



Phthalate Exposures and MicroRNA Expression in Uterine Fibroids: The FORGE Study

Ami R Zota¹, Ruth J Geller¹, Brianna N VanNoy¹ ,
Cherie Q Marfori² , Sana Tabbara³, Lisa Y Hu⁴,
Andrea A Baccarelli⁴ and Gaby N Moawad²

¹Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, Washington, DC, USA. ²Department of Obstetrics & Gynecology, The George Washington University, Washington, DC, USA. ³Department of Pathology, The George Washington University, Washington, DC, USA. ⁴Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA.

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ABSTRACT: Phthalates are associated with multiple, adverse reproductive outcomes including increased risk of uterine leiomyoma (fibroids). Phthalates can interact with epigenetic modifications including microRNAs (miRNAs), which help regulate processes crucial to fibroid pathogenesis. However, no prior study has examined the influence of phthalates on miRNA expression in fibroid tumors. We conducted a preliminary, cross-sectional study to examine the associations between phthalate exposures and miRNA expression levels in fibroid tumors and to explore potential effect modification by race/ethnicity. We quantified expression levels of 754 miRNAs in fibroid tumor samples and analyzed spot urine samples for phthalate metabolites collected from 45 pre-menopausal women undergoing surgery for fibroid treatment at an academic hospital. Associations between miRNA levels in fibroids and phthalate biomarkers were evaluated using linear regression adjusting for age, race/ethnicity, and body mass index (BMI). Statistical tests were adjusted for multiple comparisons. We also performed *in silico* Ingenuity Pathway Analysis to identify the biological pathways that are regulated by phthalate-associated miRNAs. Mono-hydroxybutyl phthalate and mono(2-ethyl-5-hydroxyhexyl) phthalate were positively associated with miR-10a-5p ($\beta=0.76$, 95% CI = [0.40, 1.11]) and miR-577 ($\beta=1.06$, 95% CI = [0.53, 1.59]), respectively. A total of 8 phthalate-miRNA associations varied by race/ethnicity ($q_{\text{interaction}} < 0.10$). Pathway analysis revealed that mRNA gene targets of phthalate-associated miRNAs were significantly associated with multiple fibroid-related processes including angiogenesis, apoptosis, and proliferation of connective tissues. Collectively, these data suggest that exposures to some phthalates are associated with miRNA in fibroids, and that associations may vary by race/ethnicity. Validation of these findings may provide insight into mechanisms underlying associations between phthalates and fibroids and contribute to novel hypotheses regarding racial/ethnic disparities in fibroids.

KEYWORDS: Endocrine-disrupting chemicals, epigenetics, gynecology, health disparities, non-coding RNAs, women's health

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CORRESPONDING AUTHOR: Ami R Zota, Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, 950 New Hampshire Avenue NW, Washington, DC 20052, USA. Email: azota@gwu.edu

Introduction

Uterine leiomyoma (fibroids)—smooth muscle tumors that develop during reproductive years in most women—are a devastating disorder for millions of women who experience symptoms (eg, heavy bleeding and pelvic pain) and/or reproductive dysfunction (eg, infertility).^{1,2} Fibroids are more common and more severe in U.S. Black women.^{3,4} Surgery remains the mainstay of fibroid treatment.⁵

Fibroid biology remains poorly understood, which has hampered progress in identifying risk factors, prognosis, and non-invasive treatments. While the exact mechanisms of fibroid pathobiology are unclear, several cellular activities have been implicated in fibroid formation and growth. Abnormal myometrial and fibroid stem cells exhibit an exaggerated response to estrogen and progesterone, stimulating processes such as cell proliferation, inhibition of apoptosis, and extracellular matrix (ECM) formation.⁶ As with other

types of tumors, these processes may contribute to cellular transformation, fibroid tumor initiation and growth.⁶ For example, large quantities of ECM (secreted by fibroblasts) separate the smooth muscle cells, contributing to tumor expansion, solid-state signaling, and the characteristic “stiffness” of fibroid tissue.^{6,7} Growth factors (eg, fibroblast growth factor 2 [FGF2] and vascular endothelial growth factor [VEGF]) embedded within the ECM may also regulate fibroid formation.^{7,8} Additional studies suggest that fibroids exhibit both pro-angiogenic and anti-angiogenic properties, since fibroids have reduced blood flow and altered vasculature compared with normal myometrium.^{9,10}

MicroRNAs (miRNAs) have the potential to accelerate prevention and treatment efforts since these epigenetic alterations help regulate mechanisms important to fibroid development. MicroRNAs are small (<22 nucleotides), non-coding RNA molecules that regulate post-transcriptional gene



expression through gene silencing.¹¹ Approximately 50 to 100 miRNAs are differently expressed in fibroids compared with myometrium in patient-matched, surgical specimens.^{12–15} Predicted gene targets of these miRNAs modulate processes that may play a significant role in fibroid pathobiology including cell proliferation, apoptosis, and ECM turnover.¹⁶ In vitro studies have validated the functional role of several miRNAs that are dysregulated in vivo.^{17–19}

MicroRNA regulation may also be involved in biological pathways linking environmental chemicals to fibroid pathogenesis. Our research group recently reported that higher exposure to certain phthalates, such as di(2-ethylhexyl) phthalate (DEHP), is associated with greater uterine volume, a measure of fibroid severity, among women with symptomatic fibroids.²⁰ Phthalates are a class of multi-functional chemicals commonly used in consumer products such as personal care products, home furnishings, and food contact materials.²¹ Phthalates can migrate out of products and enter the human body through ingestion, inhalation, or dermal application. Once inside the body, phthalates are rapidly metabolized and excreted into urine.²² Urinary concentrations of phthalate metabolites are common biomarkers of exposure²² and are detected in nearly all reproductive-aged women.^{21,23} Certain phthalates are reproductive toxicants in animal models^{24,25} and are also associated with adverse reproductive outcomes in humans.^{26–28} While the relationship between phthalates and miRNA expression in fibroids is currently unknown, phthalate exposures are associated with miRNA expression in extracellular vesicles within follicular fluid²⁹ and the placenta.³⁰

The objective of our preliminary study is to examine the association between phthalate exposures and miRNA expression in fibroid tumors among women undergoing surgical treatment for fibroids. Our secondary objective is to examine whether associations between phthalate exposure and miRNA expression vary by race/ethnicity as prior studies suggest racial/ethnic differences in both phthalate exposures^{31,32} and fibroid miRNA expression.^{13,33}

Methods

Study population

In 2014–2017, we recruited women into the Fibroids, Observational Research on Genes and the Environment (FORGE) study who were seeking care for symptomatic fibroids at the George Washington University (GW) Medical Faculty Associates and intending to undergo surgical management at GW Hospital, an urban academic hospital that serves the Washington, DC metropolitan area. Eligible women were non-pregnant, pre-menopausal, English-speaking, and ≥ 18 years of age. The sample for our current study is limited to women with phthalate metabolite and miRNA data (N=45). The GW Institutional Review Board approved the study. All participants provided written informed consent prior to enrollment.

Clinical and demographic data

We abstracted data from patients' medical records on race/ethnicity, age, body mass index (BMI), last menstrual period, and insurance type.

Phthalate exposure assessment

Sample collection and analytical methods have been previously described²⁰ and are briefly summarized below. We collected spot urine samples from participants in sterile polypropylene cups during a pre-operative appointment. For 1 participant, urine was collected after surgery. Each urine sample was analyzed for specific gravity (SG) using a handheld refractometer (Atago Co., Ltd) and then stored in polypropylene cryovials at -80°C . The Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) quantified 14 phthalate metabolites by online solid-phase extraction high-performance liquid chromatography isotope dilution tandem mass spectrometry.³⁴ This analysis includes the 13 metabolites detected in $>50\%$ of the population. The names of the phthalate metabolites, their parent compounds, and detection frequencies are presented in Table 1. Common sources of parent phthalate compounds are provided in Supplemental Table S1. Biomarker concentrations were adjusted for urine dilution using the following formula: (phthalate biomarker concentration) \times $([1.017 - 1] / [SG - 1])$, where 1.017 is the median specific gravity in this study sample and SG is the specific gravity of the individual's urine sample.³⁵ Biomarker concentrations below the limit of detection (LOD) were replaced with the LOD divided by the square root of 2 prior to SG adjustment.³⁶ In addition to examining individual metabolites, we also calculated 2 summary measures using previously described methods: the molar sum of DEHP metabolites (ΣDEHP)²¹ and a potency-weighted sum of anti-androgenic phthalate metabolites ($\Sigma\text{AA phthalates}$).³¹

Fibroid and myometrium tissue collection

Fibroid tissue was collected during hysterectomy or myomectomy procedures. For patients with multiple fibroids, we sampled the largest fibroid. Fibroid tissue samples were cut approximately 1 cm in thickness, and large fibroids were sampled 1 to 2 cm away from the peripheral zone. Samples were collected within 1 hour of surgery and snap frozen in liquid nitrogen until analysis. Pathology reports were reviewed to confirm that tumor samples were non-cancerous and actually represented leiomyomatous tissues. Among the subset of participants who underwent hysterectomy (N = 19), myometrium was also collected.

Expression analysis of miRNAs

The Laboratory of Precision Environmental Health at the Columbia University Mailman School of Public Health performed sample processing and RNA isolation. Snap-frozen tissue samples were incubated in RNAlater-ICE Frozen Tissue

Table 1. Descriptive statistics of specific gravity–adjusted phthalate biomarker concentrations in urine (N=45).

PARENT COMPOUND: PHTHALATE METABOLITE	% >LOD	GM (GSD) (NG/ML)		
		OVERALL	BLACK (N=28)	WHITE/LATINA (N=17)
Diethyl phthalate (DEP)				
Monoethyl phthalate (MEP)	100	108.15 (6.86)	205.53 (5.10) ^a	37.56 (7.04) ^a
Di- <i>n</i> -butyl phthalate (DnBP)				
Mono- <i>n</i> -butyl phthalate (MnBP)	96	8.65 (2.66)	9.07 (3.04)	8.00 (2.09)
Mono-hydroxybutyl phthalate (MHBP)	73	1.06 (1.94)	1.04 (2.00)	1.08 (1.89)
Diisobutyl phthalate (DiBP)				
Monoisobutyl phthalate (MiBP)	96	7.17 (2.26)	8.22 (2.30)	5.72 (2.12)
Mono-hydroxyisobutyl phthalate (MHiBP)	89	2.50 (2.18)	2.45 (2.24)	2.59 (2.12)
Butylbenzyl phthalate (BBzP)				
Monobenzyl phthalate (MBzP)	93	2.87 (3.13)	3.42 (3.72)	2.16 (2.07)
Di- <i>n</i> -octyl phthalate (DnOP)				
Mono(3-carboxypropyl) phthalate (MCPP)	76	1.49 (3.35)	1.29 (3.17)	1.88 (3.64)
Diisononyl phthalate (DiNP)				
Monocarboxyoctyl phthalate (MCOP)	98	13.13 (4.78)	12.16 (4.87)	14.89 (4.83)
Diisodecyl phthalate (DiDP)				
Monocarboxynonyl phthalate (MCNP)	100	1.96 (2.83)	2.16 (3.12)	1.66 (2.37)
Di(2-ethylhexyl) phthalate (DEHP)				
Mono(2-ethylhexyl) phthalate (MEHP)	62	1.78 (2.30)	1.77 (2.15)	1.80 (2.61)
Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	98	6.39 (2.49)	6.63 (2.58)	6.01 (2.39)
Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)	98	4.11 (2.65)	4.30 (2.88)	3.82 (2.32)
Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)	100	10.46 (2.31)	10.81 (2.45)	9.91 (2.14)
ΣDEHP	–	23.39 (2.29)	24.38 (2.37)	21.84 (2.22)
ΣAnti-androgenic (AA) phthalates	–	45.88 (2.40)	50.55 (2.46)	39.11 (2.32)

Abbreviations: GM, geometric mean; GSD, geometric standard deviation; LOD, limit of detection.

^a*P* < .05 from t-test by race/ethnicity.

Transition Solution (Thermo Fisher) according to the manufacturer's protocol. Tissues were homogenized using a TissueRuptor (Qiagen). Then, total RNA was isolated using miRCURY RNA Isolation Tissue Kit (Exiqon). RNA was quantified on a NanoPhotometer Pearl (Implen), and quality was verified with a Nano Assay run on a Bioanalyzer 2100 (Agilent Technologies).

All RNA samples were processed with RNA Clean & Concentrator Kits (Zymo Research). An aliquot of 100 ng of concentrated RNA from each sample was screened for 754 miRNAs and 4 internal controls (ath-miR159a, RNU48, RNU44, and U6) using TaqMan OpenArray (Thermo Fisher). QuantStudio 12K Flex is a fixed-content panel

containing validated human TaqMan assays derived from Sanger miRbase release v.14. All 754 assays have been functionally validated with miRNA artificial templates. Samples were first reverse transcribed to cDNA using Megaplex RT Primers Pools A and B. The cDNA products were pre-amplified with Megaplex PreAmp Primers, Human Pools A and B, according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies). Relative quantification cycle values (*C_q*) were defined as the amplification cycle at which the fluorescence levels for each of the miRNAs exceed the background fluorescence threshold.

Statistical analysis

We calculated descriptive statistics for demographic and environmental variables. SG-adjusted phthalate biomarkers were natural log-transformed prior to statistical analysis. Comparisons of phthalate biomarkers by race/ethnicity were performed using t-tests.

To extract miRNA data, we used Thermo Fisher Cloud Relative Quantification software. We performed quality control on raw qPCR data according to the following criteria, suggested by the manufacturer: (a) amplification scores ≥ 1.1 and (b) C_q confidence ≥ 0.8 . Data that did not meet these criteria were set to missing. C_q values < 40 were considered unexpressed. Further analyses were limited to 388 miRNAs detected in $\geq 80\%$ of fibroid samples. We used the global mean method to normalize miRNA expression data using the following formula³⁷: $\Delta C_{q,miRNAi} = C_{q,miRNAi} - C_{q,miRNAi_global_mean}$. Non-detected values were coded as 40 in the calculation. Normalization was conducted separately for miRNAs in fibroid and myometrium.

We tested differences in miRNA expression between participant-matched samples of fibroid and myometrium using paired t-tests. This analysis was limited to 377 miRNAs that were detected in $\geq 80\%$ of both fibroid and myometrium samples. We calculated the fold change in fibroid compared with myometrium miRNA expression using the $2^{-\Delta\Delta C_t}$ formula.³⁸ We further compared differences in expression of 388 miRNAs in fibroids by race/ethnicity using t-tests.

Next, we evaluated associations between phthalate biomarkers and miRNA expression in fibroids with multivariable linear regression models in which we modeled phthalate exposures as log-transformed continuous variables. All models were adjusted a priori for age (continuous), BMI (continuous), and race/ethnicity (Black vs White/Latina). White and Latina women were collapsed into 1 category since there was only 1 Latina participant.

We conducted 2 additional sets of analyses on the 285 phthalate biomarkers and miRNA combinations with significant associations ($P < .05$) in adjusted models (described above). First, we tested these associations using miRNA expression in myometrium adjusting for age, race/ethnicity, and BMI. Next, we evaluated for potential effect modification of phthalate-miRNA associations by race/ethnicity. We conducted linear regression models of fibroid miRNA expression and phthalate biomarkers adjusted for age and BMI with an interaction term for phthalate biomarker and race/ethnicity as well as the main effects of phthalate biomarker and race/ethnicity.

To control the false discovery rate (FDR) in multiple testing, we applied the Benjamini-Hochberg procedure to the number of miRNAs tested within each of the individual phthalate metabolites.³⁹ We did not apply FDR adjustment to the number of phthalate biomarkers because phthalate metabolites show moderate-to-high correlation with each other²⁰ and the conventional Benjamini-Hochberg approach assumes that all

hypotheses are independent.³⁹ Data analysis was performed in R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria). For multiple comparison adjustment, we chose a less conservative threshold of q-value < 0.10 due to our modest sample size. MicroRNA names reflect mature sequence names from miRbase release 22.

MicroRNA targets and biological network analysis

We used the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) to identify miRNA targets and explore downstream biological networks. MicroRNAs significantly associated with phthalate biomarkers (FDR $q < 0.10$) were included. We used the miRNA Target Filter tool to link messengerRNA (mRNA) targets to each miRNA from TarBase, Target Scan, miRecords, and Ingenuity Expert Findings. We filtered the pathway and network analyses using the experimentally observed confidence level. Because IPA does not identify pathways specific to fibroids, we searched for pathways known a priori to be associated with fibroid pathogenesis.^{7,16} The statistical significance of biological disease and function pathways was calculated using the Fisher exact test with an alpha of 0.05.

Results

Most women were Black (62%), overweight or obese (76%), privately insured (64%), and undergoing a myomectomy (58%). Compared with White/Latina women, Black women were more likely to be obese, publicly insured, and having a hysterectomy. Fibroid characteristics did not differ by race/ethnicity (Table 2). Phthalate exposures were ubiquitous; 9 phthalate metabolites were detected in $> 90\%$ of participants. Monoethyl phthalate (MEP) concentrations were significantly higher in Black women (Table 1).

In total, 35 miRNAs were under-expressed and 39 miRNAs were over-expressed in fibroid compared with myometrium (FDR $q < 0.10$) (Table 3). Expressions of the following miRNAs were ≥ 3 fold greater in myometrium: miR-10a-5p, miR-10a-3p, miR-140-3p, miR-144-5p, miR-150-5p, miR-205-5p, miR-27a-5p, miR-29b-2-5p, miR-29c-5p, miR-451a, and miR-95-3p. Conversely, expressions of these miRNAs were ≥ 3 fold greater in fibroid: miR-135a-5p, miR-135b-5p, miR-137-3p, miR-302b-3p, miR-335-3p, miR-34a-5p, miR-34a-3p, miR-34b-5p, miR-34c-5p, miR-483-5p, miR-488-3p, miR-488-5p, miR-508-3p, miR-577, miR-592, miR-651-5p, miR-885-5p, and miR-9-3p.

In adjusted models, there were 285 significant associations between phthalate biomarkers and miRNAs ($P < .05$) (Supplemental Table S2), and 34 of these associations were significant at $P < .005$ (Table 4). After adjusting for multiple testing, we found 2 miRNAs associated with phthalate biomarkers. A 1-unit increase in the natural-log concentrations of mono-hydroxybutyl phthalate (MHBP) was associated with a 0.76 (95% CI = [0.40, 1.11]) ΔC_q increase in miR-10a-5p

Table 2. Demographic and clinical characteristics of FORGE participants by race/ethnicity.

CHARACTERISTICS	BLACK/AFRICAN AMERICAN (N=28)	WHITE OR LATINA ^a (N=17)	TOTAL (N=45)
	N (COLUMN PERCENTAGE) OR MEDIAN (IQR)		
Age (years)	39 (33-46)	40 (36-43)	39 (36-46)
BMI (kg/m ²)	30.4 (26.6-40.7)	24.8 (21.7-27.1)	28.5 (25.1-35.5)
Private insurance	17 (61%)	12 (71%)	29 (64%)
Surgery type			
Myomectomy	12 (43%)	14 (82%)	26 (58%)
Hysterectomy	16 (57%)	3 (18%)	19 (42%)
No. of fibroids	4 (2-6)	3 (1-7)	4 (2-6)
Size of largest fibroid	7.3 (5.9-11.2)	8.3 (3.5-11.0)	7.7 (5.8-11.0)
Uterine volume	664 (239-1228)	606 (327-799)	646 (243-1082)

Abbreviations: FORGE, Fibroids, Observational Research on Genes and the Environment study; BMI, body mass index (kg/m²); IQR, interquartile range.

^aOne participant self-identified as Latina.

expression (FDR $q = 0.05$), where a higher ΔC_q indicates lower relative expression. The association of mono(2-ethyl-5-hydroxyhexyl phthalate (MEHHP) and miR-577 ($\beta = 1.06$, 95% CI = [0.53, 1.59]) was marginally significant (FDR $q = 0.10$). Phthalate biomarkers associated with miRNA targets in fibroids were not associated with these targets in myometrium (no association had FDR $q < 0.10$; data not shown).

After multiple testing adjustment, 8 phthalate-miRNA associations varied significantly between White/Latina and Black women (interaction term FDR $q < 0.10$) (Figure 1). Among Black women, there was an association between monobenzyl phthalate (MBzP) and miR-494-3p. Among white/Latina women, there were associations between: mono(3-carboxypropyl phthalate) (MCPP) and miR-337-5p; MBzP and miR-1227-3p; MEP and miR-645; MEP and miR-564; MEP and miR-374-5p; mono(2-ethylhexyl phthalate (MEHP) and miR-128-3p; and MEHP and miR-337-3p. There were no significant differences in miRNA expression by race/ethnicity after multiple testing adjustment (data not shown).

A total of 10 miRNAs that were significantly associated with phthalate biomarkers either in main analysis (miR-10a-5p, miR-577) or in certain racial/ethnic groups (miR-494-3p, miR-337-5p, miR-1227-3p, miR-645, miR-564, miR-374a-5p, miR-128-3p, miR-337-3p) were selected for downstream target prediction and functional enrichment analysis. We identified 923 mRNA targets that were experimentally observed or highly predicted targets of the 10 miRNAs. Inclusion of only experimentally observed mRNA targets yielded 26 mRNA targets for 3 miRNAs (miR-10a-5p, miR-128-3p, miR-494-3p). These mRNA targets were significantly associated with multiple fibroid-related processes including angiogenesis, apoptosis,

differentiation of muscle cells, proliferation of connective tissues, and cell viability (Table 5, Supplemental Figure S1). They were also associated with relevant disease pathways such as tumorigenesis of the reproductive tract ($P = 2.52 \times 10^{-9}$), smooth muscle tumors ($P = 4.70 \times 10^{-5}$), and differentiation of muscle cells ($P = 5.00 \times 10^{-15}$).

Discussion

In this cross-sectional study of pre-menopausal women seeking surgery for their symptomatic fibroids, we found that biomarkers of certain phthalates were associated with miRNA expression in fibroid tumors, but not in the myometrium. Furthermore, some of the associations between phthalate biomarkers and miRNAs in fibroids varied by race/ethnicity. These associations persisted even after adjustment for multiple comparisons.

Regulation of miRNAs varied between fibroid and myometrium. There were no associations between phthalate biomarkers and miRNAs in myometrium, and we found significant differences between patient-matched samples of fibroid and myometrium in expression of 74 miRNAs. Consistent with other studies, we observed that miR-10a, miR-150, miR-29b, miR-29c, and miR-451 are under-expressed and miR-34a is over-expressed in fibroids.^{12,13,15,33,40,41} (These names may not reflect current miR-Base v22 names.) These reproducible associations have emerged across studies despite the use of different technologies for miRNA quantification. While the functional significance of aberrant miRNA expression is an area of ongoing research, several studies have demonstrated that downregulation of the miR-29 family contributes to ECM formation in fibroid cells.^{17,18}

MiR-10a-5p expression was associated with concentrations of MHBP, an oxidative metabolite of DnBP (di-*n*-butyl phthalate), which is found in some personal care products (eg,

Table 3. MiRNAs showing differential expression in fibroid versus myometrium (N= 19).

UNDER-EXPRESSED		OVER-EXPRESSED	
TARGET	FOLD CHANGE	TARGET	FOLD CHANGE
let-7b-5p	-1.9	miR-1201	2.0
miR-10a-5p	-3.7	miR-130b-3p	1.5
miR-10a-3p	-3.4	miR-135a-5p	13.1
miR-1247-5p	-2.8	miR-135b-5p	17.2
miR-126-3p	-2.5	miR-137-3p	4.0
miR-126-5p	-2.0	miR-181a-2-3p	2.1
miR-139-3p	-1.7	miR-184	2.3
miR-139-5p	-2.9	miR-301b-3p	1.7
miR-140-5p	-2.1	miR-302b-3p	4.7
miR-140-3p	-3.0	miR-323-3p	2.0
miR-144-5p	-3.4	miR-335-5p	2.6
miR-149-5p	-1.9	miR-335-3p	3.9
miR-150-5p	-3.4	miR-337-3p	1.8
miR-185-5p	-1.5	miR-34a-5p	3.8
miR-193a-5p	-2.8	miR-34a-3p	3.7
miR-203a-3p	-2.2	miR-34b-3p	1.9
miR-205-5p	-3.3	miR-34b-5p	3.0
miR-22-3p	-1.7	miR-34c-5p	3.8
miR-27a-5p	-3.0	miR-369-5p	1.9
miR-29a-3p	-2.5	miR-378a-5p	1.8
miR-29b-3p	-2.7	miR-380-5p	1.7
miR-29b-2-5p	-3.3	miR-381-3p	2.0
miR-29c-3p	-2.1	miR-409-5p	2.1
miR-29c-5p	-3.2	miR-411-3p	1.6
miR-30a-3p	-1.8	miR-455-5p	2.2
miR-30e-3p	-1.7	miR-483-5p	3.5
miR-324-3p	-1.6	miR-488-3p	3.2
miR-338-3p	-2.4	miR-488-5p	3.6
miR-451a	-3.7	miR-499a-5p	1.7
miR-452-5p	-1.8	miR-503-5p	2.0
miR-511-5p	-2.2	miR-508-3p	7.3
miR-616-5p	-1.4	miR-548c-5p	1.7
miR-628-3p	-2.6	miR-551b-3p	2.9
miR-652-3p	-1.8	miR-577	5.5
miR-95-3p	-3.2	miR-592	8.8
		miR-651-5p	6.9
		miR-885-5p	3.8
		miR-889-3p	2.0
		miR-9-3p	6.0

Abbreviation: miRNAs, microRNAs.

All differences are FDR $q < 0.10$ after multiple comparison adjustment. The negative inverse fold change values are shown for targets under-expressed in fibroid tissue (-1/fold change).

Table 4. Association between phthalate biomarkers and miRNAs in fibroid tissue.^a

PHTHALATE	MIRNA NAME	ESTIMATE ^b	95% CI	P-VALUE	FDR Q-VALUE ^c
MEP	miR-429	-0.60	[-0.93, -0.27]	.0006	0.24
	miR-200b-3p	-0.46	[-0.77, -0.16]	.004	0.53
	miR-548c-5p	-0.29	[-0.47, -0.10]	.004	0.53
MnBP	miR-338-5p	1.55	[0.53, 2.58]	.004	0.73
MHBP	miR-10a-5p	0.76	[0.40, 1.11]	.0001	0.05
	miR-30a-3p	0.65	[0.25, 1.06]	.002	0.40
	miR-142-5p	0.70	[0.24, 1.15]	.004	0.40
	miR-142-3p	0.75	[0.25, 1.25]	.004	0.40
MHiBP	miR-10a-5p	0.58	[0.23, 0.93]	.002	0.61
MCPP	miR-154-5p	1.13	[0.53, 1.72]	.0004	0.17
	miR-7-5p	0.54	[0.19, 0.90]	.004	0.54
MCOP	miR-21-3p	-0.29	[-0.47, -0.12]	.002	0.35
	miR-582-3p	-0.28	[-0.45, -0.11]	.002	0.35
	miR-449b-5p	0.37	[0.13, 0.62]	.004	0.46
MCNP	miR-590-3p	0.70	[0.27, 1.14]	.002	0.87
MEHP	miR-635	0.70	[0.27, 1.14]	.002	0.67
MEHHP	miR-577	1.06	[0.53, 1.59]	.0003	0.10
	miR-1179	0.88	[0.36, 1.40]	.002	0.27
	miR-7-2-3p	0.61	[0.25, 0.98]	.002	0.23
	miR-192-3p	0.48	[0.18, 0.78]	.003	0.28
	miR-7-5p	0.65	[0.22, 1.09]	.004	0.32
MEOHP	miR-1179	0.85	[0.37, 1.33]	.0009	0.27
	miR-577	0.88	[0.36, 1.40]	.002	0.27
	miR-572	-0.50	[-0.81, -0.19]	.002	0.27
	miR-26b-3p	0.47	[0.16, 0.77]	.004	0.29
	miR-7-5p	0.62	[0.22, 1.03]	.004	0.29
MECPP	miR-577	1.01	[0.41, 1.62]	.002	0.61
	miR-93-5p	0.39	[0.13, 0.65]	.004	0.62
ΣDEHP	miR-577	1.13	[0.53, 1.72]	.0004	0.17
	miR-1179	0.92	[0.33, 1.51]	.003	0.42
ΣAA phthalates	miR-7-5p	0.80	[0.31, 1.29]	.002	0.35
	miR-7-2-3p	0.68	[0.26, 1.10]	.003	0.35
	miR-577	0.97	[0.36, 1.59]	.003	0.35
	miR-572	-0.56	[-0.93, -0.20]	.004	0.35

Abbreviations: AA, anti-androgenic; DEHP, di(2-ethylhexyl) phthalate; MCNP, monocarboxynonyl phthalate, MCOP, monocarboxyoctyl phthalate; MCPP, mono(3-carboxypropyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate; MHBP, mono-hydroxybutyl phthalate; miRNAs, microRNAs; MnBP, mono-*n*-butyl phthalate.

^aAssociation at $P < .005$ are shown. The full list of phthalate-miRNA associations significant at $P < .05$ are shown in Supplemental Table S2. All associations are adjusted for age, BMI, and race/ethnicity. Lower ΔC_q indicates higher relative miRNA expression.

^bFor every 1-unit increase in the natural-log phthalate metabolite concentration, there is an effect size increase/decrease in the miRNA ΔC_q .

^cFDR q-values are adjusted for multiple testing for the 388 miRNAs but not for the number of phthalate biomarkers.

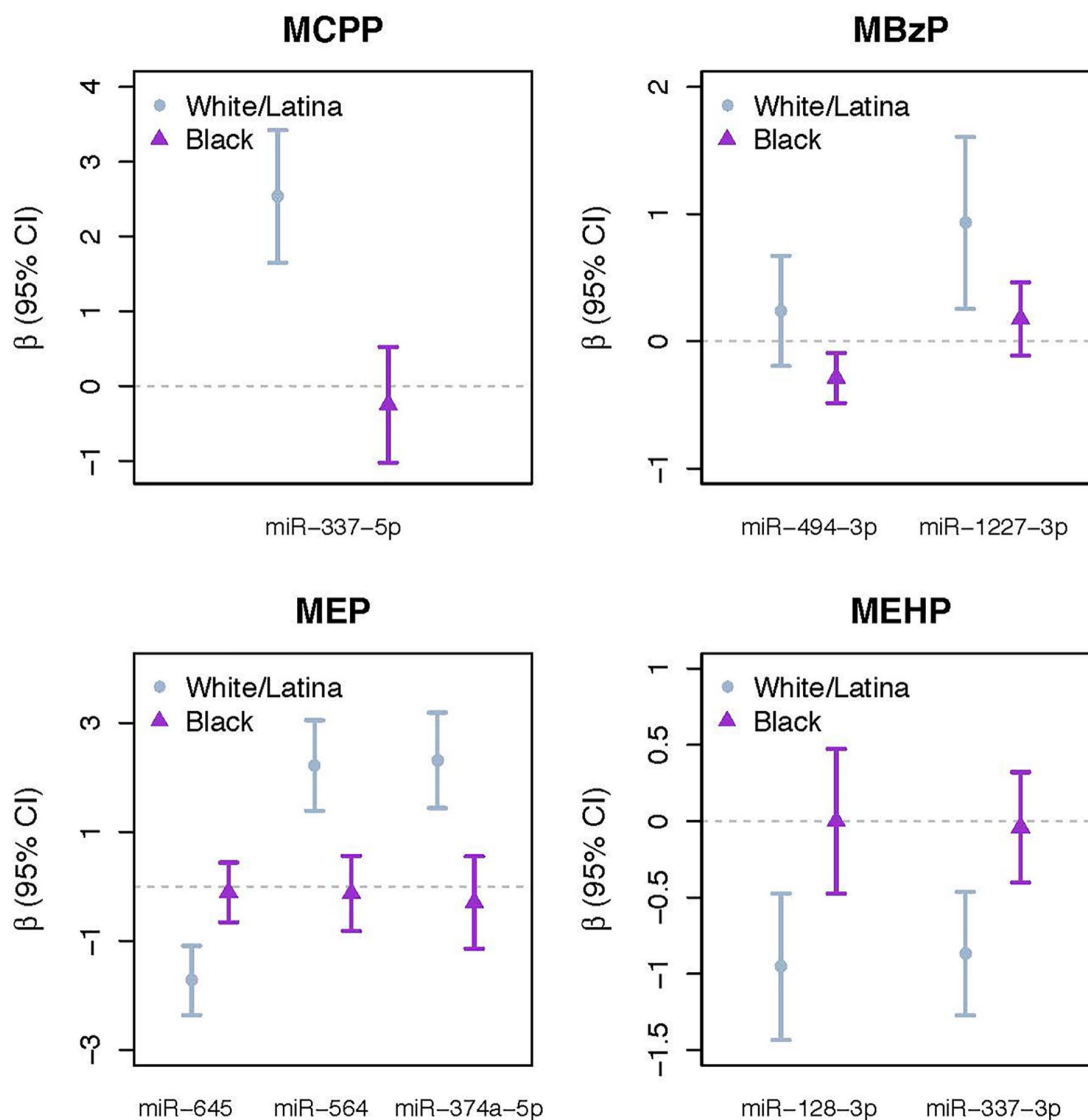


Figure 1. Association between phthalate biomarkers and miRNAs in fibroid tissue with significant effect measure modification by race/ethnicity (FDR-adjusted q value < 0.10 for race/ethnicity \times phthalate interaction term). Results are from models with an interaction term for phthalate biomarker and race/ethnicity, adjusted for age and BMI as well as including the main effects of phthalate biomarker (natural log-transformed, specific gravity-adjusted) and race/ethnicity. FDR q -values are adjusted for multiple testing for number of miRNAs but not for the number of phthalate biomarkers. Lower ΔC_q signifies higher miRNA expression. BMI indicates body mass index; FDR, false discovery rate; miRNAs, microRNAs.

nail polish) and certain medications (see Supplemental Table S1). Urinary concentrations of DnBP metabolites in this study population were generally similar to those measured from a national sample of U.S. women.⁴² DnBP is among the most potent anti-androgenic phthalates.²⁵ While DnBP metabolites were not associated with fibroid severity in our prior study, we did observe a positive association between the weighted sum of anti-androgenic phthalates, which includes MHBP and other DnBP metabolites, and greater uterine volume.²⁰

Our secondary analysis suggests that the influence of phthalate exposures on miRNA expression may vary by race/ethnicity. These results are not solely explained by biological differences between groups since we did not observe significant differences in miRNA expression by race/ethnicity. Our results are in contrast to 2 prior studies that found different miRNA

profiles in the fibroids of Black and White women.^{13,33} These discrepancies may partly be explained by methodological differences as only one of the prior studies adjusted for multiple comparisons.¹³ Similarly, these findings are not entirely explained by racial/ethnic differences in phthalate exposures since we only observed meaningful differences by race/ethnicity for 1 phthalate metabolite. Our findings may indicate that the effect of phthalates on miRNA regulation may vary by the growth status of the fibroid. Peddada et al⁴³ found that growth rates for fibroids were much higher for older (>35 years) Black women than for older (>35 years) White women, and most of our study population was >35 years of age. Alternatively, these findings may indicate that the epigenome is sensitive to interactions between chemical and non-chemical stressors. Race/ethnicity in the United States is predominately a social

Table 5. miRNA-mRNA gene targets and significant biological disease and function pathways related to fibroid pathogenesis.

BIOLOGICAL PATHWAYS	MIRNA NAME	MRNA GENE TARGET	P-VALUE
<i>Functions</i>			
Cell viability	miR-128-3p, miR-494-3p, miR-10a-5p	BMI1, E2F3, HMOX1, KIT, KLF4, NF1, NTRK3, PTEN, SNAP25, TGFBR1, TXNIP, WEE1	5.38E-06
Cell survival	miR-128-3p, miR-494-3p, miR-10a-5p	ADORA2B, BMI1, E2F3, HMOX1, KIT, KLF4, NF1, NTRK3, PTEN, SNAP25, TGFBR1, TXNIP, WEE1	1.46E-06
Cell cycle progression	miR-128-3p, miR-494-3p, miR-10a-5p	ADORA2B, BMI1, DBI, E2F3, FGF16, HMOX1, KIT, KLF4, KMT2A, NF1, NTRK3, PTEN, TGFBR1, WEE1	2.60E-10
Apoptosis	miR-128-3p, miR-494-3p, miR-10a-5p	ADORA2B, AGO1, BMI1, E2F3, HMOX1, HOXA1, KIT, KLF4, KMT2A, LDLR, NF1, NTRK3, PTEN, TGFBR1, TXNIP, VSNL1, WEE1	1.65E-06
Angiogenesis	miR-128-3p, miR-494-3p, miR-10a-5p	ADORA2B, AGO1, HMOX1, HOXA1, HOXD10, KIT, KLF4, KMT2A, NF1, PTEN, TGFBR1	2.88E-07
Cell transformation	miR-128-3p, miR-494-3p, miR-10a-5p	BMI1, KIT, KMT2A, NTRK3, PTEN, USF2	2.61E-05
Proliferation of connective tissue cells	miR-128-3p, miR-494-3p, miR-10a-5p	BMI1, HMOX1, KLF4, KMT2A, NF1, NTRK3, PTEN, TXNIP	5.35E-06
Development of connective tissue cells	miR-128-3p, miR-494-3p, miR-10a-5p	KIT, KLF4, LDLR, TGFBR1, TXNIP	4.06E-05
Proliferation of fibroblast cell lines	miR-128-3p, miR-494-3p	BMI1, E2F3, HMOX1, KIT, KMT2A, NTRK3, PTEN, TXNIP	1.65E-07
Proliferation of stem cells	miR-128-3p, miR-494-3p, miR-10a-5p	AGO1, BMI1, E2F3, HOXA1, KIT, NF1, PTEN, TGFBR1	3.90E-10
<i>Disease pathways</i>			
Tumorigenesis of reproductive tract	miR-128-3p, miR-494-3p, miR-10a-5p	AFF1, DCX, E2F3, FGF16, HOXA1, HOXD10, KIT, KLF4, KMT2A, LDLR, NF1, NTRK3, PTEN, RELN, SCN3A, TGFBR1, TXNIP, WEE1	2.52E-09
Benign tumors	miR-128-3p, miR-494-3p, miR-10a-5p	AFF1, DCX, HMOX1, KIT, KLF4, NF1, PTEN, RELN, WEE1	1.90E-06
Differentiation of muscle cells	miR-128-3p, miR-494-3p	LDLR, HMOX1	5.00E-15
Smooth muscle tumor	miR-128-3p, miR-494-3p, miR-10a-5p	DCX, KIT, NF1, PTEN, WEE1	4.70E-05
Proliferation of muscle cells	miR-128-3p, miR-494-3p, miR-10a-5p	E2F3, FGF16, HMOX1, KIT, KLF4, NF1, PTEN	2.38E-06

Abbreviations: ADORA2B, adenosine A2b receptor; AFF1, AF4/FMR2 family member 1; AGO1, argonaute RISC component 1; BMI1, BMI1 proto-oncogene, polycomb ring finger; DBI, diazepam binding inhibitor, acyl-CoA binding protein; DCX, doublecortin; E2F3, E2F transcription factor 3; FGF16, fibroblast growth factor 16; HOXA1, homeobox A1; HOXD10, homeobox D10; HMOX1, heme oxygenase 1; KIT, KIT proto-oncogene receptor tyrosine kinase; KMT2A, lysine methyltransferase 2A; KLF4, kruppel like factor 4; LDLR, low density lipoprotein receptor; NF1, neurofibromin 1; NTRK3, neurotrophic receptor tyrosine kinase 3; PTEN, phosphatase and tensin homolog; RELN, reelin; SCN3A, sodium voltage-gated channel alpha subunit 3; SNAP25, synaptosome associated protein 25; TGFBR1, transforming growth factor β receptor 1; TXNIP, thioredoxin interacting protein; USF2, upstream transcription factor 2, c-fos interaction; VSNL1, visinin like 1; WEE1, WEE1 G2 checkpoint kinase.

construct that influences a wide range of physical and social environmental exposures (eg, diet, socio-economic status, and psychosocial stress) due to residential segregation, racism, and cultural factors.^{32,44-46} Prior studies suggest that social adversity early in life can alter the biological response to environmental pollutants later in life.^{47,48} Future studies aimed at understanding racial/ethnic disparities in fibroid outcomes should consider the joint contribution of social and environmental exposures and their influence on the epigenome.

Our findings, in combination with prior experimental studies, begin to elucidate the mechanisms underlying phthalate toxicity in fibroids. We found that multiple miRNAs associated with phthalate exposures can interact with biological pathways important to fibroid pathogenesis. Moreover, several of the mRNA gene targets identified in our analysis (eg, PTEN, ADORA2B, KIT, E2F3) are associated with proteins that were over-expressed in phthalate-treated fibroid cells.⁴⁹⁻⁵² Kim and colleagues^{53,54} reported that exposing human fibroid

cells to DEHP enhanced cell proliferation, blocked apoptosis, and induced protein expression of HIF-1 α , COX-2, PCNA, and BLC2. It is plausible that the toxicity demonstrated in vitro is partly mediated through miRNA regulation. For example, PTEN is a validated target of miR-494-3p and a known tumor suppressor.^{55,56} Knockout of the PTEN gene can increase expression of COX-2,⁵⁰ which regulates proliferation of smooth muscle cells in fibroids.⁵⁷

While multiple prior studies have compared miRNA expression between fibroid and myometrium, to our knowledge, ours is the first to examine the associations between environmental chemical exposures and miRNA expression in human fibroid tissue. Our study is also novel in that we considered interactions between race/ethnicity and environmental chemical exposure. Another important strength is that we quantified the presence of a large number of miRNAs and corrected for multiple comparisons. Our study sample was diverse with respect to race/ethnicity, socio-economic status, and choice of surgical intervention, which may help account for the vast heterogeneity of this disorder.

As this is a preliminary study, there are important limitations. Our sample size was modest, which may lead to unstable or spurious results. Because all the study participants were undergoing surgery for symptomatic fibroids, these results may not be generalizable in women with asymptomatic fibroids. Our findings may be limited by residual confounding from other dietary or environmental factors. We only measured 1 tumor sample per participant so we could not examine within-individual variability in fibroid miRNA expression. We relied on a spot urine sample to estimate phthalate exposures, which may result in exposure misclassification because phthalates have a short half-life in the body.⁵⁸ In addition, our study was cross-sectional so we cannot infer causality. Future studies should prospectively assess the contribution of environmental exposures to changes in miRNA expression and development of clinically relevant outcomes through large epidemiologic studies. Finally, because most in silico programs do not have biological pathways mapped specifically for fibroids, previous studies have relied on cancer biology models to predict the biological impact of miRNAs on fibroid pathogenesis.¹⁶ Our analysis was limited to the examination of several biological pathways that we defined a priori based on this literature. While it is plausible that cellular mechanisms relevant to malignant tumor growth also apply to growth of benign tumors such as fibroids, further research is needed to validate the predicted pathways for fibroid-associated miRNA-gene targets.

In conclusion, our study suggests that regulation of miRNAs in fibroids may be influenced by exogenous factors, such as environmental chemicals. Additional multidisciplinary translational research, such as ours, that integrates population, clinical, and basic sciences has the potential to advance understanding of the biological mechanisms underlying fibroid etiology. This knowledge may help stimulate new hypotheses about drivers of racial/ethnic disparities as well as inform

prevention and treatment modalities for this complex disorder with a large public health impact.



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Author Contributions

ARZ conceived and designed the study, interpreted the data, and wrote the manuscript. RJG and BVN performed data analysis. AAB and LYH conducted miRNA analysis. CQM, AAB, ST, and GNM assisted in study design and interpretation of the results. All authors critically reviewed and approved the final manuscript.

ORCID iDs

Brianna N VanNoy  <https://orcid.org/0000-0002-0059-6268>
Cherie Q Marfori  <https://orcid.org/0000-0002-2158-723X>

Supplemental material

Supplemental material for this article is available online.

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