expression was

TABLE 2 Phenotypic traits and circulating parameters of male and female offspring at days 21 and 22 of life ($n = 8–13$)

Pups		Males				Females				Statistics		
		CD		WD		CD		WD			Two-way ANOVA	
		VEH	BET	VEH	BET	VEH	BET	VEH	BET	Three-way ANOVA	Males	Females
Day 21	Weight, g	32.8 ± 0.7	33.2 ± 0.8	47.2 ± 1.1**	48.3 ± 1.5**	33.0 ± 0.9	31.6 ± 0.7	45.7 ± 1.6**	45.2 ± 1.6**	MD	MD	MD
	Fat mass, g	2.3 ± 0.6	2.5 ± 0.2	6.4 ± 0.4**	6.8 ± 0.5**	2.8 ± 0.2	2.5 ± 0.2	6.5 ± 0.5**	6.5 ± 0.5**	MD	MD	MD
	% Body fat	7.1 ± 0.3	7.4 ± 0.3	13.5 ± 0.5**	14 ± 0.6**	8.5 ± 0.3	7.9 ± 0.4	14.0 ± 0.8**	14.2 ± 0.8**	MD	MD	MD
Day 22	rWAT, g	0.034 ± 0.002	0.039 ± 0.005	0.177 ± 0.015**	0.204 ± 0.024**	0.038 ± 0.005	0.023 ± 0.003*	0.154 ± 0.014**	0.159 ± 0.022**	MD, S	MD	MD
	BAT, g	0.139 ± 0.007	0.148 ± 0.009	0.234 ± 0.013**	0.245 ± 0.013**	0.155 ± 0.01	0.141 ± 0.009	0.219 ± 0.018**	0.236 ± 0.018**	MD	MD	MD
	Glucose, mg/dL	136 ± 6	146 ± 4	147 ± 5	149 ± 4	151 ± 4	143 ± 5	143 ± 6	133 ± 5	S × BET S × MD		
	Insulin, ng/L	40.3 ± 9.3	42.1 ± 9.1	58.2 ± 11.8	72.9 ± 23.9	31.1 ± 7.3	26.0 ± 4.9	28.8 ± 3.9	28 ± 3.4	S		
	Leptin, ng/L	1.1 ± 0.1	1.2 ± 0.1	4.5 ± 0.6**	5.3 ± 0.6**	1.5 ± 0.2	1.1 ± 0.1	4.8 ± 0.6**	5.1 ± 0.8**	MD	MD	MD

Note: Data are mean ± SEM. Three-way ANOVA was performed to analyze the effects of S, MD, and BET ($p < 0.05$). Two-way ANOVA was performed to analyze the effects of MD and BET in males and females. Mann–Whitney *U* test was performed to analyze specific differences between groups.

Abbreviations: BAT, brown adipose tissue; BET, betaine treatment; CD, control diet; MD, maternal diet; rWAT, retroperitoneal white adipose tissue; S, sex; VEH, vehicle treatment; WD, Western diet.

*Statistically significant differences compared with VEH, $p < 0.05$.

**Statistically significant differences compared with CD-fed dams, $p < 0.05$.

significantly upregulated in the offspring of maternal WD-fed dams in both sexes, whereas no significant changes were observed in phosphatidylinositol 3-kinase catalytic subunit type 3 (*Pik3c3*) expression.

Targets of let-7i-5p: *Ucp1* and *Adrb3* expression in BAT

Considering the role of *Ucp1* and *Adrb3* in thermogenesis, and given the changes in gene expression levels observed in rWAT, we analyzed the expression levels of both genes in BAT. As shown in Figure 4, the maternal WD had a general effect on increasing *Ucp1* expression in males and *Adrb3* expression in both sexes. In females, the increase in *Ucp1* mRNA levels was only observed in the betaine-supplemented group. Additionally, in males, betaine supplementation resulted in lower *Adrb3* mRNA expression levels. Notably, a significant positive correlation was found between the mRNA expression levels of *Ucp1* and *Adrb3* in BAT. UCP1 protein levels were also increased by the maternal WD when considering both sexes together (three-way ANOVA), an effect that was preserved in females when separating both sexes (two-way ANOVA).

Expression of let-7i-5p in adipose tissue

In order to elucidate whether the observed changes in the expression of the thermogenesis-related genes *Ucp1* and *Adrb3* were associated with changes in let-7i-5p levels in milk or with offspring tissue miRNA activity per se, we analyzed let-7i-5p expression in rWAT and BAT. The analysis revealed no significant variations in let-7i-5p mRNA expression levels between groups in any depot studied (data not shown).

DISCUSSION

Exploring the relationships among the maternal nutritional environment, the composition of breast milk, and the offspring phenotype may help to shape dietary recommendations that support optimal growth and development in early life. Herein, we focused on the impact of obesogenic MD on breast milk miRNA levels, the consequent effects on the offspring, and the effects of betaine supplementation during suckling, particularly on adipose tissue gene expression related to the regulation of energy metabolism and body weight.

We show that a maternal WD induces significant modifications in breast milk composition, including a distinct miRNA profile and a marked reduction in betaine. Previous work in this model of WD-fed dams [15] suggested the reduction in betaine content in milk, which we confirmed by specifically measuring betaine concentration with LC–MS/MS analysis. Our parallel determination of betaine levels in the experimental diets allowed us to directly connect the decreased betaine levels in the milk of WD-fed dams to the poor betaine content of the WD.

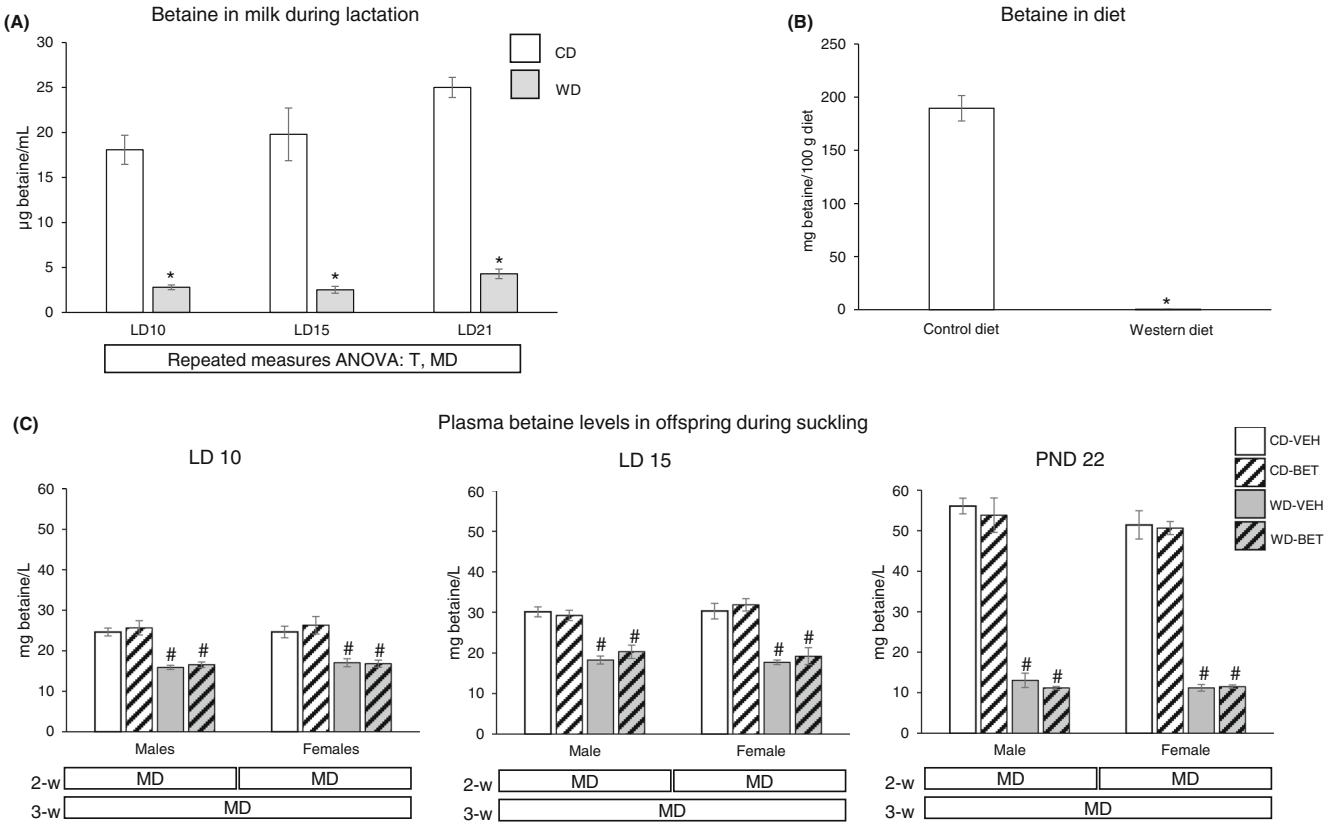

FIGURE 1 Betaine levels in (A) breast milk of dams ($n = 8$) at lactation day (LD) 10, LD 15, and LD 21, in (B) control diet (CD) and Western diet (WD), and in (C) plasma offspring at LD 10, LD 15, and postnatal day (PND) 22. Data are mean ± SEM. Three-way ANOVA was performed to analyze the effects of sex (S), maternal diet (MD), and betaine treatment (BET; $p < 0.05$). Two-way ANOVA was performed to analyze the effects of MD and BET in males and females. Repeated-measures ANOVA was conducted to analyze the potential interactive effect of time (T) and MD. Mann–Whitney *U* test was performed to analyze differences between groups (*Statistically significant difference compared with WD and CD in panels A and B, respectively; #Statistically significant difference compared with CD in panel C; $p < 0.05$).

Apart from the significant reduction in betaine, we show here that maternal WD feeding, which results in increased adiposity, profoundly affects miRNA levels in breast milk, with a general downregulation of an important number of miRNAs (36 in total). The following six are most prominently altered: miR-223-3p; miR-32-5p; let-7i-5p; miR-140-5p; miR-29c-3p; and miR-29a-3p. Obesity and exposure to an obesogenic diet have previously been associated with changes in some miRNAs in breast milk in studies in both humans and rodents [17, 30]. Our study allowed us to screen a large number of miRNAs under a controlled maternal obesogenic (i.e., Western) diet model. Interestingly, a previous study [17] suggested that the obesogenic diet per se, rather than maternal obesity, alters the milk levels of specific miRNAs.

Regarding miR-223-3p, its specific role in breast milk remains largely unknown, but fluctuations in its levels in various tissues have been linked to obesity and insulin sensitivity [31]. Studies in humans and mice have shown that upregulation of miR-223-3p in adipose tissue is associated with obesity, whereas decreased circulating levels could serve as a potential biomarker for prediabetes and adipose tissue dysfunction [31, 32]. This might be related to reduced secretion from adipose tissue associated with inflammation, a hallmark of obesity [32, 33]. However, it is important to note that the decreased expression of the *Slc2a4* gene (coding for the glucose transporter 4 [GLUT4]) in the adipose tissue of pups in the WD group does not align with the expected increase as a validated target gene of miR-223-3p [32]. Similarly, the decreased expression of another target gene, *Stat3*, in females suggests that other mechanisms beyond this miRNA must be operating.

A similar situation is observed with the targets of miR-32-5p and miR-140-5p (*Pten* and *Pdgfra*, respectively) studied here, the expression of which remained unchanged or decreased with the maternal WD. We must consider that we have focused on targets associated with fat depot regulation, thermogenic capacity, and/or adipose tissue browning. In the context of obesity, although there is little information regarding the role of miR-32-5p, it has been reported to trigger lipogenesis in the liver [34]. miR-32 is also related to BAT thermogenesis because increased levels repress its target *Tob1* (coding for the transducer 1 of Erb-B2 receptor tyrosine kinase 2), modulating fibroblast growth factor 21 (FGF21) signaling, and promoting BAT thermogenesis and subcutaneous WAT browning [35]. However, the

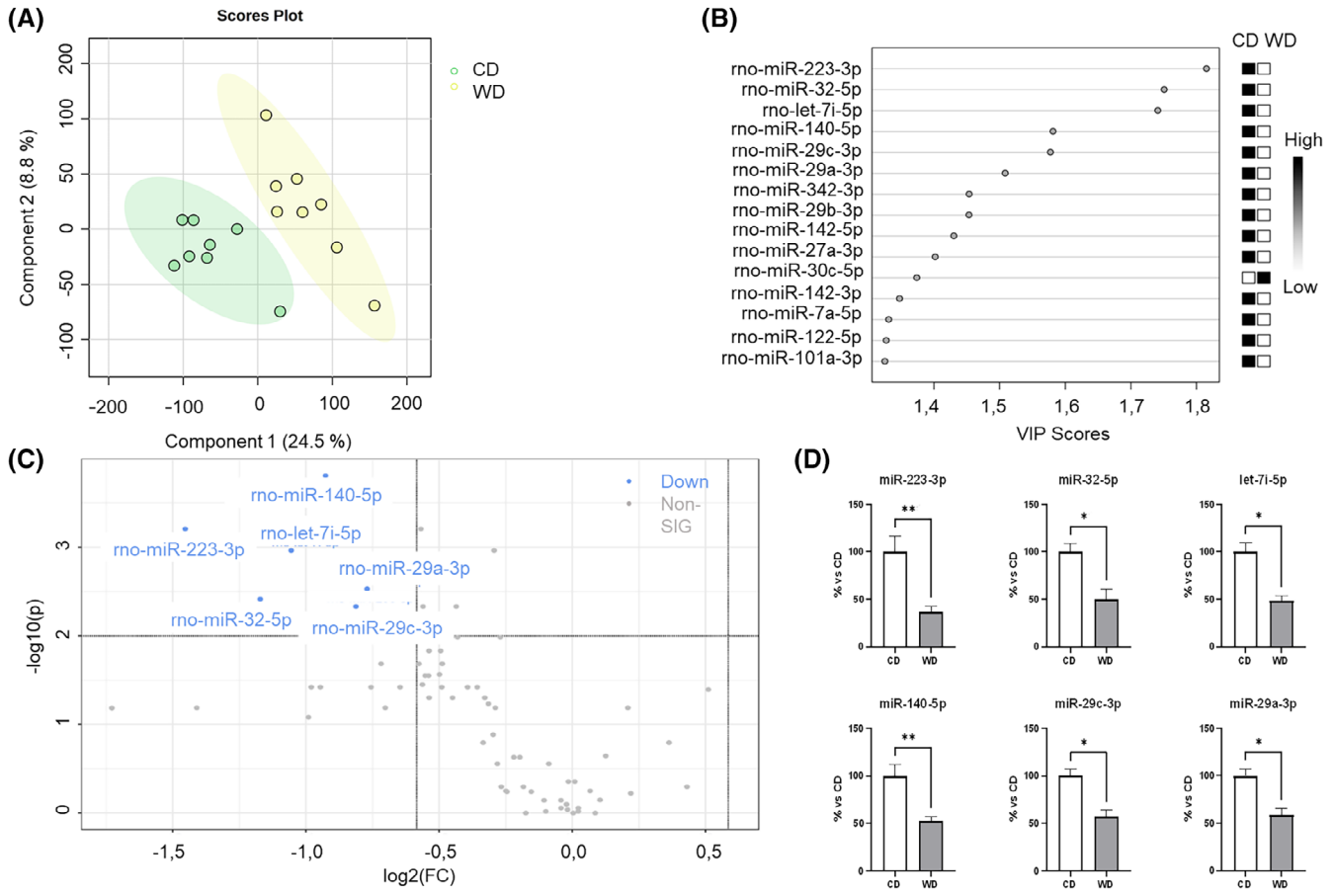

FIGURE 2 (A) Exploratory analysis of milk microRNA (miRNA) profile in control diet (CD)- and Western diet (WD)-fed dams ($n = 8$). Partial least-squares-discriminant analysis (PLS-DA). Component 1 explains 24.5% of the variation between CD- and WD-fed dams, and component 2 explains 8.8% of additional variation. CD-fed dams are represented in green, and WD-fed dams are represented in yellow. (B) Variable importance in projection (VIP) scores result from PLS-DA and top 15 miRNAs with higher VIP scores that most influenced the variation between groups. (C) Volcano plot of miRNA expression changes with *p* value of 0.01 and a fold change (FC) threshold of 1.5 (FC > 1 indicates upregulation; FC < 1 indicates downregulation). (D) Relative gene expression of the 6 outstanding miRNAs from the analysis represented in panel C that showed significantly lower levels in milk of WD-fed dams compared with those in CD-fed dams at day 21 of lactation. Gene expression is given as a percentage of the value of the CD-fed dams' group. Data are mean ± SEM. Statistics from Mann–Whitney *U* test. $^{*}p < 0.01$, $^{**}p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

decreased levels of miR-32-5p in the milk of WD-fed dams do not align with the increased thermogenic capacity observed in the offspring. Additionally, there is currently a lack of information on the presence of miR-32-5p in breast milk, making the reported decrease in its levels due to an obesogenic diet a novel finding. Regarding its direct target *Pten*, we did not observe changes in its expression, although this does not rule out the possibility that other targets might be affected. miR-140-5p has been related to adipocyte differentiation [36] and can induce lipogenesis and adipogenic differentiation by targeting *Pdgfra* [36]. Our results show a decrease in miR-140-5p levels in breast milk as a consequence of obesogenic MD. However, the expression of its target gene *Pdgfra* in the offspring rWAT followed a similar pattern to that of the miRNA in milk, contrary to the expected opposite modulation. At any rate, *Pdgfra* is known to inhibit adipocyte differentiation, and the downregulation of PDGF signaling has been suggested as a key step in adipocyte formation from precursors [37]. Therefore, the observed decrease in the expression of *Pdgfra* in male pups in the WD group aligns with an increased capacity for adipogenesis during early life in response to obesogenic MD. Even though female pups in the WD group did not exhibit the same change in *Pdgfra* expression due to MD, both sexes presented, as expected, greater body fat after weaning compared with offspring in the CD group, suggesting that sex-dependent mechanisms may be operating. Betaine supplementation does not appear to affect the expression of the target genes of these miRNAs. However, in females in the CD group, it is associated with reduced retroperitoneal fat pad.

Regarding miR-29, Shah et al. reported a nonsignificant tendency toward lower levels of miR-29a in the breast milk of women with overweight or obesity [38]. Herein, we describe a significant decrease of miR-29a levels in the milk of WD-fed dams, along with decreased levels of miR-29c. Regarding their target genes, *Lep* mRNA levels were increased in the rWAT of offspring in the WD group in both

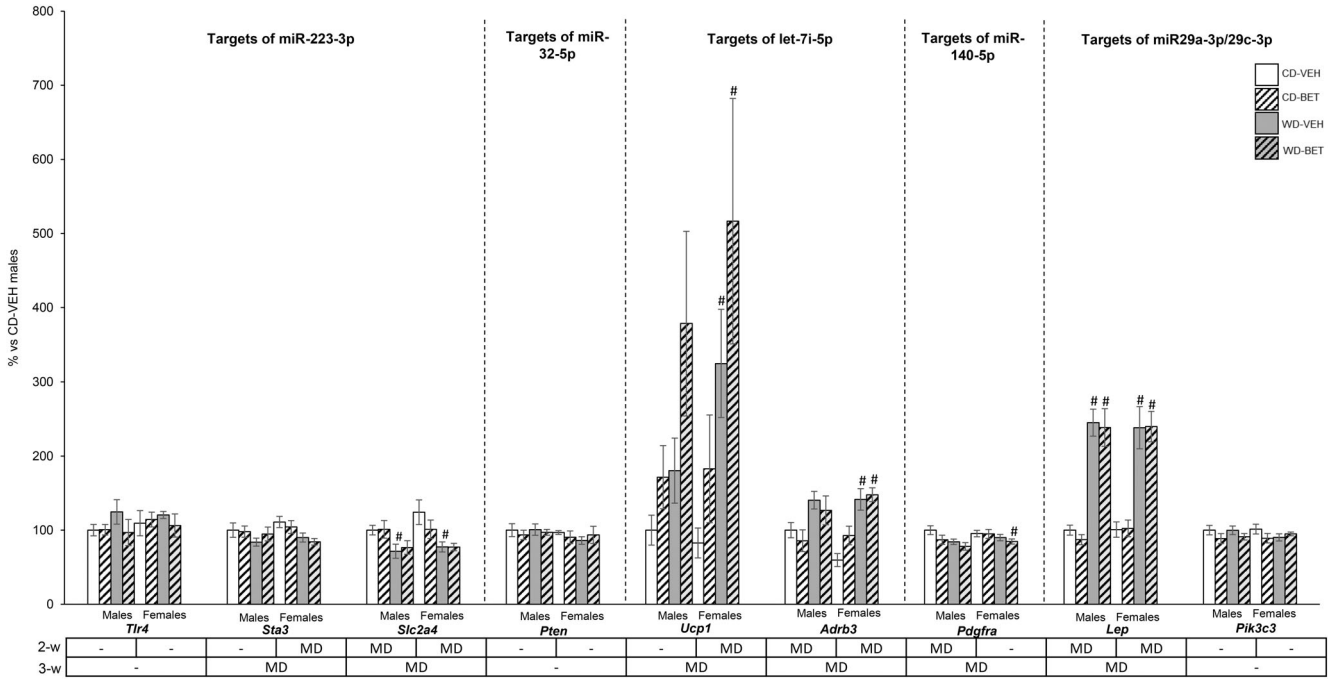

FIGURE 3 Messenger RNA (mRNA) expression of selected target genes of miR-223-3p, miR-32-5p, let-7i-5p, miR-140-5p, and miR-29a-3p/miR-29c-3p in retroperitoneal white adipose tissue (rWAT). Gene expression is given as a percentage of the value of control diet (CD)-vehicle treatment (VEH) male group. Data are mean ± SEM. Three-way ANOVA was performed to analyze the effects of sex (S), maternal diet (MD), and betaine treatment (BET; $p < 0.05$). Two-way ANOVA was performed to analyze the effects of MD and BET in males and females. Mann–Whitney *U* test was performed to analyze differences between groups (#Statistically significant difference compared with CD; $p < 0.05$). Abbreviations for gene symbols are as follows: *Adrb3*, adrenoceptor β3; *Lep*, leptin; *Pdgfra*, platelet-derived growth factor receptor α; *Pik3c3*, phosphatidylinositol 3-kinase catalytic subunit type 3; *Pten*, phosphatase and tensin homolog; *Slc2a4*, glucose transporter type 4; *Stat3*, signal transducer and activator of transcription 3; *Tlr4*, toll-like receptor 4; *Ucp1*, uncoupling protein 1.

sexes. This observation is consistent with the decrease in milk miR-29 and the plausible regulation of adipose *Lep* expression by milk miRNA changes. Overexpression of miR-29a in transgenic mice has been reported to reduce body weight gain and fat accumulation induced by a high-fat diet [39], highlighting the importance of this miRNA in preventing adiposity. Therefore, the reduction of miR-29a and miR-29c in the milk of obese dams may contribute to the epigenetic programming of the offspring, potentially modulating leptin mRNA expression. We also observed a positive correlation between milk leptin levels and miR-29a and miR-29c levels (Table S2), highlighting the relationship between them and the possibility that milk miR-29 levels might mirror variations in leptin levels. The decline of miRNA let-7i-5p in the milk of WD-fed dams is particularly noteworthy, as it targets *Ucp1* and *Adrb3* mRNAs. UCP1 (*Ucp1* gene) is the main effector of adaptive thermogenesis by increasing the conductance for H^+ through the inner mitochondrial membrane and dissipating the H^+ gradient energy as heat [40], whereas *Adrb3* encodes the β3-adrenergic receptor, which is crucial for regulating lipolysis and thermogenesis, even in humans [41]. Adrenergic activation stimulates glucose and free fatty acid uptake in brown and beige adipocytes and triggers UCP1 activation, increasing oxygen consumption and energy expenditure [41]. Apart from the expected increase of BAT mass as a physiological adaptation to combat excess calorie intake in the maternal WD groups [42], the observed decrease in let-7i-5p levels in milk, especially in males, alongside the increase in adipose *Ucp1* and *Adrb3* mRNA levels (which are positively correlated) under maternal WD feeding suggests that changes in milk miRNAs could effectively influence the thermogenic capacity of offspring, impacting both WAT and BAT thermogenic capacity. The maternal WD was also associated with increased UCP1 protein levels in BAT, especially in females. Additionally, the induction of both *Ucp1* and *Adrb3* expression in female offspring following the maternal WD was only evident in the betaine-supplemented animals. This suggests that betaine supplementation during suckling may help protect female pups from the adverse effects of a maternal WD by enhancing their thermogenic capacity. Notably, the effects of a maternal WD on the thermogenesis-related genes *Ucp1* and *Adrb3* are likely mediated primarily by let-7i-5p from the milk rather than intrinsic tissue miRNAs, as no significant changes were observed in let-7i-5p expression in rWAT and BAT of the offspring.

Overall, the response to maternal WD feeding in males and females, enhanced by betaine supplementation in females, suggests that WD offspring exhibit an adaptative response to the maternal dietary challenge. This response involves inducing browning in WAT and enhancing the thermogenic capacity in BAT. Consistent with

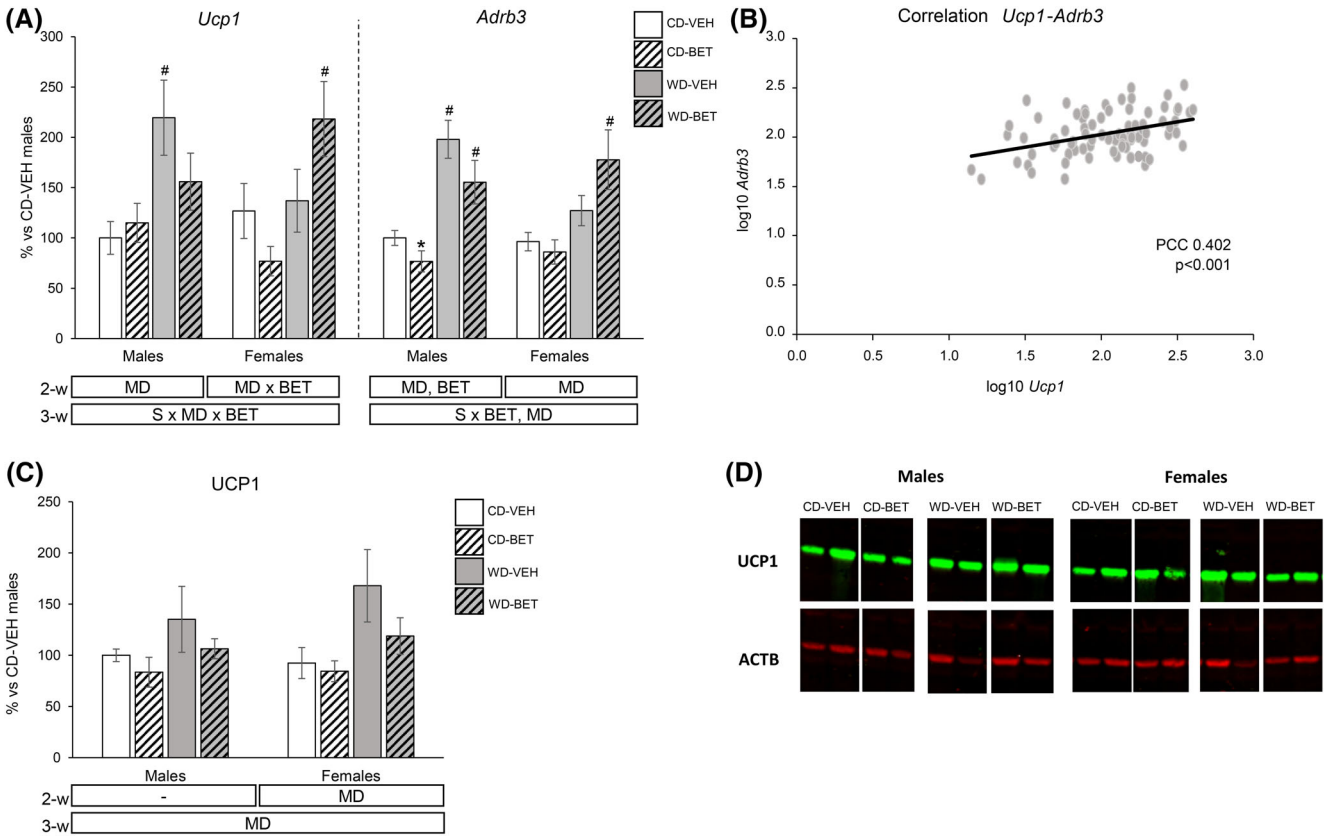

FIGURE 4 (A) Uncoupling protein 1 (*Ucp1*) and adrenoreceptor β 3 (*Adrb3*) mRNA expression in brown adipose tissue (BAT), (B) correlation between *Ucp1* mRNA and *Adrb3* mRNA levels in BAT, (C) protein levels of UCP1 in BAT, and (D) representative UCP1 and β-actin (ACTB; loading control) Western blot bands from each group. Gene expression is given as a percentage of the value of the control diet (CD)-vehicle treatment (VEH) male group. In Western blot, quantification of individual bands was corrected by ACTB signal and expressed as a percentage of the value of the CD-VEH male group. Three-way ANOVA was performed to analyze the effects of sex (S), maternal diet (MD), and betaine treatment (BET; $p < 0.05$). Two-way ANOVA was performed to analyze the effects of MD and BET in males and females. Mann–Whitney *U* test was performed to analyze differences between groups (#Statistically significant difference compared with CD; $p < 0.05$). PCC, Pearson correlation coefficient. [Color figure can be viewed at wileyonlinelibrary.com]

our findings, other studies have shown a negative association between let-7i-5p levels and UCP1 expression in brite (beige) adipocytes in both human and murine models [43]. Herein, we show a similar association with milk let-7i-5p levels. Additionally, betaine administration in other models has demonstrated potential benefits related to metabolism and thermogenic capacity. For example, studies in mice have suggested that betaine improves metabolic health and prevents high-fat-induced metabolic dysfunction-associated steatotic liver disease [44]. Moreover, increasing breast milk levels of betaine through maternal betaine supplementation during lactation has been associated with reduced adiposity and improved glucose homeostasis in adult offspring mice [27]. In a miR-143 knockout mouse model, betaine supplementation enhances energy expenditure, shown by increased oxygen consumption and carbon dioxide (CO_2) production [45]. These mice also exhibited higher body temperature at room temperature and 4°C, indicating increased thermogenesis [45].

CONCLUSION

Maternal exposure to a WD significantly alters the miRNA profile and reduces betaine concentration in milk (the latter being directly related to the low betaine content of the WD), which, in turn, can influence the metabolic programming and health of offspring. Of particular interest are the associations between decreased levels of milk let-7i-5p miRNA and the expression of key thermogenic genes in WAT and BAT, suggesting a regulatory role in the adaptative thermogenic response to obesogenic MD, as well as between decreased levels of miR-29a and the increased leptin expression in WAT. Notably, the sequences of let-7i-5p, miR-140-5p, miR-29a-3p, miR-29c-3p, and miR-32-5p are conserved between rats and humans [28], highlighting the potential relevance of these findings to humans. Although betaine supplementation during suckling did not show striking effects in our model, it might mediate an improved response in the induction of thermogenic capacity in females, revealing a sex-dependent

differential response to both MD and betaine supplementation. These findings highlight the complex interplay between MD, specific milk nutrients, epigenetic regulation, and offspring health outcomes. Further research may be of interest to elucidate the underlying mechanisms and explore potential strategies, such as enhancing milk composition with deficient compounds, to mitigate the detrimental effects of maternal exposure to obesogenic conditions on offspring health.O

ACKNOWLEDGMENTS

Rocío A. Martín-Chamorro was granted a PhD fellowship funded by the Spanish Government (grant reference PRE2019-090274). Our group is a member of Instituto de Salud Carlos III (Carlos III Health Institute), Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (Network Biomedical Research Center Pathophysiology of Obesity and Nutrition [CIBERobn]), and the European Research Network of Excellence (The European Nutrigenomics Organization, European Union [EU] contract no. FP6-506360).

FUNDING INFORMATION

Funding for this study was provided by the Spanish Government, grant/award numbers PGC2018-097436-B-I00 and PID2022-138140NBI00 (funded by the Spanish Ministry of Science, Innovation, and Universities/the Spanish State Research Agency [AEI]/10.13039/501100011033 and by the European Regional Development Fund (ERDF) - "A way of making Europe").

CONFLICT OF INTEREST STATEMENT

The authors declared no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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How to cite this article: Martín-Chamorro RA, Pomar CA, Palou A, Picó C, Rodríguez AM. Impact of Western diet on milk miRNAs and target genes in offspring adipose tissue: modulation by betaine during suckling. *Obesity (Silver Spring)*. 2025;33(4):732-742. doi:10.1002/oby.24246