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Exercise and Weight Loss Interventions and miRNA expression in Women with Breast Cancer

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Abstract

Purpose—Obesity and weight gain are associated with comorbidities including a higher risk of tumor recurrence and cancer-related deaths among breast cancer(BC) survivors, however the underlying mechanisms linking obesity and cancer are poorly understood. Given the lack of clinically validated BC biomarkers, obesity and weight loss studies utilize serum biomarkers as intermediary outcomes of tumor recurrence. Studies have indicated microRNAs(miRNA)s are reliable biomarkers for cancer. We hypothesized that miRNA expression is correlated with obesity

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Clinical Trial: This study analyzed data obtained from clinical trials Hormones and Physical Exercise (HOPE) Study; NCT02056067; <https://clinicaltrials.gov/ct2/show/NCT02056067> and Lifestyle, Exercise and Nutrition Study 1 (LEAN); NCT02109068; <https://clinicaltrials.gov/ct2/show/NCT02109068>

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and weight loss amongst BC survivors. This would yield insight into the biological pathways through which this association occurs, enabling more precise development of therapeutics.

Patients and Methods—We correlated baseline body mass index(BMI) with serum miRNA expression in 121 BC survivors enrolled in the Hormones and Physical Exercise(HOPE) trial. We then analyzed expression of the 35 most abundant miRNAs from HOPE in a six-month randomized controlled weight loss trial(Lifestyle, Exercise, and Nutrition; LEAN) in 100 BC survivors. Ingenuity Pathway Analysis(IPA) software was used to identify biological pathway targets of the BMI-associated and intervention-responsive miRNAs using predictive biomarkers.

Results—Pearson correlations in HOPE identified eight miRNAs associated with BMI, including miR_191–5p($r=-0.22$, $p=0.016$) and miR_122–5p($r=0.25$, $p=0.0048$). In the LEAN validation study, levels of miR_191–5p significantly increased during the six-month intervention($p=0.082$). Ingenuity Pathway Analysis identified “Estrogen-mediated S-phase entry”(HOPE $p=0.003$; LEAN $p<0.001$) and “Molecular mechanisms of cancer”(HOPE $p=0.02$; LEAN $p<0.001$) as the top canonical pathways significantly correlated with BMI-associated and intervention-responsive miRNAs, and contain obesity and cancer relevant genes including the E2F family of transcription factors and CCND1, which have been implicated in sporadic BC.

Conclusion—While the association between obesity and BC recurrence and mortality has been demonstrated in the literature, mechanisms underlying the link between weight gain and cancer are unclear. Using two independent clinical trials we identified novel miRNAs associative to BMI and weight loss and contribute to the development of cancer. Predictive modeling of miRNA targets identified multiple canonical pathways associated with cancer, highlighting potential mechanisms explaining the link between BMI and cancer.

Keywords

microRNA; obesity; weight loss; diet; exercise; breast cancer

Introduction:

In the United States, over 200,000 women will be diagnosed with invasive BC annually and it is the second leading cause of cancer related-deaths in women^{1–3}. Only 10% of BC cases are due to a genetic predisposition, indicating that a number of environmental and anthropometric factors affect BC risk^{4–8}. Lifestyle factors associated with higher BC risk include poor diet, alcohol intake, and smoking^{9–11}. Studies consistently indicate post-menopausal women who are obese have an increased risk for breast-cancer specific mortality as compared to women with a normal BMI both prior to- and post-diagnosis^{12–14}. This increased risk is particularly concerning since obesity rates are increasing faster in post-menopausal women with cancer than amongst women without a history of cancer(3.01% vs 2.31 % increase annually within the past ten-years)¹⁵. Adult weight-gain is also associated with a 64% increased risk of BC death amongst survivors¹⁶. Elucidating the molecular factors associated with the rise in obesity will play an important role in developing biomarkers associated with obesity-related BC recurrence.

The proposed mechanisms through which obesity and weight change affect cancer risk and survival involves an abundance of metabolically active adipose tissue that induces elevated

levels of blood glucose, insulin, free estradiol, and inflammatory cytokines^{16–19}. The heightened levels of these cytokines support a hyper-proliferative state of the surrounding epithelium, and therefore a number of regulatory checkpoints are maintained to inhibit this process. miRNAs, small non-coding RNAs, are one such vehicle that regulates various cellular processes^{20–23}, and are highly dysregulated in chronic diseases, such as cancer^{24–29}. For instance, miR-21, miR-155, and miR-10b are all deregulated in BC tissue³⁰. Additionally, forced expression of these miRNAs *in vitro* promote cell invasion, growth, and pro-survival phenotypes, and target a number of tumor suppressor genes including *PDCD4*, *PTEN*, *SOC31*, and *HOXD10*. miRNAs are also expressed in the serum of BC patients, and function as reliable biomarkers for the disease³¹. Few studies have assessed how miRNAs regulate the cellular pathways controlling weight-gain and metabolic homeostasis. For instance, serum levels of miR-122 and miR-519d are strongly correlated with obesity^{32–34}, and pathway analysis indicates these miRNAs target regulators of survival and proliferation pathways important in BC tumorigenesis. Understanding the miRNAs that correlate to BMI and weight-loss interventions will provide a unique insight into the mechanisms underlying the link between these factors and BC recurrence.

In the present study, we tested the correlation between BMI and cancer-related miRNAs with obesity and a weight-loss intervention within two completed randomized control trials amongst BC survivors. First, we used baseline serum samples from BC survivors enrolled in the HOPE study³⁵ to identify miRNAs associated with BMI in this population; second, we utilized serum samples from the LEAN³⁶ trial to assess the effect of weight-loss intervention and change in miRNA expression over six-months comparing intervention and usual care study arms. We also tested miRNA expression in relation to body composition and serum biomarkers. We hypothesized that miRNAs and the pathways they regulate correlated with BMI in HOPE would change in response to weight-loss intervention in LEAN, offering insight into the mechanisms linking obesity to BC recurrence and perhaps identifying novel targets to improve survival outcomes in this patient population.

Methods:

Participants and Study Background—The methodologies of the HOPE and LEAN trials have been previously described^{35, 36}. In brief, 121 inactive, post-menopausal BC survivors with joint pain were recruited to the HOPE trial between 2010 and 2012 from hospitals in Connecticut through the Rapid Case Ascertainment Shared Resource of the Yale Cancer Center³⁵. Participants had a history of hormone receptor-positive stage I to III BC and received aromatase inhibitor(AI) adjuvant therapy for at least six-months. Assessments of HOPE data in this manuscript evaluated blood draws and anthropometric measurements at the baseline (pre-intervention) time-point.

The LEAN trial^{36, 37}, was a three-arm weight-loss randomized study involving 100 BC survivors comparing 1) in-person counseling 2) telephone counseling and 3) usual care. Participants included in the study were diagnosed with stage 0-III BC survivors, had a BMI $\geq 25.0\text{kg/m}^2$, and completed chemotherapeutic and/or radiation therapy (Figure 1). Since no significant weight-loss occurred between the in-person and telephone counseling arm, these arms were combined into an intervention group for our analysis, while arm three

remained the usual care group. LEAN weight-loss intervention strategies were adapted from US Dietary Guidelines as well as the American Institute for Cancer Research(AICR) nutritional and physical activity guidelines^{38–42}. The usual care group was described in Irwin et al³⁷. Assessments of LEAN data in this manuscript evaluated blood draws and anthropometric measurements at both baseline and at six-months post intervention.

For both HOPE and LEAN trials, institutional review board protocol was approved by the Yale School of Medicine Human Investigation Committee, and the Connecticut Department of Public Health Human Investigation Committee. Certain data used in this study were obtained from the Connecticut Tumor Registry located in the Connecticut Department of Public Health. The author(s) assume(s) full responsibility for analyses and interpretation of these data. The clinical characteristics of HOPE and LEAN trials are outlined in Table 1.

Primary and Secondary Measures—For participants enrolled in HOPE and LEAN, height, weight, and BMI (kg/m²) were determined as previously described^{35, 36}. In LEAN weight change was additionally calculated using the baseline and six-month weight measures. In HOPE, dual-energy x-ray absorptiometry scans were performed to assess body fat, lean body mass, and bone mineral density. For biomarker analysis, fasting blood draws were obtained, and serum was barcoded, aliquoted, and stored at –80C until assayed. Circulating insulin, leptin, glucose, adiponectin concentrations, as well as IL-6, TNF- α , and C-reactive protein(CRP) levels were also measured as previously described^{35–37, 43}. Sample specimens were assayed at study endpoint and measured in duplicate, with laboratory technicians blinded to treatment assignment. Coefficient of variation for samples was under 10%.

RNA Collection and Isolation—In brief, 150ul of fasting serum from patients enrolled in HOPE with BMIs of 18.5–44kg/m² were arrayed onto 96 well plates and assayed for miRNA expression using the Firefly platform⁴⁴. A total of 68 BC-specific miRNAs were assayed in multiplex on 121 samples arrayed onto two assay plates, with each plate containing control wells for post-hybridization analysis. Data was processed as described below. After assessment of HOPE, miRNA expression was then assessed from patients enrolled in LEAN. Specifically, 100 baseline samples and 85 6-month post intervention samples were analyzed for 35 robustly detectable BC-specific miRNAs. Samples were processed similarly for Firefly detection.

RNA Processing and miRNA Expression Analysis—In HOPE, baseline serum samples were analyzed for miRNA expression. In LEAN, miRNA expression was assessed at both baseline and six-months following intervention^{35, 36}. Firefly methodology was employed as previously described^{44–46}. Samples were scanned on an EMD Millipore Guava 6HT flow cytometer, and flow cytometer output was analyzed with Firefly® Analysis Workbench software (Abcam Plc., Cambridge, MA). Target-specific background subtraction was performed to remove the average signal for each target from negative control wells. For normalization, the geNorm⁴⁷ algorithm was used to calculate probe stability, and determine expression of all targets above a defined threshold across all samples. Probes that did not meet these standards were removed. For quantification, raw signal values from each assay were assessed for expression above the calculated noise-threshold of the assay, which was a

raw mean fluorescent intensity (RMFI) of eighty, log2 scale. Expression values then underwent background subtraction and “geNorm + average analysis”. This normalized the expression of miRNA candidates within the entire sample dataset across the average signal from the twenty most invariant-expressed miRNAs. Hierarchical clustering analysis using a root mean square clustering metric with complete linkage analysis was used to assess quality of the miRNA expression dataset.

The criterion for candidate miRNA inclusion was as follows, 1) to be associative with known breast tumorigenic processes^{25, 48–50}; and/or 2) known to regulate pathways associated with metabolism^{51–53}, adipogenesis, and/or obesity^{54–56}; and/or 3) previously identified to be detectable in circulating biofluids such as plasma or serum^{57–59}. We identified 68 miRNAs eligible for analysis; these miRNA were assessed in crude serum isolates from the HOPE trial utilizing the probe-hybridization multiplex profiling assay mentioned above⁴⁴. This procedure reduces the risk of error associated with RNA extraction of clinical samples of small sample volume.

When testing miRNA expression from samples in the LEAN trial, we used the top 50% most robust and consistently expressed miRNA probes from the original 68. This was done because a number of miRNAs were barely detectable across a number of samples in HOPE (see Figure S1 and Figure S2), as well as overweight patients (BMI > 30 kg/m²). Given all samples in LEAN were from overweight patients, inclusion of those miRNAs in future analyses are inappropriate. See Figure S3 for the list of the 35 miRNAs used in the LEAN cohort analysis.

Target miRNA Analyses—miRNA targets were compared to the clinical endpoints and secondary measures from both HOPE and LEAN, as described above. Pearson correlation coefficients between each miRNA and clinical endpoint was computed using SAS software⁶⁰. $p < 0.1$ indicated statistical significance for BMI-associated miRNAs in HOPE. In LEAN, 6-month changes in miRNA expression was compared to the randomized control cohort. The changes of miRNAs were depicted as the least square means and 95% confidence interval. For exploratory purpose, $p < 0.1$ indicated a statistically significant cutoff for intervention-associated miRNAs in LEAN.

Biological Pathway Analyses

The miRNAs identified in HOPE associated with BMI ($p < 0.05$; N=8) and in LEAN associated with changes post intervention ($p < 0.05$; N=6) were uploaded into Ingenuity Pathway Analysis (IPA) Suite (Qiagen Inc., Redwood City, CA) for biological pathway analyses. IPA utilizes miRNA seed sequence binding with cognate mRNA targets to identify miRNA:mRNA target interactions across multiple available bioinformatic data sources. Since, miR_93_5p and miR_17_5p, two BMI-associated miRNAs, have identical seed sequences, miR_93_5p was considered a duplicate miRNA for predictive modeling. Therefore, the final miRNA number used for BPA predictions were N=7 for HOPE and N=6 for LEAN. We utilized a conservative approach within the IPA microRNA Target Filter for mRNA target prediction, whereby only experimentally-verified interactions, as determined by miRTarBase⁶¹, and/or highly-conserved target sites containing 8-mer seed binding, as

determined by TargetScan⁶² algorithms, were selected. This approach produced a HOPE-specific gene target list of 994 mRNAs, and a LEAN-specific gene target list of 1292 mRNAs. Each gene list was used by IPA to determine enriched canonical pathways targeted by BMI-associated and intervention-responsive miRNAs. Representation of each canonical pathway was tested using Fisher Exact Tests of ratios of miRNA targeted genes in our dataset as compared to the total number of genes in each IPA pathway.

Results:

HOPE Analysis

To identify BMI-associated miRNAs, baseline serum from those enrolled in HOPE were assayed for miRNA expression. We calculated Pearson correlation coefficients to identify miRNAs correlated with baseline BMI in HOPE (Table 2). We identified eight miRNAs significantly associated with BMI with the strongest positive associations as miR_22_3p($r=0.26$, $p=0.004$) and miR_122_5p($r=0.25$, $p=0.005$), both of which are implicated in BC metabolism^{63–65}. miRNAs with the strongest negative BMI association was miR_191_5p($r=-0.22$, $p=0.016$) and miR_17_5p($r=-0.22$, $p=0.017$), which are implicated in BC progression^{66, 67}.

To determine the genetic pathways these miRNAs regulate, we assessed the top ten target mRNA genes for each BMI-associated miRNA via TargetScan (Table S1). A number of these target genes are members of either pro-inflammatory and Notch signaling pathways, or glycolytic and lipid metabolic pathways. Utilizing formal canonical pathway analysis, we identified mRNAs targeted by these BMI-associated miRNAs also had a biological relevance for cancer. Of note, two of the eight miRNAs identified, miR_17_5p and miR_93_5p, have the same seed sequence and therefore the same mRNA targets. Probes for both miRNAs negatively associated with BMI and had the same magnitude of association, as was expected. Thus, seven miRNA targets of interest were used for pathway analysis. The most significant pathways altered were the “Estrogen mediated S-phase entry”($p=0.003$) and the “P53 signaling” pathways($p=0.006$). Specific genes in these pathways are listed in Table 3, and include a number of genes that regulate the process of cell growth, proliferation, and motility.

LEAN Analysis

We identified miRNAs responsive to weight-loss intervention by assessing the change in miRNA expression at both baseline and six-months in the diet and exercise intervention group, as compared to the usual care group within LEAN using a least square means analysis, $\pm$ 95% confidence interval. Six miRNAs had $p<0.10$ for the difference in change between the intervention and usual care arms (Table 4). Most notably, miR_106_5p decreased in expression in the intervention arm (effect change -1.0 (-9.0 to 6.9)) and increased in the usual care arm (effect change 12.5 (2.3 to 22.7)), as compared to baseline, $p=0.041$. This is in line with the finding that miR_106b_5p is a prognostic indicator of BC recurrence and also targets cell cycle regulators in BC cells^{68, 69}. Another miRNA, miR_191_5p, had the largest absolute difference in change between usual care and intervention arms, and the greatest increase in expression in the intervention arm (effect

change 32.1 (6.6 to 57.6), as compared to baseline, $p=0.082$. miR_191_5p was also significantly negatively associated with BMI in the HOPE analysis indicating this miRNA functions as a predictor of weight-loss in overweight BC survivors.

To determine the genetic pathways these miRNAs regulate we assessed the top 10 target genes for each intervention-responsive miRNA (Table S2). These target genes control either notch signaling or apoptosis pathways, indicating these miRNAs regulate a distinct set of pathways from that of HOPE-associated miRNAs. Canonical pathway analysis was performed to determine the molecular underpinnings of weight-loss responsiveness. The most significant pathways identified in LEAN included the “Molecular mechanisms of cancer” ($p=6.8 \times 10^{-7}$) and “Estrogen mediated S-phase entry” ($p=1.4 \times 10^{-6}$). Specific genes in these pathways are listed in Table 5, and include a number of genes that control cancer-related processes.

While assessing the molecular pathways regulated by BMI-associated and intervention-responsive miRNAs in HOPE and LEAN, we noticed “Estrogen mediated S-phase entry” (HOPE, $p=0.003$; LEAN, $p=1.4 \times 10^{-6}$) and the “Molecular mechanisms of cancer” (HOPE, $p=0.0016$; LEAN, $p=6.8 \times 10^{-7}$) were the most significantly modified pathways (Table S3). When assessing gene targets relevant to these pathways, we found the presence of the E2F family of transcription factors, which controls cell cycle and in tumor suppressor proteins. Other targets identified included genes related to cell cycle progression at G1, such as *CDKN1A* and *CCND1*, both of which have been linked to sporadic BC^{70–72}.

Secondary analyses(HOPE)

A secondary analysis was also performed to determine if any of the BMI-associated miRNAs in HOPE correlated to intermediary outcomes such as inflammatory serum biomarkers, and body composition (Table 6). Among the five biomarkers tested, miR_22_3p positively correlated with circulating CRP ($p=0.005$), IL6 ($p<0.001$), glucose ($p<0.001$), insulin ($p=0.003$) and leptin ($p<0.001$) levels. However, BMI-associated miRNAs such as miR_17_5p negatively correlated with IL6 ($p=0.001$) and leptin ($p=0.001$) levels, while miR_191_5p negatively correlated with IL6 levels ($p=0.05$) indicating these miRNAs predict BMI independent of glycolytic-related pathways. Similar results were observed with miR_93_5p, as expected given this miRNA has a similar seed sequence to miR_17_5p.

Analysis of body composition measures (e.g. bone mineral content, total fat, etc.) yielded a distinct subset of miRNAs not linked with BMI (Table 6). For instance, miR_27a_3p was significantly negatively associated with four of the five body measures assessed, and target genes such as NOVA1 which controls RNA splicing and is found in the serum of patients with paraneoplastic opsoclonus-ataxia and BC^{73, 74}. While miR_122_5p did not correlate with any body composition measures, it was associated with insulin ($p<0.001$) and IL6 ($p<0.001$), which is consistent with reported literature⁷⁵.

Discussion:

High BMI and weight-gain are associated with poor outcomes amongst BC survivors, yet the underlying mechanisms explaining this association are unclear. Studies indicate free fatty

acids, glucose, and eicosanoids promote cell-cycle proliferation, inhibit the activity of pro-apoptotic pathways, and/or induce changes in cellular lipid architecture that enhances cellular migration^{76–79}. Activity of these processes are regulated by the transcription factors E2F2 and PPAR γ . PPAR γ is a transcription factor that hetero-dimerizes with RXR to induce the expression of genes essential for cellular metabolism. PPAR γ expression is altered in a variety of solid carcinomas^{80, 81}, and in mouse models of sporadic BC, PPAR γ controls G1 cell-cycle progression by regulating checkpoint genes such as *CDKN1A* and *CCND1*^{82, 83}. E2F2 belongs to a family of nine transcription factors that controls cellular proliferation and apoptosis, and are also suppressed during tumorigenesis^{84, 85}. E2F2 is central to the cellular timing of G1/S phase transitions by controlling the expression of cyclins, FGF2, and SOX2⁸⁶.

E2F2, PPAR γ , and other BC-associated genes are regulated via phosphorylation of E2F-interacting proteins such as Rb and CCND2, and are tightly controlled at the epigenetic level through histone methyltransferase activity, direct methylation of the gene promoter, and/or post-transcriptional regulation via interactions with non-coding RNA^{87–91}. For instance, miR-10b and miR-122 are *bona-fide* regulators of PPAR γ and regulates the pathogenesis of non-alcoholic fatty liver disease (NAFLD) by controlling hepatocyte stenosis^{92, 93}, while the miR-17/92 family regulates the expression of E2F, and therefore the activity of the E2F/MYC signaling axis⁹¹.

miRNAs control many functions of BC etiology as well as metabolic pathways that support BC tumor growth and survival⁹⁴. However, few studies have directly assessed the role of noncoding RNAs as an indicator of obesity and weight-loss intervention. A limiting factor has been the ability to detect circulating miRNAs in biofluids such as serum. In this study, we used a miRNA detection system that obviates the use of PCR to detect raw copy numbers of particular miRNA species from crude serum extract⁴⁴. Using this system, we identified miR_122_5p as a BMI-associated miRNA, which was expected given miR_122_5p is an essential miRNA involved in regulating lipid metabolism, and circulating insulin and leptin levels⁵⁷. In fact, the original *in vivo* antisense studies targeted miR-122 in the adult liver of diet-induced obese mice effectively reducing plasma cholesterol levels, and improving liver steatosis³².

We also identified microRNAs such as, miR_191_5p and miR_17_5p to be significantly inversely correlated with BMI, both of which are involved in tumorigenic processes. For instance, the miR_17_5p/miR_93_5p family were also inversely correlated with inflammatory markers such as CRP, IL6, and leptin, and is known to inhibit tumor growth by suppressing MYC-induced cellular proliferation^{95, 66}. This miRNA cluster post transcriptionally inhibits *MYC* gene expression, while E2F and MYC induce the transcription of miR_17_5p/miR_92_5p, forming a negative feedback loop resulting in a sharp “on/off” state for pro-tumorigenic related protein expression. A related miR_17_5p/miR_93_5p family member, miR_106b_5p was unexpectedly found to be the most significant miRNA that decreased in response to diet and exercise. However, a number of groups have shown miRNAs of the same family can mediate different cellular responses through non-seed base-pairing interactions with target mRNAs⁹⁶. In support of this, miR-106b_5p is upregulated in both tissue and plasma from BC patients, and promotes cell

cycle progression by targeting CDKN1A^{68, 69}. Additional experiments are required to elucidate the molecular mechanisms behind the negative association of miR-17-5p with BMI, and the negative association of miR-106 with weight-loss intervention.

We identified that low circulating miR_191_5p levels is associative with high BMI and high circulating miR_191_5p levels is predictive of successful weight-loss post diet- and exercise-intervention. The mechanism for this is unclear given miR_191_5p associates with relatively few secondary measures in HOPE, (IL6 = $r=0.182$; $p=0.046$). However, pathway analysis, which included miR_191_5p target genes, indicated potential mechanisms may involve 17 β -estradiol signaling and S-phase entry, by targeting genes such as CCND1, E2F2, RB1, and IRS1. While it is not known if miR_191_5p directly inhibits *ESR1*, it is known that miR_191_5p is dysregulated in ER α -positive BC, a 17 β -estradiol-dependent tumor^{67, 97}. Furthermore, 17 β -estradiol promotes the expression of miR_191_5p and protects ER α -positive tumors from hormone depletion-induced apoptosis. This is relevant given all patients in HOPE and half of the patients in LEAN were on AIs, which blocks the synthesis of 17 β -estradiol. Therefore, miR_191_5p may function as an early indicator of 17 β -estradiol-specific signaling due to reduced adipose burden during weight-loss intervention, rather than as a measure of late stage inflammatory and/or metabolic activity.

Circulating miRNAs are extremely stable biomarkers that can be prognostic indicators of disease onset as well as predictive biomarkers for drug response. In a recent study involving measuring 13 a-priori selected miRNAs for change in response to a 16-week diet and exercise weight-loss intervention trial ($n=89$ men and women) identified miR_221_3p and miR_223_3p as increasing in both low- and high-weight-loss responders in response to intervention, and miR-140 increasing only in low responders⁹⁸. None of the miRNAs identified in these studies overlapped with miRNAs captured from the LEAN and HOPE datasets. The likely explanation for this is that all patients enrolled in our trials were either overweight BC-survivors or on AI therapy.

The overall strengths of our study include the utilization of two trials- one validating that our method can detect miRNAs previously associated with BMI in a population of BC-survivors, and two measuring the change in miRNA expression within a separate BC-population over a six-month weight-loss intervention. Our study is limited in that only a single intervention study was performed. Therefore, follow-up weight-loss intervention trials will be required to identify circulating miRNAs and target pathways associated with weight-loss so as to provide opportunities to develop clinical biomarkers for a physiological response to weight-loss intervention. We also identified miRNAs relating to hormone regulation, further studies are warranted to assess how miRNAs can be indicators of effective endocrine therapies in BC-survivors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Flow Chart of Inclusion Criteria for HOPE and LEAN Studies

Flow chart of participants enrolled in the HOPE and LEAN trials, and to which enrollees were assessed for BMI, serum measurements of miRNA levels and secondary biomarkers, as well as body composition measures including total fat and total mass.

Table 1.

Characteristics of participants in the HOPE and LEAN trials

Characteristic	HOPE baseline	LEAN weight loss arms	LEAN usual care arm
N	121	67	33
Age at study entry (years) (mean, SD)	61.3 (7.0)	59.5 (7.5)	58.0 (7.5)
Race/ethnicity (n), % Non-Hispanic White	102 (84.5)	61 (90.9)	30 (91)
Time since diagnosis, years (mean, SD)	3 (3.5)	2.9 (2.1)	2.8 (2.2)
Baseline BMI (kg/m²) (mean, SD)	29.4 (6.2)	32.6 (6.0)	34.0 (7.5)
Six-month weight change (kg) (Least square means from model) [LEAN Only]	N/A	-5.2 (-6.8 to -3.6)	1.7 (-3.2 to -0.3)

Abbreviations: HOPE: Hormones and Physical Exercise, LEAN: Lifestyle, Exercise and Nutrition, BMI: body mass index, SD: standard deviation

Table 2.Correlations between miRNA and BMI in the HOPE trial^a

miRNA	Correlation (rho)	<i>p</i> value
hsa_miR_191_5p	−0.22	0.016
hsa_miR_17_5p	−0.22	0.017
hsa_miR_103a_3p	−0.20	0.030
hsa_miR_93_5p	−0.18	0.048
hsa_miR_22_3p	0.26	0.004
hsa_miR_122_5p	0.25	0.005
hsa_miR_126_3p	0.22	0.015
hsa_miR_150_5p	0.19	0.037

Abbreviations: HOPE: Hormones and Physical Exercise, BMI: body mass index

^a*P* value <0.05 considered significant

Table 3.Biological pathways targeted by the 7 primary endpoint-associated miRNAs in the HOPE trial^{a,b}

Pathway Name	HOPE p-value	Ratio genes/pathway	# genes in dataset	# in IPA pathway	Genes within Respective Pathway
Estrogen mediated S-phase entry	0.003	0.38	9	24	RB1, CCNE1, E2F1, CDKN1A, E2F5, E2F3, CCND1, ESRI, E2F2
P53 signaling	0.006	0.16	18	111	PMAIP1, TP63, TNFRSF10B, PERP, BAX, CCND1, PTEN, BCL2, CCNG1, RB1, RHOF, CCNE1, NF1, IRS1, E2F1, SIRT1, AKT3, PIK3R2
Glioblastoma multiform signaling	0.011	0.13	21	158	TSC1, RHOC, CDK6, WNT16, RHOF, E2F3, CCND1, PDGFB, PTEN, PLCD1, RB1, RHOF, CCNE1, NF1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PIK3R2, E2F2
Cell cycle: G 1/S Checkpoint regulation	0.013	0.19	12	63	RB1, RBL2, CCNE1, MAX, HDAC8, E2F1, CDKN1A, E2F5, CDK6, E2F3, CCND1, E2F2
Molecular mechanisms of cancer	0.015	0.10	36	368	JAK1, BMPR2, WNT16, E2F3, MAPK11, CCND1, BCL2, TGFBR2, RB1, E2F5, AKT3, PRKCE, PIK3R2, E2F2, PMAIP1, RHOC, CDK6, RHOF, BAX, BAK1, GNAI3, CCNE1, RHOF, MAX, MAPK14, CBL, RABIF, NF1, IRS1, CDKN1A, E2F1, PAK5, BMP7, BCL2L11, PSEN1, PRKCB
Glioma signaling	0.017	0.15	16	109	RBL2, CDK6, E2F3, CCND1, PDGFB, PTEN, RB1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PRKCE, PIK3R2, E2F2, PRKCB
Chronic Myeloid Leukemia Signaling	0.022	0.15	15	103	TGFBR2, RB1, RBL2, HDAC8, IRS1, CRKL, E2F1, CDKN1A, E2F5, CDK6, AKT3, PIK3R2, E2F3, CCND1, E2F2
Role of JAK family kinases in IL 6-type Cytokine signaling	0.022	0.28	7	25	JAK1, MAPK14, OSM, OSMR, STAT3, IL6, MAPK11
Pancreatic adenocarcinoma signaling	0.025	0.14	16	118	JAK1, STAT3, E2F3, CCND1, BCL2, TGFBR2, VEGFA, RB1, CCNE1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PIK3R2, E2F2
Cell cycle regulation by BTG family proteins	0.027	0.23	8	35	RB1, CCNE1, E2F1, E2F5, BTG1, E2F3, CCND1, E2F2

Pathway analyses created using Ingenuity Pathway Analysis software (Qiagen, Inc.)

Abbreviations: HOPE: Hormones and Physical Exercise

^a miR_93_5p has the same 'seed' sequence as miR_17_5p and thus is a predictive duplicate^b *p* value <0.05 considered significant

Table 4.

Change in miRNA from baseline to end of the six-month intervention by arm in the LEAN trial^{a,b}

miRNA	Usual care	Intervention	<i>p</i> value
hsa_miR_106b_5p	12.5 (2.3 to 22.7)	−1.0 (−9.0 to 6.9)	0.041
hsa_miR_27a_3p	−12 (−19 to −4.2)	−2.6 (−8.4 to 3.2)	0.062
hsa_miR_191_5p	−4.6 (−37 to 28.0)	32.1 (6.6 to 57.6)	0.082
hsa_let_7b_5p	19.8 (3.9 to 35.7)	2.5 (−9.9 to 14.9)	0.093
hsa_miR_92a_3p	32.3 (−23 to 87.3)	−27 (−70 to 15.9)	0.094
hsa_miR_24_3p	6.7 (−13 to 26.2)	27.4 (12.2 to 42.7)	0.099

Abbreviations: LEAN: Lifestyle, Exercise and Nutrition, BMI: body mass index

^aChange in miRNA following intervention or usual care was controlled for baseline expression

^b*p* value <0.1 considered significant

Table 5.Biological pathways targeted by the 6 intervention-associated miRNAs in the LEAN trial^a

Pathway Name	LEAN p-value	LEAN Ratio genes/pathway	# genes	# in IPA pathway	Genes within Respective Pathway
Molecular mechanisms of cancer	6.8*10 ⁻⁷	0.18	67	368	JAK1, BMPR2, WNT16, E2F3, MAPK11, CCND1, BCL2, TGFB2, RB1, E2F5, AKT3, PRKCE, PIK3R2, E2F2, PMAIP1, RHOC, CDK6, RHOJ, BAX, BAK1, GNAI3, CCNE1, RHOV, MAX, MAPK14, CBL, RABIF, NF1, IRS1, CDKN1A, E2F1, PAK5, BMP7, BCL2L11, PSEN1, PRKCB
Estrogen mediated S-phase entry	1.4*10 ⁻⁶	0.67	16	24	RB1, CCNE1, E2F1, CDKN1A, E2F5, E2F3, CCND1, ESR1, E2F2
Pancreatic adenocarcinoma signaling	1.5*10 ⁻⁵	0.26	31	118	JAK1, STAT3, E2F3, CCND1, BCL2, TGFB2, VEGFA, RB1, CCNE1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PIK3R2, E2F2
Glioblastoma multiform signaling	2.0*10 ⁻⁵	0.23	36	158	TSC1, RHOC, CDK6, WNT16, RHOJ, E2F3, CCND1, PDGFB, PTEN, PLCD1, RB1, RHOV, CCNE1, NF1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PIK3R2, E2F2
Colorectal Cancer Metastasis Signaling	4.1*10 ⁻⁵	0.19	45	242	MAP2K4, FZD10, JAK1, TGFB1, MMP3, FZD3, SMAD3, MMP13, HRAS, GNG13, KRAS, IL6, CCND1, WNT8B, MYC, TGFB2, VEGFA, APPL1, RHOG, RHOB, TLR7, SMAD4, TLR3, GNG5, PTGER4, TP53, IFNG, NRAS, CASP3, GRB2, RHOC, WNT9A, ADCY3, PRKAR2A, VEGFC, MMP10, BAX, STAT3, PIK3R3, BCL2L1, TLR4, GAB1, PTGS2, TNF, WNT1
Cell cycle: G 1/S Checkpoint regulation	5.5*10 ⁻⁵	0.33	21	63	RB1, RBL2, CCNE1, MAX, HDAC8, E2F1, CDKN1A, E2F5, CDK6, E2F3, CCND1, E2F2
Chronic Myeloid Leukemia Signaling	1.3*10 ⁻⁴	0.25	26	103	TGFB2, RB1, RBL2, HDAC8, IRS1, CRKL, E2F1, CDKN1A, E2F5, CDK6, AKT3, PIK3R2, E2F3, CCND1, E2F2
Aryl Hydrocarbon Receptor Signaling	1.5*10 ⁻⁴	0.22	30	135	CDKN2A, IL1A, NFIX, CDK4, IL6, CCND1, FAS, MYC, RB1, CTSD, CCNA2, NCOA7, FASLG, TP53, CCNE2, RBL2, CDK6, BAX, NCOA3, CYP1B1, CCND2, NFIA, E2F1, CDKN1A, NFIB, DHFR, CDKN1B, RXRA, TNF, ESR1
Glioma signaling	2.2*10 ⁻⁴	0.24	26	109	RBL2, CDK6, E2F3, CCND1, PDGFB, PTEN, RB1, IRS1, E2F1, CDKN1A, E2F5, AKT3, PRKCE, PIK3R2, E2F2, PRKCB
P53 signaling	2.7*10 ⁻⁴	0.24	26	111	PMAIP1, TP63, TNFRSF10B, PERP, BAX, CCND1, PTEN, BCL2, CCNG1, RB1, RHOV, CCNE1, NF1, IRS1, E2F1, SIRT1, AKT3, PIK3R2

Pathway analyses created using Ingenuity Pathway Analysis software (Qiagen, Inc.)

Abbreviations: LEAN: Lifestyle, Exercise and Nutrition

^a *p* value <0.0005 considered significant

Table 6.miRNA correlations with secondary measures (serum markers and body composition) in HOPE^{a,b}

miRNA	Biomarkers					Body composition				
	CRP	IL6	Glucose	Insulin	Leptin	BMC	BMD	Total Fat	Total Mass	Total LMBC
hsa_miR_22_3p	0.253	0.424	0.394	0.272	0.366					
<i>p-value</i>	(0.005)	(<0.001)	(<0.001)	(0.003)	(<0.001)					
hsa_miR_126_3p	0.252		0.306	0.299	0.228					
<i>p-value</i>	(0.005)		(0.010)	(0.001)	(0.012)					
hsa_miR_93_5p ^b	−0.198				−0.214					
<i>p-value</i>	(0.030)				(0.018)					
hsa_miR_191_5p		−0.182								
<i>p-value</i>		(0.046)								
hsa_miR_122_5p		0.369		0.54628	0.182					
<i>p-value</i>		(<0.001)		(<0.0001)	(0.046)					
hsa_miR_17_5p ^b		−0.307			−0.305					
<i>p-value</i>		(<0.001)			(<0.001)					
hsa_miR_27a_3p						−0.289		−0.270	−0.301	−0.316
<i>p-value</i>						(0.001)		(0.003)	(0.001)	(<0.001)
hsa_miR_195_5p						0.235	0.384	0.189	0.198	0.206
<i>p-value</i>						(0.010)	(<0.001)	(0.040)	(0.032)	(0.024)
hsa_miR_10a_5p								−0.247	−0.2581	−0.271
<i>p-value</i>								(0.007)	(0.005)	(0.003)
hsa_miR_30d_5p								−0.274	−0.3324	−0.333
<i>p-value</i>								(0.003)	(<0.001)	(<0.001)

Abbreviations: CRP: C-reactive protein, IL6: Interleukin 6, BMC: bone mineral content, BMD: bone mineral density, LBMC: lumbar bone mineral content

^a *p* value <0.05 considered significant

^b While miR_93_5p has the same 'seed' sequence as miR_17_5p, this miRNA was not removed from analysis because variation in remaining sequence could result in different gene targets and thus contribute to differences in observed correlations with secondary serum biomarkers.