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The Relationship of DNA methylation with Healthy Longevity

A dissertation submitted in partial satisfactory of the
requirements for the degree of Doctor of Philosophy

in

Public Health (Epidemiology)

by

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Abstract of the Dissertation

The Relationship of DNA methylation with Healthy Longevity

by

Purva Jain

Doctor of Philosophy in Public Health (Epidemiology)

University of California San Diego, 2021

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In the United States, the number of individuals who are 90 and older is expected to quadruple from 1.9 million in 2010 to 7.6 million individuals in 2050. Aging into the later years is often accompanied by time spent with mobility and cognitive impairments, multiple morbidity

and disability in activities of daily living. Impairments in either the physical or mental domains contribute to declines in one's intrinsic capacity to maintain high functioning and wellbeing.

The pace of biological aging differs across older adults of the same chronological age. Faster biological aging is linked to greater accumulation of multiple morbidities, lower physical and cognitive functioning, and earlier death. Epigenetic age, a biomarker of aging, is a composite measure of DNA methylation across specific cytosine-guanine dinucleotide sites (CpG sites) associated with chronologic or phenotypic age. Epigenetic age acceleration, as measured by epigenetic clocks, is postulated to identify whether individuals are aging faster or slower when compared to their chronologic age. There have been no prospective studies that have examined the relationships between genome wide DNA methylation or epigenetic age acceleration with multiple morbidity at a specified older age or survival to 90 years of age with intact mobility and cognitive functioning.

The first chapter of this dissertation reviews the epidemiological evidence on DNA methylation and epigenetic clocks and their relationships with longevity, mobility, cognitive functioning and multiple morbidity. The second chapter assesses the associations between four DNA methylation clocks that measure epigenetic age acceleration and exceptional longevity, defined as survival to age 90 with intact mobility and cognitive function. The third chapter utilizes an epigenome wide association study to identify specific CpG sites and regions that are associated with exceptional longevity. The fourth chapter evaluates associations between the four DNA methylation clocks and multiple morbidity among women when they reach 90 years of age. The final chapter summarizes and integrates the key findings from this dissertation and highlights future directions for research on the potential of DNA methylation and epigenetic

clocks to expand knowledge of the etiology of healthy longevity and to identify targets for intervention to improve healthy aging.

1. Introduction

1.1. Overview of Epigenetics and Epigenetic Clocks

Aging is a multifactorial degenerative process defined by the accumulation of molecular changes, which eventually compromise cellular and organ functioning. Within individuals of the same chronological age, there is considerable heterogeneity in the physiological state of being.[1] Geroscience links age to chronic disease through targeting the biological aging process, which has the ability to delay or prevent multiple age-related outcomes such as cardiovascular disease, cancer, osteoporosis and Alzheimer's among others.[2,3] In order to develop interventions that can target mechanisms related to biological aging, we need to use measures that can classify individuals as biologically aged.

Epigenetic changes are a hallmark of aging and include the methylation of DNA, or acetylation and methylation of histones and chromatin-associated proteins.[1] Low levels of de-novo methylation of CpG islands occur in normal tissues and are shown to increase with age.[4-6] Changes in DNA methylation levels can change gene expression and recruitment of transcriptional factors. Alterations in the epigenome play an important role in aging and associated phenotypic changes.[7,8] Epigenetic mechanisms are involved in several age-related diseases, cellular senescence and human tumorigenesis.[9-14]

Specific patterns of DNA methylation are well-replicated biomarkers of biological age.[15] Epigenetic age, a composite measure of DNA methylation level (DNAm) across certain CpG sites associated with chronologic or phenotypic age, provides the potential to study healthy aging, disease prevention and control. Examining the difference in epigenetic age across a group of individuals of similar age can help determine the impact of endogenous or exogenous factors that have on the rates of biological aging.[16] Epigenetic age acceleration is an invaluable marker

of aging to identify those whose chronological and epigenetic ages diverge. Positive epigenetic age acceleration identifies those who have underlying tissue that is aging faster than what is expected based on an individual's chronologic age, and negative epigenetic age acceleration, the inverse.

Figure 1.1 serves as a general example of how epigenetic age is calculated across different clocks. This clock has an intercept and 8 unique CpG sites. The grey scale indicates the level of methylation, which is assigned a coefficient based on the methylation value. The epigenetic age is then the sum of the intercept and the methylation value for each CpG site. The CpGs included in the linear regression of each clock are largely built using elastic net regression, which is a form of a regularized regression. This algorithm calculates which CpG sites to use and the coefficient of each CpG site. Due to the number of potential CpG sites, this method is preferred due to its ability to minimize the cost function and thus scaling the least informative CpG with age or phenotypic outcome to 0. The coefficient estimate (B) associated with each CpG indicates the amount that age or the phenotypic outcome changes in response to a change in the methylation value. A negative coefficient indicates a decrease in the methylation level at the CpG site with age and a positive coefficient indicates the opposite. Finally, a sum of the methylation value and learned coefficient at each CpG site represents the estimated epigenetic age.[17]

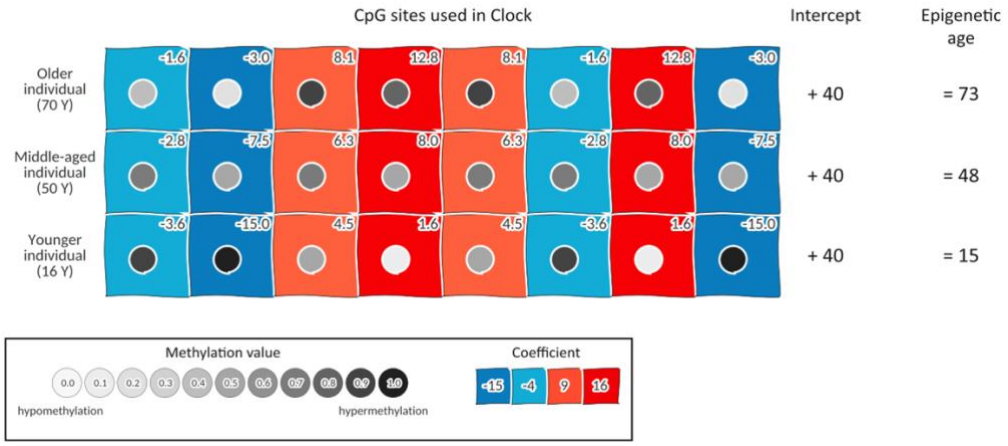


Figure 1.1: General Example of Epigenetic Age Calculation; Field et. al; 2018[17]

Chronological Age or Phenotype of Interest ~ Intercept + $B_1CpG_1 + B_2CpG_2 + \ldots + B_YCpG_Y$

There are a variety of clocks that have been created each using a unique training model with a different number of CpG sites. The clocks fall into two broad categories, the first-generation chronological clocks that use algorithms to select CpGs based on their association with chronological age and the second-generation biological clocks which have algorithms to select CpGs based on their association[18] with different aging phenotypes. Chronological clocks measure age-related changes in DNAm that are shared across individuals that occurs beyond the effect of disease. Biological clocks measure inter-individual variability in DNAm changes that contribute to declines and diseases seen in the aging process, a combination of the aging phenotypes and extrinsic drivers that influence age-related DNAm.[18] More research is required to differentiate normal biological aging versus accelerated aging due to adverse health.[1]

There are a few reasons for the selection and use of multiple epigenetic clock measures. The first reason is that genes covered by unique CpG sites within each clock have incredibly low overlap. There are only 173 genes that overlap in at least 2 DNAm clocks. More specifically, of

the four clocks (Horvath, Hannum, PhenoAge, GrimAge) being used in this study, the range in the proportion of genes that overlapped is as small as 1% (Horvath and GrimAge) up to 14% (Horvath and PhenoAge). In general, the hypermethylation of age-associated genes reduces their expression, but this is not always the case. Although the overlap can be low, there are genes that show strong relationships with certain outcomes across different clocks. As an example, there were 28 top-overlapping genes and 12 were associated with neurodegenerative disorders. This speaks to the strength of measuring epigenetic age using different clocks and the potential for DNAm clocks to be used as potential biomarkers in observational studies or clinical trials examining neurodegenerative diseases. The reason that these four clocks were chosen specifically were to have the ability to compare the results using chronological and biological clocks and to compare the results to prior literature. Prior studies that have examined aging phenotypes and longevity have used some combination of these four clocks to measure epigenetic age and epigenetic age acceleration.

1.2. Review of Epidemiologic Studies on Longevity

Exceptional longevity is an extreme phenotype, although not defined with incredibly specific constructs, possesses two major constructs. In order to experience successful aging, long lived individuals must have a biological age that is less than their chronological age and either maintain functional status or have a slowed or delayed decline in functional status. Biological age and functional status can be different ways. Evidence suggests that exceptional longevity is a combination of genetic, environmental, cultural and regional factors.[19] Rather than assessing time to death among all women who were enrolled at baseline, these studies limit the sample to women who had an opportunity to experience exceptional longevity. Focusing on this phenotype may offer strategies and insight on increasing both the health span and life span.

Studies examining the relationship of DNA methylation and human longevity are extremely limited. To the best of our knowledge, there have been only a few studies that have examined the association of epigenetic age acceleration with longevity. Horvath et. al compared the Horvath DNAmAge of 63 offspring of semi-supercentenarians (105-109 years) to 47 age-matched controls (i.e. parents were not semi-supercentenarians). The offspring of the semi-supercentenarians had a lower DNAmAge compared to controls (age difference=5.1 years, $p<.001$). In addition, the semi-supercentenarians were on average 8.6 years younger compared to their chronological age.[20] The second study published in 2017 by McEwen et. al found that among 95 participants (min age=60 years, mean=84 years) in the Costa Rican Longevity and Healthy Aging Study, the average difference between their epigenetic and chronological age was -6.9 years.[21] The final study used the Sydney Centenarian Study and found that among 23 individuals above the age of 95, the Hannum DNAmAge was on average 3.5 years (sd=3.4 years) younger than their chronological age.[22]

1.3. Review of Epidemiologic Studies on Multimorbidity

To the best of our knowledge there has been one study that examined the association between epigenetic age acceleration and comorbidity count among older adults.[23] A meta-analysis was conducted to assess the relationship of a 1-year increase in epigenetic age acceleration and comorbidity count at the time of blood draw. This analysis was conducted for each of the four clock measures (AgeAccelHorvath, AgeAccelHannum, AgeAccelPheno and AgeAccelGrim) and each analysis included the WHI BAA23, WHI EMPC, Framingham Heart Study (FHS), InCHIANTI, Jackson Heart Study (JHS). The FHS comorbidity included dyslipidemia, hypertension, cardiovascular disease (CVD) including coronary heart disease (CHD) or congestive heart failure (CHF), type 2 diabetes, cancer and arthritis. The WHI

comorbidity count included Alzheimer's disease, amyotrophic lateral sclerosis, arthritis, cancer, cataract, CVD, glaucoma, emphysema, hypertension and osteoporosis. The JHS comorbidity count included hypertension, type 2 diabetes, kidney dysfunction based on every dialysis and CVD. Finally, the InCHIANTI comorbidity count included cancer, hypertension, myocardial infarction, Parkinson's disease, stroke and type 2 diabetes. The WHI ancillary studies were stratified by race/ethnicity in the meta-analysis. Overall, all epigenetic age acceleration measures showed a significant association with cross-sectional comorbidity counts: AgeAccelGrim (P=1.1E-16), AgeAccelPheno (P=8.5E-18), AgeAccelHannum (2.1E-07) and AgeAccelHorvath (P=1.4E-06).

This study was cross-sectional and limited the comorbidities that were included in the comorbidity count for each study included. There have been no prospective studies that examined the relationship between epigenetic age acceleration and comorbidity count among women who survive to older ages (85+ years).

1.4. Review of Epigenome Wide Association Studies on Longevity

There have been two studies that have examined DNA methylation patterns associated with longevity overall. Gentilini et. al examined DNA methylation of 21 female centenarians, 21 female offspring, 21 offspring of both non-long-lived parents and 21 young women from Northern Italy. The methylated fraction of DNA was quantified using an ELISA-like reaction. While an age-related decrease in global DNA methylation was observed across groups, this process was delayed in centenarians' offspring. Genomic DNA methylation loss is associated with aging across several types of tissue in addition to age-related disease.[9-12] Additionally, there were different methylation patterns in genes associated with human longevity when comparing centenarians' offspring with age-matched control subjects born from both non-long-lived

parents. These included genes that play a role in metabolism, control of signal transmission and nucleotide biosynthesis.[24] The second study compared Nicoyans and non-Nicoyans in Costa Rica, the former a population known to have high longevity. Nicoyans were found to have several signature differences associated with slower aging including a higher proportion of CD8+ naïve cells and a lower proportion of CD8+ memory T cells.[21] Over the lifespan, naïve T cells are usually replaced by memory T cells, but this younger immune profile has been hypothesized to delay vulnerability due to infection and to increase the healthspan.[25] In addition, there was lower total mean DNAm variation in Nicoyans compared to non-Nicoyans, another marker of delayed aging.[9] Finally, the DNAm age was not significantly different between Nicoyans and non-Nicoyans.

To the best of our knowledge there have been no studies that conducted an epigenome wide association study (EWAS) on mobility and no EWAS specifically on cognitive functioning among women who were long lived. A recently published EWAS of blood DNA methylation found differentially methylated regions (DMRs) associated with hippocampal volume. The proximal genes were known to be involved in learning and memory.[26] A review of 28 studies examining the relationship of DNA methylation to Alzheimer's disease found a mix of hypermethylation, hypomethylation and inconclusive results.[27] The potential role for both hypomethylation and hypermethylation at specific gene loci has been proposed as a mechanism for dementias overall.[28]

Additionally, to the best of our knowledge there have been no studies that have used machine learning methodology to identify CpGs or sets of CpGs that are related to exceptional longevity. The benefit to using machine learning in epigenetics is to reduce collinearity of the loci within the model and prioritize the minimization of prediction error. There are two studies

worth highlighting that used similar methodology to what is being proposed for this study. Although there are several computational methods available in the analysis of DNA methylation data,[29] these studies utilized a combination of epigenome wide association and machine learning methods to identify the CpGs most predictive of the outcome of interest. The first study by Liu et. al used an EWAS and machine learning separately to identify CpG sites that were associated with alcohol intake using 13 population-based cohorts. First within each cohort, the EWAS was modeled using the DNA methylation beta value as the outcome variable with continuous alcohol measurement in grams per day and age, sex, BMI, batch effects and white blood cell counts. Alcohol consumption was also assessed categorically for both the EWAS and machine learning methods. There were then four major steps undertaken to assess if DNA methylation can be used as a biomarker in predicting alcohol consumption using machine learning. First, the whole-blood DNA samples from 10 cohorts were separated into 8 discovery and 2 replication cohorts. Then within the eight discovery cohorts, a discovery meta-analysis was performed using an inverse-variance weighted random-effects model and selected CpGs. Next, in order to minimize overfitting a LASSO regression was completed in the Family Heart Study training set to select CpGs to be used as a biomarker. The LASSO model controlled for the same confounders as the EWAS. Four sets of CpGs were selected including the largest number of CpGs and the most parsimonious set of CpGs. Then a ROC analysis was used to calculate the expected probability of being 'diseased' using a logistic regression with age, sex, and BMI with or without a set of CpGs (residuals). The sensitivity, specificity and AUC for classifying "diseased" versus "controls" were calculated. In the whole-blood samples within individuals of European ancestry, there were 363 CpGs identified that were associated with alcohol intake. The meta-analysis of discovery sets identified 361 CpGs ($p < 5x10^{-6}$). Within the FHS cohort training set, there were 5

(s=0.12), 23 (s=0.08), 78 (s='lambda.1se') and 144 (s='lambda.min'). The addition of 144 CpGs to the null model yielded a high AUC (0.90-0.99) in the ability to discriminate heavy drinkers versus non-drinkers. It is also worth noting that the models with 5 and 23 CpGs also yielded good prediction (AUC > 0.80).

The second study by Shu et. al published in 2020 identified 393 CpGs for that predicted mortality risk among an HIV-positive population. First 858 CpG sites associated with high mortality risk ($p<0.001$) among persons living with HIV (PLWH) were chosen using an epigenome wide analysis of the VACS index score in the training set. The VACS index is a well-established score to predict the risk of death among PLWH. The variable importance (0-100 score) for each selected CpG site was ranked using an elasticnet regularized generalized linear model with 100 bootstraps each containing 70% of all samples. If CpG sites had zero variable importance for 80% of the bootstraps they were removed (178 CpG sites) and the remaining 678 CpGs were ranked based on median importance ranking among 100 bootstraps and divided into 20 groups that were then used to build machine learning models. There were then three major steps used to develop machine learning prediction models for mortality risk among PLWH. Four base models (random forest, GLMNET, support vector machines and k-nearest neighbors) were used to predict mortality risk among PLWH in the training set and then aggregated. Prediction performance of each ensemble model was evaluated using the area under the receiver operating characteristic (auROC) and area under the precision recall curves. The final CpG group was based on the highest auROC curve in the validation set and independent evaluation in the testing set was conducted using balanced accuracy. Using the final ensemble model of 393 CpG sites and adjusting for baseline age, sex, race, viral load, CD4 count and antiviral medication adherence, participants who have a high risk of mortality remained to have an elevated risk of

mortality compared to those who were predicted to have a low risk of mortality (HR=1.79; 95% CI: 1.35-2.37).[30]

There have been no studies that examined DNA methylation patterns and survival to 90 years of age among women with intact mobility and cognitive functioning (absence of physician-diagnosed mild cognitive impairment and dementia). Survival to the age of 90 was selected as the primary analysis for this manuscript, because approximately one-half of WHI women in the age-eligible subgroup for survival to age 90 actually have survived. Our ability to examine survival to even older ages, is currently limited in the WHI data among women with available epigenetic data as described below.

1.5. Biological Plausibility

The biological plausibility can be defined using genes that correspond to CpGs across different clocks that are implicated in aging mechanisms and different age-related phenotypes. Using Alzheimer's disease as an example, the gene KLF14 was a hit across 6 of the 14 clocks that currently exist including Horvath, Hannum and PhenoAge, pointing towards its significance in aging mechanisms. Hypermethylation with age of KLF14 is associated with abnormal DNA repair and cell cycle control in familial early-onset Alzheimer's disease.[31] The identification of genes that are consistent across clocks in studies of the same outcome are necessary to understand the CpGs that are hypo or hypermethylated, which regions in the genome and genes the CpGs are associated with and what are the roles the genes play in disease onset and rate of progression. Additionally, BACE1 and PSEN1 which are incorporated within DNAm PhenoAge encode AB generating B- and y-secretase.[32] Clocks such as Horvath and Hannum have also been with risk factors of Alzheimer's disease including body mass index, cigarette smoking status, cholesterol and level of education.[33] The CpGs that are included in each clock can serve as a

biomarker for the complex interaction of the environment and eventual genetic pathways that lead to aging and age related outcomes.

1.6. Specific Aims

In this dissertation, I examined the relationship between DNA methylation and epigenetic age acceleration with exceptional longevity and multimorbidity. The following aims are addressed:

Aim 1.1: To determine the associations of epigenetic age acceleration (the residual variation in epigenetic age adjusting for chronologic age) and the odds of survival of to age 90 for women with intact mobility compared to women who survive without intact mobility and women who die before age 90.

Aim 1.2: To determine the associations of epigenetic age acceleration (the residual variation in epigenetic age adjusting for chronologic age) and the odds of survival of to age 90 for women with intact mobility and cognitive functioning compared to women who survive without intact mobility or without intact cognitive functioning and women who do die before age 90.

Aim 2.1: To determine significant differences in DNA methylation patterns and identify predictive CpGs and sets of CpGs for women who survive to age 90 for women with intact mobility compared to women who survive without intact mobility and women who die before age 90.

Aim 2.2: To determine significant differences in DNA methylation patterns and identify predictive CpGs and sets of CpGs for women who survive to age 90 for women with

intact mobility and cognitive functioning compared to women who survive without intact mobility or without intact cognitive functioning and women who die before age 90.

Aim 3: To determine the associations of epigenetic age acceleration (the residual variation in epigenetic age adjusting for chronologic age) and comorbidity count at age 90 years.

This research studied a population of older women living in the United States. A prospective cohort study of White, Black and Hispanic/Latino who were eligible to survive to age 90 and had DNA methylation data available was used for Aims 1, 2 and 3.

Findings from these aims provide valuable insight into the relationship between DNA methylation and epigenetic age acceleration with exceptional longevity by simultaneously assessing the relationship between lifespan and healthspan among older women in the United States.

2. The association of epigenetic age acceleration and exceptional aging in the Women's Health Initiative

Purva Jain, Alexandra Binder, Brian Chen, Humberto Parada, Linda Gallo, Steve Horvath, Parveen Bhatti, Eric A. Whitsel, Kristina Jordahl, Andrea Z. LaCroix

2.1. Abstract

Background: Faster biological aging is linked to lower cognitive functioning and physical capability, which are also strongly associated with chronic disease onset, morbidity and mortality. There have been no longitudinal studies examining the relationship between epigenetic age acceleration and exceptional longevity among older women.

Methods: This study was restricted to the 1,813 women from three Women's Health Initiative (WHI) ancillary studies with assays of genome-wide DNAm from WHI baseline who had an advanced enough age at baseline and remained an active WHI participant long enough to be eligible to survive to age 90 by the end of our observation period (September 30, 2020). Epigenetic age acceleration (EAA) was estimated using four established "clocks" (Horvath pan-tissue, Hannum, PhenoAge and GrimAge). We examined EAA as estimated by each clock in relation to these three-category exceptional longevity outcomes: women who survived to age 90 with intact physical functioning and women who survived to age 90 without intact physical functioning compared to women who did not survive to age 90. The second analysis compared women who survived to age 90 with intact physical and cognitive functioning and women who survived to age 90 without intact physical and/or cognitive functioning to women who did not survive to age 90.

Results: Compared to those that did not survive to age 90, the odds of surviving with intact physical function were reduced for every one standard deviation increase in AgeAccelHorvath (OR=0.82; 95% CI: 0.69-0.96; p=0.010), AgeAccelHannum (OR=0.67; 95% CI: 0.56-0.80; p<0.010), AgeAccelPheno (OR=0.60; 95% CI: 0.51-0.72; p<0.01) and AgeAccelGrim (OR=0.68; 95% CI: 0.55-0.84, p<0.01). The strength of these associations were similar when comparing to those that survived to age 90 with intact physical and cognitive function compared to women who did not survive to age 90.

Conclusion: Overall, our study shows that EAA is a valid biological marker of exceptional longevity among older women. As women are aging, the goal is to promote and maintain high physical and cognitive functioning so they can thrive.

2.2. Introduction

As of 2020, approximately 3.8 million individuals were aged 85 years or older in the United States (US).[34] This population is expected to quadruple over the next few decades and will comprise 10% of the US population by 2050. Traditionally, those aged 85 years or older have been considered the 'oldest old'. However, due to increases in longevity, the 90 years or older group requires additional focus. Women comprise a significantly larger proportion of long-lived individuals, and outnumber men 3 to 1 among those 90 or older.[35]

Functional status, as defined by both physical and mental capabilities, is the foundation of wellbeing in older age. These capabilities include a person's ability to meet their basic needs, learn, grow, and make decisions, and to be mobile, build and maintain relationships, and contribute to society.[36] As more women age into their 10th decade of life, living with impaired function becomes more common, but does not affect all women.[37] Impairments in either the physical or mental domains contribute to declines in one's intrinsic capacity to remain high

functioning.[38] A history of dementia or memory loss doubles the risk of disability directly through the inability to perform activities of daily living, or indirectly through decreased capability to maintain physical health.[39] These effects may be further compounded by the presence of comorbidities.[40]

Biological aging focuses on the underlying biological mechanisms of aging, such as epigenetics, that impact future health trajectories. Under the purview of geroscience, these biological mechanisms are central and fundamental to global increases in disease and disability as one ages.[41] Individuals with exceptional longevity are thought to have a biological age that is less than their chronological age. That is, women who experience exceptional longevity appear to be aging more slowly, where aging is conceptualized as a multifactorial degenerative process defined by the accumulation of molecular changes, which eventually compromise cellular and organ functioning. Within individuals of the same chronological age, there is considerable heterogeneity in the physiological state of being and the rate of biological aging.[1] Faster biological aging is linked to lower cognitive functioning and physical capability, which are also strongly associated with chronic disease onset, morbidity and mortality. Exceptional longevity may be accompanied by either maintained functional status or sufficient function to maintain independence. This concept is closely aligned with the idea of the healthspan, which is the prioritization of physiological function throughout the lifespan through effective strategies to promote primary and secondary prevention of impaired function [42].

Epigenetic age is a biomarker of aging previously reported to be associated with age-related diseases and all-cause mortality.[23,32,43,44] It is a composite measure of DNA methylation (DNAm) levels across specific cytosine-guanine dinucleotide (CpG) sites that together form a single measure that is associated with either chronologic or phenotypic age. Epigenetic age

acceleration (EAA), the residual variation in epigenetic age independent of chronological age, is one measure of whether individuals are aging faster or slower than their chronological age. EAA signifies individuals who, due to a combination of endogenous and exogenous factors, are aging faster biologically when compared to their chronological age whereas inverse or slower age acceleration signifies the opposite. Prior studies suggest reduced EAA among long-lived individuals.[20 21,22] Among long-lived individuals, older epigenetic age was also reported to be associated with lower levels of physical functioning[23,32] and declines in global cognitive functioning.[45-47]

There have been no longitudinal studies examining the relationship between EAA and exceptional longevity among older women. The aims of this study, therefore, were to examine the relationships between EAA and both survival to age 90 with intact mobility and survival to age 90 with intact mobility and cognitive functioning. We hypothesized that women who experience decelerated biological aging, as measured by epigenetic age, will be more likely to survive to age 90 with intact mobility and survive to age 90 with intact mobility and cognitive functioning.

2.3. Methods

Study Population

The Women's Health Initiative (WHI) (n=161,808) was initiated in 1993 with the goal of identifying strategies to prevent heart disease, osteoporosis and breast and colorectal cancer among postmenopausal women.[48,49] The current study included participants from three (two nested case-control and one prospective) WHI ancillary studies who had available data on DNAm. The Bladder Cancer and Leukocyte Methylation Ancillary Study (AS 311) included 468 women with and 468 without bladder cancer to identify methylation profiles associated with

bladder cancer risk using specific DNA methylation loci.[50] The Epigenetic Mechanisms of Particulate Matter-Mediated Cardiovascular Disease Ancillary Study (AS315) included a random sample of 2,200 WHI clinical trial participants to understand the pathophysiological mechanisms that underlie particulate matter-related cardiovascular disease in postmenopausal women.[51] Last, the Integrative Genomics for Risk of Coronary Heart Disease and Related Phenotypes in the WHI Cohort Ancillary Study (BA23) included 1,070 women with and 1,070 women without coronary heart disease.[52]

There were 443 women in AS311, 694 women in AS315 and 942 women in BAA23 who were eligible to survive to age 90 (total = 2,079). Of these, 395 women had information available on all longevity components in AS311, 579 in AS315 and 839 in BAA23 resulting in a final analytic sample of 1,813 women (Figure 2.1). Baseline age ranged from 63.4-81.4 years with an average of 70.5 years (i.e. follow-up ranged from 9-27 years with an average of 20 years).

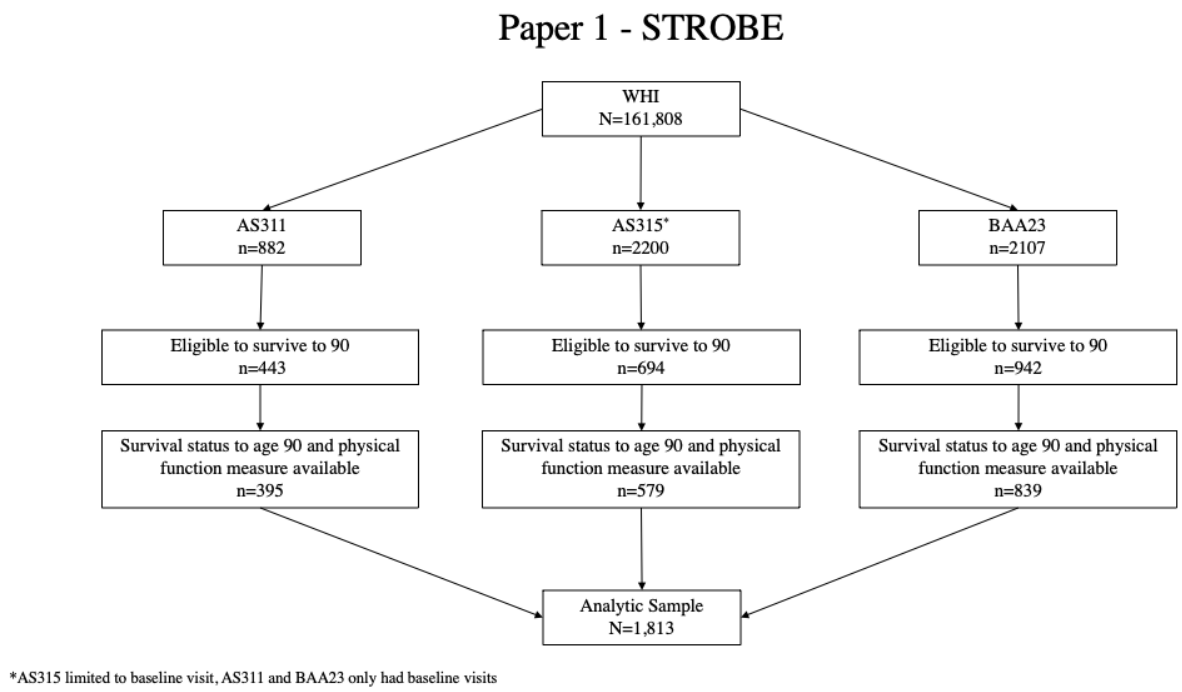


Figure 2.1: STROBE Diagram

This current study was restricted to the 1,813 women from the three WHI ancillary studies with assays of genome-wide DNAm from WHI baseline who had an advanced enough age at baseline and remained an active WHI participant long enough to be eligible to survive to age 90 by the end of our observation period (September 30, 2020).

Measures

DNAm was measured using the Illumina Infinium 450K platform (San Diego, CA, Illumina). The minfi R package was used to read in IDAT files, check for failed samples and implement normalization and quality control steps. The normal-exponential convolution using out-of-band probes method was used to correct probe intensity levels and functional normalization was used to account for type I and type II probe differences and remove technical variation and batch effects. The minfi and wateRmelon R packages were used to remove low

quality probes that interrogate non-CpG sites, are located on the X or Y chromosome or have a detection p-value above 0.01 in any sample.

Epigenetic age was estimated using four established "clocks", including the Horvath pan-tissue, Hannum, PhenoAge and GrimAge, which were then used to estimate specific EAA measures. A description of each clock analyzed is summarized in Table 2.1.[23,32,43,44] Hannum's clock used 71 CpG sites in the blood to predict age and Hannum's used 353 CpG sites to predict age across several different tissues. DNAm PhenoAge was trained on a "phenotypic age" measure created using nine clinical biomarkers associated with time-to-death. DNAm GrimAge was developed by predicting time-to-death using age, sex, DNAm-based surrogate biomarkers of plasma protein levels and a DNAm-based estimator of smoking pack-years (Table 2.1).

Table 2.1: Overview of epigenetic clocks being utilized in this study

Clock	CpGs	Genes	Age	N	Tissue	Reported Associations
Horvath[43]	353	344	0-101	8000	Various cell & tissues	Chronological age, all-cause mortality, cancer, age-related disease and several neurodegenerative phenotypes
Hannum[44]	71	94	19-101	656	Blood	Chronological age, all-cause mortality
PhenoAge[32]	513	505	>20	9926	Blood	All-cause and cause-specific mortality, survival, count of comorbidities, physical functioning, smoking status and telomere length
GrimAge[23]	1030	NA	NA (mean=66)	1731	Blood	Morbidity and mortality, survival, cognitive decline, clinical biomarkers, lifestyle factors, blood cell composition and telomere length

Table adapted from Bergsma & Rogaeva[18]

Survival outcomes

We defined three primary outcomes for this study as follows: 1) survival to age 90 with intact cognitive and physical function; 2) survival to age 90 with impairment in cognitive and/or physical function; and 3) death before age 90. Classifications were based on follow-up data through September 30th, 2020. Survival to age 90 was calculated from day of enrollment in the WHI through September 30th, 2020. The WHI ascertained death using annual mailed outcome questionnaires, systematic searches of the National Death Index, hospital records, obituaries and proxy queries.[53] Among women eligible to survive to age 90 (n=2,079), 128 (0.06%) were missing vital status at age 90. Intact physical functioning was defined using two questions from the Rand-36 Physical Function questionnaire[54] as having no self-reported limitations for both walking one block and climbing one flight of stairs from baseline to age 90. The questionnaire was administered at baseline, at 1 and 3-year follow-up assessments, and then annually after 2005. Intact cognitive functioning was defined as having no self-reported moderate or severe memory problems nor dementia or Alzheimer's Disease from baseline to age 90 and was assessed annually by questionnaire. Among women enrolled in the WHI Extension Study 1 (2005-2010) with at least 1 Form 33 collected after enrollment, the validation of Alzheimer's disease against Medicare claims was as follows (sensitivity=40%; specificity=95%) and against WHIMS (sensitivity=41%; specificity=89%).

Covariates

Covariates were measured at WHI baseline and selected due to their associations with both EAA and exceptional longevity. Covariates included age at blood draw, blood cell composition (CD8+ T Cells, CD4 T cells, Natural Killer cells, B lymphocyte cells, Monocytes, Granulocytes), race/ethnicity (White, Black, Hispanic, Other), education (high school/general education development or less, some college, college graduate or more), walking frequency

(rarely or never, 1-3 times/mo, 1 time/wk, 2-3 times/wk, 4-6 times/wk, 7+ times/wk), body mass index categories (underweight, normal, overweight, obese), alcohol consumption (non-drinker, past drinker, <1 drink/mo, <1 drink/wk, 1-<7 drinks/wk, 7+ drinks/wk), pack-years smoking (never smoker, <5, 5-20, 20+), number of chronic conditions at baseline (0, 1-2, 3+, including cancer, stroke, Alzheimer's disease, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and physical function score (RAND-36 10-item physical function subscale[54], range 0-100, higher score reflects higher function). Chronic conditions were chosen based on the high degree of impact these conditions play in the lifespan and healthspan of older women.[55-57]

Statistical Analysis

Baseline characteristics were reported by exceptional survival category. Differences by category were tested using Pearson's chi-squared tests for categorical variables and F-tests for continuous variables. The correlations between chronological age and each DNAmAge measure are reported in-text and the correlations between DNAmAge measures are reported in supplemental material. Fully-adjusted multinomial logistic regression models with a random intercept for ancillary study were used to assess the relationships between each EAA measure per standard deviation increase and exceptional longevity. The adjusted model included all covariates as described above. The weighted analysis included inverse probability weights to account for the case-control sampling of AS311 and BA23. The weights were the inverse of the selection probability for each individual and cases were down weighted. The sample was reweighted so that the sum of the weights was similar to the original sample size. Inverse probability weights were also applied to AS315 to account for the oversampling of racial/ethnic

minorities.

We examined EAA as estimated by each clock in relation to these three-category exceptional longevity outcomes as follows: 1) The first analysis compared women who survived to age 90 with intact physical functioning and separately, women who survived to age 90 without intact physical functioning to women who did not survive to age 90. 2) The second analysis compared women who survived to age 90 with intact physical and cognitive functioning and separately, women who survived to age 90 without intact physical and/or cognitive functioning to women who did not survive to age 90. 3) The final sensitivity analysis replaced the self-reported measure of intact cognitive function with the adjudicated measure of cognitive function (Table 2.2).

Table 2.2: Overview of exceptional longevity outcome components and comparison groups

Exceptional Longevity Outcome Groups			
	Group 1	Group 2	Group 3 (Ref)
Outcome 1: Longevity + Physical Health	Survival to age 90[a] with Intact Physical Function [b]	Survival to age 90 and Loss of Physical Function	Did not Survive to age 90
Outcome 2: Longevity + Physical & Cognitive Health	Survival to age 90 with Intact Physical and Cognitive Function [c]	Survival to age 90 and Loss of Physical and/or Cognitive Function	Did not Survive to age 90
Sensitivity Analysis: Longevity + Physical & Cognitive Health (WHIMS)	Survival to age 90 with Intact Physical and Cognitive Function [d]	Survival to age 90 and Loss of Physical and/or Cognitive Function	Did not Survive to age 90

[a] Survival to age 90 is defined as survival to age 90 from WHI baseline to end of follow-up

[b] Intact physical function is defined as no report of "Yes, limited a lot" to walk one block or climb one flight of stairs on annual questionnaires from WHI baseline to age 90

[c] Intact cognitive function is defined as no report of "Moderate or severe memory problems" or "Dementia or Alzheimer's" on annual questionnaires from WHI baseline to age 90

[d] Intact cognitive function is defined as no report of adjudicated diagnosis of "Probable dementia" from baseline to age 90 in WHIMS

Subgroup analyses between DNAmAge and exceptional longevity by race/ethnicity (White, Black, Hispanic, Other) and baseline age (median split = 70.5 years) were completed in the fully-adjusted and weighted pooled multinomial logistic regression models and tested using interaction terms with a Wald test at an alpha of 0.05. Additionally, the results from the fully-adjusted and weighted models stratified by ancillary study are included in supplemental material. All analyses were conducted using R Version 1.4.1106 (R Foundation for Statistical Computing, Vienna, Austria).

A sensitivity analysis was conducted by replacing the WHI self-reported measure of cognitive impairment with an adjudicated diagnosis of probable dementia from the Women's Health Initiative Memory Study (WHIMS) a nationally representative cohort study of women 65 years and older who were participating in the hormone therapy trial in the WHI.[58] The analysis was limited to women who participated in both WHI and WHIMS. WHIMS determined the incidence of all-cause dementia using cognitive functioning screening and neurologic and neuropsychological evaluations followed by surveillance for changes in cognitive functioning and use of a consensus panel to define probable dementia. The details of the design have been previously described.

2.4. Results

Of the 1,813 women included in this study, 464 experienced exceptional longevity (i.e., survived to age 90 with intact physical and cognitive functioning), 420 women survived to age 90 with loss of intact physical and/or cognitive functioning, and 929 women did not survive to age 90. Compared to women who survived to age 90 without intact function and women who did not survive to age 90, women who experienced exceptional longevity were more likely to be White, be college graduates, walk 2-3 times per week and 4-6 times per week, have a BMI in the

healthy weight range of 20-25 kg/m^2 or overweight range of 35-30 kg/m^2, have more than 1 but less than 7 alcoholic drinks per week, be never smokers, have none of the major chronic conditions examined, and have higher physical functioning (Table 2.3). Additionally, each of the four epigenetic age measures had low correlations with chronological age (Figure 2.2) and with each other (Supplementary Figure 2.1).

Table 2.3: Baseline Characteristics by Level of Outcome (n=1,813)

	90 w/ Phys & Cog Health (n=464)	90 w/o Phys/Cog Health (n=420)	Did not Survive (n=929)	p
Race/Ethnicity, n (%)				0.013
Black or African American	66 (14.3)	73 (17.4)	179 (19.4)	
Hispanic/Latino	27 (5.9)	36 (8.6)	78 (8.5)	
White	348 (75.7)	305 (72.8)	637 (69.1)	
Other	19 (4.1)	5 (1.2)	28 (3.0)	
Education, n (%)				0.003
HS/GED or Less	103 (22.3)	114 (27.3)	281 (30.5)	
Some College	181 (39.2)	177 (42.3)	369 (40.0)	
College Grad or More	178 (38.5)	127 (30.4)	272 (29.5)	
Walking Frequency, n (%)				<0.001
Rarely or Never	56 (12.1)	88 (21.2)	204 (22.2)	
1-3 times/month	66 (14.2)	51 (12.3)	144 (15.7)	
1 time/week	50 (10.8)	34 (8.2)	109 (11.9)	
2-3 times/week	144 (31.0)	118 (28.4)	231 (25.2)	
4-6 times/week	111 (23.9)	90 (21.7)	163 (17.8)	
7+ times/week	37 (8.0)	34 (8.2)	67 (7.3)	
BMI Category, n (%)				<0.001
Underweight	6 (1.3)	3 (0.7)	9 (1.0)	
Normal	167 (36.2)	101 (24.2)	251 (27.2)	
Overweight	189 (41.0)	150 (35.9)	296 (32.0)	
Obese	99 (21.5)	164 (39.2)	368 (39.8)	
Alcohol Consumption, n (%)				<0.001
Non-drinker	60 (13.1)	63 (15.1)	121 (13.2)	
Past drinker	70 (15.3)	95 (22.7)	224 (24.5)	
<1 drink/month	51 (11.1)	56 (13.4)	134 (14.6)	
<1 drink/week	99 (21.6)	93 (22.2)	167 (18.3)	
1-<7 drinks/week	120 (26.1)	71 (17.0)	173 (18.9)	
7+ drinks/week	59 (12.9)	40 (9.6)	96 (10.5)	
Smoking Pack-Years, n (%)				<0.001
Never Smoker	277 (62.0)	248 (60.6)	425 (47.6)	
<5	51 (11.4)	59 (14.4)	96 (10.8)	
5-<20	64 (14.3)	41 (10.0)	114 (12.8)	
20+	55 (12.3)	61 (14.9)	258 (28.9)	
Number of Chronic Conditions, n (%)[a]				<0.001
0	143 (30.8)	101 (24.0)	202 (21.7)	
1-2	291 (62.7)	271 (64.5)	615 (66.2)	
3+	30 (6.5)	48 (11.4)	112 (12.1)	
Age, mean (SD)	71.6 (3.5)	71.3 (3.2)	70.2 (3.4)	<0.001
Physical Function Score, mean (SD)	82.4 (20.2)	72.8 (22.7)	69.5 (24.6)	<0.001
AgeAccelHorvath	-0.6 (5.3)	0.02 (5.4)	0.09 (5.3)	0.052
AgeAccelHannum	-1.2 (4.9)	0.1 (5.0)	0.4 (5.2)	<0.001
AgeAccelGrim	-1.5 (6.8)	0.5 (6.8)	1.1 (7.0)	<0.001
AgeAccelPheno	-1.3 (3.4)	-0.6 (3.5)	0.8 (4.2)	<0.001

Note: GED=general educational development; BMI=body mass index; kg=kilograms; m=meters

Note: AgeAccel measures are the residual between chronological age and epigenetic age as measured by each individual epigenetic clock.

[a]Conditions include cardiovascular disease, cancer, cognitive impairment, depression, osteoarthritis, history of falls, chronic obstructive pulmonary disease, hypertension, diabetes, hip fracture and cerebrovascular disease.

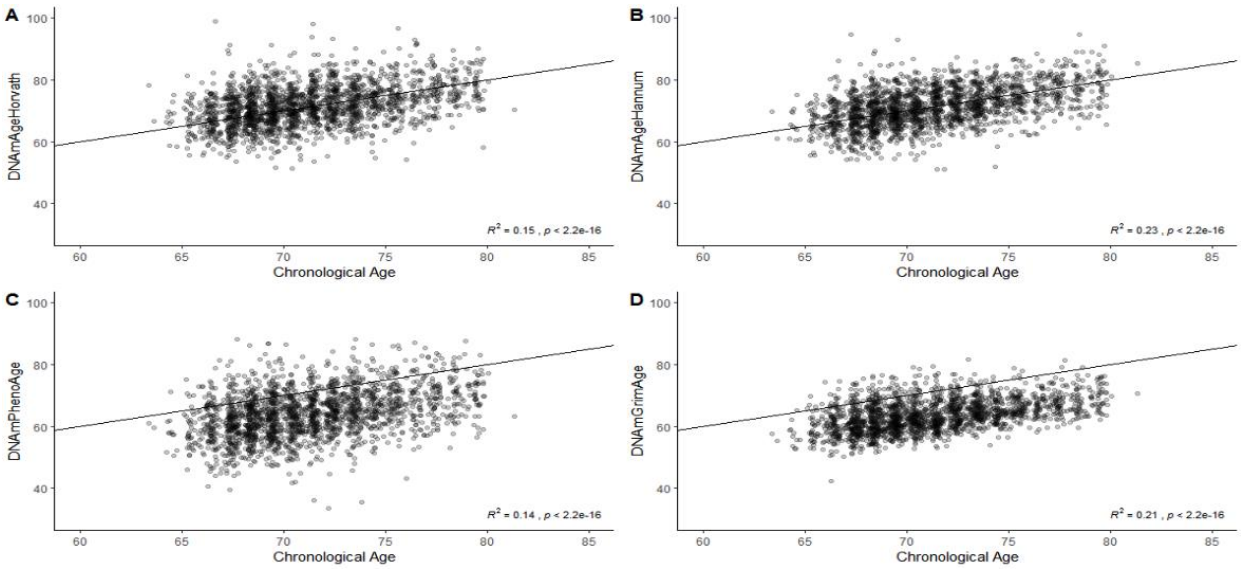


Figure 2.2: Correlation of Chronological Age and DNAmAge Measures

The results from the multinomial logistic regression models examining the association between EAA and exceptional longevity outcomes are reported in Table 2.4. Compared to those that did not survive to age 90, the odds of surviving with intact physical function were reduced for every one standard deviation increase in AgeAccelHorvath (OR=0.82; 95% CI: 0.69-0.96; p=0.010), AgeAccelHannum (OR=0.67; 95% CI: 0.56-0.80; p<0.010), AgeAccelPheno (OR=0.60; 95% CI: 0.51-0.72; p<0.01) and AgeAccelGrim (OR=0.68; 95% CI: 0.55-0.84, p<0.01). There were 29 women who were reclassified from survival to age 90 with intact physical and cognitive functioning to survival to age 90 without intact physical and cognitive functioning. The strength of these associations were similar when comparing to those that survived to age 90 with intact physical and cognitive function for every standard deviation increase in AgeAccelHorvath (OR=0.83; 95% CI: 0.71-0.98; p=0.030), AgeAccelHannum (OR=0.68; 95% CI: 0.57-0.82; p<0.001), AgeAccelPheno (OR=0.60; 95% CI: 0.50-0.72; p<0.001) and AgeAccelGrim (OR=0.73; 95% CI: 0.59-0.90, p=0.003). The odds of surviving without intact physical function were reduced for every one standard deviation increase in

AgeAccelPheno (OR=0.75; 95% CI: 0.63-0.90; p=0.002) and AgeAccelGrim (OR=0.82; 95% CI: 0.65-1.02; p=0.071) (Table 2.4, Figure 2.3). These associations were slightly more precise when comparing to the odds of surviving to age 90 without intact physical function or cognitive function for every standard deviation increase in AgeAccelPheno (OR=0.74; 95% CI: 0.62-0.88; p=0.001) and AgeAccelGrim (OR=0.75; 95% CI: 0.60-0.92; p=0.007) (Table 2.4, Figure 2.3). The results were similar when the analyses were restricted to the subgroup of women who participated in the WHI Memory Study where the outcome of intact physical and cognitive functioning was defined using an adjudicated WHIMS measure of probable dementia or mild cognitive impairment. The only differences were a strengthening of the relationship of AgeAccelPheno and likelihood to experience exceptional longevity and an attenuation for AgeAccelHorvath (Supplementary Table 2.1). In secondary analyses, we examined the interaction of each EAA measure with both race/ethnicity and length of follow-up in relation to survival to age 90 with intact physical and cognitive function and survival to age 90 without intact physical or cognitive function. Race/ethnicity and length of follow-up did not modify the association between EAA and exceptional longevity (Supplementary Table 2.2).

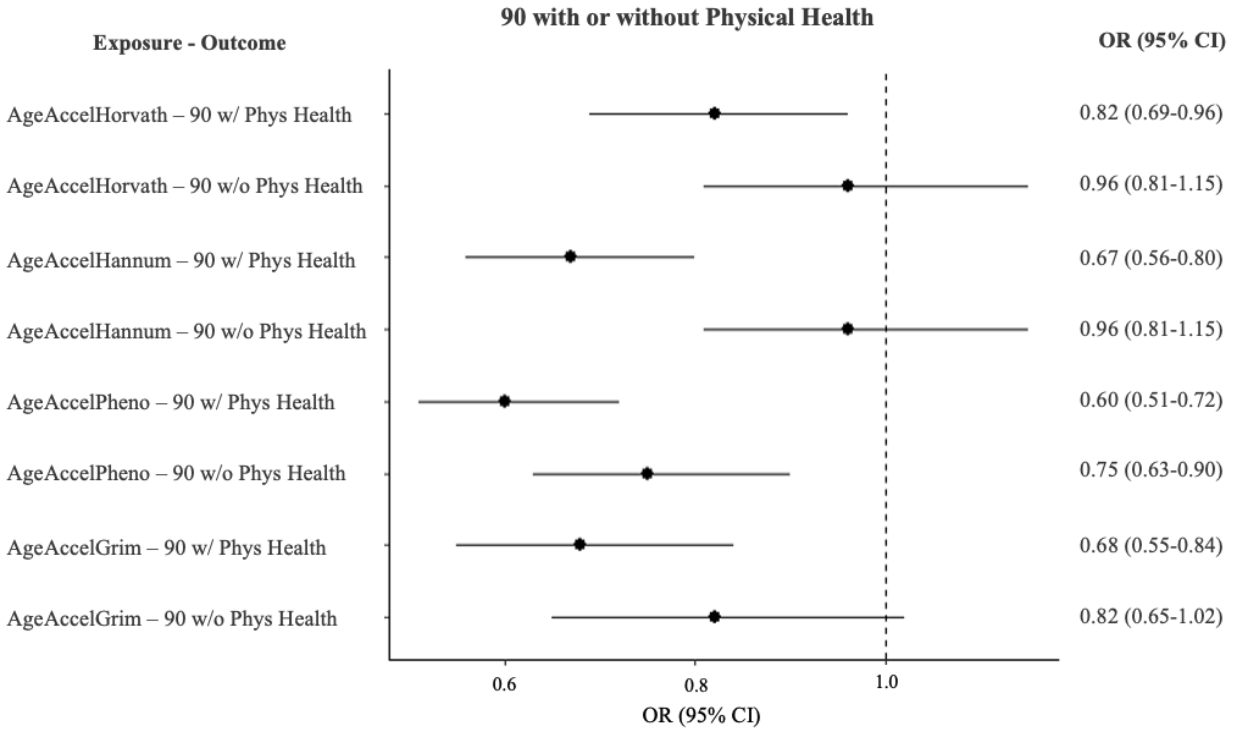
Table 2.4: Association of Epigenetic Age Acceleration and Exceptional Longevity Outcomes (N=1,813)

	90 with Intact Physical Function (n=493)[a]		90 without Intact Physical Function (n=391) [a]	
	OR (95% CI)[b]	p	OR (95% CI)[b]	p
AgeAccelHorvath	0.82 (0.69-0.96)	0.014	0.96 (0.81-1.15)	0.681
AgeAccelHannum	0.67 (0.56-0.80)	<0.001	0.96 (0.81-1.15)	0.680
AgeAccelPheno	0.60 (0.51-0.72)	<0.001	0.75 (0.63-0.90)	0.002
AgeAccelGrim	0.68 (0.55-0.84)	<0.001	0.82 (0.65-1.02)	0.071
	90 with Intact Physical & Cognitive Function (n=464) [a]		**90 without Intact Physical and/or Cognitive Function (n=420) [a]**	
	OR (95% CI)[b]	p	OR (95% CI)[b]	p
AgeAccelHorvath	0.83 (0.71-0.98)	0.030	0.93 (0.78-1.10)	0.380
AgeAccelHannum	0.68 (0.57-0.82)	<0.001	0.91 (0.77-1.09)	0.309
AgeAccelPheno	0.60 (0.50-0.72)	<0.001	0.74 (0.62-0.88)	0.001
AgeAccelGrim	0.73 (0.59-0.90)	0.003	0.75 (0.60-0.92)	0.007

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, number of chronic conditions (including cancer, stroke, Alzheimer's, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]The reference group for all comparisons is women who did not survive to age 90 (n=929).

[b]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=6.4), AgeAccelHannum (sd=6.2), AgeAccelPheno (sd=7.6) and AgeAccelGrim (sd=5.1).



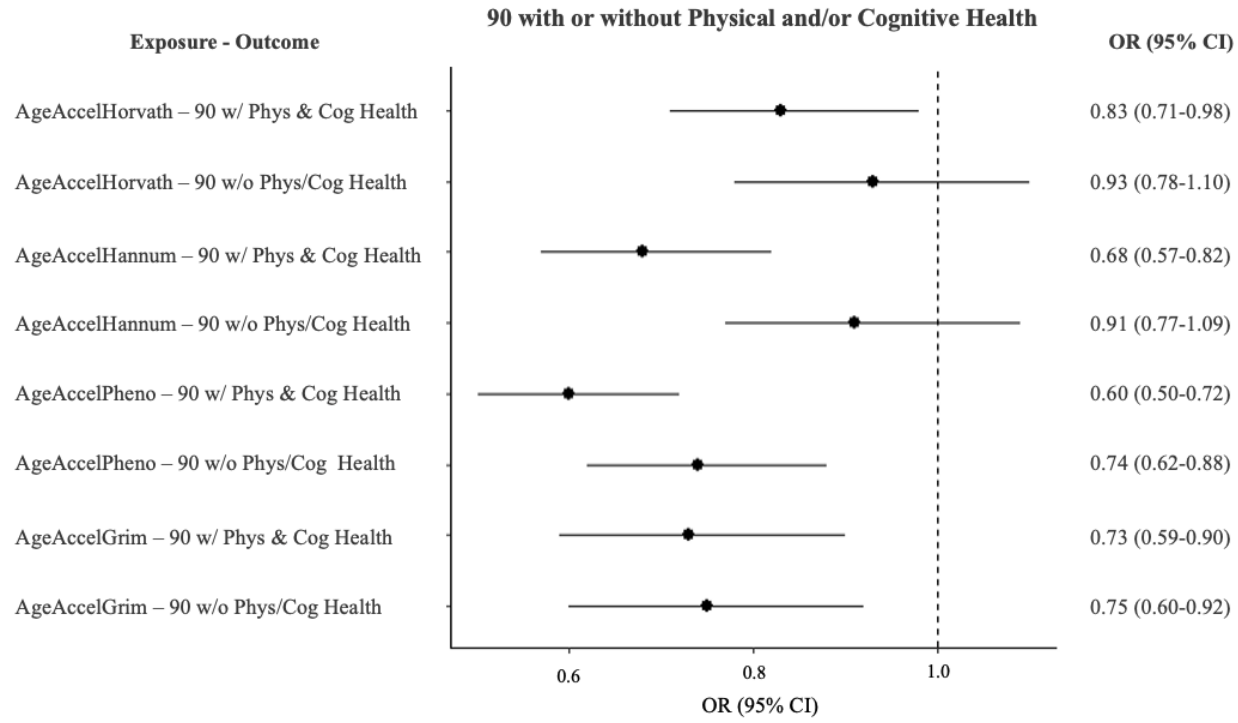


Figure 2.2: Forest Plots of the Association of Epigenetic Age Acceleration and Exceptional Longevity

2.5. Discussion

To our knowledge, this is first study examining the relationship between EAA and exceptional longevity among older women. In this racial/ethnically diverse cohort of older women, this longitudinal study showed that increased EAA as measured by the AgeAccelHorvath, AgeAccelHannum, AgeAccelPheno and AgeAccelGrim clocks resulted in decreased odds of survival to age 90 with intact physical functioning. The results were strongest in the AgeAccelHannum, AgeAccelPheno and AgeAccelGrim measures followed by AgeAccelHorvath. The newer generation PhenoAge and GrimAge clocks were also predictive of survival to age 90 without intact physical function, but not the older generation clocks. The results remained similar when the exceptional longevity outcome additionally included intact cognitive functioning, although there were only 29 number of women who were reclassified from survival to age 90 with intact physical and cognitive functioning to survival to age 90 without intact physical and cognitive functioning. Additionally, the results remained similar when the analysis was limited to the WHI Memory study and utilized an adjudicated measure for probable dementia and mild cognitive impairment to define the outcome. The interaction of each clock with both race/ethnicity and baseline age/length of follow-up did not indicate potential differences in the primary findings by these groups of interest.

To date, only two studies have examined epigenetic aging in association components of exceptional longevity (long-lived, physical and/or cognitive functioning) as separate outcomes. In the first study by McEwen et. al among 48 Nicoyans (mean age=83 years) and 47 non-Nicoyans (mean age=85 years) (Nicoyans are long-lived individuals from the Nicoya peninsula of Costa Rica) using the Horvath pan-tissue and Hannum clocks there were no statistically significant differences between Nicoyans and non-Nicoyans for either AgeAccelHorvath or

AgeAccelHannum. The authors indicated that they only had power to examine very large differences between groups.[21] Additionally, the authors discussed several known differences between Nicoyans and non-Nicoyans such as level of education, type of health insurance, longer knee height, lower BMI and waist circumference, which were not included as potential confounders in the analysis. In the second study by Horvath et al. EAA of 82 Italian semi-supercentenarians, 63 offspring of semi-supercentenarians, and 47 age-matched controls, compared to age-matched controls, the offspring of semi-supercentenarians had a lower intrinsic epigenetic aging rate.[20] A similar trend was reported in a smaller study that compared 21 offspring of female centenarians to age-matched controls and among long-lived individuals in the Sydney Centenarian Study.[22,24] This study, however, did not adjust for important covariates including blood cell counts and lifestyle factors in their analysis, did not examine the newer measures of EAA including AgeAccelPheno and AgeAccelGrim and had a small sample of long-lived individuals with DNA methylation data available (n=23).

There have been some studies of physical and cognitive functioning among older adults and to the best of our knowledge none among long-lived individuals. One study included 791 members of the Lothian Birth Cohort 1936, a cohort of 1,091 community-dwelling adults with mean age 70 years. The authors reported that a one year increase in extrinsic EAA was associated with a 6% increase in the risk of being physically frail (3+ of the following: weakness, self-reported exhaustion, slow gait speed, unintentional weight loss and low physical activity).[59] When converted to a 6 year increase, the estimated 42% increase in risk lies within the range of our estimates (5.1 to 7.6 year increases in epigenetic age). These findings were similar to a study that examined this association among 1,820 men and women aged 50-75 years.[60] Another cross-sectional study of 1,091 individuals by Marioni et. al using the Lothian Birth Cohort found a

statistically significant relationship between age acceleration and both grip strength and fluid cognitive ability.[45] The women in our study were also 70 years on average at baseline, and also had repeated measures of physical functioning that were taken into account in the assessment of exceptional longevity as they continued to age. Levine et. al conducted a study of EAA and AD-related cognitive decline and related neuropathological markers using 700 dorosolateral prefrontal cortex (DLPFC) samples from Caucasian subjects (mean age at enrollment=81.4; mean age at death=88.1) in the Religious Order Study and Rush Memory Aging Project. The authors found a statistically significant relationship between EAA of the DLPFC and a longitudinal decline in global cognitive functioning, episodic memory and working memory among individuals with AD, but not among individuals without AD.[46]

Epigenetic clocks are measures of biological aging that have been previously associated with mortality, physical functioning, and cognitive status in addition to other markers of health. These clocks measure the DNA methylation of cytosines at CpG nucleotides, which is one of the key epigenetic mechanisms involved in gene expression and splicing.[18] The training method of the clocks differed including the age range, statistical methodology, sample characteristics and technical factors. The first-generation clocks were trained to predict chronological age and the second-generation clocks predict multisystem phenotypic age and time-to-death.[18] The training of PhenoAge and Grim age to predict phenotypic age and time-to-death, respectively, versus chronological age in the first generation clocks most likely led to a stronger association using the newer clocks. Thus, there is a low overlap in the CpGs and associated genes that are included in each clock, suggesting complex and varied involvement of different biological processes during aging such as transcription, epigenomic instability, telomere biology and cellular differentiation

and senescence.[61] The associations in this study may be capturing these underlying biological processes and the influence of environmental factors as captured by the epigenetic clocks.[62]

There were several strengths and limitations to this study that should be noted. This study benefitted from a large, racial/ethnically diverse sample of women who were followed to at least 90 years of age. Women were followed for 20 years on average with low rates of loss to follow-up. There was information available on important baseline characteristics and potential confounders due to the prior data collection in the WHI. There were several clocks utilized to measure EAA with a mix of chronological and phenotypic clocks. This is currently the best practice due to the low overlap of CpG sites and associated genes between the clocks.[63] Finally, we had repeated measures of both physical and cognitive functioning from baseline to age 90 or time of death that were taken into account in the exceptional longevity classification although due to those who were unable to complete the annual survey due to their level of frailty there may the results may be attenuated. Among the limitations, the first is the nested case-control sampling of two of the ancillary studies from the larger WHI. If the sampling structure is ignored the disproportionate stratified subsamples of the study base can lead to biased estimates. Using inverse probability selection weights to account for differences in selection criteria is a recommended solution and was implemented in this study.[64] This study was limited to women and replication in cohorts that include both men and women, diverse racial/ethnic groups and represent individuals from varied regions of the world is important. Notably, few prospective studies exist for replication where sufficient numbers of participants have been followed for 20 or more years to the age of 90 or older so that healthspan related phenotypes can be defined. Additionally, epigenetic age is one measure of biological age within several spheres such as genomics, transcriptomics, proteomics, microbiomics and metabolomics to name a few. Since

there is currently no gold standard to measure biological age this research should be taken in from the systems biology purview, which attempts to understand the system as a whole rather including acknowledgement of the joint influences and interactions of several factors.[65]

In this study, we reported that increased EAA measured by both chronological age and mortality predictor clocks was associated with a decreased likelihood of experiencing exceptional longevity among older women. EAA can be used as a potential predictor of exceptional longevity among older women as it captures the effects of endogenous processes that contribute to the aging process. While future intervention upon specific CpG sites may be possible, this requires additional studies to identify the most promising targets. Overall, our study shows that EAA is a valid biological marker of exceptional longevity among older women. As women experience increases in life expectancy, there will be even larger numbers of women who are long-lived. As women are aging, the goal is to promote and maintain high physical and cognitive functioning so that they can thrive.

Supplementary Tables

Table 2.5: Association of Epigenetic Age Acceleration and Exceptional Longevity using WHIMS measure for Cognitive Impairment

	90 with Intact Physical & Cognitive Function (n=146) [a]		90 without Intact Physical and/or Cognitive Function (n=195) [a]	
	OR (95% CI)[b]	p	OR (95% CI)[b]	p
AgeAccelHorvath	0.94 (0.63-1.37)	0.695	1.29 (0.88-1.74)	0.166
AgeAccelHannum	0.60 (0.37-0.94)	0.023	0.94 (0.64-1.44)	0.844
AgeAccelPheno	0.35 (0.15-0.73)	0.008	0.68 (0.35-1.25)	0.208
AgeAccelGrim	0.69 (0.52-0.90)	0.011	0.86 (0.69-1.11)	0.218

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, number of chronic conditions (including cancer, stroke, Alzheimer's, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.
Note: This sensitivity analysis replaced the annual self-reported WHI measure of moderate or severe memory problems or Alzheimer's or dementia with the adjudicated WHIMS measure of probable dementia.

[a]The reference group for all comparisons is women who did not survive to age 90 (n=325).
Note: Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=6.4), AgeAccelHannum (sd=6.2), AgeAccelPheno (sd=7.6) and AgeAccelGrim (sd=5.1)
[b]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=6.4), AgeAccelHannum (sd=6.2), AgeAccelPheno (sd=7.6) and AgeAccelGrim (sd=5.1).

Table 2.6: Interaction of Age Accel Measures with Race/Ethnicity in Primary Analysis

Ref=White	**90 with Intact Physical & Cognitive Function (n=483)[a]**		**90 without Intact Physical and/or Cognitive Function (n=403)[a]**	
	OR (95% CI)[b]	p	OR (95% CI)[c]	p
AgeAccelHorvath*Black or AA[b]	1.23 (0.87-1.73)	0.236	1.46 (1.03-2.06)	0.032
AgeAccelHorvath*Hispanic/Latino	0.96 (0.93-1.85)	0.905	0.28 (0.14-0.54)	**<0.001**
AgeAccelHorvath*Other	2.33 (0.58-9.33)	0.237	4.04 (0.40-40.41)	0.235
AgeAccelHannum*Black or AA	1.45 (1.01-2.10)	**0.035**	1.36 (0.94-1.86)	0.097
AgeAccelHannum*Hispanic/Latino	2.70 (1.54-5.01)	**0.001**	0.73 (0.42-1.20)	0.214
AgeAccelHannum*Other	2.24 (0.73-6.42)	0.152	3.91 (0.69-22.20)	0.124
AgeAccelPheno*Black or AA	1.01 (0.68-1.46)	0.963	1.08 (0.80-1.58)	0.630
AgeAccelPheno*Hispanic/Latino	0.80 (0.37-1.58)	0.472	0.30 (0.15-0.59)	**0.001**
AgeAccelPheno*Other	1.16 (0.27-5.32)	0.816	1.70 (0.19-16.64)	0.626
AgeAccelGrim*Black or AA	1.43 (0.95-2.15)	0.088	1.17 (0.77-1.75)	0.456
AgeAccelGrim*Hispanic/Latino	1.43 (0.82-2.38)	0.218	0.77 (0.47-1.29)	0.323
AgeAccelGrim*Other	2.15 (0.70-6.60)	0.185	2.64 (0.63-11.57)	0.175

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, number of chronic conditions (including cancer, stroke, Alzheimer's, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]The reference group for all comparisons is women who did not survive to age 90 (n=929)

[b]The reference group for this comparison was White women (n=1447), Black (n=377), Hispanic/Latino (n=169), Other (n=70).

[c]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=6.4), AgeAccelHannum (sd=6.2), AgeAccelPheno (sd=7.6) and AgeAccelGrim (sd=5.1).

Table 2.7: Interaction of AgeAccel Measures with Baseline Age/Length of Follow-up (>70.6 years versus <=70.5 years) in Primary Analysis

Ref=Lower than median age at baseline (median=70.6 years)	90 with Intact Physical & Cognitive Function (n=483)[a]		90 without Intact Physical and/or Cognitive Function (n=403)[a]	
	OR (95% CI)[b]	p	OR (95% CI)[c]	p
AgeAccelHorvath*median age [a]	1.14 (0.83-1.57)	0.376	1.29 (0.94-1.78)	0.131
AgeAccelHannum*median age	1.20 (0.65-1.13)	0.303	1.13 (0.83-1.54)	0.485
AgeAccelPheno*median age	0.93 (0.68-1.26)	0.654	1.16 (0.86-1.58)	0.374
AgeAccelGrim*median age	1.11 (0.77-1.50)	0.601	1.01 (0.74-1.43)	0.905

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, number of chronic conditions (including cancer, stroke, Alzheimer's, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]The reference group for all comparisons is women who did not survive to age 90 (n=929).

[b]There were 1033 women with baseline median age <=70.5 years (reference) and 1045 women with baseline age >70.5 years.

[c]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=6.4), AgeAccelHannum (sd=6.2), AgeAccelPheno (sd=7.6) and AgeAccelGrim (sd=5.1).

Supplementary Figures

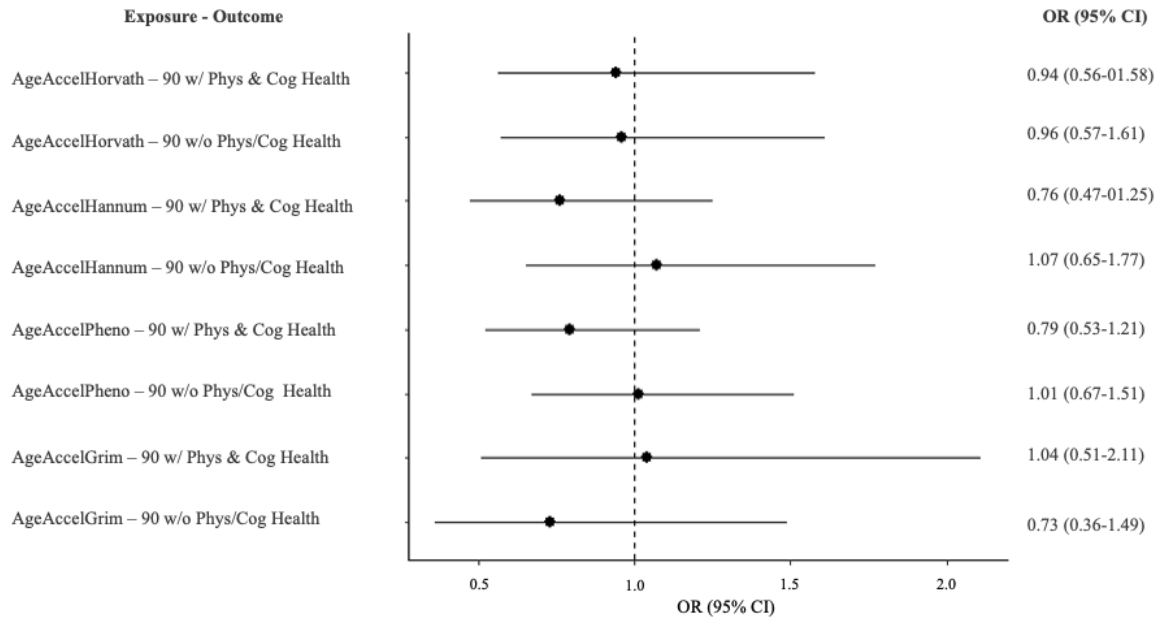


Figure 2.3: Primary Results by Ancillary Study

AS311

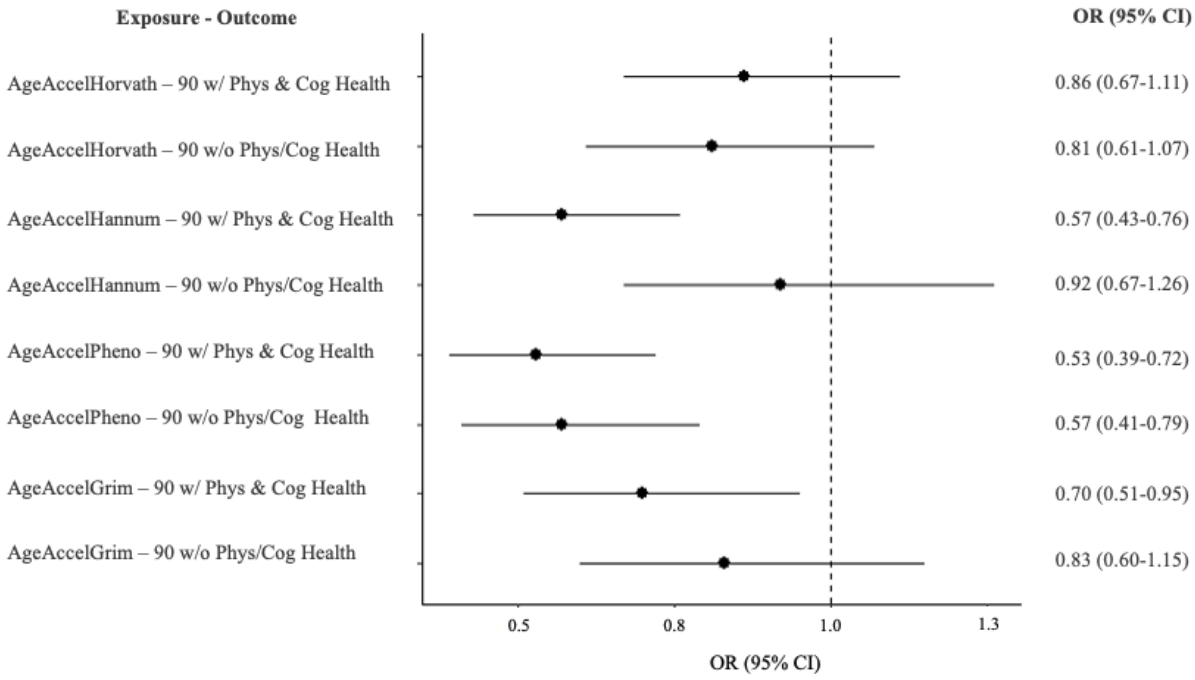


Figure 2.3: Primary Results by Ancillary Study (continued)

AS315

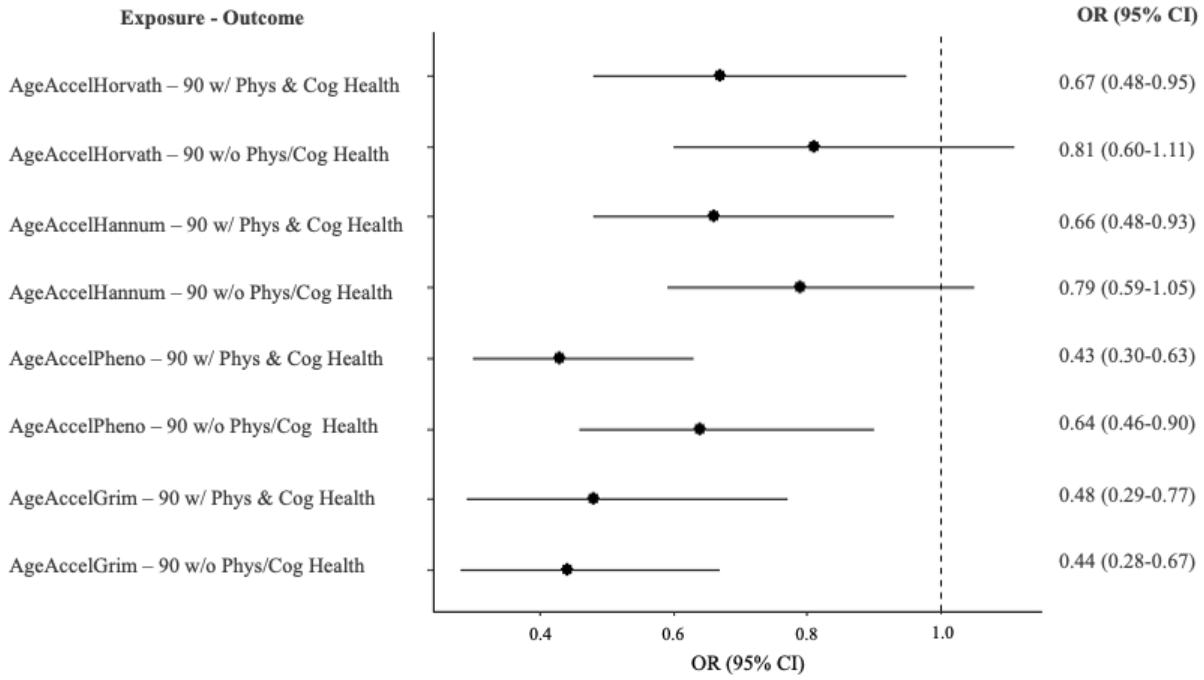


Figure 2.3: Primary Results by Ancillary Study (continued)

BAA23

Chapter 2, in full, is currently being prepared for submission for publication of the material. Jain, Purva; Binder, Alex; Chen, Brian; Parada, Humberto; Gallo, Linda; Alcaraz, John; Horvath, Steve; Bhatti, Parveen; Whitsel, Eric; Baccarelli, Andrea; Hou, Lifang; Stewart, Jay; Li, Yun; Jordahl, Kristina; LaCroix, Andrea. The dissertation author was the primary investigator and author of this paper.

3. An epigenome wide association study of exceptional aging in the Women's Health Initiative

Purva Jain, Alexandra Binder, Brian Chen, Humberto Parada, Linda Gallo, Steve Horvath, Parveen Bhatti, Eric Whitsel, Kristina Jordahl, Andrea LaCroix

3.1. Abstract

Background: The foundation of wellbeing in older age can be quantified using functional status, which is comprised of one's physical and mental capabilities. To date, there have been no longitudinal studies examining the relationship between DNA methylation (DNAm) and exceptional longevity among older women.

Methods: This study was restricted to the 1,813 women in the Women's Health Initiative (WHI) who had DNAm profiling at baseline, had an advanced enough age at baseline, and remained an active WHI participant long enough to be eligible to survive to age 90 by the end of our observation period. We conducted differential methylation analysis at 481,047 CpG sites and a genome-wide analysis of differentially methylated regions on a three-category exceptional longevity outcome: women who survived to age 90 with intact physical functioning and women who survived to age 90 without intact physical functioning compared to women who did not survive to age 90.

Results: We found 139 significantly differentially methylated positions and 210 differentially methylated regions in our analysis of which the top two are presented here. A 1% increase in Beta-value at cg07071449 was associated with a 10% (OR=1.10; 95% CI:1.07-1.14; $p=4.0 \times 10^{-12}$) increase in the odds of survival to age 90 with physical function. Cg07071449 is in the body of the RAMP1 gene, which has been associated with several chronic conditions and has been shown to be expressed at higher levels in women with knee osteoarthritis. A 1% increase in

Beta-value at cg16716449 was associated with a 5% (OR=0.95; 95% CI:0.94-0.96; p=3.6 X 10^{-12}) decrease in the odds of survival to age 90 without physical function. Cg16716449 is in the A2BP1/RBFOX1 gene, which has been implicated with several neurodevelopmental diseases in additional to Alzheimer's disease.

Conclusion: The 139 differentially methylated CpG sites and 210 differentially methylated regions across the genome identified in this study may elucidate biological mechanisms associated with exceptional longevity, serve as potential targets for intervention and be utilized in risk prediction models. These findings should be confirmed in additional studies that include both long-lived men and women and examined further using molecular studies to identify specific biological mechanisms that may be at play in these relationships.

3.2. Introduction

There were close to 4 million individuals aged 85 and older in the United States (US) as of 2020.[34] Over the next few decades, this population is expected to quadruple and comprise 10% of the US population by 2050. While those 85 and older have traditionally been considered to be the 'oldest old' taking into account the increases in longevity in the US those who are 90 years and old require additional focus. Furthermore, women outnumber men 3 to 1 among those who are 90 and older and thus comprise a significantly larger proportion of long-lived individuals.[35]

The foundation of wellbeing in older age can be quantified using functional status, which is comprised of one's physical and mental capabilities. These capabilities include a person's ability to meet their basic needs, be mobile, build and maintain relationships, learn, grow, make decisions and contribute to society.[36] Living with impaired function becomes more common as women age into their 10^{th} decade of life although this does not affect all women.[37] Declines in an

individual's intrinsic capacity to remain high functioning can be attributed to impairments in the physical or mental domains.[38]

Epigenetic changes are a hallmark of aging and include the methylation of DNA, or acetylation and methylation of histones and chromatin-associated proteins.[1] Low levels of de-novo methylation of CpG islands occur in normal tissues and are shown to increase with age.[4-6] Prior studies examining the relationship between DNAm patterns and longevity have been limited.[21,24] While these studies did find differential DNA methylation patterns among the groups that were long-lived, these studies utilized a cross-sectional design, had small sample sizes, did not include participants with diverse racial/ethnic backgrounds and were not restricted to long-lived individuals. Without control of factors associated with mortality, cross-sectional studies of loci associated with mortality are unable to identify loci that are causally related to aging.[66]

There have been no longitudinal studies that examined DNA methylation patterns and survival to 90 years of age among women with intact mobility and/or cognitive functioning (absence of physician-diagnosed mild cognitive impairment and dementia). Survival to the age of 90 was selected as the primary analysis for this manuscript, because approximately one-half of WHI women in the age-eligible subgroup for survival to age 90 have survived. Our ability to examine survival to even older ages, is currently limited in the WHI data among women with available epigenetic data as described below.

3.3. Methods

Discovery Study Population

The Women's Health Initiative (WHI) (n=161,808) was initiated in 1993 with the goal of identifying strategies to prevent heart disease, osteoporosis and breast and colorectal cancer among postmenopausal women.[48,49] The current study included participants from three (two

nested case-control and one prospective) WHI ancillary studies who had available data on DNAm. The Bladder Cancer and Leukocyte Methylation Ancillary Study (AS 311) included 468 women with and 468 without bladder cancer to identify methylation profiles associated with bladder cancer risk using specific DNA methylation loci.[50] The Epigenetic Mechanisms of Particulate Matter-Mediated Cardiovascular Disease Ancillary Study (AS315) included a random sample of 2,200 WHI clinical trial participants to understand the pathophysiological mechanisms that underlie particulate matter-related cardiovascular disease in postmenopausal women.[51] Last, the Integrative Genomics for Risk of Coronary Heart Disease and Related Phenotypes in the WHI Cohort Ancillary Study (BA23) included 1,070 women with and 1,070 women without coronary heart disease.[52]

There were 443 women in AS311, 694 women in AS315 and 942 women in BAA23 who were eligible to survive to age 90 (total = 2,079). Of these, 395 women had information available on all longevity components in AS311, 579 in AS315 and 839 in BAA23 resulting in a final analytic sample of 1,813 women (Figure 3.1). Baseline age ranged from 63.4-81.4 years with an average of 70.5 years (i.e. follow-up ranged from 9-27 years with an average of 20 years).

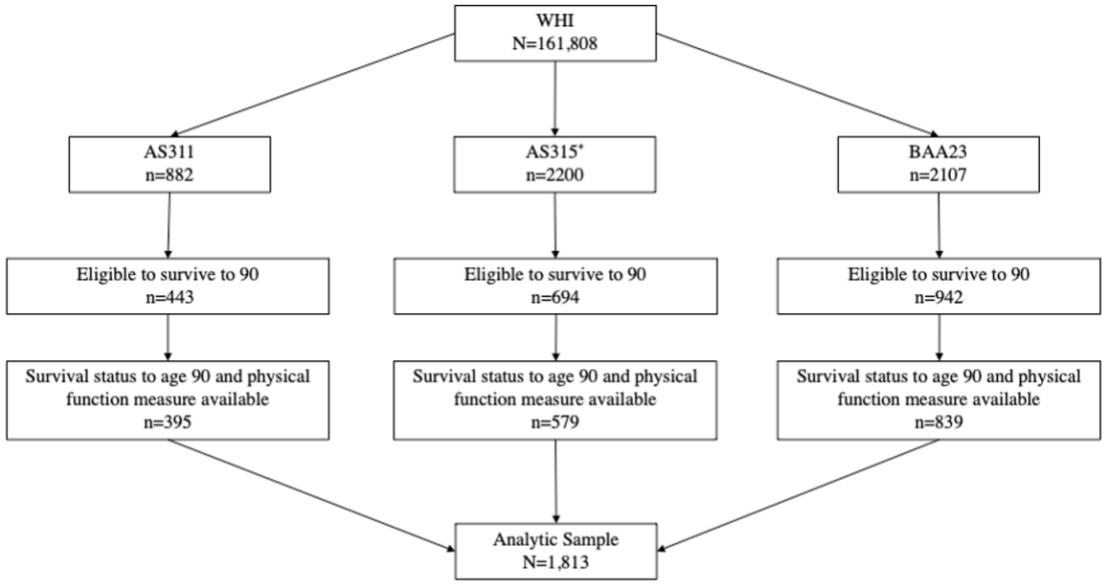


Figure 3.1: STROBE Diagram

This current study was restricted to the 1,813 women from the three WHI ancillary studies with assays of genome-wide DNAm from WHI baseline who had an advanced enough age at baseline and remained an active WHI participant long enough to be eligible to survive to age 90 by the end of our observation period (September 30, 2020).

Data and biospecimen collection

Women provided demographic and risk factor information, physical measurements and blood specimens at their baseline visit. Detailed questionnaires to collect risk factor data were also completed at each study visit. Blood samples that provided DNA methylation values were collected 12 hours after fasting and stored at -70 degrees Celsius. The WHI ascertained death using annual mailed outcome questionnaires, systematic searches of the National Death Index, hospital records, obituaries and proxy queries.[53]

Covariates

Covariates were measured at WHI baseline and selected due to their associations with both EAA and exceptional longevity. Covariates included age at blood draw, blood cell composition (CD8+ T Cells, CD4 T cells, Natural Killer cells, B lymphocyte cells, Monocytes, Granulocytes), race/ethnicity (White, Black, Hispanic, Other), education (high school/general education development or less, some college, college graduate or more), walking frequency (rarely or never, 1-3 times/mo, 1 time/wk, 2-3 times/wk, 4-6 times/wk, 7+ times/wk), body mass index categories (underweight, normal, overweight, obese), alcohol consumption (non-drinker, past drinker, <1 drink/mo, <1 drink/wk, 1-<7 drinks/wk, 7+ drinks/wk), pack-years smoking (never smoker, <5, 5-20, 20+), number of chronic conditions at baseline (0, 1-2, 3+, including cancer, stroke, Alzheimer's disease, cardiovascular disease, diabetes, history of frequent falls [2+/yr], broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and physical function score (RAND-36 10-item physical function subscale[54], range 0-100, higher score reflects higher function). Chronic conditions were chosen based on the high degree of impact these conditions play in the lifespan and healthspan of older women.[55-57]

Survival outcomes

We defined three primary outcomes for this study as follows: 1) survival to age 90 with intact cognitive and physical function; 2) survival to age 90 with impairment in cognitive and/or physical function; and 3) death before age 90. Classifications were based on follow-up data through September 30th, 2020. Survival to age 90 was calculated from day of enrollment in the WHI through September 30th, 2020. Among women eligible to survive to age 90 (n=2,079), 128

(0.06%) were missing vital status at age 90. Intact physical functioning was defined using two questions from the Rand-36 Physical Function questionnaire[54] as having no self-reported limitations for both walking one block and climbing one flight of stairs from baseline to age 90. The questionnaire was administered at baseline, at 1 and 3-year follow-up assessments, and then annually after 2005. Intact cognitive functioning was defined as having no self-reported moderate or severe memory problems nor dementia or Alzheimer's Disease from baseline to age 90 and was assessed annually by questionnaire. Among women enrolled in the WHI Extension Study 1 (2005-2010) with at least 1 Form 33 collected after enrollment, the validation of Alzheimer's disease against Medicare claims was as follows (sensitivity=40%; specificity=95%) and against WHIMS (sensitivity=41%; specificity=89%).

DNA methylation array and methylation data processing

The Illumina Infinium HumanMethylation450 Bead Array was used to measure methylation status from peripheral blood leukocytes using fasting blood draws from study participants in all three ancillary studies (San Diego, CA, Illumina). This array includes 99% of RefSeq genes that cover regions including the first exon, 3^0 and 5^0 untranslated regions, gene body and close proximity to transcription start sites. Quality control included excluding probes targeting cytosine-guanine CG sites on the Y chromosome, probes with detection p-values less than 0.001 in greater than 1% of samples, probes with a bead count less than 3 in greater than 10% of samples and probes that measure non-CpG methylation. Normalization was completed using beta-mixture quantile normalization using BMIQ and the Beta-value was calculated for the 481,440 CpG sites that met quality standards. The Beta-value represents the ratio of the methylated intensity and the overall intensity (the sum of the methylated and unmethylated intensities).[67] The details of the DNA processing have been previously described.[52,68]

Statistical analysis

The baseline characteristics were reported by exceptional survival category. The three categories were survival to age 90 with intact physical function, survival to age 90 without intact physical function and did not survive to age 90. Differences by category were tested using Pearson's chi-square tests for categorical variables and F-tests for continuous variables. An epigenome-wide association study was conducted on the 481,047 CpG loci that had methylation information available across all three ancillary studies. To account for multiple testing, a Bonferroni-adjusted p-value of 10^{-7} was used for significance testing. Fully-adjusted multinomial logistic regression models with a random intercept for ancillary study were used to assess the relationships between a 1% increase in Beta-value at each CpG and exceptional longevity. The reference group for both levels of comparison were women who did not survive to age 90. A secondary analysis was also conducted to assess the relationship of the top site-specific hits from the primary analysis with the second exceptional longevity outcome. Women who survived to age 90 with intact physical and cognitive function and women who survived to age 90 with intact physical or cognitive function were compared to women who did not survive to age 90. This outcome was also tested using a fully-adjusted multinomial logistic regression with the same covariates as the primary analysis.

A QQ-plot with lambda for inflation and Manhattan plots to display the top hits by chromosome were generated for both levels of comparisons in the primary analysis using the qqman package in R. A scatter plot of the t-stat from the top site-specific hits of each comparison were plotted to examine the overlap of both levels of comparison. The same scatter plot including the t-stat for all sites with a density overlay was also included in the supplement. A Pearson's correlation coefficient was calculated for both plots. The differentially methylated

regions were tested using the ipdmr package and a gene set enrichment analysis for biological function was completed using the methylRRA function in the methylGSA package. All analyses were completed using R Version 1.4.1106 (Vienna, Austria).

3.4. Results

This Of the 1,813 women included in this study, 464 experienced exceptional longevity (i.e., survived to age 90 with intact physical and cognitive functioning), 420 women survived to age 90 with loss of intact physical and/or cognitive functioning, and 929 women did not survive to age 90. Compared to women who survived to age 90 without intact function and women who did not survive to age 90, women who experienced exceptional longevity were more likely to be White, be college graduates, walk 2-3 times per week and 4-6 times per week, have a BMI in the healthy weight range of 20-25 kg/m^2 or overweight range of 35-30 kg/m^2, have more than 1 but less than 7 alcoholic drinks per week, be never smokers, have none of the major chronic conditions examined, and have higher physical functioning (Table 3.1).

Table 3.1: Baseline Characteristics by Level of Outcome (n=1,813)

	90 w/ Phys & Cog Health (n=464)	90 w/o Phys/Cog Health (n=420)	Did not Survive to age 90 (n=929)	p
Race/Ethnicity, n (%)				0.013
Black or African American	66 (14.3)	73 (17.4)	179 (19.4)	
Hispanic/Latino	27 (5.9)	36 (8.6)	78 (8.5)	
White	348 (75.7)	305 (72.8)	637 (69.1)	
Other	19 (4.1)	5 (1.2)	28 (3.0)	
Education, n (%)				0.003
HS/GED or Less	103 (22.3)	114 (27.3)	281 (30.5)	
Some College	181 (39.2)	177 (42.3)	369 (40.0)	
College Grad or More	178 (38.5)	127 (30.4)	272 (29.5)	
Walking Frequency, n (%)				<0.001
Rarely or Never	56 (12.1)	88 (21.2)	204 (22.2)	
1-3 times/month	66 (14.2)	51 (12.3)	144 (15.7)	
1 time/week	50 (10.8)	34 (8.2)	109 (11.9)	
2-3 times/week	144 (31.0)	118 (28.4)	231 (25.2)	
4-6 times/week	111 (23.9)	90 (21.7)	163 (17.8)	
7+ times/week	37 (8.0)	34 (8.2)	67 (7.3)	
BMI Category, n (%)				<0.001
Underweight	6 (1.3)	3 (0.7)	9 (1.0)	
Normal	167 (36.2)	101 (24.2)	251 (27.2)	
Overweight	189 (41.0)	150 (35.9)	296 (32.0)	
Obese	99 (21.5)	164 (39.2)	368 (39.8)	
Alcohol Consumption, n (%)				<0.001
Non-drinker	60 (13.1)	63 (15.1)	121 (13.2)	
Past drinker	70 (15.3)	95 (22.7)	224 (24.5)	
<1 drink/month	51 (11.1)	56 (13.4)	134 (14.6)	
<1 drink/week	99 (21.6)	93 (22.2)	167 (18.3)	
1-<7 drinks/week	120 (26.1)	71 (17.0)	173 (18.9)	
7+ drinks/week	59 (12.9)	40 (9.6)	96 (10.5)	
Smoking Pack-Years, n (%)				<0.001
Never Smoker	277 (62.0)	248 (60.6)	425 (47.6)	
<5	51 (11.4)	59 (14.4)	96 (10.8)	
5-<20	64 (14.3)	41 (10.0)	114 (12.8)	
20+	55 (12.3)	61 (14.9)	258 (28.9)	
Number of Chronic Conditions, n (%)[a]				<0.001
0	143 (30.8)	101 (24.0)	202 (21.7)	
1-2	291 (62.7)	271 (64.5)	615 (66.2)	
3+	30 (6.5)	48 (11.4)	112 (12.1)	
Age, mean (SD)	71.6 (3.5)	71.3 (3.2)	70.2 (3.4)	<0.001
Physical Function Score, mean (SD)	82.4 (20.2)	72.8 (22.7)	69.5 (24.6)	<0.001

Note: GED=general educational development; BMI=body mass index; kg=kilograms; m=meters

Table 3.2 includes the results for the 10 CpG sites with lowest p-value for each level of comparison and Supplementary Table 3.6 includes the results for all 481,047 CpGs tested in addition to the distribution of the Beta-values. There were 38 significant sites comparing women who survived to age 90 with physical function and 103 significant sites comparing women who survived to age 90 without physical function to women who did not survive to age 90. These results are also summarized in Manhattan plots in Figures 3.2 and 3.3 and the significant associations are labeled. The qq-plot for survival to age 90 with physical function (lambda=1.75) and survival to age 90 without physical function (lambda=2.13) are also presented in the supplement. A 1% increase in Beta-value at cg07071449 was associated with a 10% (OR=1.10; 95% CI:1.07-1.14; $p=4.0 \times 10^{-12}$) increase in the odds of survival to age 90 with physical function and a 1% increase in Beta-value at cg01127300 was associated with a 8% (OR=1.10; 95% CI:1.05-1.10; $p=5.8 \times 10^{-12}$) increase in the odds of survival to age 90 with physical function. Cg07071449 and cg01127300 have an average methylation of 0.84 (sd=0.06) and 0.53 (sd=0.08) for women who survived to 90 with physical function and 0.83 (sd=0.07) and 0.51 (sd=0.09) for women who did not survive to age 90, respectively. Additionally, a 1% increase in Beta-value at cg16716449 was associated with a 5% (OR=0.95; 95% CI:0.94-0.96; $p=3.6 \times 10^{-12}$) decrease in the odds of survival to age 90 without physical function and a 1% increase in Beta-value at cg22311230 was associated with a 7% (OR=0.92; 95% CI:0.89-0.94; $p=2.5 \times 10^{-11}$) decrease in the odds of survival to age 90 without physical function. Cg16716449 and cg22311230 have an average methylation of 0.83 (sd=0.11) and 0.86 (sd=0.10) for women who survived to 90 without physical function and 0.57 (sd=0.07) and 0.57 (sd=0.07) for women who did not survive to age 90, respectively

Table 3.2: Top 10 CpG Sites in tests of association between DNA methylation level and survival to age 90 with and without physical function compared to women who did not survive to age 90

Survived to age 90 with Physical Function vs. Did not Survive to Age 90							
Illumina ID	**Chr**	**Position**	**Gene**	**Gene Group**	**Relation to CpG Island**	**OR (95% CI)**	**p**
cg07071449	chr2	238777806	RAMP1	Body	OpenSea	1.10 (1.07-1.14)	4.0×10^{-12}
cg01127300	chr22	38614796	-	-	S_Shelf	1.08 (1.05-1.10)	5.8×10^{-11}
cg17242596	chr11	132527296	OPCML	Body	OpenSea	1.12 (1.08-1.17)	9.0×10^{-10}
cg11530213	chr8	42037966	PLAT	Body	OpenSea	1.05 (1.03-1.07)	1.3×10^{-9}
cg03448017	chr16	83980015	-	-	OpenSea	0.98 (0.97-0.99)	2.7×10^{-9}
cg23065768	chr11	17411712	KCNJ11	TSS1500	S_Shore	0.95 (0.94-0.97)	4.7×10^{-9}
cg11271430	chr4	187984718	-	-	Island	1.13 (1.08-1.17)	5.2×10^{-9}
cg13824991	chr18	76766250	-	-	Island	1.05 (1.03-1.07)	6.3×10^{-9}
cg13657981	chr7	134558080	CALD1	Body	OpenSea	0.93 (0.91-0.96)	1.1×10^{-8}
cg13949713	chr10	130302534	-	-	S_Shelf	0.96 (0.95-0.97)	1.4×10^{-8}
Survived to age 90 without Physical Function vs. Did not Survive to Age 90							
Illumina ID	**Chr**	**Position**	**Gene**	**Gene Group**	**Relation to CpG Island**	**OR (95% CI)**	**p**
cg16716449	chr16	6976709	A2BP1	5'UTR	OpenSea	0.95 (0.94-0.96)	3.6×10^{-12}
cg22311230	chr11	3253769	MRGPRE	TSS200	Island	0.92 (0.89-0.94)	2.5×10^{-11}
cg03492641	chr15	34807679	-	-	S_Shore	0.92 (0.90-0.95)	2.8×10^{-11}
cg18660329	chr3	8713512	-	-	OpenSea	1.08 (1.06-1.11)	6.0×10^{-11}
cg10800483	chrX	114798337	PLS3	5'UTR	S_Shelf	1.06 (1.04-1.08)	1.0×10^{-10}
cg01281718	chr6	71376634	SMAP1	TSS1500	N_Shore	0.87 (0.84-0.91)	1.6×10^{-10}
cg03623568	chr16	6915990	A2BP1	5'UTR	OpenSea	1.03 (1.02-1.03)	2.3×10^{-10}
cg23923934	chr6	31322914	HLA-B	Body	N_Shore	1.09 (1.06-1.11)	2.5×10^{-10}
cg03161606	chr19	29218774	-	-	S_Shore	0.97 (0.96-0.98)	2.7×10^{-10}
cg00754604	chr2	161230046	RBMS1	Body	OpenSea	1.13 (1.09-1.17)	3.4×10^{-10}

Note: Chr=Chromosome

Note; The odds ratio represents a 1% increase in DNA methylation at each specific CpG site.

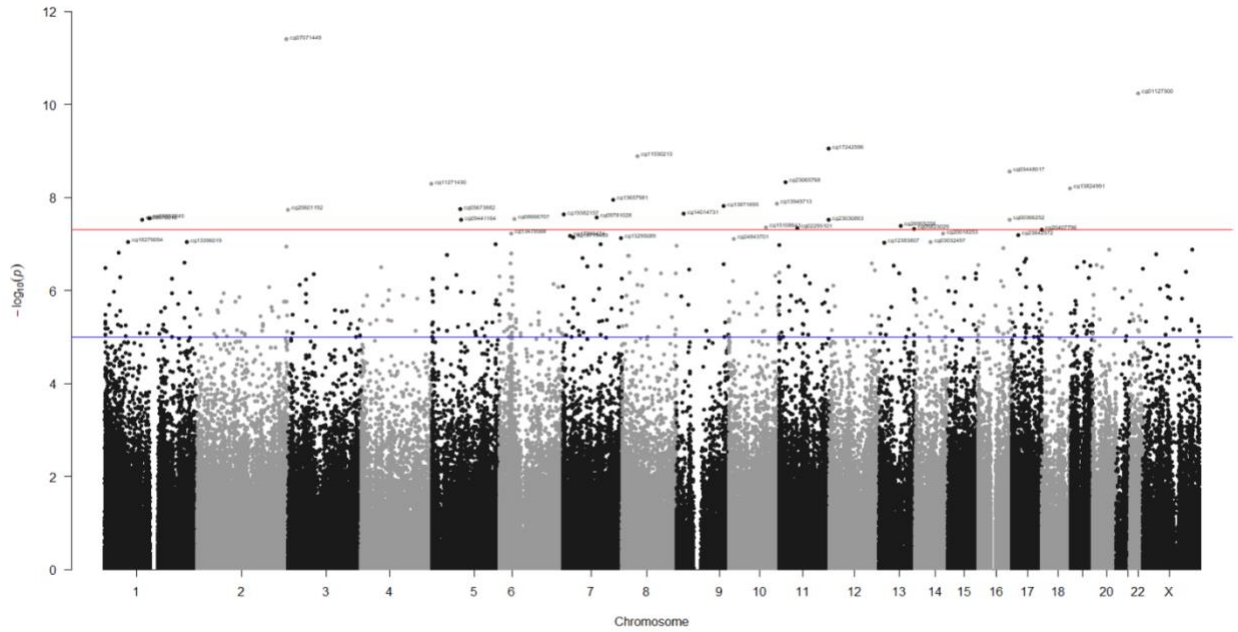


Figure 3.2: Manhattan plot displaying top CpG hits in the association between DNA methylation level and survival to age 90 with physical function compared to women who did not survive to age 90

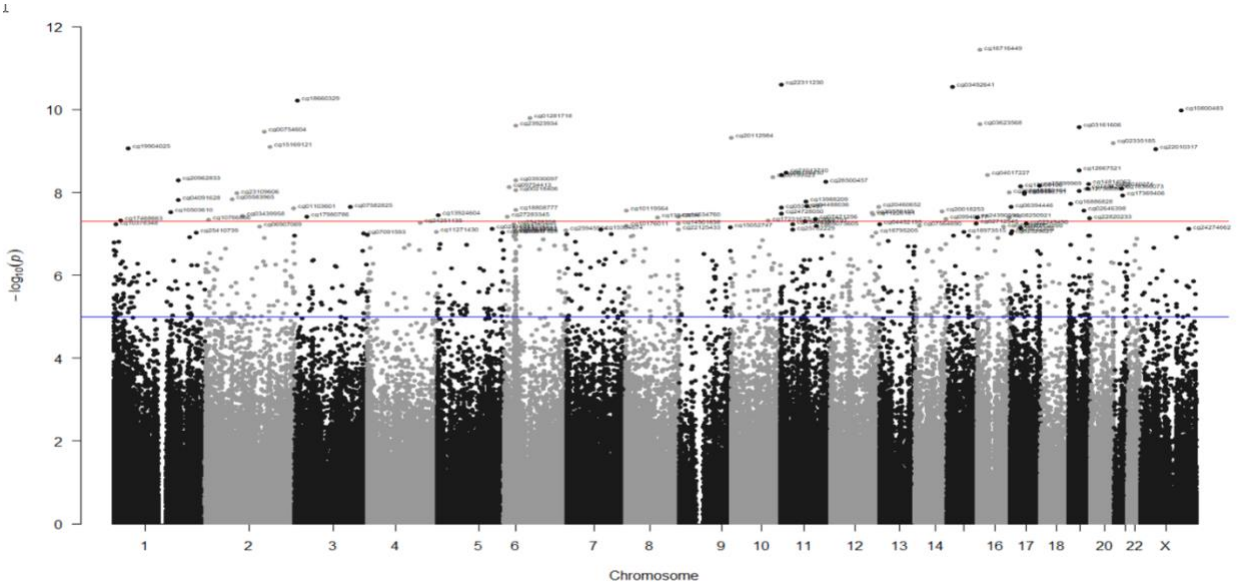


Figure 3.3: Manhattan plot displaying top CpG hits in the association between DNA methylation level and survival to age 90 without physical function compared to women who did not survive to age 90

In the secondary analysis that tested the 139 significant CpG sites in the primary analysis, there were 17 significant hits when comparing women who survived to age 90 with intact physical and cognitive function and 64 significant hits when comparing women who survived to age 90 with intact physical or cognitive function to women who did not survive to age 90. The correlation of the t-stat between the two levels of correlation was moderate (r=0.670) and there were 2 significant CpGs that overlapped. The top 10 hits are displayed in Table 3.3 and the results of full 139 CpGs. A 1% increase in Beta-value at cg11530213 was associated with a 6% (OR=1.06; 95% CI:1.04-1.07; $p=2.1 \times 10^{-10}$) increase in the odds of survival to age 90 with physical and cognitive function. A 1% increase in Beta-value at cg11271430 was associated with a 14% (OR=1.14; 95% CI:1.10-1.19; $p=2.7 \times 10^{-10}$) increase in the odds of survival to age 90 with physical and cognitive function. A 1% increase in Beta-value at cg22311230 was associated with a 9% (OR=0.91; 95% CI:0.88-0.93; $p=7.4 \times 10^{-14}$) decrease in the odds of survival to age 90 without intact physical or cognitive function. A 1% increase in Beta-value at cg18660329 was associated with a 8% (OR=1.08; 95% CI:1.06-1.11; $p=4.9 \times 10^{-12}$) decrease in the odds of survival to age 90 without physical or intact function.

Table 3.3: Top 10 CpG Sites in tests of association between DNA methylation level and survival to age 90 with and without physical and cognitive function compared to women who did not survive to age 90

Survived to age 90 with Physical and Cognitive Function vs. Did not Survive to Age 90							
Illumina ID	**Chr**	**Position**	**Gene**	**Gene Group**	**Relation to CpG Island**	**OR (95% CI)**	**p**
cg11530213	8	42037966	PLAT	Body	OpenSea	1.06 (1.04-1.07)	2.1×10^{-10}
cg11271430	4	187984718	-	-	Island	1.14 (1.10-1.19)	2.7×10^{-10}
cg07071449	2	238777806	RAMP1	Body	OpenSea	1.09 (1.06-1.12)	4.0×10^{-10}
cg13824991	18	76766250	-	-	Island	1.06 (1.04-1.07)	7.6×10^{-10}
cg01127300	22	38614796	-	-	S_Shelf	1.07 (1.05-1.06)	2.4×10^{-9}
cg14014731	9	19378679	RP56	Body	N_Shore	1.08 (1.05-1.11)	5.7×10^{-9}
cg05823029	13	114312758	ATP48	TSS1500	OpenSea	0.93 (0.91-0.96)	6.0×10^{-9}
cg23065768	11	17411712	KCNJ11	TSS1500	S_Shore	0.95 (0.94-0.97)	6.8×10^{-9}
cg03852045	1	120439870	ADAM30	TSS1500	S_Shore	0.63 (0.54-0.74)	8.5×10^{-9}
cg13295089	7	155492281	RBM33	TSS1500	OpenSea	1.02 (1.01-1.03)	9.6×10^{-9}
Survived to age 90 without Physical or Cognitive Function vs. Did not Survive to Age 90							
Illumina ID	**Chr**	**Position**	**Gene**	**Gene Group**	**Relation to CpG Island**	**OR (95% CI)**	**p**
cg22311230	11	3252769	MRGPRE	TSS200	Island	0.91 (0.88-0.93)	7.4×10^{-14}
cg18660329	3	8713512	-	-	OpenSea	1.08 (1.06-1.11)	4.9×10^{-12}
cg16716449	16	6976709	A2BP1	5'UTR	OpenSea	0.95 (0.94-0.97)	8.4×10^{-12}
cg03492641	15	34807679	-	-	S_Shore	0.92 (0.90-0.94)	1.0×10^{-11}
cg23923934	6	31322914	HLA-B	Body	N_Shore	1.09 (1.06-1.11)	3.5×10^{-11}
cg00754604	2	161230046	RBMS1	Body	OpenSea	1.13 (1.09-1.17)	4.6×10^{-11}
cg22010317	X	45060544	CXorf36	TSS1500	OpenSea	1.05 (1.04-1.07)	1.1×10^{-10}
cg20962833	1	175891165	-	-	OpenSea	1.18 (1.12-1.24)	2.4×10^{-10}
cg02249490	17	42004878	-	-	OpenSea	0.85 (0.81-0.90)	5.2×10^{-10}
cg20018253	14	94346104	-	-	OpeanSea	0.95 (0.94-0.97)	7.8×10^{-10}

Note: Chr=Chromosome

Note; The odds ratio represents a 1% increase in DNA methylation at each specific CpG site.

There were 83 DMRs comparing women who survived to age 90 with intact physical function and 127 DMRs comparing women who survived to age 90 without physical function compared to women that did not survive to age 90 that met the FDR threshold of 10^{-7}. The top 10 DMRs are in Table 3.4 and the complete table of DMRs is in Supplement Table 3.7. Women who survived to age 90 with intact physical function compared to women who did not survive to age 90 had significantly different DNA methylation at chr 10:530635-532358 ($q=3.9 \times 10^{-24}$) which includes the DIP2C gene and chr 5:1594021-1595049 ($q=3.9 \times 10^{-24}$) which includes the SDHAP3 gene. Women who survived to age 90 without intact physical function compared to

women who did not survive to age 90 had significantly different DNA methylation at chr 11:2322050-2323929 ($q=1.6 \times 10^{-34}$) and chr 11:32449163-32452840 ($q=4.8 \times 10^{-34}$).

Table 3.4: Top 10 DMRs in tests of association between DNA methylation level and survival to age 90 with and without physical function compared to women who did not survive to age 90

Survived to age 90 with Physical Function vs. Did not Survive to Age 90						
Chr	**Start**	**End**	**N Probe**	**q**	**Gene**	**Gene Group**
10	530635	532358	15	3.9×10^{-24}	DIP2C	Body
5	1594021	1595049	12	9.3×10^{-21}	SDHAP3	Body; TSS200
20	36147340	36149195	39	8.4×10^{-18}	BLCAP; NNAT	5'UTR; TSS1500
6	33871907	33873733	11	2.1×10^{-16}	-	-
14	23623480	23624789	9	1.1×10^{-15}	SLC7A8	Body; TSS200;TSS1500
15	45027253	45028596	6	6.7×10^{-14}	TRIM69	5'UTR; 1st Exon; TSS1500
10	14051636	14052029	6	3.4×10^{-13}	FRMD4A	Body
20	2729245	2731177	5	1.2×10^{-12}	EBF4	Body
11	312518	315752	18	1.4×10^{-12}	IFITM1	Body; 3'UTR; 5'UTR; 1st Exon; TSS1500
6	32847513	32847846	19	2.5×10^{-12}	PPP1R2P1	Body
Survived to age 90 without Physical Function vs. Did not Survive to Age 90						
Chr	**Start**	**End**	**N Probe**	**q**	**Gene**	**Gene Group**
11	2322050	2323939	28	1.6×10^{-34}	C11orf21; TSPAN23C	Body; 1st Exon; 5'UTR; TSS200; TSS1500
11	32449163	32452840	21	4.8×10^{-34}	WT1	Body
19	29217858	29218775	7	1.3×10^{-29}	-	-
6	33084479	33085471	20	4.3×10^{-25}	HLA-DPB2	Body
11	3253581	3254325	12	1.7×10^{-21}	MRGPRE	1st Exon; 5'UTR; TSS200; TSS1500
19	54927291	54928186	6	1.8×10^{-19}	TTYH1	Body
10	94819989	94821658	11	7.9×10^{-19}	CYP26C1	1st Exon; Body; TSS200; TSS1500
11	13983009	13984068	12	8.1×10^{-18}	SPON1	1st Exon; 5'UTR; TSS200; TSS1500
6	31431100	31431903	6	6.8×10^{-17}	HCP5	Body; 3'UTR
15	101991512	101991887	5	6.8×10^{-17}	PCSK6	Body

Note: DMR=Differentially methylated region; Chr=Chromosome

Note: q-values are p-values adjusted using the false discovery rate method.

The top 10 biological processes identified using the gene ontology for the top site-specific hits are included in Table 3.5 and the full table is in Supplemental Table 3.8. For women who survived to age 90 with physical function compared to women who did not survive to age 90 these included regulation of the postsynaptic membrane potential and central nervous system

neuron differentiation and for women who survived to age 90 without physical function compared to women who did not survive to age 90 this included locomotory behavior, telencephalon development and endochrondal bone morphogenesis.

Table 3.5: Gene set analysis for DNA methylation level and survival to age 90 with physical function compared to women who did not survive to age 90

ID	Description	Count	Size	p^{adj}
GO:0045211	postsynaptic membrane	106	329	1.3×10^{-5}
GO:0060078	regulation of postsynaptic membrane potential	58	153	2.7×10^{-5}
GO:0099055	integral component of postsynaptic membrane	47	125	4.3×10^{-5}
GO:0021953	central nervous system neuron differentiation	69	211	6.3×10^{-4}
GO:0098982	GABA-ergic synapse	34	83	6.3×10^{-5}
GO:0097060	synaptic membrane	131	470	6.3×10^{-4}
GO:0098936	intrinsic component of postsynaptic membrane	47	131	6.3×10^{-4}
GO:0099699	integral component of synaptic membrane	56	165	6.3×10^{-4}
GO:0030326	embryonic limb morphogenesis	48	135	6.3×10^{-4}
GO:0035113	embryonic appendage morphogenesis	48	135	6.3×10^{-4}
GO:0048706	embryonic skeletal system development	48	135	6.3×10^{-4}

Note: The minimum gene set size was set at 50 and the maximum gene set size was set at 500.

ID	Description	Count	Size	$p^{adj0.022}$
GO:0007626	locomotory behavior	100	202	0.022
GO:0098889	intrinsic component of presynaptic membrane	48	48	0.022
GO:0021537	telencephalon development	128	272	0.023
GO:0060350	endochondral bone morphogenesis	34	56	0.025
GO:0048168	regulation of neuronal synaptic plasticity	35	59	0.032
GO:0150034	distal axon	150	334	0.041
GO:0051966	regulation of synaptic transmission, glutamatergic	42	76	0.041
GO:0048167	regulation of synaptic plasticity	105	224	0.041
GO:0098685	schaffer collateral – CA1 synapse	46	85	0.041
GO:0030900	forebrain development	187	429	0.041
GO:0060541	respiratory system development	104	222	0.041

Note: The minimum gene set size was set at 50 and the maximum gene set size was set at 500.

3.5. Discussion

For the primary analysis that used an epigenome-wide-association study across 481,047 CpG sites comparing women who survived to age 90 with and without intact physical function to women who did not survive to age 90 there were 38 and 103 significantly differentially

methylated CpG sites identified, respectively, with 2 sites that overlapped. In the secondary analysis that tested these 139 sites comparing women who survived to age 90 with and without intact physical and/or cognitive function compared to women who did not survive to age 90 there were 17 and 64 significantly differentially methylated CpG sites, respectively. There were also 83 DMRs comparing women who survived to age 90 with intact physical function and 127 DMRs comparing women who survived to age 90 without physical function to women that did not survive to age 90.

In the site-specific analysis for both survival to age 90 with physical and survival to age 90 with physical and cognitive function, the significant sites were close to the receptor activity-modifying protein (RAMP) and plasminogen activator gene (PLAT) genes. The survival to age 90 with intact physical function additionally included opioid-binding protein/cell adhesion molecule (OPCML) in the top 3 hits. Both survival to age 90 with intact physical and cognitive function and survival to age 90 with physical or cognitive function, had significant sites that were near ataxin-2-binding protein 1 (A2BP1) and Mas-related G-protein coupled receptor member E (MRGPRE) genes. The upregulation of RAMP is associated with several conditions: heart failure, cancer, sepsis, liver cirrhosis, glomerulonephritis, Type 1 diabetes, Parkinson's.[69] RAMP-1 has also been shown to be expressed at higher levels in women compared to men with knee osteoarthritis and calcitonin gene-related peptide (CGRP) which binds to RAMP-1 was correlated with greater pain severity in women compared to men. The PLAT gene with the angiotensin converting enzyme (ACE) DD genotype have been previously associated with exceptional longevity among older women as well as centenarians.[70,71] The OPCML tends to be hypermethylated in several different types of cancer due to its role in the early stages of tumor initiation.[72] A2BP1 also known as FOX1 and RBFOX1 was identified in the secondary outcome

that included cognitive function and is a neuron-specific splicing factor that is required for proper exon usage. These splicing functions are considered important for brain development and neurodevelopmental disorders.[73] A small study also showed that there were autosomal copy number variations in A2BP1 among families with early onset – familial Alzheimer's disease.[74]

The DMRs for survival to age 90 with intact physical function included the disco-interacting protein 2 homolog (DIP2C), succinate dehydrogenase complex flavoprotein subuit A pseudogene 3 (SDHAP3), bladder cancer-associated protein (BLCAP) and neuronatin (NNAT) genes and the DMRs for survival to age 90 without intact physical function compared to women who did not survive to age 90 included the chromosome 11 open reading frame 21 (C11orf21), tetraspanin 23-C (TSPAN23C), Wilm's tumor 1 (WT1) and major histocompatibility complex, class II, DP beta 1 (HLA-DPB2) genes. DIP2C is known to play an important role in brain development and function and a gene ontology analysis indicated that differentially expressed genes in the rain are enriched in neurological functions such as memory, neuropeptide signaling pathway and response to amphetamine[75-79] BLCAP is a tumor suppressor gene associated with Wilm's tumor, bladder, cervical and breast cancer among other cancers[80-83], NNAT is associated with metabolism coronary atherosclerotic heart disease, Wilm's Tumor and several leukemias and solid tumor through control of cell growth and differentiation and HLA-DPB2 is also associated with several cancers including breast, cervical, ovarian and rectal along with hepatitis B and lupus[84-88] and finally C11orf21 has been associated with leukemia and neurodevelopmental disorders.[89,90]

The genes associated with the top individuals CpGs and DMRs with significantly differential methylation have been implicated in longevity and several diseases including many different types of cancer. Prior studies of DNA methylation and aging have also revealed

hypermethylation of CpG islands and global DNA hypomethylation although the magnitude of change was larger for cancer versus aging.[91,92] In normal adult somatic cells, most CpG islands in promoters are unmethylated and CpG sequences in heterochromatin containing repeated DNA are methylated.[93] There are two hypothesized mechanisms behind these changes. The first is epigenetic drift, the modification of epigenetic marks due to errors in epigenetic pathways, and the second is stress-induced changes.[94,95]

In McEwen's study of 48 long-lived Nicoyans and 47 non-Nicoyans in Costa Rica, there were 4 single CpGs and 20 genomic regions that were significantly differentially methylated between Nicoyans and non-Nicoyans. One DMR had six CpGs in the promoter region of the NUDT12 gene which is known to play a role in NAD metabolism, a regulatory process associated with health span and aging. The genes that were associated with these findings did not overlap with our significant findings, but this may be due to differences in the characteristics of the sample and outcome definitions.[21] Gentillini et. al tested methylation at 25,578 CpG sites comparing centenarians, centenarians' offspring and offspring of non-long-lived parents to young controls and identified 709 loci associated with 607 genes responsible for the regulation of transcription and cell differentiation among other functions. The 330 CpG loci that were hypomethylated had genes that controlled signal transduction/signaling processing and the regulation of respiratory burst involved in acute inflammatory response among additional functions.

There were several strengths and limitations to this study that should be noted. This study benefitted from a large, racial/ethnically diverse sample of women who were followed to at least 90 years of age. Women were followed for 20 years on average with low rates of loss to follow-up. There was information available on important baseline characteristics and potential

confounders due to the prior data collection in the WHI. Finally, we had repeated measures of both physical and cognitive functioning from baseline to age 90 or time of death that were taken into account in the exceptional longevity classification although due to those who were unable to complete the annual survey due to their level of frailty there may the results may be attenuated. Among the limitations, the first is the nested case-control sampling of two of the ancillary studies from the larger WHI. If the sampling structure is ignored the disproportionate stratified subsamples of the study base can lead to biased estimates. Using inverse probability selection weights to account for differences in selection criteria is a recommended solution and was implemented in this study.[64] This study was limited to women and replication in cohorts that include both men and women, diverse racial/ethnic groups and represent individuals from varied regions of the world is important. Notably, few prospective studies exist for replication where sufficient numbers of participants have been followed for 20 or more years to the age of 90 or older so that healthspan related phenotypes can be defined.

To the best of our knowledge this is the first study to investigate the potential link between genome-wide DNA methylation in blood and exceptional longevity among older women. We identified several sites and regions that had differential methylation associated with exceptional longevity among older women. These findings should be confirmed in additional studies that include both long-lived men and women and examined further using molecular studies to identify specific biological mechanisms that may be at play in these relationships. The differentially methylated CpG sites across the genome identified in this study may elucidate biological mechanisms associated with exceptional longevity, serve as potential targets for intervention and be utilized in risk prediction models.

Supplementary Figures

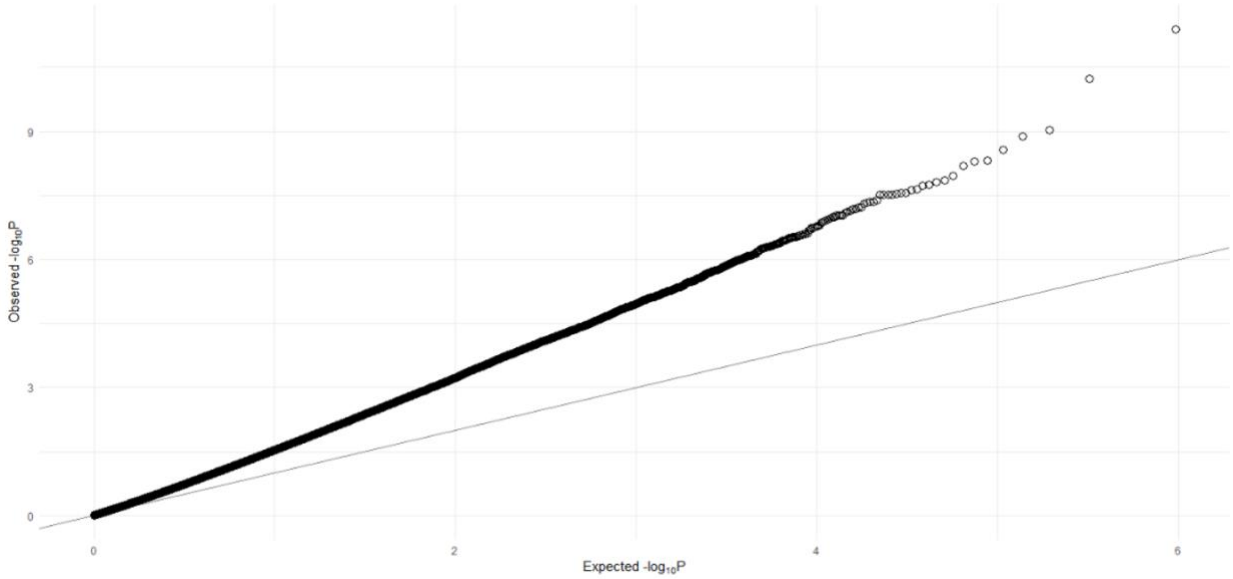


Figure 3.4: QQ plot of expected versus observed p-value comparing women who survived

to age 90 with physical function compared to women who did not survive

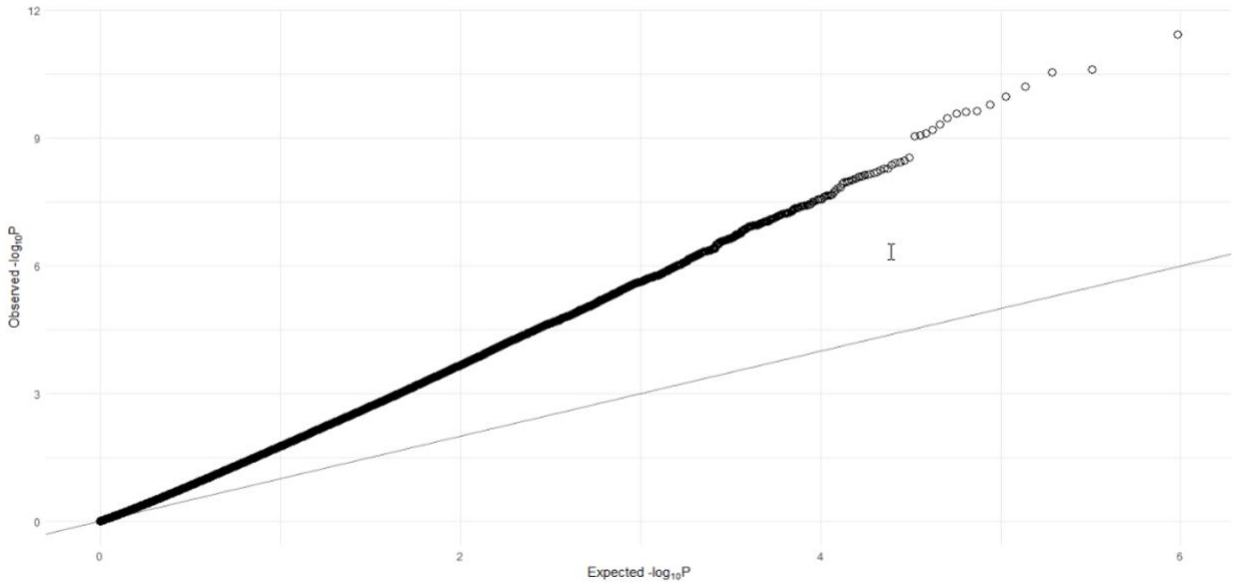


Figure 3.5: QQ plot of expected versus observed p-value comparing women who survived to age 90 without physical function compared to women who did not survive

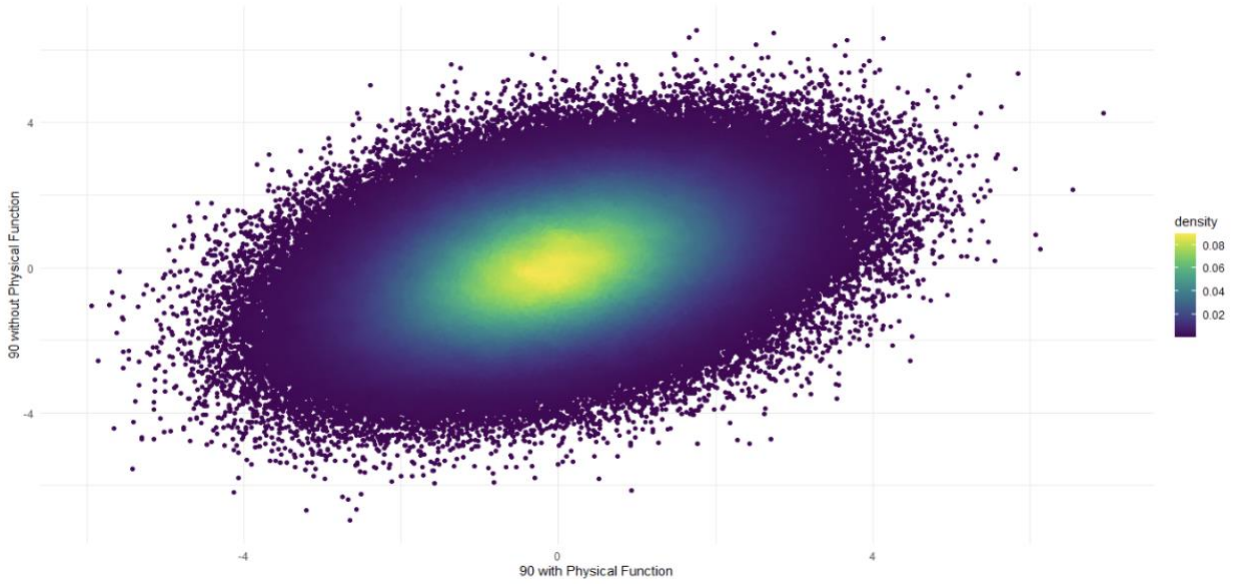


Figure 3.6: Scatter plot with density overlay displaying t-stat of association between DNA methylation level and survival to age 90 with physical function compared to women who did not survive to age 90 and DNA methylation level and survival to age 90 without physical function compared to women who did not survive to age 90

Chapter 3, in full, is currently being prepared for submission for publication of the material. Jain, Purva; Binder, Alex; Chen, Brian; Parada, Humberto; Gallo, Linda; Alcaraz, John; Horvath, Steve; Bhatti, Parveen; Whitsel, Eric; Baccarelli, Andrea; Hou, Lifang; Stewart, Jay; Li, Yun; Jordahl, Kristina; LaCroix, Andrea. The dissertation author was the primary investigator and author of this paper.

4. The association of epigenetic age acceleration and multimorbidity at age 90 in the Women's Health Initiative

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4.1. Abstract

Background: Epigenetic age acceleration (EAA), a measure of accelerated biological aging, has been associated with increased risk of several age-related chronic conditions. This was the first study to measure the relationship between EAA and both multimorbidity count and a weighted multimorbidity score among postmenopausal women using a prospective design.

Methods: This study included 1,951 women from three Women's Health Initiative (WHI) ancillary studies with genome-wide DNA methylation (DNAm) data from baseline blood samples and who could have survived to age 90 during follow-up through September 30, 2020. EAA was estimated using the Horvath pan-tissue, Hannum, PhenoAge and GrimAge "clocks." The multimorbidity score was weighted for each morbidity's relationship with mortality in the study population and included 12 age-related chronic conditions. Using mixed-effects Poisson and linear regression models with a random intercept for each ancillary study, we estimated relative risks (RRs) and 95% confidence intervals (CIs) for the relationships between each clock measured at study baseline and both multimorbidity count and weighted multimorbidity score at age 90, respectively. All models included baseline covariates associated with both EAA and multimorbidity and inverse probability weights of ancillary study selection to create a study population that was representative of the WHI overall.

Results: For every one-standard deviation increase in AgeAccelPheno, the rate of multimorbidity accumulation increased 6% (RR=1.06; 95% CI=1.01-1.12; p=0.025) and the multimorbidity

score by 7% (RR=1.07; 95% CI=1.01-1.13; p=0.014) for women who survived to age 90. The results for a one-standard deviation increase in AgeAccelHorvath, AgeAccelHannum and AgeAccelGrim with multimorbidity accumulation and score were weaker compared to AgeAccelPheno, and did not reach statistical significance.

Conclusion: Epigenetic clock PhenoAgeAccel may predict multimorbidity count and score at age 90 in older women and, thus, may be useful as a biomarker predictor of multimorbidity burden in the last decades of life.

4.2. Introduction

There are approximately 3.8 million individuals who are aged 85 and older in the United States (US) with this population expected to comprise 10% of the US population by 2050.[34] Women outnumber men 3 to 1 among those 90 or older.[35] Among Medicare beneficiaries in 2008, 82.3% of women ages 85 or older had multimorbidity. Multimorbidity as defined by US Department of Health and Human Services is the presence of 2 or more chronic conditions.[96] There is substantial evidence supporting the relationship between multimorbidity and mortality, functional status and quality of life.[97-103]

Recently, the National Institutes of Health (NIH) developed a framework highlighting the influence of factors that may cause, increase the risk for, or exacerbate multiple conditions, and the potential for these factors to inform prevention strategies to achieve significant public health impact.[104] Biological aging focuses on biological mechanisms that are fundamental and central to overall increases in disease and disability as one ages.[38] Individuals with the same chronological age may experience different rates of biological aging, and faster biological aging is associated with chronic disease onset, morbidity and mortality. Exceptional longevity can be characterized as having a biological age less than one's chronological age and is closely linked with the

concept of healthspan. Healthspan prioritizes physical and cognitive functioning with advancing age, and preservation of healthspan targets both primary and secondary prevention of impaired function.[1] Primary prevention is the prevention of disease, while secondary prevention aims for early detection and minimization of symptoms.

For a biomarker to be a useful indicator or predictor of exceptional longevity and healthspan, it should move beyond prediction of all-cause mortality and be capable of predicting multimorbidity burden at an advanced age. Epigenetic age is a composite measure of DNA methylation (DNAm) levels across specific cytosine-guanine dinucleotides (CpG) sites that are associated with chronologic and phenotypic age. These DNAm signatures are associated with age-related diseases and all-cause mortality, independent of chronologic age.[42-44] Epigenetic age acceleration (EAA) is then the difference between one's chronological age and epigenetic age predicted by chronological age and is indicative of whether one is aging slower or faster than their chronological age.

To the best of our knowledge previous studies that examined the association between EAA and multimorbidity count among older adults have been cross-sectional.[23] A meta-analysis including 9 studies from 4 unique cohorts was conducted to assess the relationship of a 1-year increase in EAA and multimorbidity count at time of blood draw. Overall, all EAA measures showed a statistically significant association with cross-sectional multimorbidity counts. The previous study limited the multimorbidities that were included in the multimorbidity count to the age-related conditions available in each cohort, did not take into consideration the risk of mortality associated with each condition, was not restricted to older age groups, and did not examine associations with multimorbidity at a specific older age when all participants would have the same amount of chronological aging for diseases to occur.

The NIH report on multimorbidity additionally recommended the use of nested-prospective, age-based, epidemiologic studies to examine potential mechanisms that may be intervened upon to target multimorbidity among older adults.[104] There have been no nested-prospective studies that have examined the relationship between EAA and multimorbidity count among women who survive to older ages (90+ years). The aims of this study, therefore, were to examine the relationships between EAA and both multimorbidity count and weighted multimorbidity score at age 90. We hypothesized that women who experienced accelerated biological aging, as measured by epigenetic age, would have higher multimorbidity counts and weighted multimorbidity scores.

4.3. Methods

Study Population

In 1993, the Women's Health Initiative (WHI) was created in order to identify strategies to prevent heart disease, osteoporosis and breast and colorectal cancers among postmenopausal women.[47,48] This study included three WHI ancillary studies, two nested case-control studies and one nested cohort, that previously assayed genome-wide DNAm. The Bladder Cancer and Leukocyte Methylation Ancillary Study (Study A) identified methylation profiles associated with bladder cancer risk among 468 women with and 468 women without bladder cancer.[50] The Integrative Genomics for Risk of Coronary Heart Disease and Related Phenotypes in the WHI Cohort Ancillary Study (Study B) included 1,070 women with and 1,070 women without coronary heart disease.[52] The Epigenetic Mechanisms of Particulate Matter-Mediated Cardiovascular Disease Ancillary Study (Study C) identified the pathophysiological mechanisms that underlie particulate matter-related cardiovascular disease in postmenopausal women using a random sample of 2,200 WHI clinical trial participants.[51]

The current study included women with baseline assays of genome-wide DNAm that were eligible to survive to age 90 between baseline and the end of the most recent observation period (September 30, 2020). There were a total of 2,079 women who were eligible to survive to age 90 (443 from AS311, 694 from AS315 and 942 from BAA23). Of these eligible women, survival status at age 90 was known for 1,951 women (94%); 1,022 women survived to age 90 while 929 women died before reaching age 90. There were 128 women who had an unknown survival status and were excluded resulting in a final analytic sample of 1,951 women. Study protocols were approved by the WHI publications and presentations committee and all women provided consent in person or by phone.

Measures

Epigenetic Age

DNAm was measured using the Illumina Infinium 450K platform (San Diego, CA, Ilumina). The minfi package in R was used to read in all DNAm data files, check for failed samples, conduct quality control and implement normalization steps. The normal-exponential convolution using out-of-band probes method was used to perform background correction. Functional normalization was used to account for type I and type II probe differences and to remove both batch effects and technical variation. The wateRmelon and minfi R packages were used to remove low quality probes that interrogate non-CpG sites, remove probes that have a detection p-value above 0.01 in any sample, and remove probes that are located on the X or Y chromosome.

Epigenetic age was estimated based on four previously developed "clocks", including the Horvath pan-tissue, Hannum, PhenoAge and GrimAge clocks, as summarized in Table 4.1.[23,32,43,44] Horvath used 353 CpG sites to predict chronological age across several different

tissues. Hannum used 71 CpG sites and also was trained to predict chronological age. PhenoAge used 513 CpG sites and was trained using a "phenotypic age" measure that was created using nine clinical biomarkers that were associated with time-to-death. GrimAge used 1,030 CpG sites derived from analyses predicting time-to-death using age, sex, DNAm-based surrogate biomarkers of plasma protein levels and a DNAm-based estimator of smoking pack-years (Table 1).

Table 4.1: Overview of epigenetic clocks being utilized in this study

Clock	CpGs	Genes	Age	N	Tissue	Reported Associations
Horvath[43]	353	344	0-101	8000	Various cell & tissues	Chronological age, all-cause mortality, cancer, age-related disease and several neurodegenerative phenotypes
Hannum[44]	71	94	19-101	656	Blood	Chronological age, all-cause mortality
PhenoAge[32]	513	505	>20	9926	Blood	All-cause and cause-specific mortality, survival, count of multimorbidities, physical functioning, smoking status and telomere length
GrimAge[23]	1030	NA	NA (mean=66)	1731	Blood	Morbidity and mortality, survival, cognitive decline, clinical biomarkers, lifestyle factors, blood cell composition and telomere length

Multimorbidity outcomes

There were 12 chronic conditions included in our multimorbidity count and weighted multimorbidity score outcomes (Table 4.2). These conditions were selected due to the their prevalence among older women in the US, their strong influence on physical functioning and quality of life, as well as guidance from current literature. Although there is currently no standardized list of conditions to include in the definition of multimorbidity, the majority of conditions were included on a list of 20 chronic conditions that were selected by the Office of the

Assistant Secretary of Health within the Health and Human Services to work towards the standardization of multimorbidity.[105] There were three conditions (sensory impairment, frequent faller, hip fracture and urinary incontinence) that were not on this list, but mirrored a previous study in the WHI that also examined multimorbidity among older women and due to their high prevalence among this population were included.

Table 4.2: Definition of 12 Chronic Conditions and Assigned Weighted Score in Multimorbidity Count and Multimorbidity Score Outcomes

Chronic Condition	Definition	Weighted Score
Stroke	One or more of the following: carotid artery disease, stroke, transient ischemic attack	18.4
Coronary disease	One or more of the following: coronary heart disease, clinical myocardial infarction, congestive heart failure, coronary artery bypass graft or percutaneous transluminal coronary angioplasty	16.5
Cancer	Any cancer (excluding nonmelanoma skin cancer)	15.8
Chronic obstructive pulmonary disease	Self-reported physician diagnosis	14.6
Sensory impairment	Self-reported moderate to severe trouble with vision or hearing loss	13.0
Diabetes	Self-reported physician diagnosis of diabetes and treatment for diabetes (pills, insulin)	11.7
Frequent faller	Self-reported ≥ 2 falls within one year	7.5
Cognitive impairment	Self-reported physician diagnosis with dementia or Alzheimer's	6.4
Hip fracture	Broken hip	4.5
Osteoarthritis	Self-reported physician diagnosis	4.0
Depression	Self-reported treatment for depression (pills or therapy)	3.1
Urinary incontinence	Self-reported very of extremely bothersome urinary leakage	1.0

These conditions were identified as part of the WHI follow-up protocol using both self-report on annual or semi-annual outcome forms, followed by physician adjudication for selected outcomes of major interest within WHI. For self-reported items, the following question was used

for ascertainment, “Since the date on the front of this form, has a doctor told you that you have any of the following conditions or have you had any of the following procedures?” The following conditions were self-reported: Alzheimer’s disease, diabetes characterized by self-reported use of diabetic medications, depression characterized by self-reported treatment of medication or therapy, sensory impairment self-reported as moderate to severe vision or hearing loss, urinary incontinence as self-reported ever leaking urine and feeling extremely bothered by it and frequent falling included a self-report of falling at least two times in the past 12 months. Participants who reported “yes” for any of the listed conditions from baseline to follow-up through reaching age 90 were classified as having the condition at age 90. This approach was taken in recognition of the chronicity of the conditions under study. The primary outcomes of the WHI study were adjudicated throughout the study by a physician using medical records including incident coronary heart disease (CHD), cerebrovascular disease, cancer and hip fracture. While conditions such as hypertension, hyperlipidemia, and obesity were considered, they were not included due their role as major risk factors for many conditions included and the focus on including conditions that were disease endpoints.

There were two outcomes for this study: multimorbidity count and weighted multimorbidity score. Multimorbidity count was defined as the total number of morbidities from baseline to follow-up to age 90, death or loss to follow-up and was used as a count. Weighted multimorbidity score was a derived score based on the association of each morbidity with survival status among women eligible to survive to age 90 who had DNA methylation data. Each morbidity was placed in an univariate model with survival status at age 90 and the weight was calculated as the beta of each condition over the beta of urinary incontinence, which had the lowest beta and served as a reference weight of 1 (Table 4.2). The final multimorbidity score was

the sum of the relative weights based on all of the conditions a woman had acquired from baseline to age 90 or her last study visit before date of death. The purpose of the weighted multimorbidity score was to capture the degree to which each disease was life threatening, and assign value accordingly using a weight as compared to a total count. This method has been previously utilized to convert the Elixhauser comorbidity measure into a single score.[106]

Covariates

Covariates were measured at WHI baseline and selected due to their associations with both EAA and multimorbidity. Covariates included age at blood draw, DNAm-based estimated blood cell composition using the Houseman method[107] (CD8+ T Cells, CD4 T cells, Natural Killer cells, B lymphocyte cells, Monocytes, Granulocytes), race/ethnicity (Black (African American), Hispanic (Latino), White, Unknown (not one of the above), education (high school/general education development or less, some college, college graduate or more), walking frequency >10 min (rarely or never, 1-3 times/mo, 1 time/wk, 2-3 times/wk, 4-6 times/wk, 7+ times/wk), body mass index categories (underweight, normal, overweight, obese), alcohol consumption (non-drinker, past drinker, <1 drink/mo, <1 drink/wk, 1-<7 drinks/wk, 7+ drinks/wk), pack-years smoking (never smoker, <5, 5-20, 20+) and physical function score (RAND-36 10-item physical function subscale[54], range 0-100, higher score reflects higher function).

Statistical Analysis

Baseline characteristics were reported by PhenoAgeAccel quartile. Differences across quartiles were tested using Pearson's chi-squared tests for categorical variables and F-tests for continuous variables. Unadjusted and fully-adjusted Poisson and linear regression models with a random intercept for ancillary study were used to estimate relative risks (RRs) and 95%

confidence intervals (CIs) for the associations between each EAA measure (one standard deviation increase) with multimorbidity count and multimorbidity score, respectively. Adjusted models included all covariates as described above and inverse probability weights to account for the case-control sampling of two ancillary studies (AS311 and BAA23) to create an analytic study population more representative of the WHI overall. The weights were the inverse of the selection probability into either AS311 or BAA23 for each individual in order to downweight cases. The sample was re-weighted so the sum of the weights approximated the original sample size of the analytic sample for AS311 and BAA23. Inverse probability weights were also applied to AS315 to account for oversampling of racial/ethnic minorities.

The primary analyses were conducted among women who survived to age 90. There were two sensitivity analyses. The first sensitivity analysis included all women eligible to survive to age 90 regardless of their survival to age 90. The purpose of this sensitivity analysis which includes women who died before reaching the age of 90 was to evaluate the robustness of the findings to selective mortality. The second sensitivity analysis repeated the primary analysis adding adjustment for baseline multimorbidity count. This analysis was done to account for the multiple morbidity count at time of blood draw that could influence the prediction of total morbidity count at age 90 and also evaluates prediction of incident multimorbidity. This second sensitivity analysis was done in both women who survived to age 90 and all women who were eligible to survive to age 90. All analyses were conducted using R Version 1.4.1106 (R Foundation for Statistical Computing, Vienna, Austria).

4.4. Results

The 1,022 women who survived to age 90 were followed for 20.7 years on average (range=10.4-25.4 years) from WHI baseline to age 90 and were. The 929 women who did not

survive to age 90 were followed for 12.8 years on average (range=0.1-24.7 years) from WHI baseline to time of death. Women who survived to age 90 had an average of 1.1 multimorbidities at baseline (range=0-5 multimorbidities) and 2.8 at age 90 (range=0-8 multimorbidities). Women who did not survive to age 90 had an average of 1.3 multimorbidities at baseline (range=0-5 multimorbidities) and 3.2 by age 90 or last-follow up before death for those women who did not survive to age 90 (range=0-9 multimorbidities). The distributions of conditions by total multimorbidity among women eligible to survive to age 90 are shown in greater detail in Figure 4.1. In brief, a large proportion of women who had 3 or fewer chronic conditions had arthritis, cancer, CVD, frequently falling or sensory impairment by age 90. Additionally, among women who had greater counts of morbidities, the conditions tended to distribute equally.

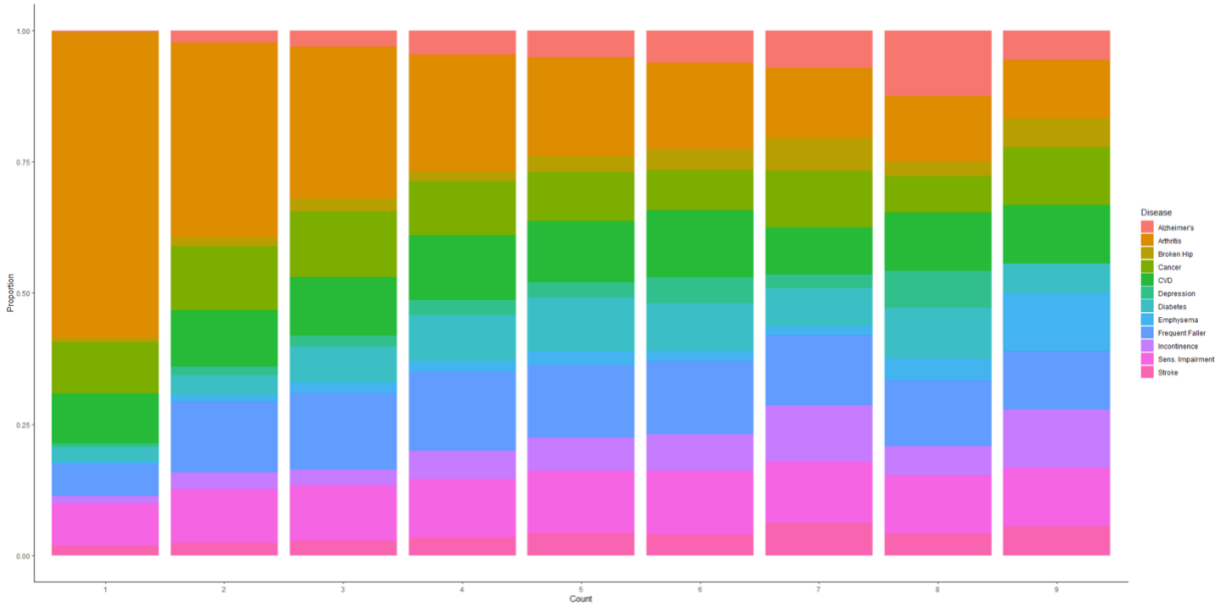

Figure 4.1: Distribution of Multimorbidities Stratified by Multimorbidity Count (N=1,951)

Note: The following is the distribution of women in each multimorbidity count group: 0 (n=60); 1 (n=264); 2 (n=446); 3 (n=512); 4 (n=355); 5 (n=197); 6 (n=90); 7 (n=16); 8 (n=9); 9 (n=2)
Note: The denominator is the distribution of the total number of conditions for all women in each comorbidity count group

Women with higher epigenetic age acceleration (accelerated biological aging) as measured by the Pheno clock were more likely to be Black or Hispanic, have lower education, be obese, drink less alcohol, have a lower physical functioning score and accumulate a greater number of multimorbidities (Table 4.3). In addition, those with higher AgeAccelPheno measures were more likely to have severe conditions such as cardiovascular disease and diabetes (Table 4.4).

Table 4.3: Baseline Characteristics by PhenoAgeAccel Quartile (N=1,951)

	Decelerated Aging		Accelerated Aging		
	-31 - -4.5 (n=493)	**-4.4-0.0 (n=516)**	**0.1-4.0 (n=411)**	**4.1-29.4 (n=529)**	**p**
Race/Ethnicity, n (%)					0.005
Black or African American	77 (15.7)	73 (14.3)	70 (17.1)	123 (23.5)	
Hispanic/Latino	32 (6.5)	41 (8.0)	34 (8.3)	47 (9.0)	
White	365 (74.6)	378 (73.8)	289 (70.5)	342 (65.3)	
Other	15 (3.1)	20 (3.9)	17 (4.1)	12 (2.3)	
Education, n (%)					0.027
HS/GED or Less	119 (24.3)	133 (25.9)	117 (28.6)	174 (33.1)	
Some College	195 (39.8)	214 (41.7)	170 (41.6)	204 (38.8)	
College Grad or More	176 (35.9)	166 (32.4)	122 (29.8)	148 (28.1)	
Walking Frequency, n (%)					0.062
Rarely or Never	81 (16.5)	87 (17.0)	75 (18.5)	131 (25.0)	
1-3 times/month	74 (15.1)	74 (14.5)	55 (13.5)	77 (14.7)	
1 time/week	53 (10.8)	59 (11.5)	40 (9.9)	53 (10.1)	
2-3 times/week	133 (27.1)	131 (25.6)	123 (30.3)	145 (27.6)	
4-6 times/week	104 (21.2)	113 (22.1)	84 (20.7)	84 (16.0)	
7+ times/week	45 (9.2)	47 (9.2)	29 (7.1)	35 (6.7)	
BMI Category, n (%)					<0.001
Underweight	5 (1.0)	4 (0.8)	5 (1.2)	5 (1.0)	
Normal	177 (36.1)	156 (30.3)	108 (26.4)	118 (22.5)	
Overweight	182 (37.1)	184 (35.7)	148 (36.2)	170 (32.4)	
Obese	126 (25.7)	171 (33.2)	148 (36.2)	231 (44.1)	
Alcohol Consumption, n (%)					0.014
Non-drinker	79 (16.1)	56 (11.1)	48 (11.8)	80 (15.3)	
Past drinker	94 (19.1)	112 (22.1)	87 (21.4)	126 (24.1)	
<1 drink/month	57 (11.6)	60 (11.9)	51 (12.6)	86 (16.4)	
<1 drink/week	96 (19.6)	116 (22.9)	82 (20.2)	93 (17.8)	
1-<7 drinks/week	112 (22.8)	114 (22.5)	89 (21.9)	81 (15.5)	
7+ drinks/week	53 (10.8)	48 (9.5)	49 (12.1)	57 (10.9)	
Smoking Pack-Years, n (%)					0.961
Never Smoker	265 (55.4)	270 (54.3)	209 (52.9)	283 (55.6)	
<5	58 (12.1)	66 (13.3)	45 (11.4)	57 (11.2)	
5-<20	62 (13.0)	60 (12.1)	52 (13.2)	60 (11.8)	
20+	93 (19.5)	101 (20.3)	89 (22.5)	109 (21.4)	

Table 4.4: Baseline Characteristics by PhenoAgeAccel Quartile (N=1,951)

Age-Related Condition					
Alzheimer's	58 (11.8)	63 (12.2)	52 (12.7)	65 (12.3)	0.982
Arthritis	387 (78.5)	406 (78.7)	320 (77.9)	426 (80.5)	0.760
Broken Hip	43 (8.7)	36 (7.0)	42 (10.2)	25 (4.7)	0.009
Cancer	155 (31.4)	154 (29.8)	130 (31.6)	187 (35.3)	0.272
Cardiovascular Disease	149 (30.2)	168 (32.6)	154 (37.5)	198 (37.4)	0.037
Depression	36 (7.3)	30 (5.8)	36 (8.8)	48 (9.1)	0.191
Diabetes	87 (17.6)	100 (19.4)	103 (25.1)	143 (27.0)	0.001
Emphysema	25 (5.1)	33 (6.4)	25 (6.1)	34 (6.4)	0.784
Frequent Faller	198 (40.2)	218 (42.2)	173 (42.1)	222 (42.0)	0.901
Sensory Impairment	154 (31.2)	179 (34.7)	136 (33.1)	168 (31.8)	0.648
Stroke	42 (8.5)	66 (12.8)	34 (8.3)	52 (9.8)	0.070
Urinary Incontinence	66 (13.4)	63 (12.2)	63 (15.3)	80 (15.1)	0.442
Age, mean (SD)	71.0 (3.5)	71.2 (3.4)	71.0 (3.6)	70.5 (3.3)	0.026
Physical Function Score, mean (SD)	76.7 (22.7)	75.0 (22.3)	72.5 (23.8)	70.0 (25.3)	<0.001
Baseline Multimorbidity Count	1.1 (0.9)	1.2 (1.0)	1.3 (1.0)	1.4 (1.1)	<0.001
Follow-up Multimorbidity Count	2.3 (1.5)	2.4 (1.5)	2.6 (1.5)	2.6 (1.5)	0.040
Total Multimorbidity Count	2.8 (1.5)	2.9 (1.5)	3.1 (1.6)	3.1 (1.6)	0.015
Multimorbidity Score	26.0 (15.9)	27.8 (17.0)	28.8 (16.9)	30.0 (1.6)	0.015
AgeAccelHorvath	-3.0 (4.8)	-0.9 (4.4)	0.4 (4.8)	2.9 (5.4)	<0.001
AgeAccelHannum	-3.2 (4.9)	-1.1 (4.4)	0.9 (4.2)	2.9 (4.6)	<0.001
AgeAccelGrim	-1.8 (3.3)	-0.8 (3.7)	0.4 (3.5)	1.9 (4.1)	<0.001

Note: GED=general educational development; BMI=body mass index; kg=kilograms; m=meters

Note: AgeAccel measures are the residual between chronological age and epigenetic age as measured by each individual epigenetic clock.

[a]Conditions include cardiovascular disease, cancer, cognitive impairment, depression, osteoarthritis, history of falls, chronic obstructive pulmonary disease, hypertension, diabetes, hip fracture and cerebrovascular disease.

The associations between baseline EAA and multimorbidity count and multimorbidity score at age 90 based on our adjusted models are reported in Table 4.5. For every one standard deviation increase in AgeAccelHorvath (5.1 years) the relative comorbidity count at age 90 was 4% higher (RR=1.04; 95% CI=1.00-1.09, p=0.074) and the weighted multimorbidity score was 3% higher (RR=1.03, 95%CI=0.98-1.07, p=0.174). For every one standard deviation increase in AgeAccelHannum (5.3 years), the relative comorbidity accumulation was 2% higher (RR=1.02; 95% CI=0.97-1.07, p=0.441) and there was no observed association between AgeAccelHannum

and the weighted multimorbidity score. For every one standard deviation increase in AgeAccelPheno (7.0 years), the relative comorbidity accumulation was 6% higher (RR=1.06; 95% CI=1.01-1.12; p=0.025) and the weighted multimorbidity score was 7% higher (RR=1.07; 95% CI=1.01-1.13; p=0.014) for women at age 90. For every one standard deviation increase in AgeAccelGrim (3.9 years), the relative multimorbidity accumulation was 2% lower (RR=0.98; 95% CI=0.93-1.03, p=0.436) and the weighted multimorbidity score was 4% lower (RR=0.96; 95% CI=0.92-1.01, p=0.117). The results were slightly attenuated for the covariate unadjusted analysis and remained similar for the sensitivity analyses that additionally adjusted for baseline multimorbidity count (Supplementary Table 4.7).

Table 4.5: Association of Epigenetic Age Acceleration with Multimorbidity Count and Multimorbidity Score Among Women Who Survived to Age 90 (N=1,022)

	Multimorbidity Count		**Multimorbidity Score**	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	1.04 (1.00-1.09)	0.074	1.03 (0.98-1.07)	0.174
AgeAccelHannum	1.02 (0.97-1.07)	0.441	1.00 (0.95-1.05)	0.947
AgeAccelPheno	1.06 (1.01-1.12)	0.025	1.07 (1.01-1.13)	0.014
AgeAccelGrim	0.98 (0.93-1.03)	0.436	0.96 (0.92-1.01)	0.117

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=5.1), AgeAccelHannum (sd=5.3), AgeAccelPheno (sd=7.0) and AgeAccelGrim (sd=3.9).

The results of the sensitivity analyses in which we examined the adjusted associations between baseline EAA and multimorbidity count and multimorbidity score among all women eligible to survived to age 90 (n=1,951 total) are reported in Table 4.6. For every one standard deviation increase in AgeAccelHorvath the rate of multimorbidity accumulation decreased 1% (RR=0.99; 95% CI=0.96-1.02; p=0.651) and the weighted multimorbidity score increased 1% (RR=1.01; 95% CI=0.98-1.04; p=0.448). For every standard deviation increase in

AgeAccelHannum, the rate of multimorbidity accumulation decreased 3% (RR=0.97; 95% CI=0.94-1.01, p=0.105) and the weighted multimoribity score decreased 2% (RR=0.98; 95% CI=0.95-1.02, p=0.360). For every standard deviation increase in AgeAccelPheno, the rate of multimorbidity accumulation increased 4% (RR=1.04; 95% CI=1.00-1.07; p=0.040) and the weighted multimorbidity score 7% (RR=1.07; 95% CI=1.04-1.10; p<0.001). One standard deviation increase in AgeAccelGrim was not associated with multimorbidity count, and a decrease in the weighted multimorbidity score by 2% (RR=0.98; 95% CI=0.95-1.01, p=0.252). The results were similar for the covariate unadjusted analysis and slightly attenuated for the sensitivity analysis that additionally adjusted for baseline multimorbidity count (Supplementary Table 4.8).

Table 4.6: Association of Epigenetic Age Acceleration with Multimorbidity Count and Multimorbidity Score Among All Women Eligible to Survive to Age 90 (N=1,951)

	Multimorbidity Count		Multimorbidity Score	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	0.99 (0.96-1.02)	0.651	1.01 (0.98-1.04)	0.448
AgeAccelHannum	0.97 (0.94-1.01)	0.105	0.98 (0.95-1.02)	0.360
AgeAccelPheno	1.04 (1.00-1.07)	0.040	1.07 (1.04-1.10)	<0.001
AgeAccelGrim	1.00 (0.97-1.04)	0.917	0.98 (0.95-1.01)	0.252

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

Note: There were 1,022 women who survived to age 90 and 929 women who died before age 90. All models included an offset for age to account for differing lengths of follow-up.

[a]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=5.1), AgeAccelHannum (sd=5.3), AgeAccelPheno (sd=7.0) and AgeAccelGrim (sd=3.9).

4.5. Discussion

To the best of our knowledge, this is the first study to examine the relationship between EAA and multimorbidity among older women at the time they reach age 90. In this racially and ethnically diverse group of older women, this prospective study showed that increased EAA as

measured by AgeAccelPheno predicted an increased risk of acquiring additional multimorbidities and more deadly multimorbidities among women who survived to age 90. A similar association was observed among all women eligible to survive to age 90. The results also remained similar in the covariate unadjusted models and the fully covariate adjusted models that additionally adjusted for baseline multimorbidity count. EAA measured by AgeAccelHorvath, AgeAccelHannum and AgeAccelGrim were not associated with either multimorbidity count or multimorbidity score over time.

To date, studies have only examined the relationship between EAA and multimorbidity count cross-sectionally. Lu et al. conducted a cross-sectional meta-analysis between each of the four EAA measures and multimorbidity count at time of blood draw. Participants were from the Framingham Heart Study (FHS), WHI, Invecchiare in Chianti (InChianti) and Jackson Heart Study (JHS). This study as well as Lu et. al's benefitted from a large sample size and racial and ethnic diversity in the sample. While the age-related conditions included in each cohort included in the Lu et. al analysis differ, the conditions across cohorts that overlapped with this analysis include stroke, coronary disease, cancer, chronic obstructive pulmonary disease, visual impairment, diabetes and cognitive impairment. The following conditions were unique to this study: hearing impairment, frequently falling, hip fracture, osteoarthritis and depression.

Although the meta-analysis estimated effect sizes were not provided to prevent comparison across EAA measures with different distributions, for each 1-year increase in EAA each of the four measures were statistically significantly associated with comorbidity count in this cross-sectional analysis. Specifically for every 1-year increase in AgeAccelPheno and its association with comorbidity count, the significant estimates within InChianti, JHS, FHS and WHI AS315 ranged from 0.01-0.03. In our study, the betas for a 1-year increase from

AgeAccelPheno and both multimorbidity count and multimorbidity score were also close to 0.01 for all analyses. It is unclear which covariates were included in each study population and if they differed across study populations in availability or measurement. Another major difference in the Lu et. al analysis was the inclusion of a broad age range of adult men and women ranging from ages 20-102. These differences in study design and populations may explain the difference in results found for AgeAccelHorvath, AgeAccelHannum and AgeAccelGrim in relation to multimorbidity count and score at age 90 in the present study.

Epigenetic clocks are thought to be a promising measure of biological age and DNA methylation the a promising age-predictive biomarker.[63] Having accelerated biological age as measured by these epigenetic clocks has been associated with increased risks of several age-related phenotypes such as Alzheimer's disease, cancer, coronary heart disease, cognitive performance, frailty, osteoarthritis, and Parkinson's disease among others.[23,32,43,44] The CpGs that were included in the clocks during the model building phase are thought to have a relationship with the epigenetic maintenance system especially at promoters and enhancers throughout the genome. More specifically for the PhenoAge clock, the CpG sites that were more prevalent among individuals with accelerated aging were associated with several pro-inflammatory signaling pathways, while those that were less prevalent among those with accelerated aging were involved in transcriptional and translational machinery and DNA damage recognition and repair.[32] Although the specific mechanisms are still under examination, the change in DNAm with age is most likely linked to declines in tissue function related to both intracellular changes that lead to a loss of cellular identity and small changes in cell composition over time.[16] Only 41 of the 513 CpGs in the Horvath pan-tissue clock and only 5 CpGs in the Hannum clock are shared with the PhenoAge clock. DNAm PhenoAge was unique among the clocks examined

because it was developed to predict phenotypic age rather than chronological age using biomarkers and risk factors related to all-cause mortality. In addition, PhenoAge was trained using longitudinal data that may better account for changes in health status over time. These differences may explain the associations between EAA measured by PhenoAge and not the other epigenetic clocks.

This study had several strengths and limitations. The study population included a large number of women who survived to age 90 and was also racially and ethnically diverse. On average, women were followed for 2 decades with low rates of loss-to-follow-up. The WHI had information on relevant baseline characteristics and potential confounders. Epigenetic age was measured using several different clocks, which is currently considered best practice due to the low overlap in CpG sites and associated genes between the clocks that potentially capture different biologic pathways.[63] Finally, there was also longitudinal measurement of several age-related chronic conditions, some of which were also adjudicated by trained physicians. There were also some limitations to note in this study. This study population included two ancillary studies that utilized nested case-control sampling of the larger WHI cohort. This study used inverse probability selection weights to account for differences in the selection criteria, which is currently the recommended approach.[64] Since this study was limited to women, it will be important to replicate the findings among both men and women with diverse race, ethnicity and geographical representation. Presently, few studies have sufficient numbers of individuals with long follow-up to age 90 or beyond with DNA methylation measures available. Another limitation to note is that biological aging can be measured in several different ways (genomics, metabolomics, proteomics, microbiomics, transcriptomics, etc). Although EAA is the focus of this study, several biological processes are likely to simultaneously contribute to age-related

disease onset and progression.[65] There is currently no gold standard to measure biological aging and thus this research should be interpreted within a larger systems biology framework, that acknowledges the influence and interaction of many underlying processes.

In this study, we report that increased EAA measured by DNAm PhenoAge was associated with an increased number and more life threatening multimorbidities at age 90 among older women. These results suggest that PhenoAgeAccel is a promising biomarker of multimorbidity burden among older women that is capturing the biological age and functional state of several organ systems and tissues beyond one's chronological age. As women continue to live to more advanced ages, it will be increasingly important to predict the overall burden of age-related diseases, to utilize that information to implement appropriate public health interventions, and to discover potential modifiable targets that can simultaneously decrease the risk of multiple morbidities.

Supplementary Tables

Table 4.7: Baseline Characteristics by Survival to Age 90 Status (N=1,951)

	Did not Survive to Age 90 (n=929)	Survived to Age 90 (n=1,022)	p
Race/Ethnicity, n (%)			0.207
Black or African American	179 (19.4)	165 (16.3)	
Hispanic/Latina	78 (8.5)	76 (7.5)	
White	637 (69.1)	738 (72.7)	
Other	28 (3.0)	36 (3.5)	
Education, n (%)			0.047
HS/GED or Less	281 (30.5)	263 (25.8)	
Some College	369 (40.0)	415 (40.8)	
College Grad or More	272 (29.5)	240 (33.4)	
Walking Frequency, n (%)			0.001
Rarely or Never	204 (22.2)	171 (16.8)	
1-3 times/month	144 (15.7)	137 (13.5)	
1 time/week	109 (11.9)	96 (9.4)	
2-3 times/week	231 (25.2)	301 (29.6)	
4-6 times/week	163 (17.8)	222 (21.9)	
7+ times/week	67 (7.3)	89 (8.8)	
BMI Category, n (%)			<0.001
Underweight	9 (1.0)	10 (1.0)	
Normal	251 (27.2)	308 (30.3)	
Overweight	296 (32.0)	389 (38.3)	
Obese	368 (39.8)	309 (30.4)	
Alcohol Consumption, n (%)			0.014
Non-drinker	121 (13.2)	142 (14.0)	
Past drinker	224 (24.5)	196 (19.3)	
<1 drink/month	134 (14.6)	120 (11.8)	
<1 drink/week	167 (18.3)	221 (21.8)	
1-<7 drinks/week	173 (18.9)	223 (22.0)	
7+ drinks/week	96 (10.5)	111 (11.0)	
Smoking Pack-Years, n (%)			<0.001
Never Smoker	425 (47.6)	603 (61.0)	
<5	96 (10.8)	130 (13.2)	
5-<20	114 (12.8)	121 (12.2)	
20+	258 (28.9)	134 (13.6)	

Table 4.8: Baseline Characteristics by Survival to Age 90 Status (N=1,951)

Age-Related Condition			
Alzheimer's	99 (10.7)	139 (13.6)	0.055
Arthritis	720 (77.5)	821 (80.3)	0.140
Broken Hip	63 (6.8)	83 (8.1)	0.300
Cancer	371 (39.9)	256 (25.0)	<0.001
Cardiovascular Disease	397 (42.7)	273 (26.7)	<0.001
Depression	76 (8.2)	74 (7.2)	0.488
Diabetes	249 (26.8)	185 (18.1)	<0.001
Emphysema	73 (7.9)	44 (4.3)	0.001
Frequent Faller	425 (45.7)	387 (37.9)	<0.001
Sensory Impairment	244 (26.3)	394 (38.6)	<0.001
Stroke	127 (13.7)	67 (6.6)	<0.001
Urinary Incontinence	127 (12.7)	67 (6.6)	<0.001
Age, mean (SD)	70.2 (3.4)	71.6 (3.4)	<0.001
Physical Function Score, mean (SD)	69.5 (24.6)	77.0 (22.2)	<0.001
Baseline Multimorbidity Count	1.3 (1.0)	1.1 (0.9)	<0.001
Follow-up Multimorbidity Count	2.6 (1.5)	2.4 (1.5)	0.001
Total Multimorbidity Count	3.2 (1.5)	2.8 (1.5)	<0.001
Multimorbidity Score	31.5 (16.5)	24.9 (16.5)	<0.001
AgeAccelHorvath	0.1 (5.3)	-0.4 (5.4)	<0.001
AgeAccelHannum	0.4 (5.1)	-0.7 (5.1)	<0.001
AgeAccelPheno	1.1 (7.0)	-1.0 (6.8)	<0.001
AgeAccelGrim	0.8 (4.3)	-0.9 (3.5)	<0.001

Note: GED=general educational development; BMI=body mass index; kg=kilograms; m=meters

Note: AgeAccel measures are the residual between chronological age and epigenetic age as measured by each individual epigenetic clock.

[a]Conditions include cardiovascular disease, cancer, cognitive impairment, depression, osteoarthritis, history of falls, chronic obstructive pulmonary disease, hypertension, diabetes, hip fracture and cerebrovascular disease.

Table 4.9: Unadjusted and Baseline Multimorbidity Adjusted Association of Epigenetic Age Acceleration with Multimorbidity Count and Multimorbidity Score Among Women Who Survived to Age 90 (N=1,022)

Unadjusted	**Multimorbidity Count**		**Multimorbidity Score**	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	1.02 (0.98-1.05)	0.460	1.02 (0.98-1.06)	0.219
AgeAccelHannum	1.00 (0.96-1.04)	0.916	0.99 (0.95-1.03)	0.773
AgeAccelPheno	1.05 (1.01-1.10)	0.025	1.05 (1.01-1.10)	0.019
AgeAccelGrim	1.03 (0.98-1.07)	0.201	1.02 (0.98-1.07)	0.217
Baseline Multimorbidity Count Adjusted[b]	**Multimorbidity Count**		**Multimorbidity Score**	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	1.03 (0.99-1.08)	0.147	1.02 (0.97-1.05)	0.459
AgeAccelHannum	1.01 (0.96-1.06)	0.691	0.99 (0.95-1.03)	0.599
AgeAccelPheno	1.07 (1.01-1.12)	0.016	1.07 (1.02-1.13)	0.005
AgeAccelGrim	1.04 (0.97-1.08)	0.500	0.99 (0.95-1.04)	0.673

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=5.1), AgeAccelHannum (sd=5.3), AgeAccelPheno (sd=7.0) and AgeAccelGrim (sd=3.9).

[b]Model included all covariates from the fully adjusted model and additionally adjusted for baseline multimorbidity count.

Table 4.10: Unadjusted and Baseline Multimorbidity Adjusted Association of Epigenetic Age Acceleration with Multimorbidity Count and Multimorbidity Score Among All Women Eligible to Survive to Age 90 (N=1,951)

Unadjusted	**Multimorbidity Count**		**Multimorbidity Score**	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	0.99 (0.96-1.02)	0.434	0.99 (0.97-1.02)	0.690
AgeAccelHannum	0.98 (0.95-1.01)	0.191	0.99 (0.96-1.02)	0.483
AgeAccelPheno	1.04 (1.01-1.07)	0.014	1.06 (1.03-1.09)	<0.001
AgeAccelGrim	1.04 (1.01-1.06)	0.012	1.04 (1.02-1.07)	0.002
Baseline Multimorbidity Count Adjusted[b]	**Multimorbidity Count**		**Multimorbidity Score**	
	RR (95% CI)[a]	p	RR (95% CI)[a]	p
AgeAccelHorvath	0.99 (0.96-1.02)	0.450	1.00 (0.98-1.03)	0.818
AgeAccelHannum	0.97 (0.94-1.00)	0.068	0.98 (0.96-1.01)	0.243
AgeAccelPheno	1.02 (0.99-1.06)	0.204	1.05 (1.01-1.08)	0.002
AgeAccelGrim	1.00 (0.97-1.04)	0.903	0.98 (0.95-1.00)	0.115

Note: All models were adjusted for the following baseline covariates: blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran), age, race/ethnicity, education, walking frequency, BMI, alcohol consumption, pack-years smoking, broken hip, emphysema, arthritis, depression, urinary incontinency and visual/auditory sensory impairment) and RAND physical functioning score.

[a]Results are presented for one standard deviation increase in DNAmAge measure: AgeAccelHorvath (sd=5.1), AgeAccelHannum (sd=5.3), AgeAccelPheno (sd=7.0) and AgeAccelGrim (sd=3.9).

[b]Model included all covariates from the fully adjusted model and additionally adjusted for baseline multimorbidity count.

Chapter 4, in full, is currently being prepared for submission for publication of the material. Jain, Purva; Binder, Alex; Chen, Brian; Parada, Humberto; Gallo, Linda; Alcaraz, John; Horvath, Steve; Bhatti, Parveen; Whitsel, Eric; Baccarelli, Andrea; Hou, Lifang; Stewart, Jay; Li, Yun; Jordahl, Kristina; LaCroix, Andrea. The dissertation author was the primary investigator and author of this paper.

5. Discussion

5.1. Summary of dissertation research

In the last decade there has been increased interest in the potential for DNA methylation to serve as a measure to define an individual's biological age, with the goal of developing interventions to slow biological aging. Epigenetic clocks are not only able to predict chronological age, but more importantly they are able to predict aging outcomes more strongly than using just chronological age.[63] Previous studies have shown first generation clocks (Hannum, Horvath) to be associated with chronological age and the second generation clocks (PhenoAge and GrimAge) to be associated with outcomes such as worse physical and cognitive functioning along with several chronic conditions.[21,23,24,32,43,44,108] Previous studies that examined the relationship with DNA methylation and epigenetic age acceleration with physical and cognitive functioning as well as multimorbidity have primarily been cross-sectional and have not focused on the oldest-old.

The purpose of this dissertation was to examine the role of DNA methylation on healthy longevity among older women. This research adds to the current body of literature by examining these relationships within an older, racially-ethnically diverse group of women using a prospective study design. The first aim of this dissertation assessed the associations between four DNA methylation clocks that measure epigenetic age acceleration and exceptional longevity, defined as survival to age 90 with intact mobility or survival to age 90 with intact mobility and and intact cognitive function. This study showed that increased EAA as measured by the AgeAccelHorvath, AgeAccelHannum, AgeAccelPheno and AgeAccelGrim clocks resulted in decreased odds of survival to age 90 with intact physical functioning. The results were strongest in the AgeAccelHannum, AgeAccelPheno and AgeAccelGrim measures followed by

AgeAccelHorvath. The newer generation PhenoAge and GrimAge clocks were also predictive of survival to age 90 without intact physical function, but not the older generation clocks. The results remained similar when the exceptional longevity outcome additionally included intact cognitive functioning.

The second aim of this dissertation utilized an epigenome wide association study to identify specific CpG positions and regions that are associated with exceptional longevity. The primary analysis that used an epigenome-wide-association study across 481,047 CpG sites comparing women who survived to age 90 with and without intact physical function to women who did not survive to age 90 identified 38 and 103 significantly differentially methylated CpG sites, respectively. In the secondary analysis that tested these 139 sites comparing women who survived to age 90 with intact physical and cognitive functioning and women who survived to age 90 with at least either intact physical or cognitive functioning compared to women who did not survive to age 90 there were 17 and 64 significantly differentially methylated CpG sites, respectively. There were also 83 DMRs comparing women who survived to age 90 with intact physical function and 127 DMRs comparing women who survived to age 90 without physical function to women that did not survive to age 90.

The final aim of this study evaluated the associations between the four DNA methylation clocks and multiple morbidity among women when they reach 90 years of age. This study showed that increased EAA as measured by AgeAccelPheno resulted in an increased risk of acquiring additional comorbidities and more lethal comorbidities among women who survived to age 90. A similar association was observed among all women eligible to survive to age 90. EAA measured by AgeAccelHorvath, AgeAccelHannum and AgeAccelGrim were not associated with either multimorbidity count or multimorbidity score over time.

This dissertation advances the field of the epigenetics of healthy aging. The findings in Chapter 2 provides additional evidence of the predictive capability of epigenetic clocks with physical and cognitive functioning over time and expand the findings to a racial and ethnically diverse group of older women. Chapter 3 identifies several CpG positions and regions across the genome that have significantly differential methylation when comparing women who are long-lived with intact physical functioning and women who are long-lived without intact physical functioning to women who are not long-lived. Chapter 4 provided evidence of the predictive capability of epigenetic age acceleration, as measured by the Pheno clock, of both multimorbidity count and multimorbidity score, while expanding the findings to a racial and ethnically diverse group of older women.

5.2. The importance of understanding the relationship between DNA methylation and healthy longevity

Epigenetic factors such as DNA methylation influence the regulation of gene expression without modification of the DNA sequence. DNA methylation is the connection between the intrinsic and extrinsic environments and the decreases in DNA methylation as one ages have been implicated in the pathogenic process of age-related diseases.[109] Identifying specific CpG sites and regions across the genome associated with specific age-related diseases may help to understand disease etiology.

There have also been public health interventions with the goal of decreasing epigenetic age that have shown changes in DNA methylation status at specific CpG sites of interest, a potential area to decrease disease and increase longevity through overall decreases in epigenetic age. Studies have shown that caloric restriction can increase the DNA methylation level within the promotor of a tumor suppressor and aging-related gene, attenuate the expression of a

molecule associated with decreased brain function and increase the promotor methylation of a DNA mismatch repair gene.[110,111] There are also additional interventions such as dietary supplementation and chemical drugs that have been associated with reversing age-associated changes in DNA methylation.[109]

5.3. Recommendations for future work in studies of DNA methylation and healthy longevity

First, there is a need for prospective studies in both the creation of epigenetic age acceleration measures as well as in studies examining their association with longevity and age-related phenotypes. Those who age faster tend to have higher mortality rates and thus create a selection bias due to non-random sampling of the cohort within cross-sectional analyses that may lend to the inclusion of more non-causative loci.[66] Although this is not a concern when the goal is to use epigenetic age acceleration to predict risk, conduct risk stratification or define an outcome of interest, this would be of concern if trying to pinpoint causal mechanisms associated with an outcome of interest. The use of longitudinal designs also allows for the exploration into how the rate of biological aging may differ between individuals and what risk factors are most strongly associated with changes in DNA methylation and the rate of biological aging. Also, when testing the relationship of specific DNA methylation and epigenetic clocks with outcomes of interest, without a prospective design it is not possible to rule out reverse causation.

Second, it is important to develop and test epigenetic age acceleration measures that are assessed using the age and racial/ethnic groups of interest. The loci that are developed using a broad age range of adults tend to degrade in their predictive capability after mid-life and may not serve to be as useful when one is interested in examining relationships among the oldest-old.[66] Although the epigenetic clocks were developed using diverse, multiethnic samples, and may show less racial/ethnic bias when compared to genetic studies, it is vital to test relationships of

interest within racial and ethnically diverse populations and examine group-specific associations when possible.

Finally, to develop a comprehensive understanding of epigenetic based biological markers in exceptional longevity it is important to consider the role of blood-based DNA methylation in the context of other factors. This may include measuring DNA methylation in other tissues to understand if the changes in DNA methylation associated with age-related phenotypes of interest are consistent in other sites in the body.

5.4. Concluding remarks

In conclusion, this dissertation offers an epidemiological examination of the relationship between DNA methylation and healthy longevity by utilizing a prospective design and a racial/ethnically diverse sample of long-lived women. This research advances the field by demonstrating the relationship between certain measures of epigenetic age acceleration and healthy longevity as well as multimorbidity, identifying specific DNA methylation positions and regions that differentiate women who experience healthy longevity and providing recommendations to guide future research.

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