



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 the intermediary outcomes of tumor recurrence. Studies have indicated microRNAs (miRNA)s are reliable biomarkers for cancer. We hypothesized that miRNA expression correlates with obesity and weight loss amongst BC survivors. This would yield insight into the biological pathways by 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 that 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 that 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 increased cancer risk.

Keywords MicroRNA · Obesity · Weight loss · Diet · Exercise · Breast cancer · miRNA · Biomarkers

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Introduction

In the United States, over 200,000 women will be diagnosed with invasive BC annually and is the second leading cause of cancer-related death in women [1–3]. Only 10% of BC cases are due to a genetic predisposition, indicating that a number of environmental and anthropometric factors influence 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 10 years) [15]. Adult weight gain is also associated with a 64% increased risk of BC death among survivors [16]. 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 involve an abundance of metabolically active adipose tissue that results in 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 entity 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 tissue [30]. Additionally, forced expression of these miRNAs in vitro promotes cell invasion, proliferation, and pro-survival phenotypes, by targeting a number of tumor suppressor genes including *PDCD4*, *PTEN*, *SOCS1*, and *HOXD10*. miRNAs are also expressed in the serum of BC patients, and function as reliable biomarkers for the disease [31]. 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, which miRNAs correlate to BMI and weight-loss intervention will provide a unique insight into the mechanisms underlying the geneticlinks 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 among BC survivors. First, we used baseline serum samples from BC survivors enrolled in the HOPE study [35] to identify miRNAs associated with BMI in this population; second, we utilized serum samples from the LEAN [36] trial to assess the effect of weight-loss intervention and the 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 certain miRNAs

and the pathways they regulate would correlate with BMI and change in response to weight-loss intervention, offering some insight into the mechanisms linking obesity to BC recurrence, and perhaps even identifying novel targets to improve survival outcomes for these patients.

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 Center [35]. 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, versus (3) usual care. Participants included in the study were diagnosed with stage 0–III BC survivors, had a BMI > 25.0 kg/m², and completed chemo- and/or radiation-therapy (Fig. 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 al. [37]. Assessments of LEAN data in this manuscript evaluated blood draws and anthropometric measurements at both baseline and 6 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) assumes full responsibility for analyses and interpretation of these data. The clinical characteristics of HOPE and LEAN trials are outlined in Table 1.

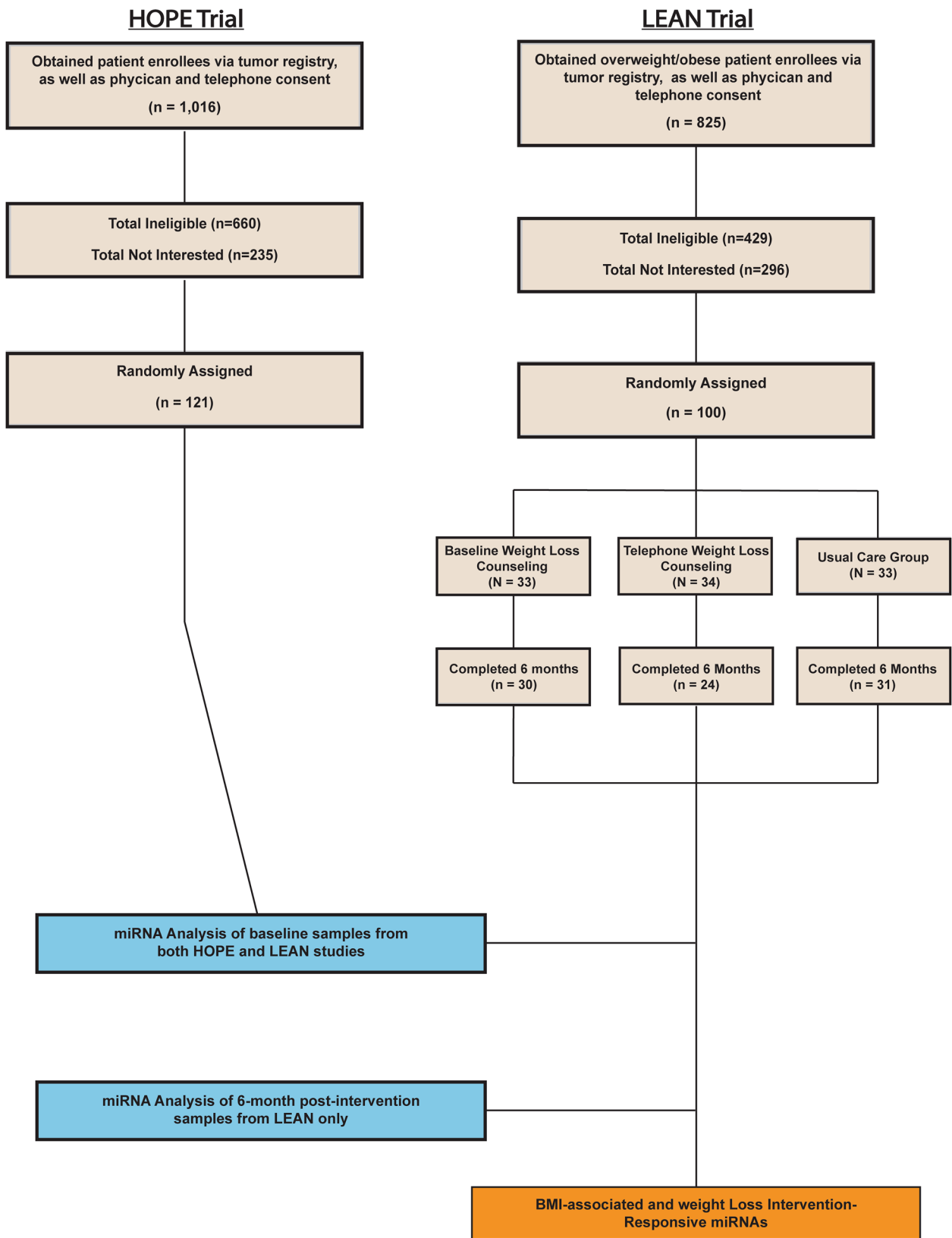


Fig. 1 Flow chart of participants enrolled in the HOPE and LEAN trials, and to which enrollees were assessed for BMI, serum measurements of miRNA levels and secondary biomarkers, as well as body composition measures including total fat and total mass

Table 1 Characteristics of participants in the HOPE and LEAN trials

Clinical Characteristics	HOPE baseline	LEAN weight loss arms	LEAN usual care arm
<i>N</i>	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/m ²) (mean, SD)	29.4 (6.2)	32.6 (6.0)	34.0 (7.5)
Six-month weight change (kg) (least square mean from model) [LEAN Only]	N/A	− 5.2 (− 6.8 to − 3.6)	1.7 (− 3.2 to − 0.3)

HOPE hormones and physical exercise, LEAN lifestyle, exercise and nutrition, BMI body mass index, SD standard deviation

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 − 80 °C 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, 150 μ l of serum from patients under fasting conditions enrolled in HOPE with a BMI of 18.5–44 kg/m² was arrayed onto 96-well plates and assayed for miRNA expression using the Firefly platform [44]. 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 were 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 signal from each target contributed by the negative control wells. For normalization, the geNorm [47] 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 underwent background subtraction and “geNorm+average analysis”, to normalize the expression of miRNA candidates within the entire sample dataset across the average signal from the 20 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 miRNAs were assessed in crude serum isolates from the HOPE trial utilizing the probe-hybridization multiplex profiling assay mentioned above [44]. This procedure reduces the risk of error associated with RNA extraction of clinical samples from small sample volumes.

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 not consistently detectable across a number of samples in the HOPE dataset (see Figure S1 and Figure S2), as well as in patients with a BMI 30 kg/m². Given all samples in LEAN were from overweight patients, the inclusion of those miRNAs in future analyses was not

performed. 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 were computed using SAS software [60]. $p < 0.1$ indicated statistical significance for BMI-associated miRNAs in HOPE. In LEAN, 6-month changes in miRNA expression were compared to the randomized control cohort. The changes in miRNA abundance were determined by a least square means analysis, with a $\pm 95\%$ confidence interval as a cut-off. For exploratory purposes, $p < 0.1$ 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 was an N of 7 for HOPE and an N of 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 miRTarBase [61], and/or highly conserved target sites containing 8-mer seed binding, as determined by Target Scan [62] 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 determined using a fisher exact test of miRNA-targeted genes within 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 was 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 were miR-191_5p ($r = -0.22$, $p = 0.016$) and miR-17_5p ($r = -0.22$, $p = 0.017$), which are implicated in influencing 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 that 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 proliferation, survival, 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, with a $\pm 95\%$ confidence interval. Six miRNAs were significantly different

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

miRNA Probe	Correlation (ρ)	p 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

HOPE hormones and physical exercise, BMI body mass index

^a p value < 0.1 considered significant

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

Pathway name	HOPE <i>p</i> value	Ratio genes/pathway	# genes in data-set	# 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, ESR1, E2F2
P53 signaling	0.006	0.16	18	111	PMAIP1, TP63, TNFRSF10B, PERP, BAX, CCND1, PTEN, BCL2, CCNG1, RB1, RHOV, CCNE1, NF1, IRS1, E2F1, SIRT1, AKT3, PIK3R2
Glioblastoma multiform signaling	0.011	0.13	21	158	TSC1, RHOC, CDK6, WNT16, RHOJ, E2F3, CCND1, PDGFB, PTEN, PLCD1, RB1, RHOV, 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, RHOJ, BAX, BAK1, GNAI3, CCNE1, RHOV, 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.)

HOPE hormones and physical exercise

^amiR_93_5p has the same ‘seed’ sequence as miR_17_5p and thus is a predictive duplicate

^b*p* value <0.05 considered significant

($p < 0.10$) 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 – 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 – 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

Table 4 Change in miRNA from baseline to end of the six-month intervention by arm in the LEAN trial^{a,b}

miRNA Probe	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

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

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, there was an association with insulin ($p<0.001$) and IL6 ($p<0.001$), consistent with the reported literature [75].

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]. Activities 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 cell-cycle progression by regulating checkpoint genes such as *CDKN1A* and *CCND1* [82, 83]. E2F2 belongs to a family of nine transcription factors that control 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 SOX2 [86].

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 regulate the pathogenesis of non-alcoholic fatty liver disease (NAFLD) by controlling hepatic stenosis [92, 93], while the miR-17/92 family regulates the expression of E2F, and subsequently the activity of the E2F/MYC signaling axis [91].

miRNAs control many functions of BC etiology as well as the metabolic pathways that support BC tumor growth and survival [94]. 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 an miRNA detection system that obviates the use of PCR to detect the copy number of a particular miRNA species from crude serum

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

Pathway name	LEAN <i>p</i> 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, TGFBR2, 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, TGFBR2, 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, TGFBR1, MMP3, FZD3, SMAD3, MMP13, HRAS, GNG13, KRAS, IL6, CCND1, WNT8B, MYC, TGFBR2, 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	TGFBR2, 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.)

LEAN lifestyle, exercise and nutrition

^a*p* value <0.05 considered significant

extract [44]. Using this system, we identified miR-122_5p as a BMI-associated miRNA, which was expected given miR-122_5p is essential in regulating lipid metabolism, and is associated with circulating insulin and leptin levels [57]. In fact, the original *in vivo* antisense studies targeted miR-122 in the adult liver of obese mice using a diet-induced model, effectively reducing plasma cholesterol levels and improving liver steatosis [32].

We also identified miRNAs 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 was 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 [66, 95]. This miRNA cluster post-transcriptionally inhibits *MYC*

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

miRNA Probe	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</i> value	(0.005)	(<0.001)	(<0.001)	(0.003)	(<0.001)					
hsa_miR_126_3p	0.252		0.306	0.299	0.228					
<i>p</i> value	(0.005)		(0.010)	(0.001)	(0.012)					
hsa_miR_93_5p ^b	– 0.198				– 0.214					
<i>p</i> value	(0.030)				(0.018)					
hsa_miR_191_5p		– 0.182								
<i>p</i> value		(0.046)								
hsa_miR_122_5p		0.369		0.54628	0.182					
<i>p</i> value		(<0.001)		(<0.0001)	(0.046)					
hsa_miR_17_5p ^b		– 0.307			– 0.305					
<i>p</i> value		(<0.001)			(<0.001)					
hsa_miR_27a_3p						– 0.289		– 0.270	– 0.301	– 0.316
<i>p</i> value						(0.001)		(0.003)	(0.001)	(<0.001)
hsa_miR_195_5p						0.235	0.384	0.189	0.198	0.206
<i>p</i> value						(0.010)	(<0.001)	(0.040)	(0.032)	(0.024)
hsa_miR_10a_5p								– 0.247	– 0.2581	– 0.271
<i>p</i> value								(0.007)	(0.005)	(0.003)
hsa_miR_30d_5p								– 0.274	– 0.3324	– 0.333
<i>p</i> value								(0.003)	(<0.001)	(<0.001)

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

^bWhile 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

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 intervention. However, a number of groups have shown that miRNAs of the same family can mediate various cellular responses through non-seed base-pairing interactions with target mRNAs [96]. 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 are associated with high BMI and high circulating miR-191_5p levels are 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*, *RBI*, 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 AI therapy, 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 fluctuations in 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. A recent study measured 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), and identified miR-221_3p and miR-223_3p as increasing in both low- and high-weight-loss responders in response to intervention, as well as miR-140

as increasing in the low responder group [98]. 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 related to hormone regulation; further studies are warranted to assess how miRNAs can be indicators of effective endocrine therapies in BC survivors.

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Compliance with ethical standards

Conflicts of interest B.D. Adams is *President and CEO* of The Brain Institute of America and holds patent interests with AUM LifeTech. Other authors have declared no conflicts of interest exist.

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

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