Original Research

Gestational Diabetes Mellitus Is Associated with Altered Abundance of Exosomal MicroRNAs in Human Milk

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ABSTRACT

Purpose: Human milk (HM) is a unique biological fluid that is enriched with a variety of factors, including microRNAs (miRNAs) that potentially provide both short- and long-term benefits to the infants. miRNAs are packaged within exosomes, making them bioavailable to infants. Gestational diabetes mellitus (GDM) may affect the abundance of exosomal miRNAs in HM, providing a mechanism for growth and adiposity variation in infants of mothers with GDM in early life. Therefore, the purposes of this study were to examine the impact of GDM on select miRNAs (miRNA-148a, miRNA-30b, miRNA-let-7a, and miRNA-let-7d) involved in metabolism and to examine the association of these miRNAs with measures of infant body composition in the first 6 months of life.

Methods: Milk samples were collected from a cohort of 94 mothers (62 mothers without GDM and 32 mothers with GDM) matched on body mass index strata at 1 month post partum. miRNA abundance was measured by real-time polymerase chain reaction. Linear regression models were used to examine potential differences in miRNA abundance in women with and without GDM, testing associations between miRNA abundance and infant growth and body composition measures from 1 to 6 months.

Findings: The abundances of miRNA-148a, miRNA-30b, miRNA-let-7a, and miRNA-let-7d were reduced in milk from mothers with GDM. Independent of GDM status, higher maternal diet quality was associated with increased abundance of each of the measured miRNAs. miRNA-148a was negatively associated with infant weight, percentage of body fat, and fat mass, whereas miRNA-30b was positively associated with infant weight and fat mass at 1 month of age. There was no association of milk miRNA-148a and miRNA-30b with infant weight at 1 month of age or with body composition measures at 3 months of age; however, miRNA-148a was negatively associated with infant weight at 6 months of age.

Abbreviations: miRNA, microRNA; GDM, Gestational Diabetes Mellitus; HM, Human milk; NW, Normal weight; OW/OB, Overweight/Obese.

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Implications: If supported by randomized dietary supplementation or other intervention trials, HM miRNAs may be a therapeutic target to mitigate risk of metabolic outcomes in offspring of women with GDM. (*Clin Ther.* 2022;44:172–185.) Published by Elsevier Inc.

Key words: breast milk microRNA, exosomes, infant body composition, infant growth, maternal gestational diabetes mellitus.

INTRODUCTION

Nutrition in the early postnatal period plays an important role in infant growth and the developmental programming of metabolic disease susceptibility later in life.[1,2] Breastfeeding is an ideal source of nutrients, playing a crucial role in nutritional programming in the growing infant.[3] The benefits of human milk (HM) may be mediated by a variety of bioactive factors, such as hormones, immunoglobulins, oligosaccharides, growth factors, and micro-RNAs (miRNAs),[4] influencing infant growth and body composition in early life.[5–7] miRNAs are small, noncoding RNAs that bind to complementary sequences within the 3' untranslated region of messenger RNAs (mRNAs), modulating protein production.[8–10] miRNAs play a role in multiple biological processes, such as adipocyte differentiation, insulin signaling, and glucose homeostasis.[11] HM is one of the richest sources of miRNAs.[12] The abundance of miRNAs in HM is affected by various maternal factors, such as nutrition, disease status, and specifically obesity during pregnancy. Our group recently found that the abundance of selected miRNAs involved in adipogenesis and insulin signaling is decreased in HM exosomes obtained from women with overweight or obesity.[7] However, to our knowledge, the impact of gestational diabetes mellitus (GDM) on HM exosomal miRNA composition and abundance has not been examined.

Evidence indicates that exposure to GDM increases the risk of obesity and type 2 diabetes (T2DM) in offspring[13,14]; however, this risk is decreased by exclusive breastfeeding for at least the first 3 months of life.[15,16] Given that infant formulas are deficient in exosomes and their miRNAs,[17] miRNAs may be one of the mechanisms by which breastfeeding decreases future risk of obesity and T2DM in infants born to mothers with GDM. Accumulating evidence indicates that diabetes during pregnancy (both pregestational and GDM) can not only alter the composition of macronutrients and total energy content of HM but also alter the composition of bioactive factors, such as insulin, adiponectin, and ghrelin, in HM.[18] However, the impact of maternal GDM on exosomal miRNAs in HM and their subsequent association with infant growth is poorly understood. Studies have found that miRNA-148a, miRNA-30b, and miRNA-let-7a are highly abundant in HM and are involved in important metabolic pathways, such as insulin signaling and adipogenesis.[7,11,19–21] We recently found that abundance of miRNA-148a, miRNA-30b, and miRNA-let-7d is lower in HM obtained from mothers with overweight or obesity.[22] Thus, in the present study, we chose these 4 miRNAs (miRNA-148a-3p, miRNA-30b-5p, miRNA-let-7a-5p, and miRNA-let-7d-5p) with the a priori hypothesis that GDM is associated with altered abundance of miRNAs that are involved in important metabolic pathways in HM. The objectives of the study were to compare the abundance of these exosomal miRNAs in HM collected at 1 month post partum from women with and without GDM, controlling for maternal prepregnancy body mass index (BMI) and other potential confounders, and to test associations between HM miRNA abundances at 1 month post partum and infant growth and body composition during the first 6 months of life.

MATERIALS AND METHODS

Study Population

All research procedures and protocols were approved by the institutional review board at the University of Minnesota. The participants in this study were a subset of a larger study entitled Maternal Metabolism, Milk, and the Microbiome (4M Study), which included 35 women with GDM and 150 women without GDM. The 4M Study included pregnant women with singleton pregnancy 21 to 45 years of age at delivery, with an intention to exclusively breastfeed for a minimum of 3 months, and, if multiparous, who achieved successful breastfeeding for at least three months. GDM was defined based on serum glucose laboratory results in the medical record following a 2-step GDM screening procedure. Specifically, at 26 to 28 weeks of gestation, a nonfasting, 1-hour, 50-g oral glucose challenge test was performed. If the serum glucose level was ≥130 mg/dL, a subsequent 3-hour, 100-g oral glucose tolerance test was performed, with GDM defined using the Carpenter-Coustan criteria

(ie, if serum glucose exceeded ≥2 of the following levels: 95 mg/dL, 180 mg/dL, 155 mg/dL, and 140 mg/dL at the fasting, 1-hour, 2-hour, and 3-hour time points, respectively).[23] Women were excluded if they used tobacco, consumed >1 alcoholic drink per week during pregnancy or lactation, or had history of preexisting diabetes. Other exclusion criteria included preterm or postterm birth (gestational age <37 or >42 weeks), an infant requiring neonatal intensive care unit admission for >48 hours, infant birth weight <2500 g, or a congenital defect in the infant that is known to impact feeding and growth. For the purposes of the present analysis, we first grouped participants by prepregnancy BMI group (obese [prepregnancy BMI ≥30 kg/m^2], overweight [prepregnancy BMI of 25–<30 kg/m^2], and normal weight [prepregnancy BMI of 18.5–<25 kg/m^2]). For each of these BMI strata, we included all women with GDM and then randomly selected twice as many women without GDM from the same stratum, for an approximately 1:2 case-control ratio in each stratum. For statistical analysis, the 2 higher BMI groups were collapsed, for a total of 4 groups: normal weight non-GDM, overweight/obese non-GDM, normal weight GDM, and overweight/obese GDM.

Maternal Characteristics and Dietary Components

Clinical characteristics of maternal participants, including parity (0 or ≥1), mode of delivery (vaginal delivery or cesarean section), first recorded maternal weight and height in the medical record within 6 weeks from conception (dated using last menstrual period), gestational weight gain (weight at admission for delivery minus the first recorded maternal weight), maternal race (White or other), and maternal age (years), were collected from the electronic health records. Maternal dietary intake data were collected during the third trimester of pregnancy and at 1 and 3 months post partum using the Diet History Questionnaire II, a food frequency questionnaire, as described previously.[24] Maternal diet quality during pregnancy and lactation was estimated using the Healthy Eating Index.[25]

Breast Milk Collection

Mother-infant pairs reported to the study site at 1, 3, and 6 months (±5 days) post partum, between 8:00 and 10:00 AM, and at least 1.5 hours since the last infant feeding. A prefeeding infant weight was obtained using a high-sensitivity scale (Seca 728, Seca, Birmingham, United Kingdom). Mothers then breastfed their infants ad libitum from both breasts. Two hours after feeding, a complete single-breast milk expression was obtained using a hospital-grade pump according to a standard protocol described previously.[5] Collected breast milk was gently mixed, aliquoted, and stored at −80 °C within 20 minutes of collection. For this study, only the 1-month milk sample was assayed for miRNA abundance.

Infant Growth and Body Composition

At birth and 1, 3, and 6 months, infant length was measured using the Seca 416 infantometer. Infant naked weight was measured using the high-sensitivity scale embedded in the Pea Pod (COSMED USA Inc., Concord, California). Age- and sex-specific length for age z score (LAZ) and weight for age z score (WAZ) were subsequently calculated using the World Health Organization z score classification system for term infants.[26] Infant percentage body fat (BF), fat mass (FM), and fat-free mass (FFM) were assessed using Pea Pod at 1 and 3 months and using dual x-ray energy absorptiometry at 6 months, with the infant in a supine position, wearing only a disposable diaper, and swaddled in a light cotton blanket.[27]

Exosome Isolation and miRNA Analysis

Samples (2 mL) of HM were thawed on ice and centrifuged twice (1200 × g, 4°C, 10 minutes) to remove fat, cells, and large debris. Defatted supernatant was centrifuged again (21,500 × g, 4°C, 50 minutes) to remove residual fat and casein and subsequently passed through 0.22-μm filters to remove residual cell debris, as previously described.[28] Exosomes were extracted from 250 μL of defatted filtered milk using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, California) according to the manufacturer's protocol. Total RNA was extracted from the isolated exosomes using the SeraMir Exosome RNA Isolation kit (System Biosciences) according to the manufacturer's instructions miRNA-specific primers were pooled according to the Applied Biosystems (Waltham, Massachusetts) protocol (publication 4465407). Reverse transcription was performed on 20 μL of total RNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Grand Island, New York). The full names, TaqMan assay

identification numbers, and sequences of each of the selected miRNAs are available in Supplemental Table I. All quantitative polymerase chain reaction (PCR) reactions were performed in triplicate using TaqMan MicroRNA Assays and TaqMan Universal Master Mix II (no UNG) using the Bio-Rad (Hercules, California) CFX96 Touch Real-Time PCR Detection System.

Statistical Analysis

Univariate analyses, including participants' demographic and anthropometric characteristics, were analyzed using GraphPad Prism statistical software, version 9.1.2 (GraphPad Software, San Diego, California) with a standard α of 0.05. Normality was determined using the Kolmogorov-Smirnov test. Between-group differences of normally distributed, continuous data were compared using the unpaired *t* test; nonnormally distributed data were analyzed by Mann-Whiney *U* test. Relative expression by quantitative PCR of individual miRNA species was calculated using the $2-\Delta\Delta Ct$ method[29] in which the geometric mean of all the miRNAs of the samples was used for normalization of the data.[30] The normal weight non-GDM group was set as the reference group (ie, this group's fold change [FC] was set as 1.0), and the FC of each of the miRNAs for the other 3 groups (normal weight GDM, overweight/obese non-GDM, and overweight/obese GDM) was calculated as compared with the normal weight non-GDM group.

Our stratified random sampling procedure was designed to achieve balance in BMI groups across the 2 GDM groups. However, residual confounding could exist, so linear regression models were constructed. Maternal age, maternal race, gestational weight gain, gestational age, maternal educational level, household income, quality of diet measured by the healthy eating index score and postpartum weight loss at 1 month, and infant sex were also included as covariates in our primary linear regression models. In the models, the FC of each miRNA served as the dependent variable, and GDM was the independent variable of primary interest. Stepwise regression was also performed, allowing independent variables to be added and/or removed from the model in a purely data-driven process until the model with the lowest Akaike information criterion confirmed whether GDM remained a significant term. The effect sizes, 95% confidence intervals (CIs), and *P* values were presented for all independent variables. To examine the effect of miRNA on infant size, we constructed 1-month infant growth (change in weight from birth to 1 month) and body composition at 1, 3, and 6 months of age linear regression models with weight, length, WAZ, WLZ, change in weight from birth to 1 month of age (Δ weight 0–1 month), percentage of BF, FM, and FFM as the dependent variables, with each miRNA FC as the primary independent variable. Models were controlled for maternal BMI, maternal age, infant sex, gestational age, birth weight, and birth length. Because these analyses were exploratory outcomes, the models were not controlled for multiple comparison tests. For the models that included Δ weight 0 to 1 month, infant birth weight and birth length were not included because Δ weight 0 to 1 month is affected by infant weight and length at birth.

RESULTS

Clinical Characteristics of Mother-Infant Dyads

Demographic and clinical information for the mother-infant dyads is presented in Table I and Table II. The WAZ and LAZ scores in Table I were calculated after adjusting for gestational age at birth. Given that our mothers with GDM were very well controlled based on their glycosylated hemoglobin level (data not shown) and did not have excessive gestational weight gain as defined by the American College of Obstetricians and Gynecologists, we were not surprised that infants born to mothers with GDM had lower WAZ scores (not statistically significant). Of 32 women with GDM, 20 received medication (insulin or an oral agent) and 8 women were diet controlled. Treatment information on 4 women was not available in the electronic medical records because of missing data. All our patients with GDM participate in educational sessions with certified diabetes educators. The patients send their blood glucose log to the practitioner on a weekly basis. In the present study, if closer follow-up to assess the level of glucose control was needed, this was done through the electronic record. When patients with GDM are taking medications such as insulin or oral agents, they are seen twice weekly for fetal surveillance and to review glucose logs. Thus, relatively lower birth weight for gestational age (not statistically significant) and lower weight at 1 month in the infants with GDM could be related to good glycemic control. Differences were observed in the racial distribution, gestational weight gain, and household income between the 2 groups. Infants born to mothers with GDM were born

Table I. Maternal and infant birth characteristics.

Characteristic	Non-GDM (n = 62)	GDM (n = 32)	*P* Value[†]
Mothers			
Age, mean (SD), y	32.58 (4.19)	34.29 (4.25)	0.071
Prepregnancy BMI, mean (SD), kg/m^2	27.98 (5.70)	29.76 (7.39)	0.245
Gestational weight gain, mean (SD), kg	12.97 (5.45)	9.83 (5.39)	0.010
Excessive gestational weight gain, %	50	32	0.124
Postpartum weight loss at 1 month, mean (SD), kg	9.50 (3.30)	10.34 (2.37)	0.164
Maternal race/ethnicity, % non-Hispanic White	87	69	0.003
Maternal educational level, %			0.123
HS graduate, GED, or associate's degree	18	25	
Bachelor's degree	34	41	
Graduate degree	48	34	
Maternal income, %			0.004
<$60,000	27	22	
$60,000–$90,000	24	9	
$90,000–$120,000	49	69	
Maternal Dietary Quality Score (HEI 2015), mean (SD)	65.68 (10.0)	62.65 (9.91)	0.200
Infants			
Sex, % male	46	45	>0.999
Birth weight, mean (SD), kg	3.66 (0.48)	3.34 (0.42)	0.054
Gestational age, mean (SD), wk	39.83 (1.00)	38.09 (1.92)	<0.001
Weight for age *z* score, mean (SD)[†]	0.76 (0.97)	0.14 (0.85)	0.058
Length for age *z* score, mean (SD)[†]	1.39 (1.21)	0.76 (1.13)	0.120
Weight for length *z* score, mean (SD)	−0.64 (1.51)	−0.90 (1.16)	0.703

BMI = body mass index; HEI = Healthy Eating Index; HS = high school; GDM = gestational diabetes mellitus; GED = General Educational Development.**P* values were calculated using the 2-sided *t* test for continuous data and the Fisher exact test used for categorical data.

[†] Weight and length for age z scores are adjusted for gestational age.

1.7 weeks earlier than infants born to mothers in the non-GDM group, but birth weight *z* score differences did not reach statistical significance.

Infants born to mothers with GDM weighed less than infants born to mothers without GDM, and had lower fat-free mass at 1 month of age, but this difference was not present at 3- or 6-months (Table II). No difference in % body fat and fat mass was observed between groups at 1-, 3-, and 6 months of age. All the infants at 1 month of age were exclusively breast fed by study design. Exclusive breastfeeding at 6 months postpartum was more common in mothers without GDM than in mothers who had GDM.

Abundance of HM miRNAs at 1 Month of Lactation in Milk of Mothers With GDM

At 1 month postpartum, the abundance of each miRNA was lower in mothers with GDM than in mothers without GDM (Figure 1). miRNA-148a was 45% lower in the normal weight GDM group and 61% lower in the overweight/obese GDM group than in the normal weight non-GDM group. miRNA-30b was 65% lower in the normal weight GDM group and 64% lower in in the overweight/obese GDM group than in the normal weight non-GDM group. miRNA-let-7a was 35% lower in the normal weight GDM group; however, no difference was observed between the overweight/obese GDM group and normal weight

Table II. Infant characteristics at 1, 3, and 6 months.

Characteristic	Non-GDM (n = 62)	GDM (n = 32)	*P* Value*
1-Month infant characteristics[†]			
Weight, mean (SD), kg	4.58 (0.53)	4.26 (0.54)	0.045
Weight for age *z* score, mean (SD)	0.29 (0.82)	−0.30 (1.17)	0.048
Δ Weight from 0–1 month, mean (SD), kg[‡]	0.89 (1.03)	0.92 (0.34)	0.860
Length, mean (SD), cm	54.71 (2.43)	53.48 (1.86)	0.022
Length for age *z* score, mean (SD)	0.20 (1.17)	−0.51 (1.16)	0.020
Weight for length *z* score, mean (SD)	0.21 (1.15)	0.17 (0.91)	0.952
Body fat, mean (SD), %	17.09 (4.17)	16.47 (5.13)	0.521
Fat mass, mean (SD), kg	0.79 (0.24)	0.71 (0.29)	0.300
Fat-free mass, mean (SD), kg	3.80 (0.39)	3.54 (0.36)	0.023
3-Month infant characteristics[†]			
Weight, mean (SD), kg	6.31 (0.71)	6.03 (0.81)	0.474
Weight for age *z* score, mean (SD)	0.16 (0.75)	−0.11 (1.00)	0.514
Δ Weight from 1–3 months, mean (SD), kg[‡]	1.98 (1.03)	1.71 (0.54)	0.534
Length, mean (SD), cm	61.19 (2.18)	60.76 (2.18)	0.993
Length for age *z* score, mean (SD)	0.19 (0.97)	0.09 (0.92)	0.822
Weight for length *z* score, mean (SD)	0.10 (0.94)	−0.21 (1.16)	0.263
Body fat, mean (SD), %	23.83 (4.12)	24.22 (5.34)	0.766
Fat mass, mean (SD), kg	1.51 (0.36)	1.49 (0.50)	0.929
Fat-free mass, mean (SD), kg	4.81 (0.51)	4.57 (0.44)	0.302
Exclusively breastfed, %[‖]	95.08	83.33	0.095
6-Month infant characteristics[§]			
Weight, mean (SD), kg	7.90 (0.87)	7.49 (1.11)	0.343
Weight for age *z* score, mean (SD)	0.21 (0.87)	−0.23 (1.19)	0.259
Δ Weight from 1–6 months, mean (SD), kg[‡]	3.32 (0.33)	3.23 (0.57)	0.298
Length, mean (SD), cm	67.06 (2.22)	67.34 (2.45)	0.196
Length for age *z* score, mean (SD)	0.11 (0.99)	0.26 (0.90)	0.150
Weight for length *z* score, mean (SD)	0.30 (0.91)	−0.44 (1.26)	0.019
Body fat, mean (SD), %	33.40 (3.25)	33.92 (3.54)	0.898
Fat mass, mean (SD), kg	2.81 (0.49)	2.73 (0.58)	0.712
Fat-free mass, mean (SD), kg	5.58 (0.65)	5.29 (0.64)	0.568
Exclusively breastfed, %[¶]	90.16	63.16	0.010

(GDM = gestational diabetes mellitus.**P* values were calculated using a 2-sided *t* test for continuous data and the Fisher exact test used for categorical data.

[†] The 1- and 3-month body compositions were assessed using air displacement plethysmography.

[‡] Δ Weight indicates change in weight for the respective ages at 1, 3, and 6 months

[§] The 6-month body composition was assessed using dual energy x-ray absorptiometry.

[‖] For exclusive breastfeeding status at 3 months, data were available from 24 of 32 infants in the GDM group and 61 of 62 infants in the non-GDM group.

[¶] For exclusive breastfeeding status at 6 months, data were available from 19 of 32 infants in the GDM group and 61 of 62 infants in the non-GDM group.

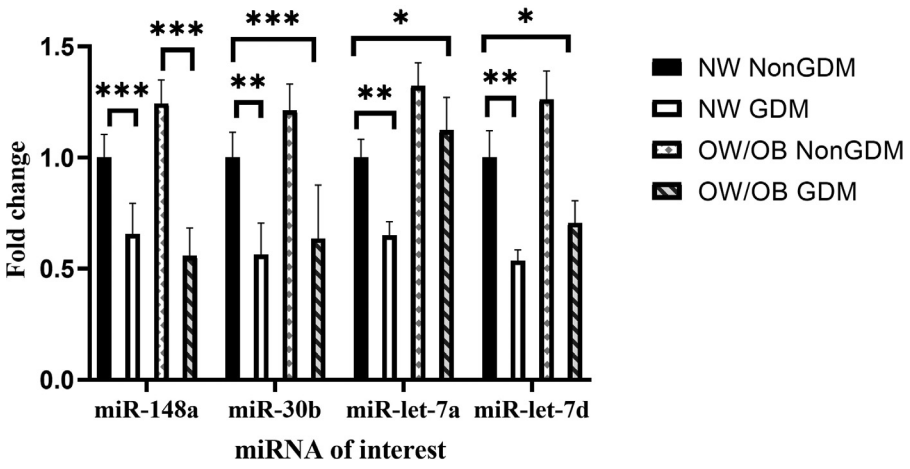

Figure 1. Relative abundances measured as fold change of miRNA-148a, miRNA-30b, miRNA-let-7a, and miRNA-let-7d are reduced in milk of mothers with gestational diabetes mellitus (GDM). Error bars indicate SEM.
(*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

non-GDM group. miRNA-let-7d was 48% lower in the normal weight GDM and 47% lower in the overweight/obese GDM than in the normal weight non-GDM group.

Abundance of HM miRNAs at 1 month of Lactation in Milk of Mothers With GDM

GDM exposure was independently associated with a lower abundance of each miRNA ($P < 0.05$) (Table III). In addition, maternal age was inversely associated with the abundance of miRNA-148a ($\beta = -0.044$) and miRNA-let-7a ($\beta = -0.041$), whereas maternal diet quality was positively associated with abundance of each of the miRNAs ($\beta = 0.023$ for miRNA-148a, $\beta = 0.024$ for miRNA-30b, $\beta = 0.018$ for miRNA-let-7a, and $\beta = 0.020$ for miRNA-let-7d), suggesting an increase in miRNA abundance with better diet. Finally, GDM exposure remained independently associated with miRNA abundances in the final stepwise regression models ($P < 0.037$ for all) (Supplemental Table II).

Association of HM miRNAs Sampled at 1 Month With Infant Weight and Adiposity at 1 Month

miR-148a and miR-30b were both significantly associated with infant weight and body composition measures at 1 month, after controlling for gestational age, birth weight, and infant sex (Table IV). With every fold increase in miRNA-148a, 1-month infant weight, WAZ, percentage of BF, and FM decreased by 0.31 kg ($P = 0.012$), 0.58 ($P = 0.032$), 3.6% ($P = 0.044$), and 0.2 kg ($P = 0.024$), respectively. In contrast, the abundance of miRNA-30b was positively associated with infant anthropometrics at 1 month of age. For every fold increase in miRNA-30b, 1-month infant weight, WAZ, and FM increased by 0.25 kg ($P = 0.003$), 0.38 ($P = 0.040$), and 0.17 kg ($P = 0.008$), respectively. There was no association any miRNA FC with Δ weight from 0 to 1 month of age.

The association of infant weight and percentage of BF with miRNA-148a and miRNA-30b did not persist at 3 months of age (Table IV). miRNA-let-7a and miRNA-let-7d were both significantly associated with percentage of BF and FM in the infant at 3 months of age, after controlling for gestational age, birth weight, and infant sex (Table IV). For every fold increase in miRNA-let-7a, percentage of BF and FM decreased by 3% ($P = 0.007$) and 0.21 kg ($P = 0.019$), respectively, at 3 months. In contrast, with every fold increase in miRNA-let-7d, percentage of BF and FM increased by 2.4% ($P = 0.038$) and 0.21 kg ($P = 0.026$), respectively, at 3 months.

The associations observed between miR-148a abundance at 1 month and infant body composition at 1

Table III. Regression model using fold change for individual miRNAs in milk exosomes.

Variable	β (95% CI)*			
	miRNA-148a	miRNA-30b	miRNA-let-7a	miRNA-let-7d
GDM	−0.474† (−0.885 to −0.062)	−0.562† (−1.109 to −0.016)	−0.493‡ (−0.824 to −0.162)	−0.667‡ (−1.113 to −0.221)
Maternal prepregnancy BMI	0.008 (−0.020 to 0.036)	0.003 (−0.034 to 0.041)	0.014 (−0.008 to 0.037)	0.007 (−0.024 to 0.038)
Maternal age	−0.044† (−0.082 to −0.005)	−0.041 (−0.092 to 0.009)	−0.041† (−0.072 to −0.010)	−0.031 (−0.073 to 0.010)
Maternal race/ethnicity (non-Hispanic White)	−0.674 (−2.215 to 0.482)	−0.718 (−2.896 to 0.686)	−0.296 (−1.360 to 0.812)	−0.317 (−1.896 to 1.030)
Household Income ($60-90K)	0.171 (−0.288 to 0.632)	0.504 (−0.107 to 1.115)	−0.032 (−0.403 to 0.338)	−0.159 (−0.459 to 0.451)
Household income ($90–$120,000)	0.309 (−0.110 to 0.730)	0.660† (0.102 to 1.218)	0.102 (−0.235 to 0.440)	−0.003 (−0.459 to 0.451)
Gestational weight gain (kg)	−0.005 (−0.039 to 0.028)	0.005 (−0.039 to 0.050)	−0.003 (−0.030 to 0.023)	−0.002 (−0.038 to 0.034)
Gestational age (weeks)	−0.020 (−0.116 to 0.076)	−0.077 (−0.205 to 0.051)	−0.041 (−0.119 to 0.036)	−0.088 (−0.193 to 0.016)
Infant Sex (females)	0.125 (−0.156 to 0.407)	0.032 (−0.341 to 0.406)	0.052 (−0.174 to 0.279)	0.139 (−0.166 to 0.445)
Maternal Dietary Quality Score (HEI 2015)	0.023‡ (0.006 to 0.040)	0.024† (0.002 to 0.046)	0.018‡ (0.004 to 0.031)	0.020† (0.001 to 0.038)
Postpartum weight loss at 1 month	−0.016 (0.073 to 0.040)	−0.050 (−0.126 to 0.026)	−0.023 (−0.069 to 0.022)	−0.007 (−0.069 to 0.054)

BMI = body mass index; GDM = gestational diabetes mellitus; miRNA = microRNA.

* Each row gives the respective β (95% CI) values for the regression model, where each miRNA is a dependent variable and each of the other variables, such as GDM, prepregnancy BMI, maternal age, and diet quality, is a predictor variable. Models were controled for maternal prepregnancy BMI, maternal age, maternal race, household income, gestational weight gain, maternal dietary quality index score (Health Eating Index), maternal postpartum weight loss at 1 month, gestational age, and infant sex.

† $P < 0.05$.

‡ $P < 0.01$.

month were observed again at 6 months of age. With every fold increase in miRNA-148a, infant weight, length, FFM, and total lean mass decreased by 0.5 kg ($P = 0.007$), 1.7 cm ($P = 0.0003$), 0.35 kg ($P = 0.022$), and 0.4 kg ($P = 0.018$), respectively (Table IV), at 6 months.

DISCUSSION

Our findings indicate that exposure to diabetes during pregnancy is associated with altered abundance of HM miRNAs known to be involved in glucose homeostasis and adipogenesis, and these miRNAs are also associated with infant weight and adiposity in the first 6 months of life. The impact of GDM on HM

Table IV. Associations between 1-month breast milk exosomal miRNA abundance and infant outcomes at 1, 3 and 6 months of age.*

miRNA	β (95% CI)						
	Weight	Length	WAZ	WLZ	Body fat	Fat mass	Fat-free mass
1-Month outcomes† (n = 84 all infants)							
miRNA-148a	−0.315§ (−0.560 to 0.069)	−0.347 (−1.372 to 0.676)	−0.588§ (−1.125 to 0.049)	−0.007 (−1.186 to 0.440)	−3.600§ (−7.115 to 0.083)	−0.222§ (−0.415 to 0.029)	−0.105 (−0.238 to 0.027)
miRNA-30b	0.253§ (0.086, 0.419)	0.420 (−0.336 to 1.176)	0.382§ (0.017 to 0.746)	−0.066 (−0.283 to 0.817)	2.960 (0.582 to 5.336)	0.178§ (0.047 to 0.308)	0.087 (−0.002 to 0.177)
miRNA-let-7a	0.080 (−0.108 to 0.270)	0.654 (−0.194 to 1.503)	0.129 (−0.286 to 0.544)	0.030 (−0.793 to 0.461)	1.39 (−1.294 to 4.082)	0.063 (−0.083 to 0.211)	0.011 (−0.090 to 0.112)
miRNA-let-7d	−0.098 (−0.304 to 0.107)	−0.545 (−1.470 to 0.380)	−0.020 (−0.472 to 0.430)	0.028 (−0.685 to 0.678)	−1.37 (−4.302 to 1.555)	−0.058 (−0.218 to 0.102)	−0.027 (−0.138 to 0.083)
3-Month outcomes† (n = 76)							
miRNA-148a	−0.027 (−0.392 to 0.336)	−0.621 (−1.777 to 0.535)	−0.262 (−0.571 to 0.390)	−0.230 (−0.646 to 0.674)	0.387 (−2.542 to 3.317)	0.013 (−0.227 to 0.253)	−0.050 (−0.280 to 0.179)
miRNA-30b	−0.158(−0.403 to 0.086)	−0.014 (−0.852 to 0.823)	0.132 (−0.455 to 0.175)	0.075 (−0.538 to 0.328)	−1.338 (−3.303 to 0.625)	−0.108 (0.270 to 0.052)	−0.061 (−0.214 to 0.091)
miRNA-let-7a	−0.126 (−0.400 to 0.148)	0.751 (−0.229 to 1.144)	−0.151 (−0.554 to 0.175)	−0.537 (−1.016 to 0.000)	−3.038‖ (−5.235 to 0.840)	−0.214§ (−0.392 to 0.035)	0.100 (−0.073 to 0.274)
miR-let-7d	0.295 (0.010 to 0.580)	−0.125 (−1.144 to 0.893)	0.085 (−0.053 to 0.694)	0.345 (−0.006 to 1.042)	2.426§ (0.137 to 4.715)	0.211§ (0.025 to 0.397)	0.082 (−0.098 to 0.264)
6-Month outcomes‡ (n = 74)							
miRNA-148a	−0.489‖ (−0.841 to 0.136)	−1.669‖ (−2.557 to 0.780)	−0.466 (−1.337 to 0.144)	−0.244 (−0.969 to 0.399)	−0.527 (−2.452 to 1.398)	−0.195 (−0.451 to 0.061)	−0.350§ (−0.651 to 0.050)
miRNA-30b	0.039 (−0.207 to 0.286)	0.871 (0.246 to 1.495)	−0.001 (−0.393 to 0.451)	−0.222 (−0.727 to 0.239)	−0.865 (−2.284 to 0.552)	−0.060 (−0.249 to 0.127)	0.157 (−0.064 to 0.379)
miRNA-let-7a	0.114 (−0.228 to 0.457)	0.620 (−0.262 to 1.504)	−0.094 (−0.595 to 0.573)	−0.592 (−1.164 to 0.176)	1.007 (−0.867 to 2.883)	0.134 (0.113 to 0.382)	−0.037 (−0.329 to 0.254)
miRNA-let-7d	0.246 (−0.058 to 0.552)	0.293 (−0.474 to 1.061)	0.364 (−0.047 to 0.983)	0.652 (0.082 to 1.251)	-0.394 (−2.029 to 1.241)	0.293 (−0.219 to 0.215)	0.135 (−0.123 to 0.386)

miRNA = microRNA; WAZ = weight for age *z* score; WLZ = weight for length *z* score.

*Each row gives the respective β (95% CI) values for the regression model, where infant outcomes are dependent variables and the miRNA fold changes are independent variables. Models were controled for maternal prepregnancy body mass index, maternal age, infant sex, gestational age, birth weight, and birth length.

†The 1- and 3-month body compositions were assessed using air displacement plethysmography.

‡The 6-month body composition was assessed using dual energy x-ray absorptiometry.

§$P < 0.05$.

‖$P < 0.01$.

miRNA abundance persists even after adjusting for a variety of potential confounders, such as maternal age, prepregnancy BMI, gestational weight gain, nutrition, gestational age, and infant sex. We also observed that higher maternal diet quality was associated with increased abundance of each of the measured miRNAs.

The literature regarding the effects of maternal diabetes on HM miRNA abundance is sparse. Xi et al[31] examined relationships between gestational metabolic diseases (GDM and gestational hypertension) and HM miRNAs (miRNA-let-7a, miRNA-30b, and miRNA-378) involved in adipogenesis. Contrary to our findings, both miRNA-let-7a and miRNA-378 were increased in gestational metabolic diseases. However, after maternal prepregnancy BMI was controlled for, the relationship was no longer statistically significant, suggesting that the difference in miRNA abundance was related to obesity rather than gestational metabolic disease. The inclusion of mothers with gestational hypertension in the gestational metabolic disease group may account for the difference because women with chronic disease beyond GDM were excluded from the present study.

Accumulating evidence suggests the systemic uptake of milk exosomes and their miRNAs in both animal models[32,33] and healthy human volunteers.[34] Milk exosomes and their miRNAs are absorbed in the systemic circulation in the infants and may impact epigenetic programming of metabolically active tissues, such as liver, pancreatic islets, and beige, brown, and white adipose tissues, in the early neonatal period and infancy.[35] miRNA-148a-3p is the most abundant miRNA in human, bovine, and porcine milk.[36–39] In the present analysis, the abundance of miRNA-148a was lower in HM of mothers with GDM during pregnancy, a new finding. Furthermore, lower abundance of miRNA-148a was associated with higher infant weight, WAZ, and percentage of BF at 1 month of life. Evidence indicates that infants born to mothers with GDM have increased adiposity after controlling for adiposity at birth, despite good maternal glycemic control during the pregnancy and predominant breastfeeding during early infancy.[40] In addition to exposure to excess nutrients that may alter hypothalamic sensing of satiety, leading to alterations in appetite, lower miRNA-148a levels in HM of mothers with GDM might be 1 mechanism for the increased adiposity and weight gain observed in previous studies of infants exposed to diabetes in utero. We observed greater miRNA-148a abundance in mothers with higher diet quality. Thus, if miRNA-148a abundance can be increased in HM from mothers with GDM by improved diet, this might be a strategy to mitigate the increased risk of obesity and T2DM in infants born to mothers with GDM. miRNA-30b in GDM HM was lower in abundance and positively associated with infant weight and FM at 1 month of age. Overexpression of miRNA-30b stimulates adipogenesis, increases the size of lipid droplets, and upregulates lipogenic genes.[41] These findings might explain the positive association of miRNA-30b with infant weight and FM at 1 month of life. An important finding is the opposite directionality of miRNA-148a and miRNA-30b with infant weight and FM at 1 month of age. It is possible that the abundance of these 2 miRNAs in HM indicate the fine-tuning by the miRNAs in HM to modulate early infant growth.

Brown adipose tissue (BAT) and beige adipose tissue (BET) play a major role in energy expenditure, and impaired BAT function is associated with obesity and metabolic disorders.[42] miRNA-30b/c is the main positive regulator of BAT and BET adipogenesis.[42] miRNAs expressed in milk-derived exosomes may have an important developmental effect on the differentiation of adipose tissue, as hypothesized recently by Melnik et al.[35] The protective role of breastfeeding in prevention of early obesity may be related to consumption of HM miRNA-30b.[43] The increase of miRNA-30b–associated FM may be related to increased BAT, which would be a favorable effect. The ratio and kinetics of postnatal miRNA-148a/miRNA-30b/c expression may tune the type and amount of adipose tissue development and thus development of obesity later in life.

Another important finding of our study is the positive association of maternal diet quality with the abundance of each of the measured miRNAs. Higher maternal diet quality during pregnancy and early lactation is associated with lower weight for length, percentage of BF, and FM in breastfed infants.[24] Current infant formulas are deficient in miRNAs,[36,44] which may have negative effects on long-term metabolic health in the infant.[45] Furthermore, formula-fed infants have more rapid weight gain out of proportion to linear growth than do breastfed infants. Formulas supplemented with particular exosomal miRNAs found to influence infant adiposity, such as

miRNA-148a and miRNA-30b, might be a way to mitigate future obesity risk. Improved diet quality might lead to increased abundance of these miRNAs in HM, decreasing adiposity among infants born to mothers with GDM.

Our study has several notable strengths, including a relatively large cohort of well-characterized lactating mothers. We used controlled and consistent sampling protocols for HM collection. Furthermore, we were able to account for a range of demographic, clinical, and lifestyle factors that may confound the association of miRNAs and offspring growth and/or body composition. Although ours is the first and 1 of the largest human studies to examine the impact of maternal GDM on specific miRNAs in HM, we acknowledge a few limitations. The first limitation is our inability to characterize miRNAs from other milk fractions, including cells and the lipid fraction of HM. However, HM exosomes contain the largest proportion of total HM miRNAs, and their miRNAs are the most bioavailable to the infant. Second, we used a targeted approach, selecting highly abundant miRNAs involved in important relevant metabolic pathways. This approach might miss other differentially abundant miRNAs in HM exosomes altered by maternal GDM but protects the study from type I error (incorrect rejection of the null hypothesis) caused by multiple comparisons. Larger studies are needed to assess the hundreds of known exosomal miRNAs. Third, we measured the abundance of miRNAs in a single HM sample at a single point in time. The extent to which a single milk sample may represent the mean output from a given individual women is unknown. Recent work by Raymond et al[46] found a dynamic change in HM miRNA composition from the second week to third month post partum. Fourth, we used 2 different methods to measure body composition: Pea Pod at 1 and 3 months and dual x-ray energy absorptiometry at 6 months of age. However, both of these methods are standard and validated to measure infant body composition.[47] Fifth, we measured infant body composition using Pea Pod and dual x-ray energy absorptiometry, which does not allow us to distinguish among the quantities of white adipose tissue, BAT, and BET. Although this is beyond the scope of the present study, this approach will be considered as part of our future studies. Furthermore, we do not have information on the maternal intake of $\omega 3$ fatty acids, which may have an impact on offspring body composition in childhood.[48] Lastly, this is an observational cohort study with strong confounder adjustment, but nonetheless we cannot establish causality of the reported associations.

CONCLUSIONS

In summary, we found that the abundance of select miRNAs is reduced in HM of mothers with GDM early in lactation, and these miRNAs are associated with infant weight and adiposity from 1 to 6 months of age. miRNA-148a was associated with decreased infant weight and adiposity, and miRNA-30b was associated with increased infant weight and adiposity at 1 month of life, suggesting fine-tuning among different miRNAs to regulate early infant weight and body composition. Furthermore, we observed that better maternal diet quality was associated with increased abundance of each of the measured miRNAs. Increased risk of obesity and T2DM in infants born to mothers with GDM might be mitigated by improving the miRNA content of the milk from these mothers through improved diet quality or by supplementing infant formulas with these biologically active components. Caution should be exercised because this is an association; therefore, causality may not be inferred. Hence, randomized trials are required to understand the role of diet and maternal clinical factors on HM miRNA abundance and to define the functional role of HM exosomal miRNAs in infant growth.

DISCLOSURE

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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APPENDIX

Supplemental Table S1. miRNA Sequences

miRNA	TaqMan Assay ID	Mature Sequence
hsa-miR-148a-3p	000470	UCAGUGCACUACAGAACUUUGU
hsa-miR-30b-5p	000602	UGUAAACAUCCUACACUCAGCU
hsa-let-7a-5p	000377	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7d-5p	002283	AGAGGUAGUAGGUUGCAUAGUU

Supplemental Table S2. Stepwise Regression Model

miRNA	Stepwise Final Model	β	p value
FC miRNA-148a	FC.148a ~ Group + mat age + mat diet	-0.310	**0.037***
FC miRNA-30b	FC.30b ~ Group + mat age + PPWL1 + mat educ cat + income cat + mat diet	-0.466	**0.026***
FC miRNA-let-7a	FC.let.7a ~ Group + mat BMI + mat age + PPWL1 + mat educ cat + mat race + mat diet	-0.356	**0.006****
FC miRNA-let-7d	FC.let.7d ~ Group + mat age + GA + mat race + mat diet	-0.527	**0.006****

β-estimate for the stepwise regression model
*__=p <0.05, **=p<0.01;__ significant values are shown in bold
FC-Fold change
Mat-Maternal
BMI-Maternal pre-pregnancy body mass index
PPWL1-post partum weight loss at 1 month of lactation
Edu cat-Education category
GA-Gestational age