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Methylation-based biological age and cardiotoxicity risk in breast cancer patients treated with trastuzumab

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Abstract

Background Trastuzumab is an effective treatment for HER2-positive cancers that has known cardiotoxic properties. Discovering biomarkers that assess cardiotoxicity risk before trastuzumab therapy is essential for protecting the cardiovascular health of cancer patients.

Objective To examine the associations between pre-treatment epigenetic age acceleration, circulating leukocyte composition, and candidate single nucleotide polymorphisms (SNPs) with cardiotoxicity risk in breast cancer patients receiving trastuzumab.

Methods Among a retrospective cohort of HER2-positive breast cancer patients treated with trastuzumab at Moffitt Cancer Center, we profiled blood DNA methylation and genetic profiles. Epigenetic clocks and circulating leukocyte subsets were derived from MethylationEPIC BeadChip data, and candidate SNPs were measured using the Global Screening Array. Cardiotoxicity events (i.e., reductions in left ventricular ejection fraction, symptomatic heart failure), were identified in medical records. Logistic regression models, adjusted for traditional risk factors, estimated odds ratios (ORs) for biomarker associations with cardiotoxicity risk.

Results Among 157 patients selected for this study, 39 (25%) experienced cardiotoxicities within one year of treatment initiation. rs776746 was inversely associated with cardiotoxicity risk (OR: 0.38, 95% CI: 0.14, 1.00, $P=0.05$). After adjusting for traditional risk factors and leukocyte composition, the Hannum AgeAccel, Horvath AgeAccel, and Horvath Skin and Blood AgeAccel metrics were significantly positively associated with cardiotoxicity risk (ORs ranging between 1.62 and 1.89). Adding Horvath Skin and Blood AgeAccel to traditional cardiotoxicity risk factors significantly improved cardiotoxicity risk prediction (AUC: 0.75 vs. 0.79; P-diff = 0.04).

Conclusions Pre-treatment epigenetic age acceleration appears to be a novel biomarker for cardiotoxicity risk that improves cardiotoxicity risk prediction.

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Keywords Epigenetic clocks, Biological age, Cardiotoxicity, Trastuzumab, Breast cancer

Introduction

Human epidermal growth factor receptor-2 (HER2) overamplification occurs in nearly 30% of breast tumors and is associated with poor clinical outcomes [1, 2]. Trastuzumab, a monoclonal antibody targeting the HER2 receptor, is a standard and effective treatment for HER2-positive breast cancer that possesses cardiotoxic properties, with decreasing rates of cardiotoxicity over time [3–5]. Trastuzumab-associated cardiac dysfunction is commonly classified as asymptomatic systolic dysfunction (i.e., decrease in left ventricular ejection fraction [LVEF]) or symptomatic heart failure [6–8]. Risk factors for cardiotoxicity include older age, higher body mass index (BMI), and prevalent chronic conditions at diagnosis [9, 10]. Although circulating biomarkers like cardiac troponins and B-natriuretic peptides can signal subclinical cardiotoxicities, they are released into the bloodstream only after cardiac injury has occurred. Identifying biomarkers that are associated with cardiotoxicity risk before treatment initiation will be crucial for protecting the cardiovascular health of cancer patients.

Blood DNA methylation (DNAm) profiles are associated with cardiovascular disease (CVD) risk factors and incidence, with findings observed at individual genomic loci and using DNAm-derived metrics of biological age ("epigenetic clocks") and leukocyte composition [11–20]. Importantly, most DNAm associations with CVD remain statistically significant even after adjusting for traditional risk factors, suggesting that DNAm profiles capture unique aspects of CVD risk. Genetic polymorphisms are also associated with CVD and are hypothesized to be potential biomarkers of trastuzumab cardiotoxicity [21–23]. Earlier studies have investigated cardiotoxicity risk associations with single nucleotide polymorphisms (SNPs) in various genes, including *ERBB2*, which encodes the HER2 receptor [21–31]. Although a small number of cardiotoxicity-associated SNPs have been identified, none have been validated in subsequent studies.

Here, using pre-treatment blood samples collected from women diagnosed with HER2-positive breast cancer and treated with trastuzumab, we examine cardiotoxicity risk associations with candidate SNPs and DNAm-derived estimates of biological age and leukocyte composition. To examine the clinical utility of these biomarkers, we test whether cardiotoxicity risk prediction can be improved beyond traditional risk factors.

Methods

Study population

This was a retrospective cohort study in a single NCI-designated Comprehensive Cancer Center. Since 2006, Moffitt Cancer Center has invited all patients aged 18 years or older to participate in the Total Cancer Care (TCC) research protocol. Participation in TCC is voluntary and includes donating biospecimens (e.g., blood) and consenting to data abstraction from electronic medical records. For this study, eligible participants included female patients diagnosed with HER2-positive breast cancer treated with trastuzumab as a first-line therapy at Moffitt between October 2006 and January 2021 ($N = 1{,}190$; 83% White, mean age at diagnosis: 56 years). Inclusion was further restricted to those with an available whole blood sample in the TCC biorepository, collected after the cancer diagnosis and within the year preceding treatment ($N = 157$; 81% White, mean age at diagnosis: 54 years). All participants provided consent under Moffitt Cancer Center's institutional TCC protocol, with additional approvals for generating DNAm and genomic data from archived samples. The study is overseen by the Advarra Institutional Review Board.

Patient characteristics

Electronic medical records were used to collect data on participants, including dates of birth, breast cancer diagnosis, blood draw, and cardiotoxicity event, race (non-Hispanic White or not), smoking status (ever, never), BMI category (< 25, 25–30, $30 + \text{kg/m}^2$) and medical history (current CVD medication use [beta-blockers, ACE inhibitors, statins], personal and family history of cancer, history of any chemotherapy exposure, prevalent chronic conditions at diagnosis [hypertension, dyslipidemia, diabetes mellitus, coronary artery disease, arrhythmia, myocardial infarction, valvular disease]). Cancer-related data included tumor stage, prior cancer diagnosis, and current breast cancer treatment details (chemotherapy [including anthracyclines], left-sided radiation therapy, and endocrine therapy).

DNA extraction, quality control, and molecular assays

DNA was extracted using the Qiagen DNA extraction kit according to the manufacturer's protocol, and DNA quantity and quality were evaluated using Tape Station. For DNAm profiling, one microgram of DNA was bisulfite converted using the EZ DNA Methylation Kit. Methylation and genotyping assays were performed at Moffitt's Molecular Genomics Core using Illumina's Infinium MethylationEPIC v2 and Global Screening Array BeadChips.

The MethylationEPIC v2 BeadChip data were preprocessed using the *ENmix* pipeline, which includes background correction, RELIC dye-bias correction, interarray normalization, and RCP probe-type bias correction,

as *ENmix* has been shown to outperform alternative pipelines [32]. Samples failing quality control measures, including bisulfate intensity < 5,000, > 5% of probes with low-quality methylation values (detection $P > 0.000001$, < 3 beads, or values outside 3 times the interquartile range), or outliers for their methylation beta value distributions, were excluded.

The Global Screening Array BeadChip data were preprocessed using GenomeStudio2.0. Probes with low call rates (< 90%) and rare SNPs (minor allele frequency < 1%) were excluded. Of the 28 candidate SNPs identified from the literature (Supplemental Table 1) [21–31], seven had minor allele frequences less than 1% (rs5762940, rs1902023, rs3892097, rs139503277, rs139944387, rs78272919, and rs1801201) and were excluded.

Biological age and leukocyte composition calculation

Processed DNAm data were used to calculate six epigenetic clocks. Epigenetic clocks use information from hundreds to thousands of genomic loci that were selected for their ability to predict chronological age (first-generation clocks: Hannum [33], Hovath [34], Horvath Skin and Blood [35]), mortality risk (second-generation clocks: PhenoAge [36], GrimAge [37]), or aging rates (DunedinPACE [38]). "Principal component" versions of epigenetic age for the Hannum, Horvath, Horvath Skin and Blood, PhenoAge, and GrimAge epigenetic clocks were derived as they are reported to be more reliable and show better agreement between replicate samples [39]. Epigenetic age acceleration (AgeAccel) represents the difference between a person's DNAm-predicted biological age and chronological age and was calculated for each of the epigenetic clocks by regressing epigenetic age on chronological age and predicting the residuals. Positive AgeAccel values represent a higher biological age relative to chronological age and are associated with disease risk factors and incidence [12–15, 40]. Unlike the other epigenetic clocks, DunedinPACE does not measure AgeAccel but estimates aging rates [38], with values above one representing faster biological aging and values less than one representing slower aging.

Processed DNAm data were also used to calculate circulating leukocyte composition. Leukocyte subset percentages were derived using the EPIC IDOL-Ext library [41], including subsets from both the myeloid (i.e., neutrophil, basophil, eosinophil, and monocyte) and lymphoid lineages (i.e., T regulatory, naïve and memory B cells, CD4 + T cells, and CD8 + T cells, and natural killer cells). In the primary analysis, the subsets were aggregated by hematopoietic lineage (major subsets): neutrophils, basophils, and eosinophils were summed to represent granulocyte percentage; naïve and memory subsets of B cells and CD8 + cytotoxic T cells were summed to represent the total percentages of those subsets. Naïve, memory, and T regulatory cells were summed to represent the total percentage of CD4 + helper T cells. Subsequent analyses were conducted using the leukocyte subsets without aggregation (refined subsets). Studies show that DNAm-based deconvolution methods are comparable in accuracy to flow cytometry for estimating leukocyte composition [41], [42]. Leukocyte subset ratios, including naïve-to-total ratios for B cells, CD4 + helper T cells, and CD8 + cytotoxic T cells and neutrophil-to-lymphocyte ratio, were also calculated.

Cardiotoxicity assessment

Each patient received cardiac evaluations before and after trastuzumab treatment using either a multigated acquisition scan or echocardiogram to measure changes in LVEF. Cardiotoxicity events within the year after treatment initiation were identified by manual chart review and were defined as new-onset symptomatic heart failure or a drop in LVEF of 15% to a value ≤ 50% (or 10% if baseline LVEF ≤ 55%) over subsequent scans.

Statistical analysis

Patient and treatment characteristics were summarized using means and standard deviations (SD) for continuous variables and counts and percentages for categorical variables. Pearson correlation coefficients were calculated to examine relationships between the epigenetic clocks and leukocyte subsets. Logistic regression models estimated odds ratios (ORs) and 95% confidence intervals (CIs) for cardiotoxicity risk associations with candidate SNPs, epigenetic clocks, and leukocyte composition. For the analyses of candidate SNPs, alleles were treated as an ordinal variable (log-additive, codominant model). For the analyses of epigenetic clocks and leukocyte subsets, to make fair comparisons of the strengths of cardiotoxicity associations across the metrics, the variables were standardized to have a mean of zero and SD of one; thus, all associations are scaled to represent cardiotoxicity risk per 1-SD increase in the DNAm metric.

Regression models of candidate SNPs were adjusted for the top five array-based principal components, while regression models of the epigenetic clocks were adjusted for age at diagnosis. Subsequent models of the epigenetic clocks and leukocyte subsets were further adjusted for smoking status (ever, never), BMI category (< 25 kg/m^2, 25–30 kg/m^2, 30 + kg/m^2), current CVD medication use (yes/no), prevalence of any chronic condition at diagnosis (hypertension, dyslipidemia, diabetes, CVD, cancer; yes/no), history of prior chemotherapy (yes/no), and treatment of this breast cancer with anthracyclines (yes/no). Epigenetic clock models were also adjusted for circulating leukocyte subsets, and leukocyte subset models were adjusted for the epigenetic clocks. To explore

the potential for effect modification, cardiotoxicity-associated DNAm biomarkers were examined in models stratified by patient characteristics. To test the predictive utility of the genetic and DNAm-based cardiotoxicity risk biomarkers, receiver operating characteristic analyses were conducted to measure discrimination by calculating the area under the curve (AUC). Statistical significance was determined at $P \leq 0.05$. All analyses were conducted using Stata version 18 and R version 4.4.

Results

Sample characteristics

The average age of the women at diagnosis was 54 years (SD = 12 years). Most were White (81%), had never smoked (62%), and were diagnosed with non-metastatic, invasive breast cancer (91%) (Table 1). In total, 39 (25%) women experienced a cardiotoxicity event (median time to event: 198 days, range: 18–365 days) (Supplemental Fig. 1), including 4 (3%) symptomatic heart failure events. Cardiotoxicity event rates appeared to vary over time, with higher rates observed before 2015 (Supplemental Fig. 2). Women who developed cardiotoxicity were more likely to have ever smoked (54% vs. 32%), have a prevalent chronic disease at diagnosis (63% vs. 42%), be using CVD medications (44% vs. 23%), have a prior cancer diagnosis (26% vs. 8%), have a history of prior chemotherapy exposure (23% vs. 6%), and been treated with anthracyclines for their current breast cancer (23% vs. 11%). The epigenetic clocks generally overestimated the ages of the women, with the exception of the PhenoAge clock (Table 1). The epigenetic age estimates from the Hannum, Horvath, Horvath Skin and Blood, PhenoAge, and GrimAge epigenetic clocks were highly correlated with age at blood draw (all $\rho > 0.8$; Supplemental Fig. 3).

As expected, the AgeAccel metrics showed no meaningful correlation with age at diagnosis (correlation range: $-0.01 < \rho < 0.06$; Supplemental Fig. 4). AgeAccel values from the first-generation epigenetic clocks were strongly correlated with one another ($0.86 < \rho < 0.95$) and moderately correlated with AgeAccel values from the second-generation clocks ($0.40 < \rho < 0.80$). The DunedinPACE clock generally showed weaker but positive correlations with the AgeAccel metrics ($0.15 < \rho < 0.62$). Women who experienced cardiotoxicity had higher pre-treatment AgeAccel based on the Hannum, Horvath Skin and Blood, PhenoAge, and GrimAge epigenetic clocks (all $P \leq 0.05$; Supplemental Table 2). No significant difference was observed for DunedinPACE ($P = 0.27$).

The six major leukocyte subsets were not strongly correlated with patient age at diagnosis (correlation range: $-0.10 < \rho < 0.22$; Supplemental Fig. 5). Granulocyte percentage was moderately inversely correlated with the other major leukocyte subsets (correlation range: $-0.74 < \rho < -0.37$). In addition, CD8 + cytotoxic T cells

Table 1 Participant characteristics of the sample population ($N = 157$)

Characteristic	Overall *N* = 157	Cardiotoxicity *N* = 39	No Cardiotoxicity *N* = 118
Patient characteristics			
Age at diagnosis (years)	54.1 (12)	56.5 (11)	53.2 (12)
Hannum (epigenetic age, years)	61.1 (9)	64.4 (9)	59.9 (9)
Horvath (epigenetic age, years)	55.9 (9)	59.4 (9)	54.7 (9)
Horvath Skin and Blood (epigenetic age, years)	58.0 (9)	61.3 (8)	57.0 (8)
PhenoAge (epigenetic age, years)	54.0 (11)	58.8 (12)	52.5 (11)
GrimAge (epigenetic age, years)	65.2 (10)	68.8 (10)	64.0 (9)
DunedinPACE (aging rate, years)	1.1 (0.1)	1.1 (0.1)	1.1 (0.1)
Race			
White	127 (81%)	31 (79%)	96 (81%)
Non-White	30 (19%)	8 (21%)	22 (19%)
Smoking status			
Ever	59 (38%)	21 (54%)	38 (32%)
Never	98 (62%)	18 (46%)	80 (68%)
BMI category			
< 25	58 (37%)	16 (41%)	42 (36%)
25–30	47 (30%)	10 (26%)	37 (31%)
30+	52 (33%)	13 (33%)	39 (33%)
Prevalent chronic disease[1]			
Yes	73 (47%)	24 (63%)	49 (42%)
No	82 (53%)	14 (37%)	68 (58%)
CVD current medication			
Yes	44 (28%)	17 (44%)	27 (23%)
No	113 (72%)	22 (56%)	91 (77%)
History of cancer			
Yes	18 (11%)	10 (26%)	10 (8%)
No	139 (89%)	29 (74%)	108 (92%)
History of chemotherapy			
Yes	16 (10%)	9 (23%)	7 (6%)
No	140 (89%)	29 (77%)	111 (94%)
CVD family history			
Yes	39 (25%)	10 (26%)	29 (25%)
No	116 (75%)	29 (74%)	87 (75%)
Clinical characteristics			
Cancer stage			
In situ	3 (2%)	1 (3%)	2 (2%)
I – III	123 (91%)	30 (91%)	93 (91%)
IV	9 (7%)	2 (6%)	7 (7%)
Endocrine therapy			
Yes	93 (60%)	20 (51%)	73 (62%)
No	63 (40%)	19 (49%)	44 (38%)
Left-sided radiation			
Yes	94 (60%)	24 (62%)	70 (59%)
No	63 (40%)	15 (38%)	48 (41%)

Table 1 (continued)

Characteristic	Overall *N* = 157	Cardiotoxicity *N* = 39	No Cardiotoxicity *N* = 118
Anthracycline treatment			
Yes	22 (14%)	9 (23%)	13 (11%)
No	135 (86%)	30 (77%)	105 (89%)

[1]Defined as hypertension, hyperlipidemia, diabetes, CVD

Missing data: history of chronic disease, 2 (1 cardiotoxicity, 1 no cardiotoxicity); CVD family history, 2 (2 no cardiotoxicity); Cancer stage, 22 (16 no cardiotoxicity, 6 cardiotoxicity); Hormone treatment, 1 (no cardiotoxicity)

were positively correlated with natural killers (ρ = 0.67) and CD4 + helper T cells were positively correlated with B cells (ρ = 0.50). Women who experienced cardiotoxicity appeared to have higher percentages of circulating monocytes (7.8% vs. 6.9%) and lower percentages of circulating naïve CD4 + helper T cells (2.2% vs. 3.4%), but associations were not statistically significant (Supplemental Table 3).

Candidate SNP associations with cardiotoxicity risk

Among the 21 candidate SNPs investigated, *CYP3A5* splice acceptor SNP rs776746 was significantly inversely associated with cardiotoxicity risk in models adjusted for the top five principal components (OR: 0.38, 95% CI: 0.14, 1.00, $P = 0.05$; Table 2). Additionally, three other SNPs were marginally associated at $P < 0.10$. Specifically, intergenic SNP rs8032978 (OR: 1.79, 95% CI: 0.95, 3.41, $P = 0.07$), *UGT1A1* intron SNP rs3755319 (OR: 0.63, 95% CI: 0.37, 1.06, $P = 0.08$), and *ERBB2* missense SNP rs1058808 (OR: 0.57, 95% CI: 0.30, 1.07, $P = 0.08$) (Table 2).

Epigenetic clock associations with cardiotoxicity risk

In age-adjusted models, all epigenetic clocks except for DunedinPACE were significantly associated with cardiotoxicity risk (Fig. 1; Supplemental Table 4). After further adjustment for cardiotoxicity risk factors, associations for Hannum AgeAccel, Horvath AgeAccel, Horvath Skin and Blood AgeAccel, and PhenoAgeAccel remained statistically significantly related to cardiotoxicity risk. In models adjusted for age and blood cell composition, associations were slightly attenuated from the age-adjusted models, and only marginal statistically significant associations were observed for Horvath Skin and Blood AgeAccel and PhenoAgeAccel (both d$P = 0.07$). Finally, in fully adjusted models including age, traditional cardiotoxicity risk factors, and blood cell composition, associations with Hannum AgeAccel, Horvath AgeAccel, and Horvath Skin and Blood AgeAccel were statistically significantly associated with cardiotoxicity risk. The strongest association was observed for the Horvath Skin and Blood AgeAccel metric (OR: 1.89, 95% CI: 1.15, 3.10, $P = 0.01$) (Supplemental Table 4).

The associations between the epigenetic clocks and cardiotoxicity risk varied by certain patient characteristics (Supplemental Table 5), with associations appearing stronger in women with healthier characteristics. For example, the association between Horvath Skin and Blood AgeAccel and cardiotoxicity risk appeared stronger in those with lower BMIs (< 25 kg/m^2, OR: 3.41, 95% CI: 1.45, 8.02, $P = 0.005$; ≥ 25 kg/m^2, OR: 1.72, 95% CI: 0.64, 4.60, $P = 0.28$), never smokers (never smokers, OR: 3.74, 95% CI: 1.21, 11.6, $P = 0.02$; smokers, OR: 1.78, 95% CI: 0.43, 7.41, $P = 0.43$), and those not using CVD medication (non-users, OR: 4.16, 95% CI: 1.62, 10.7, $P = 0.003$; users, OR: 0.83, 95% CI: 0.35, 1.98, $P = 0.68$). Similar patterns of association were observed for AgeAccel from the Hannum and Horvath epigenetic clocks.

Circulating leukocyte composition associations with cardiotoxicity risk

In age-adjusted models, none of the major leukocyte subsets were associated with cardiotoxicity risk (Fig. 2; Supplemental Table 6). In models adjusted for age and epigenetic age acceleration, circulating monocyte percentage was statistically, significantly associated with cardiotoxicity risk (OR: 1.49, 95% CI: 1.00, 2.23, $P = 0.05$) (Fig. 2; Supplemental Table 6), but this association did not reach statistical significance after additional adjustment for cardiotoxicity risk factors. No associations were found between the refined leukocyte subsets or leukocyte ratio measures and cardiotoxicity risk (Supplemental Tables 7 and 8).

Prediction of cardiotoxicity risk

Traditional cardiotoxicity risk factors (i.e., age at diagnosis, smoking status, BMI category, CVD medication use, prevalence of chronic conditions at diagnosis, prior history of cancer, history of chemotherapy, and treatment with anthracyclines) demonstrated moderate performance in discriminating women who did and did not develop cardiotoxicity (AUC: 0.749, 95% CI: 0.659, 0.840) (Table 3). When the cardiotoxicity-associated SNP or AgeAccel metrics were added to the model individually, only Horvath Skin and Blood AgeAccel significantly improved discrimination (AUC: 0.791, 95% CI: 0.709, 0.873, P-diff = 0.04). Models including all three cardiotoxicity-associated AgeAccel metrics, with and without rs776746, also performed significantly better than the traditional risk factors (all three AgeAccel metrics, AUC: 0.794, 95% CI: 0.712, 0.875, P-diff = 0.04; all three AgeAccel metrics + rs776746, AUC: 0.795, 95% CI: 0.714, 0.877, P-diff = 0.05) (Table 3), but neither provided significant improvement over the model with traditional risk factors and Horvath Skin and Blood AgeAccel alone (all three

Table 2 Candidate SNP associations with cardiotoxicity in breast cancer patients treated with trastuzumab

Chr	SNP ID	Gene name	REF	ALT	MAF	OR	95% CI	P-value
7	rs776746	*CYP3A5*	G	A	0.16	0.38	0.14, 1.00	0.05
15	rs8032978		A	G	0.195	1.79	0.95, 3.41	0.07
2	rs3755319	*UGT1A1*	A	C	0.429	0.63	0.37, 1.06	0.08
17	rs1058808	*ERBB2*	C	G	0.409	0.57	0.30, 1.07	0.08
17	rs2643195	*ERBB2*	A	G	0.375	0.67	0.38, 1.19	0.17
2	rs887829	*UGT1A1*	C	T	0.321	0.70	0.40, 1.21	0.20
17	rs7406710		C	T	0.150	0.59	0.25, 1.38	0.22
17	rs1136201	*ERBB2*	A	G	0.189	1.44	0.77, 2.67	0.25
2	rs6759892	*UGT1A6*	G	T	0.369	0.74	0.43, 1.27	0.27
2	rs7586110	*UGT1A7*	G	T	0.343	0.82	0.48, 1.39	0.47
13	rs9316695		C	A	0.149	0.81	0.41. 1.60	0.54
17	rs79747793	*ERBB2*	C	A	0.016	0.54	0.06, 5.20	0.59
4	rs11932853		T	C	0.357	1.16	0.67, 2.02	0.60
17	rs117866580	*ERBB2*	G	T	0.019	0.58	0.07, 5.13	0.62
17	rs4252633	*ERBB2*	G	T	0.006	2.07	0.10, 43.7	0.64
13	rs1751034	*ABCC4*	A	G	0.032	1.37	0.33, 5.70	0.66
17	rs35797841	*ERBB2*	C	T	0.054	0.83	0.26, 2.61	0.75
11	rs57242572		C	T	0.037	0.76	0.12, 4.79	0.77
6	rs628031	*SLC22A1*	A	G	0.349	0.92	0.52, 1.63	0.79
15	rs28415722		G	A	0.392	1.06	0.65, 1.75	0.81
11	rs61918162		T	C	0.293	1.04	0.60, 1.81	0.88

Models adjusted for top 5 principal components based on genotype array data. Abbreviations: Chr, chromosome; SNP, single nucleotide polymorphism; REF, reference allele; ALT, alternative allele; MAF, minor allele frequency

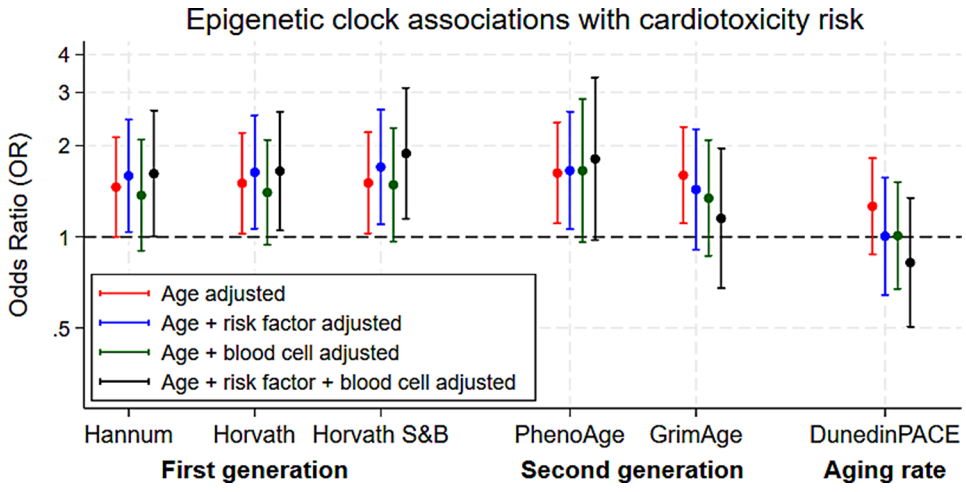

Fig. 1 Cardiotoxicity risk associations with epigenetic clocks. Odds ratios and 95% confidence intervals for the epigenetic clock associations with cardiotoxicity risk. Associations scaled per 1 standard deviation increase in epigenetic age acceleration (for the chronological age and mortality predictors) or aging rates

AgeAccel metrics, P-diff = 0.32; all three AgeAccel metrics + rs776746, P-diff = 0.63).

Discussion

Here, among a retrospective cohort of HER-2 positive breast cancer patients treated with trastuzumab at Moffitt Cancer Center, we find that epigenetic age acceleration, measured using various epigenetic clocks, is positively associated with cardiotoxicity risk. Among the metrics evaluated, Horvath Skin and Blood AgeAccel showed the strongest association, with higher values associated with nearly 90% higher odds of cardiotoxicity after adjusting for traditional risk factors and blood cell composition. Critically, integrating AgeAccel metrics into cardiotoxicity risk prediction models of traditional risk factors significantly improved prediction, highlighting the potential of epigenetic clocks to provide clinically valuable insights for early identification of high-risk patients.

This study is the first to investigate the association between cardiotoxicity risk and blood methylation

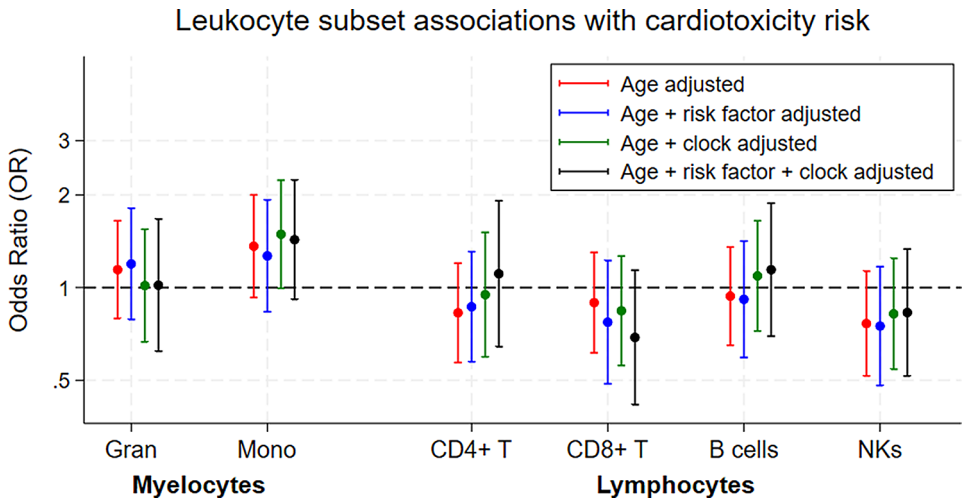

Fig. 2 Cardiotoxicity risk associations with leukocyte subsets. Odds ratios and 95% confidence intervals for the major leukocyte subset associations with cardiotoxicity risk. Associations scaled per 1 standard deviation increase in the leukocyte subset. Abbreviations: gran, granulocytes; mono, monocytes; NKs, natural killers

Table 3 Area under the curve estimates for the prediction of cardiotoxicity risk using traditional risk factors and epigenetic age acceleration metrics

Model components	AUC	95% CI		*P*-diff
Risk factors only	0.749	0.659	0.840	------
Risk factors + rs776746	0.764	0.677	0.852	0.24
Risk factors + Hannum AgeAccel	0.784	0.701	0.867	0.08
Risk factors + Horvath AgeAccel	0.781	0.679	0.864	0.12
Risk factors + Hovath Skin and Blood AgeAccel	0.791	0.709	0.873	0.04
Risk factors + three AgeAccel metrics	0.794	0.712	0.875	0.04
Risk factors + three AgeAccel metrics + rs776746	0.795	0.714	0.877	0.05

Risk factors include age at diagnosis, smoking status, BMI category, CVD medication use, prevalence of chronic conditions at diagnosis, prior history of cancer, history of chemotherapy, and treatment with anthracyclines. P-diff represents the p-value for the difference between the risk factor-only model

biomarkers in cancer patients treated with trastuzumab. Epigenetic clocks, which measure molecular changes related to aging and mortality, are robust predictors of disease incidence [43–5], making them promising tools for cardiotoxicity risk assessment [46]. We found the strongest cardiotoxicity associations for the first-generation epigenetic clocks. Given that older chronological age is a primary risk factor for trastuzumab cardiotoxicity [9], these findings suggest that accelerated biological age may further increase cardiotoxicity susceptibility. The epigenetic clock associations with cardiotoxicity were noticeably independent of blood cell composition, suggesting that these associations are not driven by changes to the peripheral immune system. Furthermore, the contrasting associations across the epigenetic clocks may provide insights into the potential mechanisms underlying accelerated aging and cardiotoxicity risk. For instance, first-generation clocks are proposed to measure stochastic age-related changes [47, 48]. Of the first-generation clocks, the Horvath Skin and Blood AgeAccel metric displayed the strongest association with cardiotoxicity risk, likely because it was optimized for blood-based applications [35], addressing limitations of the earlier Horvath clock [34]. In contrast, the second-generation clocks are proposed to measure overall physiological fitness and were designed to as proxies for blood chemistry changes associated with mortality [36, 37]. These second-generation clocks, particularly the GrimAge clock, have stronger associations than first-generation clocks with many cardiotoxicity risk factors, such as obesity and prevalent chronic disease [12, 14]. As a consequence, the diminishing cardiotoxicity association with GrimAgeAccel may be due to its inclusion of a DNAm proxy for smoking history and association with blood cell composition [37] which were included as covariates in our adjusted models. Overall, these observations suggest that the first-generation clocks' associations with cardiotoxicity risk may reflect underlying stochastic aging processes, whereas the association with PhenoAgeAccel may be more directly related to the patient's overall physical fitness.

Although epigenetic clocks have previously been linked to CVD incidence [17–20], only one study has examined their ability to improve CVD risk prediction [49]. The previous study reported limited clinical utility for most clocks, but was focused on long-term risk prediction of a composite CVD endpoint, which included conditions with distinct etiologies. In contrast, this study specifically examined the short-term risk prediction of LVEF reductions, an essential parameter for diagnosing and managing heart failure. These observations suggest that the clinical utility of epigenetic clocks may be more relevant for the prediction of short-term changes in heart

function, rather than the prediction of long-term CVD events.

Although this study is the first to evaluate epigenetic clocks as biomarkers of trastuzumab cardiotoxicity, prior research has investigated trastuzumab cardiotoxicity associations with individual SNPs [21–30]. The top-performing candidate SNP in this study, rs776749 (*CYP3A5*), affects drug metabolism and was first reported as a marker for cardiotoxicity risk in patients treated with doxorubicin and cyclophosphamide [50, 51]. More recently, its associations have been extended to those treated with trastuzumab [30]. In our analysis of 21 candidate SNPs, only rs776745 showed a statistically significant association with cardiotoxicity risk. However, we did not adjust for multiple comparisons across the tested SNPs and adding rs776746 to traditional cardiotoxicity risk factors did not improve risk prediction. Most genomic studies of trastuzumab cardiotoxicity have relied on candidate SNP approaches rather than genome-wide analyses. Furthermore, these studies have typically been limited by small sample sizes, with the largest including only a few hundred participants [21–31]. Given the modest findings from candidate SNP studies in small cohorts, larger consortia and genome-wide approaches may be required to identify robust genetic predictors of trastuzumab cardiotoxicity.

The finding that epigenetic clocks enhance cardiotoxicity risk prediction has significant clinical implications. Identifying high-risk patients before trastuzumab initiation creates opportunities for personalized treatment strategies, including dosing adjustments or considering alternative therapies. For high-risk patients, clinicians may also implement more intensive monitoring or initiate cardio-protective therapies. Additionally, our findings suggest that elevated biological age may be etiologically linked to the development of cardiotoxicity, presenting it as a potential target for intervention. Emerging evidence indicates that biological age can be reduced through medications or lifestyle interventions, offering a proactive approach to mitigating cardiotoxicity risk before treatment begins [52]. Integrating epigenetic clocks into cardio-oncology practices could enable clinicians to assess cardiotoxicity risk more effectively, protecting high-risk patients from the cardiotoxic effects of trastuzumab while identifying low-risk patients who may benefit from less intensive cardiac monitoring, reducing costs, and minimizing therapy interruptions.

This study is not without limitations. First, approximately 25% of the sample population developed cardiotoxicity within a year of starting trastuzumab. While cardiotoxicity rates have declined over time, the higher rate observed in this cohort may reflect the extended enrollment period, which began in 2006. Notably, cardiotoxicity rates in our sample appeared lower after 2015, aligning with trends reported in clinical trials. Second, we lacked detailed treatment information on other cardiotoxic therapies beyond anthracyclines or left-sided radiation. This limitation restricted our ability to evaluate how different treatment combinations might influence the observed associations. However, it does not undermine our key finding that biological age enhances cardiotoxicity risk stratification before treatment initiation. Third, Moffitt's patient population is mostly comprised of women of European ancestry, and findings will need to be validated in larger and more diverse populations. Fourth, the clinical interpretation of the cardiotoxicity association estimates is limited as the DNAm metrics were standardized; for these metrics to be clinically useful, associations with one-unit increases will need to be clarified. Finally, the study focused exclusively on breast cancer patients treated with trastuzumab. Further research is needed to determine whether epigenetic clock associations extend to other cancers treated with trastuzumab or other cardiotoxic therapies. Despite these limitations, the study is strengthened by its use of longitudinal LVEF assessments to identify cardiotoxicity events and its exploration of novel DNAm-based composite metrics, providing valuable insights into risk prediction and potential interventions.

In summary, this study demonstrates that epigenetic age acceleration, as measured by various epigenetic clocks, is positively associated with cardiotoxicity risk in breast cancer patients treated with trastuzumab. Incorporating epigenetic age acceleration into risk prediction models of traditional cardiotoxicity risk factors significantly improved discrimination. While biomarkers like cardiac troponins and B-natriuretic peptides can signal subclinical cardiotoxicity, epigenetic clocks appear to offer the advantage of detecting risk prior to treatment initiation, enabling earlier identification of high-risk patients. Additionally, these findings provide novel insights into the etiology of cardiotoxicity and highlight biological age as a potential target for interventions to reduce cardiotoxicity risk.

Abbreviations

BMI	Body mass index
CVD	Cardiovascular disease
CI	Confidence interval
DNAm	DNA methylation
HER2	Human epidermal growth factor receptor-2
LVEF	Left ventricular ejection fraction
OR	Odds ratio
SNP	Single nucleotide polymorphism
SD	Standard deviation
TCC	Total cancer care

Supplementary Information

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Supplementary Material 1

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Author contributions
J.M., A.R., M.A., and J.K.K. conceived the research question, designed the study, and drafted the manuscript. A.R., A.B., and J.Y.P. processed the molecular data. J.K.K performed the statistical analysis. All authors helped in the interpretation of the study results and reviewed the manuscript.

Data availability
De-identified data are available upon reasonable request to the senior author.

Declarations

Ethical approval
All participants provided consent under Moffitt Cancer Center's institutional Total Cancer Care protocol, with additional approvals for generating DNAm and genomic data from archived samples. The study is overseen by the Advarra Institutional Review Board.

Competing interests
The authors declare no competing interests.

Disclaimers
The authors have reported no relationships relevant to the contents of this paper to disclose.

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