Epigenetic Biomarkers of Long-term Psychosocial Stress and their Relationships with Cardiometabolic Risk Factors

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Epidemiological Sciences)
in the University of Michigan
2024

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Dedication

This dissertation is dedicated to my parents Steve and Margo, my brothers Mark and Christian, my husband Eddie, and my daughter Brooklyn.

Acknowledgements

I would like to extend my deepest gratitude to the individuals who have supported me throughout my academic journey. First, I would like to thank my dissertation chair, Jennifer Smith, for her support, mentorship, and guidance throughout my time at Michigan. Over the past three years, you have been a constant pillar in my life, motivating me to think more critically about my research and encouraging me to pursue work that I find truly fulfilling. Thanks to your constant guidance and wisdom, I have become both a better researcher, and a more well-rounded person. Without you, this dissertation would not be possible.

I would also like to thank the other members of my dissertation committee for their tremendous support and expertise. I thank Belinda Needham for all of her time and patience, helping me think critically about the many social and psychosocial aspects of my dissertation and providing me with great resources to learn more about the field. I would also like to thank Jessica Faul for helping me navigate the intricacies of the Health and Retirement Study, as well as offering thoughtful feedback that greatly improved my dissertation. And finally, I would like to thank Xiang Zhou, who helped tremendously with understanding and implementing complex statistical methods that greatly enhanced the rigor of my analyses.

I am eternally grateful to the participants of the Health and Retirement Study for their renewed participation, which has made this dissertation possible. I would also like to thank the faculty and staff of the Department of Epidemiology, as well as the Center for Social Epidemiology and Population Health (CESPH), for providing me with a supportive and positive

learning environment to grow as a researcher. I am extremely fortunate to be a part of the 2021 PhD Cohort, made up of a wonderful group of students-turned-friends. I want to specifically thank Morgan Byrd, Jillian Baker, and Jessie Ibiebele for being the best study group and a constant source of encouragement and positivity in my life over the past three years.

I also want to thank members of the Genomics Research Collaborative lab group, both past and present, for their continued feedback, encouragement, and friendship. These members include, but are not limited to, Lisha Lin, Dima Chaar, Yi Zhe Wang, Farah Ammous, Zheng Li, Hasan Abu-Amara, and Scott Ratliff. Each and every one of you has made my time at Michigan more enjoyable, and I will miss you all dearly.

Finally, I would like to thank my family for the tremendous support they have given me throughout the years. To my parents, Steve and Margo, I cannot put into words how grateful I am for all the sacrifices you have made to allow me to pursue my dreams. Your unconditional love and support means the world to me, and you are the reason for all of my success and happiness. To my brothers, Mark and Chris, thank you for all of your love and support throughout our lifetimes. Even as your older sister, you have taught me so much and shaped who I am as a person today. I want to give a special thanks to my husband, Eddie. I would not have been able to succeed in the doctoral program if it weren't for your endless support and sacrifices. I love you beyond words, and this dissertation is just as much yours as it is mine. Finally, to my daughter Brooklyn. I want this dissertation to serve as a constant reminder that you have the ability to anything in the world you put your mind to. Thank you for giving me the motivation I needed to successfully finish this dissertation. I love you more than you'll ever know.

Preface

Chapter II (Epigenome-wide Association Study of Long-term Psychosocial Stress in Older Adults) has been published in Epigenetics. The full list of authors is: Lauren A. Opsasnick, Wei Zao, Lauren L. Schmitz, Scott M. Ratliff, Jessica D. Faul, Xiang Zhou, Belinda L. Needham, and Jennifer A. Smith.

https://doi.org/10.1080/15592294.2024.2323907

Table of Contents

Dedication ii

Acknowledgements iii

Preface v

List of Tables x

List of Figures xiii

List of Abbreviations xv

Abstract xvii

Chapter 1 Introduction 1

1.1 Overview 1

1.2 Background 3

1.2.1 Psychosocial Stress 3

1.2.2 Health Consequences of Psychosocial Stress 4

1.2.3 Cardiovascular Disease and Cardiometabolic Risk Factors 6

1.2.4 The Role of Epigenetics in Psychosocial Stress and Cardiovascular Health 9

1.3 Health and Retirement Study (HRS) 9

1.3.1 Study Population 10

1.3.2 Data Collection 11

1.3.3 Epigenetic Data 12

1.4 Specific Aims and Hypotheses 13

1.4.1 Aim 1 13

1.4.2 Aim 2 14

1.4.3 Aim 3 14

1.5 References 16

Chapter 2 Epigenome-Wide Association Study of Long-term Psychosocial Stress in Older Adults 24

2.1 Abstract 24

2.2 Introduction 25

2.3 Materials and Methods 27

2.3.1 Sample 27

2.3.2 Measures 28

2.3.3 Statistical Analysis 32

2.4 Results 34

2.4.1 Descriptive Statistics 34

2.4.2 Epigenome-wide Association Analysis 35

2.4.3 Mediation Analysis 36

2.4.4 Genomic Feature Enrichment Analysis 38

2.5 Discussion 38

2.6 Conclusion 44

2.7 References 45

2.8 Tables 52

2.9 Figures 54

2.10 Supplementary Material 56

Chapter 3 Epigenome-Wide Mediation Analysis of the Relationship between Psychosocial Stress and Cardiometabolic Risk Factors in the Health and Retirement Study (HRS) 66

3.1 Abstract 66

3.2 Introduction 67

3.3 Materials and Methods 68

3.3.1 Sample 68
3.3.2 Measures 70
3.3.3 Statistical Analysis 73
3.4 Results 78
3.4.1 Descriptive Statistics 78
3.4.2 Associations between Psychosocial Stress and Cardiometabolic Risk Factors 79
3.4.3 Mediation Analysis 79
3.4.4 Overlap of CpG Mediators among Cardiometabolic Risk Factors 82
3.4.5 Genomic Feature Enrichment Analysis 83
3.4.6 Gene-set Enrichment Analysis 83
3.5 Discussion 84
3.6 Conclusion 90
3.7 References 91
3.8 Tables 100
3.9 Figures 104
3.10 Supplementary Material 106
Chapter 4 Depressive Symptoms Partially Mediate the Relationship between Psychosocial Factors and Epigenetic Age Acceleration in a Multi-Racial/Ethnic Sample of Older Adults 132
4.1 Abstract 132
4.2 Introduction 133
4.3 Materials and Methods 135
4.3.1 Sample 135
4.3.2 Measures 136
4.3.3 Statistical Analysis 140
4.4 Results 143
4.4.1 Descriptive Statistics 143

4.4.2 Correlation among Chronological Age, Epigenetic Clocks, and EAA 144
4.4.3 Associations between Psychosocial Factors/Depressive Symptoms and EAA 144
4.4.4 Effect Modification by Sex and Educational Attainment 145
4.4.5 Mediation Analysis 146
4.5 Discussion 148
4.6 Conclusion 152
4.7 References 154
4.8 Tables 161
4.9 Figures 165
4.10 Supplementary Material 167
Chapter 5 Conclusion 179
5.1 Summary and Implications of Main Findings 179
5.1.1 Summary and Implications of Chapter 2 Findings 179
5.1.2 Summary and Implications of Chapter 3 Findings 181
5.1.3 Summary and Implications of Chapter 4 Findings 183
5.2 Strengths and Limitations 186
5.3 Future Directions 189
5.4 Conclusion 192
5.5 References 194

List of Tables

Table 2-1: Characteristics of Health and Retirement Study analytic sample (N=2,689)..... 52

Table 2-2: Significant CpG sites from the epigenome-wide association study (EWAS) of cumulative psychosocial stress (Model 1: FDR q<0.10). 53

Table S2-1: Psychosocial stress measures included in the 6 stress domains. 57

Table S2-2: Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018). 58

Table S2-3: Pearson correlation coefficients between the 6 psychosocial stress domains. 59

Table S2-4: Association between adult psychosocial stress and the 9 CpG sites that were significant in cumulative psychosocial stress EWAS (Model 1; FDR q <0.10)..... 61

Table S2-5: Association between the six stress domains and the and the 9 CpG sites that were significant in cumulative psychosocial stress EWAS (Model 1; FDR q <0.10)..... 62

Table S2-6: Total variability of CpG sites identified in Model 1 (FDR q<0.10) explained by cumulative psychosocial stress. 63

Table S2-7: Mediation of health behaviors on the relationship between cumulative psychosocial stress and CpGs identified in Model 1 (FDR q<0.10)..... 64

Table S2-8: Mediation of health behaviors measured in 2014 on the relationship between cumulative psychosocial stress and CpGs identified in Model 1 (FDR q<0.10) (N=2614). 65

Table 3-1: Characteristics of the Health and Retirement Study analytic sample (N=2,668)..... 100

Table 3-2: Total effect of cumulative psychosocial stress on cardiometabolic risk factors (N=2,668). 101

Table 3-3: High-dimensional epigenetic mediation results of the association between psychosocial stress and cardiometabolic risk factors..... 102

Table 3-4: Genomic Feature Enrichment of Mediating CpG sites identified in Epigenome-wide Mediation Analysis (Model 1). 103

Table S3-1: Psychosocial stress measures and items included in the 6 stress domains. 106

Table S3-2: Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018). 107

Table S3-3: Total effect of cumulative psychosocial stress on cardiometabolic risk factors restricted to fasting participants (N=1,779). 108

Table S3-4: CpG mediators of the association between psychosocial stress and BMI identified in HDMT (N=2537). 113

Table S3-5: Mediation analysis using top 10 PCs from the 50 CpG mediators of the association between psychosocial stress and BMI identified in HDMT (Model 1). 115

Table S3-6: De-biased LASSO based high-dimensional mediation analysis on the 50 CpG mediators of the association between psychosocial stress and BMI identified in HDMT (Model 1). 116

Table S3-7: CpG mediators of the association between psychosocial stress and WC identified in HDMT (N=2346). 117

Table S3-8: Mediation analysis using top 10 PCs from the 46 CpG mediators of the association between psychosocial stress and WC identified in HDMT (Model 1). 118

Table S3-9: De-biased LASSO based high-dimensional mediation analysis on the 46 CpG mediators of the association between psychosocial stress and WC identified in HDMT (Model 1). 119

Table S3-10: CpG mediators of the association between psychosocial stress and HDL-C in HDMT (N=2668). 120

Table S3-11: Mediation analysis using top 5 PCs from the 7 CpG mediators of the association between psychosocial stress and HDL-C identified in HDMT (Model 1). 121

Table S3-12: De-biased LASSO based high-dimensional mediation analysis on the 7 CpG mediators of the association between psychosocial stress and HDL-C identified in HDMT (Model 1). 122

Table S3-13: CpG mediators of the association between psychosocial stress and CRP in HDMT (N=2666). 123

Table S3-14: Mediation analysis using top 10 PCs from the 12 CpG mediators of the association between psychosocial stress and CRP identified in HDMT (Model 1). 124

Table S3-15: De-biased LASSO based mediation analysis on the 12 CpG mediators of the association between psychosocial stress and CRP identified in HDMT (Model 1). 125

Table S3-16: Overlap of CpG mediators identified in HDMT (Model 1) across the association between stress and cardiometabolic risk factors. 126

Table 4-1: Characteristics of Health and Retirement Study analytic sample (N=2,681)..... 161

Table 4-2: Associations between psychosocial factors/depressive symptoms and epigenetic age acceleration. 162

Table 4-3: Two-way interaction between psychosocial factors/depressive symptoms and demographic characteristics on epigenetic age acceleration (Model 1; N=2,681). 163

Table 4-4: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and epigenetic age acceleration (Model 1; N=2,681). 164

Table S4-1: Eleven-item scale of loneliness derived from the 20-item Revised UCLA Loneliness Scale..... 167

Table S4-2: Eight-item scale of depressive symptoms derived from the Center for Epidemiologic Studies Depression Scale (CES-D). 168

Table S4-3: Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018). 169

Table S4-4: Pearson correlation among epigenetic clocks and chronological age. 170

Table S4-5: Pearson correlation among epigenetic age acceleration measures. 171

Table S4-6: Two-way interaction between psychosocial factors/depressive symptoms and demographic characteristics on epigenetic age acceleration (Model 2; N=2,656). 172

Table S4-7: Two-way interaction between psychosocial factors/depressive symptoms and educational attainment (no degree vs. HS degree) on epigenetic age acceleration (Model 1; N=2,681). 173

Table S4-8: Total variability in epigenetic age acceleration explained by psychosocial factors (FDR q<0.05). 174

Table S4-9: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and epigenetic age acceleration (Model 2; N=2,656) 175

Table S4-10: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and DunedinPACE in females. 176

Table S4-11: Mediation by depressive symptoms of the relationships between loneliness and epigenetic age acceleration in individuals without a college degree (Model 1; N=1,971). 177

Table S4-12: Mediation by depressive symptoms of the relationships between loneliness and epigenetic age acceleration after removing one item from CES-D measure. 178

List of Figures

Figure 1-1: Conceptual framework of dissertation aims, describing the interplay of psychosocial and epigenetic factors underlying cardiometabolic risk. 15

Figure 2-1: Manhattan plot of the EWAS for cumulative psychosocial stress in HRS. The red horizontal line indicates the epigenome-wide significance threshold (FDR q<0.10). 54

Figure 2-2: Quantile-quantile plot of the EWAS for cumulative psychosocial stress in HRS (inflation factor λ=1.09). 55

Figure S2-1: Flow diagram detailing participant inclusion criteria in primary analysis. 56

Figure S2-2: Heat map of Spearman correlations between health behaviors and BMI. 60

Figure 3-1: Flow diagram detailing participant inclusion criteria for epigenome-wide mediation analyses. 104

Figure 3-2: CpGs identified in HDMT that mediate the relationship between stress and multiple cardiometabolic risk factors. 105

Figure S3-1: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and BMI using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and BMI (inflation factor λ=1.03). 109

Figure S3-2: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and WC using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and WC (inflation factor λ=1.25). 110

Figure S3-3: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and HDL-C using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and HDL-C (inflation factor λ=0.92). 111

Figure S3-4: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and CRP using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR

q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and CRP (inflation factor λ=0.93). 112

Figure S3-5: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and BMI identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and BMI identified in high-dimensional mediation testing (Model 1). 128

Figure S3-6: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and WC identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and WC identified in high-dimensional mediation testing (Model 1). 129

Figure S3-7: Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and HDL-C identified in high-dimensional mediation testing (Model 1). 130

Figure S3-8: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and CRP identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and CRP identified in high-dimensional mediation testing (Model 1). 131

Figure 4-1: Plots of significant interactions between psychosocial factors and sex on DunedinPACE (p<0.05). Predicted DunedinPACE values for (A) cumulative psychosocial stress z-scores and (B) loneliness z-scores by sex are shown with 95% confidence intervals... 165

Figure 4-2: Plots of significant interactions between loneliness and educational attainment on epigenetic age acceleration measures (p<0.05). Predicted (A) PhenoAA, (B) GrimAA, and (C) DunedinPACE values for loneliness z-scores by educational attainment (no college degree vs. college degree or higher) are shown with 95% confidence intervals. 166

List of Abbreviations

ACH	Adrenocorticotropic hormone
AHEAD	Asset and Health Dynamics Among the Oldest Old
BASE-II	Berlin Aging Study II
BMI	Body mass index
CES-D	Center for Epidemiological Studies Depression Scale
CGI	CpG island
CHD	Coronary heart disease
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHS	DNase I hypersensitivity sites
EAA	Epigenetic age acceleration
eQTM	Expression quantitative trait methylation
EWAS	Epigenome-wide association study
FDR	False discovery rate
FHS	Framingham Heart Study
GC	Glucocorticoids
GLM	Generalized linear model
GO	Gene Ontology
HbA1c	Hemoglobin A1C
HDL-C	High-density lipoprotein cholesterol
HDMT	High dimensional multiple testing
HPA	Hypothalamic-pituitary-adrenal
HRS	Health and Retirement Study
IQR	Interquartile range
KEGG	Kyoto Encyclopedia of Genes and Genomes
KHB	Karlson-Holm-Breen
LBQ	Leave Behind Questionnaire
LDL-C	Low-density lipoprotein cholesterol
MDD	Major depressive disorder
MESA	Multi-Ethnic Study of Atherosclerosis Study
MI	Myocardial infarction
MIDUS	Midlife in the United States

NHANES	National Health and Nutrition Examination Survey
PC	Principal components
PTSD	Post-traumatic stress disorder
RPD	Reasons for perceived discrimination
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Structural equation modelling
SLE	Stressful life event
T2D	Type 2 diabetes
TC	Total cholesterol
TE	Total effect
TG	Triglycerides
VBS	Venous Blood Study
WBC	White blood cell
WC	Waist circumference
WHI	Women's Health Initiative

Abstract

Long-term exposure to psychosocial stress has been linked to an array of negative health outcomes, such as cardiovascular disease and its cardiometabolic risk factors, which include hypertension, dyslipidemia, obesity, and diabetes. Recently, epidemiological studies have begun to assess the complex relationship between psychosocial stress and cardiometabolic risk, as well as the impact of epigenomic, sociodemographic, and biological factors on this association. Nevertheless, the underlying mechanisms through which epigenomic biomarkers, including individual DNA methylation sites and epigenetic clocks, influence the effect of stress on cardiometabolic risk are not fully understood. Thus, this dissertation aims to characterize the biological mechanisms through which long-term psychosocial stress impacts cardiometabolic risk factors in a multi-ancestry population of older adults from the Health and Retirement Study (HRS).

In Aim 1, we conducted an epigenome-wide association study (EWAS) to investigate the relationship between long-term psychosocial stress and DNA methylation. For identified CpG sites, we performed formal mediation testing to examine whether smoking, alcohol use, physical activity, and body mass index (BMI) mediated the relationship between stress and DNA methylation. Nine CpG sites were associated with psychosocial stress (all $p<9E-07$; FDR $q<0.10$). Additionally, health behaviors and/or BMI mediated 9.4% to 21.8% of the relationship between stress and DNA methylation at eight of the nine CpGs. Several of the identified CpGs

were in or near genes associated with cardiometabolic traits, psychosocial disorders, inflammation, and smoking.

In Aim 2, we conducted epigenome-wide mediation analyses using the high-dimensional multiple testing (HDMT) method to examine whether DNA methylation mediates the relationship between psychosocial stress and cardiometabolic risk factors. We found that DNA methylation partially mediated the associations between psychosocial stress and BMI, waist circumference (WC), high-density lipoprotein cholesterol (HDL-C) and C-reactive protein (CRP), with the overall mediation effects across probes explaining between 35.8% and 46.3% of these associations. A subset of the mediating CpG sites were associated with the expression of genes enriched in pathways related to cytokine binding and receptor activity, chemotaxis, and neuron development.

Finally, in Aim 3, we examined whether psychosocial factors, including loneliness and psychosocial stress, and depressive symptoms were associated with epigenetic age acceleration. For identified associations, we performed formal mediation testing to characterize the role of depressive symptoms on the relationship between psychosocial factors and epigenetic aging. We discovered that psychosocial stress, loneliness, and depressive symptoms were each associated with at least one measure of epigenetic age acceleration (FDR $q<0.05$). Further, sex and educational attainment were found to modify the effects of both stress and loneliness on epigenetic aging, with females and individuals without a college degree appearing more sensitive to these effects. Depressive symptoms partially mediated the relationships between psychosocial factors and epigenetic age acceleration, with the mediation effects explaining between 24.0% and 39.9% of these associations.

Together, these studies help to enhance our understanding of the biological pathways through which long-term psychosocial stress contributes to cardiometabolic disease risk in a diverse population of older adults. Further, results from this dissertation may have a significant public health impact by highlighting the importance of individual- and community-level interventions to manage the root cause of stress before it results in complex physical and mental health conditions.

Chapter 1 Introduction

1.1 Overview

Over the past several decades, stress levels in the United States have been steadily rising. Compared to the 1990s, individuals today have reported higher levels of day-to-day stress and lower overall well-being [1]. Between 1983 and 2009, perceived stress has increased between 10 to 30 percent across all demographic groups, with highest rates of stress in women, younger adults, and those of lower socioeconomic status [2]. Stress has become such a major health concern that the World Health Organization has dubbed it "the health epidemic of the 21st century" [3]. Several factors likely contribute to this increase in stress, including family and relationship dynamics, financial concerns, work-related strain, and health problems, among others [4].

The effects of long-term stress can be detrimental to both physical and mental health. Research has linked stress to negative health outcomes, particularly cardiovascular disease (CVD) [5] and its cardiometabolic risk factors, such as hypertension [6], dyslipidemia [7], diabetes [8], and obesity [9]. Although these associations have been well-studied, less is known about the mechanisms through which they operate, particularly the epigenome. Briefly, the epigenome is comprised of biochemical modifications to DNA that regulate gene expression without altering the underlying genetic sequence [10]. DNA methylation, which is the most common epigenetic mechanism, occurs primarily on cytosines that precede a guanine nucleotide, separated by a phosphodiester bond (CpG sites) [11]. The epigenome is responsive to certain environmental stimuli and can be altered throughout the life course. Thus, epigenetic biomarkers,

including DNA methylation at individual CpG sites and epigenetic clocks, have been well-studied with respect to health outcomes. Epigenetic clocks, which are trained on DNA methylation patterns across the genome, predict chronological age and other age-related phenotypes and are used to study healthy aging across the life course [12].

Epigenetic biomarkers have well-established relationships with the development of cardiometabolic risk factors. Not only have epigenome-wide association studies (EWAS) identified relationships between individual CpGs and blood pressure [13], body mass index (BMI) [14], and blood lipid levels [15], but several epigenetic clocks have shown associations with these same risk factors [16-18]. More limited research has been conducted on the epigenetic impact of stress, with only a few studies examining the associations between epigenetic biomarkers and stress measured from multiple domains (i.e., relationship stress, financial strain, discrimination) [19-23]. Nonetheless, these studies provide preliminary evidence that stress may influence downstream health outcomes through epigenomic changes. Thus, epigenetic biomarkers may be a potential link from stress to cardiometabolic risk.

This dissertation aims to characterize the biological mechanisms through which long-term psychosocial stress impacts cardiometabolic risk factors in a multi-ancestry population of older adults from the Health and Retirement Study (HRS). First, we will perform an epigenome-wide association study to investigate the relationship between long-term psychosocial stress and DNA methylation (Aim 1) Next, we will examine whether DNA methylation mediates the relationship between psychosocial stress and cardiometabolic risk factors (Aim 2). Finally, we will evaluate the associations between psychosocial factors and epigenetic age acceleration and assess the mediating effect of depressive symptoms on those relationships (Aim 3). Together, these studies will enhance our understanding of the epigenetic mechanisms linking long-term

psychosocial stress and cardiometabolic disease risk, as well as contribute to the ongoing public health research regarding stress reduction through individual- and community- level interventions.

1.2 Background

1.2.1 Psychosocial Stress

Psychosocial stress is broadly defined as the perception of threat that results in discomfort and emotional tension [24]. Many factors influence susceptibility to stress, including genetic predisposition, coping style, personality, and social support. Thus, the extent to which an individual perceives stress may depend on their attitude toward a particular situation and the resources they have available [25]. Psychosocial stress can be triggered by a multitude of events, including difficulty in relationships, financial uncertainty, or adverse life events such as job loss, discrimination, or health issues [26]. Inherently, not all stress is considered harmful. Positive stress, termed eustress, is beneficial in terms of increasing motivation, forcing individuals to adapt, and enhancing emotional well-being. Stress becomes negative when it exceeds an individual's ability to cope, and the body can no longer tolerate the stress, leading to physical and mental health problems [27].

From a biological perspective, when a stressor is perceived and interpreted, the stress response system, also known as the hypothalamic-pituitary-adrenal (HPA) axis, is activated. The hypothalamus area of the brain sends a chemical message to the pituitary glands, which triggers the release of adrenocorticotropic hormone (ACTH). In turn, ACTH stimulates the adrenal glands to release cortisol, a steroid hormone that binds glucocorticoid receptors [28]. Because glucocorticoid receptors are found in nearly every tissue of the body, once cortisol attaches to its receptors, it interacts with several organ systems, including the nervous, immune, respiratory,

and cardiovascular systems [29]. After the stressor has been resolved, built-in regulators help return the psychological systems to usual levels, restoring the body to homeostasis. However, persistent stress can lead to long-term activation of the stress response system, resulting in elevated levels of cortisol and other stress hormones, which may disrupt the development and maintenance of brain architecture and other organ systems, increasing the risk for stress-related disease [30].

1.2.2 Health Consequences of Psychosocial Stress

Elevated levels of psychosocial stress over time, particularly in older or unhealthy individuals, have been linked to numerous health consequences. Studies have shown that psychosocial stress is strongly associated with CVD and its cardiometabolic risk factors, including hypertension [6], dyslipidemia [7], diabetes [8], and obesity [9]. In a cohort of middle-aged and older adults living in 21 low-, middle-, and high-income countries without a prior CVD diagnosis, high stress was associated with increased risk of CVD, coronary heart disease (CHD), and stroke events [31]. Similarly, in the Jackson Heart Study, a prospective cohort study of CVD among African Americans, individuals who reported moderate to high levels of perceived stress over a 7-year period had a 15% and 22% increased risk of developing hypertension, respectively, compared to those who reported consistently low perceived stress [32]. Additionally, evidence has shown that long-term exposure to stressors may cause endocrine and immune system dysfunction, leading to sustained chronic low-grade inflammation as measured by C-reactive protein (CRP) levels [33]. CRP, which is a protein synthesized by the liver, becomes elevated in response to inflammation due to a number of acute and chronic health conditions [34]. A systematic review of psychosocial stress and CRP found that various forms of stress, including

interpersonal stress, discrimination, employment-related stress, and financial burden, impacted CRP levels over time [35].

Furthermore, psychosocial stress greatly increases the risk of mental health disorders, including depression, anxiety, and psychosis [27]. In a population-based twin study from the Virginia Twin Registry, Kendler et al. (1999) concluded that stressful life events (SLEs) were associated with an increased risk of depression, and this association was due in part to the causal influence of SLEs on depression [36]. A twin-study from the Norwegian Institute of Public Health Twin Panel yielded similar results, concluding that association between SLEs and increased risk of depression is primarily causal in nature [37].

In addition to negatively impacting physical and mental health, psychosocial stress has well-established relationships with health behaviors. Research from the Midlife in the U.S. (MIDUS) study showed that increased levels of psychosocial stress over a 10-year period were associated with increased odds of smoking. Furthermore, among individuals who attempted to quit smoking during the study, those with higher levels of stress were more likely to be unsuccessful in their quit attempts [38]. The British Whitehall II study, a longitudinal cohort of British civil servant workers, found that the likelihood of alcohol consumption increased with the number of stressful life events [39]. Additionally, a systematic literature review examining the influence of stress on physical activity showed an inverse relationship between stress and physical activity in the vast majority of studies, indicating that stress impedes efforts to be more physically active [40]. A large body of evidence has shown that adverse health effects due to stress are incredibly common; however, more research needs to be conducted to understand the molecular mechanisms behind these associations.

1.2.3 Cardiovascular Disease and Cardiometabolic Risk Factors

CVD is a class of disorders that affects the heart and blood vessels, including coronary heart disease, stroke, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, deep vein thrombosis, and pulmonary embolism [41,42]. CHD is the most common type of CVD, accounting for one-third to one-half of all CVD cases. CHD involves the progressive narrowing of coronary arteries due to the build-up of plaque along the artery walls. Over time, CHD weakens the heart muscle and can contribute to heart failure or arrhythmias [43]. Myocardial infarction (MI), commonly referred to as a heart attack, is an acute event that occurs when the coronary arteries are blocked, cutting off the flow of blood to the heart and causing the heart muscle to die. Most MIs are due to underlying CHD that goes undetected. Although not always fatal, MIs can lead to lifetime complications, including arrhythmias, heart failure, or inflammation of tissue surrounding the heart [44].

Data from the National Health and Nutrition Examination Survey (NHANES) showed that between 2017 and 2020, the overall prevalence of CVD (including CHD, heart failure, stroke, and hypertension) in the U.S. was 48.6%, with nearly 128 million Americans affected by CVD [45]. In general, men had a slightly higher prevalence of CVD than women (52.4% vs. 44.8%). Additionally, Black men and women had the highest prevalence of CVD among racial/ethnic groups, at 58.9% and 59.0%, respectively.

The development and progression of CVD is heavily influenced by a group of cardiometabolic risk factors, which include hypertension, dyslipidemia, diabetes, and obesity. Hypertension, or high blood pressure, results when the force of the blood pushing against the artery walls is consistently elevated. Blood pressure readings are comprised of two numbers, systolic blood pressure (SBP), which measures the pressure on the artery walls when the heart

beats or contracts, and diastolic blood pressure (DBP), which measures the pressure when the heart is relaxing [46]. Although guidelines used to diagnose hypertension vary widely across the literature, the 2017 Hypertension Clinical Practice Guidelines criteria defined hypertension as either an SBP ≥ 130 mm Hg and/or DBP ≥ 80 mm Hg, self-reported antihypertensive medication use, or having been told at least twice by a health professional that one has hypertension [47]. Based on this definition, NHANES found that the age-adjusted prevalence of hypertension among U.S. adults was 46.7% between 2017 and 2020. This equates to an estimated 122.4 million Americans affected by hypertension. Males had a higher overall prevalence than females (50.4% vs. 43.0%). Furthermore, the prevalence of hypertension in Black Americans was among the highest in the world, with an age-adjusted prevalence of 55.8% among Black males and 56.9% among Black females [45].

Dyslipidemia, a disorder of lipoprotein metabolism, is defined as increased levels of serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), or a decreased level of high-density lipoprotein cholesterol (HDL-C) [48]. According to NHANES data from 2017-2020, the age-adjusted prevalence of moderately and significantly elevated TC levels (≥ 200 and ≥ 240 mg/dL) was 34.7% and 10.0%, respectively. The prevalence of high LDL-C, defined as ≥ 130 mg/dL, was 25.5%, while the prevalence of low HDL-C, defined as < 40 mg/dL, was 16.9%. Additionally, 19.9% of adults had high TG levels (≥ 150 mg/dL) [45]. Sex and racial/ethnic disparities were observed for each of the four age-adjusted prevalences.

Diabetes is a metabolic disease characterized by blood sugar (glucose) dysregulation. Type 2 diabetes (T2D), which occurs when the body is unable to process insulin efficiently and thus cannot regulate glucose levels, accounts for between 90% and 95% of all diabetes cases in the United States [49]. Diabetes is diagnosed based on a fasting plasma glucose ≥ 126 mg/dl, a

2-hour post-challenge glucose ≥ 200 mg/dL during an oral glucose tolerance test, a random glucose ≥ 200 mg/dL with presentation of hyperglycemic symptoms, or a hemoglobin A1c (HbA1c) ≥ 6.5% [50]. According to the 2017-2020 NHANES data, the age-adjusted prevalence of diagnosed diabetes in the U.S. was 10.6%. Additionally, 46.4% of the population had prediabetes, defined as having a fasting glucose between 100 and 125 mg/ dL or an HbA1c between 5.7% and 6.4%. Males had a slightly higher prevalence of diagnosed diabetes than females (12.2% vs. 9.1%). Additionally, the age-adjusted prevalence of diagnosed diabetes varied by race/ethnicity and years of education. Non-Hispanic White Americans with greater than a high school education had the lowest prevalence, while Hispanic adults with less than a high school education had the highest (7.9% vs. 16.2%) [45].

Obesity is a complex condition defined by abnormal or excessive accumulation of fat deposits that presents risk to one's health and overall quality of life [51]. A common tool for measuring obesity is the use of BMI, calculated as an individual's weight in kilograms divided by the square of height in meters (kg/m^2). In the U.S., being overweight is defined as BMI ≥ 25 kg/m^2, and obesity is defined as BMI ≥ 30 kg/ m^2 [52]. A limitation of BMI, however, is its inability to accurately measure abdominal adiposity [53], which is a known risk factor for CVD, independent of overall adiposity [54]. Thus, waist circumference (WC) is a tool used to specifically measure abdominal obesity, defined as a WC measurement of ≥ 102 cm in men and ≥ 88 cm in women [55]. NHANES data assessing trends in general and abdominal obesity in US adults from 2001-2018 found that the age-adjusted prevalence of general obesity increased from 33.1% to 41.4% in females and 26.9% to 42.4% in males over the 17-year study period. Further, the age-adjusted prevalence of abdominal obesity increased from 57.6% to 67.3% in females and 39.1% to 49.7% in males during that same time [56].

1.2.4 The Role of Epigenetics in Psychosocial Stress and Cardiovascular Health

To date, there have been a number of candidate gene studies and EWAS that have examined the relationship between individual psychosocial stressors and the epigenome. For instance, Alasaari et al. (2012) found that work stress and burnout in a cohort of nurses were associated with DNA methylation in several CpG sites in the *SLC6A4* promoter region [57]. Tyrka et al. (2012) found that having a history of childhood adversity was associated with increased methylation of the promoter region of the glucocorticoid receptor gene *NR3C1* in healthy adults [58]. Additionally, individual psychosocial stressors, including low socioeconomic status, childhood adversity, and work-related strain, have established relationships with both DNA methylation [59-64] and epigenetic clocks [65-67]. However, only a few studies have examined the relationship between epigenetic biomarkers and psychosocial stress defined more broadly, capturing multiple aspects of stress [20-24]. Further, these studies were conducted in small samples, and most were performed in a single ancestry population. Nevertheless, findings from these studies provide preliminary evidence that psychosocial stress may influence the epigenome.

The role of epigenetics on the development of CVD and its cardiometabolic risk factors is far more established. Several large-scale EWAS have identified associations between blood pressure [13], BMI [14], blood lipid levels [15], and individual CpGs, some of which have a potential causal role in regulating genes that impact development of cardiometabolic risk factors [68]. Furthermore, some epigenetic clocks have strong associations with these same risk factors [16-18], indicating that the aging epigenome may play a potential role in shaping CVD risk.

1.3 Health and Retirement Study (HRS)

All three aims of this dissertation use data from the Health and Retirement Study (HRS). HRS is a longitudinal cohort study of U.S. adults over age 50 that began in 1992 [69]. Upon creation, the primary objective of this study was to understand the changing health, economic, and social circumstances associated with aging. HRS captures broad topics, including income and wealth, cognition, use of healthcare services, work and retirement, and psychosocial well-being. Additionally, HRS measures extensive blood biomarker and genetic data on a subset of participants. The combination of economic, health, and psychosocial information provides a unique opportunity to study complex research questions regarding aging and retirement [70].

1.3.1 Study Population

HRS is a longitudinal survey consisting of over 43,000 individuals from U.S. households. Beginning in 1992, the initial HRS cohort consisted of individuals aged 51-61 (born 1931-1941) and their spouses. The following year, a second cohort study, Asset and Health Dynamics Among the Oldest Old (AHEAD), was conducted to capture information on individuals 70 years and older (born 1890-1923). The two studies merged in 1998, and in order to make the sample representative of the U.S. population over age 50, two new cohorts were enrolled: the Children of the Depression, consisting of individuals born 1924-1930, and the War Babies, made up of individuals born 1942-1947. HRS continues to employ a steady-state design by replenishing the sample every 6 years with younger cohorts [71].

The HRS sample is selected based on a multi-stage area probability sample design, which involves geographical stratification and oversampling of certain demographic populations. To determine eligibility, a screening interview is administered to each sampled housing unit. From all age-eligible household members, a primary respondent is randomly selected, and if that person has a spouse or partner, they are also included in the sample, regardless of age [72].

1.3.2 Data Collection

Core interviews are conducted every 2 years using a mixed-mode design of in-person, phone, or web interviews. The core content areas in HRS include information regarding employment history and retirement, income and wealth, health conditions and health status, sociodemographic characteristics, cognitive status, physical function, and family structure.

A. Psychosocial and Lifestyle Questionnaire

Starting in 2006, HRS selected a random one-half of the sample to complete an enhanced face-to-face interview, which included a set of physical performance measures, and a self-administered psychosocial questionnaire provided to participants at the end of the interview [73]. The Psychosocial and Lifestyle Questionnaire captures information on six substantive areas of interest, including: 1) subjective well-being; 2) lifestyle and experience of stress; 3) quality of social ties; 4) personality traits; 5) work-related beliefs; and 6) self-related beliefs [74]. In 2008, an enhanced face-to-face interview was conducted on the remaining half of the sample, and longitudinal data for the enhanced interviews is available at four-year intervals.

B. Venous Blood Study (VBS)

In 2016, all panel respondents who completed the HRS core interview were invited to participate in an ancillary study, the Venous Blood Study (VBS). Consenting HRS participants had venous blood collected by a trained phlebotomist via an in-home visit. In total, 9,934 respondents provided whole blood samples with valid results, which consisted of a large number of assays, including lipid panels, comprehensive metabolic panels, complete blood counts, cytokine panels, and immune system markers [75]. Furthermore, a subsample (n=4,104) of HRS respondents who completed the VBS were selected to perform additional assays reflecting molecular, cellular, and epigenetic processes of aging, including DNA methylation. In total,

4,018 samples passing quality control. The sample, which has a median age of 68.7 years and is 58% female, is racially and socioeconomically diverse.

1.3.3 Epigenetic Data

A. DNA Methylation

DNA methylation was assessed from whole blood samples using Illumina Infinium HumanMethylationEPIC Beadchip v.1.0 at the University of Minnesota. Whole blood samples were randomized across plates by key demographic variables, including age, sex, education, and race/ethnicity. The *minfi* R package was used for data preprocessing and quality control [76]. A detection p-value <0.01 was used to remove 3.4% of methylation probes (n=29,431) due to suboptimal performance. Additionally, sex mismatches and any controls (cell lines, blinded duplicates), were removed prior to analysis [77]. At each CpG site, DNA methylation was quantified using beta values, which approximates the proportion of methylation.

B. Epigenetic Clocks

Research has shown that methylation changes at specific portions of the genome are related specifically to age or age-related health outcomes. Thus, using information from a small number of CpGs (~100-500 sites), epigenetic clocks have been developed as indicators of epigenetic aging [78]. Epigenetic clocks are estimated in epigenetic years, with the difference between epigenetic and chronological age indicating whether individuals are biologically aging faster or slower than their chronological age [78].

Thirteen epigenetic clocks have been constructed using DNA methylation data from HRS. An initial set of clocks deemed “first-generation clocks” were designed to predict chronological age, including Horvath 1 [79], Hannum [80], Lin [81], Weidner [82], VidalBralo

[83], Horvath 2 [84], epiTOC [85], Bocklandt [86], and Garagnani [87]. However, because they were trained exclusively on age, they may not have selected the most health-relevant DNA methylation sites, as evidenced by their relatively weak associations with measures of morbidity and mortality [88]. Thus, second-generation clocks, including PhenoAge [89], GrimAge [90] and Zhang [91], were derived from a combination of health biomarkers to better predict age-related disease status and mortality. Finally, third-generation clocks, including DunedinPoAm38 and DunedinPACE [92], were developed using longitudinal change of age-related biomarkers to assess physiological decline in a cohort of individuals with the same chronological age. Unlike the first- and second- generation clocks, which measure biological age in years, DunedinPoAm38 and DunedinPACE measure pace of aging.

1.4 Specific Aims and Hypotheses

The three aims for this dissertation are detailed below and summarized in Figure 1.1:

1.4.1 Aim 1

Conduct an epigenome-wide study to identify DNA methylation sites associated with long-term psychosocial stress in older adults and assess the mediating effect of health behaviors and BMI on the relationship between stress and DNA methylation.

Hypothesis 1.1: Long-term psychosocial stress is associated with DNA methylation at a subset of CpG sites.

Hypothesis 1.2: Health behaviors and BMI partially mediate the relationship between psychosocial stress and DNA methylation.

1.4.2 Aim 2

Evaluate the epigenetic mediation of the relationship between psychosocial stress and cardiometabolic risk factors in older adults.

Hypothesis 2.1: Psychosocial stress is positively associated with cardiometabolic risk factors.

Hypothesis 2.2: The associations of psychosocial stress with cardiometabolic risk factors are partially mediated by DNA methylation levels at CpG sites throughout the epigenome.

1.4.3 Aim 3

Examine the associations between psychosocial factors, as well as their interactions with sociodemographic characteristics, and epigenetic age acceleration and evaluate the mediating role of depressive symptoms on the relationship between psychosocial factors and epigenetic aging in older adults.

Hypothesis 3.1: Psychosocial factors, including psychosocial stress and loneliness, are associated with epigenetic age acceleration.

Hypothesis 3.2: The associations between psychosocial factors and epigenetic age acceleration are modified by sociodemographic characteristics, including sex and educational attainment.

Hypothesis 3.3: Depressive symptoms partially mediate the relationship between psychosocial factors and epigenetic age acceleration.

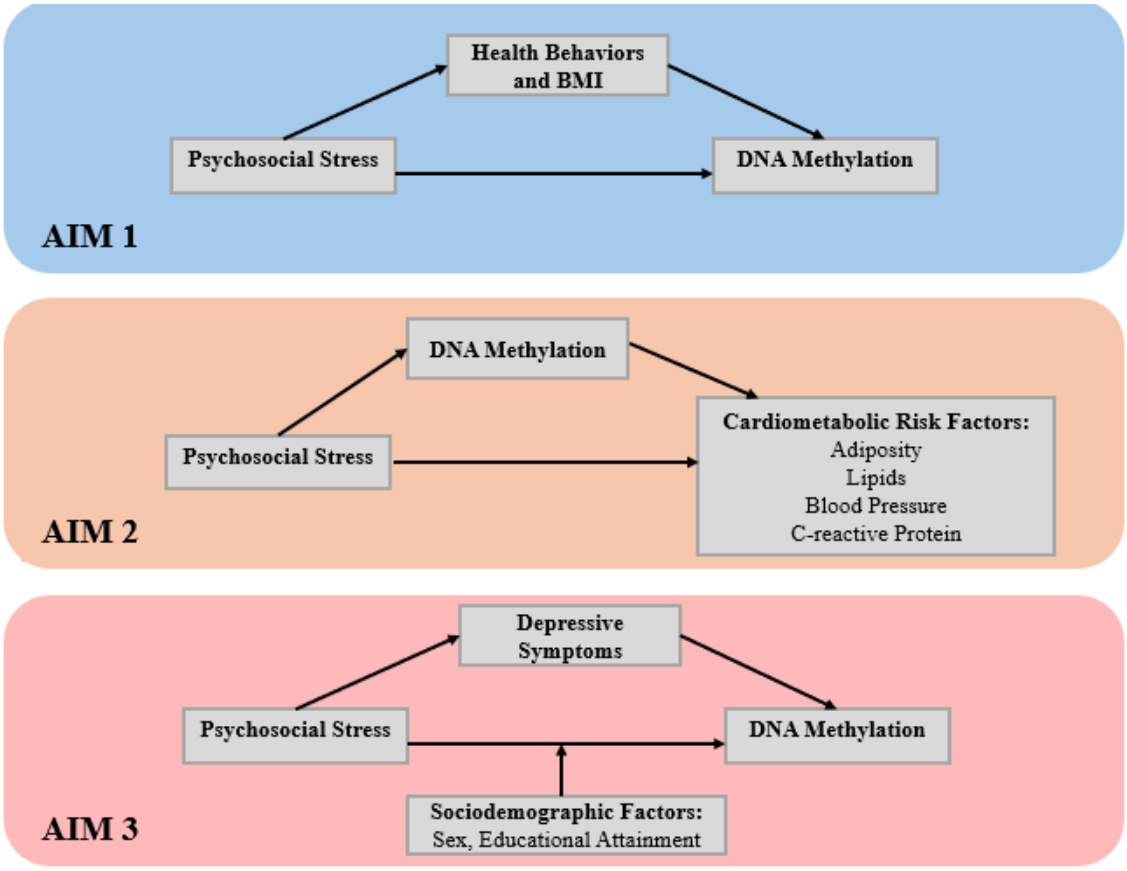


Figure 1-1: Conceptual framework of dissertation aims, describing the interplay of psychosocial and epigenetic factors underlying cardiometabolic risk.

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Chapter 2 Epigenome-Wide Association Study of Long-term Psychosocial Stress in Older Adults

2.1 Abstract

Long-term psychosocial stress is strongly associated with negative physical and mental health outcomes, as well as adverse health behaviors; however, little is known about the role that stress plays on the epigenome. One proposed mechanism by which stress affects DNA methylation is through health behaviors. We conducted an epigenome-wide association study (EWAS) of cumulative psychosocial stress (n=2,689) from the Health and Retirement Study (mean age=70.4 years), assessing DNA methylation (Illumina Infinium HumanMethylationEPIC Beadchip) at 789,656 CpG sites. For identified CpG sites, we conducted a formal mediation analysis to examine whether smoking, alcohol use, physical activity, and body mass index (BMI) mediate the relationship between stress and DNA methylation. Nine CpG sites were associated with psychosocial stress (all $p<9E-07$; FDR $q<0.10$). Additionally, health behaviors and/or BMI mediated 9.4% to 21.8% of the relationship between stress and methylation at eight of the nine CpGs. Several of the identified CpGs were in or near genes associated with cardiometabolic traits, psychosocial disorders, inflammation, and smoking. These findings support our hypothesis that psychosocial stress is associated with DNA methylation across the epigenome. Furthermore, specific health behaviors mediate only a modest percentage of this relationship, providing evidence that other mechanisms may link stress and DNA methylation.

2.2 Introduction

Psychosocial stress, or the perception of threat that results in discomfort and emotional tension, can be triggered by a multitude of stressors, including difficulty in relationships, financial uncertainty, or adverse life events such as job loss, discrimination, or health issues [1,2]. Previous research has shown that elevated levels of stress over time, especially in older and unhealthy individuals, is linked to serious health consequences, including a weakened immune system, hypertension, hyperglycemia, and increased risk for heart attack [3–5]. Additionally, psychosocial stress adversely impacts health behaviors, leading to increased rates of smoking and alcohol consumption, as well decreased rates of physical activity [6,7].

Furthermore, the co-occurrence of psychosocial stressors has been proven to have negative additive effects on health outcomes due to the multiple pathways by which the stressors can operate [8]. The cumulative stress hypothesis postulates that accumulation of psychosocial stressors, or repeated exposure to the same stressor, is particularly harmful to one's health and may result in greater likelihood of disease, as compared to a single stressor [9]. Thus, it is crucial to capture different sources of psychosocial stressors in order to accurately characterize the overall impact of psychosocial stress on health outcomes. Assessing multiple stressors simultaneously may lend insight into possible mechanisms for how stress affects one's health.

Although the associations between stress and negative health outcomes have been well-established, less is known about how psychosocial stress impacts the epigenome. Broadly, the epigenome is comprised of biochemical modifications to DNA and its related proteins that regulate gene expression without altering the underlying genetic sequence [10]. Epigenetic mechanisms, such as DNA methylation, can change throughout the life course under certain environmental stimuli, and they may help to explain the biological link between psychosocial

stress and adverse health outcomes. To date, there have been a large number of candidate gene and epigenome-wide association studies (EWAS) examining the relationship between individual psychosocial stressors and DNA methylation, including low socioeconomic status [11,12], maternal anxiety and depression [13,14], childhood adversity [15,16], and work-related strain [17,18]. However, there have only been a few studies that have examined the relationship between methylation and psychosocial stress defined more broadly, capturing multiple aspects of stress [19–21]. Further, these studies were conducted in relatively small samples, and most were performed in a single ancestry population. Nonetheless, results from these studies showed associations between stress and CpG sites that mapped to genes related to hypertension and heart disease, providing preliminary evidence that long-term psychosocial stress may influence downstream health outcomes through epigenomic changes.

One proposed mechanism by which psychosocial stress affects DNA methylation is through health behaviors [22]. As previously stated, psychosocial stress is known to negatively impact health behaviors [6,7]. There is also strong evidence that these same health behaviors, including smoking, alcohol, and physical activity, lead to significant changes in DNA methylation across the epigenome [23,24]. Thus, health behaviors may mediate the relationship between psychosocial stress and DNA methylation to a certain extent. Although alternative relationships between stress and health behaviors are possible, including the impact that smoking and alcohol use may have on relationship stress or financial strain, it is less likely that health behaviors influence psychosocial stress, particularly chronic stressors across the life course [25]. Further, there may be additional mechanisms by which psychosocial stress affects the epigenome, such as increased inflammatory response [26], elevated cortisol levels [27], and

psychiatric health conditions that trigger changes to the brain [28]. Nonetheless, these pathways are not well-established.

In this study, we conducted an epigenome-wide association analysis to identify CpG sites associated with long-term cumulative psychosocial stress in a large sample of older adults, which is critical for understanding the biological consequences of psychosocial stress. For CpG sites associated with psychosocial stress, we next conducted a formal mediation analysis to understand if health behaviors, including smoking, alcohol use, and physical activity, or body mass index (BMI), mediate the relationship between stress and DNA methylation.

2.3 Materials and Methods

2.3.1 Sample

Data are from the Health and Retirement Study (HRS), a nationally representative longitudinal cohort study of individuals over age 50. Information regarding the HRS study design has been described elsewhere [29,30]. In brief, HRS, which began in 1992, consists of biennial interviews that assess metrics of physical and mental health, employment, family characteristics, and wealth. In 2006, HRS added the Psychosocial and Lifestyle Questionnaire, or Leave Behind Questionnaire (LBQ), which is a self-reported psychosocial survey administered to a random 50% of the total HRS sample at each biennial interview [31]. This survey captures information regarding participants' well-being, lifestyle, and social relationships. In addition, all panel respondents who completed an HRS interview in 2016 were invited to participate in an ancillary study, the Venous Blood Study (VBS, n = 9,934) [32]. DNA methylation was measured in a sample of participants who completed the VBS blood draw (n = 4,018). This analysis was restricted to 2,689 participants who had data on psychosocial stress (2010 or 2012), all

covariates, and DNA methylation (2016). Figure S2-1 provides a flow diagram detailing participant inclusion criteria for our primary analysis.

To assess the potential impact of restricting our analysis to complete case data, we compared participant characteristics between those who were included ($n = 2689$) and excluded ($n = 1329$) in the primary analysis from the total DNA methylation sample. Student's t-tests and Chi-squared tests were used, as appropriate. Further, Cohen's d and Cramer's V effect sizes were calculated for continuous and categorical variables, respectively, to quantify the size of the difference between the two groups.

2.3.2 Measures

A. DNA Methylation

DNA methylation was assessed from whole blood samples using Illumina Infinium HumanMethylationEPIC Beadchip in a subset of racially and socioeconomically diverse HRS participants. Samples were randomized across plates by key demographic variables, including age, sex, education, and race/ethnicity. The *minfi* R package was used for data preprocessing and quality control [33]. Sex mismatches and probes with an average median intensity < 8.5 were removed, as were probes with missing data for > 5% of the sample [34]. A detection p-value <0.01 was used to remove samples and probes with a detection rate < 5%. Additionally, we removed cross-reactive probes that target repetitive sequences that may map to multiple places in the genome [35]. After quality control, a total of 789,656 CpG in 4,018 samples were available for analysis. At each CpG site, DNA methylation was quantified using beta values, which approximate the proportion of methylation, making them biologically interpretable. To ensure that strong outliers were not driving the results, we winsorized methylation values that were

greater than three times the interquartile range (IQR) of the 25th and the 75th percentiles [36,37]. The Houseman Method was used to estimate white blood cell proportions [38].

B. Psychosocial Stress

The Psychosocial Leave Behind Questionnaire collects data on a variety of psychosocial stress measures [31]. Because this survey was administered to only half of participants at each wave, we utilized data from the 2010 and 2012 interviews to ensure all participants had the opportunity to respond to the psychosocial survey. We examined six psychosocial stress domains, consistent with previous studies: 1) acute life events, 2) financial stress, 3) neighborhood stress, 4) relationship stress, 5) lifetime discrimination, and 6) childhood adversity [8,39]. Because we are interested in understanding how lifetime stress exposure affects DNA methylation, we examined both acute and chronic measures of stress from childhood throughout adulthood [40]. Each stress domain included one or more stress measures, as described below and in Table S2-1. For a measure to be considered complete, participants had to have answered at least 80% of the questions within the measure, unless otherwise noted in the measurement scoring instructions [41]. Only participants with completed stress measures were included in the analysis. We transformed each stress measure into a z-score. For domains that included multiple stress measures, we summed the individual z-scores together and then standardized the summed score into a z-score to allow for cross-domain comparisons [42,43].

In total, we assessed six stress domains. Acute life events included two measures: (1) acute lifetime traumas (seven items) and (2) acute stressful life events in the past 5 years (six items). Financial stress included two measures: (1) financial strain (two items) and 2) lack of financial autonomy (two items). Neighborhood stress included one measure (four items), focusing on neighborhood safety, vandalism, and cleanliness. Relationship stress included four

measures: (1) spouses (four items), (2) children (four items), (3) other family members (four items), and friends (four items). Lifetime discrimination, which measures racial and nonracial discrimination, included two measures: (1) perceived everyday discrimination (five items) and (2) major discriminatory events (six items). Childhood adversity included one measure related to lifetime traumas in adolescence (four items).

Using the six stress domains, we created a cumulative psychosocial stress score by summing the standardized scores from the six stress domains and then re-standardizing the summed scores [8]. This cumulative stress score represents the total stress exposure across all stress domains, and it is the main exposure of interest in our analyses. Furthermore, because the effects of childhood adversity may operate differently on the epigenome than the other five stress domains measured in adulthood, we created an additional variable to represent adult psychosocial stress by summing the standardized score from the five stress domains measured in adulthood (acute life events, neighborhood stress, financial stress relationship stress, and lifetime discrimination) and re-standardizing the summed score [44]. This allowed us to compare results when assessing cumulative stress from adulthood versus throughout the life course.

C. Covariates

Sociodemographic factors were collected at the 2010 or 2012 interview, concurrent with the timing of the Psychosocial Leave Behind Questionnaire. Consistent with previous literature, we included the following variables in our adjusted regression models: age, sex (male or female), educational attainment (no degree, high school degree, college degree or higher), employment status (yes/no), marital status (married/partnered or single/widowed/divorced), having any children (yes/no), and self-reported total household wealth in US dollars [8,39–42]. Total wealth was calculated as the sum of all assets (stocks, mutual funds, investments, bonds, checking and

savings accounts, and all other savings) minus all debt [41,42]. To avoid large standard errors, we log-transformed total household wealth using the modulus transformation, sign(total wealth)*log(|total wealth|+1) [45]. The top 10 principal components (PCs) of genetic ancestry were estimated using genome-wide genotype data and were used as model covariates to account for population stratification and admixture [46].

D. Potential Mediators

Like sociodemographic factors, health behaviors, including smoking (never, former, current), alcohol use (never, moderate, heavy), and moderate or vigorous physical activity (active, inactive), and BMI were collected at the 2010 or 2012 interview and were considered potential mediators of the association between psychosocial stress and DNA methylation. The same set of variables was also collected on a subset of participants who had available data in 2014 (n = 2,614). Based on the National Institute on Alcohol Abuse and Alcoholism criteria, moderate drinking was defined as 1–7 drinks per week for females and 1–14 drinks per week for males, while heavy drinking was defined as > 7 drinks per week for females and > 14 drinks per week for males [47]. Physical activity was calculated based on the self-reported frequencies of moderate or vigorous activities. Participants reporting that they engaged in moderate or vigorous physical activity at least once per week were classified as active, while those reporting physical activity less than once per week were classified as inactive [48]. BMI was calculated from participant's weight in kilograms divided by the square of their height in meters, measured during the physical health section of their interview [49]. For participants who did not have their height and weight measured during their interview, we used their self-reported BMI. We performed Spearman's rank-order correlations to assess the strength of associations between

health behaviors. Physical activity, alcohol use, and smoking status were analyzed as ordinal variables.

2.3.3 Statistical Analysis

A. Epigenome-wide Association Analysis

We conducted an epigenome-wide association study (EWAS) of cumulative psychosocial stress using multivariable linear regression to estimate associations of stress with DNA methylation at each CpG site, adjusting for potential confounders, including age, sex, educational attainment, top 10 genetic ancestry PCs, year of psychosocial stress measurement, white blood cell proportions, and technical covariates, including sample plate, plate row, and plate column (Model 1). To correct for multiple testing, the Benjamini-Hochberg procedure was applied to control the false discovery rate (FDR) at a threshold of $q < 0.10$. The minimally adjusted multivariable model (Model 1) was considered our primary model. We investigated the attenuation of effects on all significant CpG sites identified in our primary model by further adjusting for marital status, employment status, total income, and having children (Model 2).

As a sensitivity analysis, we conducted an EWAS of the psychosocial stress score comprised only of the five stress domains measured in adulthood (i.e., excluding childhood adversity) using Model 1. This allowed us to understand if the time period where stress was experienced (adulthood vs. life course) influenced the associations between stress and DNA methylation.

Next, to assess how much each of the six stress domains were driving the association between cumulative psychosocial stress and DNA methylation, we examined the associations of each stress domain separately with the significant CpG sites identified in the primary analysis (Model 1). Analyses were conducted using R software (version 4.1.3).

B. Mediation Analysis

To investigate the mediating effect of health behaviors on the relationship between psychosocial stress and DNA methylation, we conducted a formal mediation analysis for all significant CpG sites identified in the EWAS of cumulative stress (Model 1) using the Karlson-Holm-Breen (KHB) method [50]. The KHB method, which has a structural equation modelling (SEM) framework, decomposes the total effect of an independent variable into the sum of the direct and indirect effects [51]. Although this method was primarily developed for non-linear models, it is also appropriate for linear models [52]. A major advantage of KHB, as opposed to other mediation methods, is that it provides unbiased estimates for the overall, direct, and indirect effects on the same scale, thus achieving reliable comparisons across different mediators of different variable types [53]. Additionally, this method allows for the examination of multiple mediators simultaneously and has the ability to disentangle the effects of correlated mediators, thereby quantifying the relative contribution of each mediator to the total effect [54,55].
We used the *khb* R package to test mediation by smoking, alcohol use, physical activity, and BMI simultaneously [50]. Due to computational constraints of KHB, we pre-adjusted DNA methylation for white blood cell proportions and batch effects using linear models (sample plate, plate row, plate column), and we used the residuals as the outcome in the mediation models. For each CpG site, we conducted a generalized linear model (GLM) following a Gaussian (normal) distribution [51,52]. All mediation models were adjusted for age, sex, educational attainment, employment status, marital status, having any children, total income, genetic ancestry PCs, and year of psychosocial stress measurement, consistent with the adjustment variables in Model 2 of our EWAS. In the mediation analysis, both psychosocial stress and health behaviors were

collected at least four years prior to DNA methylation, thereby reducing the concern for reverse causation, where DNA methylation could influence either stress or health behaviors.

Additionally, we conducted a secondary mediation analysis on all significant CpG sites identified in the EWAS of cumulative psychosocial stress for participants who had available health behaviors and BMI data in 2014 (n = 2,614). In this sensitivity analysis, the potential mediators were collected after psychosocial stress, as opposed to concurrently, further establishing temporality between psychosocial stress and health behaviors.

C. Enrichment of Genomic Features

For CpG sites that were significantly associated with psychosocial stress, we examined whether their genomic locations were enriched for features, including gene promoters, enhancers, DNase I hypersensitivity sites (DHS), CpG islands, CpG island flanking shores, or CpG island flanking shelves. We considered a CpG site to be in the promoter region if it was located less than 1.5 kb upstream of a transcriptional start site. A CpG site was designated as a CpG island (CGI) flanking shore or shelf if it was within 2 kb or between 2–4 kb of a CGI, respectively. Annotation files from Illumina and the UCSC genome browser were used to identify the target genes and genomic features associated with each CpG [56,57]. Enrichment analysis was performed using a two-sided Fisher's exact test.

2.4 Results

2.4.1 Descriptive Statistics

Sample characteristics are shown in Table 2-1. The mean age of participants was 70.4 years (SD: 9.5), the majority were female (59%), and a quarter of participants had a college degree or higher. Overall, 74% of participants were non-Hispanic white, 13% were non-Hispanic

Black, 10% were Hispanic, and 3% were another race or ethnicity. Slightly less than half of the participants were working for pay at the time the psychosocial battery was administered (43.7%). Approximately 70% of participants were either married or had a partner, while the remaining 30% were either single, widowed, or divorced. Additionally, 89% had at least one child. Average household wealth for participants was $442,619 (SD: 952,905). In terms of participants' health behaviors, 10% were current smokers, 40% were moderate to heavy drinkers, and 59% participated in moderate or vigorous exercise at least once per week. The mean measured BMI for participants was 30.4 kg/m2 (SD: 6.7). Participants from the DNA methylation sample with complete data on stress and covariates tended to be older, have higher education, and have healthier behaviors than those who were excluded, although the effect sizes between the two groups were relatively small (Cramer's V and Cohen's $d < 0.30$ for all covariates) (Table S2-2).

All six of the psychosocial stress domains were correlated with one another at $p < 0.001$ (Table S2-3). The correlation coefficients ranged from 0.10 to 0.39, with the highest measures of correlation occurring between lifetime discrimination and relationship stress, as well as lifetime discrimination and financial stress. Health behaviors and BMI were weakly, but significantly, correlated with one another (Figure S2-2). BMI and physical activity had the strongest correlation (Spearman's =-0.19, $p < 0.001$).

2.4.2 Epigenome-wide Association Analysis

In the primary EWAS (Model 1), we identified nine CpG sites associated with psychosocial stress (FDR $q < 0.10$, all $p < 9E–07$; Figures 2-1 and 2-2). Eight of the CpGs were less methylated with higher levels of cumulative stress. When adding additional sociodemographic characteristics that may be on the causal pathway from early life stress exposure to later life DNA methylation to the primary model (Model 2), effect estimates of the

nine CpG sites were slightly attenuated but remained at least nominally associated with psychosocial stress (all $p < 2E–05$; Table 2-2).

In the sensitivity analysis that excluded childhood adversity from the cumulative psychosocial stress score, we observed very similar results to those obtained from our original EWAS in Model 1 (Table S2-4). Five CpG sites were associated with cumulative psychosocial stress in adulthood (FDR $q < 0.10$), and those CpG sites matched the top five results from the cumulative stress EWAS in Model 1. Furthermore, the remaining four CpG sites that were significant in our original EWAS were all at least nominally associated with cumulative psychosocial stress in adulthood (all $p < 9E–06$).

When assessing the relationship between the six individual stress domains and the nine CpG sites, we found that acute life events, financial stress, and lifetime discrimination domains had at least nominally significant associations with all CpG sites ($p < 0.05$), while neighborhood stress, relationship stress, and childhood adversity each had nominal associations with at least six CpG sites (Table S2-5). These results indicate that the associations between DNA methylation and the domains of stress that contributed to the cumulative psychosocial stress score were relatively consistent.

2.4.3 Mediation Analysis

We next investigated the mediating effect of health behaviors on the association between psychosocial stress and DNA methylation. Cumulative psychosocial stress explained between 0.6% and 4.8% of the total variability of the nine identified CpG sites in our primary model (Model 1) and between 0.5% and 4.5% of the total variability after adjusting for additional sociodemographic covariates (Model 2; Table S2-6). When assessed simultaneously, health behaviors (smoking, alcohol use, physical activity) and BMI significantly mediated 9.4% to

21.8% of the relationship between stress and methylation at eight of the nine CpG sites (Table S2-7). Specifically, current smoking mediated between 5.4% and 8.8% of the total effect of psychosocial stress on DNA methylation at four CpG sites (cg17565444, cg01695954, cg01766737, cg12581512; all $p < 0.05$). Moderate alcohol use mediated 3.9% of the total effect in cg04803208 ($p = 0.04$). Finally, between 4.0% and 10.4% of the total effect of psychosocial stress on DNA methylation was mediated by BMI at seven CpGs (cg25737313, cg08274633, cg01695954, cg24704287, cg01766737, cg12581512, cg04803208; all $p < 0.05$).

Physical activity did not significantly mediate the relationship between stress and methylation at any of the nine CpG sites. It should be noted that the total effects in these mediation models were substantively similar to those in the Model 2 EWAS (Table 2-2). Any slight differences observed between the two sets of analyses are due to the pre-adjustment of methylation beta values for batch effects and white blood cell proportions prior to conducting the mediation analysis.

In the sensitivity analysis testing for mediation by health behaviors collected in 2014, we observed similar results to our primary mediation analysis (Table S2-8). Health behaviors and BMI significantly mediated 10.1% to 26.0% of the relationship between stress and methylation at all nine CpG sites, with similar contributions from smoking, alcohol, and BMI. One difference between the two analyses was the impact of physical activity on the relationship between stress and methylation. When physical activity was collected in 2014, it mediated between 3.8% and 5.1% of the total effect of stress on DNA methylation at four CpGs (cg01695954, cg24704287, cg12581512, cg20559385; all $p < 0.05$), which is slightly more than observed in the primary mediation model for those CpG sites (0.7%-2.1%).

2.4.4 Genomic Feature Enrichment Analysis

Using the Illumina annotation gene mapping, we found that CpGs associated with psychosocial stress (FDR $q < 0.10$) were enriched for enhancers (OR:8.38, $p = 0.034$), but not for promoters, DHS, CGIs, or CGI flanking shores or shelves.

2.5 Discussion

This large-scale EWAS of long-term psychosocial stress in whole blood samples from a multi-ancestry cohort of older adults identified nine CpG sites. We found that health behaviors and BMI may partially mediate the relationship between psychosocial stress and DNA methylation; however, given that these factors explained a relatively low proportion of the relationship, it is likely that stress also impacts methylation through mechanistic pathways independent of these factors.

Our most significant CpG associated with psychosocial stress, cg01695954, is in the promoter region of the ribonucleotide reductase regulatory subunit M2 (RRM2) gene on chromosome 2. This gene encodes one of two non-identical subunits for ribonucleotide reductase (RNR), which is important for DNA synthesis and DNA repair by producing deoxynucleoside triphosphate (dNTP) [58]. Additionally, its expression is regulated by both transcription and protein degradation. Studies have found that RRM2 plays a crucial role in cell death, affecting autoimmune disorders such as rheumatoid arthritis, as well as several types of cancer [59]. [60]. In particular, the inhibition of RRM2 results in a decrease in cellular proliferation and in increase in apoptosis, thus representing a potential chemotherapeutic target and treatment for RA. DNA methylation in or near RRM2 has also been associated with schizophrenia and post-traumatic stress disorder (PTSD), two psychosocial disorders related to psychosocial stress [61,62]. In an EWAS analysis, PTSD was associated with methylation of cg18623836, a CpG site located in

the promoter region of RRM2, 112 bps from our significant CpG, cg01695954 [61]. This finding supports the hypothesis that the epigenetic patterning of the RRM2 genomic region may be impacted by long-term psychosocial distress.

Another CpG associated with psychosocial stress in our study is cg08274633, located in the promoter region of an uncharacterized gene, C15orf53. This CpG has been associated with aging [63], clear cell renal carcinoma [64], incident chronic obstructive pulmonary disease (COPD) [65], and incident type 2 diabetes [65] in previous large-scale EWAS. The observed directions of cg08274633 methylation on COPD and type 2 diabetes indicate a potential pathway where elevated psychosocial stress results in decreased DNA methylation at cg08274633, which is in turn associated with increased risk of COPD and type 2 diabetes. Future studies can examine this pathway in greater detail to understand the underlying mechanisms linking stress, DNA methylation, and chronic diseases.

Two identified CpG sites, cg17565444 and cg12581512, map to the gene body of the ankyrin repeat and SOCS box containing 2 (ASB2) gene on chromosome 14. This gene has been associated with BMI [66], smoking [67], waist circumference [68], cognition [69], inflammation [70], and HIV infection [71] ASB2 encodes a protein that plays a crucial role in protein degradation by coupling cytokine signaling protein suppressors with the elongin BC complex [72]. Additionally, this gene plays a role in growth inhibition induced by retinoic acid, a metabolite of vitamin A that is essential for embryogenesis and the regulation of regeneration and plasticity in the adult brain [73].

Several EWAS have failed to detect significant associations between psychosocial stressors and DNA methylation, and those that detected associations had relatively poor consistency across studies [74]. This may be due to challenges faced when conducting EWAS of

psychosocial stress, including small sample sizes, lack of appropriate adjustment covariates, and inconsistent methods for measuring stress. Kalinowski et al. (2022), which examined the relationship between stress overload and DNA methylation among 228 African American women, did not identify any CpGs at the epigenome-wide significance threshold, likely due to the small sample size; however, the top CpGs identified mapped to genes related to heart disease and hypertension [19]. Lam et al. (2012) found associations between DNA methylation and several stress measures including socioeconomic status, perceived stress, and cortisol levels, in a community cohort of 92 individuals, but this analysis did not adjust for any potential confounders [20]. Our study aimed to address these challenges by conducting an EWAS in a diverse population of 2,689 older adults, where we captured both a comprehensive set of objective and subjective stress measures across the life course and adjusted for relevant potential confounders. In a recent systematic review investigating DNA methylation signatures of broad psychosocial stressors (Zhang et al. (2022)), none of our findings overlapped with those from the candidate gene studies and EWAS identified in the review, likely due to the reasons stated above [74].

Health behaviors and BMI explained a relatively modest, but significant, percentage of the relationship between stress and DNA methylation. Cumulatively, smoking, alcohol consumption, physical activity, and BMI mediated between 9% and 22% of the relationship between stress and methylation at the nine CpG sites identified in the EWAS. Further, we achieved similar results in a sensitivity analysis using a subset of participants who had health behavior and BMI data measured in 2014. One explanation is that we may not have fully captured the mediating effect of health behaviors on the relationship between stress and DNA methylation. The health behaviors we assessed as potential mediators were only measured at a

single timepoint. Because this is a population of older adults, health behaviors from earlier life, which were not captured in our analysis, could have mediated the association between stress and DNA methylation. Furthermore, the health behaviors selected for mediation testing may not have been comprehensive. Other health behaviors, aside from those included in our analysis, could be potential mediators on the relationship between stress and DNA methylation. Alternatively, psychosocial stress may affect DNA methylation through additional underlying pathways independent of health behaviors.

One pathway through which long-term psychosocial stress may influence DNA methylation is through increased levels of cortisol. When a stressor is perceived and interpreted, the stress response system, or the hypothalamic-pituitary-adrenal axis (HPA axis), is activated [3]. The hypothalamus area of the brain sends a chemical message to the pituitary glands, which triggers the release of adrenocorticotropic hormone (ACTH). In turn, ACTH stimulates the adrenal glands to release cortisol, a steroid hormone that binds glucocorticoid receptors. Because glucocorticoid receptors are found in nearly every tissue of the body, once cortisol attaches to its receptors, it interacts with many organ systems, including the nervous, immune, respiratory, and cardiovascular systems [75]. After the stressor has been resolved, built-in regulators help return the psychological systems to usual levels, restoring the body to homoeostasis. However, chronic stress can lead to long-term activation of the stress response system, resulting in elevated levels of cortisol and other stress hormones, which may disrupt the development of brain architecture and other organ systems, potentially leading to changes in DNA methylation. Glad et al. (2017) found that excessive cortisol exposure over time prompted changes in global DNA methylation patterns, particularly in genes known to induce hyperactivity of the HPA-axis [76].

Another mechanism by which psychosocial stress may affect DNA methylation is through psychiatric health conditions. Long-term psychosocial stress may trigger certain psychiatric disorders, including anxiety and depression, which in turn may affect DNA methylation through alterations to brain anatomy [77,78]. Neuroimaging studies of individuals with major depressive disorder (MDD) found significant alterations to brain regions, including the frontal lobe, hippocampus, parietal lobe, striatum, and thalamus, as well as impairment of crucial circuits in the brain [77]. For example, the prefrontal-subcortical circuit, which is comprised of the striatum, thalamus, and prefrontal cortex and is involved heavily in emotional and cognitive processing, may be impaired in individuals with MDD, leading to emotional and cognitive dysfunction. Furthermore, a prior EWAS found that MDD was associated with differently methylated CpG sites through mechanisms involved in neuronal synaptic plasticity and calcium signaling, which are involved in the neurobiology of depressive disorders [79].

As with any study, there are limitations. First, due to the novel research question being studied, we were unable to replicate our findings in an independent cohort. Additionally, we only used self-reported psychosocial stress data to create the cumulative stress score. Future analyses can utilize multiple sources of stress, including changes in physiological markers and behaviors, to get a more comprehensive definition of cumulative stress. Furthermore, we weighed all six psychosocial stress domains equally when calculating cumulative stress score, but certain stress domains may have a greater impact on DNA methylation than others.

Although we implemented a longitudinal study design, we cannot rule out the possibility of reverse causality due to the small timeframe between measurement of the exposure, mediators, and outcome variables. For future analyses, it would be useful to measure both DNA methylation and psychosocial stress at multiple time points to assess changes in the epigenome in

response to stress. Additionally, we only examined the mediating role of BMI on the association between stress and DNA methylation, although it is possible that other relationships exist. For example, BMI may be a downstream consequence of methylation changes. Future research should further explore whether BMI, as well as other comorbidities, lie in the causal pathway between stress and DNA methylation or are subsequent to methylation changes.

Since data were collected from a cohort of older adults, there is the possibility of selection bias in our study. Participants who had severe life stressors may have been less likely to participate or remain in the cohort. Thus, the psychosocial stress scores that we calculated based on our sample may differ slightly from what we would observe in the general U.S. population, although the literature is mixed on whether psychosocial distress and mental health influence study participation or attrition [79–82]. Finally, because health behaviors were measured concurrently with psychosocial stress, the associations between stress and certain health behaviors may have been bi-directional. Consequently, in addition to mediators, these health behaviors could have also been confounders on the relationship between stress and DNA methylation. To help establish a temporal relationship between the exposure (stress) and mediators (methylation), we conducted a sensitivity mediation analysis using health behaviors and BMI measured in 2014, two to four years after psychosocial stress measurement, and the results were substantively similar to our primary analysis.

This study also has several key strengths. First, we examined psychosocial stress in both childhood and adulthood, which allowed us to better understand the impact of cumulative stress on DNA methylation across the life course, not just at a specific period in time. Furthermore, we examined the stress domains in sensitivity analyses. We found that although there were similarities among the domains, certain stress domains were driving the associations between

cumulative stress and DNA methylation more than others. Finally, we conducted our research in a multi-ancestry population, including African, Hispanic/Latino, and European ancestries, allowing us to study the effects of cumulative stress in a diverse population of older adults.

2.6 Conclusion

In summary, our results support the hypothesis that cumulative psychosocial stress is associated with a subset of CpG sites in the epigenome. Additionally, health behaviors mediate only a small percentage of this relationship, providing evidence that independent mechanisms may link psychosocial stress and DNA methylation. Future studies are needed to replicate our findings in an independent cohort in order to confirm the observed associations between psychosocial stress and DNA methylation.

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2.8 Tables

Table 2-1: Characteristics of Health and Retirement Study analytic sample (N=2,689).

Characteristics	N (%) or mean (SD)
Age, years	70.4 (9.5)
Female	1590 (59.1)
Race/Ethnicity	
Hispanic	272 (10.1)
Black	350 (13.0)
White	1997 (74.3)
Other	69 (2.6)
Education	
No degree	346 (12.9)
HS degree	1631 (60.7)
College degree or higher	712 (26.5)
Employment Status	
Working for pay	1175 (43.7)
Not working for pay	1514 (56.3)
Marital Status	
Married/Partnered	1905 (70.8)
Single/Widowed/Divorced	784 (29.2)
Have Children	2390 (88.9)
Total Household Wealth ($)	442,619 (952,905)
Smoking Status	
Never smoker	1208 (45.2)
Former smoker	1138 (42.6)
Current smoker	327 (12.2)
Alcohol Use	
Never drinker	1588 (59.1)
Moderate drinker	914 (34.0)
Heavy drinker	186 (6.9)
Physical Activity	
Active	1591 (59.2)
Inactive	1096 (40.8)
BMI (kg/m^2)	30.4 (6.7)

Table 2-2: Significant CpG sites from the epigenome-wide association study (EWAS) of cumulative psychosocial stress (Model 1: FDR q<0.10).

CpG Site	Functional Annotation				Model 1			Model 2	
	Chr	*Gene*	*Relation to CpG Site*	*Genomic Feature*	*Beta*	*P-value*	FDR q	*Beta*	*P-value*
cg01695954	2	*RRM2*	S_Shore	Promoter	-0.40	5.7E-09	0.0045	-0.38	8.4E-08
cg08274633	15	*C15orf53*	OpenSea	Promoter	-0.42	1.5E-08	0.0058	-0.42	4.4E-08
cg17565444	14	*ASB2*	OpenSea	Body	-0.32	3.3E-08	0.0086	-0.30	4.8E-07
cg24704287	19	*MIR23AHG*	N_Shore	Body	-0.66	5.5E-08	0.011	-0.59	2.9E-06
cg01766737	22	*CENPM*	N_Shore	Body	-0.13	4.2E-07	0.063	-0.11	1.1E-05
cg25737313	19	*HOOK2*	N_Shore	Body	-0.28	4.7E-07	0.063	-0.27	2.8E-06
cg12581512	14	*ASB2*	OpenSea	Body	-0.29	6.7E-07	0.075	-0.27	8.2E-06
cg20559385	6		OpenSea		-0.61	8.8E-07	0.083	-0.67	1.6E-07
cg04803208	8	*LOC100130298*	OpenSea	Body	0.51	9.4E-07	0.083	0.51	2.1E-06

Beta is the regression coefficient representing the percent change in DNA methylation associated with a 1 standard deviation increase in the cumulative psychosocial stress score

Model 1: CpG Site ~ Psychosocial Stress Score + Age + Sex + Educational Attainment + Interview Year + Genetic Ancestry PC 1-10 + White Blood Cell Proportions + Row Position on Chip + Column Position on Chip + Sample Plate

Model 2: Model 1 + Marital Status + Employment + Has Children + Total Household Wealth

Associations were only evaluated in Model 2 if they had an FDR q <0.10 in Model 1.

2.9 Figures

Figure 2-1: Manhattan plot of the EWAS for cumulative psychosocial stress in HRS. The red horizontal line indicates the epigenome-wide significance threshold (FDR q<0.10).

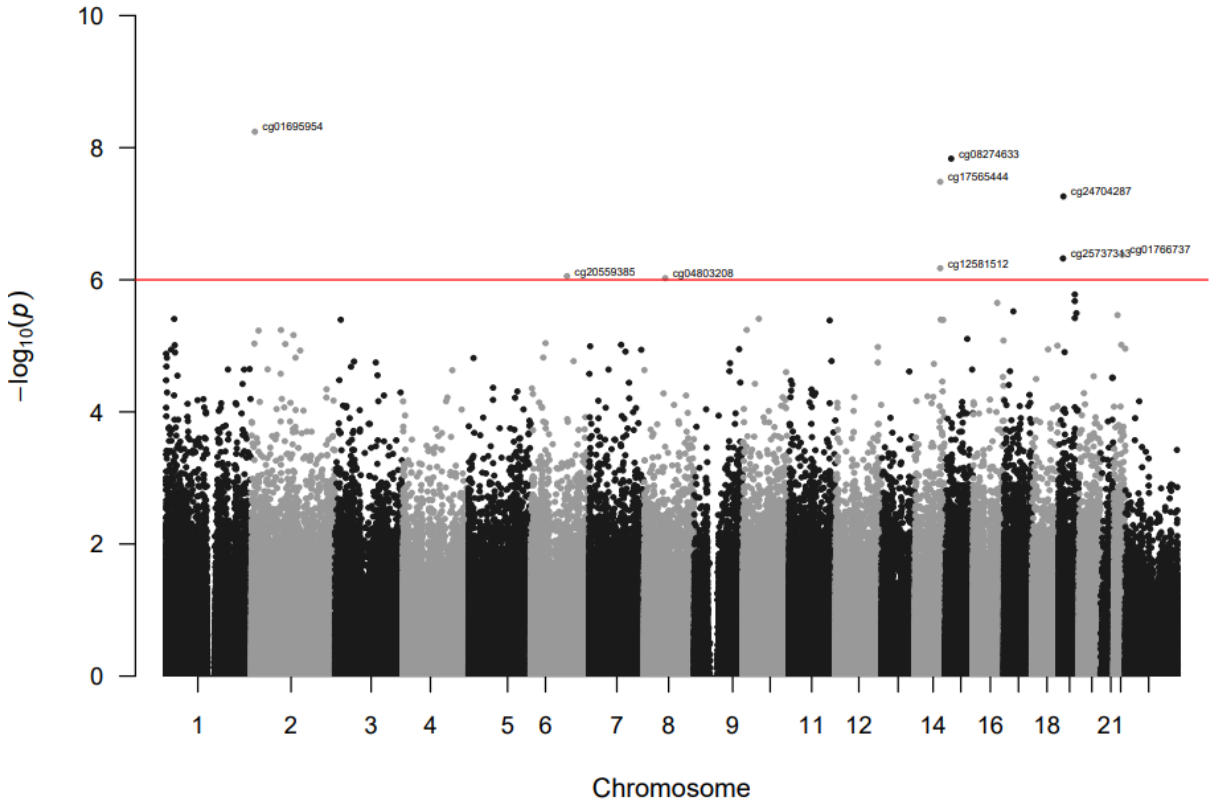
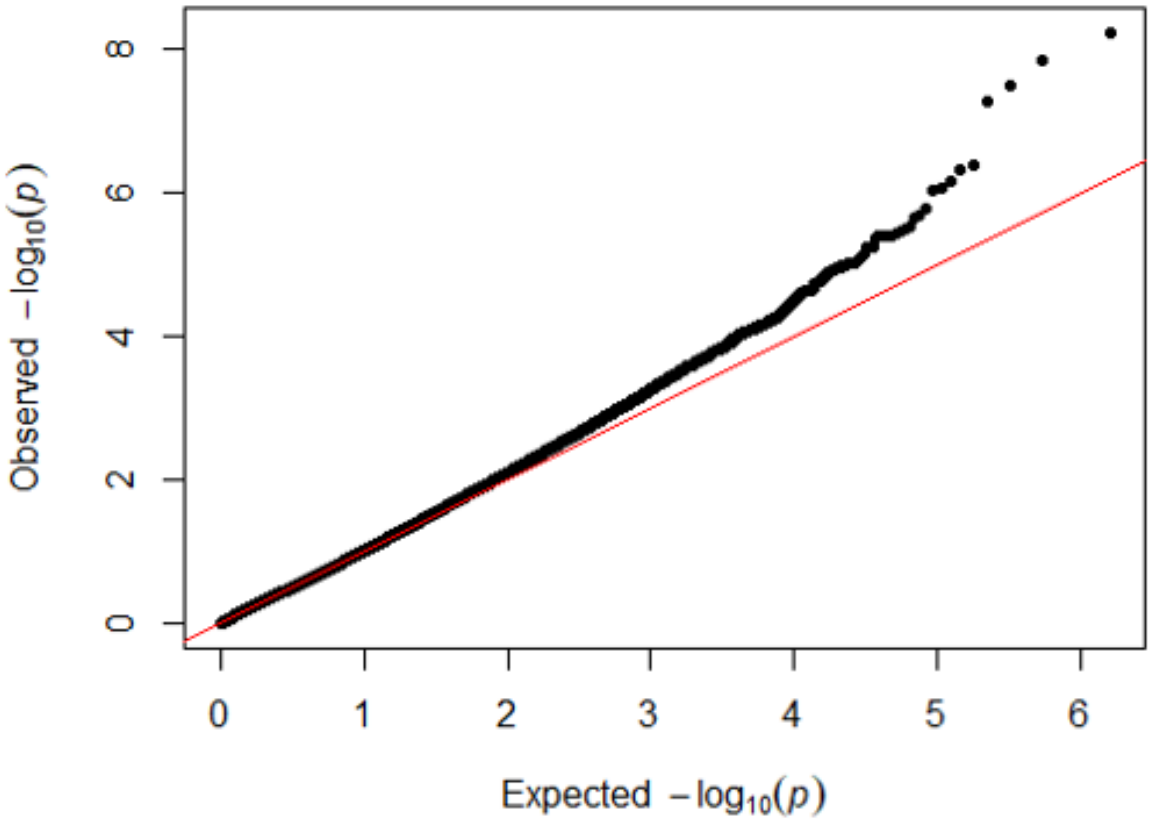


Figure 2-2: Quantile-quantile plot of the EWAS for cumulative psychosocial stress in HRS (inflation factor λ=1.09).



2.10 Supplementary Material

Figure S2-1: Flow diagram detailing participant inclusion criteria in primary analysis.

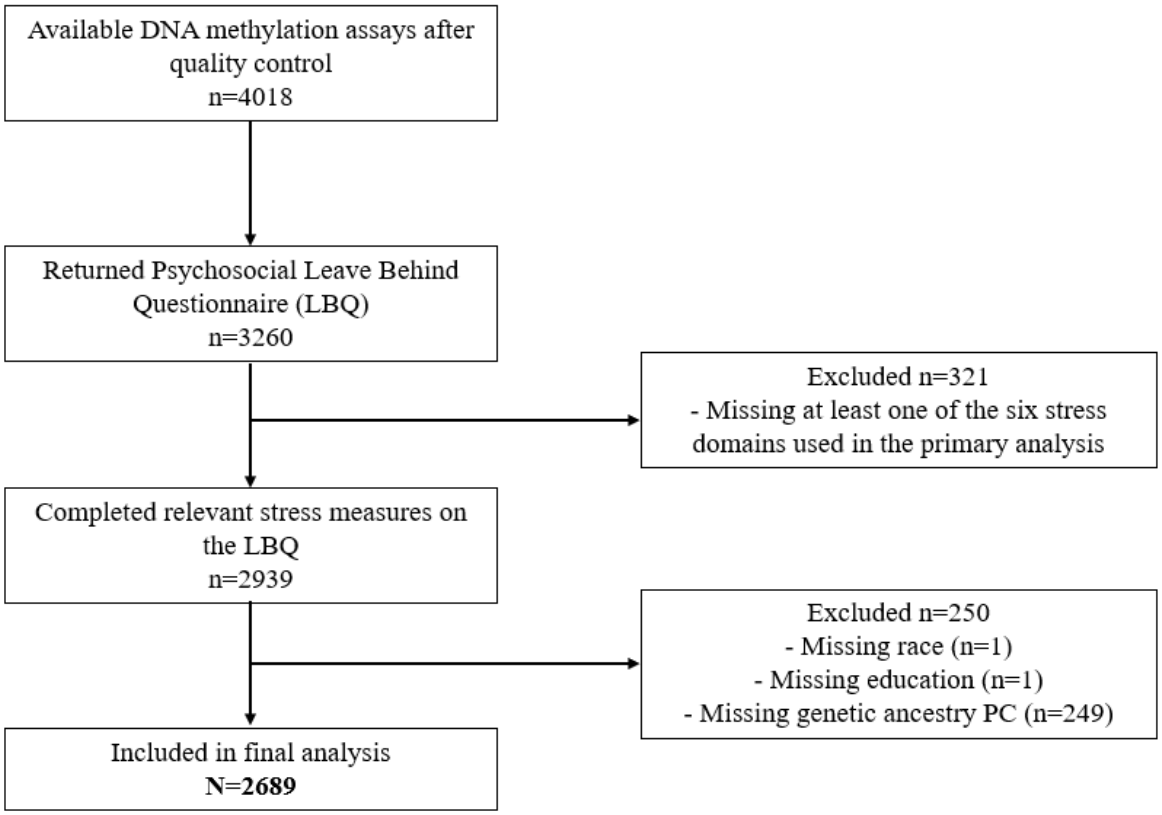


Table S2-1: Psychosocial stress measures included in the 6 stress domains.

Stress Domain	Stress Measure	Individual Items
Acute Life Events	Lifetime Traumas	(1) Has experienced the death of child; (2) has ever been in a major fire, flood, earthquake or other natural disaster; (3) has ever fired a weapon in combat or been fired upon in combat; (4) has a spouse, partner, or child has ever been addicted to drugs or alcohol; (5) was a victim of a serious physical attack or assault; (6) has ever had a life-threatening illness or accident; (7) has spouse or a child has ever had a life-threatening illness or accident.
	Stressful Life Events in Past 5 Years	(1) Has involuntarily lost a job for reasons other than retirement; (2) has been unemployed and looking for work for longer than 3 months; (3) was anyone in household unemployed and looking for work for longer than 3 months; (4) have moved to a worse residence or neighborhood; (5) have been robbed or home has been burglarized; (6) has been victim of fraud.
Financial Stress	Financial Strain	(1) Difficulty meeting monthly payments on bills; (2) distress caused by ongoing financial strain.
	Lack of Financial Autonomy	(1) Satisfaction with his/her current financial situation; (2) amount of control over his/her financial situation (*reverse-coded*).
Neighborhood Stress	Neighborhood Disorder	(1) Vandalism and graffiti are a big problem in this area; (2) People would be afraid to walk alone in this area after dark; (3) This area is always full of rubbish and litter; (4) There are many vacant or deserted houses or storefronts in this area.
Relationship Stress	Marital Stressors Child-Related Stressors Other Family-Related Friend Stressors	(1) How often do they make too many demands on you? (2) How much do they criticize you? (3) How much do they let you down when you are counting on them? (4) How much do they get on your nerves?
Lifetime Discrimination	Major Discriminatory Events	(1) Have been unfairly dismissed from a job; (2) have been unfairly not hired for a job; (3) have been unfairly denied a promotion; (4) have been unfairly prevented from moving into a neighborhood because the landlord or a realtor refused to sell or rent you a house or apartment; (5) have been unfairly denied a bank loan; (6) have been unfairly stopped, searched, questioned, physically threatened or abused by the police.
	Perceived Everyday Discrimination	(1) Have been treated with less courtesy or respect than other people; (2) have received poorer service than other people at restaurants or stores; (3) people act as if they think you are not smart; (4) people act as if they are afraid of you; (5) you are threatened or harassed.
Childhood Adversity	Childhood Adversity Before Age 18	(1) Had to do a year of school over again; (2) was ever in trouble with the police; (3) either of his/her parents drank or used drugs so often that it caused problems in the family; (4) was ever physically abused by his/her parents.

Table S2-2: Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018).

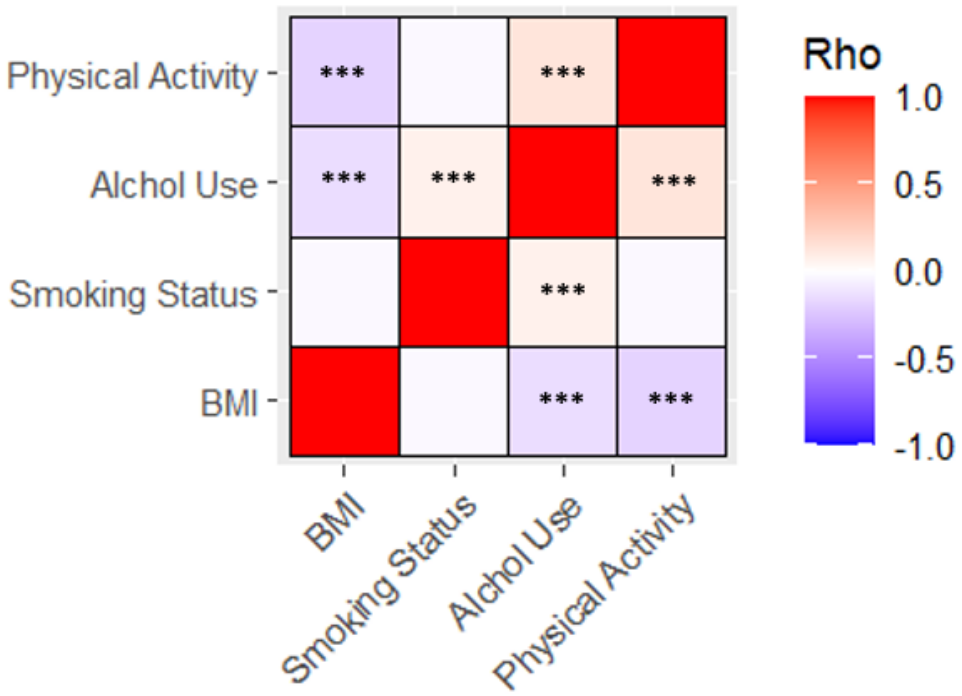
Characteristics, N(%) or mean (SD)	Included (n=2689)	Excluded (n=1329)	Effect Size (Cohen's d or Cramer's V)	P-value
Age, years	70.4 (9.5)	67.5 (9.6)	0.30	<0.001
Female	1590 (59.1)	759 (57.1)	0.02	0.22
Race/Ethnicity			0.24	
Hispanic	272 (10.1)	295 (22.2)		<0.001
Black	350 (13.0)	308 (23.2)		
White	1997 (74.3)	672 (50.6)		
Other	69 (2.6)	53 (4.0)		
Education			0.16	
No degree	346 (12.9)	329 (24.8)		<0.001
HS degree	1631 (60.7)	744 (56.0)		
College degree or higher	712 (26.5)	255 (19.2)		
Employment Status			0.26	
Working for pay	1175 (43.7)	917 (71.5)		<0.001
Not working for pay	1514 (56.3)	365 (28.5)		
Marital Status			0.08	
Married/Partnered	1905 (70.8)	812 (63.2)		<0.001
Single/Widowed/Divorced	784 (29.2)	473 (36.8)		
Have Children	2390 (88.9)	1170 (88.0)	0.08	0.41
Total Household Wealth ($)	442,619 (952,905)	304,965 (795,877)	0.15	<0.001
Smoking Status			0.07	
Never smoker	1208 (45.2)	539 (42.1)		<0.001
Former smoker	1138 (42.6)	523 (40.9)		
Current smoker	327 (12.2)	218 (17.0)		
Alcohol Use			0.06	
Never drinker	1588 (59.1)	826 (64.4)		0.002
Moderate drinker	914 (34.0)	365 (28.5)		
Heavy drinker	186 (6.9)	91 (7.1)		
Physical Activity			0.05	
Active	1591 (59.2)	692 (53.8)		0.001
Inactive	1096 (40.8)	594 (46.2)		
BMI (kg/m^2)	30.4 (6.7)	29.2 (6.5)	0.19	<0.001

Table S2-3: Pearson correlation coefficients between the 6 psychosocial stress domains.

	Acute Life Events	Financial Stress	Neighborhood Stress	Relationship Stress	Lifetime Discrimination	Childhood Adversity
Acute Life Events	1.00	0.33	0.11	0.21	0.35	0.20
Financial Stress	0.33	1.00	0.24	0.35	0.37	0.14
Neighborhood Stress	0.11	0.24	1.00	0.21	0.21	0.10
Relationship Stress	0.21	0.35	0.21	1.00	0.39	0.15
Lifetime Discrimination	0.35	0.37	0.21	0.39	1.00	0.20
Childhood Adversity	0.20	0.14	0.10	0.15	0.20	1.00

All correlations between pairs of stress domains had $p<0.001$.

Figure S2-2: Heat map of Spearman correlations between health behaviors and BMI.



***p<0.001; all other correlations had p>0.10.

Table S2-4: Association between adult psychosocial stress and the 9 CpG sites that were significant in cumulative psychosocial stress EWAS (Model 1; FDR q <0.10).

CpG Site	Functional Annotation						
	Chr	*Gene*	*Relation to CpG Site*	*Genomic Feature*	*Beta*	*P-value*	*FDR q*
cg01695954	2	*RRM2*	S_Shore	Promoter	-0.42	8.0E-10	0.00063
cg08274633	15	*C15orf53*	OpenSea	Promoter	-0.37	3.4E-07	0.076
cg17565444	14	*ASB2*	OpenSea	Body	-0.29	3.8E-07	0.076
cg24704287	19	*MIR23AHG*	N_Shore	Body	-0.63	2.1E-07	0.076
cg01766737	22	*CENPM*	N_Shore	Body	-0.12	5.9E-07	0.093
cg25737313	19	*HOOK2*	N_Shore	Body	-0.25	3.5E-06	0.26
cg12581512	14	*ASB2*	OpenSea	Body	-0.25	9.2E-06	0.28
cg20559385	6		OpenSea		-0.58	2.1E-06	0.22
cg04803208	8	*LOC100130298*	OpenSea	Body	0.49	2.2E-06	0.22

Beta is the regression coefficient representing the percent change in DNA methylation associated with a 1 standard deviation increase in the adult psychosocial stress score.

Model: CpG Site ~ Adult Psychosocial Stress Score + Age + Sex + Educational Attainment + Interview Year + Genetic Ancestry PC 1-10 + White Blood Cell Proportions + Row Position on Chip + Column Position on Chip + Sample Plate

Table S2-5: Association between the six stress domains and the and the 9 CpG sites that were significant in cumulative psychosocial stress EWAS (Model 1; FDR q <0.10).

CpG Sites	Cumulative Stress		Acute Life Events		Financial Stress		Neighborhood Stress		Relationship Stress		Lifetime Discrimination		Childhood Adversity	
	Beta	P-value	Beta	P-value	Beta	P-value	Beta	P-value	Beta	P-value	Beta	P-value	Beta	P-value
cg17565444	-0.32	3.3E-08	-0.32	1.3E-09	-0.18	0.001	-0.066	0.22	-0.13	0.023	-0.16	0.004	-0.18	0.002
cg25737313	-0.28	4.8E-07	-0.22	9.9E-06	-0.17	0.001	-0.14	0.009	-0.056	0.27	-0.16	0.002	-0.15	0.004
cg08274633	-0.42	1.5E-08	-0.28	3.1E-05	-0.3	2.1E-05	-0.12	0.093	-0.16	0.027	-0.25	4.6E-04	-0.25	3.4E-04
cg01695954	-0.40	5.7E-09	-0.26	3.6E-05	-0.34	1.9E-07	-0.17	0.008	-0.20	0.003	-0.27	5.1E-05	-0.059	0.36
cg24704287	-0.66	5.5E-08	-0.37	8.8E-04	-0.48	4.3E-05	-0.31	0.009	-0.28	0.017	-0.42	2.2E-04	-0.29	0.013
cg20559385	-0.61	8.8E-07	-0.52	5.5E-06	-0.25	0.038	-0.36	0.002	-0.25	0.031	-0.34	0.004	-0.25	0.032
cg01766737	-0.13	4.2E-07	-0.10	1.8E-05	-0.095	9.4E-05	-0.039	0.11	-0.069	0.004	-0.069	0.005	-0.044	0.064
cg12581512	-0.29	6.7E-07	-0.23	2.3E-05	-0.21	1.4E-04	-0.071	0.20	-0.13	0.021	-0.12	0.036	-0.18	0.001
cg04803208	0.51	9.4E-07	0.32	8.1E-04	0.31	0.003	0.32	0.001	0.14	0.17	0.38	1.8E-04	0.21	0.034

Beta is the regression coefficient representing the percent change in DNA methylation associated with a 1 standard deviation increase in the psychosocial domain.

Models: CpG sites ~ Stress Domain + Age + Sex + Educational Attainment + Interview Year + Genetic Ancestry PC 1-10 + White Blood Cell Proportions + Row Position on Chip + Column Position on Chip + Sample Plate

Table S2-6: Total variability of CpG sites identified in Model 1 (FDR q<0.10) explained by cumulative psychosocial stress.

CpG Sites	Percent of Variance Explained by Cumulative Psychosocial Stress[a]	
	Model 1	*Model 2*
cg17565444	0.97%	0.80%
cg25737313	0.72%	0.61%
cg08274633	1.91%	1.78%
cg01695954	1.19%	1.00%
cg24704287	0.88%	0.65%
cg20559385	1.72%	0.90%
cg01766737	1.65%	1.22%
cg12581512	0.62%	0.49%
cg04803208	4.82%	4.49%

Model 1: CpG Site ~ Psychosocial Stress Score + Age + Sex + Educational Attainment + Interview Year + Genetic Ancestry PC 1-10 + White Blood Cell Proportions + Row Position on Chip + Column Position on Chip + Sample Plate

Model 2: Model 1 + Marital Status + Employment + Has Children + Total Household Wealth

[a] Variance explained is the difference in R^2 between a full model (includes cumulative psychosocial stress score) and a reduced model (excludes cumulative psychosocial stress score).

Table S2-7: Mediation of health behaviors on the relationship between cumulative psychosocial stress and CpGs identified in Model 1 (FDR q<0.10).

	cg17565444		cg25737313		cg08274633		cg01695954		cg24704287	
	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated
Total Effect	**-0.29**	**100%**	**-0.25**	**100%**	**-0.41**	**100%**	**-0.36**	**100%**	**-0.55**	**100%**
Direct Effect	**-0.24**	**82.76%**	**-0.22**	**88.00%**	**-0.37**	**90.24%**	**-0.29**	**80.56%**	**-0.43**	**78.18%**
Indirect Effect	**-0.05**	**17.24%**	**-0.04**	**12.00%**	**-0.04**	**9.76%**	**-0.06**	**19.44%**	**-0.12**	**21.82%**
Via Former Smoker	-0.005	1.66%	-0.0009	0.38%	-0.002	0.60%	-0.0069	1.93%	-0.02	2.98%
Via Current Smoker	**-0.03**	**8.77%**	-0.01	4.44%	-0.008	1.84%	**-0.0246**	**6.89%**	-0.04	7.45%
Via Moderate Alcohol Use	-0.003	0.98%	-0.007	2.67%	-0.01	2.94%	0.0026	-0.72%	0.001	0.21%
Via Heavy Alcohol Use	-0.0003	0.11%	-0.0008	0.33%	-0.001	0.24%	0.0006	-0.16%	0.0003	-0.05%
Via Physically Active	-0.07	2.28%	0.002	-0.81%	-0.005	1.16%	-0.0076	2.14%	0.005	0.89%
Via BMI	-0.005	2.73%	**-0.02**	**6.67%**	**-0.02**	**3.96%**	**-0.0260**	**7.30%**	**-0.06**	**10.37%**

	cg01766737		cg12581512		cg20559385		cg04803208	
	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated
Total Effect	**-0.11**	**100%**	**-0.26**	**100%**	**-0.64**	**100%**	**0.49**	**100%**
Direct Effect	**-0.09**	**81.87%**	**-0.21**	**80.77%**	**-0.58**	**90.63%**	**0.40**	**81.63%**
Indirect Effect	**-0.02**	**18.13%**	**-0.05**	**19.23%**	**-0.06**	**9.38%**	**0.09**	**18.37%**
Via Former Smoker	-0.002	1.72%	-0.002	0.88%	0.004	-0.69%	0.005	1.05%
Via Current Smoker	**-0.006**	**5.42%**	**-0.02**	**7.67%**	-0.02	2.56%	0.008	1.65%
Via Moderate Alcohol Use	-0.003	2.45%	-0.002	0.93%	-0.01	1.87%	**0.02**	**3.90%**
Via Heavy Alcohol Use	-0.0002	0.22%	-0.0003	0.10%	0.0007	-0.11%	0.00006	0.01%
Via Physically Active	0.0007	0.62%	0.002	0.67%	-0.01	1.69%	0.01	3.00%
Via BMI	**-0.008**	**7.03%**	**-0.021**	**8.49%**	-0.02	3.44%	**0.04**	**8.38%**

Beta is the regression coefficient representing the percent change in DNA methylation associated with a 1 point increase in health behavior. The CpG sites have been pre-adjusted for bath effects(row position on chip, column position on chip, sample plate) and white blood cell proportions.

Bolded values represent significant associations at p<0.05.

Models: CpG Residuals~ Psychosocial Stress Score + Age + Sex + Educational Attainment + Marital Status + Employment + Has Children + Total Household Wealth + Smoking + Alcohol + Physical Activity + BMI + Interview Year + Genetic Ancestry PC 1-10

Table S2-8: Mediation of health behaviors measured in 2014 on the relationship between cumulative psychosocial stress and CpGs identified in Model 1 (FDR q<0.10) (N=2614).

	cg17565444		cg25737313		cg08274633		cg01695954		cg24704287	
	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated
Total Effect	**-0.29**	**100%**	**-0.26**	**100%**	**-0.42**	**100%**	**-0.36**	**100%**	**-0.55**	**100%**
Direct Effect	**-0.24**	**81.19%**	**-0.22**	**84.17%**	**-0.36**	**85.71%**	**-0.28**	**77.66%**	**-0.41**	**74.02%**
Indirect Effect	**-0.05**	**18.81%**	**-0.04**	**15.83%**	**-0.06**	**14.29%**	**-0.08**	**22.34%**	**-0.14**	**25.98%**
Via Former Smoker	-0.004	1.21%	-0.001	0.39%	-0.003	0.80%	-0.007	1.95%	-0.01	2.59%
Via Current Smoker	**-0.03**	**11.53%**	**-0.01**	**5.45%**	-0.008	2.03%	**0.03**	**8.26%**	**-0.05**	**8.98%**
Via Moderate Alcohol Use	-0.004	-1.33%	-0.001	0.56%	-0.005	1.32%	0.002	-0.58%	0.004	-0.80%
Via Heavy Alcohol Use	-0.001	0.34%	-0.002	0.75%	-0.005	1.15%	0.0008	-0.21%	0.004	-0.65%
Via Physically Active	-0.009	3.23%	-0.004	1.65%	-0.01	3.36%	**-0.01**	**4.00%**	**-0.03**	**5.09%**
Via BMI	-0.01	3.83%	**-0.02**	**7.04%**	**-0.02**	**5.64%**	**-0.03**	**8.91%**	**-0.06**	**10.98%**
	cg01766737		cg12581512		cg20559385		cg04803208			
	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated	Beta	% Mediated		
Total Effect	**-0.10**	**100%**	**-0.26**	**100%**	**-0.65**	**100%**	**0.49**	**100%**		
Direct Effect	**-0.08**	**81.64%**	**-0.19**	**74.06%**	**-0.58**	**89.93%**	**0.39**	**80.01%**		
Indirect Effect	**-0.02**	**18.36%**	**-0.07**	**25.94%**	**-0.07**	**10.07%**	**0.10**	**19.99%**		
Via Former Smoker	-0.002	1.58%	-0.001	0.57%	0.005	-0.73%	0.006	1.14%		
Via Current Smoker	**-0.006**	**5.57%**	**-0.03**	**10.40%**	-0.02	3.36%	0.008	1.67%		
Via Moderate Alcohol Use	-0.0005	0.51%	-0.0008	0.32%	-0.004	0.65%	0.006	1.34%		
Via Heavy Alcohol Use	-0.0007	0.71%	-0.003	1.05%	0.006	-0.92%	0.007	1.51%		
Via Physically Active	-0.003	3.06%	**-0.01**	**4.71%**	**-0.03**	**3.79%**	0.02	4.11%		
Via BMI	**-0.007**	**6.93%**	**-0.02**	**8.89%**	-0.03	3.92%	**0.05**	**10.23%**		

Beta is the regression coefficient representing the percent change in DNA methylation associated with a 1 point increase in health behavior. The CpG sites have been pre-adjusted for bath effects(row position on chip, column position on chip, sample plate) and white blood cell proportions.

Bolded values represent significant associations at p<0.05.

Models: CpG Residuals~ Psychosocial Stress Score + Age + Sex + Educational Attainment + Marital Status + Employment + Has Children + Total Household Wealth + Smoking + Alcohol + Physical Activity + BMI + Interview Year + Genetic Ancestry PC 1-10

Chapter 3 Epigenome-Wide Mediation Analysis of the Relationship between Psychosocial Stress and Cardiometabolic Risk Factors in the Health and Retirement Study (HRS)

3.1 Abstract

Exposure to psychosocial stress is linked to a variety of negative health outcomes, including cardiovascular disease and its cardiometabolic risk factors. DNA methylation has been associated with both psychosocial stress and cardiometabolic disease; however, little is known about the mediating role of DNA methylation on the association between stress and cardiometabolic risk. Thus, using the high-dimensional mediation testing (HDMT) method, we conducted an epigenome-wide mediation analysis of the relationship between psychosocial stress and ten cardiometabolic risk factors in a multi-racial/ethnic population of older adults (n=2,668) from the Health and Retirement Study (mean age=70.4 years).

A total of 50, 46, 7, and 12 CpG sites across the epigenome mediated the total effects of stress on body mass index, waist circumference, high-density lipoprotein cholesterol, and C-reactive protein, respectively. When reducing the dimensionality of the CpG mediators to their top 10 uncorrelated principal components (PC), the cumulative effect of the PCs explained between 35.8% and 46.3% of these associations.

A subset of the mediating CpG sites were associated with the expression of genes enriched in pathways related to cytokine binding and receptor activity, as well as neuron development. Findings from this study help to elucidate the underlying mechanisms through which DNA methylation partially mediates the relationship between psychosocial stress and cardiometabolic risk factors.

3.2 Introduction

Cardiovascular disease (CVD), which is a class of disorders affecting the heart and blood vessels [1,2], is the leading cause of death in the United States, accounting for nearly 930,000 deaths in 2020 alone [3]. Due to the high rates of CVD and subsequent CVD-related mortality in the U.S. and globally, substantial research has been conducted to understand risk factors associated with this disease. Several studies found that individuals at risk of CVD may have a cluster of cardiometabolic risk factors, which include hypertension, dyslipidemia, dysglycemia, and obesity [4]. Aside from these traditional risk factors, emerging evidence has shown that inflammatory profiles are independent cardiometabolic risk factors for CVD [5,6], including increased levels of C-reactive protein (CRP), a sensitive and nonspecific marker of inflammation produced in the liver [7,8].

Psychosocial stress, defined as the perception of threat that may result in discomfort and emotional strain [9], is a well-established determinant of cardiometabolic risk [10,11]. Several studies have identified associations between psychosocial stress and traditional cardiometabolic risk factors including hypertension [12], dyslipidemia [13], dysglycemia [14], and obesity [15], as well as CRP [16]. Additionally, prior research has linked psychosocial stress to epigenetic mechanisms, including DNA methylation, a biochemical modification to DNA and its related proteins that regulates gene expression without altering the underlying genetic sequence [17]. Both candidate gene studies and epigenome-wide association studies (EWAS) have examined the associations between psychosocial stress and DNA methylation [18-23]. Results from these studies showed relationships between psychosocial stress and CpG sites that mapped to genes associated with inflammation, hypertension, and coronary heart disease.

DNA methylation is also associated with the development of cardiometabolic risk factors. Large-scale EWAS have identified associations between individual CpGs and blood pressure [24], body mass index (BMI) [25], blood lipid levels [26], and CRP [27], and some of the identified CpG sites have a potential causal role in regulating genes that may influence the development of these cardiometabolic risk factors [28]. Thus, DNA methylation may act as both an upstream regulator and a downstream marker of cardiometabolic processes. Limited research suggests that DNA methylation may mediate the relationship between individual psychosocial stressors, including neighborhood disadvantage, prenatal adversity, and childhood trauma, and cardiometabolic risk [29-31]. However, to our knowledge, no study has examined the mediating effect of DNA methylation across the genome on the association between cumulative psychosocial stress across the life-course and cardiometabolic risk factors.

In this study, we conducted a high-dimensional mediation analysis to identify whether DNA methylation mediates the relationship between cumulative psychosocial stress and ten cardiometabolic risk factors. Further, we tested for enrichment of functional elements and biological pathways of the mediating CpG sites to investigate the functional role of identified genes and better understand the mechanisms through which stress may influence cardiometabolic processes in older adults.

3.3 Materials and Methods

3.3.1 Sample

Participant data are from the Health and Retirement Study (HRS), a longitudinal cohort comprised of approximately 20,000 participants in each wave that is nationally representative of Americans over age 50. Detailed information regarding the HRS protocol and study design can be found elsewhere [32,33]. In brief, HRS, which originated in 1992, consists of biennial surveys

that collect information regarding income, employment, physical and mental health, and cognitive functioning. Beginning in 2006, a random one-half of the HRS sample was selected to complete an enhanced face-to-face interview, which captured participants' physical and biological measures, including blood pressure, height, weight, and waist circumference [34]. Participants who complete the enhanced face-to-face interview are also given the Psychosocial and Lifestyle Questionnaire, a self-administered psychosocial survey capturing information on participants' well-being, lifestyle, and social support [35]. In 2008, an enhanced face-to-face interview was administered to the remaining half of the sample. Data from the enhanced interview is available longitudinally every four years for all participants.

Additionally, all panel respondents who completed an HRS interview in 2016 were invited to participate in an ancillary study, the Venous Blood Study (VBS, n=9,934), where blood-based biomarkers were assayed [36]. DNA methylation was measured in a sample of participants who completed the VBS blood draw (n=4,018). Of the 4,018 participants who had DNA methylation data, 3,260 completed and returned the Psychosocial and Lifestyle Questionnaire. Subsequently, 343 participants were removed due to missing data on relevant psychosocial stress, sociodemographic, or blood biomarker variables, leaving 2,668 participants in the final analytic sample. A flow diagram detailing participant inclusion criteria is provided in Figure 3-1.

To understand how using complete cases data impacts the representativeness of the final sample, we compared participant characteristics between those who were included (n=2668) and excluded (n=1350) in the analysis from the total DNA methylation sample. Chi-squared tests and student's t-tests were used, as appropriate. Further, to quantify the size of the difference between

the two groups, Cramer's V and Cohen's d effect sizes were calculated for categorical and continuous variables, respectively.

3.3.2 Measures

A. DNA Methylation

DNA methylation data from HRS were assessed from whole blood samples using the Illumina Infinium HumanMethylationEPIC v.1.0 chip. Samples were randomized across plates by key demographic variables, including age, cohort, sex, education, and race/ethnicity with 39 pairs of blinded duplicates [37]. The *minfi* R package was used for data preprocessing and quality control, resulting in the removal of 3.4% of methylation probes due to their detection p-value falling below the threshold of 0.01 (n = 29,431 out of 866,091) [38]. Sex mismatched samples and any controls (cell lines, blinded duplicates) were removed from the analysis, as were probes with missing data for >5% of the sample. Additionally, we removed cross-reactive probes that target repetitive sequences, potentially mapping to multiple places in the genome [39]. Missing DNA methylation data was imputed using the mean of each CpG [40]. Finally, to ensure that strong outliers were not impacting the results, we winsorized methylation values greater than 3 times the interquartile range (IQR) of the 25th and the 75th percentiles [41,42]. In total, 789,656 CpGs were available for analysis after quality control. DNA methylation was quantified using beta values, which approximate the proportion of methylation, making them biologically interpretable. To account for cell-type heterogeneity, we pre-adjusted DNA methylation by regressing each methylation beta value on white blood cell proportions estimated by the Houseman method [43]. Additionally, methylation sample plate and position (sample plate row and column) were added as random effects to account for potential batch effects.

B. Psychosocial Stress

Data from the Psychosocial and Lifestyle Questionnaires were used to construct a cumulative psychosocial stress score [35]. Because this survey was administered to only half the HRS participants at each wave, we utilized data from both the 2010 and 2012 psychosocial surveys to ensure all participants had the opportunity to respond. Furthermore, we measured psychosocial stress at these specific timepoints as they were the only set of two consecutive timepoints that captured all of the individual stress measures needed to create the cumulative stress score.

Based on previous HRS studies, we examined six domains of psychosocial stress: 1) acute life events, 2) financial stress, 3) neighborhood stress, 4) relationship stress, 5) lifetime discrimination, and 6) childhood adversity [23,44,45]. Because the focus of this study was to understand how lifetime stress exposure affects DNA methylation and subsequent cardiometabolic risk, we evaluated both acute and chronic stressors spanning the life course. Each domain included one or more individual stress measures, detailed below and in Table S3-1. For an individual measure to be considered complete, participants had to have answered at least 80% of the questions within the measure, unless otherwise noted in the measurement scoring instructions [46]. Only participants who had completed all stress measures were included in the analysis. Each stress measure was transformed into a z-score. For domains comprised of multiple stress measures, z-scores from individual measures were summed together and subsequently standardized, allowing for cross-domain comparisons [47,48].

The six domains of stress are as follows: 1) *Acute life events,* which included two measures: (i) acute lifetime traumas (seven items) and (ii) acute stressful life events in the past 5 years (six items); 2) *Financial stress,* which included two measures: (i) financial strain (two

items) and (ii) lack of financial autonomy (two items); *Neighborhood stress*, included one measure (four items), related to neighborhood safety, vandalism, and cleanliness; *Relationship stress*, which included four measures: (i) spouses (four items), (ii) children (four items), (iii) other family members (four items), and (iv) friends (four items) 5) *Lifetime discrimination*, which included two measures: (i) perceived everyday discrimination (five items) and (ii) major discriminatory events (six items); and 6) *Childhood adversity*, which included one measure related to lifetime traumas in adolescence (four items).

Finally, we calculated a cumulative psychosocial stress score by summing the standardized scores from each of the six domains and re-standardizing the summed scores [44]. This cumulative score represents the total stress exposure across all stress domains, and it is the primary exposure of interest in this study.

C. Cardiometabolic Risk Factors

We examined ten cardiometabolic risk factors, including body mass index (BMI), waist circumference (WC), systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), and high-sensitivity C-reactive protein (CRP). Measurement methods have been described elsewhere [34,36]. Glucose, TC, LDL-C, HDL-C, TG, and CRP measurements were collected from the VBS blood draw (2016). Fasting was recommended, but not required, and participant's fasting status was recorded by the phlebotomist. We log-transformed glucose and CRP measures due to the skewed nature of the data. BMI, WC, SBP, and DBP were measured during the participant's enhanced face-to-face interview (2016 or 2018). BMI was calculated from the participant's weight in kilograms divided

by the square of their height in meters. For participants who did not have their height and weight measured during the enhanced interview, we used their self-reported BMI.

For each cardiometabolic risk factor, we removed extreme outliers, defined as values greater than 3 times the IQR of the 25th and the 75th percentiles. Additionally, we adjusted select cardiometabolic risk factors for participant medication use, as appropriate, based on previously reported studies. For participants who self-reported taking hypertension medication, we added 15 Hg and 10 Hg to their SBP and DBP measures, respectively [49,50]. For participants who reported using statin medication, we divided their LDL-C levels by 0.7 and their TC levels by 0.8 [51,52]. HDL-C and TG were not adjusted for lipid-lowering medication use [52,53]. Finally, when assessing the total effect of psychosocial stress on glucose, we removed any participants who self-reported use of diabetes medication [54,55].

D. Covariates

Sociodemographic characteristics were collected at the 2010 or 2012 interview, concurrent with the timing of the participant's psychosocial stress assessment. These variables included: age, sex (male or female), educational attainment (no degree, high school degree, college degree or higher), and smoking status (never, former, current). Additionally, the top 10 principal components (PCs) of genetic ancestry, which were estimated using genome-wide genotype data [56], were used as model covariates to account for population stratification and admixture.

3.3.3 Statistical Analysis

A. Associations between Psychosocial Stress and Cardiometabolic Risk Factors

We first examined whether cumulative psychosocial stress was associated with the ten cardiometabolic risk factors (total effects), using linear regression models adjusted for age, sex, educational attainment, year of psychosocial stress measurement, and genetic ancestry PCs; fasting status was also included in models for glucose, HDL-C, LDL-C, and TG (Model 1). Furthermore, since smoking is a known risk factor for cardiometabolic disease and may be on the causal pathway between psychosocial stress and cardiometabolic risk factors, we were interested in the associations between stress and cardiometabolic risk both before and after controlling for any mediating effects of smoking. Thus, we further adjusted the total effect models for smoking (Model 2). Associations between psychosocial stress and cardiometabolic risk factors with $p<0.05$ in Model 1 were selected for mediation analysis.

Additionally, because fasting status is known to greatly affect the measurement of certain blood-based cardiometabolic risk factors, we conducted a sensitivity analysis to examine the associations between cumulative psychosocial stress and glucose, HDL-C, LDL-C, and TG, restricting the sample to participants who fasted for their VBS blood draw.

B. Mediation Analysis

For significant associations identified between psychosocial stress and cardiometabolic risk factors, we conducted an epigenome-wide mediation analysis to identify CpG sites that may mediate the relationship between stress and the relevant risk factors. The mediation effect was defined as the product of the exposure-mediator effect (α) and mediator-outcome effect (β), adjusting for the exposure [57]. We used regression models to estimate: (1) the association of psychosocial stress with each DNA methylation site (Equation 1) and (2) the association of each DNA methylation site with each cardiometabolic risk factor, adjusting for psychosocial stress (Equation 2).

$$M_j = \alpha_{0,j} + \alpha_j X + \alpha_{C,j}^T C + \varepsilon_{M,j}\,, (\boldsymbol{Equation\ 1})$$

$$Y = \beta_{0,j} + \beta_{X,j} X + \beta_j M_j + \beta_{C,j}^T C + \varepsilon_{Y,j}. (\boldsymbol{Equation\ 2})$$

In these equations, which represent the exposure-mediator and mediator-outcome relationships for a single individual, M_j represents DNA methylation (beta value) for j=1,2…J mediators, X represents cumulative psychosocial stress, C is the set of potential confounders (age, sex, educational attainment, 10 genetic ancestry PCs), Y represents a single cardiometabolic risk factor, and $\varepsilon_{M,j}$ and $\varepsilon_{Y,j}$ are residual errors, following an independent and normal distribution.

We evaluated the epigenetic mediation effect by testing: $H_{0,j}$: $\alpha_j * \beta_j = 0$ vs. $H_{1,j}$: $\alpha_j * \beta_j \neq 0$ for j=1,2,…J mediators using the high-dimensional mediation testing (HDMT) method. This method uses a corrected reference distribution for the MaxP statistic, max ($p_{\alpha_j}, p_{\beta_j}$), with p_{α_j} and p_{β_j} representing the p-values for testing $\alpha_j = 0$ and $\beta_j = 0$ in Equations 1 and 2, respectively [58]. HDMT accounts for the compositional nature of the null hypothesis through a mixture reference distribution. In low power settings (i.e. over half a million CpG sites being investigated, each likely with a small mediation effect), HDMT is advantageous because it increases power by providing finite sample-size adjustment for p-values. Additionally, this method controls for false positive rates while also yielding high true positive rates better than other high-dimensional mediation methods that do not use a mixture reference distribution, including Sobel's test and MaxP test [59]. Epigenome-wide mediation analysis was conducted using the *medScan* package in R. We corrected for multiple testing using the false discovery rate (FDR), where a CpG was considered a mediating site if FDR q<0.05.

Due to the possible correlation between CpGs within close proximity to one another, we were unable to quantify the overall mediation effects of each set of CpG mediators using the HDMT method. Thus, we performed a PC analysis on the identified CpG mediators for each

cardiometabolic risk factor [29]. PC analysis reduces the dimensionality of the CpG mediators and generates methylation PCs that are uncorrelated. Therefore, the cumulative effect of the top PCs represents the lower bound of the mediation effect across all CpG mediators. After calculating the top 10 PCs for each set of CpG mediators, we used the *mediate* package in R to test and estimate the mediation effect of each methylation PC individually [60]. Using non-parametric bootstrapping with 10,000 iterations, we obtained the average mediation effect estimate, as well as the proportion of the total effect explained by each methylation PC.

Additionally, for each set of CpG mediators identified in HDMT, we conducted a penalized regression based high dimensional mediation analysis using the *hima2()* function in R [61], which allowed us to determine the CpG mediators that remain significant after adjusting for all other mediating CpGs. For each cardiometabolic risk factor, we fit a multivariate mediation model, assigning all CpGs identified in HDMT as the mediators. A de-biased LASSO regression was applied to estimate the regression parameters of each CpG site on a given cardiometabolic risk factor, controlling for the other CpGs in the model [62]. To determine the significance of the mediation effect, we performed a joint significance test with a mixture of null distributions from the exposure-mediator and mediator-outcome models to accurately control for the false discovery rate. A CpG site was considered significant when FDR $q<0.05$.

Finally, to better understand the epigenetic mediation of the association between psychosocial stress and cardiometabolic risk factors independent of smoking, we conducted sensitivity analyses by further adjusting all HDMT, PC, and de-biased LASSO based mediation models for smoking status.

C. Functional Characterization of CpG Mediators

We performed genomic feature enrichment analysis to examine whether the locations of each set of mediating CpGs were enriched for genomic features, including gene promoters, enhancers, DNase I hypersensitivity sites (DHS), CpG islands (CGI), and CpG island flanking shores or shelves. CpG sites were considered to be in the promoter region if they were located less than 1.5 kb upstream of a transcriptional start site. CpGs were designated as CGI flanking shores or shelves if they were within 2 kb or between 2-4 kb of a CGI, respectively. We used annotation files from both Illumina and the UCSC genome browser to identify the proximal genes and genomic features associated with each CpG [63,64].

Additionally, to assess whether the identified CpGs were associated with gene expression, we used empirical data from Framingham Heart Study (FHS) [65]. Keshawarz et al. (2023) performed expression quantitative trait methylation (eQTM) analysis to detect CpG sites whose methylation levels were associated with gene expression. In total, they identified 70,047 cis-eQTMs (CpG-transcript pairs located <1 Mb apart) and 246,667 trans-eQTMs (CpG-transcript pairs located >1 Mb apart) in whole blood samples at significance levels of $P \leq 1E\text{-}07$ and $P \leq 1E\text{-}14$, respectively. To test whether each set of mediating CpGs were more likely to be enriched as eQTMs, we compared the number of significant CpG sites that mapped to gene transcripts in the eQTMs versus the number of non-significant CpGs that mapped to transcripts. Enrichment analyses were performed using a two-sided Fisher's exact test.

To better understand the underlying biological pathways of the CpG mediators, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses for each cardiometabolic risk factor. We first performed a gene-set analysis using the gometh() function from the R package *missMethyl* [66]. This method accounts for potential sources of bias, including the uneven distribution of CpGs across the genome, as well

as CpGs that are annotated to multiple genes. However, one constraint of this method is that it uses annotation files to map CpG sites to their proximal genes based on chromosomal position. Because CpGs do not always act on the nearest gene, but instead may affect the expression of distal genes, we performed a second enrichment analysis using enrichGO() and enrichKEGG() functions in the *clusterProfiler* R package [29,67]. This method allows us to pre-define the gene list based on existing CpG-gene expression pairs, providing a more robust examination of functional pathways. Using the FHS eQTM dataset, genes known to be associated with each set of CpG mediators were extracted and used as the signal gene list. The background gene list included all genes associated with CpG sites that appear in both the FHS study and our study. GO terms and KEGG pathways with FDR $q<0.01$ were considered significant for all gene-set analyses.

3.4 Results

3.4.1 Descriptive Statistics

Sample characteristics are displayed in Table 3-1. The mean age of participants was 70.4 years (SD: 9.5) and the majority were female (59%). Over a quarter of participants had a college degree or higher (26%). Additionally, 74% of participants were non-Hispanic white, 13% were non-Hispanic Black, 10% were Hispanic, and 3% were another race or ethnicity. Approximately 45% of the participants never smoked, while 43% formerly smoked and 12% currently smoked at the time of interview. Half the participants reported taking lipid lowering medication, 57% reported taking antihypertensive medication, and 21% reported taking diabetes medication. Participants from the DNA methylation sample with complete data on stress, blood biomarkers, and covariates were older, had higher educational attainment, and had healthier smoking habits

than those who were excluded; however, the effect sizes were relatively small, with a Cramer's V and Cohen's d <0.30 for all covariates (Table S3-2).

3.4.2 Associations between Psychosocial Stress and Cardiometabolic Risk Factors

After adjusting for age, sex, education, year of psychosocial stress measurement, and top 10 genetic PCs, along with fasting status, when appropriate, an increase in cumulative psychosocial stress was associated with higher BMI, WC, DBP, and CRP, in addition to lower HDL-C (Model 1; Table 3-2). All of these associations remained significant after further adjusting for smoking (Model 2; Table 3-2).

When restricting the sample to only participants who fasted for the VBS blood draw, the associations between psychosocial stress and glucose, TC, HDL-C, LDL-C, and TG were substantively similar to results from the main total effect models (Table S3-3).

3.4.3 Mediation Analysis

For associations between stress and cardiometabolic risk factors with $p<0.05$, we tested for epigenetic mediation, adjusting for the same covariates used in Model 1. We identified 50, 46, 7, and 12 CpGs that significantly mediated the associations between psychosocial stress and BMI, WC, HDL-C, and CRP, respectively (Figures S3-1 to S3-4). No CpGs were found to mediate the relationship between stress and DBP. A summary of the mediation results for each cardiometabolic risk factor can be found in Table 3-3.

A. Psychosocial Stress and BMI

Cumulative psychosocial stress explained 16.5% of the total variability in BMI after adjusting for age, sex, education, year of psychosocial stress measurement, and 10 genetic PCs. Through high-dimensional mediation testing, we identified 50 CpG sites that mediated between

4.3% and 11.0% of the total effect of stress on BMI. Complete summary statistics and gene annotations for these CpG mediators are provided in Table S3-4. Additionally, Manhattan and QQ-plots from the epigenome-wide mediation analysis can be found in Figure S3-1.

The first 10 epigenetic PCs accounted for 51.2% of the variability across the 50 mediating CpGs. After performing individual mediation analyses on each of the PCs, 5 of the 10 PCs (PC1, PC3, PC5, PC6, PC8) significantly mediated the association between stress and BMI, explaining between 2.0% and 25.4% of the total effect (Table S3-5). Cumulatively, these 5 PCs mediated 46.3% of the association between stress and BMI. When performing the de-biased LASSO based mediation model, 9 of the 50 CpG sites showed evidence of mediation after adjusting for the other CpG mediators (Table S3-6). Individually, these 9 CpGs mediated between 3.3% and 9.2% of the total effect of psychosocial stress on BMI.

B. Psychosocial Stress and WC

After adjusting for Model 1 covariates, cumulative psychosocial stress explained 16.7% of the total variability in WC. Between 4.2% and 10.6% of this total effect of stress on WC was mediated by 46 CpG sites (Table S3-7). Corresponding Manhattan and QQ-plots can be found in Figure S3-2.

The first 10 epigenetic PCs accounted for 55.7% of the variability across the 46 mediating CpGs. Five PCs (PC1, PC4, PC5, PC8, PC9) significantly mediated the association between psychosocial stress and WC, explaining between 1.8% and 17.5% of the total effect (Table S3-8). Cumulatively, these 5 PCs mediated 35.9% of the relationship between psychosocial stress and WC. Results from the penalized-based high dimensional mediation analysis showed that 6 of the 46 CpG sites identified through HDMT were mediators of the

stress-WC association after adjusting for all other CpGs (Table S3-9). Individually, these 6 CpGs mediated between 3.7% and 8.4% of the total effect of psychosocial stress on WC.

C. Psychosocial Stress and HDL-C

Cumulative psychosocial stress explained 3.1% of the total variability in HDL-C after adjusting for Model 1 covariates. Of this relatively small total effect, between 9.4% and 14.5% was mediated by 7 CpGs (Table S3-10 and Figure S3-3).

Due to the small number of mediating CpGs identified in the epigenome-wide mediation analysis, we extracted only the top 5 epigenetic PCs, which accounted for 87.7% of the variability across the 7 mediating CpGs. We found that PC1 and PC2 significantly mediated 22.9% and 8.5% of the association between stress and HDL-C, respectively (Table S3-11). Cumulatively, 31.4% of the relationship between psychosocial stress and HDL-C was due to the epigenetic mediation of the 7 identified CpGs. In the penalized-based mediation analysis, 3 of the 7 CpGs showed evidence of mediation after adjusting for the other CpG mediators (Table S3-12). Cg04803208, cg25607249, and cg08274633 mediated 13.0%, 8.4%, and 6.8% of the total effect of psychosocial stress on HDL-C, respectively.

D. Psychosocial Stress and CRP

After adjusting for Model 1 covariates, cumulative psychosocial stress explained 15.6% of the total variability in CRP. Of this total effect, between 6.5% and 11.4% was mediated by 12 CpG sites identified by HDMT (Table S3-13 and Figure S3-4).

The first 10 epigenetic PCs accounted for 92.3% of the variability across the 12 mediating CpGs. PC1, PC2 and PC3 significantly mediated the association between stress and CRP, explaining 27.5%, 14.0%, and 3.6% of the total effect, respectively (Table S3-14).

Cumulatively, 45.1% of the relationship between psychosocial stress and CRP could be attributed to epigenetic mediation by the 12 identified CpGs. Furthermore, results from the de-biased LASSO mediation analysis showed that 7 of the 12 CpG sites mediated between 4.0% and 9.3% of the relationship between psychosocial stress and CRP after adjusting for all other CpG mediators (Table S3-15).

E. Sensitivity Analysis Adjusting for Smoking Status

Because we were interested in the epigenetic mediation of the association between stress and cardiometabolic risk factors independent of smoking, we further adjusted all mediation models for smoking status. Compared to results from the primary epigenome-wide mediation models, the mediation effects of CpGs across all cardiometabolic outcomes were slightly attenuated after accounting for smoking; however, all CpGs remained nominally significant ($p<5.6E-05$). Additionally, 25, 9, 3, and 2 CpGs found to mediate the relationships between psychosocial stress and BMI, WC, HDL-C, and CRP, respectively, remained FDR significant after adjusting for smoking (FDR $q<0.05$). Further adjusting the methylation PC and penalized-based mediation models for smoking yielded substantively similar results across all cardiometabolic outcomes (Model 2; Tables S3-4:S3-15).

3.4.4 Overlap of CpG Mediators among Cardiometabolic Risk Factors

In total, we identified 75 distinct CpGs that mediated the relationship between stress and at least one cardiometabolic risk factor (Table S3-16). Overall, 22, 17, and 4 of these CpGs uniquely mediated the relationships between stress and BMI, WC, and CRP, respectively. No CpGs exclusively mediated the association between stress and HDL-C. The remaining 32 CpGs were identified as mediators in at least two epigenome-wide mediation analyses (Figure 3-2).

Two CpGs in particular, cg04803208 and cg25607249, were found to mediate the relationship between stress and all four cardiometabolic risk factors.

3.4.5 Genomic Feature Enrichment Analysis

Based on the Illumina annotation mapping, compared to non-mediating CpG sites, CpGs that mediated the relationships between psychosocial stress and BMI, WC, and CRP were more likely to reside in enhancer regions, suggesting these CpGs may be involved in the regulation of nearby gene expression (Table 3-4; all $p<0.001$). We observed no significant enrichment of these CpGs in promoter regions, DHS, CGIs, or CGI flanking shores or shelves, nor did we detect enrichment of any genomic features for the CpGs that mediated the association between stress and HDL-C.

Overall, 36,662 of the 789,656 (4.6%) CpGs considered in the epigenome-wide mediation analysis mapped to at least one gene transcript using eQTM data from Keshawarz et al. (2023). In our study, 16 (36.0%), 12 (26.1%), 2 (28.6%) and 5 (41.7%) of the identified CpG mediators for BMI, WC, HDL-C, and CRP, respectively, mapped to gene transcripts in the eQTMs. Compared to the non-mediating CpG sites, CpG mediators of all four cardiometabolic risk factors were enriched for eQTMs, suggesting the mediating CpGs identified by HDMT were more likely to influence gene expression (Table 3-4; $p<0.05$).

3.4.6 Gene-set Enrichment Analysis

CpGs found to mediate the relationships between stress and BMI, WC, HDL-C, and CRP were located throughout the genome, mapping to 37, 30, 4, and 10 unique genes, respectively. When using the Illumina annotation files for gene mapping, we did not observe enrichment of GO terms or KEGG pathways for CpGs mediating the associations between stress and any cardiometabolic risk factor (FDR $q<0.01$). However, when performing the gene-set analysis

using eQTM data from FHS, we found that CpGs mediating the relationship between stress and each cardiometabolic risk factor were associated with the expression of genes enriched for several GO terms and KEGG pathways. CpGs that mediated the relationship between stress and BMI were associated with genes enriched for 10 GO terms and 2 KEGG pathways related to cytokine binding and receptor activity, cell surface, peptide receptor activity, and coreceptor activity (Figure S3-5). Similarly, CpG mediators of the relationship between stress and WC were associated with genes enriched for 7 GO terms and 1 KEGG pathway related to cytokine binding and receptor activity, cell surface, and transmembrane signaling activity (Figure S3-6). The mediating CpGs of the stress and HDL-C relationship were associated with genes enriched in 7 GO terms, including biological processes related to cytokine binding activity, cell surface, and neuron development (Figure S3-7). We did not observe over-representation of KEGG pathways for these genes. Finally, the CpG mediators of the relationship between stress and CRP were associated with genes enriched for 11 GO terms and 2 KEGG pathways (Figure S3-8), including pathways involving cytokine binding and receptor activity, cell surface, cell morphogenesis, and neuron development.

3.5 Discussion

This study evaluated the role of epigenetic mediation on the relationship between cumulative psychosocial stress and cardiometabolic risk factors in a multi-racial/ethnic cohort of older adults. Epigenome-wide mediation analysis identified 50, 46, 7, and 12 CpG sites that mediated the total effect of stress on BMI, WC, HDL-C, and CRP, respectively. When reducing the dimensionality of the CpG mediators to their top uncorrelated PCs, the overall mediation effects across probes explained between 35.8% and 46.3% of these associations. Additionally, a number of independent CpGs were found to mediate the relationship between stress and each of

the cardiometabolic risk factors, even after adjusting for all other mediating CpGs. Further adjustment of the mediation models for smoking slightly attenuated the mediation effects, indicating that smoking may be part of the pathway between stress and these risk factors; however, CpG mediators across all cardiometabolic risk factors remained nominally significant. In epigenome-wide mediation analysis, we identified 32 distinct CpGs that mediated the association between psychosocial stress and multiple cardiometabolic risk factors. In particular, two CpGs, cg04803208 and cg25607249, mediated the relationships between stress and BMI, WC, HDL-C, and CRP, suggesting these methylation sites may play an important role on influencing cardiometabolic risk. Cg04803208 annotates to the *LOC100130298* gene on chromosome 8, which is a member of the long non-coding class of RNAs (lncRNA) [68]. LncRNAs play a critical role in gene expression through transcriptional, translational, and epigenetic regulation [69]. In large-scale EWAS, this CpG has been previously associated with type 2 diabetes, ischemic heart disease, and inflammatory Crohn's disease [70,71]. Cg25607249 maps to the gene body of the solute carrier family 1 member 5 (*SLC1A5*) gene, located on chromosome 19. *SLC1A5*, which encodes for the ASCT2 protein, is a sodium-dependent neutral amino acid transporter that plays a role in cellular glutamine homeostasis and is a critical regulator for cancer development and tumor growth [72,73]. Prior research has established a link between *SLC1A5* and cardiovascular disease. Kennel et al. (2019) found that the mRNA levels and protein expression of *SLC1A5* in heart failure patients were suppressed compared to healthy controls [74]. Additionally, Westerman et al. (2019) discovered a region in *SLC1A5* (chr19:47287777-47288263) to be differentially methylated with respect to incident cardiovascular disease in the Women's Health Initiative cohort, with results replicated in the Framingham Heart Study [75]. Interestingly, cg25607249, in addition to two other CpG

mediators found in our HDMT results, cg24507742 (significant in WC and HDL models) and cg01406381 (significant in BMI and WC models), fall within this differentially methylated region, providing further evidence that these CpGs may be associated with cardiometabolic risk factors.

Several top CpGs reported in this study have been previously associated with CVD outcomes and their corresponding risk factors. Cg00420390, which was found to independently mediate the relationships between stress and BMI, WC, and CRP in the penalized-based mediation models, annotates to the mitotic arrest deficient 1 like 1 (*MAD1L1*) gene on chromosome 7. *MAD1L1* is a mitotic spindle assembly checkpoint gene that plays a critical role in cell cycle regulation by inhibiting the transition to anaphase until all chromosomes are properly aligned during metaphase [76]. This gene has been linked to a number of psychiatric conditions, including anxiety and depressive phenotypes [77,78], schizophrenia [79], and bipolar disorder [80]. Additionally, emerging research has found *MAD1L1* to be a potential candidate gene for cardiovascular disease [81,82].

Another CpG that independently mediated the relationships between stress and both BMI and WC, cg02370100, maps to the ATP binding cassette subfamily G member 1 (*ABCG1*) gene on chromosome 21. *ABCG1* is a transporter gene that plays a critical role in cellular lipid regulation by promoting the outflow of cholesterol to high density lipoproteins [83]. Further, this gene participates in glucose metabolism by regulating insulin secretion. Due to its significant involvement in cholesterol homeostasis, *ABCG1* has been identified as a potential therapeutic target for atherosclerotic CVD [84]. Large-scale EWAS found cg02370100 to be associated with type 2 diabetes [70], serum triglycerides [85], and fasting insulin [86].

A subset of the mediating CpG eQTMs were associated with the expression of genes enriched in cytokine activity, including cytokine binding, cytokine receptor activity, and cytokine-cytokine receptor interaction. These findings support existing evidence that the upregulation of inflammatory cytokines predicts both cardiovascular disease and its cardiometabolic risk factors [87,88]. Wu et al. (2020) found that obesity is associated with low-grade inflammation resulting from an increase in inflammatory cytokines in a variety of tissues, including adipose, skeletal muscle, and brain [89]. In turn, this inflammation has been thought to cause obesity-linked metabolic dysregulation, leading to insulin resistance and type 2 diabetes. Additionally, prior research has shown that chronic stress increases inflammatory cytokine activity in both animals and humans [90-92]; however, the association between stress and inflammatory pathways, particularly involving cytokines, is complicated, and more research needs to be done to better elucidate that relationship [93]. Overall, this study provides evidence that the methylation of inflammatory genes may mediate the relationship between psychosocial stress and cardiometabolic risk factors through mechanisms related to cytokine activity. Additionally, CpG mediators of the relationships between stress and both HDL-C and CRP were associated with expression of genes enriched in neuron development, including cell morphogenesis involved in neuron differentiation, axon development, synapse organization, and axonogenesis. Chronic stress has been found to influence neuronal structure and function through the release of glucocorticoids (GCs), which are hormones secreted from the adrenal gland in response to stress [94]. Although GCs normally regulate themselves through negative feedback mechanisms to restore homeostasis, elevated GC levels due to prolonged stress exposure provoke structural remodeling of neurons and synaptic structure [95]. Further, hypersecretion of GCs due to chronic stress has been found to negatively impact health

outcomes, including increased cardiovascular risk [96]. Future research should be conducted to better characterize the pathways of genes related to neuron development involved in mediating the relationship between stress and cardiometabolic risk factors.

Due to the novelty of our research question, we were unable to replicate our findings in an independent cohort of multi-ancestry older adults. However, we did observe overlap between our findings and those of Wang et al. (2022), which examined the epigenetic mediation of the relationship between neighborhood disadvantage, a single psychosocial stressor, and cardiovascular disease risk factors [29]. Specifically, two CpGs (cg02508743 and cg09315878) that were identified as mediators of the relationship between cumulative psychosocial stress and BMI in our study (p=1.2E-06 and p=2.7E-06, respectively) were also found to mediate the relationship between adult socioeconomic status and BMI in Wang et al. (p=3.5E-09 and p= 3.9E-06, respectively). Both cg02508743, which maps to the *LYN* proto-oncogene on chromosome 8, and cg09315878, which maps to the stromal cell derived factor 4 (*SDF4*) gene on chromosome 1, have been discovered in multiple EWAS of BMI and maternal obesity [97-100]. As a result, these methylation sites should be considered in future studies to confirm their role in the epigenetic mediation of psychosocial stressors and cardiometabolic risk.

There are limitations to this study that should be acknowledged. First, the cumulative psychosocial stress score was comprised of only self-reported stress measures, most of which captured stress retrospectively (i.e., childhood adversity, lifetime trauma, stressful life events). Because of the time lag between when participants may have experienced a stressful event and when they were administered the survey, there is potential for differential misclassification of the stress exposure, where individuals who experienced greater cumulative stress across the life course may be more likely to report past stressors than those who were relatively unaffected by

stress. In addition, some of the downstream health consequences of psychosocial stress, including depression and post-traumatic stress disorder, may influence the likelihood of a participant endorsing a stress exposure [101]. To get a more comprehensive definition of psychosocial stress, future analyses should include additional measures of stress, as well as objective biological markers such as cortisol, alpha-amylase, and pro-inflammatory cytokines [102]. Additionally, the cardiometabolic risk factors examined in this study are often on the same disease pathway [103-106], suggesting that the CpG mediators identified in epigenome-wide mediation analysis are likely not independent for each outcome.

Due to the short timeframe between the measurement of psychosocial stress, DNA methylation and cardiometabolic risk factors, and because DNA methylation and cardiometabolic risk factors assessed through the VBS were measured concurrently (2016), we cannot confidently assert the causal direction of effect between the exposure, mediator, and outcome variables. As additional epigenetic, blood biomarker, and cardiometabolic risk factor data becomes available in HRS and other studies, future analyses should assess measures across multiple time points to establish a temporal relationship between psychosocial stress and cardiometabolic risk factors mediated by the epigenome.

Finally, CpG mediators that are in close proximity to one another and on the same causal pathway may be highly correlated. However, most mediation methods that examine high-dimensional DNA methylation data, including HDMT, do not explicitly model the correlation among potential CpG mediators and instead assess their contributions univariately [107]. Because we were interested in the epigenetic mediation of the association between stress and cardiometabolic risk factors across the epigenome, we did not have the computational power to account for the correlations between the nearly 800,000 CpGs assessed in high-dimensional

mediation analyses. To address this limitation, we conducted PC analysis to characterize the overall mediation effect across identified CpGs in HDMT and account for possible correlation among those CpG sites [29].

This study had several key strengths. To our knowledge, this was the first study to utilize a high dimensional mediation testing method, HDMT, to formally test the mediation effect of CpGs across the epigenome on the relationship between cumulative stress and cardiometabolic risk factors. This method used a corrected mixture reference distribution utilizing the composite structure of the null hypothesis, thus providing well-calibrated p-values under the null. Further, we utilized a composite psychosocial stress score that captured several stress domains across the life course, allowing us to assess the cumulative impact of stress on DNA methylation and subsequent cardiometabolic risk factors, as opposed to the influence of a single stressor. Lastly, this research was conducted in a multi-racial/ethnic population of European, African, and Hispanic/Latino participants, which allowed us to examine the epigenetic mediation of psychosocial stress on cardiometabolic risk in a diverse population.

3.6 Conclusion

In summary, results from this study demonstrate that DNA methylation partially mediates the relationship between cumulative psychosocial stress and several cardiometabolic risk factors. Furthermore, a subset of the mediating CpG eQTMs found in the epigenome-wide analysis were associated with expression of genes enriched in pathways related to cytokine binding and receptor activity, as well as neuron development. Future studies should be conducted to replicate these findings and further elucidate the mechanisms through which DNA methylation mediates the effect of psychosocial stress on cardiometabolic risk.

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3.8 Tables

Table 3-1: Characteristics of the Health and Retirement Study analytic sample (N=2,668).

Characteristics	N (%) or mean (SD)
Age, years	70.4 (9.5)
Female	1575 (59.0)
Race/Ethnicity	
Hispanic	269 (10.1)
Black	349 (13.1)
White	1981 (74.3)
Other	68 (2.5)
Education	
No degree	346 (13.0)
HS degree	1617 (60.6)
College degree or higher	705 (26.4)
Smoking Status	
Never smoker	1207 (45.2)
Former smoker	1134 (42.5)
Current smoker	327 (12.3)
Medication Use	
Lipid lowering medication (n=2665)	1335 (50.1)
Antihypertensive medication (n=2352)	1354 (57.6)
Diabetes medication (n=2654)	546 (20.6)
Cardiometabolic Risk Factors	
Body mass index (*kg/m*^{2}, n=2537)	30.1 (6.7)
Waist circumference (*cm*, n=2346)	40.8 (6.2)
Glucose (*mg/dL*, n=2100)	99.7 (21.3)
Systolic blood pressure (*mm Hg*, n=2363) [a]	127.0 (17.9)
Diastolic blood pressure (*mm Hg*, n=2363) [a]	75.7 (10.4)
Total cholesterol (*mg/dL*, n=2664) [a]	186.8 (41.3)
High-density lipoprotein cholesterol (*mg/dL*)	57.7 (19.1)
Low-density lipoprotein cholesterol (*mg/dL*, n=2611) [a]	100.6 (34.7)
Triglycerides (*mg/dL*, n=2631)	138.7 (68.8)
C-reactive protein (*mg/dL*, n=2666)	4.8 (10.1)

[a] Values reported are prior to adjustment for medication use

Table 3-2: Total effect of cumulative psychosocial stress on cardiometabolic risk factors (N=2,668).

Cardiometabolic Risk Factors	*Cumulative Psychosocial Stress*			
	Model 1		*Model 2*	
	Beta	**P-value**	**Beta**	**P-value**
BMI	**0.80**	**1.15E-08**	**0.87**	**6.29E-10**
WC	**0.92**	**3.79E-12**	**0.95**	**1.28E-12**
SBP[a]	0.38	0.399	0.44	0.335
DBP[a]	**0.58**	**0.026**	**0.60**	**0.021**
Log (Glucose)[a,b]	0.006	0.181	0.006	0.187
TC[a,b]	-1.74	0.058	-1.71	0.064
LDL-C[a,b]	-1.09	0.203	-1.01	0.238
HDL-C[b]	**-1.48**	**7.67E-05**	**-1.46**	**1.25E-04**
TG[b]	2.70	0.056	2.71	0.056
Log (CRP)	**0.10**	**3.34E-06**	**0.10**	**3.68E-05**

Abbreviations: Body mass index (BMI); Waist circumference (WC); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Total cholesterol (TC); Low-density lipoprotein cholesterol (LDL-C); High-density lipoprotein cholesterol (HDL-C); Triglycerides (TG); C-reactive protein (CRP)

Model 1: Cardiometabolic Risk Factor ~ Cumulative Psychosocial Stress + Age + Sex + Education + Year of psychosocial stress measure + Top 10 genetic PCs

Model 2: Model 1 + Smoking

[a] Cardiometabolic risk factors were pre-adjusted for medication use (antihypertensive, lipid-lowering, and diabetes medication)

[b] Fasting status (yes/no) was included as an additional covariate in the models.

Bold text indicates $p<0.05$.

Table 3-3: High-dimensional epigenetic mediation results of the association between psychosocial stress and cardiometabolic risk factors.

Cardiometabolic Risk Factor	% variance explained by psychosocial stress[a]	# of mediating CpGs identified in HDMT[b]	% Mediated by individual CpGs in HDMT[b]	Cumulative % Mediated by methylation PCs[c]	CpGs identified in de-biased LASSO mediation analysis[d]	% Mediated by individual CpGs in LASSO[d]
BMI	16.5%	50	4.3-11.0%	46.3%	cg00420390, cg19748455, cg02370100, cg08366476, cg13770461, cg09885325, cg09971499, cg04881642, cg02508743	3.3-9.2%
WC	16.7%	46	4.2-10.6%	35.9%	cg19748455, cg11454468, cg03037271, cg02370100, cg04803208, cg00420390	3.7-8.4%
HDL-C	3.1%	7	9.4-14.5%	31.4%	cg04803208, cg25607249, cg08274633	6.8-13.0%
CRP	15.6%	12	6.5-11.4%	45.1%	cg02508743, cg03699074, cg00420390, cg15781610, cg26010590, cg11956636, cg24837149	4.0-9.3%

Abbreviations: Body mass index (BMI); Waist circumference (WC); High-density lipoprotein cholesterol (HDL-C); C-reactive protein (CRP); High-dimensional mediation testing (HDMT); Principal component (PC)

All models were adjusted for Model 1 covariates: age, sex, education, year of psychosocial stress measure, top 10 genetic PCs, and fasting status (when appropriate).

[a] Calculated as the change in R-squared values from Model 1 with and without including the psychosocial stress variable divided by the R-squared value of Model 1 including the psychosocial stress variable.

[b] Results from the epigenome-wide mediation analysis using the HDMT method. The percent mediated by each individual CpG was not controlled for the other mediating CpGs.

[c] Results from the methylation PC analysis, which reduced the dimensionality of the CpGs identified in HDMT to top uncorrelated PCs. The cumulative percent mediated by all PCs approximates the lower bound of the overall mediation effect of the CpGs on the relationship between stress and each cardiometabolic risk factor.

[d] Results from de-biased LASSO mediation analysis, which implemented a penalization-based approach to identify CpGs from HDMT that remained significant after adjusting for other mediating CpGs. The percent mediated by each individual CpG was controlled for the other mediating CpG

Table 3-4: Genomic Feature Enrichment of Mediating CpG sites identified in Epigenome-wide Mediation Analysis (Model 1).

Genomic Feature	BMI		WC		HDL-C		CRP	
	OR	*P-value*	*OR*	*P-value*	*OR*	*P-value*	*OR*	*P-value*
DHS	1.11	0.78	1.92	0.051	5.43	0.094	2.04	0.38
Enhancer	**5.59**	**0.0002**	**6.17**	**1.19E-04**	3.66	0.26	**9.77**	**0.006**
Promoter	0.82	0.74	0.49	0.12	2.46	0.21	0.66	0.74
Shore/shelf	0.92	0.99	1.03	0.87	2.20	0.38	2.09	0.20
CpG Island	0.46	0.11	0.40	0.091	0.52	0.98	0.32	0.33
eQTM	**9.67**	**5.03E-10**	**7.25**	**8.85E-07**	**8.22**	**0.039**	**14.67**	**1.30E-04**

Abbreviations: Odds ratio (OR); DNAse hypersensitivity site (DHS); Expression quantitative trait methylation site (eQTM)
Bold text indicates associations present at $p<0.05$.

3.9 Figures

Figure 3-1: Flow diagram detailing participant inclusion criteria for epigenome-wide mediation analyses.

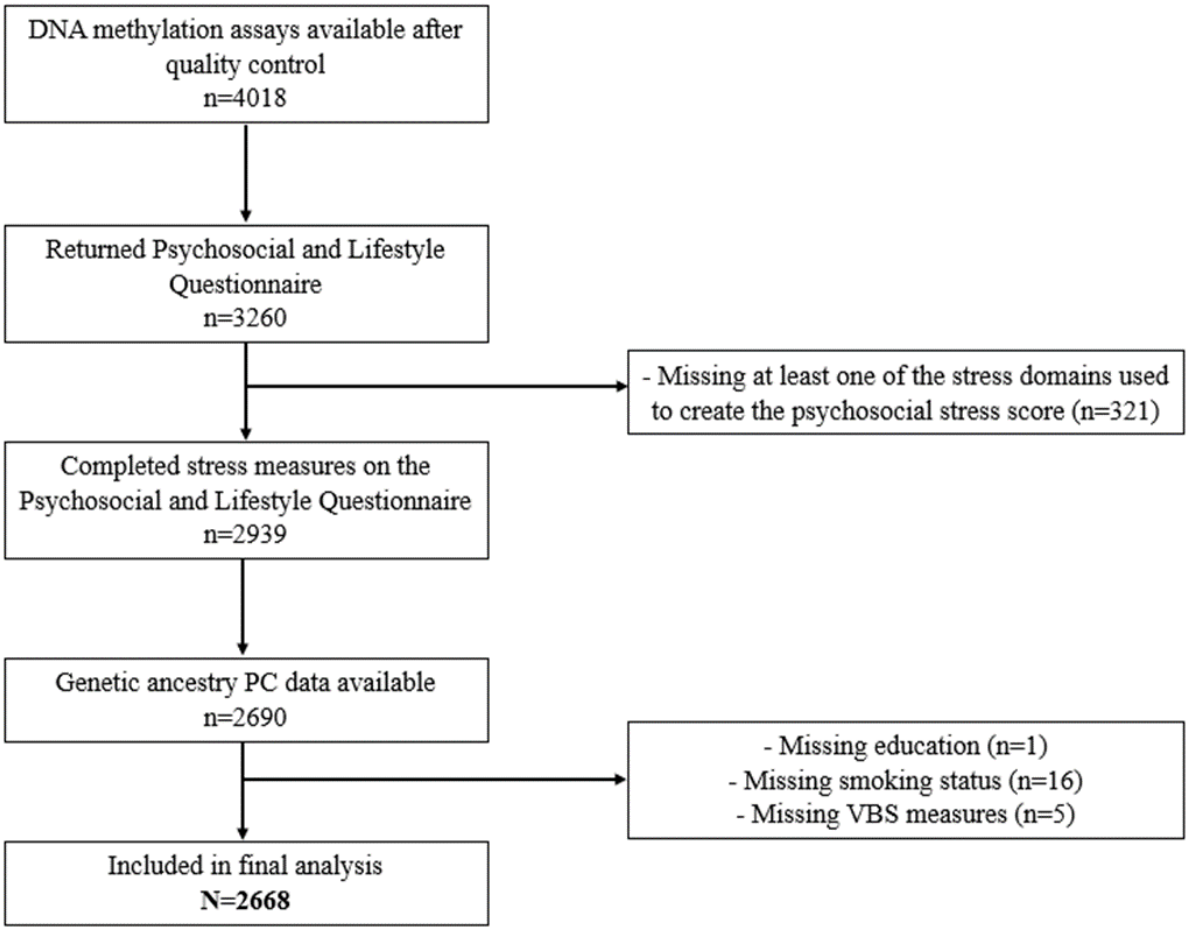
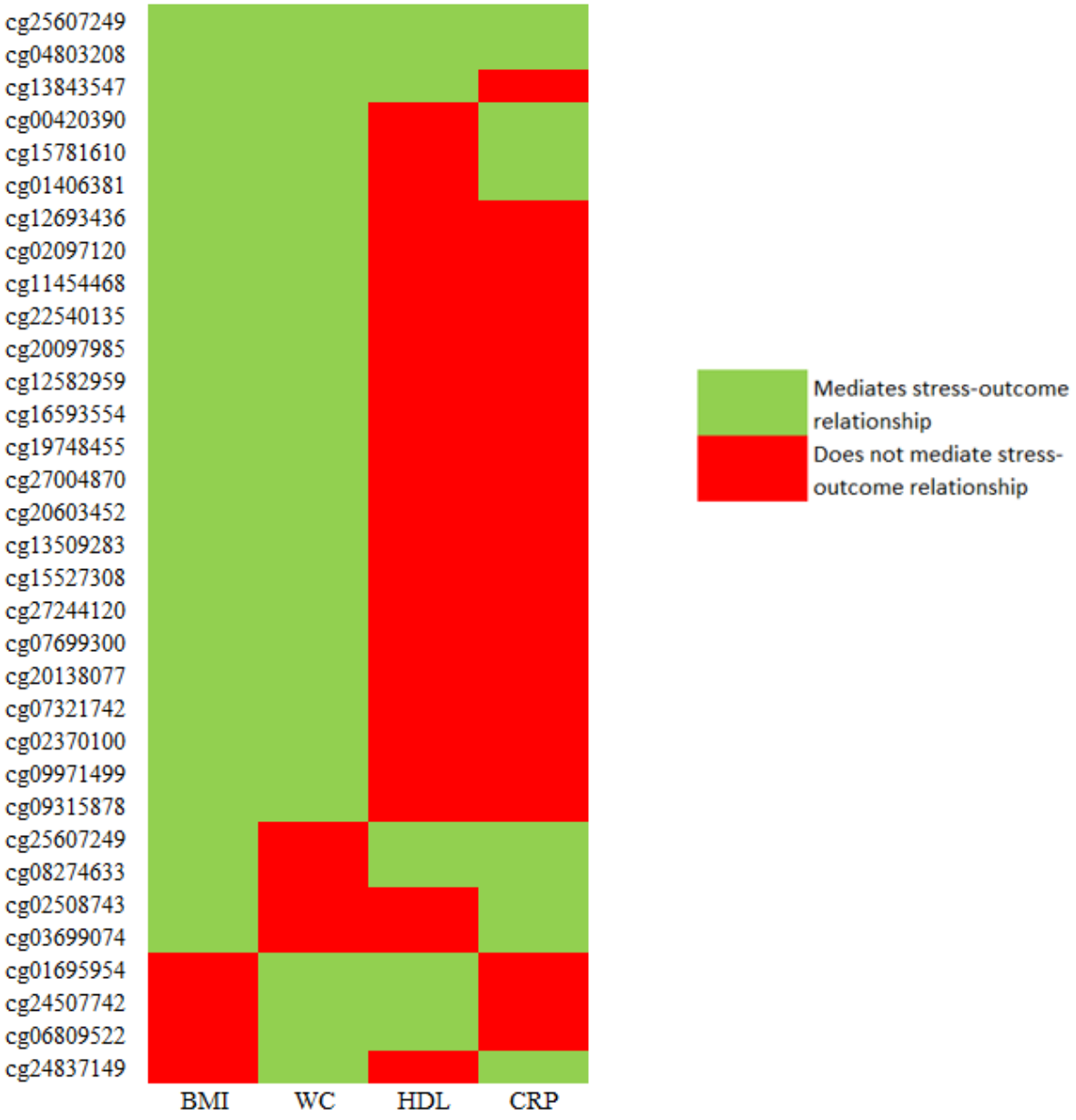


Figure 3-2: CpGs identified in HDMT that mediate the relationship between stress and multiple cardiometabolic risk factors.



Abbreviations: Body mass index (BMI); Waist circumference (WC); High-density lipoprotein cholesterol (HDL-C); C-reactive protein (CRP); High-dimensional mediation testing (HDMT)

3.10 Supplementary Material

Table S3-1: Psychosocial stress measures and items included in the 6 stress domains.

Stress Domain	Stress Measure	Individual Items
Acute Life Events	Lifetime Traumas	(1) Has experienced the death of child; (2) has ever been in a major fire, flood, earthquake or other natural disaster; (3) has ever fired a weapon in combat or been fired upon in combat; (4) has a spouse, partner, or child has ever been addicted to drugs or alcohol; (5) was a victim of a serious physical attack or assault; (6) has ever had a life-threatening illness or accident; (7) has spouse or a child has ever had a life-threatening illness or accident.
	Stressful Life Events in Past 5 Years	(1) Has involuntarily lost a job for reasons other than retirement; (2) has been unemployed and looking for work for longer than 3 months; (3) was anyone in household unemployed and looking for work for longer than 3 months; (4) have moved to a worse residence or neighborhood; (5) have been robbed or home has been burglarized; (6) has been victim of fraud.
Financial Stress	Financial Strain	(1) Difficulty meeting monthly payments on bills; (2) distress caused by ongoing financial strain.
	Lack of Financial Autonomy	(1) Satisfaction with his/her current financial situation; (2) amount of control over his/her financial situation (*reverse-coded*).
Neighborhood Stress	Neighborhood Disorder	(1) Vandalism and graffiti are a big problem in this area; (2) People would be afraid to walk alone in this area after dark; (3) This area is always full of rubbish and litter; (4) There are many vacant or deserted houses or storefronts in this area.
Relationship Stress	Marital Stressors Child-Related Stressors Other Family-Related Friend Stressors	(1) How often do they make too many demands on you? (2) How much do they criticize you? (3) How much do they let you down when you are counting on them? (4) How much do they get on your nerves?
Lifetime Discrimination	Major Discriminatory Events	(1) Have been unfairly dismissed from a job; (2) have been unfairly not hired for a job; (3) have been unfairly denied a promotion; (4) have been unfairly prevented from moving into a neighborhood because the landlord or a realtor refused to sell or rent you a house or apartment; (5) have been unfairly denied a bank loan; (6) have been unfairly stopped, searched, questioned, physically threatened or abused by the police.
	Perceived Everyday Discrimination	(1) Have been treated with less courtesy or respect than other people; (2) have received poorer service than other people at restaurants or stores; (3) people act as if they think you are not smart; (4) people act as if they are afraid of you; (5) you are threatened or harassed.
Childhood Adversity	Childhood Adversity Before Age 18	(1) Had to do a year of school over again; (2) was ever in trouble with the police; (3) either of his/her parents drank or used drugs so often that it caused problems in the family; (4) was ever physically abused by his/her parents.

Table S3-2: Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018).

Characteristics N (%) or mean (SD)	Included (n=2668)	Excluded (n=1350)	Effect Size	P-value
Age, years	70.4 (9.5)	67.6 (9.6)	0.29	<0.001
Female	1575 (59.0)	774 (57.3)	0.016	0.30
Race/Ethnicity			0.24	
Hispanic	269 (10.9)	298 (22.1)		<0.001
Black	349 (13.1)	309 (22.9)		
White	1981 (74.3)	688 (51.0)		
Other	68 (2.5)	54 (4.0)		
Education			0.15	
No degree	346 (13.0)	329 (24.4)		<0.001
HS degree	1617 (60.6)	758 (56.2)		
College degree or higher	705 (26.4)	262 (19.4)		
Smoking Status			0.06	
Never smoker	1207 (45.2)	540 (42.0)		<0.001
Former smoker	1134 (42.5)	527 (41.0)		
Current smoker	327 (12.3)	218 (17.0)		

*Effect sizes were measured by either Cohen's d or Cramer's V.

Table S3-3: Total effect of cumulative psychosocial stress on cardiometabolic risk factors restricted to fasting participants (N=1,779).

	Cumulative Psychosocial Stress			
	Model 1[a]		**Model 2[b]**	
Cardiometabolic Risk Factors	*Beta*	*P-value*	*Beta*	*P-value*
Log (Glucose)[c]	0.0020	0.649	0.0014	0.747
TC[c]	-1.83	0.101	-1.92	0.085
LDL-C[c]	-1.16	0.266	-1.21	0.247
HDL-C	**-1.57**	**4.28E-04**	**-1.56**	**5.02E-04**
TG	3.15	0.055	3.03	0.066

Abbreviations: Total cholesterol (TC); Low-density lipoprotein cholesterol (LDL-C); High-density lipoprotein cholesterol (HDL-C); Triglycerides (TG)

[b] Model 2: Model 1 + Smoking

[c] Cardiometabolic risk factors were adjusted for medication use (antihypertensive, lipid-lowering, and diabetes medication) prior to analysis.

Bold text indicates $p<0.05$.

Figure S3-1: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and BMI using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and BMI (inflation factor λ=1.03).

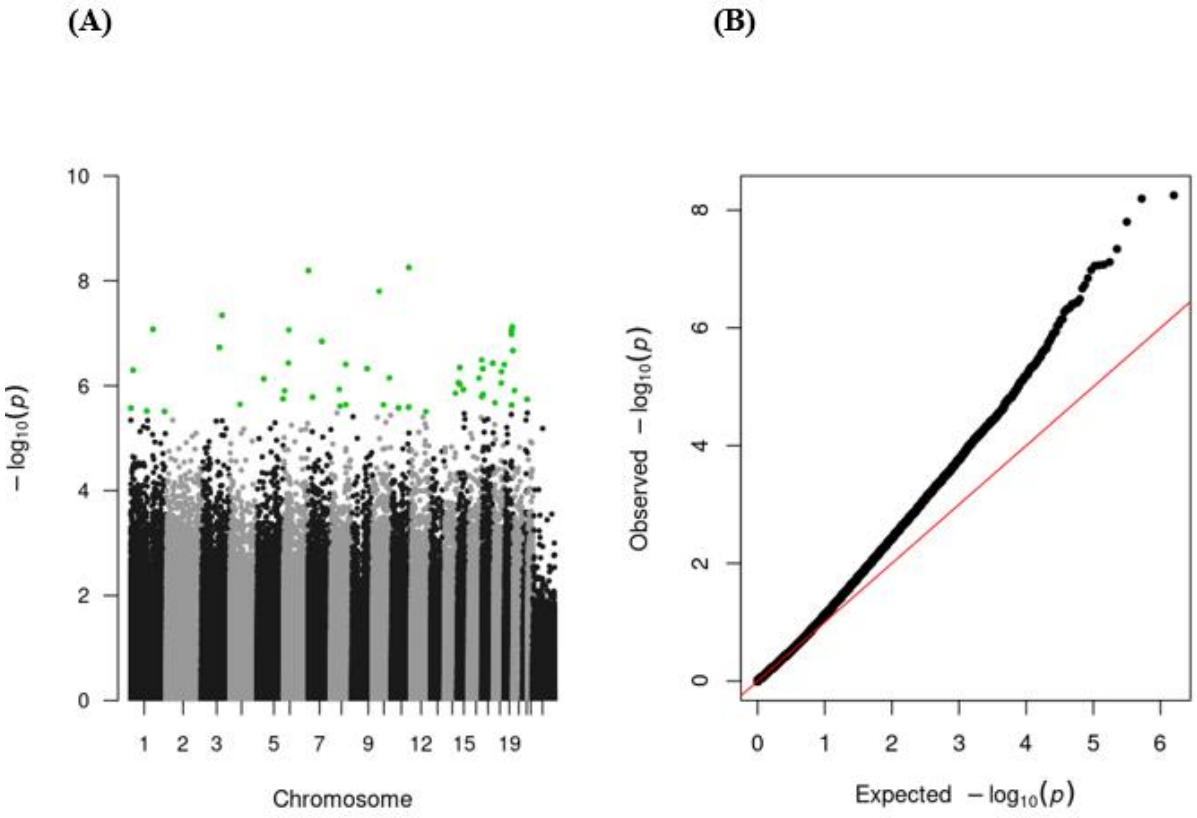


Figure S3-2: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and WC using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and WC (inflation factor λ=1.25).

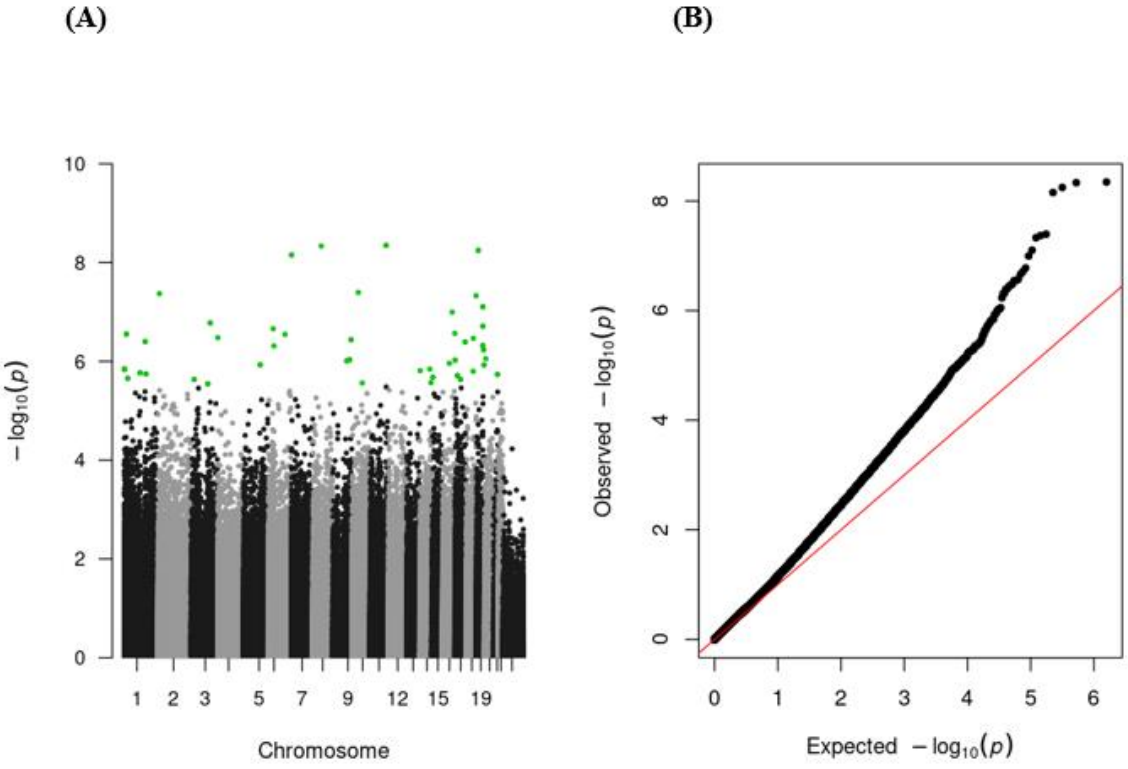


Figure S3-3: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and HDL-C using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and HDL-C (inflation factor λ=0.92).

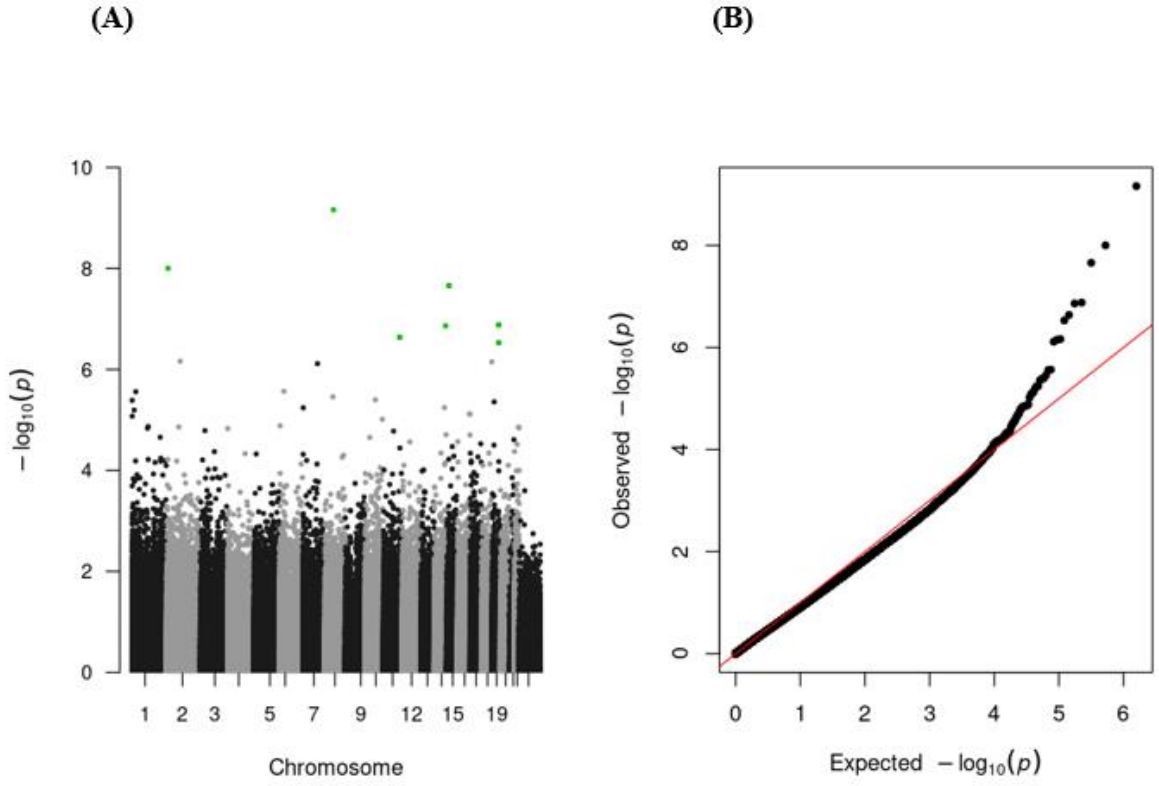


Figure S3-4: (A) Manhattan plot of the epigenetic mediation effects from the association between psychosocial stress and CRP using the high-dimensional mediation testing method. The green dots represent CpGs that meet the epigenome-wide significance threshold (FDR q<0.05). (B) Quantile-quantile plot of the epigenetic mediation effects from the association between psychosocial stress and CRP (inflation factor λ=0.93).

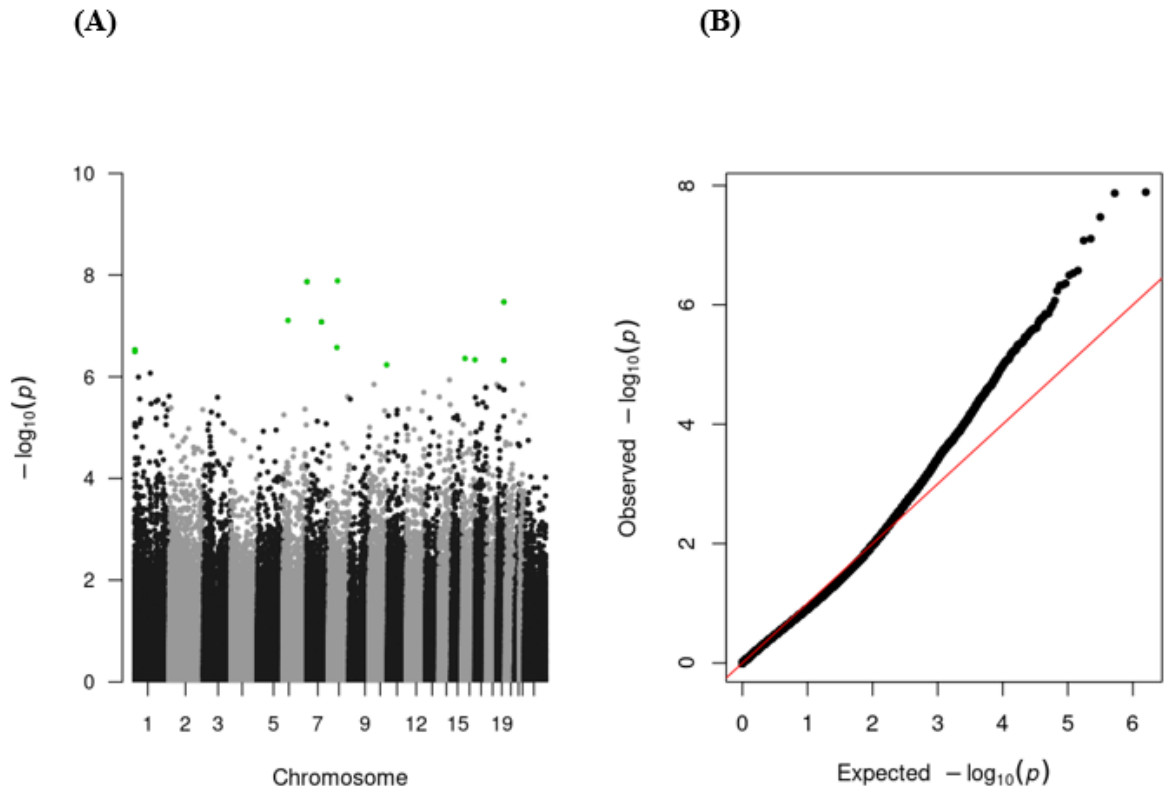


Table S3-4: CpG mediators of the association between psychosocial stress and BMI identified in HDMT (N=2537).

CpG	Model 1[a]					Model 2[b]				
	Mediation Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value	Mediation Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value
cg13843547	0.057	0.801	0.071	5.59E-09	**0.003**	0.058	0.866	0.067	1.11E-07	**0.019**
cg00420390	0.072	0.801	0.090	6.37E-09	**0.003**	0.072	0.866	0.083	2.57E-08	**0.019**
cg12693436	0.053	0.801	0.066	1.58E-08	**0.004**	0.054	0.866	0.062	1.18E-07	**0.019**
cg11454468	0.055	0.801	0.069	4.55E-08	**0.009**	0.055	0.866	0.064	3.09E-07	**0.029**
cg22540135	0.050	0.801	0.062	7.61E-08	**0.009**	0.050	0.866	0.058	7.95E-08	**0.019**
cg02097120	0.050	0.801	0.062	8.42E-08	**0.009**	0.051	0.866	0.058	8.39E-07	**0.042**
cg15781610	0.058	0.801	0.072	8.62E-08	**0.009**	0.058	0.866	0.067	9.13E-07	**0.042**
cg25607249	0.060	0.801	0.075	8.78E-08	**0.009**	0.060	0.866	0.069	2.19E-06	0.052
cg01406381	0.047	0.801	0.058	1.04E-07	**0.009**	0.048	0.866	0.056	2.59E-06	0.057
cg11961918	0.045	0.801	0.056	1.43E-07	**0.011**	0.045	0.866	0.052	1.89E-07	**0.025**
cg20097985	0.044	0.801	0.055	1.85E-07	**0.013**	0.045	0.866	0.052	3.45E-07	**0.029**
cg12582959	0.044	0.801	0.055	2.14E-07	**0.014**	0.044	0.866	0.050	4.05E-07	**0.029**
cg27004870	0.054	0.801	0.068	3.22E-07	**0.018**	0.054	0.866	0.063	1.10E-06	**0.042**
cg20445774	0.043	0.801	0.054	3.70E-07	**0.018**	0.043	0.866	0.050	3.86E-07	**0.029**
cg19748455	0.088	0.801	0.110	3.74E-07	**0.018**	0.085	0.866	0.098	3.51E-06	0.068
cg24990400	0.042	0.801	0.053	3.92E-07	**0.018**	0.042	0.866	0.049	9.63E-07	**0.042**
cg16593554	0.045	0.801	0.056	3.96E-07	**0.018**	0.046	0.866	0.053	1.03E-06	**0.042**
cg08274633	0.056	0.801	0.070	4.51E-07	**0.019**	0.057	0.866	0.066	2.55E-07	**0.029**
cg20603452	0.048	0.801	0.060	4.71E-07	**0.019**	0.049	0.866	0.056	1.41E-06	**0.044**
cg04257841	0.041	0.801	0.051	4.75E-07	**0.019**	0.042	0.866	0.048	2.24E-06	0.052
cg13509283	0.042	0.801	0.053	5.07E-07	**0.019**	0.042	0.866	0.049	1.29E-06	**0.042**
cg15527308	0.044	0.801	0.054	5.40E-07	**0.019**	0.043	0.866	0.050	6.12E-07	**0.040**
cg17489748	0.043	0.801	0.054	7.10E-07	**0.023**	0.043	0.866	0.050	8.56E-07	**0.042**
cg27244120	0.042	0.801	0.052	7.18E-07	**0.023**	0.043	0.866	0.049	1.16E-06	**0.042**
cg13808803	0.047	0.801	0.058	7.38E-07	**0.023**	0.047	0.866	0.054	2.31E-06	0.052
cg07699300	0.042	0.801	0.053	8.78E-07	**0.026**	0.043	0.866	0.049	6.43E-06	0.091
cg20138077	0.039	0.801	0.049	8.89E-07	**0.026**	0.039	0.866	0.045	2.18E-06	0.052
cg16760163	0.041	0.801	0.051	9.39E-07	**0.026**	0.041	0.866	0.048	2.21E-06	0.052
cg02508743	0.051	0.801	0.064	1.17E-06	**0.031**	0.051	0.866	0.059	3.46E-06	0.068
cg08366476	0.060	0.801	0.074	1.18E-06	**0.031**	0.059	0.866	0.068	8.20E-06	0.094
cg07321742	0.040	0.801	0.050	1.23E-06	**0.031**	0.041	0.866	0.047	7.99E-07	**0.042**
cg02120866	0.048	0.801	0.060	1.25E-06	**0.031**	0.048	0.866	0.055	8.76E-06	0.095
cg23919111	0.038	0.801	0.048	1.39E-06	**0.033**	0.040	0.866	0.046	7.05E-06	0.094
cg15095917	0.037	0.801	0.046	1.48E-06	**0.034**	0.038	0.866	0.044	1.21E-05	0.102
cg03699074	0.040	0.801	0.050	1.62E-06	**0.036**	0.040	0.866	0.047	1.29E-05	0.102
cg12937391	0.045	0.801	0.056	1.66E-06	**0.036**	0.045	0.866	0.052	4.60E-06	0.081
cg22108563	0.038	0.801	0.047	1.77E-06	**0.038**	0.038	0.866	0.044	2.02E-06	0.052
cg02370100	0.047	0.801	0.059	1.82E-06	**0.038**	0.047	0.866	0.055	7.28E-07	**0.042**
cg18129971	0.038	0.801	0.047	2.12E-06	**0.043**	0.038	0.866	0.044	1.22E-05	0.102
cg04881642	0.038	0.801	0.047	2.26E-06	**0.043**	0.037	0.866	0.043	1.72E-06	**0.049**

cg03483314	0.039	0.801	0.048	2.29E-06	**0.043**	0.039	0.866	0.045	7.70E-06	0.094
cg09971499	0.052	0.801	0.065	2.30E-06	**0.043**	0.053	0.866	0.061	1.21E-06	**0.042**
cg09885325	0.037	0.801	0.046	2.34E-06	**0.043**	0.037	0.866	0.043	6.06E-06	0.090
cg04803208	0.047	0.801	0.059	2.44E-06	**0.044**	0.048	0.866	0.055	1.49E-06	**0.045**
cg23111342	0.037	0.801	0.046	2.55E-06	**0.045**	0.037	0.866	0.043	6.76E-06	0.094
cg13770461	0.050	0.801	0.062	2.64E-06	**0.045**	0.050	0.866	0.057	7.49E-06	0.094
cg09315878	0.041	0.801	0.051	2.66E-06	**0.045**	0.041	0.866	0.048	3.71E-06	0.070
cg21327712	0.048	0.801	0.060	3.04E-06	**0.049**	0.048	0.866	0.055	1.51E-05	0.107
cg00851028	0.035	0.801	0.043	3.10E-06	**0.049**	0.035	0.866	0.040	7.06E-06	0.094
cg18944984	0.044	0.801	0.055	3.11E-06	**0.049**	0.044	0.866	0.051	1.16E-05	0.102

Abbreviations: Body mass index (BMI); High dimensional mediation testing (HDMT)

[a] All mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.
[b] All mediation models were adjusted for Model 1 covariates + smoking.
Associations were only evaluated in Model 2 if they had an FDR q <0.01 in Model 1.
FDR q values in Models 1 and 2 were bolded if they met the epigenome-wide mediation significance threshold of FDR q<0.05.

Table S3-5: Mediation analysis using top 10 PCs from the 50 CpG mediators of the association between psychosocial stress and BMI identified in HDMT (Model 1).

	Model 1[a]			Model 2[b]		
Methylation PC	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)
PC1	**0.204 (0.139, 0.279)**	**<2.22E-16**	**0.254 (0.160, 0.426)**	**0.207 (0.140, 0.282)**	**<2.22E-16**	**0.238 (0.152, 0.379)**
PC2	-3.82E-05 (-0.007, 0.007)	0.985		-5.54E-04 (-0.009, 0.007)	0.905	
PC3	**0.017 (0.002, 0.040)**	**0.025**	**0.020 (0.002, 0.057)**	**0.0170 (0.001, 0.040)**	**0.027**	**0.018 (0.001, 0.051)**
PC4	0.004 (-0.004, 0.017)	0.370		0.004 (-0.005, 0.017)	0.416	
PC5	**0.112 (0.064, 0.168)**	**<2.22E-16**	**0.138 (0.076, 0.244)**	**0.112 (0.063, 0.167)**	**<2.22E-16**	**0.128 (0.070, 0.219)**
PC6	**0.027 (0.006, 0.057)**	**0.007**	**0.033 (0.007, 0.080)**	**0.027 (0.003, 0.057)**	**0.024**	**0.029 (0.004, 0.072)**
PC7	0.014 (-0.0005, 0.035)	0.062		0.014 (-0.0007, 0.036)	0.071	
PC8	**0.016 (0.0005, 0.038)**	**0.041**	**0.018 (0.0006, 0.052)**	**0.016 (0.0004, 0.039)**	**0.041**	**0.017 (0.0005, 0.048)**
PC9	5.85E-04 (-0.008, 0.010)	0.883		7.47E-04 (-0.007, 0.010)	0.854	
PC10	3.85E-05 (-0.006, 0.006)	0.995		-6.70E-05 (-0.007, 0.006)	0.992	

Abbreviations: Body mass index (BMI); Principal component (PC); Confidence interval (CI); High dimensional mediation testing (HDMT)

[a] PC mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] PC mediation models were adjusted for Model 1 covariates + smoking.

PCs were constructed from the 50 CpGs that mediated the association between stress and BMI (Model 1).

Bolded values are significant PC mediators at $p<0.05$.

Table S3-6: De-biased LASSO based high-dimensional mediation analysis on the 50 CpG mediators of the association between psychosocial stress and BMI identified in HDMT (Model 1).

	Model 1[a]				Model 2[b]			
CpG	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value
cg00420390	0.054	0.801	0.068	4.43E-05	0.052	0.866	0.060	5.03E-05
cg19748455	0.074	0.801	0.092	8.15E-05	0.068	0.866	0.079	2.90E-04
cg02370100	0.048	0.801	0.060	3.62E-04	0.049	0.866	0.057	1.53E-04
cg08366476	0.036	0.801	0.045	1.17E-03	0.034	0.866	0.039	1.06E-03
cg13770461	0.033	0.801	0.041	1.62E-03	0.031	0.866	0.036	1.81E-03
cg09885325	0.030	0.801	0.038	2.50E-03	0.029	0.866	0.034	2.47E-03
cg09971499	0.030	0.801	0.038	4.37E-03	0.029	0.866	0.034	9.37E-03
cg04881642	0.026	0.801	0.033	6.86E-03	0.027	0.866	0.031	7.88E-03
cg02508743	0.030	0.801	0.038	8.78E-03	0.029	0.866	0.034	8.79E-03

Abbreviations: Body mass index (BMI); High dimensional mediation testing (HDMT)

[a] De-biased LASSO based mediation models were adjusted for age, sex, education, year of psychosocial stress measure, top 10 genetic PCs, and all other mediating CpGs.

[b] De-biased LASSO based mediation models were adjusted for Model 1 covariates + smoking.

Results represent CpGs identified in HDMT that remain significant mediators of the association between stress and BMI after adjusting for other mediating CpGs (FDR q<0.05).

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Table S3-7: CpG mediators of the association between psychosocial stress and WC identified in HDMT (N=2346).

CpG	Model 1[a]					Model 2[b]				
	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value
cg13843547	0.066	0.922	0.072	4.50E-09	**0.001**	0.064	0.950	0.068	6.45E-08	**0.013**
cg04803208	0.058	0.922	0.063	4.63E-09	**0.001**	0.058	0.950	0.061	1.15E-08	**0.008**
cg24704287	0.057	0.922	0.062	5.66E-09	**0.001**	0.055	0.950	0.058	1.83E-07	**0.029**
cg00420390	0.062	0.922	0.068	7.00E-09	**0.001**	0.062	0.950	0.065	2.76E-08	**0.008**
cg12693436	0.057	0.922	0.062	4.02E-08	**0.005**	0.056	0.950	0.059	2.28E-07	**0.029**
cg01695954	0.056	0.922	0.061	4.26E-08	**0.005**	0.055	0.950	0.057	3.20E-08	**0.008**
cg16593554	0.061	0.922	0.067	4.68E-08	**0.005**	0.058	0.950	0.062	8.48E-07	0.052
cg24507742	0.050	0.922	0.054	7.89E-08	**0.008**	0.047	0.950	0.050	2.03E-06	0.069
cg27244120	0.056	0.922	0.061	1.01E-07	**0.009**	0.055	0.950	0.057	5.48E-07	**0.048**
cg11454468	0.063	0.922	0.069	1.67E-07	**0.013**	0.061	0.950	0.065	9.59E-07	0.054
cg25607249	0.070	0.922	0.076	1.94E-07	**0.014**	0.066	0.950	0.070	2.89E-06	0.071
cg15781610	0.062	0.922	0.068	2.19E-07	**0.014**	0.060	0.950	0.063	1.57E-06	0.065
cg27004870	0.057	0.922	0.062	2.71E-07	**0.015**	0.057	0.950	0.060	7.32E-07	0.051
cg13509283	0.043	0.922	0.046	2.80E-07	**0.015**	0.042	0.950	0.045	6.59E-07	0.051
cg05308643	0.046	0.922	0.050	2.85E-07	**0.015**	0.046	0.950	0.048	2.88E-07	**0.029**
cg25922180	0.047	0.922	0.051	3.32E-07	**0.016**	0.045	0.950	0.048	2.87E-06	0.071
cg15527308	0.044	0.922	0.048	3.42E-07	**0.016**	0.045	0.950	0.047	2.94E-07	**0.029**
cg05241923	0.065	0.922	0.070	3.64E-07	**0.016**	0.061	0.950	0.064	4.20E-06	0.083
cg02097120	0.054	0.922	0.058	3.98E-07	**0.016**	0.052	0.950	0.055	2.34E-06	0.069
cg19748455	0.098	0.922	0.106	4.05E-07	**0.016**	0.093	0.950	0.098	2.72E-06	0.071
cg01406381	0.056	0.922	0.061	4.80E-07	**0.017**	0.052	0.950	0.055	1.10E-05	0.130
cg23733945	0.042	0.922	0.046	4.87E-07	**0.017**	0.041	0.950	0.043	6.83E-06	0.108
cg22540135	0.041	0.922	0.044	5.83E-07	**0.020**	0.040	0.950	0.042	1.86E-06	0.068
cg07321742	0.043	0.922	0.047	8.86E-07	**0.029**	0.042	0.950	0.045	2.25E-06	0.069
cg08848140	0.041	0.922	0.045	9.33E-07	**0.029**	0.041	0.950	0.043	2.35E-06	0.069
cg23691006	0.041	0.922	0.044	9.48E-07	**0.029**	0.040	0.950	0.042	3.74E-06	0.078
cg20603452	0.046	0.922	0.049	9.81E-07	**0.029**	0.045	0.950	0.047	2.73E-06	0.071
cg10529845	0.040	0.922	0.043	1.10E-06	**0.031**	0.038	0.950	0.040	7.48E-06	0.112
cg07787851	0.042	0.922	0.045	1.17E-06	**0.031**	0.041	0.950	0.043	3.74E-06	0.078
cg12582959	0.041	0.922	0.045	1.18E-06	**0.031**	0.041	0.950	0.044	2.09E-06	0.069
cg12581512	0.038	0.922	0.042	1.43E-06	**0.035**	0.037	0.950	0.039	5.40E-06	0.099
cg24837149	0.040	0.922	0.044	1.43E-06	**0.035**	0.039	0.950	0.041	3.20E-06	0.073
cg09315878	0.040	0.922	0.043	1.46E-06	**0.035**	0.040	0.950	0.042	1.85E-06	0.068
cg06088445	0.040	0.922	0.044	1.55E-06	**0.036**	0.040	0.950	0.042	1.90E-06	0.068
cg20138077	0.040	0.922	0.043	1.59E-06	**0.036**	0.040	0.950	0.042	2.67E-06	0.071
cg23086613	0.047	0.922	0.051	1.72E-06	**0.038**	0.044	0.950	0.047	1.97E-05	0.144
cg00950718	0.039	0.922	0.043	1.80E-06	**0.038**	0.039	0.950	0.041	1.57E-06	0.065
cg02370100	0.039	0.922	0.043	1.84E-06	**0.038**	0.040	0.950	0.043	7.76E-07	0.051
cg04051206	0.043	0.922	0.046	1.94E-06	**0.039**	0.042	0.950	0.044	9.36E-06	0.123
cg07699300	0.041	0.922	0.044	2.10E-06	**0.041**	0.040	0.950	0.042	1.22E-05	0.132
cg20745693	0.039	0.922	0.042	2.22E-06	**0.043**	0.038	0.950	0.040	1.37E-06	0.065
cg03037271	0.048	0.922	0.052	2.32E-06	**0.043**	0.047	0.950	0.049	9.06E-06	0.121
cg21646392	0.039	0.922	0.042	2.34E-06	**0.043**	0.039	0.950	0.041	4.14E-06	0.083
cg06809522	0.039	0.922	0.042	2.71E-06	**0.048**	0.038	0.950	0.040	1.53E-06	0.065
cg09971499	0.048	0.922	0.052	2.75E-06	**0.048**	0.050	0.950	0.052	1.30E-06	0.065
cg20097985	0.041	0.922	0.044	2.85E-06	**0.049**	0.040	0.950	0.042	5.15E-06	0.097

Abbreviations: Waist circumference (WC); High dimensional mediation testing (HDMT)

[a] All mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] All mediation models were adjusted for Model 1 covariates + smoking.

Associations were only evaluated in Model 2 if they had an FDR q <0.01 in Model 1.

FDR q values in Models 1 and 2 were bolded if they met the epigenome-wide mediation significance threshold of FDR q<0.05

Table S3-8: Mediation analysis using top 10 PCs from the 46 CpG mediators of the association between psychosocial stress and WC identified in HDMT (Model 1).

	Model 1[a]			Model 2[b]		
Methylation PC	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)
PC1	**0.161 (0.104, 0.226)**	**<2.22E-16**	**0.175 (0.109, 0.278)**	**0.157 (0.102, 0.220)**	**<2.22E-16**	**0.166 (0.103, 0.262)**
PC2	0.011 (-0.002, 0.031)	0.104		0.011 (-0.002, 0.032)	0.125	
PC3	-7.97E-05(-0.010, 0.010)	0.988		-9.22E-04 (-0.012, 0.009)	0.848	
PC4	**0.049 (0.018, 0.088)**	**0.002**	**0.053 (0.019, 0.103)**	**0.049 (0.017, 0.087)**	**0.002**	**0.051 (0.018, 0.098)**
PC5	**0.083 (0.045, 0.129)**	**<2.22E-16**	**0.090 (0.048, 0.154)**	**0.084 (0.045, 0.128)**	**<2.22E-16**	**0.088 (0.047, 0.149)**
PC6	0.012 (-0.004, 0.033)	0.145		0.011 (-0.007, 0.032)	0.228	
PC7	0.008 (-0.002, 0.024)	0.157		0.008 (-0.02, 0.025)	0.136	
PC8	**0.018 (0.0004, 0.044)**	**0.043**	**0.018 (0.001, 0.050)**	**0.018 (0.0001, 0.043)**	**0.0482**	**0.017 (0.0002, 0.049)**
PC9	**0.022 (0.002, 0.048)**	**0.035**	**0.023 (0.002, 0.056)**	0.019 (-0.002, 0.045)	0.081	
PC10	-1.96E-04 (-0.007, 0.006)	0.955		3.61E-06 (-0.007, 0.007)	0.998	

Abbreviations: Waist circumference (WC); Principal component (PC); Confidence interval (CI); High dimensional mediation testing (HDMT)

[a] PC mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] PC mediation models were adjusted for Model 1 covariates + smoking.

PCs were constructed from the 46 CpGs that mediated the association between stress and WC (Model 1).

Bolded values are significant PC mediators at $p<0.05$.

Table S3-9: De-biased LASSO based high-dimensional mediation analysis on the 46 CpG mediators of the association between psychosocial stress and WC identified in HDMT (Model 1).

	Model 1[a]				Model 2[b]			
CpG	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value
cg19748455	0.084	0.922	0.091	7.36E-05	0.078	0.950	0.082	2.23E-04
cg11454468	0.044	0.922	0.048	7.57E-05	0.042	0.950	0.044	2.01E-04
cg03037271	0.054	0.922	0.058	2.17E-04	0.052	0.950	0.055	4.61E-04
cg02370100	0.037	0.922	0.040	2.76E-04	0.039	0.950	0.041	2.62E-04
cg04803208	0.040	0.922	0.044	1.97E-03	0.039	0.950	0.041	2.03E-03
cg00420390	0.038	0.922	0.041	3.50E-03	0.037	0.950	0.039	3.40E-03
cg19748455	0.084	0.922	0.091	7.36E-05	0.078	0.950	0.082	2.23E-04

Abbreviations: Waist circumference (WC); High dimensional mediation testing (HDMT)

[a] De-biased LASSO based mediation models were adjusted for age, sex, education, year of psychosocial stress measure, top 10 genetic PCs, and all other mediating CpGs.

[b] De-biased LASSO based mediation models were adjusted for Model 1 covariates + smoking.

Results represent CpGs identified in HDMT that remain significant mediators of the association between stress and WC after adjusting for other mediating CpGs (FDR $q<0.05$).

Table S3-10: CpG mediators of the association between psychosocial stress and HDL-C in HDMT (N=2668).

CpG	Model 1[a]					Model 2[b]				
	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value
cg04803208	-0.22	-1.48	0.145	6.89E-10	**0.0005**	-0.21	-1.46	0.144	2.02E-09	**0.002**
cg01695954	-0.18	-1.48	0.125	9.96E-09	**0.004**	-0.16	-1.46	0.112	9.97E-09	**0.004**
cg08274633	-0.19	-1.48	0.126	2.19E-08	**0.006**	-0.18	-1.46	0.124	2.24E-08	**0.006**
cg25607249	-0.19	-1.48	0.129	1.32E-07	**0.022**	-0.17	-1.46	0.115	8.69E-06	0.429
cg06809522	-0.14	-1.48	0.098	1.37E-07	**0.022**	-0.14	-1.46	0.093	1.22E-06	0.193
cg13843547	-0.14	-1.48	0.094	2.30E-07	**0.030**	-0.12	-1.46	0.085	8.16E-06	0.429
cg24507742	-0.15	-1.48	0.099	2.95E-07	**0.033**	-0.12	-1.46	0.085	3.31E-05	1.000

Abbreviations: High-density lipoprotein cholesterol (HDL-C); High dimensional mediation testing (HDMT)
[a] All mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.
[b] All mediation models were adjusted for Model 1 covariates + smoking.
Associations were only evaluated in Model 2 if they had an FDR q <0.01 in Model 1.
FDR q values in Models 1 and 2 were bolded if they met the epigenome-wide mediation significance threshold of FDR q<0.05

Table S3-11: Mediation analysis using top 5 PCs from the 7 CpG mediators of the association between psychosocial stress and HDL-C identified in HDMT (Model 1).

	Model 1[a]			Model 2[b]		
Methylation PC	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)
PC1	**-0.333 (-0.487, -0.202)**	**<2.22E-16**	**0.229 (0.121, 0.489)**	**-0.304 (-0.453, -0.177)**	**<2.22E-16**	**0.210 (0.109, 0.453)**
PC2	**-0.126 (-0.222, -0.053)**	**<2.22E-16**	**0.085 (0.034, 0.199)**	**-0.129 (-0.225, -0.054)**	**<2.22E-16**	**0.088 (0.035, 0.206)**
PC3	-0.007 (-0.045, 0.022)	0.629		-0.009 (-0.049, 0.023)	0.579	
PC4	-0.002 (-0.028, 0.019)	0.819		-0.002 (-0.026, 0.018)	0.860	
PC5	-0.001 (-0.015, 0.022)	0.879		6.06E-04 (-0.016, 0.019)	0.945	

Abbreviations: High-density lipoprotein cholesterol (HDL-C); Principal component (PC); Confidence interval (CI); High dimensional mediation testing (HDMT)

[a] PC mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] PC mediation models were adjusted for Model 1 covariates + smoking.

PCs were constructed from the 7 CpGs that mediated the association between stress and HDL-C (Model 1).

Bolded values are significant PC mediators at $p<0.05$.

Table S3-12: De-biased LASSO based high-dimensional mediation analysis on the 7 CpG mediators of the association between psychosocial stress and HDL-C identified in HDMT (Model 1).

	Model 1[a]				Model 2[b]			
CpG	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value
cg04803208	-0.192	-1.478	0.130	4.93E-07	-0.186	-1.464	0.127	9.69E-07
cg25607249	-0.124	-1.478	0.084	3.33E-03	-0.108	-1.464	0.074	3.66E-03
cg08274633	-0.101	-1.478	0.068	1.77E-02	-0.097	-1.464	0.067	1.81E-02

Abbreviations: High-density lipoprotein cholesterol (HDL-C); High dimensional mediation testing (HDMT)

[a] De-biased LASSO based mediation models were adjusted for age, sex, education, year of psychosocial stress measure, top 10 genetic PCs, and all other mediating CpGs.

[b] De-biased LASSO based mediation models were adjusted for Model 1 covariates + smoking.

Results represent CpGs identified in HDMT that remain significant mediators of the association between stress and HDL-C after adjusting for other mediating CpGs (FDR $q<0.05$).

Table S3-13: CpG mediators of the association between psychosocial stress and CRP in HDMT (N=2666).

CpG	Model 1[a]					Model 2[b]				
	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value	Mediating Effect	Total Effect	Proportion Mediated	Mediation p-value	Mediation q-value
cg04803208	0.010	0.102	0.095	1.30E-08	**0.005**	0.009	0.095	0.098	1.83E-08	**0.014**
cg00420390	0.011	0.102	0.110	1.35E-08	**0.005**	0.011	0.095	0.112	4.27E-08	**0.017**
cg25607249	0.008	0.102	0.081	3.38E-08	**0.009**	0.007	0.095	0.071	5.60E-07	0.098
cg15781610	0.012	0.102	0.114	7.80E-08	**0.013**	0.010	0.095	0.108	6.40E-07	0.098
cg04051649	0.008	0.102	0.076	8.37E-08	**0.013**	0.007	0.095	0.072	5.67E-07	0.098
cg02508743	0.011	0.102	0.109	2.66E-07	**0.031**	0.010	0.095	0.110	7.44E-07	0.098
cg24837149	0.008	0.102	0.077	2.95E-07	**0.031**	0.007	0.095	0.071	2.35E-06	0.153
cg09276842	0.008	0.102	0.077	3.19E-07	**0.031**	0.007	0.095	0.075	1.42E-06	0.140
cg26010590	0.007	0.102	0.065	4.37E-07	**0.034**	0.006	0.095	0.066	9.20E-07	0.104
cg03699074	0.009	0.102	0.092	4.64E-07	**0.034**	0.008	0.095	0.087	2.94E-06	0.153
cg01406381	0.007	0.102	0.071	4.74E-07	**0.034**	0.006	0.095	0.060	3.01E-06	0.153
cg11956636	0.008	0.102	0.080	5.83E-07	**0.038**	0.007	0.095	0.073	5.47E-06	0.180

Abbreviations: C-reactive protein (CRP); High dimensional mediation testing (HDMT)

[a] All mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] All mediation models were adjusted for Model 1 covariates + smoking.

Associations were only evaluated in Model 2 if they had an FDR q <0.01 in Model 1.

FDR q values in Models 1 and 2 were bolded if they met the epigenome-wide mediation significance threshold of FDR q<0.05

Table S3-14: Mediation analysis using top 10 PCs from the 12 CpG mediators of the association between psychosocial stress and CRP identified in HDMT (Model 1).

	Model 1[a]			**Model 2[b]**		
Methylation PC	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)	Mediation Effect (95% CI)	Mediation p-value	Proportion Mediated (95% CI)
PC1	**0.027 (0.018, 0.037)**	**<2.22E-16**	**0.275 (0.165, 0.512)**	**0.023 (0.015, 0.032)**	**<2.22E-16**	**0.258 (0.148, 0.498)**
PC2	**0.014 (0.007, 0.021)**	**<2.22E-16**	**0.140 (0.071, 0.278)**	**0.013 (0.007, 0.021)**	**<2.22E-16**	**0.147 (0.075, 0.310)**
PC3	**0.004 (0.0003, 0.008)**	**0.028**	**0.036 (0.003, 0.097)**	**0.004 (0.0002, 0.008)**	**0.035**	**0.037 (0.003, 0.103)**
PC4	2.11E-04 (-0.0008, 0.002)	0.732		9.72E-05 (-0.001, 0.001)	0.874	
PC5	4.21E-04 (-0.0008, 0.002)	0.532		4.55E-04 (-0.0008, 0.002)	0.525	
PC6	4.76E-04 (-0.0009, 0.002)	0.506		4.82E-04 (-0.0008, 0.002)	0.504	
PC7	-3.78E-05 (-0.001, 0.0009)	0.933		-2.94E-05 (-0.001, 0.0009)	0.965	
PC8	-1.65E-05 (-0.001, 0.0009)	0.961		-3.09E-05 (-0.001, 0.0009)	0.947	
PC9	-1.85E-04 (-0.002, 0.001)	0.824		-2.23E-04 (-0.002, 0.001)	0.763	
PC10	-6.03E-04 (-0.003, 0.001)	0.501		-6.12E-04 (-0.003, 0.001)	0.502	

Abbreviations: C-reactive protein (CRP); Principal component (PC); Confidence interval (CI); High dimensional mediation testing (HDMT)

[a] PC mediation models were adjusted for age, sex, education, year of psychosocial stress measure, and top 10 genetic PCs.

[b] PC mediation models were adjusted for Model 1 covariates + smoking.

PCs were constructed from the 12 CpGs that mediated the association between stress and CRP (Model 1).

Bolded values are significant PC mediators at $p<0.05$.

Table S3-15: De-biased LASSO based mediation analysis on the 12 CpG mediators of the association between psychosocial stress and CRP identified in HDMT (Model 1).

	Model 1[a]				Model 2[b]			
CpG	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value	Mediation Effect	Total Effect	Proportion Mediated	FDR-based joint p-value
cg02508743	0.009	0.102	0.093	5.08E-05	0.009	0.095	0.095	1.16E-04
cg03699074	0.007	0.102	0.064	1.04E-04	0.006	0.095	0.062	4.18E-04
cg00420390	0.008	0.102	0.077	1.32E-04	0.008	0.095	0.080	1.19E-04
cg15781610	0.007	0.102	0.071	2.63E-04	0.007	0.095	0.069	2.68E-04
cg26010590	0.006	0.102	0.055	6.38E-04	0.005	0.095	0.056	7.71E-04
cg11956636	0.004	0.102	0.041	2.50E-02	0.004	0.095	0.039	2.49E-02
cg24837149	0.004	0.102	0.040	3.39E-02	0.004	0.095	0.038	3.58E-02

Abbreviations: C-reactive protein (CRP); High dimensional mediation testing (HDMT)

[a] De-biased LASSO based mediation models were adjusted for age, sex, education, year of psychosocial stress measure, top 10 genetic PCs, and all other mediating CpGs.

[b] De-biased LASSO based mediation models were adjusted for Model 1 covariates + smoking.

Results represent CpGs identified in HDMT that remain significant mediators of the association between stress and CRP after adjusting for other mediating CpGs (FDR $q<0.05$).

Table S3-16: Overlap of CpG mediators identified in HDMT (Model 1) across the association between stress and cardiometabolic risk factors.

Distinct CpG Mediators [a]	Stress-BMI Mediator	Stress-WC Mediator	Stress-HDL-C Mediator	Stress-CRP Mediator	# of outcomes CpG is identified as a mediator	CHR	BP	UCSC RefGene Name[b]
cg25607249	TRUE	TRUE	TRUE	TRUE	4	19	47288039	SLC1A5
cg04803208	TRUE	TRUE	TRUE	TRUE	4	8	61878906	LOC100130298
cg00420390	TRUE	TRUE	FALSE	TRUE	3	7	2256023	MAD1L1
cg13843547	TRUE	TRUE	TRUE	FALSE	3	11	117873636	
cg15781610	TRUE	TRUE	FALSE	TRUE	3	6	36992554	FGD2
cg01406381	TRUE	TRUE	FALSE	TRUE	3	19	47288263	SLC1A5
cg12693436	TRUE	TRUE	FALSE	FALSE	2	10	47130971	
cg02097120	TRUE	TRUE	FALSE	FALSE	2	1	154492952	TDRD10
cg11454468	TRUE	TRUE	FALSE	FALSE	2	3	142569221	PCOLCE2
cg22540135	TRUE	TRUE	FALSE	FALSE	2	19	51898904	
cg20097985	TRUE	TRUE	FALSE	FALSE	2	3	124003076	KALRN
cg12582959	TRUE	TRUE	FALSE	FALSE	2	19	56159199	CCDC106
cg16593554	TRUE	TRUE	FALSE	FALSE	2	18	74813401	MBP
cg19748455	TRUE	TRUE	FALSE	FALSE	2	17	76274856	LOC100996291
cg27004870	TRUE	TRUE	FALSE	FALSE	2	16	88850384	FAM38A
cg08274633	TRUE	FALSE	TRUE	FALSE	2	15	38988533	C15orf53
cg20603452	TRUE	TRUE	FALSE	FALSE	2	9	104181324	
cg13509283	TRUE	TRUE	FALSE	FALSE	2	1	15742829	EFHD2
cg15527308	TRUE	TRUE	FALSE	FALSE	2	18	54813067	
cg27244120	TRUE	TRUE	FALSE	FALSE	2	16	70610434	SF3B3
cg07699300	TRUE	TRUE	FALSE	FALSE	2	15	29392964	APBA2
cg20138077	TRUE	TRUE	FALSE	FALSE	2	18	53587802	LINC01416
cg02508743	TRUE	FALSE	FALSE	TRUE	2	8	56903623	LYN
cg07321742	TRUE	TRUE	FALSE	FALSE	2	20	8696066	PLCB1
cg03699074	TRUE	FALSE	FALSE	TRUE	2	16	88849875	FAM38A
cg02370100	TRUE	TRUE	FALSE	FALSE	2	21	43655256	ABCG1
cg09971499	TRUE	TRUE	FALSE	FALSE	2	10	76631573	KAT6B
cg09315878	TRUE	TRUE	FALSE	FALSE	2	1	1152580	SDF4
cg01695954	FALSE	TRUE	TRUE	FALSE	2	2	10262019	RRM2
cg24507742	FALSE	TRUE	TRUE	FALSE	2	19	47288244	SLC1A5
cg24837149	FALSE	TRUE	FALSE	TRUE	2	1	1333778	CCNL2;LOC148413
cg06809522	FALSE	TRUE	TRUE	FALSE	2	14	103031976	
cg11961918	TRUE	FALSE	FALSE	FALSE	1	7	94295627	PEG10;PEG10
cg20445774	TRUE	FALSE	FALSE	FALSE	1	6	33400021	SYNGAP1
cg24990400	TRUE	FALSE	FALSE	FALSE	1	8	102006838	
cg04257841	TRUE	FALSE	FALSE	FALSE	1	17	5976451	WSCD1
cg13808803	TRUE	FALSE	FALSE	FALSE	1	5	42549944	GHR
cg17489748	TRUE	FALSE	FALSE	FALSE	1	10	117887197	GFRA1
cg16760163	TRUE	FALSE	FALSE	FALSE	1	15	38901688	
cg02120866	TRUE	FALSE	FALSE	FALSE	1	6	6804127	
cg08366476	TRUE	FALSE	FALSE	FALSE	1	15	63664748	CA12
cg23919111	TRUE	FALSE	FALSE	FALSE	1	14	95983256	
cg15095917	TRUE	FALSE	FALSE	FALSE	1	17	7482694	CD68
cg12937391	TRUE	FALSE	FALSE	FALSE	1	7	29900403	WIPF3
cg22108563	TRUE	FALSE	FALSE	FALSE	1	5	176936877	DOK3
cg03483314	TRUE	FALSE	FALSE	FALSE	1	8	102083111	

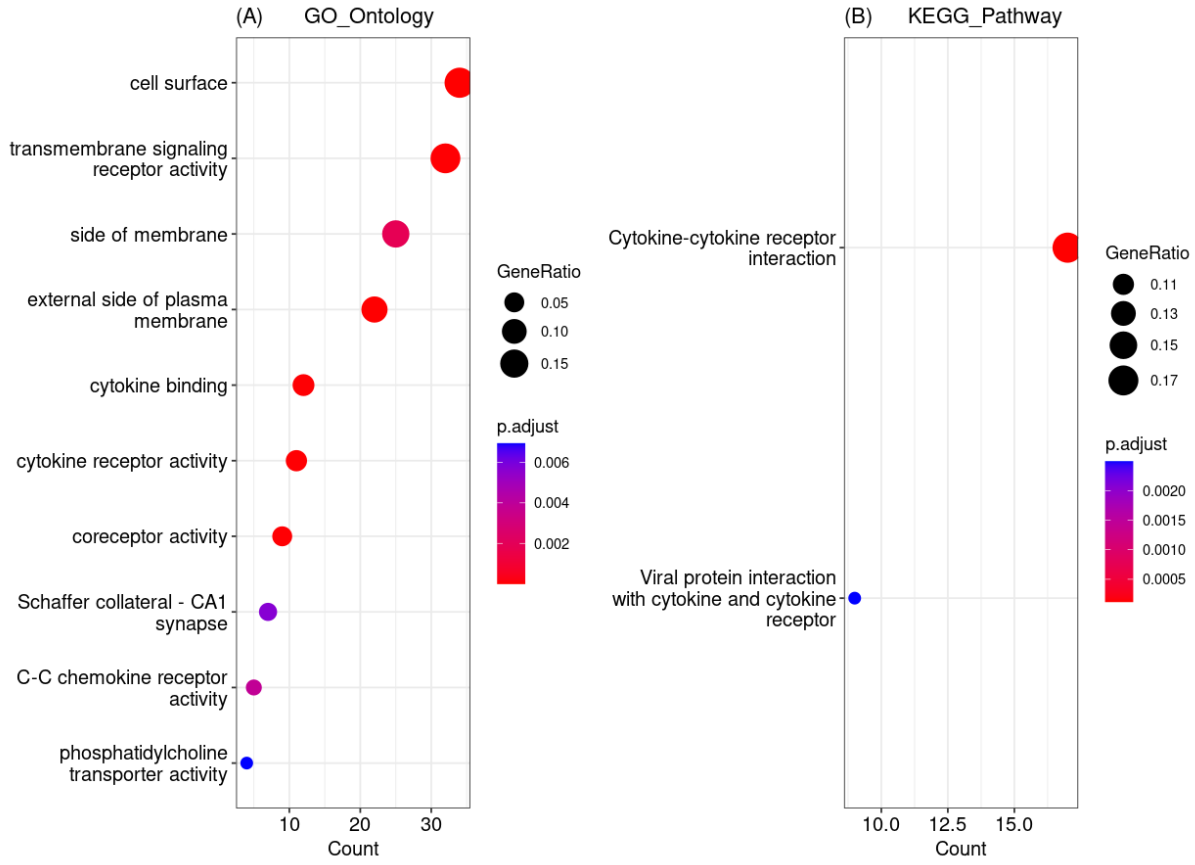
cg04881642	TRUE	FALSE	FALSE	FALSE	1	4	69048156	LOC100130017
cg09885325	TRUE	FALSE	FALSE	FALSE	1	19	47814494	C5AR1
cg18129971	TRUE	FALSE	FALSE	FALSE	1	18	9884912	TXNDC2
cg13770461	TRUE	FALSE	FALSE	FALSE	1	11	46016312	PHF21A
cg23111342	TRUE	FALSE	FALSE	FALSE	1	11	116857480	SIK3
cg00851028	TRUE	FALSE	FALSE	FALSE	1	1	234905772	
cg18944984	TRUE	FALSE	FALSE	FALSE	1	12	100866566	NR1H4
cg21327712	TRUE	FALSE	FALSE	FALSE	1	1	111744503	DENND2D
cg24704287	FALSE	TRUE	FALSE	FALSE	1	19	13951481	
cg05308643	FALSE	TRUE	FALSE	FALSE	1	6	124982296	NKAIN2
cg25922180	FALSE	TRUE	FALSE	FALSE	1	4	693321	
cg05241923	FALSE	TRUE	FALSE	FALSE	1	9	136010810	RALGDS
cg23733945	FALSE	TRUE	FALSE	FALSE	1	6	43056688	PTK7
cg08848140	FALSE	TRUE	FALSE	FALSE	1	9	124982018	LHX6
cg23691006	FALSE	TRUE	FALSE	FALSE	1	17	1510666	SLC43A2
cg10529845	FALSE	TRUE	FALSE	FALSE	1	16	49522337	
cg07787851	FALSE	TRUE	FALSE	FALSE	1	5	121488147	ZNF474
cg12581512	FALSE	TRUE	FALSE	FALSE	1	14	94422013	ASB2
cg06088445	FALSE	TRUE	FALSE	FALSE	1	14	23837451	
cg23086613	FALSE	TRUE	FALSE	FALSE	1	1	117047419	
cg00950718	FALSE	TRUE	FALSE	FALSE	1	1	159859904	CCDC19
cg04051206	FALSE	TRUE	FALSE	FALSE	1	17	17750855	TOM1L2
cg20745693	FALSE	TRUE	FALSE	FALSE	1	1	26909537	
cg03037271	FALSE	TRUE	FALSE	FALSE	1	17	40548565	
cg21646392	FALSE	TRUE	FALSE	FALSE	1	3	23710303	
cg04051649	FALSE	FALSE	FALSE	TRUE	1	7	105088822	
cg09276842	FALSE	FALSE	FALSE	TRUE	1	1	1155026	SDF4
cg26010590	FALSE	FALSE	FALSE	TRUE	1	16	17412395	XYLT1
cg11956636	FALSE	FALSE	FALSE	TRUE	1	10	126330161	FAM53B

Abbreviations: Body mass index (BMI); Waist circumference (WC); High-density lipoprotein cholesterol (HDL-C); C-reactive protein (CRP); High-dimensional mediation testing (HDMT)

[a] Distinct CpGs that mediated the association between stress and at least one cardiometabolic risk factor.

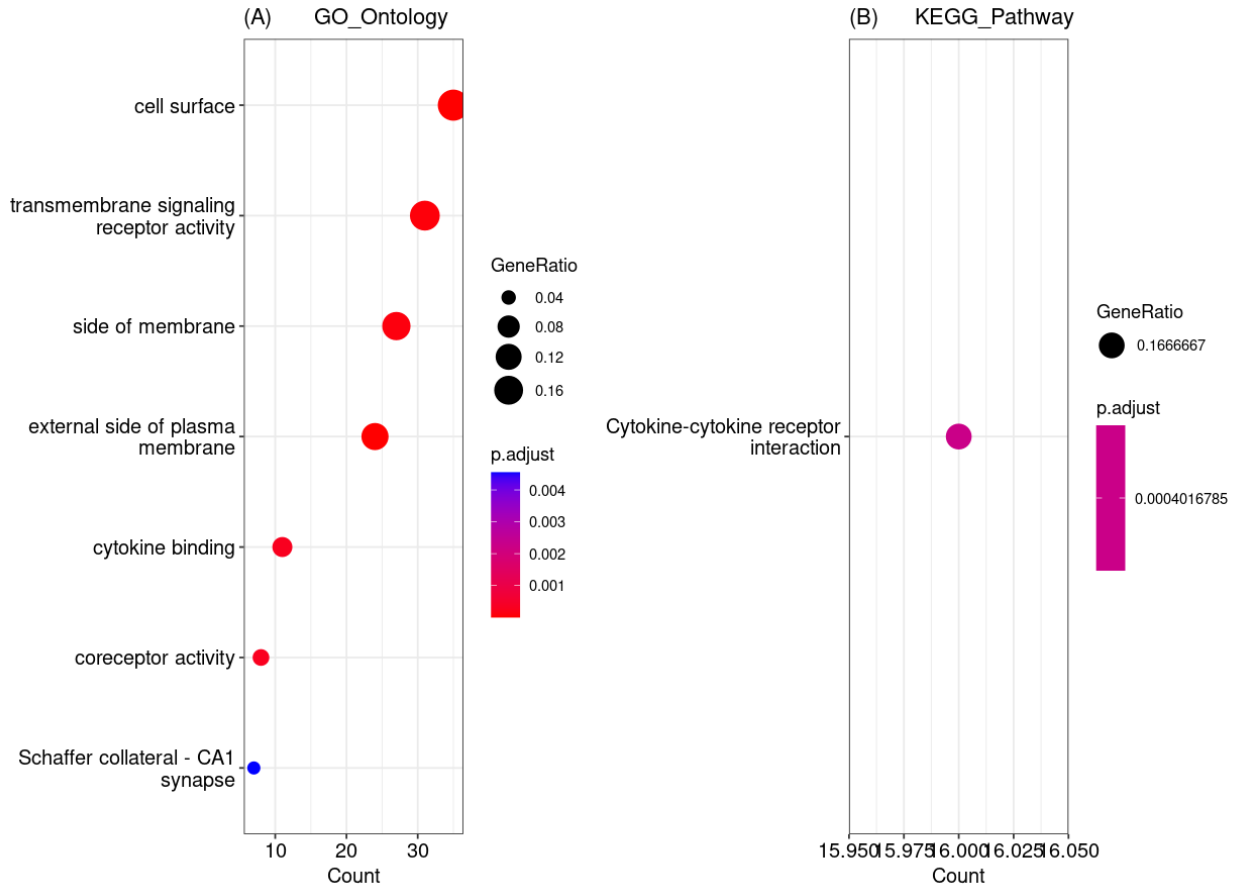
[b] Gene mapping and functional annotations were based on Illumina annotation files.

Figure S3-5: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and BMI identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and BMI identified in high-dimensional mediation testing (Model 1).



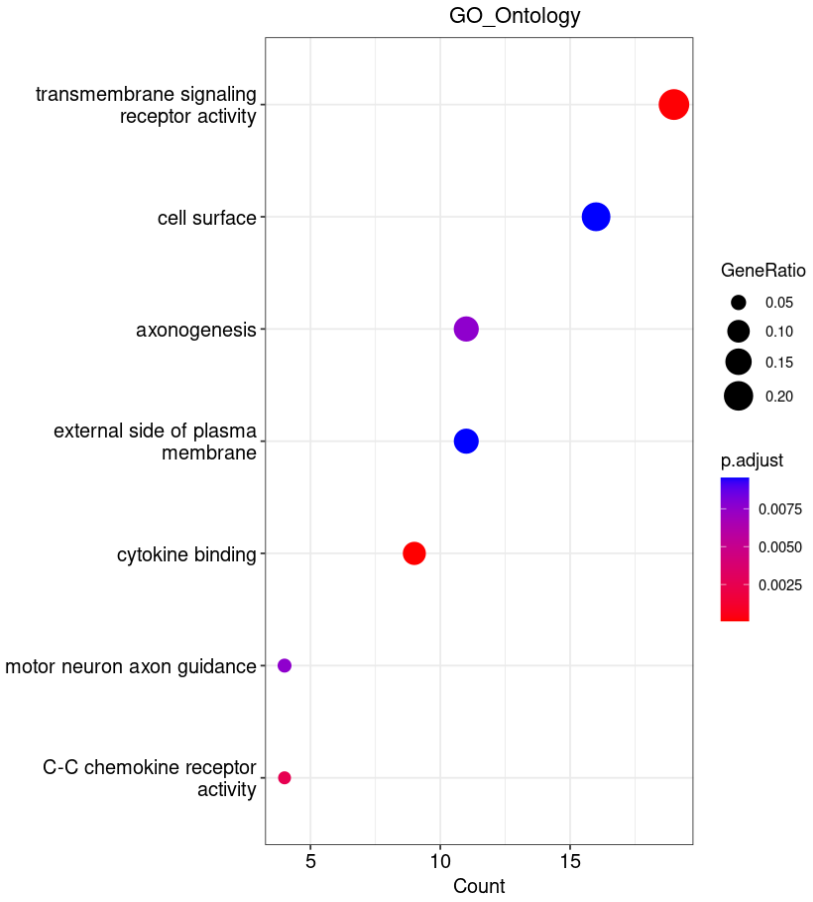
Dot size represents the fraction of differentially expressed genes found in the gene set. Dot color represents the FDR q-value, with a significance level of FDR q<0.01.

Figure S3-6: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and WC identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and WC identified in high-dimensional mediation testing (Model 1).



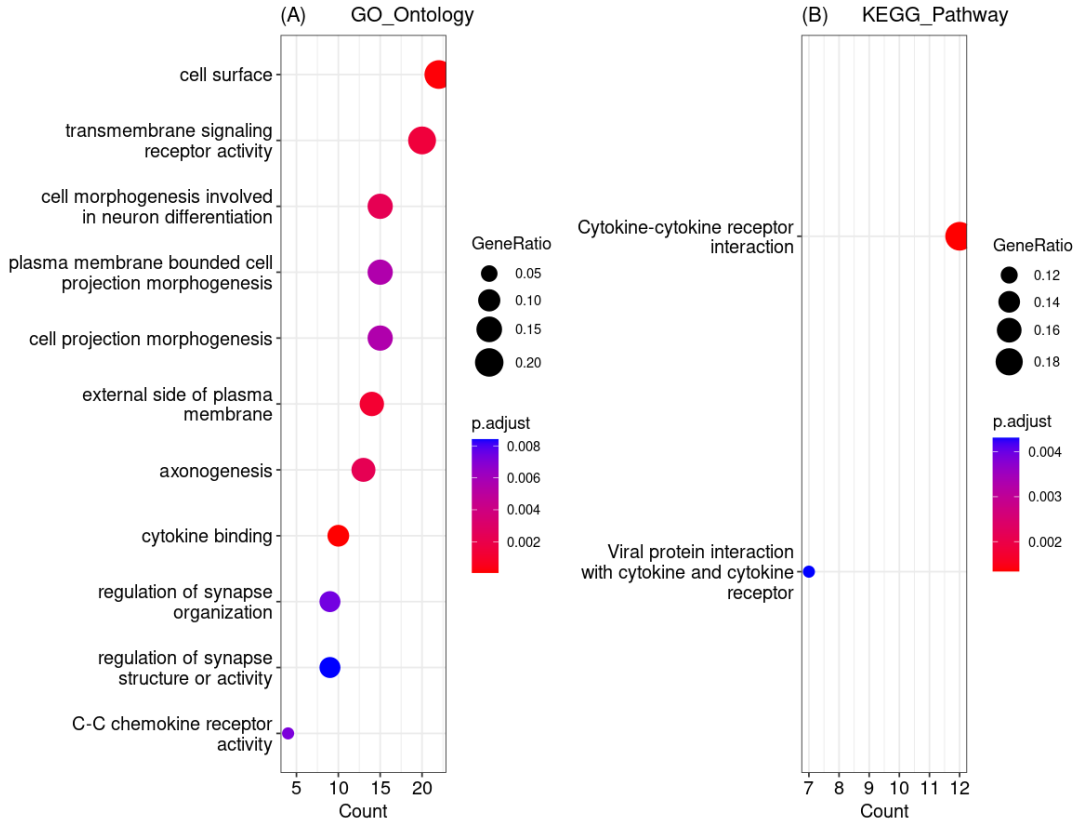
Dot size represents the fraction of differentially expressed genes found in the gene set. Dot color represents the FDR q-value, with a significance level of FDR q<0.01.

Figure S3-7: Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and HDL-C identified in high-dimensional mediation testing (Model 1).



Dot size represents the fraction of differentially expressed genes found in the gene set. Dot color represents the FDR q-value, with a significance level of FDR q<0.01.

Figure S3-8: (A) Dotplot showing the Gene Ontology (GO) biological processes and molecular function enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and CRP identified in high-dimensional mediation testing (Model 1) (B) Dotplot showing the KEGG pathway enrichment results for genes whose expression was associated with CpGs mediating the relationship between stress and CRP identified in high-dimensional mediation testing (Model 1).



Dot size represents the fraction of differentially expressed genes found in the gene set. Dot color represents the FDR q-value, with a significance level of FDR q<0.01.

Chapter 4 Depressive Symptoms Partially Mediate the Relationship between Psychosocial Factors and Epigenetic Age Acceleration in a Multi-Racial/Ethnic Sample of Older Adults

4.1 Abstract

Psychosocial factors, including cumulative psychosocial stress and loneliness, have been linked to epigenetic aging. Further, depressive symptoms have established relationships with both psychosocial factors and epigenetic aging. However, it is not known whether depressive symptoms mediate the association between psychosocial factors and epigenetic aging.

We conducted linear regression models to examine associations between psychosocial stress, loneliness, and depressive symptoms and five epigenetic age acceleration (AA) measures estimated by DNA methylation in a multi-racial/ethnic sample of 2,681 older adults from the Health and Retirement Study (mean age: 70.4 years). For all identified associations, we tested for effect modification by sex and educational attainment and performed mediation analysis to characterize the role of depressive symptoms on these associations.

Psychosocial stress, loneliness, and depressive symptoms were each associated with at least one measure of epigenetic AA (FDR $q<0.05$). Further, we observed interactions between loneliness, psychosocial stress, and sex on DunedinPACE, as well as loneliness and educational attainment on GrimAA, PhenoAA, and DunedinPACE, with females and individuals without a college degree appearing more sensitive to the psychosocial effects on epigenetic aging. Depressive symptoms mediated between 24% and 35% of the relationships between psychosocial stress and HannumAA, GrimAA, and DunedinPACE, as well as 40% and 37% of the relationships between loneliness and both GrimAA and DunedinPACE, respectively.

Results from this study may help elucidate the relationship between psychosocial factors and epigenetic aging, which is critical in understanding the biological mechanisms through which psychosocial factors may contribute to age-related disease.

4.2 Introduction

Psychosocial factors, defined as social, cultural, or environmental phenomena that impact an individual's thoughts or behaviors [1], have been associated with a range of mental and physical health outcomes, including age-related diseases [2,3]. One such factor is psychosocial stress, broadly defined as the perception of threat that results in discomfort and emotional tension [4]. Psychosocial stress has well-established relationships with cardiometabolic disease and its corresponding risk factors, including hypertension and diabetes [5-7], as well as autoimmune disorders [8,9]. Similarly, loneliness, which is a state of distress that arises from the perception that one's social needs are not being met by their relationships [10], is associated with an increased risk of cardiovascular disease [11-12], functional disability [13], cognitive decline, and dementia [14,15].

More recently, evidence has linked psychosocial factors to biological aging, defined as the amount of damage that has occurred to the body's cellular functions, including changes to the epigenome [16]. Epigenetic mechanisms, such as DNA methylation, are biochemical modifications to DNA that alter gene activity without changing the underlying genetic sequence [17]. Epigenetic clocks are measures of biological aging developed from DNA methylation patterns across the genome [18]. First-generation clocks, which include HannumAge [19] and HorvathAge [20], were designed to predict chronological age. However, because they were trained exclusively on age, they may not have selected the most relevant DNA methylation sites, as evidenced by their relatively weak associations with measures of morbidity and mortality [21].

Second-generation clocks, such as GrimAge [22] and PhenoAge [23], were trained on a combination of health biomarkers or outcomes and designed to predict age-related disease status and mortality. Finally, third-generation clocks, including DunedinPACE [24], were developed using longitudinal change of age-related biomarkers to assess physiological decline in a cohort of individuals with the same chronological age. HorvathAge, HannumAge, PhenoAge, and GrimAge all measure biological age in years, while DunedinPACE measures the pace of aging, such that each unit represents an average rate of one year of biological aging per year of chronological aging. Because each set of epigenetic clocks were trained on different outcomes and markers, differences in the associations between individual clocks and sociodemographic characteristics have been observed within the same study population [25]. Thus, when studying epigenetic aging, it is important to consider multiple clocks to better understand the biological impact of specific exposures on each epigenetic aging measure.

Previous studies have identified associations between measures of psychosocial stress and epigenetic aging measured by several clocks [26-28]. Yet, results have been somewhat inconsistent, with other studies reporting no significant relationships [29,30]. In a systematic review of the impact of psychosocial stress on epigenetic aging, two studies found positive associations between lifetime stress and epigenetic aging measured by GrimAge and HorvathAge, while the remaining four studies did not observe any significant associations [31]. Recent studies have also discovered associations between loneliness and accelerated epigenetic aging, measured by GrimAge and DunedinPACE [32,33]. However, these studies were only conducted in participants during midlife. Because the epigenome can change substantially during the lifespan, it is not clear whether these findings would persist in a population of older adults.

Furthermore, the pathway linking these psychosocial factors to epigenetic aging is not fully understood. One possible mechanism that may elucidate the relationship between psychosocial factors and epigenetic aging is mental health outcomes, including depression and its symptoms [34]. There are well-established relationships between psychosocial factors and depressive symptoms, with studies finding that an increase in both psychosocial stress and loneliness is associated with elevated depressive symptoms [35,36]. Additionally, extensive research has shown associations between depressive symptoms and accelerated epigenetic aging measured by multiple clocks [37,38]. Thus, depressive symptoms may mediate the relationship between psychosocial factors and epigenetic aging.

In this study, we examined associations between psychosocial stress, loneliness, and depressive symptoms and five measures of epigenetic age acceleration (EAA) in a multi-racial/ethnic sample of older adults. Additionally, we tested for effect modification by sex and educational attainment to assess sociodemographic differences in the effects of psychosocial factors and depressive symptoms on epigenetic aging. Finally, we performed mediation analysis to understand the role of depressive symptoms in the associations between psychosocial factors and epigenetic aging. Results from this study may help to characterize the relationship between psychosocial factors and epigenetic aging, which is crucial in understanding the biological mechanisms through which psychosocial factors may contribute to age-related disease.

4.3 Materials and Methods

4.3.1 Sample

The Health and Retirement Study (HRS) is a longitudinal cohort study of U.S. individuals over age 50 and their spouse/partner. HRS, which began in 1992, consists of biennial surveys covering topics such as income and wealth, physical and mental health, cognition, work,

retirement, and family support [39,40]. Beginning in 2006, a random 50% of participants were administered the Psychosocial and Lifestyle Questionnaire, or Leave Behind Questionnaire (LBQ), which is a self-completed survey capturing information on participants' psychological well-being, lifestyle, and social relationships [41]. The remaining half of the sample was administered the LBQ in 2008, and data from this survey is available for all participants longitudinally every four years. Furthermore, in 2016, all panel participants who completed the HRS survey were invited to participate in an ancillary study, the Venous Blood Study (VBS, n=9,934), where blood-based biomarkers were collected [42]. A subsample of participants who completed the VBS blood draw had their DNA methylation measured (n=4,104). This analysis was restricted to 2,681 participants who had complete data on psychosocial factors, sociodemographic covariates, and epigenetic clocks.

To evaluate the impact of restricting the analysis to complete case data, we compared sociodemographic characteristics and health behaviors between those who were included (n=2681) and excluded (n=1337) in the primary analysis from the total DNA methylation sample, using student's t-tests and Chi-squared tests, as appropriate. Further, we calculated Cohen's d and Cramer's V effect sizes for continuous and categorical variables, respectively, to quantify the size of the differences between the two groups.

4.3.2 Measures

A. DNA Methylation

DNA methylation was measured from whole blood in a sample of racially and socioeconomically diverse HRS participants using the Illumina Infinium HumanMethylationEPIC Beadchip v.1.0 [43]. DNA samples were randomized across plates by demographic variables, including age, sex, cohort, education, and race/ethnicity. Data processing

and quality control were performed using the *minfi* R package [44]. Approximately 3.4% of methylation probes (n=29,431 out of 866,091) were removed because the detection p-value fell below a threshold of 0.01. Further, sex mismatched samples and any controls (cell lines, blinded duplicates) were removed prior to analysis. Methylation data was available for 97.9% of participants (n=4,018) who passed quality control.

B. Epigenetic Clocks and Age Acceleration

Five epigenetic clocks estimated from the HRS methylation data were examined as outcomes in this study [43]. HorvathAge, developed in samples of 51 healthy tissues and cell types, was defined based on 353 CpGs sites, while HannumAge was developed from whole blood samples using 71 CpG sites. PhenoAge, which included 513 CpGs sites, was developed to estimate mortality risk using nine aging biomarkers in whole blood samples. GrimAge, which was developed to capture seven methylation-based surrogate markers of plasma protein and smoking-pack years in whole blood samples, included 1,030 unique CpG sites. Finally, DunedinPACE, which measured within-individual decline across 19 biological markers in whole blood samples, included 173 CpG sites. DunedinPACE represents years of accelerated aging per chronological year, with values larger than zero indicating a faster pace of aging. All clocks are publicly available (see https://hrsdata.isr.umich.edu/data-products/epigenetic-clocks), aside from DunedinPACE, which was created using the DunedinPACE algorithm in Belsky et al. [24].

EAA for each clock (excluding DunedinPACE) was quantified by the residuals from regressing epigenetic age on chronological age and chronological age squared. Both age terms were included to account for potential non-linear relationships between age and the epigenetic clocks [45]. Because both age and sex are used in the GrimAge calculation, sex is also regressed

out of the measure for GrimAge [46]. A positive EAA measure indicates that an individual's epigenetic age is greater than their chronological age.

C. Psychosocial Factors

Cumulative Psychosocial Stress Psychosocial stress was calculated using data from the 2010 and 2012 Psychosocial Leave Behind Questionnaires [41]. To investigate the impact of lifetime stress exposure on epigenetic aging, we created a cumulative score that captured both acute and chronic stressors that spanned the life course. Six domains of psychosocial stress were included in the cumulative stress score, including: 1) acute life events, 2) financial stress, 3) neighborhood stress, 4) relationship stress, 5) lifetime discrimination, and 6) childhood adversity [47-48]. Each domain was made up of one or more stress measures. Details regarding the individual stress measures, as well as the construction of the cumulative stress score, can be found elsewhere [47-49]. In summary, each stress measure was transformed into a z-score, and z-scores from the individual stress measures within each domain were summed and re-standardized to obtain domain specific standardized scores. Finally, the standardized scores from each of the six domains were summed and subsequently standardized to calculate the cumulative psychosocial stress score.

Loneliness Loneliness was captured in either 2010 or 2012 using the 11-item Revised UCLA Loneliness Scale (R-UCLA) from the Psychosocial Leave Behind Questionnaire [50,51]. The full list of items can be found in Table S4-1. Participants were asked to respond (1=often, 2=some of the time, 3=hardly ever or never) to each of the items, and the scores from each item were averaged to create a loneliness index. To allow for comparisons between psychosocial factors, we then standardized the index to create a loneliness z-score.

D. Depressive Symptoms

Depressive symptoms were assessed in 2014 using the modified 8-item Center for Epidemiological Studies Depression (CES-D) scale [52], which can be found in Table S4-2. Participants were asked to respond (yes/no) to having experienced 8 depressive symptoms. The individual responses were summed for a final score ranging from 0-8, with a higher score indicating a greater number of depressive symptoms. Subsequently, participants' scores were standardized to create depressive symptoms z-scores. For participants who did not complete the CES-D scale in 2014, their 2016 scores were instead used.

E. Covariates

Sociodemographic factors and health behaviors were collected during the participant's baseline interview, as well as their 2010 or 2012 HRS core interview, in parallel with the Psychosocial Leave Behind Questionnaire. The following sociodemographic variables were included as covariates: sex (male or female), educational attainment (no degree, high school degree, or college degree or higher), marital status (married/partnered, single, or widowed/divorced), having children (yes/no), employment status (yes/no), and total household wealth in US dollars. The top 10 principal components (PCs) of genetic ancestry, which were estimated using genotype data, accounted for population stratification and admixture [53]. Furthermore, the Housman method was used to adjust for white blood cell (WBC) proportions [54].

Health behaviors, including smoking status (never, former, current), alcohol use (never, moderate, heavy), and BMI (normal, overweight, obese class I, obese class II) were additional covariates included in the analysis. Based on standards from the National Institute on Alcohol Abuse and Alcoholism, moderate drinking was defined as between 1 and 7 drinks per week for females and between 1 and 14 drinks per week for males, whereas heavy drinking was defined as

greater than 7 drinks for females and greater than 14 drinks for males per week [55]. BMI, which was calculated from participant's weight in kilometers divided by the square of their height in meters, was categorized according to the World Health Organization guidelines as either normal (BMI < 25 kg/m^2), overweight (BMI: 25.0 – 29.9 kg/m^2), obese class I (BMI: 30.0 – 34.9 kg/m^2), or obese class II (BMI ≥ 35 kg/m^2) [56]. If individuals did not have their measurement recorded during their interview, we used their self-reported weight and height.

4.3.3 Statistical Analysis

A. Correlation among Chronological Age, Epigenetic Clocks, and EAA

We first assessed the relationship between five epigenetic clocks (HorvathAge, HannumAge, PhenoAge, GrimAge, DunedinPACE) and chronological age using Pearson correlations. Additionally, we calculated the correlations among HorvathAge acceleration (HorvathAA), HannumAge acceleration (HannumAA), PhenoAge acceleration (PhenoAA), GrimAge acceleration (GrimAA), and DunedinPACE.

B. Associations between Psychosocial Factors/Depressive Symptoms and EAA

Next, we examined the relationships between the exposures of interest (psychosocial factors, depressive symptoms) and each EAA measure separately using linear regression models adjusted for sex, educational attainment, marital status, having children, employment status, total wealth, year of psychosocial stress measurement, white blood cell proportions, and genetic ancestry PCs (Model 1). Further, because health behaviors may be on the causal pathway between psychosocial factors and epigenetic aging, we were interested in understanding the direct relationship between the exposures of interest and epigenetic aging independent of these health behaviors. Thus, as a secondary analysis, we adjusted the primary models for smoking

status, alcohol use, and BMI (Model 2). To account for multiple testing, we applied a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure, and associations with an FDR $q < 0.05$ were considered significant.

C. Effect Modification by Sex and Educational Attainment

We then assessed sex and educational attainment as possible effect modifiers of the relationship between the exposures of interest and epigenetic aging by including a multiplicative interaction term for each demographic factor in the linear regression models. To better characterize the effect of education on these relationships, we dichotomized educational attainment into two separate variables: 1) no degree vs. high school degree or higher and 2) less than college degree vs. college degree or higher [57]. We only tested for interactions when both the exposure of interest and the demographic factor were associated with EAA in Model 1. For this analysis, $p<0.05$ was considered significant.

D. Mediation Analysis

i. Mediation by depressive symptoms on associations between psychosocial factors and EAA

Finally, for the significant associations between psychosocial factors and EAA measures identified in Model 1, we conducted a formal mediation analysis using the *regmedint* package in R to examine the mediating effect of depressive symptoms on the association between psychosocial factors and EAA [58]. This method is an extension of the regression-based causal mediation analysis using the counterfactual framework, which decomposes the total effect into the sum of the direct effect and indirect effect [59]. In causal mediation analysis, the total, direct, and indirect effects are defined relative to a contrast in an exposure X, denoted as x vs. x^*, where x^* is the reference level. Because both cumulative psychosocial stress and loneliness are standardized, we set $x^*=0$ and $x=1$ to represent a change in one standard deviation of the

psychosocial exposure. The total effect (TE) represents the change in an outcome, if the exposure were to change from *x** to *x* for the entire population. The natural direct effect (NDE) expresses the change in an outcome from *x** to *x*, allowing the mediator for each individual to be kept at the level it would have taken under exposure *x**. Finally, the natural indirect effect (NIDE) is the change in outcome, exclusively quantified by the exposure induced change of the mediator, meaning the exposure is controlled at *x*, but the mediator is changed from the level it would take at *x** to the level it would take at *x* [60].

In single mediator models with a continuous mediator and outcome, traditional mediation methods, including the difference and product method, provide similar effect estimates as causal mediation analysis. However, one advantage of causal mediation analysis is that it allows for exposure-mediator interactions, which capture any changes in the strength or direction of mediation by level of the exposure [61]. In this analysis, interactions between psychosocial factors and depressive symptoms were included in all mediation models.

We used linear regression models to estimate: (1) the association of each psychosocial factor with depressive symptoms (Equation 1) and (2) the association of depressive symptoms with EAA, adjusting for the psychosocial factor and the psychosocial factor-depressive symptoms interaction (Equation 2).

$$M = \alpha_0 + \alpha_1 X + \alpha_C^T C + \varepsilon_M, (\boldsymbol{Equation\ 1})$$

$$Y = \beta_0 + \beta_1 X + \beta_2 M + \beta_3 X * M + \beta_C^T C + \varepsilon_Y. (\boldsymbol{Equation\ 2})$$

In Equations 1 and 2, which reflect the mediator and outcome models for a single individual, M represents depressive symptoms, X represents the psychosocial factor (psychosocial stress, loneliness), C is the set of potential confounders (sex, educational attainment, marital status, having children, employment status, total wealth, year of psychosocial stress measurement, white

blood cell proportions, and genetic ancestry PCs), Y represents a single EAA measure, X*M represents the interaction between the psychosocial factor and depressive symptoms, and ε_M and ε_Y are residual errors, following an independent and normal distribution. Subsequently, TE, NDE, and NIDE, as well as the proportion mediated, were calculated for each exposure-outcome pair.

ii. Mediation Stratified by Sex and Educational Attainment

We then conducted stratified mediation analyses for any significant interactions observed between sociodemographic characteristics and psychosocial factors on EAA in the full sample. This allowed us to understand whether the mediation effect of depressive symptoms on the relationship between each psychosocial factor and EAA differed by sociodemographic characteristics.

iii. Mediation Sensitivity Analysis after Removing Loneliness Item from CES-D

Finally, one item in the CES-D explicitly asks participants the question, "Do you feel lonely?" (yes/no). Thus, as a sensitivity analysis, we removed that item when assessing the mediation effect of depressive symptoms on the relationship between loneliness and EAA to ensure that it was not significantly driving the mediation results. For all mediation analyses, $p<0.05$ was considered significant.

4.4 Results

4.4.1 Descriptive Statistics

Sample characteristics are shown in Table 4-1. The mean age of participants (n=2,681) was 70.4 years (SD: 9.5) and the majority were female (59%). Approximately three-quarters of the sample were non-Hispanic White (74%), 13% were non-Hispanic Black, 10% were Hispanic, and 3% were another race/ethnicity. Over a quarter of participants had a college degree or higher

(26%) and nearly half were working for pay (44%). Seventy percent of the sample was either married or had a partner, and the vast majority of participants had at least one child (89%). In terms of health behaviors, 45% of participants never smoked, 43% were former smokers, and 12% reported currently smoking at the time of their interview. A total of 59% reported not drinking alcohol, while 34% were moderate drinkers, and 7% were heavy drinkers. Finally, approximately 80% of the sample was classified as either overweight or obese, having a BMI greater than 25 kg/m^2. Participants with complete data on stress and adjustment covariates tended to be older, have higher education, and have healthier behaviors than those from the DNA methylation sample who were excluded, though the effect sizes between the two groups were relatively small (Cramer's V and Cohen's d <0.30 for all covariates) (Table S4-3).

4.4.2 Correlation among Chronological Age, Epigenetic Clocks, and EAA

Participants' epigenetic age ranged from 55.3 to 68.6 years based on the four epigenetic clocks. Additionally, DunedinPACE had a mean of 1.03 (SD: 0.15), suggesting this sample had a slightly accelerated pace of aging. Correlations among the five epigenetic clocks were between 0.06 and 0.77, and each clock was highly correlated with chronological age (all $p<0.001$; Table S4-4). When adjusting the clocks for chronological age, the strength of the associations diminished, with correlations among EAA measures ranging from 0 to 0.62 (Table S4-5). These correlations remained nominally significant ($p<0.05$), aside from DunedinPACE and Horvath age acceleration.

4.4.3 Associations between Psychosocial Factors/Depressive Symptoms and EAA

In the primary analysis (Model 1), a 1-standard deviation (SD) increase in cumulative psychosocial stress was associated with accelerated aging in HannumAA (β=0.33 years [0.14, 0.52]), PhenoAA (β=0.42 years [0.16, 0.69]), and GrimAA (β=0.42 years [0.26, 0.58]), as well

as a faster pace of aging in DunedinPACE (β=0.013 [0.007, 0.019]; Table 4-2). Similarly, a 1-SD increase in loneliness was associated with accelerated aging in HannumAA (β=0.32 years [0.13, 0.51]), PhenoAA (β=0.49 years [0.23, 0.75]), and GrimAA (β=0.22 years [0.06, 0.37]), and a faster pace of aging in DunedinPACE (β=0.01 [0.004, 0.015]). Finally, an increase in depressive symptoms was associated with higher HannumAA (β=0.27 years [0.08, 0.46]), GrimAA (β=0.36 years [0.20, 0.53]), and DunedinPACE (β=0.017 [0.012, 0.023]).

After including smoking status, alcohol use, and BMI as additional covariates (Model 2), we observed an attenuation of beta estimates for all three exposures (Table 4-2). However, the observed associations remained significant at an FDR $q<0.05$, except for associations between cumulative stress and both PhenoAA and GrimAA, loneliness and GrimAA, and depressive symptoms and HannumAA.

4.4.4 Effect Modification by Sex and Educational Attainment

We next assessed whether the associations between our exposures of interest and EAA were modified by specific sociodemographic characteristics, including sex and educational attainment. We found significant interactions between sex and both cumulative psychosocial stress and loneliness on DunedinPACE (Table 4-3 and Figure 4-1). A 1-SD increase in psychosocial stress was associated with a 0.019 increase in pace of aging for males versus a 0.036 increase in pace of aging for females. Similarly, a 1-SD increase in loneliness was associated with a 0.015 increase in pace of aging for males versus a 0.027 increase in pace of aging for females. These interactions remained significant after adjusting for Model 2 covariates (Table S4-6).

Additionally, after dichotomizing educational attainment to be less than college degree vs. college degree or higher, we observed significant interactions between loneliness and

educational attainment on EAA in PhenoAge and GrimAge, and on DunedinPACE (Table 4-3 and Figure 4-2). A 1-SD increase in loneliness was associated with a 0.061 increase and 0.027 decrease in PhenoAA and GrimAA, respectively, for those with a college degree, as opposed to a 0.66 increase in PhenoAA and a 0.32 increase in GrimAA for those without a college degree. Additionally, a 1-SD increase in loneliness was associated with a 0.001 versus a 0.013 increase in pace of aging for individuals with and without a college degree, respectively, as measured by DunedinPACE. In Model 2, these interactions were no longer significant (Table S4-6). When dichotomizing educational attainment to be no degree vs. high school degree or higher, we did not observe significant interactions for any of the EAA measures (Table S4-7).

4.4.5 Mediation Analysis

A. Mediation by Depressive Symptoms on Associations between Psychosocial Factors and EAA

Finally, we investigated the mediating effect of depressive symptoms on the associations between psychosocial factors and EAA. Cumulative psychosocial stress explained between 1.5% and 4.7% of the total variability of HannumAA, PhenoAA, GrimAA, and DunedinPACE after adjusting for sociodemographic covariates in the primary model (Model 1), and between 0.1% and 0.9% of the total variability after further adjusting for health behaviors (Model 2; Table S4-8). Loneliness explained between 1.4% and 4.1% of the total variability in Model 1 and between 0.1% and 2.4% of the total variability in Model 2. We found that depressive symptoms mediated 25.1%, 24.0%, and 35.3% of the relationship between cumulative psychosocial stress and HannumAA, GrimAA, and DunedinPACE, respectively (Table 4-4). Furthermore, depressive symptoms mediated 39.9% of the relationship between loneliness and GrimAA, as well as 36.5% of the relationship between loneliness and DunedinPACE (all $p<0.05$). After further adjusting for health behaviors in the mediation models, depressive symptoms mediated 49.9% of the

association between cumulative stress and DunedinPACE, and 33.2% of the association between loneliness and DunedinPACE (Table S4-9).

B. Mediation Stratified by Sex and Educational Attainment

We then conducted stratified mediation analyses when significant interactions between sociodemographic characteristics and psychosocial factors on EAA were present. Specifically, we examined the mediating effect of depressive symptoms on the relationship between psychosocial factors and DunedinPACE stratified by sex. In females, depressive symptoms mediated 27.6% of the relationship between cumulative stress and DunedinPACE, as well as 24.8% of the relationship between loneliness and DunedinPACE (Model 1; Table S4-10). After adjusting for health behaviors, the mediation effects persisted, with depressive symptoms mediating 30.6% and 19.9% of the associations between cumulative stress and loneliness, respectively, and DunedinPACE (Table S4-10). Conversely, in males, the total effects of both cumulative stress and loneliness on DunedinPACE were not significant in Model 1. As a result, the mediation effect of depressive symptoms on these relationships was not evaluated.

Additionally, we examined the mediating effect of depressive symptoms on the associations between loneliness and PhenoAA, GrimAA, and DunedinPACE stratified by educational attainment (college degree vs. no college degree). For those without a college degree, depressive symptoms mediated 36.5% and 31.1% of the relationships between loneliness and both GrimAA and DunedinPACE, respectively (Model 1; Table S4-11). However, the total effects of loneliness on GrimAA and DunedinPACE were no longer significant after adjusting for health behaviors (Model 2). Additionally, for those with a college degree, the total effects of loneliness on all EAA measures were not significant in Model 1.

C. Mediation Sensitivity Analysis after Removing Loneliness Item from CES-D

Finally, we assessed the mediating effect of depressive symptoms on the association between loneliness and EAA, excluding the loneliness-specific item from the CES-D. Results obtained for both Models 1 and 2 were substantively similar to results from the primary mediation analysis (Table S4-12).

4.5 Discussion

In this study, psychosocial factors, including cumulative psychosocial stress and loneliness, and depressive symptoms were associated with EAA in a multi-racial/ethnic cohort of older adults. We found that females, as well as those with no college degree, were more sensitive to the effects of psychosocial factors on epigenetic aging. Furthermore, depressive symptoms partially mediated the relationship between psychosocial factors and EAA for a number of epigenetic clocks. To our knowledge, this was the first study to examine the mediating role of mental health on the relationships between cumulative psychosocial stress, loneliness, and epigenetic aging in a diverse sample of older adults.

Several studies examined the association between individual psychosocial stress measures and epigenetic aging; however, results were mixed. In the Berlin Aging Study II (BASE-II), a cohort of middle-aged and older adults comprised mostly of European ancestry, Vetter et al. (2022) found that psychosocial stress, evaluated using the Perceived Stress Scale, was not associated with HorvathAA, HannumAA, PhenoAA, and GrimAA. Skinner et al. (2023) looked at those same EAA measures in a cohort of postmenopausal women from the Women's Health Initiative (WHI) and only observed associations between stressful life events and GrimAA. A systematic review by Lim et al. (2022) provided further evidence of discordant findings when they reported that only two of the six studies included in the review had positive associations between either lifetime trauma or discrimination and EAA. A possible explanation

for why we are observing stronger associations between psychosocial stress and several EAA measures in our study is because the psychosocial stress measure captures multiple stress domains across the life course. Research has shown that the accumulation of stress results in worsened physical and mental health outcomes compared to individual stressors [62,63]. Thus, it is reasonable to assume cumulative psychosocial stress has a similar dose-response relationship on epigenetic aging. Future analyses should examine the associations between individual stress domains and EAA in the same sample to assess if particular domains are driving the relationships.

In terms of loneliness, findings from this study are fairly consistent with those from previous literature. In a cohort of middle-aged adults from the Midlife in the United States (MIDUS) study, Freilich et al. (2024) found that loneliness, measured by a single, self-reported item, was associated with EAA estimated by GrimAge and DunedinPACE after adjusting for sex, race and education [32]. The addition of behavioral characteristics, including smoking status, alcohol use, and BMI, attenuated their results, though the associations did remain nominally significant ($p<0.05$). Although associations with HannumAA and PhenoAA were not significant, as they were in our study, the beta coefficients were in the same direction. Similarly, Beach et al. (2022) examined the association between loneliness and DunedinPACE in a sample of middle-aged Black adults using two items from the UCLA Loneliness Scale [33]. Over an 11-year period, they found that change in loneliness was associated with a change in DunedinPACE, after adjusting for sociodemographic characteristics, as well as smoking and alcohol use.

When we assessed sociodemographic characteristics as possible effect modifiers of the relationship between the psychosocial factors and epigenetic aging, interactions revealed that the effects of psychosocial stress and loneliness on epigenetic aging measured by DunedinPACE

were driven by females. Prior research has determined differences in stress response between males and females due to a variety of biological and psychological mechanisms [64-66]. Two functional magnetic resonance imaging studies performed by Goldstein et al. (2010) and Cohen et al. (2023) demonstrated that response to stress activated different regions of the brain for men and women [67,68]. Specifically, they found that sex differences in stress were regulated through hormones via the impact of subcortical brain activity. Further, from a psychological perspective, females are often less resilient to stress [69,70] and have stronger perceptions of stress [71,72] than their male counterparts. This may partially explain why females experience higher rates of mental health disorders, including anxiety [73,74] and depression [75,76]. Thus, results from our study, which found that females experienced a sharper acceleration in epigenetic aging in the presence of stress, are logical in the context of this research.

Mediation analyses revealed that depressive symptoms mediated between 24% and 35% of the relationship between cumulative psychosocial stress and HannumAA, GrimAA, and DunedinPACE, in addition to 40% and 37% of the relationships between loneliness and GrimAA and DunedinPACE, respectively. When significant interactions between sociodemographic characteristics and psychosocial factors on EAA were present, we performed stratified mediation analyses and found that the mediation effects persisted only in females and those without a college degree. A study by Beydoun et al. (2022), which examined the mediating role of depressive symptoms on the associations between perceived discrimination and epigenetic aging in HRS, yielded similar results [34]. After adjusting the models for birth cohort, sex, and race/ethnicity, they found that depressive symptoms mediated roughly 39% and 32% of the relationships between reasons for perceived discrimination (RPD) and GrimAge and DunedinPoAm38, a pace of aging measure that preceded DunedinPACE, respectively. Further,

when stratified by sex, the mediating effects of depressive symptoms on the associations between RPD and these two epigenetic clocks were only present in females. As both of these studies were conducted in HRS, a future next step would be to replicate these findings in an independent cohort of older adults.

The strength of associations between psychosocial factors and EAA varied across epigenetic clocks. We observed the weakest associations with first-generation clocks, though psychosocial factors and depressive symptoms were associated with HannumAA. Alternatively, cumulative psychosocial stress, loneliness, and depressive symptoms were strongly associated with second- and third-generation clocks. A systematic review conducted by Oblak et al. (2021), which examined general trends regarding the associations between epigenetic clocks and several biological, social and environmental factors, supported our findings [77]. This review concluded that differences between chronological age-trained (first-generation) clocks and mortality-trained (second- and third-generation) clocks were more prominent in mental health outcomes, including post-traumatic stress disorder, schizophrenia, and depression, than physical outcomes. Thus, future research should focus on exploring distinct biological mechanisms that may link individual epigenetic clocks to mental health outcomes.

There are limitations to this study that must be acknowledged. First, psychosocial factors, depressive symptoms, and epigenetic aging were all assessed at a single time point. Although we implemented a longitudinal study design, the exposures, mediator, and outcomes were only measured four to six years apart, therefore introducing the possibility of reverse causation. Future studies would benefit from examining these variables at multiple time points to assess changes in epigenetic aging resulting from psychosocial factors. Additionally, although the CES-D shows high diagnostic accuracy and is often used as a first step in screening for depression

[78], this subjective measure assesses depressive symptoms over a short period of time and does not represent a diagnosis of clinical depression. Therefore, these findings should be replicated in a population that has clinical depression diagnoses available. Finally, when we stratified the mediation models by sex and educational attainment, we observed different sample sizes in each of the strata, which may have impacted the magnitude of the total effects. As a result, these analyses should be replicated in a population with larger strata to ensure the lack of association in certain sociodemographic groups was not due to statistical power.

This study also had several strengths. First, we examined the association between psychosocial factors and epigenetic aging using validated, self-report measures of loneliness and depressive symptoms, as well as a cumulative psychosocial stress score that comprised several domains of stress across the life course. Examining multiple psychosocial measures allowed us to compare the strength in which specific factors influenced epigenetic aging. Second, we implemented a causal mediation method that accounted for interactions between psychosocial factors and depressive symptoms in the mediation models. Thus, unlike more traditional mediation methods, this method captured differences in the strength of the mediation effect by levels of psychosocial stress and loneliness. Finally, this research was conducted in a sample comprised of African, European, and Hispanic race/ethnicities, which allowed us to examine the associations between psychosocial factors and epigenetic aging, as well as the mediating role of depressive symptoms on those relationships, in a diverse population of older adults.

4.6 Conclusion

Results from this study demonstrate that cumulative psychosocial stress, loneliness, and depressive symptoms are associated with epigenetic aging in a multi-racial/ethnic population of older adults, with females and those without a college degree appearing more sensitive to these

effects. Furthermore, depressive symptoms partially mediate the relationship between psychosocial factors and epigenetic aging. Future research should be conducted to replicate these findings and further characterize the biological mechanisms through which psychosocial factors influence mental health outcomes and may contribute to age-related disease.

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4.8 Tables

Table 4-1: Characteristics of Health and Retirement Study analytic sample (N=2,681).

Characteristic	N (%) or mean (SD)
Age, years	70.4 (9.5)
Female	1585 (59.2)
Race/Ethnicity	
Hispanic	270 (10.1)
Black	349 (13.0)
White	1992 (74.3)
Other	69 (2.6)
Education	
No degree	346 (12.9)
High school degree	1625 (60.6)
College degree or higher	710 (26.5)
Employment Status	
Working for pay	1173 (43.8)
Not working for pay	1508 (56.2)
Marital Status	
Married/Partnered	1876 (70.0)
Separated/Divorced/Widowed	680 (25.4)
Never married	125 (4.7)
Have children	2384 (88.9)
Total Wealth	$443,612 ($954,116)
Smoking Status	
Never smoked	1205 (45.2)
Former smoker	1134 (42.6)
Current smoker	326 (12.2)
Alcohol Use	
Non-drinker	1582 (59.1)
Moderate drinker	912 (34.0)
Heavy drinker	186 (6.9)
BMI (kg/m^2)	
Underweight/Normal	525 (19.6)
Overweight	896 (33.5)
Obese class I	728 (27.2)
Obese class II	524 (19.6)
Epigenetic Age	
HorvathAge, years	66.4 (9.5)
HannumAge, years	55.3 (9.2)
PhenoAge, years	58.1 (10.0)
GrimAge, years	68.6 (8.6)
Epigenetic Age Acceleration (AA)	
HorvathAA, years	0.0 (6.5)
HannumAA, years	0.0 (5.4)
PhenoAA, years	0.0 (6.9)
GrimAA, years	0.0 (4.4)
DunedinPACE, accelerated aging per year	1.03 (0.15)

Table 4-2: Associations between psychosocial factors/depressive symptoms and epigenetic age acceleration.

	HorvathAA	HannumAA	PhenoAA	GrimAA[a]	DunedinPACE[b]
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Model 1 (N=2,681)					
Psychosocial Stress	-0.10 (-0.35, 0.15)	**0.33 (0.14, 0.53)**	**0.42 (0.15, 0.69)**	**0.41 (0.25, 0.58)**	**0.013 (0.007, 0.019)**
Loneliness	0.18 (-0.06, 0.42)	**0.32 (0.13, 0.51)**	**0.49 (0.23, 0.75)**	**0.21 (0.05, 0.37)**	**0.010 (0.004, 0.015)**
Depressive Symptoms	-0.004 (-0.25, 0.24)	**0.27 (0.08, 0.46)**	0.24 (-0.02, 0.50)	**0.36 (0.20, 0.52)**	**0.017 (0.012, 0.023)**
Model 2 (N=2,656)					
Psychosocial Stress	-0.14 (-0.39,0.12)	**0.26 (0.07, 0.46)**	0.26 (-0.001, 0.53)	0.08 (-0.06, 0.22)	**0.007 (0.002, 0.012)**
Loneliness	0.14 (-0.10, 0.38)	**0.26 (0.07, 0.45)**	**0.41 (0.15, 0.67)**	0.08 (-0.06, 0.21)	**0.007 (0.002, 0.012)**
Depressive Symptoms	-0.05 (-0.30, 0.19)	0.20 (0.08, 0.40)	0.12 (-0.14, 0.38)	**0.16 (0.03, 0.30)**	**0.013 (0.008, 0.018)**

Model 1: Epigenetic age acceleration ~ Psychosocial factor + Sex + Educational attainment + Marital status + Employment + Has child + Top 10 genetic ancestry PCs + WBC proportions + Year of psychosocial battery

Model 2: Model 1 + Smoking status + Alcohol use + BMI

[a] Models were not adjusted for sex, as GrimAge was regressed upon sex to calculate GrimAA.

[b] Age was included as an additional covariate in these models.

For HorvathAA, HannumAA, PhenoAA, and GrimAA, β represents change in years of epigenetic age for 1 SD increase in the mean psychosocial factor score; for DunedinPACE, it represents the per-year acceleration of aging for 1 SD increase in mean psychosocial factor score.

FDR q-value < 0.05 is bolded.

Table 4-3: Two-way interaction between psychosocial factors/depressive symptoms and demographic characteristics on epigenetic age acceleration (Model 1; N=2,681).

Interaction Term	Exposure	EAA measure	$\beta_{Psychosocial}$	$P_{Psychosocial}$	β_{Demo}	P_{Demo}	$\beta_{Interaction}$	$P_{Interaction}$
*Exposure*Sex*	Psychosocial Stress	Hannum	0.15	0.31	**-1.07**	**1.07E-07**	0.33	0.08
		DunedinPACE	0.004	0.33	**-0.02**	**8.34E-04**	**0.016**	**0.004**
	Loneliness	Hannum	**0.31**	**0.03**	**-1.08**	**9.34E-08**	0.01	0.94
		DunedinPACE	0.003	0.53	**-0.02**	**4.02E-04**	**0.01**	**0.003**
	Depressive Symptoms	Hannum	0.20	0.23	**-1.18**	**5.91E-09**	0.11	0.59
		DunedinPACE	**0.012**	**0.01**	**-0.02**	**2.47E-05**	0.008	0.13
Exposure College Degree*	Psychosocial Stress	PhenoAge	**0.49**	**0.001**	**-0.64**	**0.04**	-0.32	0.29
		GrimAge	**0.44**	**2.84E-06**	**-1.54**	**1.07E-15**	-0.08	0.66
		DunedinPACE	**0.01**	**1.46E-05**	**-0.04**	**1.99E-10**	-0.002	0.73
	Loneliness	PhenoAge	**0.66**	**1.59E-05**	**-0.68**	**0.03**	**-0.60**	**0.04**
		GrimAge	**0.32**	**5.68E-04**	**-1.62**	**2.00E-16**	**-0.35**	**0.049**
		DunedinPACE	**0.01**	**1.28E-05**	**-0.04**	**9.42E-12**	**-0.01**	**0.03**
	Depressive Symptoms	GrimAge	**0.42**	**2.70E-06**	**-1.56**	**1.28E-15**	-0.21	0.31
		DunedinPACE	**0.02**	**1.57E-10**	**-0.04**	**6.46E-10**	-0.004	0.56

Interaction Model: Epigenetic age acceleration ~ Psychosocial factor + Sex + Educational attainment + Marital status + Employment + Has child + Top 10 genetic ancestry PCs + WBC proportion + Year of psychosocial battery + Psychosocial factor*Demographic characteristic

$\beta_{Psychosocial}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in the mean psychosocial factor score; $\beta_{Demographic}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for females/those with a college degree; $\beta_{Interaction}$ represents difference in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in mean psychosocial factor score between levels of demographic variables.

We only tested for interactions when the main effects of both the exposure of interest (psychosocial stress, loneliness, depressive symptoms) and demographic factor were associated with EAA in Model 1.

P-value <0.05 is bolded.

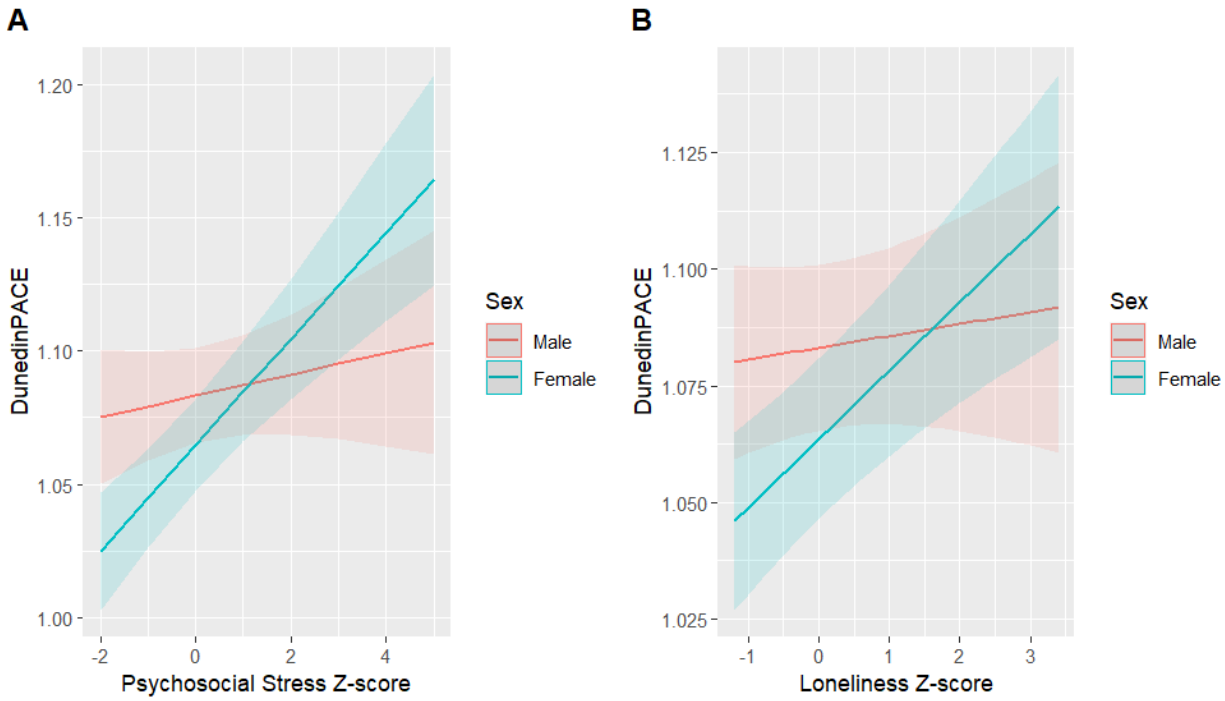
Table 4-4: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and epigenetic age acceleration (Model 1; N=2,681).

	HannumAA		PhenoAA		GrimAA		DunedinPACE	
	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value
Psychosocial Stress								
Total Effect	**0.31 (0.12, 0.51)**	**0.002**	0.44 (0.17, 0.71)	0.001	**0.40 (0.24, 0.57)**	**1.65E-06**	**0.013 (0.007, 0.019)**	**7.04E-06**
Direct Effect	**0.23 (0.02, 0.44)**	**0.028**	0.42 (0.14, 0.71)	0.004	**0.31 (0.13, 0.48)**	**5.53E-04**	**0.008 (0.002, 0.014)**	**0.006**
Indirect Effect	**0.08 (0.01, 0.14)**	**0.016**	0.02 (-0.07, 0.10)	0.75	**0.09 (0.04, 0.15)**	**4.09E-04**	**0.004 (0.003, 0.006)**	**1.30E-06**
Proportion Mediated	**0.25**	--	0.03	--	**0.24**	--	**0.35 (0.14, 0.56)**	--
Loneliness								
Total Effect	0.32 (0.13, 0.51)	9.06E-04	0.50(0.24, 0.76)	1.61E-04	**0.21 (0.05, 0.37)**	**0.008**	**0.010 (0.005, 0.015)**	**2.85E-04**
Direct Effect	0.26 (0.06, 0.46)	0.01	0.49 (0.22, 0.76)	4.39E-04	**0.13 (-0.04, 0.29)**	**0.13**	**0.006 (0.0007, 0.011)**	**0.03**
Indirect Effect	0.06 (-0.0007, 0.11)	0.05	0.01 (-0.07, 0.09)	0.81	**0.09 (0.04, 0.13)**	**5.49E-04**	**0.004 (0.002, 0.005)**	**1.26E-05**
Proportion Mediated	0.18	--	0.02	--	**0.40**	--	**0.36**	--

Associations with significant indirect effects (p-value <0.05) are bolded.

4.9 Figures

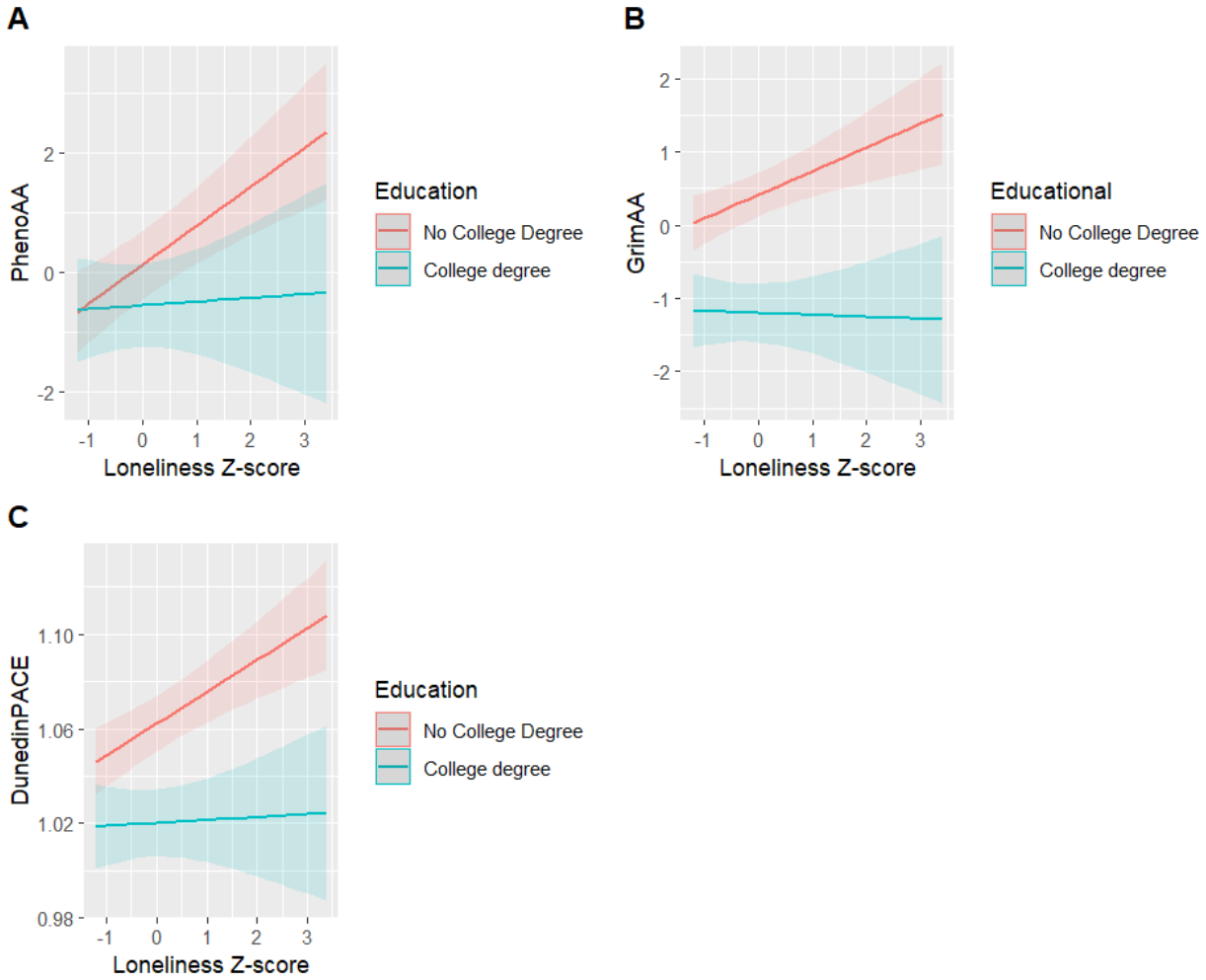
Figure 4-1: Plots of significant interactions between psychosocial factors and sex on DunedinPACE (p<0.05). Predicted DunedinPACE values for (A) cumulative psychosocial stress z-scores and (B) loneliness z-scores by sex are shown with 95% confidence intervals.



95% confidence intervals intend to capture the true line in the population 95% of the time across repeated samples.

Interaction models: DunedinPACE ~ psychosocial factor + sex + educational attainment + marital status + employment + has child + top 10 genetic ancestry PCs + WBC proportion + year of psychosocial battery + psychosocial factor*sex.

Figure 4-2: Plots of significant interactions between loneliness and educational attainment on epigenetic age acceleration measures (p<0.05). Predicted (A) PhenoAA, (B) GrimAA, and (C) DunedinPACE values for loneliness z-scores by educational attainment (no college degree vs. college degree or higher) are shown with 95% confidence intervals.



95% confidence intervals intend to capture the true line in the population 95% of the time across repeated samples. Interaction models: DunedinPACE ~ psychosocial factor + sex + educational attainment + marital status + employment + has child + top 10 genetic ancestry PCs + WBC proportion + year of psychosocial battery + psychosocial factor*educational attainment.

4.10 Supplementary Material

Table S4-1: Eleven-item scale of loneliness derived from the 20-item Revised UCLA Loneliness Scale.

The next questions are about how you feel about different aspects of your life. HOW MUCH OF THE TIME DO YOU FEEL...
1. You lack companionship?
2. Left out?
3. Isolated from others?
4. That you are “in tune” with the people around you?
5. Alone?
6. That there are people you can talk to?
7. That there are people you can turn to?
8. That there are people who really understand you?
9. That there are people you feel close to?
10. Part of a group of friends?
11. That you have a lot in common with the people around you?

Coding: 1 = Often, 2 = Some of the time, 3 = Hardly ever or never

Scaling: The index of loneliness was created by reverse coding items 1, 2, 3, and 5 and averaging the scores across all 11 items. The final loneliness score was set to missing if there were greater than 5 items with missing values.

Table S4-2: Eight-item scale of depressive symptoms derived from the Center for Epidemiologic Studies Depression Scale (CES-D).

Now think about the past week and the feelings you have experienced. Please tell me if each of the following was true for you much of the time during the past week. Much of the time during the past week…..
1. You felt depressed?
2. You felt that everything you did was an effort?
3. Your sleep was restless?
4. You were happy.
5. You felt lonely.
6. You enjoyed life.
7. You felt sad?
8. You could not get going?

Coding: 0=No, 1=Yes

Scaling: The depressive symptoms score was created by reverse coding items 4 and 6 and summing the scores across all 8 items.

Table S4-3: **Participant characteristics comparing those included and excluded in the primary analysis from the full DNA methylation sample (N=4018).**

Characteristics	Included (n=2681) N(%) or mean (SD)	Excluded (n=1337) N(%) or mean (SD)	Effect Size (Cohen's d or Cramer's V)	P-value
Age, years	70.4 (9.5)	67.5 (9.6)	0.30	<0.001
Female	1585 (59.2)	764 (57.1)	0.02	0.20
Race/Ethnicity				
Hispanic	270 (10.1)	297 (22.2)		
Black	349 (13.0)	309 (23.1)		
White	1992 (74.3)	677 (50.7)	0.24	<0.001
Other	69 (2.6)	53 (4.0)		
Education				
No degree	346 (12.9)	329 (24.6)		
HS degree	1625 (60.6)	750 (56.1)	0.15	<0.001
College degree or higher	710 (26.5)	257 (19.2)		
Employment Status				
Working for pay	1173 (43.8)	919 (71.2)	0.26	<0.001
Not working for pay	1508 (56.2)	371 (28.8)		
Married/Partnered	1876 (70.0)	818 (63.3)	0.07	<0.001
Have Children	2384 (88.9)	1176 (88.0)	0.07	0.40
Total Household Wealth ($)	$443,612 ($954,116)	$303,758 ($793.648)	0.15	<0.001
Smoking Status				
Never smoker	1205 (45.2)	542 (42.1)		
Former smoker	1134 (42.6)	527 (40.9)	0.10	<0.001
Current smoker	326 (12.2)	219 (17.0)		
Alcohol Use				
Never drinker	1582 (59.1)	832 (64.5)		
Moderate drinker	912 (34.0)	367 (28.5)	0.06	0.002
Heavy drinker	186 (6.9)	91 (7.1)		
BMI (kg/m^2)	30.4 (6.7)	29.2 (6.5)	0.18	<0.001

Table S4-4: Pearson correlation among epigenetic clocks and chronological age.

Epigenetic Age	Age	HorvathAge	HannumAge	PhenoAge	GrimAge	DunedinPACE
Age	1.00					
HorvathAge	0.72	1.00				
HannumAge	0.81	0.77	1.00			
PhenoAge	0.72	0.64	0.76	1.00		
GrimAge	0.84	0.63	0.75	0.74	1.00	
DunedinPACE	0.12	0.06	0.22	0.35	0.44	1.00

All correlations are significant at $p<0.001$.

Table S4-5: Pearson correlation among epigenetic age acceleration measures.

Epigenetic Age Acceleration	HorvathAA	HannumAA	PhenoAA	GrimAA	DunedinPACE
HorvathAA	1.00				
HannumAA	0.46	1.00			
PhenoAA	0.25	0.43	1.00		
GrimAA	0.04	0.17	0.35	1.00	
DunedinPACE	-0.03	0.21	0.38	0.62	1.00

All correlations are significant at $p<0.05$ except for DunedinPACE and HorvathAA ($p=0.091$).

Table S4-6: Two-way interaction between psychosocial factors/depressive symptoms and demographic characteristics on epigenetic age acceleration (Model 2; N=2,656).

Interaction Term	Exposure	EAA measure	$\beta_{Psychosocial}$	$P_{Psychosocial}$	β_{Demo}	P_{Demo}	$\beta_{Interaction}$	$P_{Interaction}$
*Exposure*Sex*	Psychosocial Stress	Hannum	0.06	0.66	**-1.08**	**1.75E-07**	0.35	0.06
		DunedinPACE	-0.001	0.71	-0.009	0.09	**0.02**	**0.002**
	Loneliness	Hannum	0.23	0.11	**-1.07**	**2.34E-07**	0.05	0.77
		DunedinPACE	0.004	0.91	-0.009	0.10	**0.01**	**0.019**
	Depressive Symptoms	Hannum	0.13	0.42	**-1.15**	**2.73E-08**	0.10	0.60
		DunedinPACE	0.007	0.09	**-0.012**	**0.03**	0.009	0.09
Exposure College Degree*	Psychosocial Stress	PhenoAge	**0.33**	**0.03**	-0.49	0.11	-0.30	0.33
		GrimAge	0.06	0.44	**-0.93**	**1.30E-08**	0.10	.0.55
		DunedinPACE	**0.007**	**0.02**	**-0.03**	**2.39E-05**	-0.001	0.84
	Loneliness	PhenoAge	**0.56**	**2.51E-04**	-0.51	0.10	-0.52	0.07
		GrimAge	0.14	0.07	**-0.97**	**2.14E-09**	-0.23	0.13
		DunedinPACE	**0.01**	**7.48E-04**	**-0.03**	**7.86E-05**	-0.009	0.11
	Depressive Symptoms	GrimAge	**0.18**	**0.02**	**-0.93**	**2.02E-08**	-0.05	0.78
		DunedinPACE	**0.01**	**1.18E-06**	**-0.02**	**8.34E-05**	0.0001	0.99

Interaction Model: Epigenetic age acceleration ~ Psychosocial factor + Sex + Educational attainment + Marital status + Employment + Has child + Smoking status + Alcohol use + BMI + Top 10 genetic ancestry PCs + WBC proportion + Year of psychosocial battery + Psychosocial factor*Demographic characteristic

$\beta_{Psychosocial}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in the mean psychosocial factor score; $\beta_{Demographic}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for females/those with a college degree; $\beta_{Interaction}$ represents difference in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in mean psychosocial factor score between levels of demographic variables.

We only tested for interactions when the main effects of both the exposure of interest (psychosocial stress, loneliness, depressive symptoms) and demographic factor were associated with EAA in Model 1.

P-value <0.05 is bolded.

Table S4-7: Two-way interaction between psychosocial factors/depressive symptoms and educational attainment (no degree vs. HS degree) on epigenetic age acceleration (Model 1; N=2,681).

Interaction Term	Exposure	EAA measure	$\beta_{Psychosocial}$	$P_{Psychosocial}$	β_{Demo}	P_{Demo}	$\beta_{Interaction}$	$P_{Interaction}$
*Exposure*HS Degree*	Psychosocial Stress	PhenoAge	0.42	0.20	-0.64	0.13	0.03	0.94
		GrimAge	0.39	0.06	**-1.16**	**7.42E-06**	0.09	0.67
		DunedinPACE	0.009	0.16	**-0.03**	**4.08E-05**	0.006	0.38
	Loneliness	PhenoAge	0.26	0.47	-0.62	0.14	0.27	0.48
		GrimAge	**0.49**	**0.03**	**-1.06**	**5.95E-05**	-0.29	0.23
		DunedinPACE	0.005	0.51	**-0.03**	**6.27E-05**	0.006	0.43
	Depressive Symptoms	GrimAge	0.33	0.07	**-1.08**	**6.44E-05**	0.11	0.61
		DunedinPACE	**0.014**	**0.02**	**-0.03**	**4.57E-04**	0.005	0.43

Interaction Model: Epigenetic age acceleration ~ Psychosocial factor + Sex + Educational attainment + Marital status + Employment + Has child + Top 10 genetic ancestry PCs + WBC proportion + Year of psychosocial battery + Psychosocial factor*Demographic characteristic

$\beta_{Psychosocial}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in the mean psychosocial factor score; $\beta_{HSdegree}$ represents change in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for those with a HS degree; $\beta_{Interaction}$ represents difference in years of epigenetic age (or per-year acceleration of aging for DunedinPACE) for 1 SD increase in mean psychosocial factor score between individuals with and without a HS degree.

We only tested for interactions when the main effects of both the exposure of interest (psychosocial stress, loneliness, depressive symptoms) and educational attainment were associated with EAA in Model 1.

P-value <0.05 is bolded.

Table S4-8: Total variability in epigenetic age acceleration explained by psychosocial factors (FDR q<0.05).

	Percent Variance Explained (PVE)			
	HannumAA	**PhenoAA**	**GrimAA**	**DunedinPACE**
Model 1 (N=2,681)				
Psychosocial Stress	1.47%	2.86%	4.68%	1.99%
Loneliness	1.46%	4.08%	1.37%	1.50%
Model 2 (N=2,656)				
Psychosocial Stress	0.83%	0.93%	0.13%	0.07%
Loneliness	0.90%	2.35%	0.13%	0.36%

Model 1: Epigenetic age acceleration ~ Psychosocial determinant + Gender + Educational attainment + Marital Status + Employment + Has child + Top 10 genetic ancestry PCs + WBC proportion + Year of psychosocial battery

Model 2: Model 1 + Smoking status + Alcohol use + BMI

Table S4-9: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and epigenetic age acceleration (Model 2; N=2,656)

	HannumAA		PhenoAA		GrimAA*		DunedinPACE	
	β (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value
Psychosocial Stress								
Total Effect	0.23 (0.03, 0.43)	0.02	0.29 (0.02, 0.57)	0.03	--	--	**0.007 (0.002, 0.012)**	**0.01**
Direct Effect	0.17 (-0.04, 0.38)	0.11	0.30 (0.01, 0.59)	0.04	--	--	**0.003 (-0.002, 0.009)**	**0.23**
Indirect Effect	0.06 (-0.002, 0.12)	0.06	-0.01 (-0.10, 0.07)	0.80	--	--	**0.003 (0.002, 0.005)**	**5.82E-05**
Proportion Mediated	0.26	--	-0.04	--	--	--	**0.50**	--
Loneliness								
Total Effect	0.25 (0.06, 0.44)	0.009	0.43 (0.17, 0.69)	0.001	--	--	**0.007 (0.002, 0.012)**	**0.004**
Direct Effect	0.21 (0.01, 0.41)	0.04	0.44 (0.17, 0.72)	0.002	--	--	**0.005 (-0.0003, 0.01)**	**0.06**
Indirect Effect	0.04 (-0.02, 0.10)	0.16	-0.01 (-0.09, 0.06)	0.67	--	--	**0.002 (0.001, 0.004)**	**7.45E-04**
Proportion Mediated	0.16	--	-0.04	--	--	--	**0.33**	--

Indirect effects with p-value <0.05 are bolded.

* Mediation was not assessed because the total effects of psychosocial factors and GrimAA were not significant (p-value>0.05).

Table S4-10: Mediation by depressive symptoms of the relationships between psychosocial stress/loneliness and DunedinPACE in females.

	DunedinPACE			
	Model 1 (N=1,585)		**Model 2 (N=1,565)**	
	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value
Psychosocial Stress				
Total Effect	**0.018 (0.010, 0.025)**	**6.05E-05**	**0.011 (0.004, 0.019)**	**0.002**
Direct Effect	**0.013 (0.005, 0.021)**	**0.002**	**0.008 (0.0005, 0.015)**	**0.04**
Indirect Effect	**0.005 (0.002, 0.007)**	**3.03E-04**	**0.004 (0.001, 0.006)**	**0.004**
Proportion Mediated	**0.28**	**--**	**0.31**	**--**
Loneliness				
Total Effect	**0.012 (0.004, 0.019)**	**0.002**	**0.010 (0.003, 0.017)**	**0.004**
Direct Effect	**0.009 (0.001, 0.016)**	**0.02**	**0.008 (0.001, 0.015)**	**0.03**
Indirect Effect	**0.003 (0.0007, 0.005)**	**0.009**	**0.002 (0.0001, 0.004)**	**0.04**
Proportion Mediated	**0.25**	**--**	**0.20**	**--**

Indirect effects with p-value <0.05 are bolded.

Table S4-11: Mediation by depressive symptoms of the relationships between loneliness and epigenetic age acceleration in individuals without a college degree (Model 1; N=1,971).

	PhenoAA		GrimAA		DunedinPACE	
	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value
Total Effect	0.61 (0.30, 0.92)	9.82E-05	**0.28 (0.08, 0.48)**	**0.005**	**0.012 (0.006, 0.018)**	**2.37E-04**
Direct Effect	0.63 (0.31, 0.95)	1.23E-04	**0.18 (-0.02, 0.38)**	**0.08**	**0.008 (0.002, 0.015)**	**0.01**
Indirect Effect	-0.02 (-0.12, 0.09)	0.75	**0.10 (0.04, 0.17)**	**0.002**	**0.004 (0.002, 0.006)**	**5.49E-04**
Proportion Mediated	-0.03	--	**0.36**	**--**	**0.31**	**--**

Indirect effects with p-value <0.05 are bolded.

Table S4-12: Mediation by depressive symptoms of the relationships between loneliness and epigenetic age acceleration after removing one item from CES-D measure.

	HannumAA		PhenoAA		GrimAA*		DunedinPACE	
	β (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value	Mediation Effect (95% CI)	P-value
Model 1 (N=2,681)								
Total Effect	0.32 (0.13, 0.51)	8.70E-04	0.50 (0.24,0.76)	1.73E-04	**0.21 (0.05, 0.37)**	**0.009**	**0.010 (0.004, 0.015)**	**3.14E-04**
Direct Effect	0.28 (0.09, 0.48)	0.004	0.50 (0.23, 0.77)	2.86E-04	**0.15 (-0.02, 0.31)**	**0.08**	**0.007 (0.001, 0.012)**	**0.02**
Indirect Effect	0.06 (-0.01, 0.09)	0.13	-0.0009 (-0.06,0.06)	0.98	**0.07 (0.03, 0.11)**	**0.001**	**0.003 (0.002, 0.005)**	**8.72E-06**
Proportion Mediated	0.12	--	-0.001	--	**0.31**	--	**0.32**	--
Model 2 (N=2,656)								
Total Effect	0.25 (0.06, 0.44)	0.009	0.43 (0.17, 0.69)	0.001	--	--	**0.007 (0.002, 0.012)**	0.004
Direct Effect	0.22 (0.03, 0.42)	0.03	0.44 (0.17, 0.72)	0.001	--	--	**0.005 (0.00001, 0.010)**	**0.04**
Indirect Effect	0.03 (-0.02, 0.07)	0.25	-0.01 (-0.08, 0.05)	0.62	--	--	**0.002 (0.0009, 0.003)**	**5.14E-04**
Proportion Mediated	0.11	--	-0.04	--	--	--	**0.30**	--

Indirect effects with p-value <0.05 are bolded.

*Mediation was not assessed in Model 2 because the total effect of loneliness and GrimAA was not significant (p-value > 0.05).

Chapter 5 Conclusion

5.1 Summary and Implications of Main Findings

The primary goals of this dissertation were to: (1) identify associations between long-term psychosocial stress and individual DNA methylation sites in older adults; (2) examine epigenetic mediation of the relationship between psychosocial stress and cardiometabolic risk factors; and (3) evaluate the mediating role of depressive symptoms on the relationships between psychosocial factors and epigenetic aging. Furthermore, this dissertation aimed to identify differences in stress-induced epigenetic changes across sociodemographic groups to better characterize health disparities. Together, these studies provide significant contributions to the field of social epigenomics by advancing our understanding of the interplay between psychosocial factors, mental health conditions, and cardiometabolic risk factors, and the influential role of epigenetics on those relationships. Findings from this dissertation further support the need to intervene on psychosocial factors in their earliest stages to improve long-term physical and mental health in the most vulnerable populations.

5.1.1 Summary and Implications of Chapter 2 Findings

In Chapter 2, we conducted an EWAS of long term-psychosocial stress in a multi-racial/ethnic sample of older adults, assessing DNA methylation from whole blood. After adjusting for age, sex, educational attainment, year of psychosocial stress measurement, genetic ancestry PCs, white blood cell proportions, and technical covariates, we identified nine CpGs: *cg01695954, cg08274633 cg17565444 cg24704287 cg01766737 cg25737313 cg12581512*

cg20559385 cg04803208 at FDR q<0.10. These associations remained nominally significant after adjusting for additional sociodemographic covariates, including marital status, employment status, having children, and total wealth (all p<2E-05).

Next, for the nine identified CpGs, we conducted a formal mediation analysis to examine whether health behaviors, including smoking, alcohol use, and physical activity, as well as body mass index (BMI), mediated the relationship between stress and DNA methylation. When assessed simultaneously, health behaviors and BMI significantly mediated between 9.4% and 21.8% of the relationship between stress and DNA methylation at eight of the nine CpG sites.

Findings from this study are important, as they address gaps in the literature regarding the relationship between psychosocial stress and DNA methylation patterns across the epigenome. Prior studies have examined associations between individual psychosocial stressors, including low socioeconomic status, childhood adversity, and work stress, and DNA methylation [1-6]; however, only a few studies have examined this relationship when defining psychosocial stress more broadly, capturing multiple domains of stress [7-9]. Our study was the first to do this in a multi-racial/ethnic sample with a large enough sample size to adequately detect epigenome-wide associations. Further, we were able to quantify the mediating effects of health behaviors and BMI on the associations between psychosocial stress and individual CpGs identified from the EWAS.

These findings have important implications for understanding the relationship between psychosocial stress and DNA methylation. Several of the identified CpGs were in or near genes associated with cardiometabolic traits, inflammation, and smoking, with one particular gene, RRM2, having established associations with mental health conditions, including schizophrenia and PTSD [10,11]. Results from this study further support the hypothesis that epigenetic

patterning of the RRM2 genomic region may be impacted by long-term psychosocial distress. Furthermore, health behaviors and BMI explained a relatively modest, but significant, percentage of the relationship between stress and DNA methylation, providing evidence that stress may affect DNA methylation through underlying pathways independent of health behaviors. Thus, emphasis should be placed on understanding other potential mechanisms through which stress impacts the epigenome, including mental health disorders. This research question was further explored in Chapter 4, where we examined the mediating role of depressive symptoms on the relationship between stress and epigenetic aging.

5.1.2 Summary and Implications of Chapter 3 Findings

In Chapter 3, we conducted an epigenome-wide mediation analysis of the relationship between psychosocial stress and ten cardiometabolic risk factors, including blood pressure, blood lipids, blood sugar, inflammation, and obesity, in a multi-racial/ethnic sample of older adults using the high-dimensional multiple testing (HDMT) method. Additionally, to quantify the overall mediation effects of each set of CpG mediators, we performed principal component (PC) analysis, with the cumulative effect of the top PCs representing the lower bound of the mediation effect across all identified mediators. We observed significant epigenetic mediating effects on the associations between psychosocial stress and body mass index (BMI), waist circumference (WC), high-density lipoprotein cholesterol (HDL-C), and C-reactive protein (CRP) at FDR $q<0.05$.

Psychosocial stress explained 16.5% of the total variability in BMI, with 50 CpGs mediating between 4.3% and 11.0% of this association. When reducing the CpG mediators to their top 10 PCs, we found that at least 46.3% of the association between stress and BMI was due to epigenetic mediation. Similarly, psychosocial stress explained 16.7% of the total variability in

WC, with 46 CpGs partially mediating between 4.2% and 10.6% of this relationship. At least 35.9% of the relationship between psychosocial stress and WC was mediated by the epigenome. Approximately 3.1% of the total variability in HDL-C was explained by psychosocial stress. Of this relatively small total effect, between 9.4% and 14.5% was mediated by 7 CpGs. PC analysis revealed that 31.4% of the relationship between psychosocial stress and HDL-C was due to epigenetic mediation. Finally, psychosocial stress explained 15.6% of the total variability in CRP, with 12 CpGs mediating between 6.5% and 11.4% of this association. Cumulatively, 45.1% of the relationship between stress and CRP was mediated by the epigenome.

Next, we performed functional enrichment analyses to understand the functional consequences of each set of CpG mediators. CpGs that mediated the relationship between psychosocial stress and BMI, WC, and CRP were more likely to reside in enhancer regions, indicating these CpGs may be involved in the regulation of nearby genes. Additionally, the CpG mediators of all four cardiometabolic risk factors were enriched for expression quantitative trait methylation sites (eQTMs), suggesting that the CpG mediators were more likely to influence gene expression. Finally, the identified CpG mediators were associated with the expression of genes enriched in pathways related to cytokine binding and receptor activity, cell surface, and neuron development.

Findings from this study advance our understanding of the role of epigenome on the associations between stress and cardiometabolic risk factors. To our knowledge, this was the first study to examine the mediating effect of DNA methylation on the relationship between psychosocial stress across the life-course and cardiometabolic risk factors. We identified 75 distinct CpGs that mediated the associations between psychosocial stress and either BMI, WC, HDL-C, or CRP. Further, two CpGs (*cg04803208* and *cg25607249*) mediated the relationship

between stress and all four outcomes, suggesting these methylation sites may play an important role on influencing cardiometabolic risk. Additionally, we compared our findings to those of Wang et al., who examined the epigenetic mediation of the relationship between neighborhood disadvantage, a single psychosocial stressor, and cardiovascular disease risk factors in the Multi-Ethnic Study of Atherosclerosis (MESA) study [12]. Two CpGs (*cg02508743* and *cg09315878*) that were identified as mediators of the relationship between stress and BMI in our study were also found to mediate the relationship between adults SES and BMI in Wang et al. Furthermore, both of these CpGs were discovered in multiple EWAS of BMI and maternal obesity [13-16]. Thus, our study provides additional evidence that *cg02508743* and *cg09315878* may be involved in the epigenetic mechanisms through which psychosocial stressors operate to influence cardiometabolic risk.

5.1.3 Summary and Implications of Chapter 4 Findings

In Chapter 4, we examined the associations between stress, loneliness, and depressive symptoms and five epigenetic age acceleration (EAA) measures estimated by DNA methylation in a multi-racial/ethnic sample of older adults. Additionally, for all identified associations, we tested for effect modification by sociodemographic characteristics, including sex and educational attainment. We found that a 1 SD increase in psychosocial stress, loneliness, and depressive symptoms were associated with 0.22 to 0.49 years of accelerated aging in most second generation clocks, as well as an increased pace of aging when measured by DunedinPACE. When examining sex and educational attainment as potential modifiers, we identified significant interactions between sex and both psychosocial stress and loneliness on DunedinPACE, with females experiencing a more accelerated pace of aging as psychosocial factors worsened. Furthermore, we observed significant interactions between loneliness and educational attainment

on PhenoAA, GrimAA, and DunedinPACE, with individuals having less than a college degree experiencing more accelerated aging as loneliness increased.

Next, for all identified associations between psychosocial factors and EAA, we conducted mediation analyses to characterize the role of depressive symptoms on those relationships. Depressive symptoms mediated between 24.0% and 35.3% of the relationships between psychosocial stress and HannumAA, GrimAA and DunedinPACE, while mediating 39.9% and 36.5% of the relationships between loneliness and both GrimAA and DunedinPACE, respectively (all $p<0.05$).

Finally, we conducted stratified mediation analyses when significant interactions between sociodemographic characteristics and psychosocial factors on EAA were present. When stratifying by sex, depressive symptoms mediated 27.6% of the relationship between psychosocial stress and DunedinPACE, as well as 24.8% of the relationship between loneliness and DunedinPACE in females. In males, the total effects of both stress and loneliness on DunedinPACE were not significant, so mediation was not evaluated. Furthermore, when stratifying by educational attainment, depressive symptoms mediated 36.5% and 31.1% of the relationship between loneliness and both GrimAA and DunedinPACE, respectively, for individuals without a college degree. For those with a college degree, the total effects of loneliness on all EAA measures were not significant. Thus, health disparities across key sociodemographic groups may exist in the effect of psychosocial factors on epigenetic aging in older adults.

Results from this study have important implications on our understanding of the associations between psychosocial factors and epigenetic aging, as well as the mediating role of depressive symptoms on those relationships. Although previous studies have identified

associations between individual measures of psychosocial stress and epigenetic aging, results have been relatively inconsistent [17]. This study found stronger relationships between stress and EAA measures than most previous studies, suggesting that the accumulation of stress across the life course may be more detrimental to epigenetic aging than individual stressors. Further, associations between loneliness and epigenetic aging have only been examined in middle-aged adults [18,19]. Because the epigenome can change substantially across the lifespan, it was not clear whether these findings would persist in older adults; however, results from our study show consistent associations between loneliness and EAA, particularly GrimAA and DunedinPACE.

Mediation results revealed that not only did depressive symptoms partially mediate the relationship between psychosocial factors and multiple measures of EAA, but also when stratified by sociodemographic characteristics, many of these mediation effects only persisted in females and those without a college degree. Our findings suggest that certain sociodemographic groups are more sensitive to the epigenetic effects of psychosocial factors and depressive symptoms. Previous studies have identified differences in stress response between males and females due to a variety psychological mechanisms, including that females are often less resilient to stress [20,21] and have stronger perceptions of stress [22,23] than their male counterparts. These differences have been shown to result in more severe physical and mental health outcomes in females, including higher levels of anxiety and depression as well as increased heart rate [24,25]. Additionally, differences in the effects of stress on health outcomes by socioeconomic status (SES) have been observed. In a nationally-representative study of middle-aged to older adults from England, psychological distress was associated with increased mortality from both stroke and coronary heart disease (CHD), with the strongest associations observed in the low SES group [26]. One possible explanation for these findings is that higher-SES individuals have

a larger social support network and have a greater abundance of resources available to help cope with stressful events [27]. Results from our study were consistent with the literature, and they warrant the need for future research to better understand why health disparities exist in the context of psychosocial stress and how to adequately address these disparities to improve health outcomes.

5.2 Strengths and Limitations

Although this dissertation provides important insights into the epigenetic mechanisms linking psychosocial stress and cardiometabolic risk factors, there are limitations that should be acknowledged. First, in all three studies, the psychosocial stress score we created was comprised of only self-reported stress measures that captured stress retrospectively. Due to the time lag between when participants may have experienced a stressful event and when they were administered the survey, there is potential for misclassification of the stress exposure, where individuals who experienced greater cumulative stress across the life course may be more likely to report past stressors than those who were relatively unaffected by stress. Additionally, certain mental health consequences of psychosocial stress, including depression and PTSD, may influence the likelihood of participants endorsing a particular stress exposure [28]. To capture a more objective measure of psychosocial stress, biological markers such as cortisol, alpha-amylase, and pro-inflammatory cytokines could be utilized [29].

Because data was collected from a cohort of older adults, there is the possibility for selection bias in our studies. Participants who had significant life stress may have been less likely to participate or remain in the cohort. Therefore, the psychosocial stress scores that we calculated based on our sample may differ slightly from what we would observe in the general U.S.

population, although the literature has been mixed on whether psychosocial distress influences study participation or attrition [30-33].

Furthermore, although we implemented a longitudinal study design, we cannot rule out the possibility of reverse causation due to the short timeframe between measurement of the exposure, mediators, and outcome variables in each study, particularly the cardiometabolic risk factors. In this dissertation, we have positioned DNA methylation as a mechanism, whereby changes in methylation levels affect the expression of genes that subsequently influence the development of cardiometabolic risk factors. However, there is existing evidence that shows DNA methylation may instead be a biomarker of cardiometabolic risk factors. For example, although some research points to BMI as a downstream consequence of changes to DNA methylation patterns [16,34], several studies have presented evidence that BMI is a driver of methylation changes at some specific methylation sites [13,15]. Nonetheless, even if DNA methylation is only a biomarker of cardiometabolic risk, this dissertation may provide novel insights into cardiovascular disease processes. The epigenome is often good at capturing exposures that cannot be measured well, including smoking and environmental toxins [35,36]. Additionally, methylation risk scores (MRS), which are a linear combination of DNA methylation effects across the epigenome, are often used as biomarkers to estimate disease risk and have been shown to improve phenotype inference [37]. For example, several studies have found that CRP MRS, comprised of CpGs known to be associated with CRP, can better predict cognition than serum CRP alone [38,39]. Therefore, DNA methylation regions identified in this dissertation could also serve as potential biomarkers for psychosocial cardiometabolic risk factors in future studies, particularly in studies that have not measured psychosocial factors.

This dissertation also had several key strengths. First, we utilized a comprehensive psychosocial stress score that captured several stress domains across the life course, enabling us to assess the cumulative effect of stress, as opposed to the impact of a single psychosocial stressor. Additionally, we conducted this research in a multi-racial/ethnic population of European, African, and Hispanic/Latino participants, which allowed us to examine the effects of psychosocial stress on DNA methylation and subsequent cardiometabolic risk in a diverse population.

Lastly, in each study, we performed formal mediation testing to characterize the mechanisms linking psychosocial stress, DNA, methylation, and cardiometabolic risk factors. To assess the mediating effects of health behaviors and BMI on the associations between stress and DNA methylation (Chapter 2), we implemented the KHB mediation method, which not only allowed for the examination of multiple mediators simultaneously, but also disentangled the effects of correlated mediators. To examine the epigenetic mediation of psychosocial stress on cardiometabolic risk factors (Chapter 3), we implemented a high-dimensional mediation method, HDMT, which used a corrected mixture reference distribution to account for the compositional nature of the null hypothesis, thereby providing well calibrated p-values under the null. Finally, to understand the mediating role of depressive symptoms on the associations between psychosocial factors and epigenetic aging (Chapter 4), we utilized a regression-based causal method that allowed for interactions between psychosocial factors and depressive symptoms. It should be noted that in all mediation analyses, we implemented an analytic approach requiring the total effect of the exposure on the outcome to be significant in order to move forward with formal mediation testing. Although the need to test for a total effect has been heavily debated in

the literature, we chose to employ this method, as our main goal was to characterize the mechanisms linking stress to the outcomes of interest (cardiometabolic risk factors, epigenetics). However, as a result, our findings are conservative and may result in missed indirect effects. Additionally, in some of the mediation models, the total variability of the outcomes explained by psychosocial stress was quite low. Therefore, even if significant mediation effects were detected, stress itself had a relatively weak impact on those particular outcomes. Nonetheless, even with their limitations, these studies help to establish the many behavioral and biological pathways that link psychosocial stress to cardiometabolic risk.

5.3 Future Directions

The studies from this dissertation build upon previous work that examined associations of psychosocial stress and DNA methylation across the epigenome [7-9], evaluated the epigenetic mediation of individual stressors and cardiometabolic risk factors [12], and characterized the mediating role of depressive symptoms on the relationship between discrimination, a domain of psychosocial stress, and epigenetic aging [40]. In Chapter 2, we discovered CpG sites across the epigenome that were associated with psychosocial stress. As a next step, our study should be replicated in an independent cohort of older adults to further validate these findings. Additionally, as transcriptomic data becomes available in HRS, future analyses should examine the associations between psychosocial stress and gene expression. CpGs identified in our study could be tested as mediators of the identified stress-gene associations to determine if the epigenetic effects of stress influence the transcription levels of specific genes. This multi-omics approach would help to disentangle the biological mechanisms linking psychosocial stress and cardiometabolic disease risk by identifying the specific genes that are impacted by stress and assessing how changes in gene expression lead to particular cardiometabolic risk factors.

In Chapter 3, we identified a subset of CpGs that mediated the relationship between psychosocial stress and BMI, WC, HDL-C, and CRP. However, because DNA methylation and cardiometabolic risk factors were measured concurrently, we were not able to confidently state causal directions of effect between psychosocial stress, DNA methylation, and cardiometabolic risk. As additional epigenetic and blood biomarker data becomes available in HRS, future analyses should assess these measures across multiple timepoints to establish a temporal relationship between psychosocial stress and cardiometabolic risk factors operating through the epigenome. Furthermore, results from this study identified a select number of CpGs that showed strong evidence of mediation in both our study, as well as previous literature. Two of these CpGs included *cg02508743*, which maps to the *LYN* proto-oncogene on chromosome 8, and *cg09315878*, which maps to the stromal cell derived factor 4 (*SDF4*) gene on chromosome 1. The next step would be to investigate the relationship between DNA methylation and cardiometabolic risk factors in an independent cohort of older adults, focusing on the genomic regions surrounding these CpGs, as well as genes identified through eQTM analysis. This would allow us to examine DNA methylation patterns more thoroughly in the region and establish stronger epigenetic biomarkers of cardiometabolic disease.

In Chapter 4, we identified associations between psychosocial factors, including psychosocial stress and loneliness, and EAA, which were partially mediated by depressive symptoms. Although the CES-D shows high diagnostic accuracy and is often used as a first step in screening for depression [41], this measure examines depressive symptoms over a short period of time and thus does not represent a clinical diagnosis of depression. Future analyses should replicate these findings in a population that has clinical depression diagnoses available to ensure we are capturing both the upstream influences and downstream consequences of mental health

appropriately. We also observed strong differences in the effect estimates of psychosocial factors on EAA when stratifying by sex and educational attainment. In particular, women and individuals without a college degree were far more sensitive to the effects of stress and loneliness on epigenetic aging, as well as the mediating effects of depressive symptoms on those relationships. Future work should be conducted to understand if these findings persist in different populations, particularly in individuals in early to midlife, and determine the underlying factors that contribute to these disparities.

Prior research has shown that both psychosocial stress and cardiovascular disease are racially/ethnically patterned, where non-Hispanic Black individuals are more likely to experience both increased levels of stress and elevated risk for cardiovascular disease [42,43] compared to non-Hispanic Whites. Further, Black men in particular have been found to underreport their psychological stress due ideals regarding culture, fearlessness, and toughness [44], making it difficult to accurately capture the relationship between stress and cardiovascular disease operating through the epigenome. Because this dissertation focused on a multi-racial/ethnic population of older adults, we were unable to investigate differences in epigenetic impacts by racial/ethnic groups. Thus, future studies should investigate similar hypotheses in single racial/ethnic groups to better understand how the impacts of psychosocial stress on the epigenome, as well as downstream health consequences, differ by race/ethnicity.

Finally, although the cumulative stress score used in this dissertation is multi-faceted, allowing us to capture several domains of psychosocial stress, it does not easily lend itself to developing a public health intervention or policy. Because stress is examined across the life course, we are not able to pinpoint where a successful intervention could be implemented for a specific individual. For instance, economic interventions, including a larger child tax credit or a

housing stipend, could be implemented to combat high levels of childhood adversity due to socioeconomic status [45]. However, more targeted interventions, including individual or couples therapy with a licensed professional, would better address stress that arose from acute life events or personal relationships [46]. Thus, to develop interventions that target the appropriate stressor, future studies should examine each stress domain separately to better understand the impact of each individual stress domain on cardiometabolic risk factors operating through the epigenome.

5.4 Conclusion

In conclusion, the studies from this dissertation provide valuable insights into the role of DNA methylation as both a biomarker of psychosocial stress, as well as a potential biological mechanism through with stress contributes to cardiometabolic disease risk in a diverse population of older adults. Chapters 2 and 3 highlights the epigenetic impacts of psychosocial stress and its subsequent influence on cardiometabolic risk factors. Chapter 4 characterizes the mediating role of depressive symptoms on associations between psychosocial factors and epigenetic aging. Together, findings from this dissertation may have an important public health impact, as they highlight the importance of individual- and community-level interventions to adequately address stress before it compounds, resulting in complex physical and mental health conditions. Preliminary evidence has suggested that stress reduction techniques, including meditation and yoga, contribute to epigenetic changes in genes related to inflammation and immune response [47]. Thus, future work should be conducted in larger and more diverse samples to better characterize the epigenetic consequences of stress reduction interventions.

Furthermore, this dissertation has the potential to provide meaningful advances in the field of precision medicine, where epigenetic biomarkers can serve as tools for early diagnosis

and treatment of age-related disease. Previous studies have already used DNA methylation as biomarkers to predict treatment efficacy in patients with mental health conditions, including anxiety disorder and PTSD [48]. Additionally, established epigenetic biomarkers of CVD have been used to create novel therapies targeting specific epigenetic modifications, designed to prevent vascular inflammation, endothelial dysfunction, and atherosclerosis [49]. Findings from this dissertation add to the growing body of literature regarding the utility of epigenetic biomarkers and provide insights that may ultimately help to improve mental health outcomes, reduce health disparities, and lessen the burden of age-related chronic disease in vulnerable populations.

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