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Randomized trial of weight loss in primary breast cancer: Impact on body composition, circulating biomarkers and tumor characteristics

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Obesity adversely impacts overall and cancer-specific survival among breast cancer patients. Preclinical studies demonstrate negative energy balance inhibits cancer progression; however, feasibility and effects in patients are unknown. A two-arm, single-blinded, randomized controlled weight-loss trial was undertaken presurgery among 32 overweight/obese, Stage 0–II breast cancer patients. The attention control arm (AC) received basic nutritional counseling and upper-body progressive resistance training whereas the weight loss intervention (WLI) arm received identical guidance, plus counseling on caloric restriction and aerobic exercise to promote 0.68–0.92 kg/week weight loss. Anthropometrics, body composition, blood and survey data were collected at baseline and presurgery ~30 days later. Tumor markers (e.g., Ki67) and gene expression were assessed on biopsy and surgical specimens; sera were analyzed for cytokines, growth and metabolic factors. Significant WLI *vs.* AC differences were seen in baseline-to-follow-up changes in weight (−3.62 *vs.* −0.52 kg), %body fat (−1.3 *vs.* 0%), moderate-to-vigorous physical activity (+224 *vs.* +115 min/week), caloric density (−0.3 *vs.* 0 kcal/g), serum leptin (−12.3 *vs.* −4.0 ng/dl)

Additional Supporting Information may be found in the online version of this article.
Key words: breast cancer, clinical trial, diet, exercise, Ki67
Abbreviations: AC: attention control; ACSM: American College of Sports Medicine; BMI: body mass index; CC-ARG: cell cycle-apoptosis related genes; DCIS: ductal carcinoma *in situ*; DE: differential expression; DXA: dual-energy absorptiometry; FACT-B: Functional Assessment of Cancer Therapy Questionnaire for Breast Cancer; FFA: free fatty acids; FGF: fibroblast growth factor; IL: interleukin; PA: physical activity; RD: registered dietitian; SD: standard deviation; SHBG: sex hormone binding globulin; TNF: tumor necrosis factor; UAB: University of Alabama at Birmingham; VEGF: vascular endothelial growth factor; WLI: weight loss intervention
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and upregulation of tumor PI3Kinase signaling and cell cycle-apoptosis related genes (CC-ARG; all p-values <0.05). Cytolytic CD56^{dim}NK cell expression was positively associated with weight loss; CC-ARG increased with physical activity. Increased tumor (nuclear) TNFα and *IL-1β*, *CX3CL1* and *CXCL1* gene expression was observed in the WLI. Tumor Ki67 did not differ between arms. Feasibility benchmarks included 80% accrual, 100% retention, no adverse effects and excellent adherence. Short-term weight loss interventions are feasible; however, mixed effects on tumor biology suggest unclear benefit to presurgical caloric restriction, but possible benefits of physical activity.

What's new?

Obesity adversely impacts survival among breast cancer patients. Preclinical studies demonstrate negative energy balance inhibits cancer progression; however, effects in patients are unknown. This is the first randomized controlled trial to assess the impact of a pre-surgical weight loss intervention among early-stage breast cancer patients. Results show the effects of acute negative energy balance on tumor biology, circulating biomarkers, and quality-of-life. Short-term weight loss interventions are feasible; however, mixed effects on tumor biology suggest unclear benefit to pre-surgical caloric restriction. Such interventions may be better timed after surgical resection, though cell cycle-apoptosis and DNA damage-repair scores support increasing physical activity.

Introduction

Recent US estimates indicate that roughly 63,410 new cases of ductal carcinoma *in situ* (DCIS) and 252,710 new cases of invasive breast cancer are diagnosed annually, with deaths due to breast cancer occurring in approximately 40,610 women.[1] Obesity is related to poorer prognosis, with a meta-analysis of 82 studies among 213,075 breast cancer patients showing significantly reduced overall and disease-specific survival in both pre and postmenopausal obese women, with increased risks ranging from 8% to 29%.[2] A variety of mechanisms that link obesity to cancer progression have been identified and include inflammation, glucoregulation and sex hormones and their interconnecting signaling pathways.[3] Therefore, weight loss is a potential multitargeted therapeutic strategy to slow disease progression, and has been suggested for women with precursor lesions (DCIS), and those with invasive disease.[4]

Limited research shows that weight loss interventions (WLIs) are safe for breast cancer survivors and improve health-related quality-of-life in the short-term.[5] A few trials also suggest improvements in circulating inflammatory markers and adipokines, insulin, insulin-like growth factors, sex steroid hormones and associated binding proteins.[5] However, to date, no trials have assessed the effects of negative energy balance directly on breast tumor tissue, hence potential benefits on the tumor microenvironment and breast cancer biology remain unclear.

Our NCI-funded weight-loss trial conducted among women awaiting surgery for Stage 0–II breast cancer, was designed to address this knowledge gap. The primary aim of this trial was to test the feasibility of a WLI that promoted a calorically restricted diet and increased physical activity (PA) among overweight and obese breast cancer patients during the time that they awaited lumpectomy or mastectomy, *vs.* an attention control (AC). Other aims were to quantify and compare between-arm differences in body weight (and other measures of adiposity); tumor cell proliferation rate (Ki67); candidate biomarkers (e.g., estradiol, estrone, sex hormone-binding globulin [SHBG], growth hormones [vascular endothelial growth factor and fibroblast growth factor-β (FGFβ)]; cytokines [tumor necrosis factor-α (TNFα)]; adipokines [leptin]; insulin; tumor gene expression); and quality-of-life. We hypothesized that in comparison to the AC, the WLI arm would lose weight, exhibit decreases in tumor cell Ki67 and demonstrate improvements in known biomarkers of antitumor immunity and breast cancer progression.

Materials and Methods

Detailed methods have been published previously[6] and are summarized.

Study design

This trial was an open-label, two-arm, Phase II randomized controlled trial that was approved and monitored by the University of Alabama at Birmingham (UAB) Institutional Review Board (Protocol: F130325009). Participants were stratified by body mass index (BMI; overweight *vs.* obese) and randomly assigned electronically in blocks of four (equal allocation by study biostatistician) to the WLI or AC arm immediately after the baseline appointment and then followed until the time of their surgery.

Study setting/participants

Recruitment occurred at the UAB Interdisciplinary Breast Health Clinic. Patients receiving surgery as the first course of treatment for stage 0-II breast cancer and scheduled at least 3 weeks out were approached and screened. Other eligibility criteria were (*i*) BMI = 25–60 kg/m^2; (*ii*) no contraindications to unsupervised exercise using the Physical Activity Readiness Questionnaire[7]; (*iii*) no medical conditions affecting weight status; (*iv*) no other active malignancies; (*v*) English speaking/reading; (*vi*) not currently enrolled in a weight loss program; and (*vii*) no previous or scheduled chemotherapy during the presurgical period. Written informed consent was

obtained from all study participants. Data on BMI, age, ethnic and distance from home to UAB were collected on all patients approached to determine if women who refused or were ineligible differed from enrollees.

After obtaining consent, a registered dietitian (RD) conducted a 24-hr, multiple-pass dietary recall with each participant.[8] To collect objective data on PA, participants were given a programmed accelerometer (wGT3X: Actigraph, LLC, Pensacola, FL) and instructed to wear it at the hip during waking hours until their baseline appointment (3–5 days later). Participants were instructed to report to the baseline appointment after a 12-hr fast.

Baseline appointment

Another dietary recall was taken ensuring that dietary intakes from a weekday and a weekend day were represented; recalls were analyzed using the Nutrition Data System for Research (NDSR 2014, Minneapolis, MN). Other survey data were collected using validated instruments, that is, Older American Resources and Services (OARS) comorbidity scale[9]; Functional Assessment of Cancer Therapy for Breast Cancer (FACT-B) quality-of-life questionnaire[10]; and the Godin Leisure-Time Exercise Questionnaire.[11]

Systolic and diastolic blood pressure was measured using a conventional mercury sphygmomanometer with the participant seated. Submaximal exercise testing (Trackmaster Treadmills Model TMX425CP, Full Vision Inc., Newton, KS) was performed using the Naughton protocol.[12] Cardiorespiratory fitness was estimated using the treadmill incline and speed achieved at 85% of age-predicted maximal heart rate.[12]

Weight, height and waist circumference were measured in accordance with the Anthropometric Standardization Manual.[13] BMI was calculated as kg/m^2. Body composition was assessed *via* dual-energy X-ray absorptiometry (DXA [Lunar iDXA, GE Healthcare, Waukeha, WI]).

Blood (20 ml) collected from venipuncture was separated into sera, plasma and buffy coat. All samples were stored at −80°C until batch analysis at study completion.

AC intervention

An AC condition was employed to improve study accrual and retention. Participants in this arm received dietary counseling by an RD to correct nutrient deficiencies that were detected from their two-day dietary recalls and encouraged to do so using food sources, rather than supplements.[14] They also received "prehabilitation" instruction from an American College of Sports Medicine (ACSM)-certified Cancer Exercise Trainer (CET) to strengthen their triceps, biceps and deltoids prior to surgery using three resistance band exercises (Therabands®, Hygenic Corporation, Akron, OH).[15] The CET and RD interacted with patients weekly to answer questions, provide support, problem-solve and set goals for the upcoming week. Potential adverse events were solicited weekly along with information to ascertain severity and attribution.

Weight loss intervention

WLI participants received identical dietary and PA guidance as to the AC, plus additional counseling to achieve a weight loss of 0.68–0.92 kg/week. Energy needs were estimated using the Mifflin–St. Jeor equation: 1.2 [10 × (body weight (kg)) + 6.25 (height (cm)) − 5(age)] and then subtraction of 750–1,000 kcal/day.[16] Participants were coached to achieve this energy deficit *via* caloric restriction and increased PA. Participants could either count their calories, follow a prescribed meal plan, or use an exchange list system; weekly guidance was provided on portion control, dietary restraint, dietary choices aligned with national guidelines and substituting nutrient-rich and low-energy foods for nutrient-poor and high-energy foods.[17]

An exercise program to achieve an expenditure of 200–400 kcal/day *via* aerobic training of large leg muscles was emphasized, with ramping of intensity and volume over time as per ACSM guidelines.[18] Participants trained once-to-twice weekly under the supervision of the CET, and were encouraged to exercise at home using a pedometer to assure fidelity to protocol.

Social cognitive theory served as the behavioral framework to guide the WLI.[19] Participants were provided with scales, and pedometers or Fitbits®, and self-monitored their body weight, foods eaten with accompanying calorie or exchange list values and PA behaviors daily. The CET and RD interacted with patients at least twice weekly to measure weight, review logged behaviors, answer questions, provide support, problem-solve and set goals for the upcoming week.

Follow-up appointment

All participants were scheduled for a follow-up appointment on the business day prior to or the day of surgery. Identical procedures performed at baseline were repeated, though height and demographic measures were not collected again. Paraffin-embedded tissue was obtained postsurgery from the Department of Pathology.

Assessment of proliferation index and protein expression in tumor tissue

All tumor blocks were reviewed by a pathologist (blinded to study arm) to confirm atypical lesions. Immunostaining for Ki67, insulin receptor (I-R), VEGF, TNFα, NFκB, 4E-BP1, proliferating cell nuclear antigen (PCNA), activated caspase-3, pSK6 and p16 were performed as described previously.[6]

nanoString gene expression analysis of tumor tissue

Paraffin-embedded blocks from the preintervention biopsy and the subsequent postintervention surgical resection were assessed visually for the presence of adequate tumor tissue that could be macrodissected for gene expression analysis using the nanoString nCounter system (Seattle, WA); macrodissection occurred on the surgical specimens from 24 participants (11 AC and 13 WLI participants). Many biopsy specimens were devoid of adequate tumor; however, ample tumor on both biopsy and surgical

specimens was identified from six participants in the WLI arm, which were then matched to six weight stable ACs based on ethnic and age (±5 years). Tumor RNA was extracted using High Pure FFPET RNA Isolation kits (Roche Diagnostics, Indianapolis, IN) and the nanoString nCounter Flex system was used to run the PanCancer Pathways Panel (770 essential genes representing 13 canonical pathways in cancer, 606 Pathway genes, 124 cancer driver genes and 40 reference genes panels). Ample RNA remained to run the PanCancer Immune Profiling Panel (770 genes combining markers for 24 different immune cell types and populations, 30 common cancer antigens and genes that represent all categories of immune response including key checkpoint blockade genes) on surgical specimens from 10 WLI and 10 ACs.

Circulating biomarkers

Assays were performed in the UAB Physiology and Metabolism Core. Sera were analyzed for (*i*) leptin *via* radioimmunoassay (Millipore, Billerica, MA); (*ii*) free fatty acids (FFA)s using a Wako reagent (Richmond, VA) and on a Sirrus Stanbio Analyzer (Boerne, TX); (*iii*) insulin and SHBG using immunofluorescence on a TOSOH 900 Automated Immunoassay Analyzer (AIA; TOSOH Bioscience, Inc., South San Francisco, CA); and (*iv*) bFGF, Interleukin (IL)-1β, IL-6, VEGF-C and TNFα using multiplex proinflammatory assays on the SECTOR imager 2400 (Meso Scale Diagnostics, Rockville, MD).

Assays for sex hormones were performed *via* mass spectrometry by the UAB Targeted Metabolomics and Proteomics Laboratory. Levels of estradiol, estrone and testosterone were determined by isotope dilution High-Performance Liquid Chromatography (HPLC)-electrospray ionization-multiple reaction ion mass spectrometry adapted from the method of Tai and Welch,[20] and run against known standards. Chromatography was carried out using an Ace Excel C_{18}-Aromatic 1.7 μm 50 × 3.0 mm IS column at 50°C using a 20 AD Prominence HPLC (Shimadzu, Kyoto, Japan) in tandem with 6500 Qtrap mass spectrometer (SCIEX, Framingham, MA). LC–MS operation and data collection were captured using Analyst 1.6.2 software (SCIEX).

Power and sample size calculations

Data from our previous weight loss programs were used to estimate power and suggested a mean weight loss of 2.82 ± 1.57 kg over a 3–4 week period. Initially, 40 participants with 20/arm were planned with forecasts of 20% attrition to yield a final sample size of 16/arm. This sample size would yield >90% power to detect a difference in the weight change noted above using a two-group, two-sided *t*-test and a significance level of 5%. The trial was halted after 32 participants completed the trial.

Statistical analysis

Descriptive statistics were calculated for each arm. Continuous variables were assessed for normality and $\log_{10}$ transformed as needed. All analyses were performed on an intention-to-treat basis. Given the exploratory nature of our study, imputation was not performed for missing data. Statistical tests were two-sided and were performed using a 5% significance level. Comparisons of baseline characteristics between enrollees and nonenrollees, as well as participants in the two study arms (Table 1), and those for whom tumor markers were assessed only in surgical specimens (Table 3), were performed using the two-group *t*-test for continuous variables, and the chi-square test, or Fisher's exact test as appropriate. Baseline to follow-up changes and between-arm differences were assessed simultaneously using mixed models repeated measures analysis for weight status, body composition, diet, PA, fitness and quality-of-life variables (Table 2) and for serum and tumor biomarkers (Table 3). An unstructured covariance matrix was assumed for these analyses. The Tukey–Kramer multiple comparisons test was used for *post hoc* comparisons. These models included terms for study arm, time (baseline, follow-up) and the study arm by time interaction. Analyses were repeated for fitness, and systolic and diastolic blood pressure while adjusting for the use of beta-blockers (yes/no), and for tumor markers adjusting for the use of stage (DCIS [Stage 0]/Invasive [Stages 1–2]). The results obtained with these adjusted analyses were similar to those displayed in Tables 2 and 3, and as such, are not presented. SAS software (version 9.4; SAS Institute, Cary, NC) was used to conduct all statistical analyses except gene expression. These analyses were performed using the nSolver software that assessed *p* values and fold change values calculated from nCounter default settings that accounted for background signals measured in negative controls and normalization factors derived from housekeeping genes and positive controls. Genes identified as having $p < 0.05$ and fold change greater than ±1.5 were further evaluated and reported based on statistical and biological significance. Differentially expressed (DE) gene-associated pathways (Fig. 2*a*) were reported from nanoString designated gene sets. Identified DE genes were separated into groups of upregulated or downregulated genes, then subjected to gene ontology enrichment analysis to identify associated biological processes (Fig. 2*b*).[21–23] nanoString-identified cell type scores and pathway scores were analyzed for potential associations with degree of weight loss or accelerometer-measured PA *via* linear regression using GraphPad Prism, version 7.0 (Figs. 2*c* and 2*d*).

Data availability

Data are not publicly available given that National Institutes of Health funding was below the cap. However, data and materials are available upon request to the corresponding senior author.

Results

Trial status

This trial was conducted between August 21, 2014, and October 18, 2016, during which time 100 patients were approached and screened (Fig. 1). Roughly one-third of patients approached refused to participate (leading reasons were "too busy," "overwhelmed," or

Table 1. Baseline characteristics of participants

	Total (n = 32)	Control (n = 15)	Weight loss (n = 17)	p-value
Stage/biopsy grade n (%)				
DCIS	10 (31.2%)	3 (20.0%)	7 (41.2%)	0.265[1]
Low to low-intermediate	1 (10.0%)	1 (33.3%)	0	
Intermediate	2 (20.0%)	0	2 (28.6%)	
Intermediate-high	4 (40.0%)	1 (33.3%)	3 (42.8%)	
High	3 (30.0%)	1 (33.3%)	2 (28.6%)	
Invasive	22 (68.8%)	12 (80.0%)	10 (58.8%)	
Stage 1	18 (81.8%)	8 (66.7%)	10 (100%)	
Grade I	4 (22.2%)	3 (37.5%)	1 (10.0%)	
Grade II	9 (50.0%)	5 (62.5%)	4 (40.0%)	
Grade III	5 (27.8%)	0	5 (50.0%)	
Stage 2	4 (18.2%)	4 (33.3%)	0	
Grade I	0	0	0	
Grade II	2 (50.0%)	2 (50.0%)	0	
Grade III	2 (50.0%)	2 (50.0%)	0	
Histology				
Invasive ductal carcinoma	16 (72.7%)	8 (66.7%)	8 (80%)	
Lobular Tubular	3 (13.6%)	1 (8.3%)	2 (20%)	
	2 (9.1%)	2 (16.7%)	0 (0%)	
Invasive micropapillary carcinoma	1 (4.5%)	1 (8.3%)	0 (0%)	
Hormone receptor status n (%)				
ER+	29 (90.6%)	14 (93.3%)	15 (88.2%)	1.0
PR+	23 (71.9%)	11 (73.3%)	12 (70.6%)	1.0
Amplified HER2 (invasive cancers only)	1 (4.5%)	0 (0)	1 (10%)	0.262
Body mass index				
Mean (SD)	34.8 (5.7)	34.9 (5.6)	34.7 (6.0)	0.944
Overweight [n (%)]	6 (18.8%)	3 (20.0%)	3 (17.6%)	0.587
Obese class I [n (%)]	12 (37.5%)	4 (26.7%)	8 (47.1%)	
Obese class II [n (%)]	8 (25.0%)	5 (33.3%)	3 (17.6%)	
Obese class III [n (%)]	6 (18.8%)	3 (20.0%)	3 (17.6%)	
Numbers of comorbidities n (%)				
Up to 2	18 (56.3%)	11 (40.0%)	12 (70.6%)	0.082
3 or more	14 (43.8%)	9 (60.0%)	5 (29.4%)	
Cardiovascular disease n (%)	5 (15.6%)	3 (20.0%)	2 (11.8%)	0.645
Diabetes n (%)	9 (28.1%)	5 (33.3%)	4 (23.5%)	0.699
Age (years)				
Mean (SD)	60.9 (9.4)	59.7 (7.8)	62.1 (10.7)	0.480
Ethnic n (%)				
African American or mixed ethnic	15 (46.9%)	5 (33.3%)	10 (58.8%)	0.149
Caucasian	17 (53.1%)	10 (66.7%)	7 (41.2%)	
Education n (%)				
High school graduate or less	8 (25.0%)	4 (26.7%)	4 (23.5%)	1.0
Some college or more	24 (75.0%)	11 (73.3%)	13 (76.5%)	
Current smoker n (%)	2 (6.3%)	2 (13.3%)	0 (0.0%)	0.212
Duration of implementation (days)	29.6 (8.8)	30.1 (8.4)	29.1 (9.4)	0.702

[1]Between-arm p-value for DCIS *vs*. invasive disease; note the p-value exploring between-arm differences for stage 0, 1, or 2 also was not statistically significant (p = 0.073), nor were there differences I histology (p = 0.261).

Table 2. Baseline to follow-up change in weight status, body composition, diet, physical activity, fitness and quality-of-life

	Control		Weight loss intervention				
	Baseline (n = 15) mean (SD)	**Follow-up (n = 15) mean (SD)**	**Baseline (n = 17) mean (SD)**	**Follow-up (n = 17) mean(SD)**	**Between arm p-value**	**Within arm p-value**	**Between* within arm interaction p-value**
Weight (kg)	92.7 (16.8)	92.2 (16.4)	89.9 (16.7)	86.2 (16.3)	0.46	**<0.001**[1]	**<0.001**
BMI (kg/m^2)	34.9 (5.6)	34.7 (5.5)	34.7 (6.0)	33.3 (6.0)	0.72	**<0.001**[1]	**<0.001**
Body fat (%)	47.7 (4.2)	47.7 (4.2)	48.1 (4.3)	46.8 (4.6)	0.83	**<0.001**[1]	**<0.001**
Android body fat (%)	53.5 (5.2)	54.1 (5.8)	53.9 (6.5)	51.6 (6.3)	0.61	**0.003**[1]	**<0.001**
Gynoid body fat (%)	48.1 (5.7)	47.7 (6.1)	49.2 (5.4)	48.0 (5.5)	0.68	**<0.001**[1]	0.16
Lean body mass (kg)	46.5 (7.1)	46.1 (6.9)	44.5 (5.60)	43.8 (5.6)	0.33	**0.004**[1]	0.36
Caloric intake (kcal/day)	1,530 (415)	1,174 (520)	1,366 (374)	825 (234)	**0.039**	**<0.001**[2]	0.22
Macronutrient distribution (% of kcal)							
Total fat	38.9 (4.8)	38.5 (7.6)	38.3 (7.3)	32.3 (8.1)	0.11	**0.035**[3]	0.068
Carbohydrate	42.2 (6.6)	41.9 (9.3)	42.6 (7.5)	40.8 (11.0)	0.89	0.57	0.67
Protein[4]	16.4 (4.6)	18.3 (6.4)	18.1 (3.8)	26.1 (7.1)	**0.005**	**<0.001**[4]	**0.018**
Energy density (kcal/g)	1.1 (0.4)	1.1 (0.45)	1.2 (0.4)	0.9 (0.2)	0.60	0.15	**0.037**
Self-reported physical activity (PA)							
MVPA min/week	8.7 (13.8)	123.5 (356.0)	42.9 (68.4)	267.1 (209.9)	**0.001**	**<0.001**	**0.006**
Accelerometer assessed PA[5]							
MVPA min/week	79.8 (45.5)	91.0 (74.9)	156.1 (113.4)	205.8 (191.1)	**0.023**	0.98	0.68
Fitness[6] (ml/kg/min)	18.7 (5.5)	20.9 (4.7)	18.6 (5.7)	21.4 (5.7)	0.99	**0.002**[7]	0.37
Blood pressure							
Systolic	132.4 (9.4)	130.0 (9.8)	132.3 (10.6)	132.8 (12.5)	0.68	0.45	0.90
Diastolic	79.4 (8.0)	76.4 (7.5)	77.9 (7.7)	74.9 (10.2)	0.42	0.17	0.56
Quality of life (FACT B)							
Summary score	113.8 (14.2)	114.7 (15.5)	117.4 (20.3)	117.7 (19.1)	0.58	0.67	0.82
Physical well-being	24.1 (4.6)	24.5 (4.8)	24.1 (4.8)	24.4 (4.6)	0.98	0.37	0.96
Social well-being	22.5 (4.8)	22.8 (3.9)	22.8 (5.4)	22.1 (6.1)	0.91	0.66	0.27
Emotional well-being	20.0 (2.6)	19.5 (3.3)	18.6 (4.5)	18.6 (4.8)	0.37	0.67	0.73
Functional well-being[6]	20.5 (5.3)	22.3 (4.1)	23.2 (5.1)	23.8 (4.9)	0.22	**0.009**	0.16
Additional concerns	26.5 (5.1)	25.6 (7.0)	28.6 (5.5)	28.8 (5.0)	0.19	0.46	0.31

Bold values indicate the findings of statistical significance ($p < 0.05$).
[1]For the weight loss intervention arm only, the follow-up mean is significantly less than the baseline mean.
[2]For both arms, the follow-up mean is significantly less than the baseline mean.
[3]For the weight loss intervention arm only, the follow-up mean is significantly less than the baseline mean; in addition, the follow-up mean for the weight loss intervention arm is significantly less than the follow-up mean for the wait-list control arm.
[4]For the weight loss intervention arm only, the follow-up mean is significantly greater than the baseline mean; in addition, the follow-up mean for the weight loss intervention arm is significantly greater than the follow-up mean for the wait-list control arm.
[5]Cell sizes vary from values in the heading. For the wait-list control n = 14 at baseline and n = 12 at follow-up and for the WLI, n = 16 at baseline and follow-up.
[6]For the wait-list control arm only, the follow-up mean is significantly greater than the baseline mean.
[7]Cell sizes vary from values in the heading. For the wait-list control n = 13 at baseline and follow-up, and for the WLI, n = 16 at baseline and n = 14 at follow-up.

travel), roughly one-third were ineligible (leading reasons were <3 weeks prior to surgery and neoadjuvant treatment), and roughly one-third were enrolled. Analyses between trial enrollees (n = 33) *vs.* nonenrollees (n = 67) determined that there were no statistically significant differences between participants *vs.* nonparticipants regarding age, BMI, menopausal status and ethnic; however, a previously reported analysis showed that women who lived 25 miles or more from UAB were significantly less likely to enroll (p = 0.034).[24] Thirty-three participants were enrolled and randomized, and 32 completed the trial. One participant was excluded soon after randomization to the AC due to new evidence of advanced cancer. Based on the eligibility criteria, she was classified as a post-randomization exclusion. Surgical specimens were released from the Department of Pathology 1-year after surgery.

Characteristics of study participants are reported in Table 1. The study sample was diverse, with roughly half of the participants self-identifying as non-Hispanic White and the other half self-reporting as African American or mixed ethnic. While most had some college education, only one-quarter were college graduates. The mean age of participants was 60.9 years; most had

Table 3. Baseline to follow-up changes in circulating and tumor biomarkers

	Control		Weight Loss Intervention				
	Baseline (n = 14) mean (SD)	Follow-up (n = 14) mean (SD)	Baseline (n = 17) mean (SD)	Follow-up (n = 17) mean (SD)	Between arm p-value	Within arm p-value	Between * within arm interaction p-value
Circulating serum biomarkers							
Estradiol (pg/ml)	18.5 (20.0)	15.4 (13.0)	14.9 (8.8)	18.9 (23.0)	0.94	0.98	0.68
Estrone (pg/ml)	209.7 (136.1)	193.6 (125.3)	170.2 (95.8)	171.3 (91.5)	0.43	0.84	0.39
Testosterone (pg/ml)	1,089.3 (666.5)	979.2 (595.3)	1,001.9 (908.8)	1,076.6 (941.8)	0.85	0.90	0.095
SHBG (nmol/L)	48.9 (21.5)	53.0 (24.8)	41.3 (15.5)[1]	48.6 (16.1)[1]	0.58	**0.001**[2]	0.12
Free fatty acids (mEQ)	0.7 (0.3)	0.9 (0.4)	0.8 (0.3)[1]	0.8 (0.2)[1]	0.43	**0.040**	0.12
Insulin (mg/dl)	13.2 (7.8)	11.6 (7.4)	13.4 (7.6)[1]	11.5 (8.4)[1]	0.81	**0.016**	0.61
Leptin (ng/ml)	62.4 (18.1)	58.4 (13.9)	65.6 (20.0)	53.3 (25.9)	0.62	**<0.001**[3]	**0.004**
Fibroblast growth factor-β (pg/ml)	2.4 (1.5)	2.1 (2.0)	2.9 (3.4)	5.2 (10.4)	0.82	0.49	0.30
Interleukin-6 (pg/ml)	1.3 (0.7)	2.9 (0.6)	2.8 (0.7)	1.5 (1.2)	0.94	0.40	0.91
TNFα (pg/ml)	3.1 (0.7)	2.9 (0.6)	2.8 (0.7)	3.0 (0.7)	0.56	0.85	0.057
VEGF-C (pg/ml)	207.6 (68.0)	201.0 (82.0)	258.8 (146.1)	242.6 (120.0)	0.38	0.66	0.76
Immunohistochemistry-based tumor markers assessed in both biopsy and surgical specimens[4,5]							
Ki-67 (primary endpoint)	38.6 (34.1)	31.5 (33.1)	31.8 (29.9)	30.6 (27.6)	0.91	0.35	0.58
Immunohistochemistry-based tumor markers assessed only in surgical specimens[4]							
PCNA[6]		93.54 (10.17		95.36 (2.50)	0.54		
I-R cytoplasm		0: 3/10 (30%)		0: 0/12 (0%)	0.10		
		0.5:2/10 (20%)		0.5: 2/12 (17%)			
		1.0:5/10 (50%)		1.0:10/12 (83%)			
I-R membrane		0: 3/10 (30%)		0: 6/12 (50%)	0.088		
		0.5: 0/10 (0%)		0.5: 1/12 (8%)			
		1.0:4/10 (40%)		1.0: 2/12 (17%)			
		1.5:1/10 (10%)		1.5: 0/12 (0%)			
		2.0: 0/10 (0%)		2.0: 3/12 (25%)			
		3.0:2/10 (20%)		3.0: 0/12 (0%)			
I-R nuclear		0.5:2/10 (20%)		0.5: 0/12 (0%)	0.19		
		1.0:8/10 (80%)		1.0:12/12 (100%)			
TNFα cytoplasm[7]		1.2 (0.2)		1.2 (0.2)	0.93		
TNFα membrane[7]		1.3 (0.1)		1.4 (0.2)	0.68		
TNFα nuclear[7]		0.7 (0.2)		1.0 (0.2)	**<0.001**		

Bold values indicate the findings of statistical significance ($p < 0.05$).
[1]Sample sizes for these cells were 16, due to insufficient sera on one participant at baseline and follow-up.
[2]For the weight loss intervention arm only, the follow-up mean is significantly greater than the baseline mean.
[3]For the weight loss intervention arm only, the follow-up mean is significantly less than the baseline mean.
[4]All tumor-related analyses were repeated adjusting for stage (DCIS/Invasive); results were very similar to values in this table, and are not presented separately.
[5]Based on the availability of tissue, sample sizes for controls were n = 12 at baseline and n = 14 at follow-up, and n = 16 for intervention arm at both timepoints.
[6]Based on the availability of tissue, sample sizes for controls were n = 13 and for the intervention arm n = 11.
[7]Based on the availability of tissue, sample sizes for controls were n = 12 and for the intervention arm n = 13.

obesity, and reported one or more comorbidities. Roughly, one-third of participants had DCIS, with most tumors graded as intermediate-to-high; the other two-thirds of women had invasive cancers, with most having Stage I (Grade II) disease. Most participants had estrogen receptor-positive (ER+) and progesterone receptor-positive (PR+) disease. There were no significant differences between study arms and the average duration on study was 29.6 ± 8.8 days.

Table 2 provides data and p values for within- and between-arm comparisons, and interaction terms over time for weight, body composition, diet, PA, fitness, blood pressure and quality-of-life. As compared to AC, the WLI arm significantly reduced their body weight, BMI and % body fat (especially in the upper body region); significant reductions in lean mass also were observed. Data suggest that the WLI arm consumed fewer Calories primarily through reductions in dietary fat as a

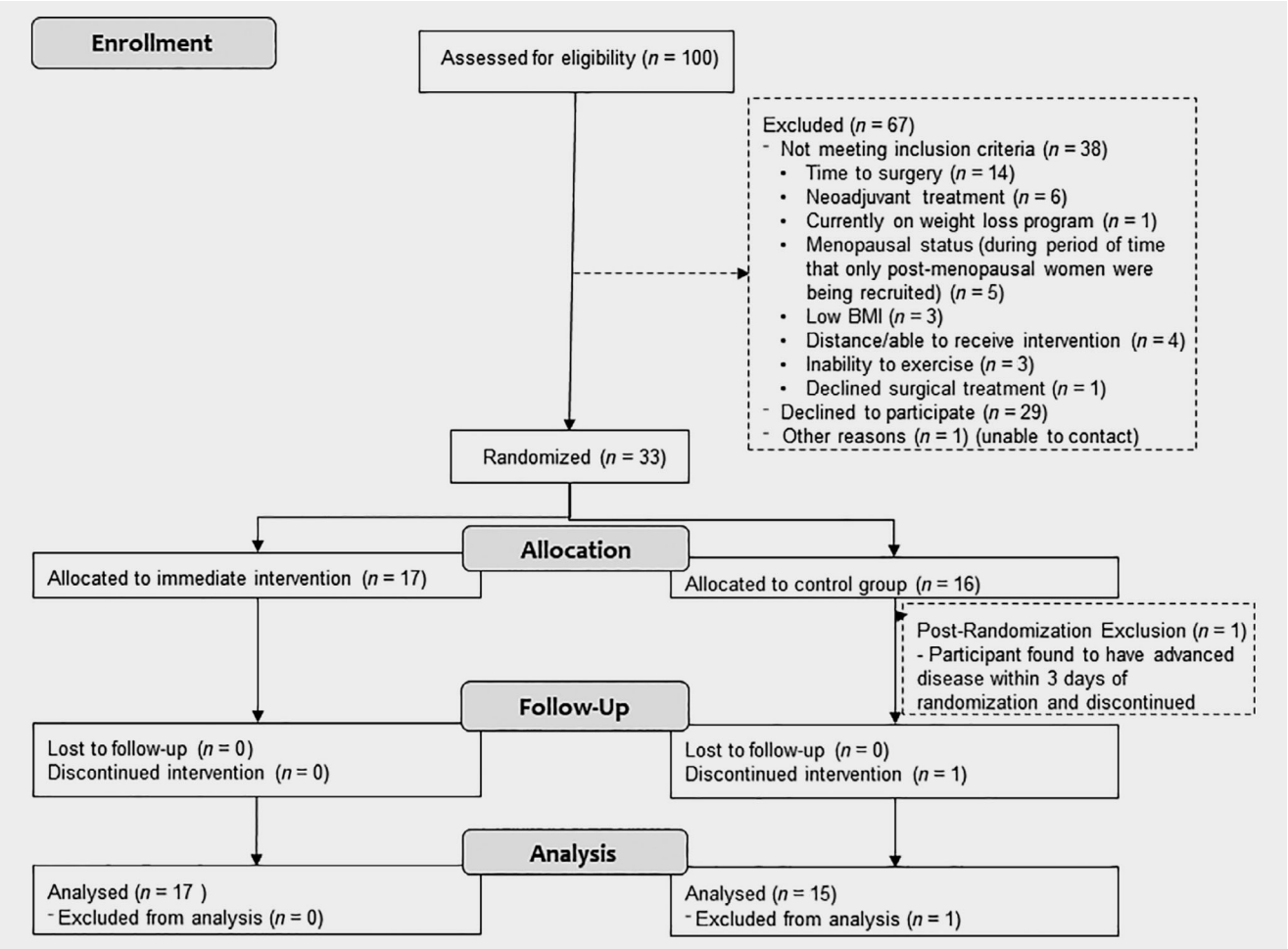

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) diagram.

proportionately higher percentage of protein was consumed. These dietary changes resulted in a significant reduction in diet energy density among the WLI as compared to AC. WLI arm participants also reported significant increases in moderate-to-vigorous PA that were moderately supported by the accelerometer data. Fitness improved in the WLI arm, but there were no between- or within-arm interactions for fitness, blood pressure or quality-of-life, though the AC reported higher functional well-being at follow-up than the WLI arm.

Table 3 shows data and *p* values for within- and between-arm comparisons and interaction terms for circulating and tumor markers. Of all serum biomarkers, changes in leptin were the most remarkable, with both arms showing significant decreases, however, an even larger reduction was detected in the WLI arm. Significant within-arm decreases also were detected for serum insulin, and trends toward DE of the I-R were observed in the histopathological staining of tumor specimens with increased levels in the cytoplasm and decreased levels in the membrane seen in the WLI as compared to AC. For SHBG and FFAs, significant within-arm differences supported by trends for time × arm interaction were observed, that is, serum SHBG increased to a larger degree within the WLI arm and serum FFAs were stable in the WLI but increased within the AC. Finally, the time x arm interaction for serum TNFα approached significance as levels of this cytokine increased in the WLI, but decreased in the AC; TNFα nuclear staining also was significantly higher in the tumor specimens from WLI participants as compared to AC. No significant within- or between-arm differences or interactions were seen in serum levels of any of the sex hormones, FGFβ, IL-6 or VEGF-C, moreover, other immunohistopathological data (i.e., caspase 3, NFkB, p16, 4E-BP, pSK6 and VEGF) did not differ between study arms. No between-arm differences were seen in proliferative markers, that is, PCNA or Ki67 staining. A positive correlation of borderline significance ($r = 0.39$; $p = 0.051$) was found between baseline-to-follow-up changes in serum TNFα and tumor cell Ki67, though correlations between TNFα in the sera and TNFα tumor staining, as well as TNFα tumor staining and Ki67 were nonsignificant (*p*-values ranging between 0.50 and 0.58).

In comparing postintervention surgical samples to preintervention biopsies (Fig. 2*a*), DE of 20 genes (*p* values <0.05 and fold changes > ±1.5) was detected specifically within

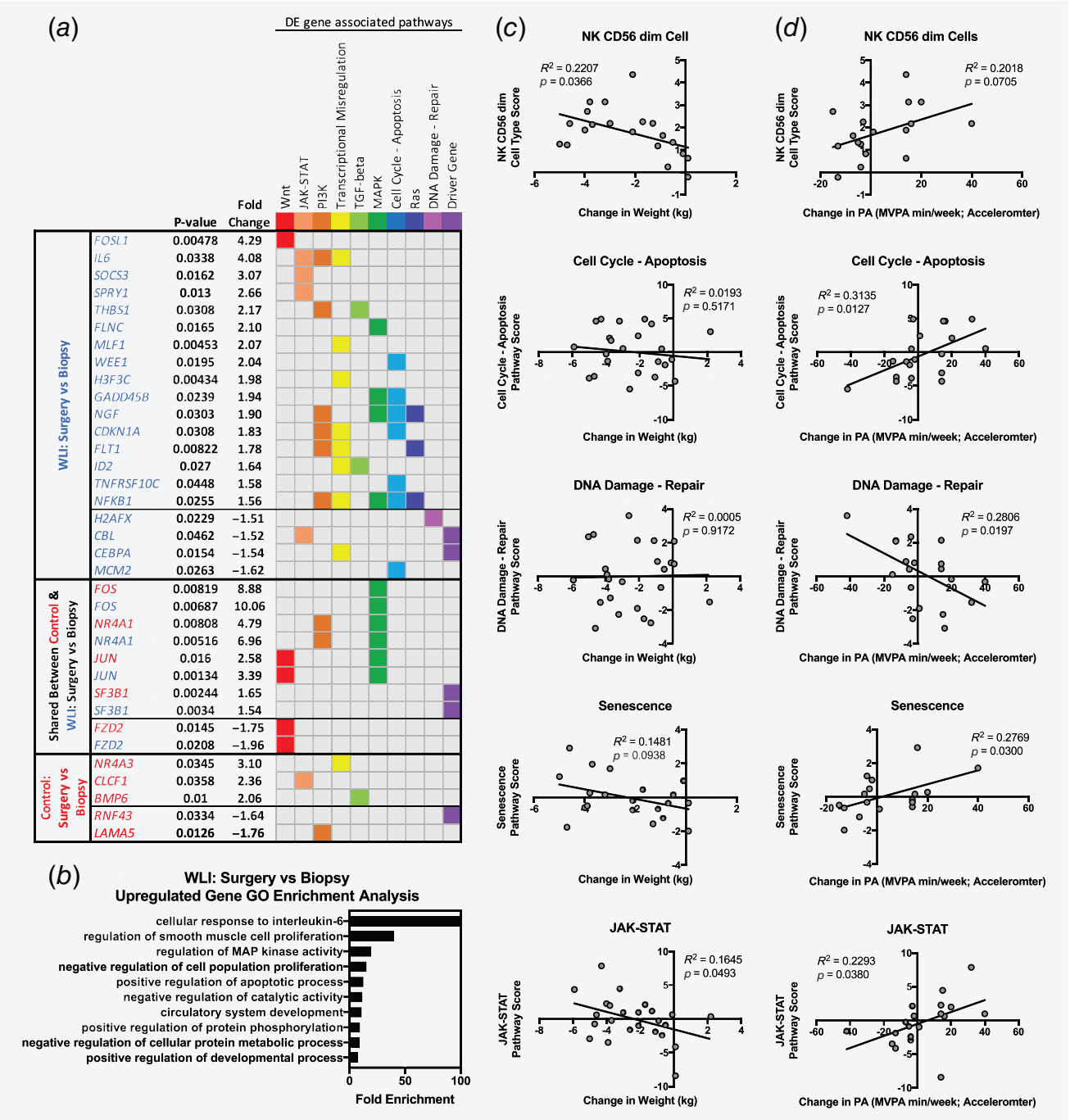

Figure 2. Effects of WLI, weight loss and physical activity on differentially expressed (DE) genes and associated pathways, biological processes and cell types in breast tumor tissues. (*a*) DE gene-associated pathways reported from nanoString-designated gene sets for WLI ($n = 12$) and AC ($n = 12$) participants at time of surgery *vs*. preintervention biopsy. (*b*) Gene Ontology (GO) Enrichment Analysis and associated GO biological processes of 16 upregulated DE genes in tumor tissue of WLI participants at time of surgery *vs*. preintervention biopsy. Association of (*c*) degree of weight loss or (*d*) accelerometer-measured physical activity (PA) with nanoString-identified cell type scores and pathway scores.

the WLI group. These DE genes reflected the involvement of 10 different nanoString-identified biological pathways, with transcriptional misregulation, cell cycle-apoptosis and PI3Kinase signaling pathways being the most heavily altered (with 8, 7 and 6 DE genes, respectively). The majority of DE genes were upregulated (16 of 20) with the WLI. The gene encoding transcriptional regulator FOSL1, a subunit of the AP-1 transcription factor complex and downstream target of WNT signaling, showed the highest fold change increase (p-value 0.00478, fold change 4.29). Four DE genes in the WLI group were downregulated from

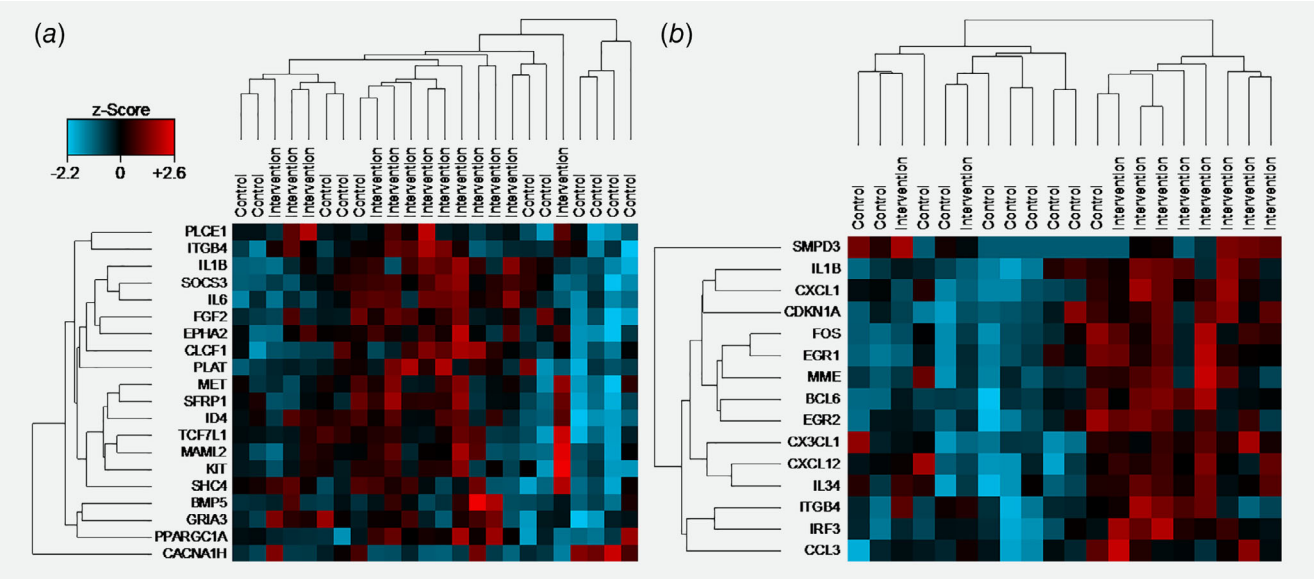

Figure 3. DE genes in WLI *vs*. AC tumor specimens at time of surgery. Heatmaps depicting DE genes identified from nanoString (*a*) PanCancer Pathways and (*b*) PanCancer Immune Profiling panels.

biopsy to surgery; these included *CBL* (*p*-value 0.0462, fold change −1.52), which encodes a proto-oncogene previously associated with breast cancer progression, and CCAAT Enhancer Binding Protein Alpha (*CEBPA*) which regulates leptin expression.[25] An additional five DE genes were comparably altered in both the WLI and AC groups, in surgery *vs*. biopsy biospecimens (Fig. 2*a*). Five DE genes were uniquely altered in surgical tumor specimens *versus* biopsies from the AC. Thus, the WLI as compared to AC was associated with a greater number of gene expression changes over the course of the trial.

To further interrogate the biological relevance of the 16 upregulated genes that were DE specifically in the WLI group from biopsy to surgery, we performed Gene Ontology pathway analysis. Doing so revealed that the "Cellular response to Interleukin-6" pathway was most highly upregulated (>100-fold enrichment, Fig. 2*b*) in the tumor microenvironment postintervention. We next evaluated gene expression data from all participants at time of surgery (combined from both WLI and AC) for potential associations between nanoString-identified cell type scores and pathway scores, and either change in weight (Fig. 2*c*) or change in accelerometer-measured PA (Fig. 2*d*). Analyzing data in this way revealed that as weight is lost, regardless of treatment arm, expression of genes associated with cytolytic $CD56^{dim}$ NK cells increased. Genes associated with JAK–STAT signaling showed similar trends, increasing with both heightened weight loss and increased PA. In contrast, genes related to Cell Cycle-Apoptosis pathways showed a positive association only with increased PA, but no association with weight loss. Likewise, genes related to DNA Damage-Repair and Senescence pathways showed significant associations with PA, but not weight loss.

Heat maps depicting DE genes in surgically resected tumors from the WLI *vs*. AC are shown in Figure 3 (left panel: PanCancer Pathways/right panel: PanCancer Immune Profiling/supporting data shown in Supporting Information Table S1). These data indicate that a clear majority of WLI biospecimens (8 of 10) grouped together during immune profiling and showed upregulation of genes including *IL-1β*, *CX3CL1* and *CXCL1*. Strong concordance was found between the PanCancer Pathways panel and the PanCancer Immune panels, in terms of results for *IL1B* and Integrin β4 (*ITGB4*).

Discussion

This is the first trial to assess the effects of a presurgical WLI among women with early-stage breast cancer. Hence, for the first time, we provide results on the impact of acute negative energy balance on the biology of the tumor and select gene expression, while also providing information on related circulating biomarkers and quality-of-life.

The trial met well-established benchmarks for feasibility, that is, a racially diverse enrollment that achieved 80% of the target, the absence of serious adverse events and excellent adherence and retention. Participants assigned to the WLI demonstrated significant increases in PA (observed by both subjective and objective measures) and reductions in caloric density of the diet, though minor "drop-in" was evidenced among the AC for overall caloric restriction. As a result, significant between-arm differences were apparent for weight and other measures of adiposity.

With this loss of weight, favorable changes were seen for two biomarkers that are prevalently measured in weight loss trials among breast cancer patients, as well as in the general population, that is, SHBG and leptin. Like others,[26,27] we show that serum SHBG significantly increases and leptin significantly decreases with weight loss. Since SHBG binds both estrogen

and testosterone and reduces the pool of free hormone that can interact with the breast tissue, this effect is considered beneficial. Also favorable are the concomitant decreases in leptin which have been postulated as a critical lynchpin connecting obesity to breast cancer progression.[28] Our findings showing decreased leptin levels with weight loss corroborate the results of other weight loss trials among breast cancer survivors,[27,29,30] though we are the first to document leptin-associated changes in tumor gene expression of *CEBPA*.

While data for the impact of weight loss on circulating insulin levels are not as strong, the concordant effects observed in the sera and in tumor gene expression of the I-R provides evidence that negative energy balance exerts an impact on glucoregulation which may have been heightened further had the intervention period been longer. For TNFα, our results differ from the significant reductions reported by Pakiz *et al.*[31] in a 16-week weight-loss trial among 68 breast cancer survivors, as well as the nonsignificant trend toward a reduction in TNFα with a 12-week weight loss regimen among 28 triple-negative breast cancer survivors reported by Swisher *et al.*[32] Instead, our data suggest a trend toward an increase in serum levels of this cytokine within the WLI arm as compared to AC and significantly higher levels of nuclear TNFα detectable by immunohistochemistry upon surgery. While the impact of weight loss on levels of TNFα within tumor specimens has never been reported, our results (particularly since increases in serum TNFα appeared correlated with increases in tumor Ki67) are unexpected and of concern.

Our transcriptomic analysis of gene expression changes in the tumor microenvironment also showed both expected and unexpected changes with the WLI. Comparing preintervention biopsies to postintervention surgical samples revealed that the WLI produced significant changes in a greater number of genes (20 in total) than occurred in the AC (5 in total). An additional 5 genes showed similar biopsy-to-surgery changes in both groups, likely illustrating changes that occur with neoplastic progression or response of the tumor to biopsy (Fig. 2*a*). Upregulated WLI-specific DE genes included *FLT1* (*VEGFR1*),[33] *IL6*,[34] *SPRY1*[35] and *THBS1*,[36] all of which have been linked to breast cancer progression. The most highly upregulated gene was *FOSL1*, a transcription factor and downstream target of WNT signaling. Increased WNT signaling has recently been linked to the presence of decreased T cell-related gene signatures in breast and other tumor types.[37] The mixed effects of WLI on tumor biology were additionally evident from examination of downregulated genes: both *CBL*, which encodes a proto-oncogene, and *CEBPA*, which encodes a transcription factor that functions as both a tumor suppressor and regulator of leptin expression,[38] were decreased post-WLI. Despite no between-arm differences in serum IL-6, we found a strong IL-6 signature within the tumor as evidenced by a >100-fold upregulation of genes associated with cellular responses to this cytokine (Fig. 2*b*). Although IL-6 can have protumorigenic effects, it has also been linked to increased mobilization of cytolytic NK cells into tumors after exercise,[39] a finding that concurs with our observation that protective $CD56^{dim}$ cytolytic NK cell scores increased in surgical samples as weight loss increased (Figs. 2*c* and 2*d*). Of note, our analysis of surgical samples revealed that most (8 of 10) WLI biospecimens grouped together when immune-related DE genes were analyzed; these samples showed upregulation of *CCL3*, *CXCL1*, *CXCL12*, *CX3CL1* and *IL-34* which collectively would be associated with increased leukocytic infiltration into tumors. Further analysis of nanoString cell type scores and biological pathways across surgical samples from all study participants suggested that increased PA, but not weight loss, was the primary factor driving observed changes in cell cycle-apoptosis and DNA damage-repair scores (Figs. 2*c* and 2*d*). These findings stand in stark contrast to the conclusions of a systematic review by Campbell *et al.*[40] of 19 studies that evaluated gene expression in relation to weight loss and PA interventions in an effort to derive implications for carcinogenesis. They concluded that weight loss, but not PA, was responsible for far more changes in gene expression. However, this review did not focus on tumor gene expression, but instead focused on gene expression within subcutaneous adipose tissue—a tissue that obviously would be directly affected by weight loss. The discrepancy between our data and the data emanating from studies using normal tissue underscores the tissue-specific nature of microenvironment remodeling, and the importance of directly assessing tumor tissue. Of note, Ligibel *et al.*[41] reported gene expression changes in a presurgical weight-loss trial that was in concordance with our findings. In particular, these include induction of JAK–STAT signaling and NK cytotoxicity, as well as unaltered Ki67 and decreased serum leptin.

Given mixed effects, it is unsurprising that we were unable to detect between-arm differences in tumor proliferation rates, either detected by Ki67 or PCNA. These data which show the lack of impact of diet, exercise or weight loss on Ki67 join the three aforementioned exercise interventions,[41–43] as well as a presurgical diet and exercise trial among 34 men with prostate cancer,[44] and a presurgical metformin trial that produced modest weight loss among 200 breast cancer patients,[45] all showing no significant differences in markers of tumor proliferation. However, data from the current study clearly differ from a forerunning presurgical WLI that our group conducted on 34 men with prostate cancer,[46] and another conducted among 87 Barrett's esophagus patients,[47] that showed increased rather than decreased Ki67 among patients assigned to the diet and exercise interventions *vs.* controls. Thus, in each of these trials, including the current investigation, the hypothesized effect that weight loss would result in decreased proliferation was unsupported.

Clinically, improvements in fitness were seen within the WLI arm, though no differences were observed for blood pressure. Such effects are associated with weight loss; however, the brief duration of the intervention may have interfered with our ability to detect differences in blood pressure which may have occurred over a longer time period. The AC as compared to the WLI arm reported superior improvements in functional

outcomes. It is unknown whether this outcome was spurious, or may be the result of increased soreness involved with an exercise regimen; however as reported previously,[48] quality-of-life outcomes are not always positively influenced by weight loss regimens as was demonstrated by increasing levels of depression over the 2-year period of the ENERGY trial (n = 692).

Our primary study limitation is a lack of statistical power, common to many feasibility trials. Moreover, the large number of assessments in combination with the small sample size further increases the risk of a type 1 error. In addition, the selection of patients may have masked effects; first, because of the broad heterogeneity, and second, because the tendency toward lower tumor proliferation rates (and hence floor effects) among patients with early-stage disease. Furthermore, because the trial was integrated within a presurgical clinical care timeline, it was necessarily brief. Certainly, much more could be learned about the impact of negative energy balance on tumor biology given a longer intervention period affording multiple serial measures. Finally, because of cost and a limited supply of biospecimens, we are unable to report findings on all adipokines and cytokines.

In summary, this trial showed feasibility and achieved significant weight loss, and changes in caloric consumption and expenditure during the presurgical period. The WLI also resulted in significant reductions in serum leptin and increases in SHBG, and the upregulation of several genes in tumor tissue consistent with enhanced immune function and apoptosis, and decreased insulin- and WNT-signaling. However, other effects were less positive, for example, increased expression of several genes related to cytokines, growth factors and proliferative markers. Ultimately, there was no net effect on the proliferation rates of tumors among women assigned to the WLI *vs.* AC arms. Hence, the results of our study do not support the large body of preclinical research supporting the role of caloric restriction on neoplastic progression. While it is currently unknown whether a presurgical trial of longer duration or within a particular subset of breast cancer patients would yield different findings, the fact remains that this is the fifth trial to assess changes in Ki67 in response to a WLI, and to date, none have shown a significant reduction in proliferation rate,[45,49] and two have shown significant increases.[47,50] Thus, while the results suggest a positive effect of PA that merits further investigation, it may be prudent to delay weight loss among cancer patients, at least until after the tumor is resected.

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