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Physical Activity and Differential Methylation of Breast Cancer Genes Assayed from Saliva: A Preliminary Investigation

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Abstract

Purpose—Individuals who exercise are at lower risk for breast cancer and have better post-diagnosis outcomes. The biological mechanisms behind this association are unclear, but DNA methylation has been suggested.

Methods—We developed a composite measure of DNA methylation across 45 CpG sites on genes selected a priori. We examined the association of this measure to self-reported physical activity and objectively measured cardiovascular fitness in a sample of healthy nonsmoking adults (n = 64) in an exercise promotion intervention.

Results—Individuals who were more physically fit and who exercised more minutes per week had lower levels of DNA methylation. Those who increased their minutes of physical activity over 12 months experienced decreases in DNA methylation.

Conclusions—DNA methylation may be a mechanism linking exercise and cancer incidence, and could serve as a biomarker for behavioral intervention trials. Studies with larger samples, objectively measured exercise, and more cancer-related markers are needed.

Keywords

DNA methylation; breast cancer; fitness; physical activity; biomarker

Introduction

Across numerous meta-analyses and reviews, physical activity has been associated with reduced risk of developing breast cancer [1–3]. Physical activity *after* a cancer diagnosis also has implications for psychological and mortality outcomes [4–6]. For example, a recent meta-analysis of physical activity and breast cancer survival found that post-diagnosis physical activity reduced breast cancer deaths by 34% [5]. Clearly, physical activity plays some role in breast cancer prevention as well as outcomes following diagnosis. However, the mechanisms behind this relationship are unclear, although roles for the immune system, hormonal influences and body weight have been suggested [7, 8]. In this study, we consider another plausible biological mechanism that may explain the effect of exercise behavior on

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breast cancer prevention: mediation by DNA methylation of genes associated with breast cancer.

DNA methylation is an epigenetic process, meaning it is a genomic factor that—unlike genotype—can change in response to the passage of time, development, or exposures in the environment. Methylation is typically studied either at a global level [e.g., examining many repetitive DNA elements such as the long interspersed repeat sequences (LINE-1)], or at a gene-specific level (e.g., examining methylation at specific genes). The present study examines gene-specific methylation, in which a methyl group (one carbon atom bonded to three hydrogen atoms) is added to DNA at a cytosine-guanine (CpG) dinucleotide: where a cytosine (C) nucleotide is located next to a guanine (G) nucleotide linked by a phosphase (p) molecule on a particular gene. At gene promoters, CpG dinucleotides often cluster together in what are called 'CpG islands' [9]. In normal, healthy cells, CpG islands are unmethylated. This allows the information in DNA to be copied into a new molecule of messenger RNA (mRNA) that is then used in protein synthesis. CpG dinucleotides at locations other than CpG islands are typically methylated.[10]. This pattern is disrupted in cancer, such that cancer cells are characterized by an overall loss of DNA methylation compared to normal cells (global hypomethylation at sites other than CpG islands), as well as increased methylation (hypermethylation) at the CpG islands of genes involved in cell cycle regulation, tumor cell invasion and DNA repair. These abnormalities lead to instability in the gene and, ultimately, the production of tumors [i.e., production of new tumors; 11, 12].

A considerable body of work to date has focused on DNA methylation at CpG sites in genes associated with breast cancer [6, 13–16]. While the evidence for DNA methylation effects on breast cancer development and prognosis are increasingly clear, the next step is to understand the behavioral and environmental stimuli that may mitigate or exacerbate the epigenetic modifications that increase the risk for breast cancer. A number of environmental and behavioral factors that may alter methylation have been postulated including cigarette smoking [17], dietary habits [18], and exposure to infectious agents, nonsteroidal anti-inflammatory drugs, and toxins [19]. Perhaps because of the compelling epidemiological evidence that exercise is linked to lower incidence of breast cancer as well as better treatment outcomes after breast cancer diagnosis [5], a growing number of breast cancer researchers have focused on the relationship between physical activity and methylation. Much of this work has utilized tumor cells in cancer patients [13], or breast tissue in cancer cases versus controls [6, 14]. Studies on cancer-free populations suggest that lifestyle factors may be associated with increased methylation of cancer genes [20], indicating that assessment of degree of methylation in currently healthy individuals could potentially serve as a preclinical biomarker of risk for the development of cancer.

Two studies to date have empirically tested the effects of exercise on DNA methylation. The first compared six months of regular moderate exercise to six months of no exercise among older adults aged 40–87 [21]. After six months of regular exercise, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) methylation was higher among participants in the exercise condition compared to participants in the control condition. Note that methylation of ASC is associated with lower levels of inflammation [22] and ASC methylation may decrease with age, thus *higher* ASC methylation is desirable. Importantly, *change* in methylation was not measured in this study, but rather methylation was measured only at posttest, after six months of the program. The second study examined global methylation at LINE-1 sequences via peripheral blood among healthy older adults in the North Texas Healthy Heart study [23]. In this case, *higher* levels of LINE-1 methylation are desirable as lower methylation of LINE-1 elements is associated with cancer. Physical activity was measured via accelerometry and individuals with more than 26 minutes per day of physical activity had higher LINE-1 methylation than individuals with less than 10

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minutes per day. Unfortunately the effect was reduced to non-significance in multivariate models adjusting for covariates. As with the ASC study, these were cross-sectional data only, and the degree of physical activity in this sample was very low (mean moderate physical activity per day was 17.5 minutes). In sum, the existing links in the literature between physical activity and lower breast cancer incidence, DNA methylation and breast cancer, and physical activity and DNA methylation led to our focus in this analysis on the specific association of exercise with the methylation of CpG sites on genes associated with breast cancer in a currently cancer-free sample.

While discovery of a biologically plausible mechanistic association between exercise and reduced cancer risk via changes in DNA methylation is interesting from a basic science perspective, this question has the potential to directly inform applied intervention work to increase a range of health behaviors including physical activity. In the science of behavioral medicine, particularly with regard to behavior change in service of the prevention of illness, it can be difficult to quantitatively capture the efficacy of interventions. Disease outcomes are often in the distant future and are probabilistic in nature (e.g., sedentary young adults may not develop cancer until middle age, and even people with many risk factors for a disease are not guaranteed to get the disease). Even at the proximal level we are sometimes limited to the use of self-reported behavior change to document effectiveness. DNA methylation might serve as an objective tool behavioral scientists can utilize to quantitatively assess biological change occurring as a result of behavior change in a relatively short period of time. Methylation could be a biological outcome that could bolster self-reported behavior change findings in studies with the goal of prevention such as interventions to increase physical activity in currently disease free sedentary populations. More broadly, such a measure could also be used as an additional measure of the success of interventions to change a range of other behaviors (e.g., fruit and vegetable consumption, cigarette smoking) that may influence disease risk through DNA methylation.

At yet another level, the measurement of changes in DNA methylation as a result of exercise behavior change may also have the potential to encourage the maintenance of these changes. Informing individuals of their genetic risk for disease has been utilized to motivate initial behavior changes, and has shown some success, particularly in the area of cancer screening behavior [24]. It may be possible to utilize DNA methylation levels in a similar fashion, but in this case by providing a biologically based indicator that initial behavioral changes are successfully reducing risk for disease. Whereas other benefits of exercise behavior such as weight loss or reduction in cardiovascular risk may take longer to accrue or be difficult to perceive, DNA methylation might provide relatively more proximate and objective evidence of the success of initial behavior change.

We present a preliminary investigation to determine whether there is promise to the approach of examining *change* in methylation in the context of a behavior change intervention with healthy participants. With this objective in mind, the first goal of this paper is to explore the relationship between current level of self-reported physical activity (7-Day Physical Activity Recall, PAR) [25] and current fitness objectively measured by maximal oxygen consumption (VO_2 max) with methylation of a set of CpG islands on genes with the most *a priori* support for being involved in the pathophysiology of breast cancer. Second, we will examine the degree to which change in methylation at this set of sites is associated with changes in fitness and physical activity during an exercise promotion intervention trial. Third, in accord with studies that have explored methylation at a global level [e.g., 23] we will test the specificity of the relationship of physical activity behavior and fitness to methylation theoretically related to breast cancer by comparing these same variables to overall levels of DNA methylation across a large number of CpG islands.

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Methods

Participants

Participants were a subset of 238 participants from a larger exercise intervention study (COSTRIDE) [26]. Limitations in funding prevented us from completing methylation analyses on all participants. Those included in the analysis investigating the relationship between current physical activity, fitness, and DNA methylation included 100 individuals (84.0% female; 68.0% White) who were randomly selected from all individuals having DNA at baseline, completing a successful VO_2 max test, and a having a complete PAR assessment. Their mean age was 29.08 years, they reported an average of 105.31 minutes of physical activity in the past week, and reached an average VO_2 max of 33.13 ml/kg/min. Participants for the analysis investigating whether change in physical activity and fitness is associated with changes in DNA methylation included 64 of the 100 individuals (mean age=29.38 years; 82.8% female; 65.6% White) who had both baseline and 12-month DNA methylation data and who either increased their VO_2 max (increasers) over the course of the study or did not increase their VO_2 max (non-changers). Individuals in this subsample (n = 64) did not significantly differ by treatment condition on PAR, VO_2 max, or body mass index (BMI) at baseline (see Table 1). Additionally, VO_2 max increasers versus non-changers did not significantly differ on PAR, VO_2 max, or BMI at baseline. VO_2 max and PAR minutes were significantly and positively correlated at baseline ($r = .36$, $p < .001$). This research was approved by the University of Colorado Human Research Committee, the Scientific Advisory Committee of University of Colorado General Clinical Research Center (GCRC) and the University of New Mexico’s Human Research Review Committee for the protection of human participants in research.

Procedures

Intervention procedures—Colorado STRIDE (COSTRIDE) was a 12-month randomized controlled trial (RCT) testing an exercise promotion intervention [27]. Participants for the main study (n = 238) were selected because they reported being physically inactive at the time of recruitment (i.e., 90 minutes or less of voluntary moderate or greater physical activity per week on average over the past three months). After giving informed consent, participants completed three sessions: an orientation (baseline) session, a VO_2 max fitness assessment, and a submaximal exercise session. Participants were then randomized into either the STRIDE exercise intervention condition (n = 123) or a health-and-wellness contact control condition (n = 115). Follow-up assessments were conducted at 3, 6, 9, and 12 months following randomization. Baseline and follow-up questionnaires measured psychosocial motivational constructs and self-reported physical activity. Cardiorespiratory fitness (i.e., VO_2 max) was again assessed at the 12-month follow-up session. Full detail regarding participant recruitment and study procedures are available elsewhere [26]. In the analysis of the complete intervention dataset, we showed significantly greater increases in exercise, but not in VO_2 max, in the STRIDE condition as compared to the health-and-wellness contact control condition [28] and thus we controlled for intervention condition in the longitudinal analyses included in this paper.

Individuals were eligible if they were physically inactive, had a BMI between 18 and 37.5, were physically capable of engaging in moderate-intensity physical activity, had a regular menstrual cycle (if female), and were willing to be randomly assigned to one of the two interventions. Individuals were excluded if they smoked cigarettes, were on a restricted diet, were currently taking psychotropic medications, were receiving treatment for a psychiatric disorder, were diabetic, reported having a flu or illness in the previous month, had a history of cardiovascular or respiratory disease, or were pregnant (if female). Thus, individuals were inactive but otherwise healthy.

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Measures—Epidemiological studies typically utilize broad self-reports of physical activity status, assessed retrospectively over long time periods (e.g., lifetime), as opposed to assessments of recent and current activity level and fitness. In this study, *self-reported physical activity* was measured using the 7-day Physical Activity Recall (PAR) [25], a widely-used and validated [29, 30] researcher-administered interview assessing minutes and intensity of self-reported physical activity over the previous seven days. We utilized total minutes of physical activity including all intensities and activities. One week after the self-report interview, c*ardiorespiratory fitness* (VO_2 max) was assessed by measuring oxygen uptake using a Balke protocol [31] (a graded, incremental exercise test) on a motorized treadmill. Prior to the fitness test, saliva samples (5ml) were collected for DNA extraction and measurements of height and weight were taken for calculation of BMI.

DNA processing and SNP selection—Though still somewhat controversial, there is increasing evidence that DNA methylation patterns associated with cancer are detectable in peripheral tissues [32–36]. In some cases of breast cancer, for example, low-level promoter methylation of BRCA1 has been detected in buccal mucosal DNA and is associated with the development of BRCA1-like breast cancer [37]. Further, a recent study of two monozygotic twins demonstrated that the twin affected with breast cancer had significantly higher DNA methylation at BRCA1 assayed from saliva than did her cancer unaffected co-twin [38]. Therefore, we utilized DNA extracted from buccal cells taken from saliva. Quantitative DNA methylation measurements of purified DNA were performed using the Illumina Infinium Human Methylation27 BeadChip assay (Illumina, San Diego, CA). This platform targets approximately 27,578 CpG sites per sample at a single-nucleotide resolution, including about 144 CpG sites in methylation ‘hot spots’ in cancer genes (~ 7.6 sites per gene) and about 982 CpG sites in ‘cancer-related targets’ (approximately 1.9 sites per gene). The CpG sites represented on the HumanMethylation27 panel are located within the proximal promoter regions of 14,475 consensus coding sequences in the NCBI Database (Genome Build 36) [39]. Genomic DNA samples (1 μg) were treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA) to convert unmethylated cytosines to uracil, while methylated cytosines remain unchanged. Bisulfite deaminates unmethylated cytosine to uracil, while methylated cytosine is protected from deamination. Bisulfite treatment thus converts epigenetic information to sequence-based information, which can be measured with methods similar to those used to distinguish single nucleotide polymorphisms. The bisulfite-converted DNA was then whole-genome amplified, fragmented by an enzymatic process and hybridized to BeadChip arrays. Two oligonucleotide probes interrogated each CpG site, one probe with sequences targeting methylated DNA and the other containing sequences targeting unmethylated DNA. Using the Illumina Genome Studio software package, average beta values were calculated by dividing the methylated probe signal intensity by the sum of methylated and unmethylated probe signal intensities. Average beta values range from 0 (completely unmethylated) to 1 (fully methylated) and provide a quantitative readout of relative DNA methylation for each CpG site within the cell population interrogated.

Our goal was to select, *a priori* CpG sites linked to breast cancer acquisition and progression and to develop a composite measure [40, 41] of these sites. We first selected six genes with a total of 11 markers based on the extant literature identifying sites linked with breast cancer [13–15]. Given that the genes identified in the existing literature come almost exclusively from tumor cells in patients with active breast cancer (e.g., FBLN2, RUNX3, BRCA1) [13, 15], we thought it reasonable to also include genes associated more broadly with breast cancer, as it is possible that methylation at these sites has the potential to serve as a preclinical marker of breast cancer in individuals who do not currently have cancer. In order to find additional CpG sites meeting this criterion, we used the Illumina annotation file available on the Illumina web site (Illumina_HumanMethylation27-v1.2) to search the

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function of each of the remaining markers for "breast cancer" or "breast tumor" and eliminated any located on the X chromosome. Twenty-one genes with a total of 34 markers which had appropriate distributional properties (skew < 2, kurtosis < 4, $SD > .06$) were retained. A list of the 45 markers tested in this study is included in Table 2. This set of 45 markers was selected prior to any analysis of their association to physical activity. We hypothesized that DNA methylation across this set of markers would be negatively associated with self-reported level of physical activity and cardiovascular fitness.

Results

Correlation of Methylation with Baseline Fitness/Physical Activity

To control for the probability of Type I error inflation [40], we created a linear combination of the 45 markers identified *a priori* as potentially being associated with breast cancer by averaging beta methylation values of all 45 markers across all participants. Using a linear combination is an approach similar to the genetic prediction strategy taken in genome wide association studies [41], is similar to regression-based approaches using cross-validation in DNA methylation analyses of case-control samples [42, 43], and is the approach also taken in Nakajima et al. [21]. The average baseline methylation of these 45 markers was .456 (SD=.020; range: .417 to .496). Baseline average methylation was significantly related to baseline PAR minutes ($r = -.20$, $p = .05$) and VO_2 max ($r = -.31$, $p = .002$). The association between VO_2 max and methylation remained significant after controlling for BMI and age; however, the correlation of PAR minutes to average methylation did not remain after controlling for BMI ($r = -.19$, $p = .06$) and age ($r = -.16$, $p = .12$). Table 2 presents the individual correlations of VO_2 max and PAR minutes to each of the 45 individual markers.

Given that breast cancer primarily (but not exclusively) [44] affects women, we also computed these correlations excluding males. When excluding men from the analysis, baseline average methylation was no longer significantly related to baseline PAR minutes ($r = -.10$, $p = .38$), but the relationship with VO_2 max remained significant ($r = -.28$, $p = .009$). This association between VO_2 max and methylation remained significant after controlling for BMI and age. Overall, consistent with our hypothesis, the pattern of results suggests that higher fitness and more physical activity at baseline are associated with less average methylation in breast cancer genes.

Correlation of Change in Fitness/Physical Activity with Methylation

Among the individuals included in the longitudinal analysis, VO_2 max and PAR minutes were significantly and positively correlated at baseline ($r = .42$, $p < .001$) and marginally related at 12-months ($r = .24$, $p = .06$). There was no significant relationship between change in PAR minutes and change in VO_2 max ($r = .04$, $p = .71$).

To test whether changes in fitness variables were related to changes in methylation over the 12 months of the study, average methylation of the same 45 markers assessed at baseline was computed at 12-months (see Supplementary Table 1 for mean methylation values at baseline and 12 months for each individual CpG site along with change score means). For those participants included in the longitudinal analysis, average methylation of these 45 CpG sites increased from baseline .459 (SD=.017; range: .421 to .496) to 12 months .471 (SD=.010; range: .452-.495), $F(1,62) = 31.07$, $p < .001$, and this effect was not moderated by intervention condition ($p = .99$). The mean change in methylation (12-month minus baseline) was .012 (SD=.017; range: −.024 to .052). Baseline to 12-month change scores for both VO_2 max and PAR minutes values were also created. The 12-month average methylation composite was regressed on change in VO_2 max or PAR minutes controlling for baseline methylation. Thus, the regression analyses addressed the conceptual question of

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whether changes in VO_2 max and/or PAR minutes predicted changes in methylation. Increasing PAR minutes was significantly associated with a decrease in methylation at 12 months ($\beta = -.25$, $t = 2.09$, $p = .04$). This association remained significant controlling for BMI, age, and intervention condition in the model ($p = .02$), and when controlling for baseline VO_2 max ($p = .04$). Additionally, there was not a significant PAR minutes X intervention condition interaction ($p = .32$) nor was change in VO_2 max associated with methylation at 12 months ($p = .99$). This association remained nonsignificant when controlling for BMI, age, intervention condition, and baseline PAR minutes and there was no significant interaction with intervention condition ($p = .74$). Table 3 details the outcomes of the longitudinal regression analyses.

As with the baseline analysis, we also conducted the longitudinal analysis excluding men. Consistent with the above outcomes, increasing minutes of physical activity was significantly associated with a decrease in methylation at 12 months ($\beta = -.27$, $t = 2.11$, $p = .04$), and remained after controlling for BMI, age, and condition. Condition assignment did not moderate this relationship ($p = .32$). Change in VO_2 max was not associated with methylation at 12 months ($p = .66$) nor was there a significant interaction with intervention condition ($p = .86$).

To examine the specificity of increases in physical activity being associated with methylation of CpG sites on breast cancer genes, as opposed to DNA methylation more broadly, the average baseline methylation value was compared to the average 12 month methylation value. The average methylation value was computed by averaging the methylation values for each person across all CpG sites in the Illumina assay (excluding those on the X chromosome). There was a significant increase in average methylation from baseline ($M = .160$, $SD = .014$, range: .136-.205) to 12 months ($M = .172$, $SD = .011$, range: .162-.235), $F(1,62) = 26.57$, $p < .001$, and this effect was not moderated by intervention condition ($p = .66$). Although there was an overall increase in average methylation, change in PAR minutes and change in VO_2 max was not associated with average 12 month methylation when controlling for baseline methylation ($p = .19$ and $p = .53$, respectively).

Discussion

The connection between physical activity, cardiovascular fitness, and DNA methylation in humans was examined. This is only the second study of which we are aware examining DNA methylation before and after a period of increasing versus stable physical activity in healthy individuals (the first was Nakajima et al., 2010) [21]. These findings provide prospective preliminary evidence that a potential biological mechanism underlying the epidemiological association between exercise and decreased risk of various cancers is DNA methylation, and lay the foundation for larger intervention trials of this phenomenon.

A strength of this work is that we were able to control for both age and BMI in our analyses, as well as conduct analyses in a sample of nonsmokers, given that age, BMI, and smoking status are all associated with DNA methylation. Further, an advantage of this study is that each of the CpG sites used in this analysis and included in the linear combination was selected *a priori* on the basis of empirical literature supporting its association to methylation in breast cancer or on the basis of its function as being associated with the breast. In other words, this study was not a “discovery” study wherein each of the ~27,000 markers on the array were agnostically interrogated and chance findings resulting from Type I error, particularly in a sample as small as ours, would be highly problematic.

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The findings demonstrated significant associations between DNA methylation and both objectively measured cardiovascular fitness and self-reported physical activity at baseline, although the correlation was slightly larger with cardiovascular fitness. At 12-months, only change in physical activity minutes was associated with decreases in methylation. One interpretation may be that number of physical activity minutes more strongly influences changes in methylation than fitness. However, this interpretation disagrees with data suggesting that increases in cardiovascular fitness, resulting from high intensity exercise, are a better predictor of all-cause mortality than differences in physical activity minutes [45, 46].

A more likely interpretation is a lack of variability in VO_2 max change in this sample (average change = 2.32 ml/kg/min, *SD* = 2.87). VO_2 max is influenced by many factors including genetic variation [47] and physical activity level, and fairly intense, sustained physical activity is necessary to substantially increase VO_2 max. The intervention described here was intended to increase low to moderate-level exercise and focused almost entirely on walking behavior. Thus the activity level increases may not have been enough to result in substantial changes in VO_2 max, and in fact this assumption is supported by the finding that despite significant intervention effects on physical activity minutes in the larger trial, there was no significant intervention effect on changes in VO_2 max. Meanwhile, there was considerable variability in the change in reported PAR minutes (average change = 61.98, *SD* = 109.80), which potentially allowed for more power to detect an association. Future research should randomly assign and tightly control the intensity and duration of prescribed physical activity to examine whether it is intensity, duration, or some optimal combination of the two that most influences DNA methylation. This is crucial information for understanding the exercise prescriptions we ought to be recommending in behavioral interventions to increase physical activity [48].

Our analysis regarding breast-cancer specific versus global methylation changes suggests that our effects are stronger for the CpG sites chosen a priori for their association with breast-cancer. At first blush our findings seem at odds with the global methylation findings of studies such as Zhang and colleagues' [23] who found that global methylation was associated with physical activity. Our first conclusion is that in broad terms our findings are actually consistent with Zhang et al., in that in both studies a higher level of physical activity was associated with a "healthier" methylation profile. At DNA repetitive elements as examined in Zhang et al, *higher* global methylation is desirable and in that study higher methylation was associated with higher levels of physical activity. At CpG islands of genes associated with carcinogenic processes as examined in our study, *lower* methylation is desirable and in our study lower methylation was associated with higher levels of physical activity. The second conclusion is that the two studies examined two different types of methylation, i.e., methylation at multiple DNA repetitive long interspersed nucleotide elements (LINE) in Zhang et al. versus methylation change at the single nucleotide level across specific genes in our work, so it might be this measurement issue that accounts for the discrepancy in our findings. In truth, the field of epigenomics is really only beginning to understand the full range and complexity of methylation processes at work in the development of negative health outcomes [48], whether the effects are global or specific, and whether physical activity can significantly influence those processes. Additional research is needed to examine which forms of methylation are both related to disease and affected by behavior or the environment.

Clearly, the preliminary nature and small sample size of our study are significant limitations of the work. Our lack of direct control over duration and intensity of exercise was a further limitation. It is important that our results be replicated with a much larger sample size under more carefully controlled conditions. Our use of buccal cells from saliva samples is also a

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potential limitation of our work, as there is inconsistency in the literature regarding the conservation of DNA methylation across tissue types [49]. In addition, little is known about the temporal effects of physical activity on DNA methylation. The epigenome is known to be most labile in early development [50]. There is evidence that DNA methylation may be altered in response to short-term interventions [51], including a 6-month intervention of moderate-intensity aerobic exercise in breast cancer patients [6]; however, studies are needed to examine the longer-term effects of physical activity programs on DNA methylation on healthy individuals to determine the value of this marker for understanding the mechanism by which exercise is translated into reduced incidence of the occurrence of breast cancer, and for use as biomarker in longitudinal behavior change intervention trials.

We also chose to include males in our analysis. There is a much lower incidence of breast cancer among men than among women [44]. Yet males are not immune from breast cancer and many develop and die from breast cancer in the U.S. every year. To address the possibility that exercise may differentially influence methylation of genes associated with breast cancer among men and women, we repeated our analyses excluding males. The results, in large part, were identical to the main analysis (with the exception of a weaker relationship between PAR minutes and methylation at baseline), suggesting that the same mechanisms are likely at play among men and women regarding the relationship between physical activity, fitness, and methylation of breast cancer genes.

Our findings are important as they provide preliminary evidence of a relationship between exercise behavior, cardiovascular fitness, and methylation on CpG sites on a number of genes associated with breast cancer. These effects appear to be specific to genes associated with breast cancer, rather than applying to overall average methylation of CpG sites across a range of different types of genes. Larger studies with more diverse samples might fruitfully explore additional specific panels of markers associated with other cancers that have strong epidemiological links with exercise behavior such as cancer of the colon [52]. Our data are insufficient for this purpose. From a basic science perspective, data such as ours aid in the explication of the etiology of cancer and lifestyle factors that may disrupt its development. A better understanding of the modifiable lifestyle behaviors that limit the development of aberrant methylation patterns associated with cancer will enable recommendations of specific preventive behaviors and possibly result in more effective treatment of cancer and other methylation-associated diseases (e.g., neurological disorders, cardiovascular disease) [53, 54]. Further work is needed to examine the cellular mechanisms by which physical activity may modify DNA methylation. DNA methylation is primarily mediated by three known DNA methyltransferases (DNMT1, DNMT3a and DNA3b) that catalyze the transfer of a methyl group from S-adenosyl methionine to DNA [55]. Speculatively, the physiological consequences of regular physical activity may inhibit DNMT activity and thus disrupt methylation processes that may promote gene instability and cancer development. While our study cannot speak to this issue, it is clearly an important research question to pursue at the molecular level.

We speculated at the outset that DNA methylation may have the potential to serve as a quantifiable, biological outcome in preventive interventions such as interventions to increase physical activity in sedentary populations. Our findings indicate that this may be possible, although certainly additional work is necessary to clarify the clinical and practical importance of different magnitudes of change in methylation vis a vis disease prevention. What is perhaps more promising at this stage is the idea that DNA methylation might be used as a relatively immediate objective reinforcement that initial behavioral changes have resulted in noticeable changes in risk for disease. We acknowledge that this application of DNA methylation information is highly speculative and as yet untested, but given that the maintenance of exercise behavior change—or most other domains of behavior change for

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that matter—seems far more difficult to achieve than initial behavior change, any tool that may bolster motivation during the early stages of change is potentially highly valuable [48].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Baseline characteristics among participants randomly assigned to the STRIDE exercise intervention versus health and wellness (HW) control intervention

	HW (*n* = 27)	**STRIDE (*n* = 37)**	**Total (*n* = 64)**	***p*-value**
Baseline				
Age	28.44 (8.92)	30.03 (7.46)	29.38 (8.08)	.44
% female	85.2	81.1	82.8	.68
VO_2 Max (ml/kg/min)	32.60 (8.77)	33.62 (8.16)	33.19 (8.37)	.63
Total PAR minutes	77.67 (86.67)	77.46 (85.83)	77.13 (185.50)	.97
BMI	24.73 (5.44)	24.69 (4.18)	24.70 (4.71)	.98

Note: PAR = Physical Activity Recall, BMI=body mass index

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Table 2

Correlation of methylation at 45 sites with cardiorespiratory fitness (VO_2 max) and minutes of physical activity (PAR).

Marker	Gene	Chromosome	Location	VO_2 max	*p*	PAR	*p*
cg19094438	*BCAR1*	16	73843510	−.22	.02	−.15	.13
cg08927738	*BCAS1*	20	52120801	−.18	.08	−.07	.49
cg18917378	*BCAS1*	20	52120414	−.16	.12	.08	.44
cg14826456	*BCAS4*	10	115794085	−.32	.001	−.13	.20
cg21041127	*BCMP11*	7	140271834	−.04	.66	.01	.96
cg10590292	*BIN2*	12	50003941	.18	.08	.01	.90
cg21022247	*BIN2*	12	50004577	.21	.04	.07	.50
cg06973652	*BRCA1*	17	38532148	−.36	.000	−.17	.09
cg14048487	*BRCA1*	17	38526965	−.17	.09	.02	.81
cg19088651	*BRCA1*	17	38530739	.05	.64	−.03	.78
cg27383744	*BRCA1*	17	38507849	−.10	.33	.03	.79
cg12836863	*BRCA2*	13	31787023	.16	.12	.00	.98
cg05338167	*CALML4*	15	66285305	.17	.09	.00	.97
cg09099177	*CALML4*	15	66286421	.16	.11	.04	.73
cg08952029	*CHRDL2*	11	74120548	−.02	.83	−.07	.47
cg09949775	*COMP*	19	18763107	−.20	.04	−.13	.19
cg25935911	*DBC1*	9	121172082	−.20	.04	−.04	.99
cg15312298	*FAM84B*	8	127640090	−.13	.20	−.14	.16
cg00201234	*FBLN2*	3	13565968	−.24	.02	−.04	.68
cg10612997	*GREB1*	2	11591379	−.14	.17	.10	.30
cg10849854	*GREB1*	2	11592008	.13	.23	−.06	.58
cg09226684	*KLK10*	19	56211905	−.20	.04	−.21	.04
cg19356189	*KLK10*	19	56216502	−.16	.12	−.06	.54
cg26060255	*MRPS26*	20	2973968	.19	.05	.00	.69
cg19794490	*NCOA6*	20	32876604	.06	.57	.04	.70
cg25242557	*PAX6*	11	31789840	−.24	.02	−.17	.09
cg01775265	*RP11-49G10.8*	20	31244543	−.32	.001	−.10	.31
cg11854007	*RP11-49G10.8*	20	31245358	.12	.23	−.08	.44

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Marker	Gene	Chromosome	Location	VO_2 max	*p*	PAR	*p*
cg03171924	*RUNX3*	1	25130919	–.11	.29	.01	.90
cg04757093	*RUNX3*	1	25101779	.18	.08	.15	.13
cg13461622	*RUNX3*	1	25163972	–.22	.03	–.14	.16
cg14182690	*RUNX3*	1	25163534	–.22	.03	–.04	.69
cg24019564	*RUNX3*	1	25130153	–.21	.04	–.02	.82
cg25433648	*S100A14*	1	151855406	–.15	.13	.02	.85
cg19663795	*SEPT1*	16	30301355	.18	.08	.02	.82
cg04452095	*SEPT9*	17	72827463	–.05	.61	–.01	.91
cg12865837	*SIM1*	6	101018247	–.15	.15	–.23	.02
cg05046097	*SNCG*	10	88707906	.12	.22	–.14	.12
cg21012874	*SNCG*	10	88708373	–.13	.20	–.06	.57
cg05517572	*STAP2*	19	4289769	–.17	.08	.01	.92
cg21348412	*STAP2*	19	4290217	–.12	.22	.00	.97
cg05471521	*STK6*	20	54402011	–.16	.11	–.26	.008
cg25912611	*STK6*	20	54402033	–.24	.01	–.23	.02
cg02643667	*TFFI*	21	42659768	–.19	.06	.03	.75
cg18729973	*TFFI*	21	42658854	.16	.11	.02	.82
Average baseline methylation of the 45 sites				–.31	.002	–.20	.05

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Table 3

Predicting 12-month Methylation from VO_2 max change and change in PAR (Physical Activity Recall) minutes change controlling for baseline methylation.

	Mean (SD)	Range	B	SE B	β	*p*-value
Independent Variable						
VO_2 max change	2.32 (2.87)	−1.1 to 10.6	.000004	.0004	.001	.993
Controlling baseline PAR minutes			.00001	.0004	.003	.977
Controlling age, gender, & condition			.00006	.0004	.017	.895
VO_2 max change X condition effect			.0003	.0008	.07	.737
PAR total minutes change	61.98 (109.80)	−150 to 340	−.00002	.00001	−.25	.041
Controlling baseline VO_2 max			−.00002	.00001	−.26	.035
Controlling age, gender, & condition			−.00003	.00001	−.29	.016
PAR change X condition effect			.00002	.00002	.23	.324

NOTE: All analyses control for baseline methylation on the linear composite of methylation on breast cancer-related CpG sites.