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Recreational and household physical activity at different time points and DNA global methylation

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Abstract

Background—DNA methylation patterns are heritable but can change over time and in response to exposures. Lower global DNA methylation, which may result in increased genomic and chromosomal instability, has been associated with increased cancer risk. Physical activity is a modifiable factor that has been inversely related to the risk of cancer. Changes in DNA methylation may be a mechanism by which lifestyle and environment factors influence disease. We investigated the relationship between DNA methylation and physical activity in a sample of women enrolled in The Sister Study, a large U.S. cohort study of women aged 35–74 years with a family history of breast cancer.

Methods—Global DNA methylation was measured using bisulfite converted DNA and pyrosequencing of a LINE-1 repetitive sequence in the peripheral blood of 647 non-Hispanic white women. Physical activity (average hours per week) was retrospectively assessed for three time periods: childhood (ages 5–12), teenage years (ages 13–19) and the previous twelve months.

Findings—Compared with women with physical activity levels below the median for all three time periods, those at or above the median physical activity for one ($\beta = 0.20$, 95% CI: -0.10 , 0.49), two ($\beta = 0.22$, 95% CI: -0.08 , 0.52) or all three ($\beta = 0.33$, 95% CI: 0.01 , 0.66) time periods had increased global methylation.

Interpretation—Maintaining higher levels of physical activity over these three time periods was associated with increased global DNA methylation, consistent with reported associations between exercise and decreased cancer risk.

Keywords

DNA methylation; LINE-1; physical activity; pyrosequencing; Sister Study

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Introduction

DNA methylation is an epigenetic mechanism associated with altered gene expression patterns in cells. Aberrant methylation patterns have been associated with numerous diseases, including cancer. Epidemiologic and animal studies have shown that DNA methylation patterns are heritable but also change over the course of the lifetime.¹ Differences between monozygotic twins were larger for older twin pairs compared to younger twin pairs,² suggesting that lifestyle factors or environmental exposures, or both, may alter methylation levels.

The study of the role of DNA methylation in the development of cancer and other health outcomes and how exposures and lifestyle factors may influence these processes is a relatively new area of research. In cancer, the epigenetic profile is characterized by genome-wide under- or hypomethylation, which may be involved in tumorigenesis by promoting genomic³ and chromosomal instability,⁴ and over- or hypermethylation of CPG island promotor sites of specific genes, sometimes resulting in gene silencing.⁵ The methylation of repetitive elements, such as long interspersed repeat sequences (LINE-1), has been used as a surrogate measure of global methylation.⁶ DNA methylation in both target body tissues and in white blood cells has been associated with the risk of cancer,⁷ with lower levels of global methylation observed in cancer tissues⁸ and in the white blood cells of cancer patients compared to controls.⁹

There is a growing interest in identifying exposures, particularly modifiable environmental or lifestyle factors, that influence DNA methylation.¹⁰ Differences in global methylation in blood DNA have been observed by sex, age, race, body size, and alcohol consumption, but the patterns of association have been inconsistent across studies, and most studies have had small numbers of subjects.^{11, 12} Dietary exposures may be important; the results of some folate intervention studies suggest that adequate folate is necessary to maintain global methylation^{13, 14} but other studies have found no association.¹⁵ There has been special interest in studying exposures occurring in early life when the epigenome is thought to be most labile, with the idea that their effect on methylation levels may persist into adulthood.^{1, 15}

Physical activity is a modifiable lifestyle factor that has been associated with a reduced risk of colon, endometrial, and pre- and postmenopausal breast cancer.^{16, 17} Physical activity may influence cancer risk by reducing circulating levels of sex hormones.^{18, 19} Long-term exposure to estrogen is an established risk factor for breast, ovarian and endometrial cancers, and several possible mechanisms for this relationship have been postulated. Estrogen stimulates cell proliferation,²⁰ which may increase the opportunity for genetic errors during DNA replication and eventually lead to neoplastic transformation after sufficient numbers of mutations accumulate over time;²¹ estrogen has also been shown to silence tumor suppressor genes in normal breast cell lines.^{22, 23} Physical activity may also affect cancer risk by decreasing inflammation, which is an important factor in tumor progression.²⁴ Inflammation also has been shown to induce DNA methylation.^{25, 26} Physical activity has been shown to decrease chronic inflammation by reducing amplification of inflammatory mediators and initiating cytokine inhibitors.²⁷ In this study, we consider the possibility that the effects of physical activity may be mediated by changes in LINE-1 methylation across the genome, providing a possible biological mechanism linking physical activity and cancer and resulting in a more favorable methylation profile in regard to cancer risk.

Previous evidence suggests that increased physical activity, itself, may affect DNA methylation. A clinical trial of breast cancer survivors (n=12) found that after six months of moderate-intensity aerobic exercise, DNA methylation changes were observed in 43

genes.²⁸ Another study (n=64) observed that increased physical activity over 12 months was related to decreases in average DNA methylation across 45 CpG sites on genes related to breast cancer acquisition and progression.²⁹ A study of older adults (n=1016) found that levels of recent physical activity were inversely correlated with global methylation.³⁰

We investigated the association between self-reported physical activity at different times in life (childhood, teenage years and past 12 months) and LINE-1 methylation measured using pyrosequencing in a sample of women enrolled in the Sister Study, a large U.S. cohort study of women with a family history of breast cancer.

Methods

Study population

The Sister Study is a long-term prospective cohort study of genetic and environmental risk factors for breast cancer and other health conditions. Participants are 50,884 women living in the United States and Puerto Rico, ages 35 to 74, who had a sister diagnosed with breast cancer but did not have the disease themselves when they joined the study (www.sistersudy.org); study enrollment took place 2004–2009. For this analysis, we used a sample of participants because the time and cost for conducting methylation assays for all cohort members would have been prohibitive. Participants were randomly selected from 29,026 women who had completed baseline study activities by June 1, 2007 (n=705). Because of the possibility that effects could differ by race/ethnicity^{12, 31} the sample was further limited to non-Hispanic White women who were breast cancer-free at the time of sampling in May of 2008 (n=647). The Sister Study was approved by the Institutional Review Board (IRB) of the NIEHS and the Copernicus Group IRB, and participants provided informed consent.

Data collection

Participants completed computer-assisted telephone interviews at baseline covering demographic characteristics, environmental exposures, lifestyle factors including physical activity, medical history, and other possible risk factors for breast cancer. Trained examiners measured participant's height and weight and collected a blood sample during a home visit at enrollment. Body mass index (BMI) was calculated by dividing weight in kilograms by height squared in meters. Assessment of physical activity included sports and exercise activities, other recreational activities and physically active chores during three time points: childhood, teenage years, and the past 12 months. Specifically, for ages 5 to 19, women were asked to describe sports and exercise activities they participated in at least once a week for at least two months. For each activity, they were asked to report the age(s) they participated, and the average number of months per year (<3, 3–6, 7–9, >9) and hours per week (<1, 1–2, 3–6, >6) spent doing the activity. For ages 10 and 16, additional information was collected on the hours per week engaged in play activities, chores and other physical activities that were not part of an organized sports team or regular exercise program. For age 10 years, these included activities such as physically active play, bike riding, hiking, skating, dancing and playing ball, and for age 16, bike riding, hiking, skating, and dancing. For both ages 10 and 16, women also reported the average hours per week spent doing household or farm chores that caused sweating or increased heart rate.

For the past 12 months, women were asked to report sports or exercise activities they participated in at least once a week for at least one month. For each activity, women were asked how many months per year and how many days per week they engaged in the activity and the average minutes per day spent doing the activity on the days they did it (<20, 20–29, 30–59, 60–90 and >90). Women also reported how many minutes per day on average they

spent walking (<20, 20–29, 30–59, 60–90 and >90), and how many flights of stairs on average they climbed daily. The latter was converted to total number of flights climbed per week, multiplied by 10 seconds/flight and then converted to hours to give total hours per week spent stair climbing. In addition, women were asked the average hours per week they spent doing chores that would increase heart rate slightly such as vacuuming, mopping, scrubbing, or washing cars, as well as chores that would cause sweating and increase the heart rate substantially such as moving furniture or doing yard work.

For each time period (ages 5 through 12, 13 through 19 and currently), the average hours per week of recreational and household physical activity was calculated by adding the average hours/week across all activities. Mid-points of reporting categories were used in calculations. For chores, play, and other activities, data collected for age 10 was used as a proxy measure for childhood (ages 5 through 12) and data for age 16 was used to estimate these activities for the teenage years (ages 13 through 19). Using the data from all women, quartiles of activity were calculated for the 3 time periods. In addition, a summary variable was created to identify the number of time periods (childhood, teenage years, past 12 months) that a woman was at or above the median hours per week of physical activity (range 0–3).

Global DNA Methylation

Global DNA methylation was assessed by LINE-1 pyrosequencing. Briefly, genomic DNA (gDNA) was extracted using automated equipment (Autopure LS, Gentra Systems). Three DNA aliquots from each subject were independently bisulfite converted in a total of 49 separate batches. DNA was denatured by adding 5.5 μ L 2 M NaOH and incubating the sample at 37°C for 10 minutes. The cytosine deamination reaction occurs by adding 30 μ L freshly prepared 10 mM hydroquinone, followed by 520 μ L 3 M sodium bisulfite, pH 5.0, and incubating the sample at 50°C overnight. The samples were desalted using the Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA) and washed twice with 80% isopropanol. Column purified DNA was desulfonated by adding 5.5 μ L 3 M NaOH for 5 minutes. Samples were neutralized by the addition of 33 μ L 10 M NH_4Ac and ethanol precipitated overnight at –20°C. DNA was pelleted by centrifuging at maximum speed for 30 minutes at 4°C, followed by a wash with 70% ethanol and centrifugation at maximum speed at 4°C for 10 minutes. The bisulfite converted DNA was air-dried and resuspended in 10 μ L DEPC H_2O . Bisulfite-converted DNA was amplified using PCR with primers designed to recognize a consensus LINE-1 sequence (modified from Yang et al., 2004⁶): LINE-1-F, 5'-TTTTTTGAGTTAGGTGTGGG -3'; LINE-1-R, 5'- Biotin-TCTCACTAAAAAATACCAAACAA -3'; and, LINE-1-Seq, 5'-AGTTAGGTGTGGGATATAGT -3' (IDT, Coralville, Iowa, USA). The 25 μ L PCR reaction mix contained 1 $\times$ reaction buffer (Qiagen, Germantown, Maryland, USA), 1.5 mM MgCl_2 , 800 nM dNTPs, 5 pmol of forward and reverse primers, 0.8 U HotStarTaq polymerase (Qiagen), and 1 μ L bisulfite-converted DNA. Bisulfite-converted DNA was amplified using a 95°C hot start for 15 minutes, 45 cycles of amplification (95°C for 20 seconds, 50°C for 20 seconds, 72°C for 20 seconds), and a final extension at 72°C for 5 minutes. Following PCR, the biotin-labeled DNA product was bound to streptavidin-coated Sepharose beads (GE Healthcare, United Kingdom), purified, and denatured (0.2 M NaOH) to a single-stranded template. Pyrosequencing primers (0.3 μ mol/L) were annealed to the single-stranded template and the pyrosequencing run was carried out using PSQ HS 96 System (Biotage, Charlotte, North Carolina, USA). Percentage methylation was quantified using the PSQ Software (Biotage).

To adjust for day-to-day variability in bisulfite conversions and pyrosequencing (batch effect), a maximum of 3 different control samples were evaluated with study samples in each batch. A total of 10 control samples were used. They included MCF-7 breast cancer

cell line DNA (ATCC, Manassas, Virginia, USA), peripheral blood lymphocyte (PBL) DNA from a commercial source and peripheral blood lymphocytes (PBL) DNA from healthy female donors (Promega). Only the commercially available PBL DNA (Promega) was represented in all batches. To estimate an average control methylation level for each batch, we employed a linear mixed model with random batch effects using LINE-1 methylation data from all control samples across all batches. We then adjusted all raw sample methylation measures accordingly so that all averaged control methylation levels were the same.

Statistical analyses

We used linear mixed models, adjusting for batch effects, to examine associations between quartiles of physical activity (hours/week) at each of the three time points (childhood, teenage years and previous 12 months) and global methylation. In a separate linear mixed model, we assessed the cumulative effect of physical activity by comparing women who were at or above the median at 1, 2, or 3 of the time points to those who were below the median at all three time points. All models were adjusted *a priori* for age at blood draw. Effect measure modification was assessed for current BMI at an *a priori* alpha level of 0.10, but it was not found to be a significant modifier of the relationship between physical activity and LINE-1. We examined possible confounding by variables that, based on published studies, may be associated both physical activity levels and methylation. These included socioeconomic factors, relative body size during the childhood and teen years, current BMI, current folate consumption (units), alcohol consumption, smoking history and family history of breast cancer; none of these variables substantially affected the estimates and they were not retained in the models.

Results

The women sampled for this analysis were similar to other non-Hispanic white women in the Sister Study cohort with respect to demographic and lifestyle factors, including levels of physical activity (data not shown). Participants had median age of 55 years and were highly educated (over 50% reporting a bachelor's degree or higher), mostly non-smokers, and primarily light consumers of alcohol (<1 drink/day) (Table 1). Most women were overweight or obese. By design, all women had at least one sister with breast cancer; 28% had more than one first degree relative with breast cancer.

Median physical activity in hours per week was 12.5 [interquartile range (IQR 7.5–18.0)] for past 12 months, 5.9 (IQR 2.8–10.4) for teenage years, and 9.8 (SD 4.6) for childhood. The mean LINE-1 DNA methylation level was 76.20% (SD 1.2%). LINE-1 methylation appeared to increase with increasing quartiles of physical activity at all ages although no individual quartile association was statistically significant (Table 2). Comparing women in the highest quartile of physical activity to the lowest, differences in percent LINE-1 methylation were 0.26% (95% CI – 0.04, 0.55) for past 12 months, 0.24% (95% CI – 0.05, 0.53) for teenage years, and 0.17% (95% CI – 0.12, 0.46) for childhood.

Women who reported physical activity levels at or above the median at all 3 time periods had significantly increased methylation compared with those below the median at all time periods (0.33%; 95% CI: 0.01, 0.66). There was also a trend of increasing level of methylation with increasing number of time periods with exercise at or above the median (Table 3).

Discussion

Measuring global methylation using pyrosequencing of the LINE-1 element, we found that maintaining higher physical activity across three time periods (childhood, teenage years, and past 12 months) was associated with a statistically significant increase in DNA LINE-1 methylation in middle-aged white women with a history of breast cancer. Women who reported physical activity levels at or above the median for all 3 of the time periods (9.8, 5.9 and 12.5 hours per week for childhood, teenage years, and past 12 months, respectively) had significantly increased percent global methylation compared with those below the median for those 3 time periods. Women who were at or above the median for 1 or 2 of the time periods also had greater global methylation but the results were not statistically significant.

This study investigated childhood and teenage physical activity, along with recent adult physical activity, in association with global methylation. Previous studies have focused on recent physical activity. Zhang et al.³² investigated the relationship between physical activity and LINE-1 methylation in cancer-free adults in the North Texas Healthy Heart Study (n=161) using an accelerometer to measure current physical activity. Subjects who were physically active 26 to 30 minutes a day had higher levels of global methylation compared to those active 10 minutes or less per day, but the results were not statistically significant after adjustment for age, sex, and race/ethnicity. In the Commuting Mode and Inflammatory Response Study, New York, levels of LINE-1 and IL-6 promoter methylation were not correlated with levels of various physical activity measures including minutes per day of moderate leisure-time physical activity, vigorous leisure-time physical activity and job-related physical activity in a cancer-free subjects aged 18–78 years (n=165).³³ A study of 70 year-olds in Sweden (n=1016) found that amount and intensity of self-reported physical activity, measured as the number of times per week engaged in non-sweat-inducing and sweat-inducing activities, was inversely correlated with global methylation measured in leukocytes with the LUMA assay; results remained statistically significant after adjustment for sex, body mass index, blood pressure, cholesterol, and serum triglycerides.³⁰

Although currently unknown, mechanisms linking physical activity to DNA methylation might include physical activity altering sex hormone levels, which then alters DNA methylation, or physical activity changing inflammation, which could also lead to changes in DNA methylation. There is some evidence that physical activity may affect genes related to inflammation. After a 6-month intervention study of high intensity walking, older adults had increased promoter methylation of the ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) gene, which facilitates cytokine release, with methylation levels reaching those in younger adults.³⁴ It has been suggested that physical activity may reduce levels of inflammatory cytokines by increasing promoter methylation of ASC and suppressing transcription.³⁵

The differences in methylation we found were quantitatively small, but they are similar in magnitude to those reported in studies comparing people with and without cancer and previous reports on current physical activity and global methylation.^{30, 32} Previous studies did not take into account past levels of physical activity. If physical activity influences DNA methylation by decreasing inflammation or the amount of circulating sex hormones, processes that occur over long-periods of time, it is feasible that previous levels of physical activity are relevant to current DNA methylation status. Our study subjects were non-Hispanic white women aged 35 to 74 years with a family history of breast cancer, and therefore these results are not necessarily generalizable to the general population.

The strengths of this study include the use of a pyrosequencing method that is highly quantitative, detects small changes in methylation and used multiple replicates with independent bisulfite conversions for each sample. This study had more subjects than most previous studies examining factors associated with global methylation.¹¹ Collection of self-reported physical activity at different times during life allowed us to examine differences in percent global methylation that we would not have detected if we had assessed physical activity in the past 12 months only. We were also able to examine the possible confounding effects of several demographic, anthropometric, and lifestyle variables.

Our study also had some limitations. Information on physical activity was self-reported. In a systematic review of studies comparing direct measures such as accelerometry with questionnaire and other self-report physical activity measures, correlations were generally low to moderate.³⁶ The limited reliability and validity of questionnaires is a common concern for epidemiologic studies of physical activity, but more direct measures are generally not feasible for the large numbers of subjects in these studies.³⁷ Direct measures are also not feasible in studies attempting to collect retrospective data or cover extended periods of time. Duration of activity tends to be over-reported, perhaps because people tend to include time preparing for the activity or, for organized activities, time spent listening to trainer instructions or socializing. Despite these limitations, there is evidence that standardized questionnaires have practical value in broadly assigning subjects to physical activity categories that can be used to monitor populations. We would expect any misreporting to be independent of methylation status in our sample and for women in the top quartile to have spent more time participating in physical activity than the lower quartiles. Physical activity information was also collected retrospectively, and women were asked to recall childhood and adolescent activities that occurred many years in the past. The collection of physical activity history is challenging, and the extent to which current physical activity influences recall is unknown.³⁸

While we collected extensive information on recreational and household physical activity in childhood, teenage years and the previous 12 months, this measure does not encompass any occupational activities or transportation that might have contributed to total physical activity. For these analyses, we studied hours per week of physical activity and did not take into account intensity of the physical activity. It is not known whether the duration of activity, intensity of activity, general fitness or some combination of these measures most influences methylation. In a separate analysis, we examined methylation by quartiles of MET (metabolic equivalent) values³⁹ for the past 12 months, the only time period for which they were available (data not shown). Differences in methylation by quartiles of MET values were very similar to those for quartiles of hours per week of activity, suggesting that the two measures are correlated and that the amount of time spent in activity captured the relevant exposure. Although our measures of physical activity were likely imprecise, we may have minimized misclassification by categorizing women in broad categories by quartiles of activity levels.

In summary, we found that women who maintained higher physical activity levels across three time periods had small, but statistically significant increases in levels of percent global methylation compared to women who had lower activity levels. These results are consistent with the possibility that physical activity associated changes in DNA methylation may act as a possible intermediate of cancer risk. Physical activity and DNA methylation is a relatively new topic of inquiry, and further research is needed to establish the association between physical activity and methylation with more certainty and to elucidate the mechanisms underlying it. More work is needed to better understand what dimensions of physical activity are most relevant and what magnitude of change in global methylation may be important for disease prevention. Physical activity is an important modifiable risk factor and

further understanding of how physical activity is related to methylation may ultimately lead to better cancer prevention strategies.

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Table 1

Adult and childhood characteristics among a sample (n=647) of non-Hispanic white women aged 35–74 years in the Sister Study

	N	%
Age		
35–39	26	4.0
40–49	178	27.5
50–59	247	38.2
60–69	161	24.9
70–74	35	5.4
Education		
High school or less	81	12.5
Some college	113	17.5
Associates or technical degree	97	15.0
Bachelor's degree	183	28.3
Advanced degree	172	26.6
Current smoking		
Nonsmoker	359	55.5
Past	244	37.7
Current	44	6.8
Current alcohol		
Never	14	2.2
Former	80	12.4
Current, < 1 drink/day	445	68.8
Current, 1 drink/day	108	16.7
BMI		
Normal/underweight	277	42.7
Overweight	212	32.8
Obese	158	24.5
Weight relative to peers, age 10		
Lighter	216	33.5
Same weight	302	46.8
Heavier	127	19.7
Missing	2	
Weight relative to peers, age 16		
Lighter	202	31.3
Same weight	304	47.1
Heavier	139	21.6
Missing	2	
Number of first degree relatives with breast cancer		
1	453	72.0
2	154	24.5
3	20	3.2

	N	%
4	2	0.3
Missing	18	

Table 2

Associations between quartiles of physical activity (hours per week) during childhood, teenage years, and past 12 months and current age-adjusted¹ global methylation in a sample (n=647) of non-Hispanic white women aged 35–74 years in the Sister Study

Quartile	Range (hrs/week)	β^1	95% CI	p-value
Childhood, ages 5–12				
1	7	0	referent	--
2	7.01–9.80	0.04	–0.25, 0.34	0.8
3	9.81–12.43	0.11	–0.17, 0.40	0.4
4	12.44	0.17	–0.12, 0.46	0.2
Teenage, ages 13–19				
1	2.85	0	referent	--
2	2.86–5.90	0.17	–0.12, 0.46	0.3
3	5.91–10.43	0.11	–0.18, 0.40	0.5
4	10.44	0.24	–0.05, 0.53	0.1
Past 12 months				
1	7.52	0	referent	--
2	7.52–12.51	–0.05	–0.34, 0.24	0.7
3	12.51–18.03	0.13	–0.16, 0.42	0.4
4	18.03	0.26	–0.04, 0.55	0.09

¹ Adjusted for age at baseline data collection (continuous)

Associations between a summary physical activity variable¹ and current global methylation in a sample (n=647) of non-Hispanic white women aged 35 to 74 years in the Sister Study

	N	%	β #	95% CI	p-value
0	128	19.8	0		
1	205	31.7	0.20	-0.10, 0.49	0.2
2	178	27.5	0.22	-0.08, 0.52	0.2
3	136	21.0	0.33	0.01, 0.66	0.04

¹ Defined as the number of time periods (range 0 to 3) a woman was at greater than or equal to the median of physical activity (hrs/week)

² Adjusted for age at baseline data collection (continuous)