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DNA methylome analysis identifies accelerated epigenetic aging associated with postmenopausal breast cancer susceptibility

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Running title: Epigenetic age analysis of breast cancer

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Abstract

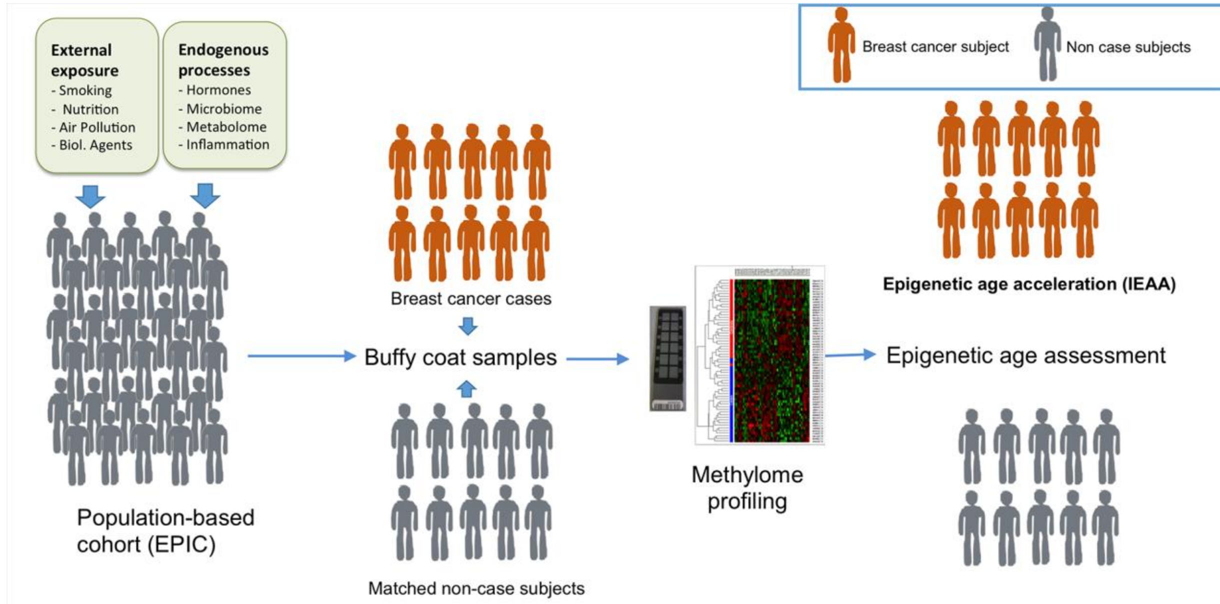
Aim of the study. A vast majority of human malignancies are associated with aging and age is a strong predictor of cancer risk. Recently, DNA methylation-based marker of aging, known as "epigenetic clock", has been linked with cancer risk factors. This study aimed to evaluate whether the epigenetic clock is associated with breast cancer risk susceptibility and to identify potential epigenetics-based biomarkers for risk stratification.

Methods. Here, we profiled DNA methylation changes in a nested case-control study embedded in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (n=960) using the Illumina HumanMethylation 450K BeadChip arrays and used the Horvath age estimation method to calculate epigenetic age for these samples. Intrinsic epigenetic age acceleration (IEAA) was estimated as the residuals by regressing epigenetic age on chronological age.

Results. We observed an association between IEAA and breast cancer risk (OR, 1.04; 95% CI, 1.007-1.076, P= 0.016). One unit increase in IEAA was associated with a 4% increased odds of developing breast cancer (OR, 1.04; 95% CI, 1.007-1.076). Stratified analysis based on menopausal status revealed that IEAA was associated with development of postmenopausal breast cancers (OR, 1.07; 95% CI, 1.020-1.11, P=0.003). In addition, methylome-wide analyses revealed a higher mean DNA methylation at CpG islands was associated with increased risk of breast cancer development (OR per 1 SD =1.20; 95 %CI: 1.03-1.40, P=0.02) whereas mean methylation levels at non island CpGs were indistinguishable between cancer cases and controls.

Conclusion. Epigenetic age acceleration and CpG island methylation has a weak but statistically significant association with breast cancer susceptibility.

Graphical abstract



Highlights

- Genome-wide DNA methylation in blood was measured in a large nested case-control study of breast cancer

- Epigenetic age acceleration is associated with risk of postmenopausal breast cancer

- Higher CpG island methylation leads to increased risk of developing breast cancer

Keywords: DNA methylation; epigenomics; age acceleration; breast cancer; biomarkers; prospective studies

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Introduction

Aging is a major risk factor for most neoplasms (1). In particular, breast cancer is an age-associated disease whose incidence raises sharply after menopause (1). This increased risk was proposed to be the consequence of accumulation of genetic changes (mutations) associated with deregulation of cellular processes and genomic instability. However, accumulation of genetic changes exhibits striking inter-individual differences (2), and differences in biological aging processes may only be partly explained by genetic determinants (3).

A recent study demonstrates that DNA methylation data lend themselves for developing a highly accurate multi-tissue biomarker of aging (4). The DNA methylation-based marker of aging (known as "epigenetic clock") derived from several tissues can be used to accurately estimate the chronological age of all tissues and cell types (4). This composite biomarker of aging, which is defined as a weighted average across 353 specific CpG sites, produces and estimate of age (in units of years), referred to as "epigenetic age" or "DNA methylation age" (DNAm age)". Recent studies demonstrate that DNAm age is at least a passive biomarker of biological age: the epigenetic age of blood has been found to be predictive of all-cause mortality (5-9), frailty (10), cognitive and physical functioning (5). Further, the utility of the epigenetic clock method using various tissues and organs has been demonstrated in applications surrounding Alzheimer's disease (11), centenarian status (8), prenatal and early life influences (12), Down syndrome (13), HIV infection (14), Huntington's disease (15), obesity (16), lifetime stress (17), menopause (18), and Parkinson's disease (19). Departures of methylation-estimated age from chronological age can be used to define intrinsic epigenetic age

1 acceleration (IEAA) that measures cell-intrinsic aging effects that are independent of
2 chronological age and blood cell composition.
3 A recent study suggests that IEAA can be used to predict lung cancer risk (20). However,
4 it is not yet known whether IEAA lends itself for predicting breast cancer susceptibility in a
5 prospective case-control study. To test this hypothesis, we analyzed blood
6 methylation data from incident breast cancer cases and matching controls of a large
7 prospective study within the European Prospective Investigation into Cancer and
8 Nutrition (EPIC) cohort.
9

1 **Materials and Methods**

2 **Selection of incident cancer and control participants**

3 The present study was conducted on nested case-control samples from the European
4 Prospective Investigation into Cancer and Nutrition (EPIC) cohort, a large prospective
5 study conducted in 23 centers across 10 European countries (Denmark, France,
6 Germany, Greece, Italy, Norway, Spain, Sweden, The Netherlands, and the UK), aiming
7 to investigate the relationship between diet, lifestyle, metabolism and cancer risk (21). In
8 brief, the EPIC cohort includes a total of about 315,000 women and 200,000 men. At
9 baseline recruitment, all study participants provided extensive questionnaire information
10 about nutrition and other lifestyle factors. All study participants also provided a blood
11 sample, which was processed, divided into aliquots of plasma, serum and buffy coat and
12 frozen at -196°C (under liquid nitrogen) for later use in specific research projects. In all
13 EPIC centers an identical protocol for subject recruitment, sample collection and storage
14 was followed. Detailed information on the subject recruitment, baseline data, and blood
15 collection protocols have been reported previously (22). All participants gave written,
16 informed consent for data and biospecimen collection and storage, as well as follow-
17 up. The study was approved by the local ethics committees and the Institutional Review
18 Board of the International Agency for Research on Cancer (IARC, Lyon, France).
19 During prospective follow-up of the EPIC cohort, a very large number (>11,000) of newly
20 diagnosed, invasive breast cancer cases were confirmed histologically or cytologically
21 as primary breast cancers according to the International Classification of Diseases for
22 Oncology, Second Edition (ICD-O-2) and included all breast cancer subsites (ICD C50.0-
23 C50.9). A representative sub-set of these cases was used for studies comparing a variety
24 of biomarker measurements with a set of control subjects, matching the cases by

recruitment centre. Incident patients with cancer were identified at regular intervals through population-based cancer registries (in Denmark, Italy except Naples, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) or by active follow-up (France, Germany, Greece, and Naples), which involved a combination of methods, including a review of health insurance records, cancer and pathology registries, and direct contact with participants and their next-of-kin.

For the purpose of this study, we included 960 females from the EPIC cohort including 480 incident breast cancer cases. Our main criteria for selection of case/control pairs included: (i) a balanced representation of the main subtypes of breast cancer, and (ii) representation of recruiting centres. One control participant was randomly assigned for each patient with breast cancer from appropriate risk sets consisting of all cohort participants alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis (and hence, age) of the index case. Matching criteria were: center, length of follow-up, age at blood collection (3 months relaxed up to 2 years for sets without available controls), time of blood collection, fasting status, menopausal status, menstrual cycle day and current use of contraceptive pill/ hormone replacement therapy.

Twenty technical replicates were included to compare inter and intra-array batch variation. Technical replicates and 38 samples or their matched counterparts which failed the quality control criteria were excluded from the analysis leaving 902 participants (451 controls and 451 cases) (Table1).

Bisulfite conversion and genome-wide DNA methylation analysis

The DNA was isolated as per the standard DNA extraction procedure from the from the buffy coat samples (Autopure LS, Qiagen). DNA methylome profiling was carried out using Illumina Infinium HumanMethylation450 (HM450) as previously described (23).

Bioinformatics analysis

Data pre-processing and analyses were performed using R 3.2.3 (https://www.r-project.org/) and Bioconductor 3.2 (24) as described before (23). DNA methylation level was described as a β value, which is a continuous variable ranging between 0 (no methylation) and 1 (full methylation). To avoid spurious associations, we excluded the cross-reactive probes and probes overlapping with a known single nucleotide polymorphism (SNPs) with an allele frequency of at least 5% in the overall population (European ancestry, (25)), leaving 423,066 probes. In any given sample, probes with a detection P-value (a measure of an individual probe's performance) of more than 0.05 were assigned missing status. If a probe was missing in more than 5% of samples, it was excluded from all samples. According to this criterion, we excluded 1483 probe, leaving 421,583 probes available for the analyses. We applied color bias correction followed by quantile and beta-mixture quantile normalization (BMIQ) to align Type I and Type II probe distributions (26).

White blood cell count estimates

Quantile normalized data were used to infer blood cell proportions. We estimate blood cell counts using two different software tools. First, Houseman's estimation method (27) was used to estimate the proportions of CD8+ T cells, CD4+ T, natural killer, B cells, and granulocytes (also known as polymorphonuclear leukocytes). Second, the advanced analysis option of the epigenetic clock software (4, 14) was used to estimate the

1 percentage of exhausted CD8+ T cells (defined as CD28-CD45RA-) and the number
2 (count) of naïve CD8+ T cells (defined as CD45RA+CCR7+). We and others have shown
3 that the estimated blood cell counts have moderately high correlations with corresponding
4 flow cytometric measures (27, 28). For example, flow cytometric measurements correlate
5 strongly with DNA methylation based estimates: r=0.63 for CD8+T cells, r=0.77 for CD4+
6 T cells, r=0.67 for B cell, r=0.68 for naïve CD8+ T cell, r=0.86 for naïve CD4+ T, and
7 r=0.49 for exhausted CD8+ T cells (28).
8
9 **Global and mean methylation analysis**

10 For the global DNA methylation analyses, mean methylation of the DNA methylation
11 probes (421,583) was calculated for cases and control samples. Human cancers are
12 characterized by global hypomethylation and a loci-specific DNA hypermethylation (29).
13 We hypothesized that DNA methylation of probes would vary based on their physical
14 location. To this end, the probes were classified into different categories either reflecting
15 their physical location in relation to CpG islands (island, shore, shelf and open sea) or
16 based on a functional criterion (DP: distal promoter, DS: distal sequence, GB: gene body,
17 IG: intergenic, and PP: proximal promoter) as previously described (30). A CpG shore is
18 defined as the area 2 kb on either side of the CpG island, and a CpG shelf is defined as
19 the area 2 kb outside of the CpG shore (31, 32). while the regions in the genome
20 containing isolated CpG sites outside CpG islands, shores and shelves, that do not have
21 a specific designation are referred to as open seas (33).
22 **Epigenetic clock of aging**

23 The epigenetic clock is a prediction method of chronological age based on the DNA
24 methylation levels of 353 CpGs (4). The predicted (estimated) age resulting from the
25 epigenetic clock is referred to as "DNA methylation age". Epigenetic age acceleration is

defined as the DNAm age left unexplained by chronological age where intrinsic denotes a modification to this concept. In addition to adjusting for chronologic age, IEAA also adjusts the DNAm age estimate for blood cell count estimates, arriving at a measure that is unaffected by both variation in chronologic age and blood cell composition.

We focused on IEAA in our blood based methylation study since this measure of age acceleration is significantly correlated with epigenetic age acceleration in (non-malignant) female breast tissue (9).

Formally, IEAA is defined by regressing DNAm age on chronological age and seven measures of blood cell count abundances (naive CD8 T cells, exhausted CD8 T cells (defined as CD28-CD45RA-), plasma blasts, CD4 T cells, NK cells, monocytes, granulocytes. IEAA is automatically calculated using the advanced analysis option of the epigenetic clock software (where IEAA is denoted as "AAHOAdjCellCounts"). By definition, IEAA is not correlated with chronological age or blood cell counts. A positive or negative value of IEAA indicates that the woman is older or younger than expected based on chronological age at the time of the blood draw.

Statistical analysis

For the mean methylation analysis, average methylation over all probes within each category was calculated and the odds ratios (per one standard deviation of global methylation) were estimated by conditional logistic regression model with case-control status as the outcome and the epigenome-wide methylation measurement as continuous predictor adjusting for surrogate variables (technical batch effects such as sample plate, array chips), alcohol consumption (g/day) and body mass index (as continuous variable).

1 Odds ratios (ORs) for breast cancer and 95% CIs were calculated by using logistic
2 regression for IEAA. Initial analysis was done using unconditional logistic regression to
3 allow calculation of OR. Multivariate logistic regression was performed by including
4 known breast cancer risk factors including alcohol consumption (g/day), full term
5 pregnancy (ever/ never), body mass index (as continuous variable and as
6 categorical variable: underweight, normal, overweight and obese), level of education
7 (none, primary, technical/profession, secondary, higher education), age at menarche,
8 Cambridge physical activity index (inactive, moderately inactive, moderately active and
9 active) stratified by clustering variable. A stratified multivariate conditional logistic
10 regression analysis based on the menopausal status was performed using the
11 aforementioned models.

Results

Baseline characteristics

The baseline characteristics of samples at the time of recruitment are listed in Table 1. Women were between 26 and 73 years of age with a mean age of 52.3 years for cases and controls. The majority of breast cancer cases were hormone receptor (ER and PR) positive (83%) while 17% of the breast cancers were triple negative (Table 1). There was a very high correlation between the intra- and inter-plate technical replicates (average correlation coefficient r^{2}=0.98 and 0.97, respectively, data not shown).

Hypermethylation of CpG islands is associated with breast cancer risk

We compared the global mean methylation across 421,583 probes and observed no difference between prospectively collected cases and matched controls (51.82% vs 51.86%, *P* =0.68). Our analysis showed that each unit (95% CI/1SD, 1.03-1.40, *P*=0.02) increase in methylation at CpG islands sites increased the risk of being a case by 20% (Table 2). While $P < 0.05$, it should be noted that the results would be marginally significant allowing for 4 sub-sets (CpG islands, CGI shores, CGI shelves, and open sea). No change in breast cancer risk was observed for other regions (shore, shelf and open sea) (Table 2), nor did we find an association of individual CpG site or region with breast cancer status.

Postmenopausal breast cancer cases exhibit DNA methylation age acceleration

Epigenetic age had a strong positive correlation with chronological age in both case and control samples (Figure 1a). We observed a marginally significant difference in age acceleration between prospective cases compared to matched controls (Figure 1b,

P=0.05, Supplementary Figure 1). Stratified analysis based on time from blood collection to disease diagnosis revealed that prospective breast cancers exhibited age acceleration 10 years prior to diagnosis compared to matched control samples (Figure 1c, *P*=0.01).

A conditional logistic regression model that relates breast cancer status to IEAA showed that IEAA was associated (Table 3) with breast cancer status. The results were not attenuated after adjusting for known breast cancer factors (Supplementary Table 1). Each unit increase in IEAA led to 4% increased odds of being a breast cancer case (OR, 1.04; 95% CI, 1.007-1.076, *P* = 0.016) (Table 3). IEAA follows an approximately normal distribution with mean zero, variance=28.2, standard deviation of 5.31. The following quantiles describe the empirical distribution of IEAA: minimum= -24.2, maximum 24.4, median=-0.12, first quartile=-3.0, third quartile=3.0). Thus, 25% of women had an IEAA value >3.

A very high value of IEAA=10 is associated with a doubling of odds of developing postmenopausal breast cancer (OR=1.97 calculated as 1.06^10 from our multivariate logistic regression model Table 3). Twenty five percent of all women exhibit an age acceleration larger than 3 which is associated with 22% increase in the odds of developing postmenopausal breast cancer (OR=1.22=1.07^3).

None of the blood cell count measures were associated with disease status in prediagnostic blood samples (Supplementary Figure 2). Interestingly, high physical activity was associated with decreased odds of being a breast cancer case (Supplementary Table 1).

A recent study demonstrated that menopause has a weak but statistically significant effect on epigenetic age acceleration. Further, menopause has been known to accelerate age-

related diseases including breast cancer (34, 35). To adjust for menopausal status, we evaluated the association between IEAA and breast cancer in separate strata defined by menopausal status (premenopausal and postmenopausal). The baseline characteristics of premenopausal and postmenopausal breast samples are shown in Supplementary Table 2. We observed a positive correlation between epigenetic and chronological age in postmenopausal samples (Figure 2a). Stratified analysis of postmenopausal breast cancers based on the lead-time between blood collection and cancer diagnosis revealed that breast cancers had a higher IEAA compared to non-cancer samples (Figure 2b, Supplementary Figure 3).

We found that breast cancer that developed within 10 years from date of recruitment had a stronger association with IEAA (Figure 2c). However, the results of this secondary analysis should be interpreted with caution due to an inflated false positive rate resulting from multiple comparisons. We did not observe such associations in premenopausal breast samples (Supplementary Figure 4, Supplementary Figure 5). Similar to our findings in all breast samples high physical activity was associated with decreased odds of being a breast cancer case in postmenopausal women (Supplementary Table 3).

Interestingly, we observed a highly significant association between IEAA and incident postmenopausal breast cancers (OR, 1.07; 95% CI, 1.020-1.11, P = 0.003). By contrast, no significant association could be observed for incident premenopausal breast cancers (OR, 1.00; 95% CI, 0.9510-1.056, $P = 0.94$) (Table 3).

Discussion

Using a rigorous and large scale nested prospective case-control study, we demonstrate a) that intrinsic epigenetic age acceleration in blood is predictive of postmenopausal breast cancers, and b) that genome-wide hypermethylation in CpG islands is associated with incident breast cancer cases. While several articles have studied blood methylation data versus breast cancer risk (36-39), it appears that ours is the first study to detect a weak but significant association with breast cancer susceptibility. Our study stands out in terms of its large sample size, its use of a robust epigenome wide technology (Illumina 450K array), the careful matching of breast cancer cases with controls in a prospective case-control study, and its use of a powerful epigenetic biomarker of aging which is independent of blood cell counts (IEAA).

Our finding regarding the association between global CpG island methylation levels and breast cancer risk is congruent with the findings from our earlier retrospective study on breast cancer (39) and supports the notion that regulatory regions of the genome are often hypermethylated in cancer cells (29). But it is noteworthy that we observed CpG island hypermethylation in blood tissue samples of incident breast cancer patients. Several epidemiologic case–control studies have reported global genomic hypomethylation in peripheral blood of cancer patients, suggesting a systemic effect of hypomethylation on disease predisposition (40, 41). In addition, two recent studies reported a lower global methylation levels in prospectively collected blood samples from breast cancer cases compared to controls (38, 42). However, we did not find any change in global DNA methylation levels between cases

and controls. These discrepancies may be due to technical and biological variations attributable to the low power of the studies.

Epigenetic changes are ubiquitous in primary breast cancers, although the role of deregulation of the epigenome is largely unknown. It has been suggested that a gradual accumulation of methylation changes (“epigenetic drift”) may occur through stochastic events, resulting in clonal expansion of the stem/progenitor cells, and that this process may contribute to the age-associated increase in risk of developing breast cancer (43-45). DNA methylation age is highly correlated to chronological age across sorted cell types (CD4 T cells, monocytes, B cells, glial cells, neurons), complex tissues (e.g. blood) and organs (brain, breast, kidney, liver, lung) (4). Our findings were consistent with the previous studies in different tissues (4, 16). The epigenetic clock derived from the DNA methylation age is robust with respect to the batch effects and can be applied to all Illumina array platforms: the EPIC chip (850K), the Illumina 450K array and the 27K array (4) and possibly measures a cell intrinsic and tissue independent epigenetic drift (46). For blood derived DNA measured on the Illumina 450K array, the epigenetic clock algorithm provides not only several measures of age acceleration but also estimates of blood cell counts. One of the major concerns regarding age-associated DNA methylation signatures is the influence of tissue’s cellular composition which may alter with age. We found no differences in leukocyte subpopulations between cases and controls. By definition, our intrinsic measure of epigenetic age acceleration (IEAA) is not confounded by changes in the proportion of blood cell counts (Methods). We focused on IEAA since it has been shown to be correlated with epigenetic age acceleration in breast tissue (9). Future research could investigate whether epigenetic age acceleration of breast tissue is predictive of breast cancer (11).

We can only speculate when it comes to explaining why IEAA was only predictive of postmenopausal breast cancer but not of pre-menopausal breast cancer. Breast cancers developing in postmenopausal women are influenced by specific polymorphisms in endogenous steroid hormone metabolic pathways and exogenous administration of hormones at menopause (hormone replacement therapy). Our observed age acceleration in postmenopausal breast cancers might reflect differences in hormone exposure. In this context, it is noteworthy that both natural and surgical menopause are associated with an increase in intrinsic age acceleration (18). In addition, age associated compromised detoxification, DNA repair mechanisms and immune surveillance may add to the endogenous factors which could lead to postmenopausal breast cancer development (1). It is unlikely that smoking and body mass index confound the relationship between epigenetic age and breast cancer risk because a) body mass index and smoking have only a very weak effect on the epigenetic age acceleration of blood tissue (correlation $r<0.10$) (16, 20), and b) we could detect accelerated aging effects in multivariate regression models that adjusted for these potential confounders. Our results based on a prospective study cohort points to a higher rate of aging in the blood samples from individuals who develop breast cancer compared to the controls. While the results from our epigenetic age analysis are biologically meaningful, the association between DNA methylation age and disease risk is probably too weak for prognostic purposes.

In the present study we demonstrated that a surrogate tissue (blood) captures accelerated aging effects and relates to an effector (breast cancer) of aging. We have demonstrated that IEAA was associated with postmenopausal breast cancer susceptibility and identified potential epigenetics-based biomarkers for risk stratification. Because menopause has been known to accelerate age-related diseases including

1 cancer, our finding also suggest potential underlying mechanism and provides biological
2 plausibility to the association between menopause and cancer risk. Further research
3 aimed at understanding epigenome deregulation in cancer causation, risk stratification
4 and the mechanism underlying accelerated epigenetic clock is warranted.

5

Table 1: Characteristics of incident breast cancer and control participants at baseline (i.e. time of blood collection).

		All samples	
		Controls (%)	Cases (%)
Sample size		451	451
Mean methylation (in %)		51.86	51.82
Age (years)			
	Mean (SD)	52.3 (8.94)	52.3 (8.97)
	Median	53.4	53.5
Alcohol consumption	Mean(SD)	8.2 (11.82)	10.0 (12.98)
Age at menarche	Mean (SD)	12.9 (1.34)	12.7 (1.59)
BMI	Mean (SD)	25.5 (4.22)	26.0 (4.72)
Physical activity (Cambridge index)			
	Sedentary	99 (22.0)	121 (26.8)
	Moderately sedentary	187 (41.5)	178 (39.5)
	Moderately active	76 (16.9)	87 (19.3)
	Active	78 (17.3)	62 (13.7)
	Missing	11 (2.4)	3 (0.7)
Hormone receptor			
	$ER^+/PR^+/Her2^+$	-	85 (18.8)
	$ER^+/PR^+/Her2^-$	-	290 (64.3)
	$ER^-/PR^-/Her2^-$	-	76 (16.9)
Country			
	Italy	160 (35.5)	160 (35.5)
	Spain	27 (6.0)	27 (6.0)
	UK	38 (8.4)	38 (8.4)
	The Netherlands	66 (14.6)	66 (14.6)
	Greece	25 (5.5)	25 (5.5)
	Germany	135 (29.9)	135 (29.9)

SD: Standard deviation

Table 2: Association between global methylation and breast cancer risk by CpG genomic feature per 1 SD of β methylation values in the EPIC study.

	Context	# CpGs	Std. dev.	OR (95% CI)*	P value
	All CpG sites	421 583	3.45E-04	1.09 (0.94-1.25)	0.21
CpG context	Islands	130 982	5.87E-04	1.20 (1.03-1.40)	0.02
	Open Sea	150 852	4.50E-03	1.49 (0.36-6.24)	0.58
	Shelf	40 948	4.88E-04	0.89 (0.78-1.02)	0.10
	Shore	98 801	5.40E-04	1.00 (0.87-1.16)	0.97
Genic context	Distal promoter	19 990	5.42E-04	1.06 (0.92-1.21)	0.44
	Distal sequence	7 828	6.68E-04	0.96 (0.84-1.09)	0.52
	Gene Body	168 460	3.80E-04	1.02 (0.89-1.18)	0.76
	Intergenic	56 903	5.35E-04	1.02 (0.89-1.17)	0.76
	Proximal promoter	168 337	5.26E-04	1.15 (0.99-1.34)	0.07

*** Odds ratio and confidence interval were calculated per 1 standard deviation**

*Odds ratios were adjusted for body mass index (continuous variable) and daily alcohol intake.

OR- Odds ratio, CI: confidence interval

Table 3: Logistic regression analysis of IEAA versus incident breast cancer status

		Univariate analysis	**Multivariate analysis***
All samples			
	IEAA	1.04 (1.007-1.075)	1.04 (1.007-1.076)
Premenopausal samples			
	IEAA	1.00 (0.9572-1.06)	1.00 (0.9510-1.056)
Postmenopausal samples			
	IEAA	1.06 (1.019-1.11)	1.07 (1.020-1.11)

OR: Odds Ratio; CI: Confidence Interval

IEAA: Intrinsic Epigenetic Age Acceleration

*Odds ratios were adjusted for physical activity (inactive, moderately inactive, moderately active and active)

Role of funding resource

The funders of the study had no role in study design, data collection, data analysis, data interpretation or writing of the manuscript.

Conflict of interest statement

The Regents of the University of California is the sole owner of a patent application directed at the invention of measures of epigenetic age acceleration for which Steve Horvath is a named inventor. The other authors declare no conflict of interest.

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Figure legends

Figure 1. Epigenetic clock analysis.

a) DNA methylation age (y-axis) versus chronological age (x-axis). Points correspond to female subjects. Red indicates breast cancer case, black control. The dashed line indicates a regression line, b) Epigenetic age acceleration versus breast cancer status. Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group comparison test p-value (Wilcoxon test), c) Epigenetic age acceleration versus breast cancer status (developed within 10 years post blood draw). Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group comparison test p-value (Wilcoxon test).

Figure 2. Epigenetic clock analysis for postmenopausal breast samples.

a) DNA methylation age (y-axis) versus chronological age (x-axis). Points correspond to female subjects. Red indicates breast cancer case, black control. The dashed line indicates a regression line, b) Epigenetic age acceleration versus breast cancer status. Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group comparison test p-value (Wilcoxon test), c) Epigenetic age acceleration versus breast cancer status (developed within 10 years post blood draw). Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group comparison test p-value (Wilcoxon test).

Figure_1
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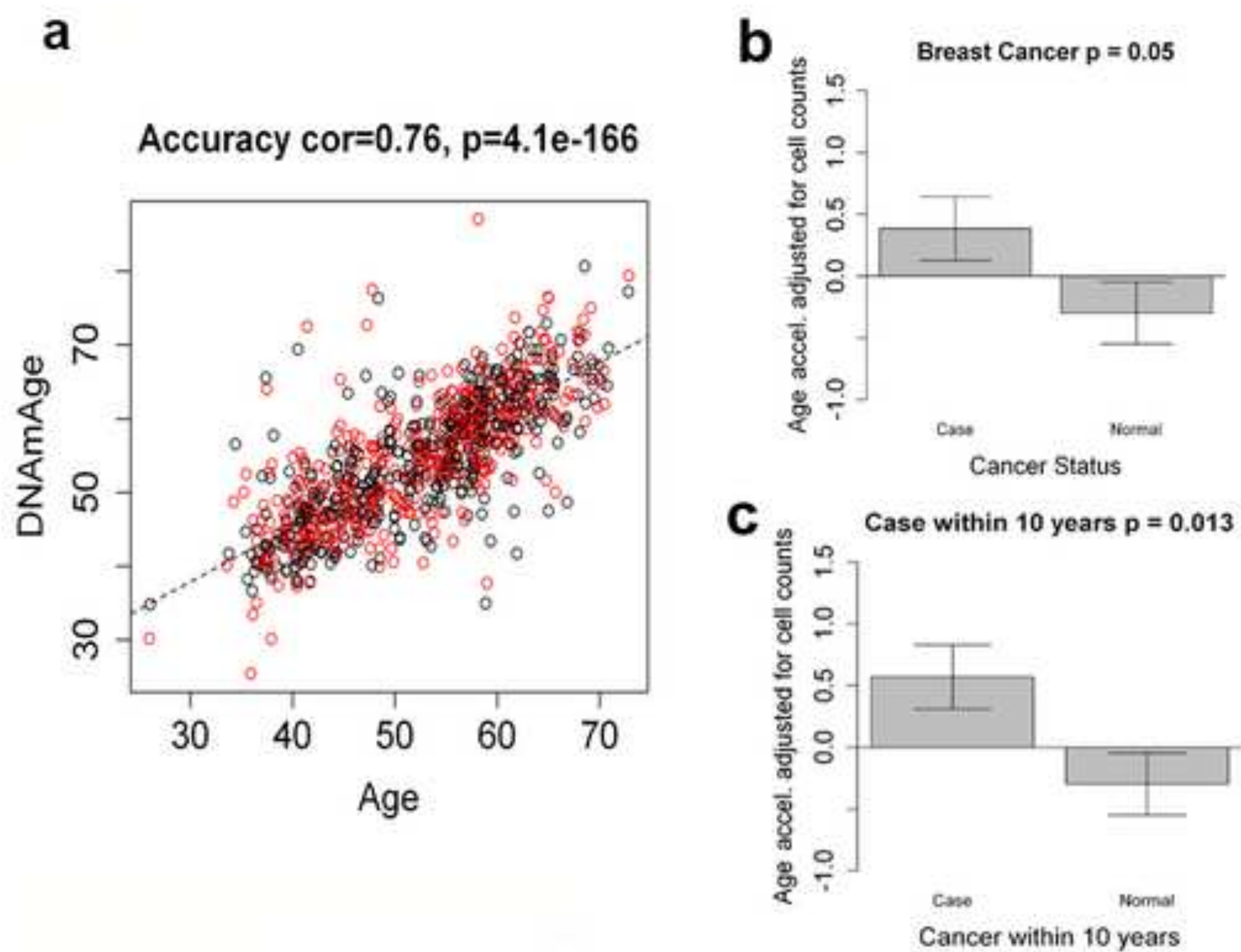

Figure 1

Figure_2
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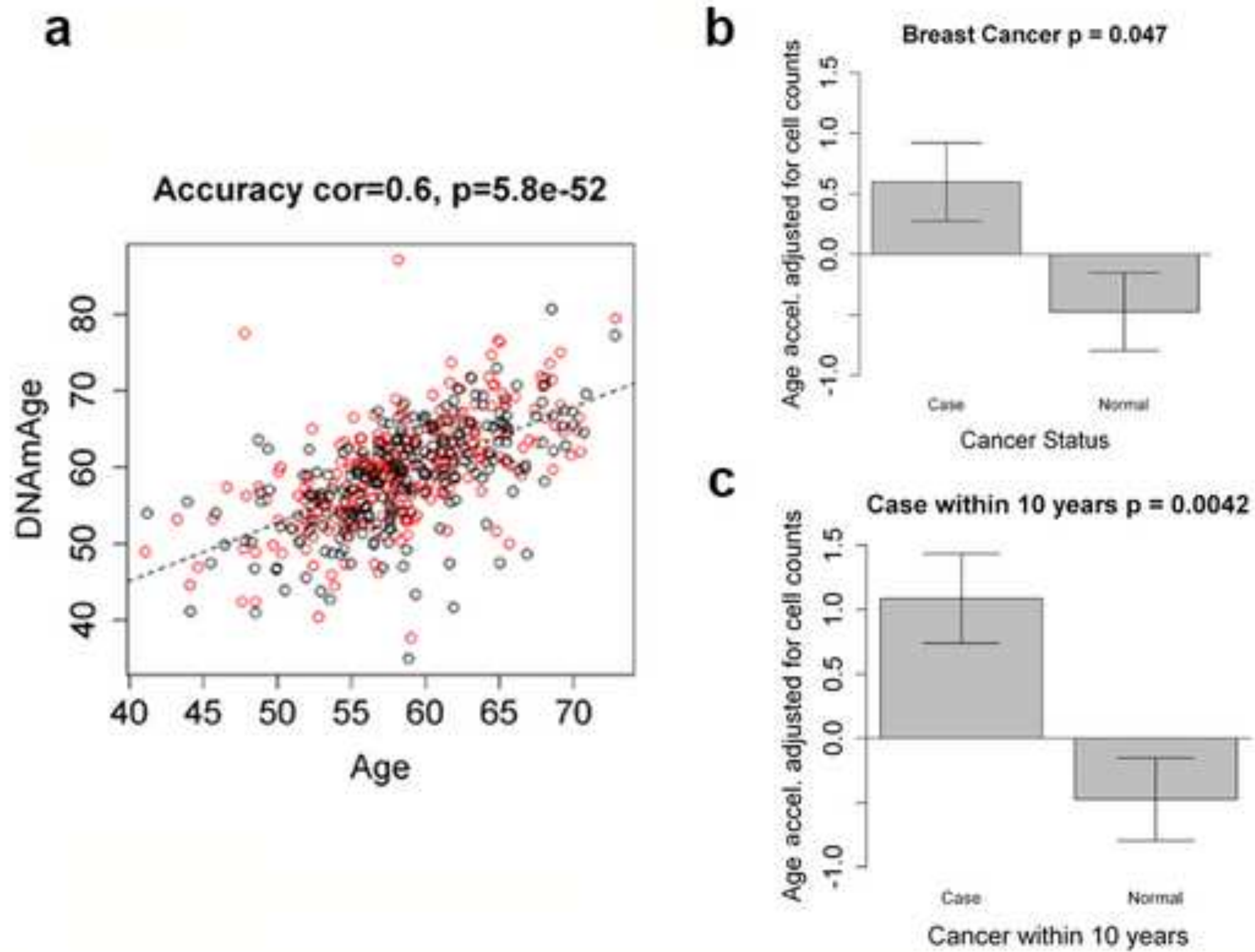

Figure 2

Conflict of interest statement

The authors declare that they have no actual or potential competing financial interests.

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Supplementary Figure (online publication only)

Supplementary Material

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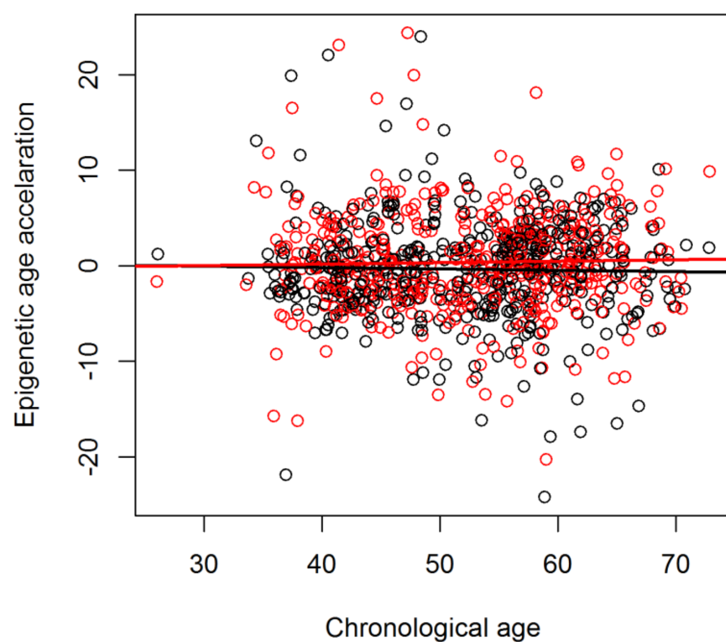

Supplementary Figure 1: Epigenetic age accelaration of breast samples.

Epigenetic age accelaration (IEAA) (Y-aixs) versus chronological age. Points correspond to female subjects. Red colored circles indicates breast cancer case while the black circles represent non-case samples. The solid solid linesss indicates a regression lines for cases (in red) and non-case samples (in black)s for cases (in red) and non-case samples(in black).

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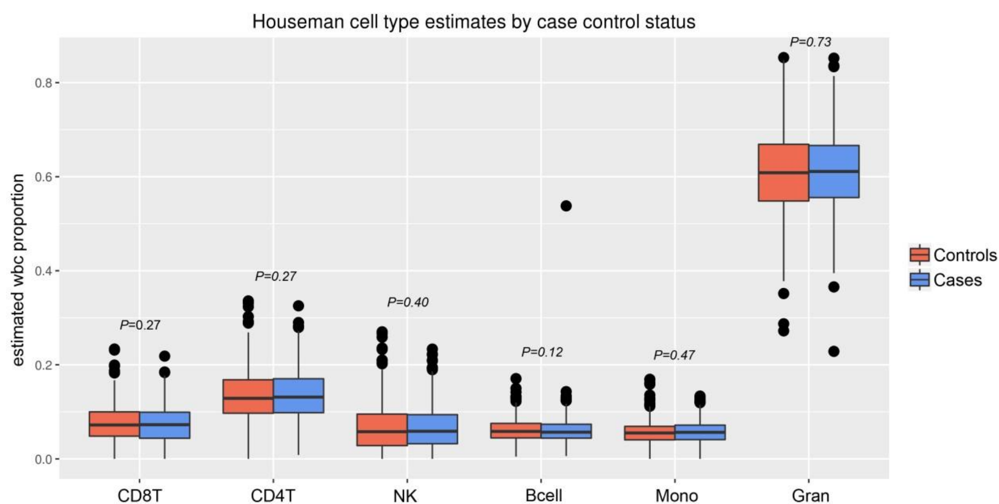
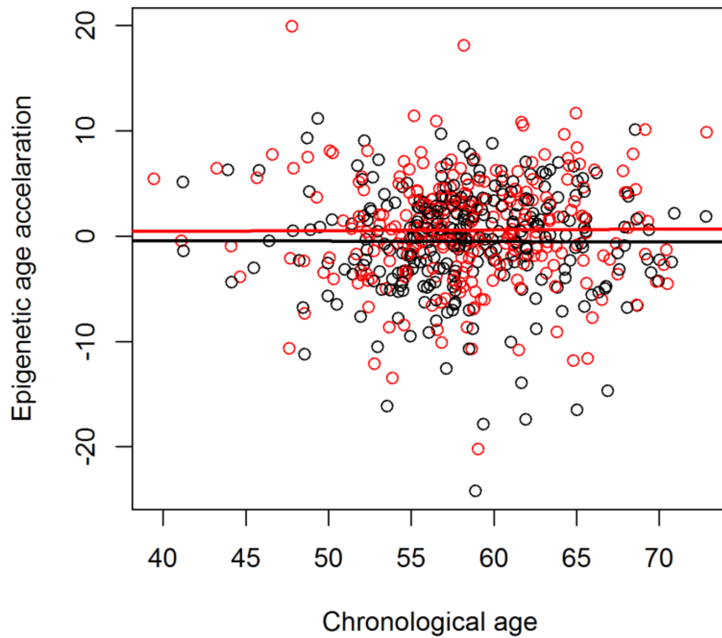


Supplementary Figure 2~~1~~: Distribution of inferred leucocyte cell subpopulation. Proportion of leukocyte subtypes derived from DNA methylation data. Inferred data were plotted by sample groups (breast cancer cases and controls) where X-axis shows leucocyte subtypes and Y-axis shows proportion of estimated leucocytes.



Supplementary F~~f~~igure 3~~3~~: Epigenetic age accelaration of postmenopausal breast samples.

Epigenetic age accelaration (IEAA) (Y-aixs) versus chronological age. Points correspond to female subjects. Red colored circles indicates breast cancer case while the black circles represent non-case samples. The solid lines indicates a regression lines for cases (in red) and non-case samples (in black).~~The solid lines indicates a regression lines for cases (in red) and non-case samples(in black)~~.

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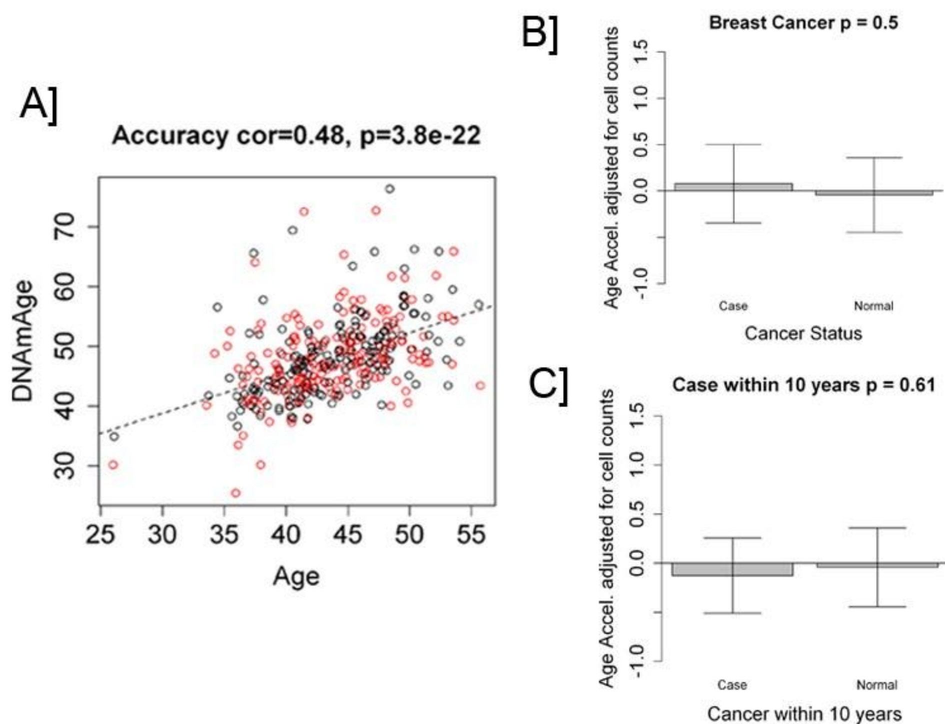
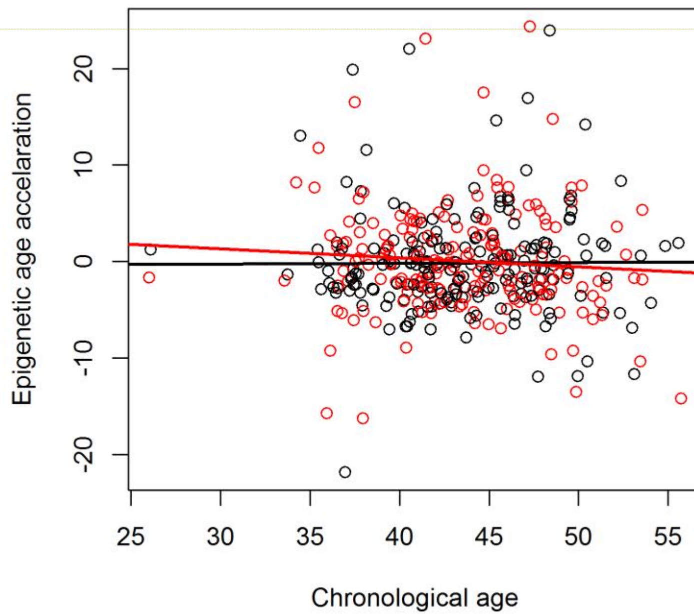


Supplementary Figure 4: Epigenetic clock analysis for premenopausal breast samples. A) DNAm age (Y-aixs) versus chronological age. Points correspond to female subjects. Red colored circles indicates breast cancer case while the black circles represent non-case samples. The dashed line indicates a regression line. B) Epigenetic age accelaration versus breast cancer status. Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group test p-value (Wilcoxon test). C) Epigenetic age accelaration versus breast cancer status (developed within 10 years post blood draw). Each bar plot depicts the mean value, standard deviation, and reports a non-parametric group comparison test p- value (Wilcoxon test).



Supplementary Figure 5: Epigenetic age accelaration of premenopausal breast samples. Epigenetic age accelaration (IEAA) (Y-aixs) versus chronological age. Points correspond to female subjects. Red colored circles indicates breast cancer case while the black circles represent non-case samples. The solid lines indicates a regression lines for cases (in red) and non-case samples(in black).

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Supplementary Table 1. Conditional logistic regression model of epigenetic age acceleration in all samples

	OR (95% CI)	P value
IEAA	1.05 (1.01-1.09)	0.01
Alcohol at the time of recruitment	1.01 (1.00-1.02)	0.06
Level of education (Ref. No education)		
Primary	1.53 (0.63-3.69)	0.34
Technical/professional	1.14 (0.44-2.97)	0.79
Secondary	1.92 (0.72-5.08)	0.19
Higher education	1.69 (0.63-4.51)	0.29
Full term pregnancy (Ever/never)	0.96 (0.66-1.41)	0.85
Physical activity (Cambridge index, Ref.		
Moderately inactive	0.71 (0.48-1.06)	0.10
Moderately active	0.77 (0.48-1.24)	0.29
Active	0.54 (0.33-0.91)	0.02
Age at menarche	0.93 (0.85-1.02)	0.14
BMI (Categorical, Ref. Normal)		
Underweight	0.61 (0.14-2.68)	0.51
Overweight	1.01 (0.72-1.42)	0.95
Obese	1.15 (0.75-1.78)	0.52

Conditional logistic regression was performed using known breast cancer risk factors highlighted in bold

IEAA: Intrinsic Epigenetic Age Acceleration

BMI: Body Mass Index

Supplementary Table 2. Demographic and lifestyle factor details of pre and postmenopausal samples

		Premenopausal samples		Postmenopausal samples	
		Controls (%)	Cases (%)	Controls (%)	Cases (%)
Sample size		180	180	259	259
		Demographic and lifestyle factors			
Age (years)					
	Mean (SD)	43.6 (4.73)	43.6 (4.74)	58.5 (5.50)	58.5 (5.50)
	Median	43.5	43.4	58.3	58.3
Smoking					
	Never	90 (50.0%)	85 (47.3%)	158 (61.0%)	171 (66.1%)
	Former	37 (20.5%)	46 (25.5%)	50 (19.3%)	47 (18.1%)
	Current	51 (28.4%)	49 (27.2%)	50 (19.3%)	40 (15.4%)
	Not known	2 (1.1%)	-	1 (0.4%)	1 (0.4%)
Alcohol					
	Mean(SD)	8.1(11.06)	10.3 (12.12)	8.1 (12.15)	9.5 (13.55)
	Median	4.4	5.3	3.0	4.0
Age at					
	Mean (SD)	12.9 (1.34)	12.7 (1.59)	13.3 (1.64)	13.3 (1.71)
	Median	13.0	13.0	13.0	13.0
BMI					
	Mean (SD)	24.7 (4.14)	24.8 (4.12)	26.1 (4.25)	26.9 (4.95)
	Median	23.88	23.98	25.56	25.97
IEAA					
	Mean (SD)	-0.042 (5.39)	0.079 (5.67)	-0.47 (5.16)	0.60 (5.19)

IEAA: Intrinsic Epigenetic Age Acceleration

Supplementary Table 3: Conditional logistic regression model of epigenetic age acceleration in postmenopausal samples

	OR (95% CI)	P value
IEAA	1.08 (1.03-1.13)	0.003
Alcohol at the time of recruitment	1.01 (0.99-1.02)	0.424
Level of education (Ref. No education)		
Primary	2.94 (0.75-11.46)	0.121
Technical/professional	1.46 (0.34-6.20)	0.609
Secondary	2.51 (0.57-11.13)	0.226
Higher education	2.98 (0.67-13.20)	0.151
Full term pregnancy (Ever/never)	0.94 (0.56-1.58)	0.827
Physical activity (Cambridge index, Ref.		
Moderately inactive	0.78 (0.47-1.29)	0.334
Moderately active	0.51 (0.26-0.99)	0.046
Active	0.39 (0.19-0.80)	0.011
Age at menarche	0.98 (0.86-1.11)	0.759
BMI (Categorical, Ref. Normal)		
Underweight	0.61 (0.05-7.77)	0.707
Overweight	1.11 (0.71-1.74)	0.653
Obese	1.08 (0.62-1.88)	0.791

Conditional logistic regression was performed using known breast cancer risk factors highlighted in bold

IEAA: Intrinsic Epigenetic Age Acceleration

BMI: Body Mass Index