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BRIEF REPORT

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Maternal glucose levels and late pregnancy circulating extracellular vesicle and particle miRNAs in the MADRES pregnancy cohort

Elizabeth C. Anderson [a], Helen B. Foley[b], Joshua J. Levy[c,d], Megan E. Romano[a], Jiang Gui[e], Jessica L. Bentz[f], Luis E. Maldonado[b], Shohreh F. Farzan[b], Theresa M. Bastain[b], Carmen J. Marsit[g], Carrie V. Breton[b], and Caitlin G. Howe[a]

[a]Department of Epidemiology, Geisel School of Medicine at Dartmouth, Lebanon, NH, USA; [b]Division of Environmental Health, Department of Population and Public Health Sciences, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA; [c]Emerging Diagnostic and Investigative Technologies, Department of Pathology and Laboratory Medicine, Dartmouth Hitchcock Medical Center, Lebanon, NH, USA; [d]Department of Pathology and Laboratory Medicine and the Department of Computational Biomedicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA; [e]Department of Biomedical Data Science, Geisel School of Medicine at Dartmouth, Hanover, NH, USA; [f]Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA; [g]Gangarosa Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, GA, USA

ABSTRACT

Maternal hyperglycemia during pregnancy adversely affects maternal and child outcomes. While mechanisms are not fully understood, maternal circulating miRNAs may play a role. We examined whether continuous glucose levels and hyperglycemia subtypes (gestational diabetes, type 2 diabetes, and glucose intolerance) were associated with circulating miRNAs during late pregnancy. Seven miRNAs (hsa-miR-107, hsa-let-7b-5p, hsa-miR-126-3p, hsa-miR-181a-5p, hsa-miR-374a-5p, hsa-miR-382-5p, and hsa-miR-337-5p) were associated ($p < 0.05$) with either hyperglycemia or continuous glucose levels prior to multiple testing correction. These miRNAs target genes involved in pathways relevant to maternal and child health, including insulin signaling, placental development, energy balance, and appetite regulation.

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Introduction

Hyperglycemia during pregnancy impacts the health of both mother and child. [1–3] Although these outcomes have primarily been investigated in the context of overt diabetes (i.e., preexisting and gestational diabetes), linear relationships between glucose levels and adverse health outcomes have been reported even within the subclinical range. [2] The mechanisms by which hyperglycemia during pregnancy elicit these adverse outcomes are currently unknown. However, a growing body of research supports a possible role of microRNAs (miRNAs).

MiRNAs are short, non-coding RNAs that post-transcriptionally regulate gene expression. [1,4] MiRNAs can be released into circulation and transported by extracellular vesicles and particles (EVPs), which protect them from degradation, and influence gene expression in both proximal and distant target cells. During pregnancy, the placenta is a major source of EVP miRNAs in maternal circulation. These miRNAs influence maternal-offspring communication, regulate placental function and growth, and hold promise as potential early and minimally invasive biomarkers of placental health. [1]

Growing evidence suggests that maternal circulating EVP miRNAs, including placenta-specific miRNAs from the Chromosome 14 and 19 miRNA clusters (C19MC and C14MC) are sensitive to hyperglycemia during pregnancy. [1] Of note, these miRNAs have been implicated in the dysregulation of glucose metabolism, insulin signaling and resistance and increased inflammation. [1] However, prior studies have focused on gestational diabetes mellitus (GDM). Thus, whether these miRNAs are similarly sensitive to glucose levels in the subclinical range is unknown.

CONTACT Elizabeth C. Anderson Elizabeth.c.anderson.gr@dartmouth.edu Department of Epidemiology, Geisel School of Medicine at Dartmouth, 1 Medical Center Drive, Lebanon, NH 03756, USA

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The objective of the current study was to determine if hyperglycemia, both in the clinical and subclinical range, influences late pregnancy maternal circulating EVP miRNAs. Given prior findings for overt diabetes, we hypothesized that miRNAs involved in glucose homeostasis, insulin signaling, inflammation, and placental function would be sensitive to higher maternal glucose levels during pregnancy.

Methods

The Maternal And Developmental Risks from Environmental and Social Stressors study (MADRES), initiated in 2015, is a longitudinal pregnancy cohort of >1,000 mother-child pairs. [5] Participants were recruited from prenatal providers that primarily care for medically underserved populations in Los Angeles, California, and are therefore predominantly low-income and Hispanic.

In the U.S., pregnant individuals without type 1 or type 2 diabetes are typically screened for GDM through a glucose challenge test (GCT) at 24–28 weeks gestation. [6] In MADRES, continuous glucose measures from the GCT were abstracted from maternal electronic medical records (EMRs). If a participant had more than one GCT measure recorded for the index pregnancy (50% of participants), the first measure was used. In MADRES, the median (IQR) gestational age of the first GCT measure was 10 (8–16) weeks. As described previously, MADRES participants were classified as having the following hyperglycemia subtypes: type 2 diabetes, GDM, glucose intolerance, or normal glucose using information from maternal EMRs. [4] All type 2 diabetes classifications and the majority (90%) of GDM classifications were based on a physician diagnosis, while 10% of GDM classifications and all glucose intolerance and normal glucose classifications were based on laboratory measures. Participants classified as having normal glucose served as the reference group.

Methods for extracting and profiling EVP miRNAs have been described previously. [4] Briefly, during the third trimester (median (IQR): 32 (30–33) weeks of gestation), peripheral blood samples were collected from participants instructed to fast for 10 h beforehand. Samples were transported on ice to the laboratory. Upon receipt, samples were fractionated, and plasma aliquots were stored at −80°C. EVP RNA was extracted from 500 µl of plasma using the Qiagen ExoRNeasy kit. While this kit enriches extracellular vesicles, it is important to note that it may co-isolate other small particles that carry miRNAs, such as lipoproteins. [7] We therefore use the term extracellular vesicles and particles ('EVPs'). MiRNAs were quantified using a BioAnalyzer small RNA kit (Agilent Technologies, Inc. USA). A total of 798 human miRNAs were profiled by the University of Southern California Genomics Core using the NanoString nCounter Human v3 miRNA expression assay (NanoString Technologies, Inc.) as described previously. [4] Normalization methods and quality control assessment of resulting miRNA counts are described in Figure S1. We did not observe evidence of hemolysis for any of the included samples based on levels of nine miRNAs identified as reliable biomarkers of hemolysis during pregnancy. [8] We used ComBat (R package 'sva') to correct for batch. [9] Primary outcomes included 90 miRNAs exceeding sample-specific detection thresholds for ≥60% of participants. Normalized and batch-corrected counts were log-transformed and modeled continuously in statistical analyses. Secondary outcomes included all C19MC and C14MC miRNAs that were above sample-specific detection thresholds for the limit of detection in 20–60% of participants, which were modeled as binary variables (detectable vs. not detectable).

Potential confounders were identified using a Directed Acyclic Graph (DAG) based on *a priori* evidence from published literature (Figure S2). Information on infant sex assigned at birth and pre-pregnancy BMI was abstracted from maternal EMRs. Maternal pre-pregnancy BMI was determined using self-reported weight and measured height. Maternal prenatal vitamin use, perceived stress, and depression were determined by questionnaires administered during pregnancy in either Spanish or English depending on the preferred language. Psychosocial stress and depression were measured using the Perceived Stress Scale (PSS) and Center for Epidemiological Studies – Depression (CES-D) questionnaires, respectively.

Although prenatal vitamin use was identified as a potential confounder, all participants in the analytic sample reported using prenatal vitamins during pregnancy, so this variable was excluded from statistical models. Smoking was also identified as a potential confounder. However, only a small number (n = 7) of participants reported smoking during pregnancy. We therefore did not include this variable in primary models but conducted a sensitivity analysis in which results were compared including versus excluding these participants (Table S3). In this cohort, the CES-D and PSS measures are collinear. Therefore, the PSS was selected as a covariate for primary models, and the CES-D measure was assessed separately as a covariate in sensitivity analyses (Table S3). In addition to the potential confounders and precision variables identified by the DAG, two variables related to the MADRES study design were included in all models: recruitment site and the timing of enrollment (<20 weeks versus 20–30 weeks gestation). The final covariates for primary models included maternal age, PSS score, infant sex, pre-pregnancy BMI, recruitment site, and timing of enrollment. In sensitivity analyses, results were compared after adjustment for a number of additional variables not included in primary models (Table S3).

Statistical analyses were conducted in R (v4.2.2). [10] Separate covariate-adjusted robust linear regression models were used to assess associations between (1) continuous glucose and (2) each hyperglycemia subtype and continuous levels of each widely detected miRNA (Figure S3). C19MC and C14MC miRNAs that were detectable for 20–60% of participants were modeled as binary variables using logistic regression. Secondary analyses investigated potential interactions with infant sex, maternal pre-pregnancy BMI, gestational age at GCT, and the time between GCT and gestational age at miRNA assessment using cross-product terms. P-values were adjusted for multiple testing using the Benjamini & Hochberg FDR approach.

Experimentally validated genes for all miRNAs associated with measures of glucose and hyperglycemia at a $p < 0.05$ level were identified using miRTargetLink (v 2.0). [11] For target genes classified as having 'strong' experimental validation, over- or underrepresentation ($P_{FDR} < 0.05$) in PANTHER pathways was determined using EnrichR. [12]

Results

Participant characteristics for the analytic subset ($n = 328$) are shown in Table S1 and separately by hyperglycemia subtype in Table S2. The median (IQR) maternal age was 28.5 (23.9–33.0) y, and the median (IQR) pre-pregnancy BMI was 27.8 (24.6–32.0) kg/m [2]. Most participants (75%) enrolled before 20 weeks gestation. In the analytic subset, 76 participants (23.2%) were classified as having glucose intolerance, 30 participants (9.1%) were classified as having GDM, and six participants (1.8%) were classified as having type 2 diabetes. More than half (57%) of participants had a high-school education or less. Demographics of the analytic subset were similar to those of the full cohort (Table S1). On average, participants with hyperglycemia were older and had a higher BMI and an earlier GCT than those without hyperglycemia.

In primary analyses, continuous glucose was inversely associated with hsa-miR-107 (Table 1). When each hyperglycemia subtype was compared to the reference group, glucose intolerance was inversely associated with one miRNA (hsa-let-7b-5p), and type 2 diabetes was inversely associated with three miRNAs (hsa-miR-126-3p, hsa-miR-181a-5p, and hsa-miR-374a-5p). Type 2 diabetes was also inversely associated with two of the placenta-specific miRNAs that did not meet the detection threshold for primary analyses (hsa-miR-382-5p and hsa-miR-337-5p). No statistically significant associations were identified between GDM and the miRNAs evaluated. None of the associations were statistically significant after multiple testing correction.

Pathway analyses were conducted separately for miRNAs associated with continuous glucose versus any hyperglycemia (Figure 1). For both sets of analyses, predicted target genes were overrepresented in the CCKR signaling map ST pathway, angiogenesis, the p53 pathway, and the VEGF signaling pathway ($P_{FDR} < 0.05$).

Table 1. Associations between glucose measures or hyperglycemia subtypes and circulating EVP miRNAs in late pregnancy.

Predictor	Outcome	Primary or Secondary Outcome*	Effect Estimate (95% Confidence Interval)	P-value	FDR Adjusted P-value
Continuous Glucose					
Glucose, mg/dL	hsa-miR-107	Primary	−0.12 (−0.22, −0.01)	0.03	0.98
*Hyperglycemia Subtypes***					
Glucose Intolerance	hsa-let-7b-5p	Primary	−0.08 (−0.15, 0.00)	0.04	1.00
Type 2 Diabetes	hsa-miR-126-3p	Primary	−0.40 (−0.75, −0.06)	0.02	1.00
Type 2 Diabetes	hsa-miR-181a-5p	Primary	−0.41 (−0.77, −0.05)	0.03	1.00
Type 2 Diabetes	hsa-miR-374a-5p	Primary	−0.30 (−0.59, −0.02)	0.04	1.00
Type 2 Diabetes	hsa-miR-382-5p	Secondary	−0.43 (−0.14, −0.13)	0.04	0.56
Type 2 Diabetes	hsa-miR-337-5p	Secondary	−0.41 (−0.06, 0.19)	0.04	0.56

*The primary outcome analysis examined the $n = 90$ widely detected circulating miRNAs in late pregnancy. Secondary outcomes included the $n = 26$ placenta-specific miRNAs that were not widely detectable but exceeded the limit of detection for 20–60% of participants.

**The reference group for the hyperglycemia subtypes analysis are the participants with normal glucose levels.

Effect estimates and p-values are from robust linear regression models for the widely detected analysis and logistic regression for the placenta-specific analysis which adjust for the following covariates: maternal age, PSS score, infant sex, maternal pre-pregnancy BMI, recruitment site, and timing of enrollment. The effect estimate for the continuous glucose analysis was scaled by 100. The interpretation is therefore the difference in log counts for the specified miRNA for a 100 mg/dL increase in glucose.

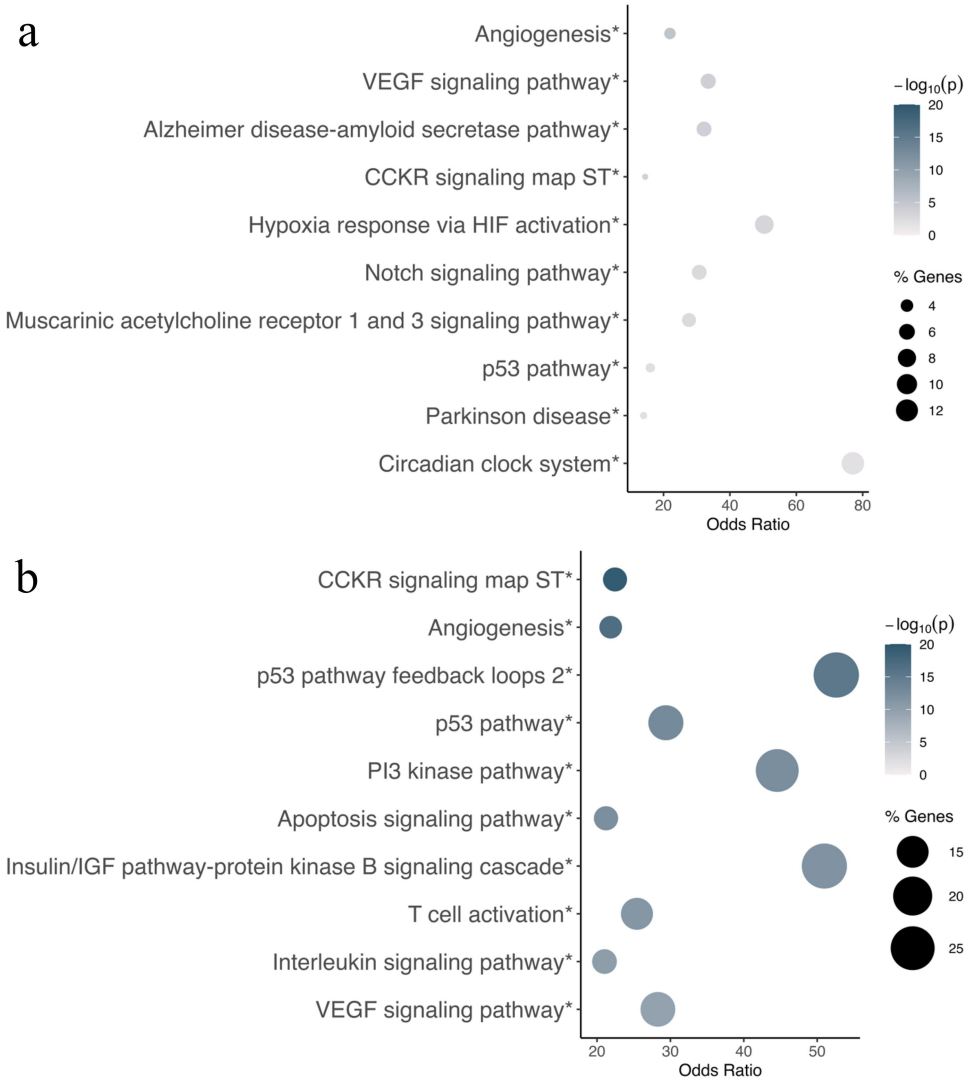

Figure 1. Bubble plots showing the top 10 PANTHER pathways for continuous glucose (a) and hyperglycemia subtypes (b). The color of each bubble shows the -log10(P-value) from Fisher's exact tests. As the color gets darker, the p-value gets smaller. The size of each bubble represents the percentage of genes in each pathway that are identified for the relevant EVP miRnas.

In secondary analyses, we did not observe statistically significant interactions for infant sex, maternal BMI, gestational age at GCT, or the gestational duration between the GCT and miRNA assessment ($P_{FDR} \geq 0.05$ level). Results from sensitivity analyses were generally consistent with results from primary models (Table S3). However, when participants with type 2 diabetes and GDM were excluded, the association between continuous glucose and hsa-miR-107 became stronger, and when participants using metformin or insulin during pregnancy were excluded, associations between type 2 diabetes and hsa-miR-126-3p and hsa-miR-181a-5p became stronger, while its association with miR-374a-5p somewhat attenuated (Table S3).

Discussion

In this predominantly lower-income Hispanic population in Los Angeles, we identified miRNAs in maternal circulating EVPs during late pregnancy that may be sensitive to maternal glucose levels. Predicted target genes of these miRNAs are involved in pathways relevant to glucose metabolism, energy balance, appetite regulation, and angiogenesis.

Notably, the six miRNAs (hsa-let-7b-5p, hsa-miR-126-3p, hsa-miR-181a-5p, hsa-miR-374a-5p, hsa-miR-337-5p, and hsa-miR-382-5p) that were associated with a hyperglycemia subtype have all been associated with diabetes in other populations, although these prior studies focused on non-pregnant populations. [13–15] Experimentally validated target genes of these miRNAs were enriched in pathways relevant to diabetes, placental and fetal development, and regulation of energy balance and appetite, such as the insulin/IGF pathway-protein kinase B signaling cascade and the CCKR signaling map pathways, which are highly relevant to adverse maternal and child outcomes that have been associated with hyperglycemia during pregnancy. [13–15]

Additionally, we found that participants with higher continuous glucose levels had lower levels of hsa-miR-107 which is involved in insulin resistance and is upregulated among non-pregnant individuals with type 2 diabetes. [16] We have previously reported a positive association between maternal circulating hsa-miR-107 and gestational duration in MADRES. [4] Interestingly, this miRNA was not associated with either type 2 diabetes or GDM, and the association for continuous glucose remained after excluding participants with diabetes. One possibility is that distinct biological pathways are affected by overt hyperglycemia versus elevated glucose within the normal range, which occurs in healthy pregnancies to support fetal growth. One of the pathways targeted by hsa-miR-107 (Notch signaling) is not targeted by any of the miRNAs that were associated with the hyperglycemia subtypes. This pathway is upregulated in response to high glucose levels and is involved in placental development. [17,18]

This study had several limitations that are important to acknowledge. First, because the GCT is conducted in a non-fasting state, results may have been influenced by recent diet. However, any measurement error was likely non-differential, which would have biased results toward the null. While it is possible that some of the results for type 2 diabetes may be explained by changes in lifestyle or medication use made by participants after an early pregnancy diagnosis, the majority of associations were found to be independent of physical activity level, diet quality, or diabetes-related medication use in late pregnancy. Although the NanoString nCounter platform measures the majority of high-confidence human miRNAs annotated by miRBase, it is possible that other miRNAs not covered on this platform are sensitive to glucose. Additionally, glucose may differentially affect miRNAs carried by different EVP subtypes, which we were unable to assess in the current study. Major strengths of the study include the prospective design, the large sample size, the inclusion of participants with type 2 diabetes in addition to GDM, the large number of miRNAs evaluated, and the assessment of glucose in the subclinical range, which has not been widely investigated in relation to circulating miRNAs.

In conclusion, we identified seven miRNAs in maternal circulating EVPs in late pregnancy that may be sensitive to higher glucose levels, including one miRNA (hsa-miR-107) that was associated with glucose continuously, including in the subclinical range, and one miRNA (hsa-let-7b-5p) that was higher among participants who did not have overt diabetes but were classified as having possible glucose intolerance. Although these associations were not

statistically significant after multiple testing correction ($P_{FDR} < 0.05$), results from pathway analyses and concordance with prior literature indicate that these findings may have important implications for both maternal and child health and merit further investigation.

Ethics approval and consent to participate

Written informed consent and HIPAA authorization to obtain medical records from each participant were obtained at study entry for each participant and their child. The protocol was approved by the University of Southern California's Institutional Review Board.

Data availability statement

Raw NanoString miRNA data for this research were deposited in the NCBI Gene Expression Omnibus and are publicly available at the accession number GSE168788 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168788)

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

ECA, CGH, and CVB originated the analysis conception and design. JJL, JLG, MER, and JLB provided feedback on the analysis plan. ECA completed the analysis with supervision from CGH. All the authors approved the final analysis plan. ECA wrote the manuscript with CGH. All authors provided feedback on and approved the final manuscript. CVB, TMB, CJM, CGH, and SFF acquired funding support, and CVB, TMB, SFF, LEM, and HBF generated data for the analysis.

Abbreviations

EVP	Extracellular vesicles and particles
GCT	Glucose challenge test
OGTT	Oral glucose tolerance test
GDM	Gestational diabetes mellitus
MADRES	Maternal And Developmental Risks from Environmental and Social Stressors Study
CES-D	Center for Epidemiologic Studies – Depression
PSS	Perceived Stress Scale
C19MC	Chromosome 19 microRNA Cluster
C14MC	Chromosome 14 microRNA Cluster

ORCID

Elizabeth C. Anderson http://orcid.org/0000-0002-1050-5470

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