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# Impact of a Pre-Operative Exercise Intervention on Breast Cancer Proliferation and Gene Expression: Results from the Pre-Operative Health and Body (PreHAB) Study

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## Abstract

**Purpose:** Exercise after breast cancer diagnosis is associated with lower cancer-specific mortality, but the biological mechanisms through which exercise impacts breast cancer are not fully understood. The Pre-Operative Health and Body (PreHAB) Study was a randomized window-of-opportunity trial designed to test the impact of exercise on Ki-67, gene expression, and other biomarkers in women with breast cancer.

**Experimental Design:** Inactive women with newly diagnosed breast cancer were randomized to an exercise intervention or mind–body control group, and participated in the study between enrollment and surgery (mean 29.3 days). Tumor and serum were collected at baseline and surgery.

**Results:** Forty-nine women were randomized (27 exercise, 22 control). At baseline, mean age was 52.6, body mass index was 30.2 kg/m$^2$, and exercise was 49 minutes/week. Exercise participants significantly increased exercise versus controls (203 vs. 23 minutes/week, $P < 0.0001$). There were no differences in changes of expression of Ki-67, insulin receptor, and cleaved caspase-3 in exercise participants versus controls. KEGG pathway analysis demonstrated significant upregulation of 18 unique pathways between the baseline biopsy and surgical excision in exercise participants and none in control participants ($q < 0.1$). Top-ranked pathways included several implicated in immunity and inflammation. Exploratory analysis of tumor immune infiltrates demonstrated a trend toward a decrease in FOXP3$^+$ cells in exercise versus control participants over the intervention period ($P = 0.08$).

**Conclusions:** A window-of-opportunity exercise intervention did not impact proliferation but led to alterations in gene expression in breast tumors, suggesting that exercise may have a direct effect on breast cancer.

*See related commentary by Koelwyn and Jones, p. 5179*

## Introduction

Physical activity is associated with breast cancer risk and prognosis (1). Observational studies have demonstrated that physically active women are at lower risk of developing breast cancer, with most studies suggesting a 20% reduction in breast cancer risk in the most versus least active women (2, 3). Data from a number of prospective observational studies also demonstrate an inverse relationship between physical activity and risk of breast cancer–specific and all-cause mortality in individuals with early stage, potentially curable, breast cancers (4). A recent meta-analysis of 16 cohort studies, including 42,602 patients with breast cancer, demonstrated that individuals who engaged in the highest levels of physical activity after breast cancer diagnosis had a 29% lower risk of

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## Translational Relevance

Exercise after breast cancer diagnosis is associated with better disease-specific mortality, but the biological mechanisms through which exercise impacts breast cancer are not fully understood. The Pre-Operative Health and Body Study was a randomized window-of-opportunity trial designed to test the impact of a preoperative exercise intervention on tissue and serum biomarkers in women with breast cancer. Results demonstrated that exercise did not alter proliferation, but impacted tumor gene expression, especially in pathways involved in inflammation and immune regulation. These findings are analogous to animal models, where exercise induces activation of immune pathways within breast cancers. This trial provides some of the first evidence in humans that exercise could directly affect human breast cancer, providing insight into the biologic pathways through which exercise could impact breast cancer and novel information to guide the design of future trials testing the impact of exercise on breast cancer recurrence and mortality.

breast cancer–specific mortality [RR, 0.71; 95% confidence interval (CI), 0.58–0.87; $P < 0.01$] and a 43% lower risk of all-cause mortality (RR, 0.57; 95% CI, 0.45–0.72; $P < 0.01$), compared with inactive individuals (4).

The mechanisms underlying the relationship between exercise and breast cancer outcomes are poorly understood. Current hypotheses suggest that metabolic, inflammatory, and immune pathways may underlie the connection between physical activity and breast cancer (5–8). In animal models, exercise has been shown to reduce breast tumor formation and slow tumor progression (9–12). Some studies have suggested that exercise may exert these effects through impacting PI3K and other metabolic pathways (12), whereas other studies suggest that effects of exercise on breast cancer may be exerted through upregulation of immune pathways (13). Despite these emerging data from preclinical studies, there is almost no information regarding the direct effects of exercise on breast cancer in humans.

The Pre-Operative Health and Body (PreHAB) Study was a randomized window-of-opportunity trial designed to test the impact of a pre-operative exercise intervention on tissue and serum biomarkers in women with newly diagnosed breast cancer. The trial was designed to provide some of the first information regarding the impact of exercise on human breast cancer, providing insight into the biologic pathways through which exercise could impact cancer risk and outcomes.

## Materials and Methods

### Study population

Participants were recruited from the Breast Oncology Clinics at the Dana-Farber Cancer Institute, the Brigham and Women's Hospital (Boston, MA) and the Smilow Cancer Hospital at Yale Cancer Center (New Haven, CT) between December 2011 and April 2015. Because of slow accrual, enrollment was halted at Yale in 2014. Eligibility criteria included having a new histologic diagnosis of invasive breast cancer, planning to undergo primary breast surgery, and baseline participation in fewer than 90 minutes of moderate or vigorous physical activity per week. Patients were excluded if they were taking hormonal agents at the time of breast cancer diagnosis, had diabetes or distant breast cancer metastases, were planning to undergo neoadjuvant systemic therapy, or had uncontrolled cardiac disease or other contraindications to moderate-intensity exercise.

Medical clearance was obtained from all participants' medical or surgical oncologists. The study was approved by the Institutional Review Board at the Dana-Farber/Harvard Cancer Center and registered on the clinicaltrials.gov website (NCT01516190). Informed consent was obtained from all participants before enrollment. All research was conducted in accordance with the United States Common Rule.

### Study design

This was a randomized window-of-opportunity trial evaluating the impact of a preoperative exercise intervention on tissue and serum biomarkers in women with newly diagnosed breast cancer (Supplementary Fig. S1). The primary outcome of the study was to evaluate the impact of the exercise intervention on Ki-67. Although there have been no prior trials evaluating the impact of exercise on Ki-67 in breast cancer in humans, exercise interventions have been shown to reduce breast cancer proliferation in animal models (9–12). In addition, Ki-67 is a proliferative marker linked to breast cancer prognosis that has been shown to decrease in response to short exposures of pharmacologic interventions in prior window-of-opportunity trials (14, 15). Secondary outcomes included tissue expression of cleaved caspase-3 and insulin receptor, tissue gene expression, tissue immune markers, and serum metabolic and inflammatory biomarkers.

Participants were randomized 1:1 to an exercise intervention or mind–body control. All participants from both institutions were randomized through the Quality Assurance for Clinical Trials (QACT) Core at Dana-Farber, which served as the coordinating center of the study. The exercise intervention group participated in an aerobic and strength training exercise intervention and participants randomized to the mind–body control group received a self-directed surgical preparation program consisting of a book and a relaxation audioguide. Study participation took place between enrollment and the time of surgery. Participants were stratified by tumor hormone receptor status (estrogen and/or progesterone receptor positive vs. both negative) and menopausal status (pre- or perimenopausal vs. postmenopausal). Tumor tissue was collected through a baseline biopsy and at the time of surgery. Participants also underwent fasting blood collection and assessment of physical activity and anthropometric measures at the time of study enrollment and again a few days before breast surgery. Changes in measures over time were compared between participants randomized to the intervention and control groups.

### Exercise intervention

Participants randomized to the exercise intervention received social/behavioral support to increase physical activity to 220 minutes of exercise per week, including 40 minutes of strength training and 180 minutes of moderate-intensity, aerobic exercise. Participants were required to take part in two 60–90 minutes supervised exercise sessions, led by American College of Sports Medicine–certified exercise trainers, per week and performed the remainder of their exercise as unsupervised aerobic training. As in prior trials conducted by our group (6, 16), and in accordance with exercise guidelines for cancer survivors from the American

College of Sports Medicine (17), sessions began with 30–45 minutes of at least moderate-intensity aerobic exercise, followed by 20 minutes of strength training. Six common strength-training exercises (for muscles of the chest, back, shoulders, quadriceps, hamstrings and gluteals, as well as biceps and triceps) were performed using variable resistance machines and free weights. The supervised sessions concluded with approximately 10 minutes of cool down and stretching.

Participants were required to complete additional aerobic exercise on their own. They were given a pedometer to help track home-based exercise and were asked to record the duration and type of aerobic exercise in a home exercise log. Pedometer measures and exercise journals were used to track progress and set weekly exercise goals for exercise participants.

### Mind–body control

Participants randomized to the mind–body control arm were given the book *Prepare for Surgery, Heal Faster*, as well as accompanying relaxation audioguide (18). The program focused on relaxation and visualization. Participants were instructed to read the book and listen to the audioguide twice daily.

### Measurements

Demographic data and disease information were collected at the time of enrollment. Study measures were collected at baseline and at the end of the intervention period, immediately before surgery. Anthropometric measures (weight and height) were used to calculate BMI ($kg/m^2$). Exercise was assessed with the 7-Day Physical Activity Recall (7-Day PAR) interview, an interviewer-administered instrument that has been demonstrated to correlate with changes in $VO_2$ max, body composition (19–21), and activity patterns generated through direct observation or activity monitors (21, 22). A fasting blood draw was collected for analysis of metabolic and inflammatory biomarkers previously linked to breast cancer. Serum samples were stored at $-80°C$ until assayed. Serum insulin, leptin, and adiponectin concentrations were quantified with a radioimmunoassay; C-Reactive Protein (CRP) concentration was quantified with an automated chemistry analyzer; and IL6 concentration was quantified with an ELISA. Baseline and postintervention serum samples were assayed simultaneously and in duplicate at the end of the study (coefficients of variation for all assays were $\leq$10%).

***Tissue collection.*** Participants underwent tumor tissue collection at baseline, before participation in the exercise or mind–body interventions, and again at the time of surgery. Initially, all participants were required to undergo a baseline research biopsy, to allow for collection of paraffin-fixed and fresh tumor tissue. The protocol was amended in 2013 to allow use of the diagnostic biopsy as a source of baseline tumor tissue, given technological advances allowing RNA sequencing (RNA-seq) from paraffin-fixed tissue. Tumor tissue collected at the time of surgery was processed within 30 minutes of excision to limit ischemic time and was cut into cores to allow similar processing for surgical and core tissue samples.

***Tissue analysis.*** Slides were made from the formalin-fixed tissue samples according to standard procedures. Three slides were made from each specimen for IHC: Ki67 (MIB-1, DAKO), insulin receptor (mouse monoclonal, 1:50 dilution, EMD Millipore), cleaved caspase-3 (Asp175,5A1E). Ki67 and CC3 were scored quantitatively using the Aperio Systems. Images were acquired using an Aperio ScanScope Digital Slide Scanner and ImageScope Viewer and automated scoring was performed within tumor areas designated by a breast pathologist, who was blinded to participant group assignment. Results were tabulated as the percentage positive nuclei for Ki67 and as H-scores (sum of percent $\times$ intensity levels) for CC3. Given the lack of a standardized scoring system for insulin receptor, results were tabulated as the percentage positive nuclei, staining intensity, H scores, Allred, and Allred intensity. H scores are included in the article with other measures available in Supplementary Materials.

Tumor samples were reviewed by a central breast pathologist, who was blinded to participant group assignment, and were macrodissected to include areas with at least 10% tumor cellularity. RNA was isolated using the RNeasy FFPE kit (Qiagen). Capture RNA-seq of the transcriptome coding regions was performed using the Illumina Truseq RNA access platform. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis was conducted using the Bioconductor package GAGE (Generally Applicable Gene-Set Enrichment for Pathway Analysis) from the R Studio. RNA-seq data were uploaded into the Gene Expression Omnibus (GEO) database (accession number GSE129508).

***Fluorescence IHC.*** A multiplexed fluorescence IHC assay was performed on 4-μm slides using the Leica Bond Rx autostainer. The antibody panel for detection of immune cells included CD4 (EP204, rabbit monoclonal, Cell Marque), CD8 (C8/144B, mouse monoclonal, Dako), FOXP3 (D2W8E, rabbit monoclonal, Cell Signaling Technology), CD56 (123C3.D5, mouse monoclonal, Cell Marque), and CD163 (NCL-L-CD163, mouse monoclonal, Leica Biosystems), along with Cytokeratin (CAM5.2, mouse monoclonal, Cell Marque) for masking the epithelial cells. Sequential tyramine signal amplified immunofluorescence was performed for each target, with DAPI counterstain. Each labeling cycle consisted of application of primary antibody, secondary antibody conjugated to HRP, and an opal fluorophore (Opal 520, Opal 540, Opal 570, Opal 620, Opal 650 and Opal 690, PerkinElmer, Inc.), respectively.

***Tumor annotation and digital image analysis for immunologic markers.*** Whole-slide images were acquired from stained slides using a PerkinElmer Vectra 3 imaging system (PerkinElmer, Inc.) and analyzed using Halo Image Analysis platform (Indica Laboratories). Image annotations were performed by a breast pathologist, blinded to participants group assignment, on hematoxylin and eosin–stained slides with the marked areas containing invasive carcinoma used for image analysis. An algorithm learning tool using the Halo image software was trained for glandular and stroma regions and cell segmentation was subsequently completed. Thresholds for the markers were set based on staining intensity by cross reviewing more than 20 images. Cells with intensity above threshold were defined as positive.

### Statistical analysis

The primary endpoint of the study was to assess changes in Ki-67 between baseline and the end of the study intervention (post–pre). Secondary outcomes measured over this same time period included changes in: serum metabolic and inflammatory biomarkers; tumor apoptosis (CC3); expression of insulin receptor (IR) and immune markers (CD8, CD4, FOXP3, CD56 and CD163) in breast tumor tissue; and tumor gene expression.

The planned sample size for the study was 50 patients. Patients were randomized 1:1 to an exercise intervention or mind–body control intervention. Participants were stratified by menopausal status (pre- or perimenopausal vs. post) and tumor hormone receptor status (estrogen and/or progesterone receptor positive vs. both negative). Because of the short duration of the intervention, loss of measurement was estimated to be 5%, resulting in an evaluable sample of 48 women (24 per arm). With an evaluable sample of 48 women, the study would have 85% power to detect a 5.7% difference in change of Ki-67 between the exercise and control groups, assuming a two-sided alpha of 0.1 and a SD of 7.3% for Ki-67 change that was observed over a 21-day period in the nonintervention arm of a chemoprevention trial reported by Dowsett and colleagues (23).

Data were analyzed according to the intention-to-treat principle. Baseline demographic, disease characteristics, prior treatment, and comorbidities were compared using the relevant descriptive methods for continuous and categorical variables. The primary outcome of change in Ki-67 (post–pre) was analyzed using the Wilcoxon rank-sum test. Analyses of secondary endpoints correlated the effect of the intervention with biomarker outcomes and changes in anthropometrics relative to baseline. Comparisons between treatment groups were conducted using the Wilcoxon rank-sum test or Fisher exact test for continuous or categorical outcomes, respectively.

RNA-seq analysis and quality control steps were performed using the VIPER, Visualization Pipeline for RNA-seq (24). This pipeline uses STAR (25) for the alignment and cufflinks (26) for the assembly of transcripts and normalization of the read counts per gene. Deseq2 (27) was used for differential gene expression and the GAGE package (28) was used for testing differential KEGG pathways.

## Results

Forty-nine participants were randomized, 27 to exercise and 22 to control (Fig. 1). Forty-two participants were enrolled at Dana-Farber Cancer Institute (Boston, MA) and 7 participants at Yale (New Haven, CT). One patient was found to be ineligible after randomization and two patients withdrew consent after randomization, resulting in a total sample size of 46 participants. Baseline characteristics were well balanced in the two arms (Table 1). Average age was 52, average BMI was approximately 30 kg/m$^2$, and the majority of participants were white and had hormone receptor-positive, HER-2–negative breast cancers.

### Physical activity and anthropometric outcomes

Participants were inactive at baseline, engaging in an average of 49 minutes of moderate or vigorous intensity physical activity/week. Mean time between enrollment and surgery was 29.3 days (range, 10–70). Participants randomized to the exercise intervention significantly increased exercise versus controls (increase of 203 $\pm$ 129 vs. 23 $\pm$ 76 minutes/week, $P < 0.0001$). There was no significant change in BMI over the intervention period in exercise or control participants ($-0.34 \pm 0.41$ kg/m$^2$ vs. $-0.07 \pm 0.83$ kg/m$^2$, $P = 0.27$).

### Serum biomarkers

Paired pre- and postintervention fasting serum samples were available from 44 study participants (Table 2). There was a significant reduction in leptin ($P = 0.008$) and a trend toward a decrease in IGF-1 ($P = 0.08$) over the intervention period in exercise participants compared with controls.

### Tissue biomarkers

Paired tissue samples from baseline biopsy and surgical excisions specimens were available from 39 participants. Missing samples occurred due to insufficient tumor tissue in biopsy samples and inability to obtain baseline biopsy tissue collected at outside hospitals. At baseline, Ki-67 was expressed in an average of 21% of cells (Table 3). There was no change in Ki-67 expression in exercise participants versus controls ($-1.1\% \pm 12.9\%$ vs. $-0.7\% \pm 8.1\%$, $P = 0.77$). There were also no significant changes

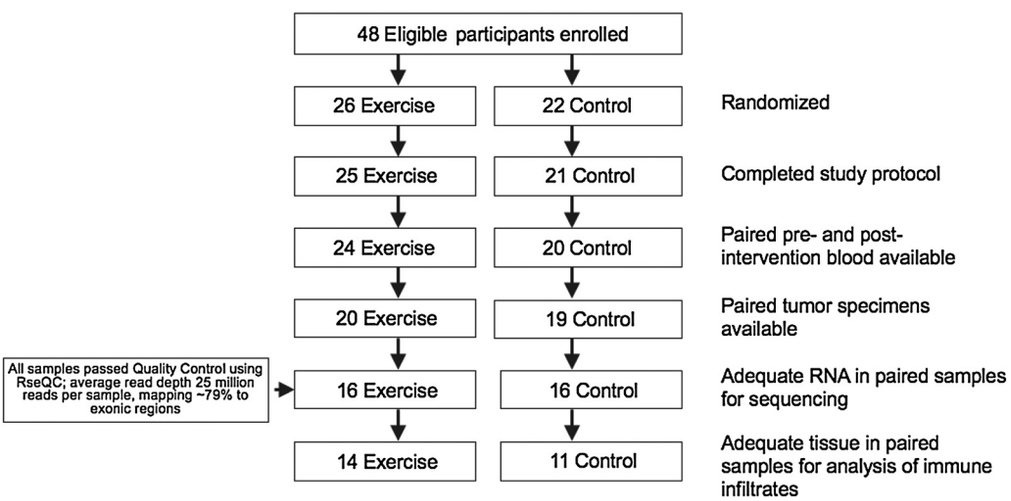


**Figure 1.**
Consort diagram.

**Table 1.** Baseline demographic, disease, and prior treatment characteristics

| | Exercise ($N = 26$) | Control ($N = 22$) | $P^a$ |
|---|---|---|---|
| Age (y, SD) | 52.3 (9.6) | 53.1 (7.9) | 0.80 |
| Body mass index (BMI; kg/m$^2$, SD) | 30.7 (6.1) | 29.1 (7.4) | 0.22 |
| Menopausal status | | | 0.78 |
| Pre/perimenopausal | 12 (46%) | 9 (41%) | |
| Postmenopausal | 14 (54%) | 13 (59%) | |
| Race | | | 0.90 |
| White | 22 (85%) | 18 (82%) | |
| Black | 1 (4%) | 1 (4%) | |
| Asian | 0 (0%) | 1 (4%) | |
| Other | 3 (12%) | 2 (9%) | |
| Ethnicity | | | 0.88 |
| Hispanic | 2 (8%) | 3 (14%) | |
| Non-Hispanic | 21 (80%) | 17 (77%) | |
| Unknown | 3 (12%) | 2 (9%) | |
| Estrogen receptor status | | | |
| Positive | 23 (88%) | 18 (82%) | |
| Negative | 3 (12%) | 4 (18%) | |
| HER-2 status | | | 0.46 |
| HER-2 positive | 4 (15%) | 1 (5%) | |
| HER-2 negative | 20 (77%) | 18 (82%) | |
| Unknown | 2 (8%) | 3 (13%) | |
| Tumor size (CM, SD) | 2.6 (2.2) | 2.1 (1.3) | 0.41 |
| Tumor stage | | | 0.60 |
| Stage I | 10 (38%) | 10 (45%) | |
| Stage II | 9 (35%) | 8 (37%) | |
| Stage III | 6 (23%) | 2 (9%) | |
| Unknown | 1 (4%) | 2 (9%) | |
| Tumor grade | | | 0.43 |
| Well differentiated | 4 (15%) | 4 (18%) | |
| Moderately differentiated | 8 (31%) | 11 (50%) | |
| Poorly differentiated | 13 (50%) | 6 (27%) | |
| Unknown | 1 (4%) | 1 (5%) | |
| Mean days between enrollment and surgery (SD) | 25.5 (12.2) | 33.7 (18.7) | 0.27 |
| Weekly minutes of moderate/vigorous physical activity (SD) | 49 (70) | 49 (52) | 0.51 |

Abbreviations: CM, centimeters; y, years.
[a]*P* values are estimated using Wilcoxon rank-sum tests for continuous measures and Fisher exact tests for categorical measures.

in expression of CC3 or insulin receptor in exercise participants versus controls (Table 2; Supplementary Table S1).

### Gene expression analyses

Sufficient tumor tissue was available from paired pre- and postintervention samples for transcriptomic analysis from 32 patients (16 exercise and 16 control). Quality control analysis of the RNA-seq data showed an average read depth of 25 million reads per sample, mapping approximately 79% to exonic regions. Principal Component Analysis revealed no read bias or batch effects. Unsupervised sample-to-sample clustering showed that the pre- and postoperative matched samples from the same patient co-aggregated at the level of the first dendrogram, with the exception of one matched pair (Fig. 2). The samples also clustered together by PAM50 subtype. There was no clustering by intervention group, menopausal status, or tumor cellularity. Differential gene expression analysis by DEseq2 did not reveal statistically significant changes in individual genes in the exercise group versus controls after correction for multiple comparisons using a statistical cutoff of log2 FC $\geq$1.5 or $\leq$−1.5, adjusted *P* value of <0.05 (data not shown). KEGG pathways analysis demonstrated upregulation of 18 unique pathways between the baseline biopsy and surgical excision in exercise participants and none in controls ($q < 0.2$; Fig. 3). The top-ranked upregulated pathway was cytokine–cytokine receptor interactions ($q$ = 6.93E−05, set size = 238 genes). Other pathways upregulated in the exercise group included the NF-κB signaling pathway, chemokine signaling pathway, natural killer (NK) cell–mediated cell cytotoxicity, Jak–STAT signaling pathway, antigen processing and presentation, and T-cell receptor signaling pathway.

### Tumor immune markers

Sufficient tissue was available to conduct exploratory analyses of immune infiltrates in pre- and postintervention tumor specimens from 25 participants (14 exercise and 11 control). Baseline expression of T-cell markers in tumor samples was low (Table 3),

**Table 2.** Serum and tissue biomarkers

| | | Intervention | | Wilcoxon rank-sum |
|---|---|---|---|---|
| | Assessment | Exercise Mean (SD) | Control Mean (SD) | *P* value |
| **Serum biomarkers ($n = 46$)** | | | | |
| Insulin, pmol/L | Baseline | 7.6 (5.1) | 8.0 (7.2) | 0.94 |
| | Change from baseline | −1.6 (2.9) | −0.8 (4.0) | 0.26 |
| Leptin, pg/mL | Baseline | 349.2 (231.0) | 264.8 (185.1) | 0.23 |
| | Change from baseline | −41.6 (81.3) | 2.1 (41.8) | 0.008 |
| Adiponectin, ng/mL | Baseline | 115.8 (44.2) | 107.6 (35.6) | 0.66 |
| | Change from baseline | −3.3 (14.3) | −2.2 (14.5) | 0.97 |
| IGF-1, ng/mL | Baseline | 1.0 (0.3) | 1.0 (0.2) | 0.41 |
| | Change from baseline | −0.1 (0.3) | 0.0 (0.1) | 0.08 |
| CRP, ng/mL | Baseline | 22.7 (12.8) | 19.6 (15.0) | 0.33 |
| | Change from baseline | −0.4 (12.7) | −1.5 (7.5) | 0.33 |
| IL6, pg/mL | Baseline | 2.0 (1.0) | 2.2 (1.6) | 0.63 |
| | Change from baseline | −0.0 (0.7) | −0.2 (1.1) | 0.82 |
| **Tissue biomarkers ($n = 39$)** | | | | |
| Ki-67, staining (%) | Baseline | 23.7 (14.6) | 18.5 (18.3) | 0.13 |
| | Change from baseline | −1.1 (12.9) | −0.7 (8.1) | 0.77 |
| Insulin receptor, H score | Baseline | 33.7 (34.8) | 56.0 (51.3) | 0.15 |
| | Change from baseline | 0.6 (22.7) | −1.7 (38.4) | 0.21 |
| Cleaved caspase 3, H score | Baseline | 45.5 (39.7) | 36.1 (34.9) | 0.65 |
| | Change from baseline | −6.6 (20.7) | 6.2 (29.4) | 0.13 |

**Table 3.** Tumor immune IHC biomarkers

| | | Intervention | | Wilcoxon rank-sum |
|---|---|---|---|---|
| | Assessment | Exercise Mean (SD) | Control Mean (SD) | *P* value |
| $CD4^+$ (%) | Baseline | 0.04 (0.05) | 0.36 (0.90) | 0.33 |
| | Fold change[a] | 36.57 (133.42) | 0.80 (0.82) | 0.64 |
| $CD56^+$ (%) | Baseline | 1.86 (2.15) | 2.14 (3.94) | 0.95 |
| | Fold change | 1.33 (2.34) | 0.90 (0.81) | 0.53 |
| $FOXP3^+$ (%) | Baseline | 0.44 (0.53) | 0.45 (0.77) | 0.68 |
| | Fold change | 0.76 (1.42) | 2.68 (3.57) | 0.08 |
| $CD8^+$ (%) | Baseline | 1.36 (1.38) | 1.43 (1.13) | 0.76 |
| | Fold change | 1.60 (2.51) | 1.44 (2.08) | 0.89 |
| $CD163^+$ (%) | Baseline | 5.85 (5.40) | 6.01 (7.85) | 0.89 |
| | Fold change | 1.40 (1.32) | 1.14 (1.27) | 0.85 |

[a]Fold change in the percentage of cell staining between baseline and post-intervention (exercise vs. control).

with less than 2% of cells staining for CD4, CD8, and FOXP3. A slightly higher proportion of cells stained for the macrophage marker CD163 at baseline (5.85% of cells in the exercise group vs. 6.01% in controls, $P = 0.89$). Fold changes (postintervention/baseline) in percent of cells staining demonstrated a trend toward a decrease in $FOXP3^+$ cells in the exercise group versus controls (0.76 vs. 2.86, $P = 0.08$). Changes in other markers were similar between groups. Sensitivity analyses including only ER and/or $PR^+$, $HER\text{-}2^-$ cancers (22 of the 25 of the paired samples) were similar to analyses including all evaluable tumor specimens (data not shown).

## Discussion

A preoperative exercise intervention led to changes in tumor gene expression but no change in Ki-67 in women with newly diagnosed breast cancer. Adherence to the exercise intervention was excellent, with participants randomized to exercise increasing physical activity by more than 200 minutes/week, and study attrition was low (<5%). Despite the short duration of the exercise intervention, physiologic effects were seen with changes in serum metabolic markers in exercise participants versus controls. Although no changes were detectable in expression of proliferative or apoptotic markers, pathway analysis of tumor gene expression demonstrated that exercise participants experienced upregulation of pathways related to inflammation and immunity,

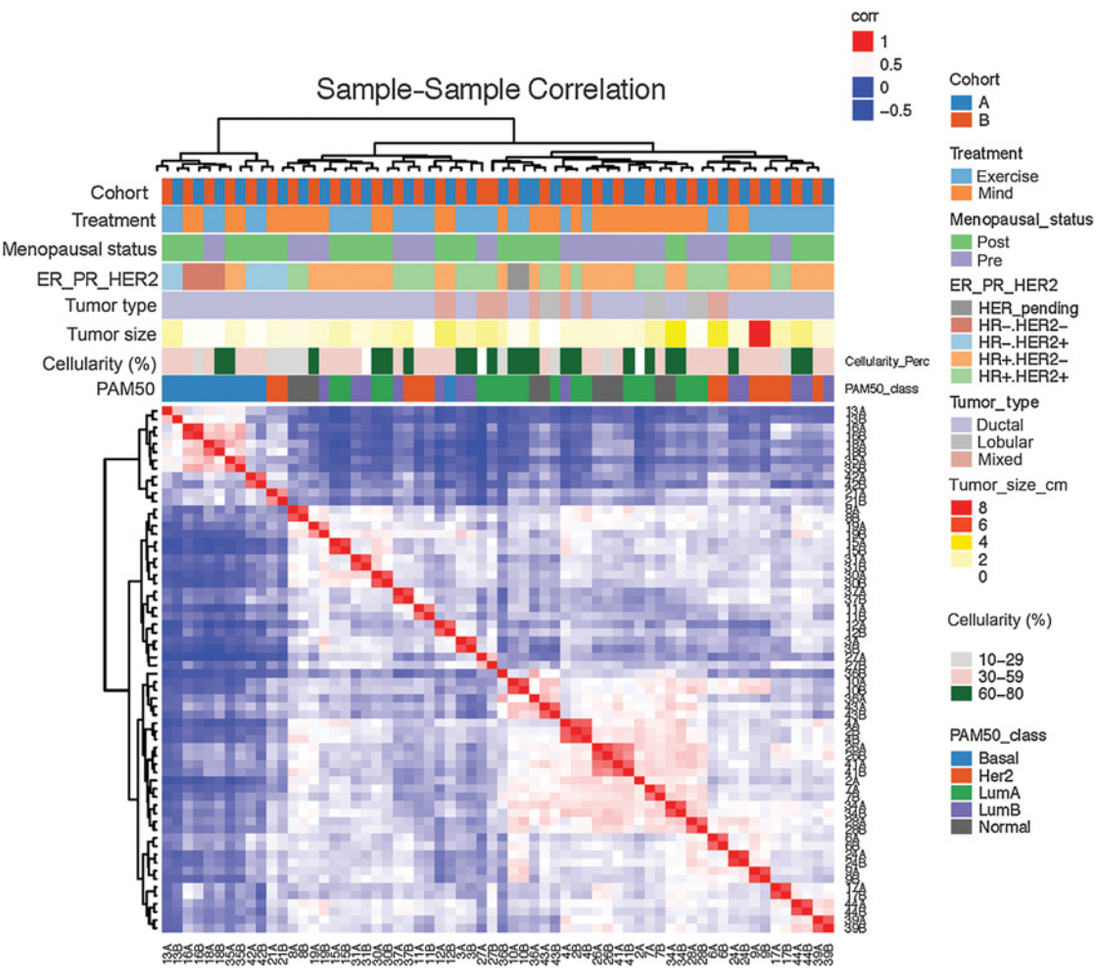


**Figure 2.**
Unsupervised sample-to-sample clustering with detailed clinical annotation.

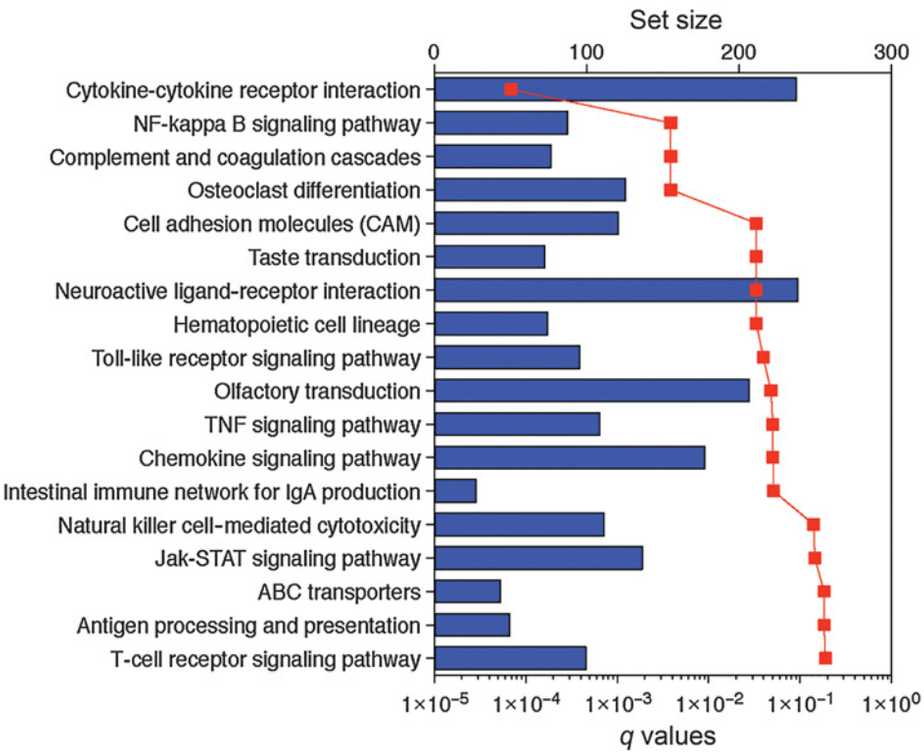


**Figure 3.**
Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of upregulated pathways in the exercise intervention group.

whereas controls experienced no pathway upregulation. Exploratory analysis looking at immune infiltrates also suggested changes related to exercise, with a trend toward a reduction in $FOXP3^+$ cells in response to the exercise intervention.

Acute and chronic exercise alter the quantity and function of immune cell types that comprise the innate and adaptive immune system in both the circulation and within certain tissue compartments (29). NK cells appear to be the most responsive immune cells to exercise, displaying an acute mobilization to the circulation during physical exertion (30). Emerging evidence from animal models suggests that NK cells play a pivotal role linking exercise to cancer control (13, 31). A recent preclinical study evaluating the impact of a voluntary wheel running intervention in 5 different murine tumor models demonstrated that the exercise intervention led to a 60% reduction in tumor incidence and growth (13). Gene expression analysis showed that the exercise intervention–induced upregulation of pathways associated with immune function and led to an IL6-mediated NK-cell infiltration of tumor tissue. A second experiment using a mouse model of breast cancer demonstrated that exercise delayed tumor development and promoted M1 activation of isolated peritoneal macrophages, whereas peritoneal macrophages from mice not exposed to exercise exhibited a pronounced protumorigenic M2 phenotype (32).

Less is known regarding the impact of exercise on immune function in women with breast cancer. A recent study in postmenopausal women with early-stage breast cancer demonstrated that 15 weeks of moderate-intensity aerobic exercise training enhanced the *ex vivo* cytotoxic capacity of NK cells in peripheral blood mononuclear cells (33). In our trial, exercise led to upregulation of pathways such as NK-mediated cytotoxicity, cytokine–cytokine receptor interactions (including upregulation of IL6), T-cell receptor signaling, and antigen processing, providing some of the first evidence that exercise could modulate the immune environment in human breast cancer. Exploratory evaluation of the impact of exercise on immune infiltrates also suggested that exercise led to a trend toward downregulation of immunosuppressive $FOXP3^+$ regulatory T cells. Although requiring validation in a larger dataset, our trial provides some of the first evidence that exercise could have a direct effect on human breast cancer, providing insight into the biological basis for the lower risk of breast cancer and improved outcomes in women who engage in regular exercise.

A number of limitations of our study must be acknowledged. Our study did not meet its primary endpoint, as exercise was not found to impact expression of Ki-67. Our sample size was small, especially for RNA-seq and immune marker analyses, and our patient population was heterogenous, potentially limiting our power to detect changes in biomarker expression between the exercise and control groups. Also, given that our trial used a window-of-opportunity model, the intervention period was short. The observed changes in serum markers suggest a physiologic effect of the exercise program despite the short duration of the intervention, but it is possible that more significant changes would have been seen with longer exposure to exercise. Participants also engaged in the exercise or control programs between the time of enrollment and the time of surgery, resulting in different lengths of exposure. Finally, our study compared two interventions, exercise and a mind–body (control) program, rather than looking at exercise versus usual care. An active control arm was chosen for this study to reduce the possibility that women assigned to the control arm would increase exercise, as well as to make the study more appealing to potential participants. A mind–body control program was chosen based on prior data, suggesting

that mind–body programs were unlikely to impact serum biomarkers and other physiologic outcomes (34–37). Of note, we did not see any impact of the mind–body program on biomarkers or gene expression, but it is possible that using an active control biased our findings toward the null.

In conclusion, we enrolled 49 women with newly diagnosed breast cancer to a novel randomized window-of-opportunity trial testing the impact of an exercise intervention on tissue and serum biomarkers linked to breast cancer prognosis. Exercise participants significantly increased exercise and experienced favorable changes in serum metabolic markers and upregulation of genes in pathways involved in inflammatory and immune response in tumor tissue. These findings are analogous to findings in animal models, where exercise interventions induce activation of immune pathways, reducing carcinogenesis. Although validation is needed in additional studies, this trial provides some of the first evidence in humans that exercise could directly affect human breast cancer.

## Disclosure of Potential Conflicts of Interest

D. Dillon is a consultant/advisory board member for Novartis and Oncology Analytics. M. Brown reports receiving commercial research grants from Novartis, and is a consultant/advisory board member for Kronos Bio, Aleta Biotherapeutics, and GTx, Inc. E.P. Winer is a consultant/advisory board member for Genentech, LEAP, Lilly, Carrick Therapeutics, GlaxoSmithKline, Seattle Genetics, and DragonFly. R. Jeselsohn reports receiving commercial research grants from Pfizer. No potential conflicts of interest were disclosed by the other authors.

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