ARIC Manuscript Proposal #3552

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1.a. Full Title:
Epigenetic variation of metabolically healthy and unhealthy BMI

b. Abbreviated Title (Length 26 characters):
DNAm of metabolically healthy obesity

2. Writing Group:
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ARIC investigators welcome
WHI EMPC, BAA23, and AS311 investigators are welcome

I, the first author, confirm that all the coauthors have given their approval for this manuscript proposal. WLD **[please confirm with your initials electronically or in writing]**

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3. Timeline:
Data analysis: January - April, 2020
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4. Rationale:
The obesity epidemic continues to rise with more than 41.1% of women obese in the United States in 2016 (1). With an increasing obese population, a growing body of evidence have found heterogeneity in obesity, with some phenotypes exhibiting differential risk for cardiovascular outcomes. Obesity in isolation of additional metabolic parameters (*metabolically healthy obesity*) has been shown to exhibit reduced or null risk of cardiovascular outcomes and mortality compared to obesity with additional metabolic health risk factors (*metabolically unhealthy obesity*). Indeed, the latter has consistently been associated with significantly poorer outcomes including cardiovascular outcomes and all-cause mortality compared to metabolically healthy obesity (2-6). These differences are persistent across all BMI categories with normal weight individuals with an excess of metabolic abnormalities exhibiting similar risk of chronic disease outcomes as their overweight and obese counterparts (6). These findings suggest that metabolic health status differentially influences the relationship between body mass index (BMI) and health outcomes while hinting at biomolecular differences underlying these phenotypes. BMI is intended to be a measure of adiposity and risk for chronic disease. However, BMI does not capture differences in body composition, particularly for athletes, individuals with high muscle mass, elderly individuals, and racial and ethnic minorities (7, 8). Thus examining BMI in combination with additional metabolic parameters can be more sensitive to body composition and improve predictive capacity.

Biological mechanisms contributing to these differences have been well defined. For example, many rodent models have found several features that may be present in metabolically healthy obese individuals including low lipid deposition in the liver, a metabolically advantageous adipokine ratio (9) and an overexpression of GLUT4 transporter increasing de-novo lipogenesis (10). Similarly in humans, there appears to be substantial differences in liver fat content with metabolically healthy individuals having more favorable adipokine ratios including higher levels of adiponectin (11-13) and lower levels of ghrelin (14). However, it is unknown whether these phenotypes may be exhibiting differential epigenetic profiles which may be contributing to the differences in outcomes.

Epigenetic mechanisms, such as DNA methylation (DNAm) are important biological features to examine in the context of chronic diseases such as obesity and metabolic health. Particularly in the role that they may play mediating health outcomes, since changes to DNAm can induce changes in gene expression in causal disease pathways. Obesity has been widely examined and shown to exhibit prolific methylation changes (15-17). Similarly, metabolic syndrome and metabolic outcomes have additionally been shown to be associated with differential methylation (18).

However, no studies have integrated these phenotypes to examine how BMI-associated methylation varies by metabolic health status. Particularly since DNAm has been shown to play a mediating role with obesity and cardiovascular outcomes (19), evaluating the epigenome may provide insight into pathways contributing to the differences in outcomes. The purpose of this study is to examine the whether metabolic health status differentially influences BMI-associated methylation. In the significant sites identified through the epigenome-wide association study (EWAS), longitudinal analysis of association between metabolic health status and change in methylation will be explored as well as the association between DNAm and coronary heart disease (CHD).

5. Main Hypothesis/Study Questions:

- **Aim:** Examine whether metabolic health status differentially influences the relationship between BMI and DNA methylation.
- **Hypothesis**: We will identify BMI-associated methylation that is influenced by metabolic health status.

6. Design and analysis (study design, inclusion/exclusion, outcome and other variables of interest with specific reference to the time of their collection, summary of data analysis, and any anticipated methodologic limitations or challenges if present).

Study population

Two cohorts will be used in the discovery phase: the Women's Health Initiative (WHI) and the Atherosclerosis Risk in Communities study (ARIC). Data from three WHI ancillary studies will be included: *Epigenetic Mechanisms of Particulate Matter-Mediated Cardiovascular Disease* (EMPC, aka AS315), the *Integrative Genomics for Risk of Coronary Heart Disease and Related Phenotypes in WHI cohort* (BAA23), and *Bladder Cancer and Leukocyte Methylation* (AS311). EMPC assessed epigenetic mechanisms underlying associations between ambient particulate matter air pollution and cardiovascular disease within the WHI Clinical Trials (CT, n=2200). BAA23 was a case-control study assessing predictors of CHD within the WHI CT (n=1664) and OS (n=442), where cases were identified using eight biomarkers of CHD. AS311 is a matched case-control study of bladder cancer among women within the WHI CT (n = 405) and OS (n = 455). Secondary analyses will include a subset of individuals from EMPC and AS534 cohort.

ARIC includes data from two ancillary studies of 2,879 African Americans (AA) and 1,100 European Americans (EA). ARIC is an ongoing prospective cohort study investigating the etiology of CHD in four US communities: Forsyth County, NC; Jackson, MS; Minneapolis, MN; Washington County; MA. Participants were aged 45 to 64 and followed up for trends in coronary heart disease in each community over 15 years with 7 study visits (20). DNAm are available in a subset (n= 3979) of participants at visit 2 (1990-1992) or visit 3 (1993-1995).

The replication cohort will be derived from the Multi-Ethnic Study of Atherosclerosis (MESA) study. MESA is a longitudinal, population cohort study designed to examine risk factors for and the progression of cardiovascular disease (CVD). Participants aged 45-84 years without clinically apparent CVD were recruited between July 2000 and August 2002 from 6 regions in the US: Winston-Salem, NC; Northern New York, NY; Baltimore, MD; St. Paul, MN; Chicago, IL; and Los Angeles, CA. DNA methylation was derived from monocyte samples at Exam 5 (April 2010-

February 2012) in a random sample of 1,264 non-Hispanic white, African American and Hispanic participants (21, 22).

Measurements

Weight, height, waist circumference and blood pressure (BP) were measured at the physical exam. BMI was calculated as weight (kg)/height $(m)^2$. Waist circumference was measured to the nearest 0.5 cm. Two BP measurements were collected (systolic/diastolic). Biochemical measurements were analyzed in blood samples collected after a 12-hour fast. These include triglycerides (TG), high-density lipoprotein cholesterol (HDL), and fasting glucose.

Metabolic Health Exposures

Metabolic risk will be determined by presence of three or more components of metabolic syndrome using the Adult Treatment Panel III (ATP III) criteria listed in Table 1. Thus, *metabolically unhealthy* and *healthy* will refer to the presence of three or more and less than three components, respectively. BMI will be examined continuously.

Table 1. ATP III Clinical Identification of Metabolic Syndrome

Clinical Measure	Defining Level
Waist Circumference (WC)	≥102 cm in men or ≥88 cm in women
Triglycerides	≥150 mg/dL or drug treatment for elevated triglycerides
High Density Lipoprotein (HDL)	<40 mg/dL in men or <50 mg/dL in women or drug treatment for reduced HDL
Blood Pressure (BP)	≥130/85 mmHG or antihypertensive drug treatment in patient with a history of hypertension
Glucose	≥110 mg/dL or drug treatment for elevated glucose

Covariates

Covariates in our analysis will include race/ethnicity, age, smoking status, and physical activity level. Race/ethnicity, smoking and physical activity were self-reported. Smoking status will be defined as current, former or never. Physical activity was measured using a physical activity questionnaire and will be defined by total energy expended from recreational physical activity which includes walking, mild, moderate and strenuous physical activity in kcal/week/kg (MET-hours/week).

Exclusions

Individuals will be excluded if metabolic health parameters and DNA methylation were not measured within the same year. Additionally, secondary analyses involving CHD will exclude participants with a history of (or incident) myocardial infarction or coronary revascularization (angioplasty; stent; bypass) before measurement of DNA methylation.

Outcome in Secondary Analyses

In significant sites identified through EWAS, DNA methylation at cytosine and guanine nucleotide pair (CpG) sites will be examined as a predictor of incident CHD. CHD was defined by incident myocardial infarction or CHD death. In WHI and ARIC, medical records were reviewed and acute, hospitalized myocardial infarction was identified on the basis of cardiac pain, electrocardiogram, and biomarker data; then physician-adjudicated. Further details regarding the review, classification, and adjudication of CHD in WHI (23) and ARIC (24) have been described.

DNA methylation

In the WHI and ARIC cohorts, DNA was extracted from peripheral blood leucocytes collected at visit-specific fasting blood draws (25). In the MESA cohort, DNA was extracted from peripheral blood monocytes (26). DNA methylation in all three cohorts was measured using the Illumina

450K Infinium Methylation BeadChip. DNA methylation was estimated as the proportion of methylated probes relative to combined unmethylated and methylated probes for a specific CpG sites defined as the β-value. All methylation data will be quality controlled and normalized using beta-mixture quantile normalization. Technical covariates will include plate, chip, and row to adjust for batch effects and cell composition, which was estimated using the reference-based Houseman method (27). Additional quality control procedures will include exclusion of probes with multi-modal signals as detected by the *gaphunter* function in the *minfi* package.

Statistical Analysis

A summary of the proposed analyses is provided in **Figure 1**.

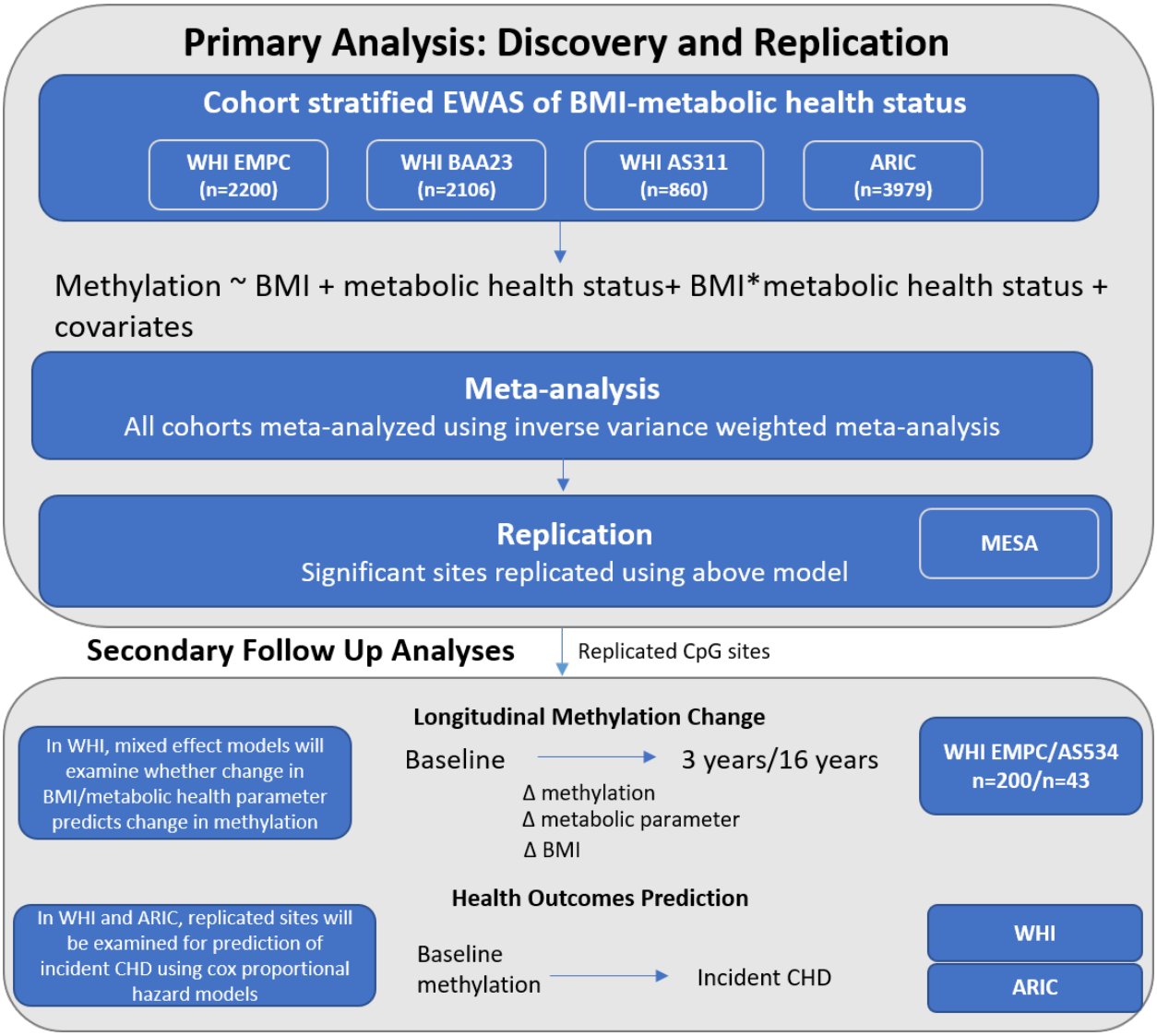

Figure 1. Proposed Analysis Plan

We will be using R for all analyses. Demographic characteristics will be defined by means and counts. In the EWAS, all models will be stratified by cohort (EMPC, BAA23, AS311 in WHI) and race (AA and EA in ARIC) and pooled using inverse-variance weighted analysis. Since BMI has been shown to linearly associate with methylation, we will examine BMI continuously. To examine the differential impact of metabolic health status on BMI, linear regression models will be used regressing the methylation β-value on BMI with an interaction term included for BMI and metabolically unhealthy status, adjusting for covariates (**Figure 2**). Covariates in all models will include cell composition, principal components of genetic relatedness, race/ethnicity (WHI), sex (ARIC), age, alcohol consumption and smoking. Study-specific covariates will include trial arm (EMPC, BAA23, AS311), case-control status (BAA23, AS311) and U.S. Census region (WHI) or study site (ARIC). To account for potential chip-to-chip differences in measurement and to adjust for batch effects, we will include a fixed effect for each BeadChip in our model. Significant CpG sites will be identified by the interaction p-value (β_3 in Figure 2) at a false discovery rate (FDR) q-value <0.05. At significant sites, metabolically healthy status will be examined for interaction with physical activity. In non-significant interaction models, physical activity will be examined as a confounder for each CpG site.

Figure 2. Linear models examined in discovery and replication cohorts

$$\text{Methylation}_i = \beta_0 + \beta_1(\text{BMI}) + \beta_2(\text{Metabolically Unhealthy}) + \beta_3(\text{BMI*Metabolically Unhealthy}) + PC_i\gamma + X_i\alpha + C_i\theta$$

- $PC_i\gamma$=First four principal components of genetic relatedness matrix
- $X_i\alpha$ = Control variables
- $C_i\theta$= Cohort specific controls and technical covariates such as chip position and chip

Results identified in the discovery cohorts will be replicated in the MESA cohort using linear regression models. Significant CpG sites will be examined using linear regression models regressing methylation β-value on BMI with an interaction for metabolically unhealthy status. Models will be adjusted for chip number and location, cell composition, principal components of genetic relatedness, ethnicity, age, sex, alcohol consumption and smoking. Significance is defined at Bonferroni-corrected p-value < 0.05.

Longitudinal and Outcomes Analyses

Table 2. Number of Adjudicated CHD Cases in WHI

	Cases	Non-Cases
Total	313	2010
EMPC	49	1133
BAA23	255	673
AS311	9	204

Among significant sites, using longitudinal data from EMPC and AS534, we will examine the association between change in metabolic health parameters and BMI and methylation in 200 women with methylation and metabolic health status measured 3 or 6 years and 43 women with methylation and metabolic health status measured on average 16 years after baseline methylation measurement. Using mixed effect models, we will examine methylation (outcome) regressed on metabolic health status and BMI (exposures) adjusting for age, ethnicity, chip, plate and cell composition as fixed effects with a random effect for time and subject. Models examining change in metabolic health status will be examined adjusted and unadjusted for BMI.

Given the association between metabolic health, BMI, and differential health outcomes, multivariate cox proportional hazard ratios will be used to examine whether significant sites identified through EWAS (exposure) are associated with incident CHD in the WHI (Table 2) and ARIC cohort. Covariates will include age, ethnicity, smoking status, chip, plate, and cell composition in the reduced model. The full model will additionally adjust for triglycerides, HDL cholesterol, blood pressure and CHD medications use.

Enrichment Tests

In the identified sites, we will conduct several analyses including Kytoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis utilizing several publically available resources (28). To assess the functional enrichment of differentially methylated sites and identify relevant pathways associated with metabolic health, a KEGG pathway analysis will be conducted on significant CpG sites using the *missMethyl* package (29). Threshold for the above analyses will be $p<0.05$.

Power Calculation

Table 3. Minimum Effect Size for Interaction Analysis

# of DM CpG sites	10	50	1000
Discovery r^2	0.0048	0.0041	0.0032
Replication r^2	0.0105	0.013	0.0187

DM=differentially methylated

Using the method of Liu and Hwang (30), we tested several scenarios to ensure adequate power to identify the minimum effect size (r^2) and achieve the desired power (>80%) with a FDR q-value<0.05 for 450,000 tests (Table 3). We tested the minimum effect size for the above aims with a total sample of ~3500 to account for any exclusions. We have >80% power to detect methylation changes associated with the interaction between metabolic health and BMI with an r^2 detecting effects as low as 0.3% of the variance in methylation. Interaction effects are often subtle, but this analysis shows we'll have power to detect very subtle effects. In the replication analysis, we calculated the minimum effect size we will be able to identify for replicating 10, 50 or 1000 CpG sites correcting for multiple testing using Bonferroni correction (0.05/# of tests). We will be able to detect effects if as low as 1% of the variance in methylation can be explained by the interaction.

Sensitivity analyses

As metabolic health status is constructed from a number of metabolic parameters, differences in methylation may be driven by individual metabolic parameters. To assess the degree that individual metabolic parameters influence methylation at significant sites, we will reanalyze associations between BMI*metabolic health status and methylation adjusting for each individual metabolic parameter and compare the effects to the original estimates obtained through EWAS. Among replicated sites identified in the main analysis, we will examine categorical BMI and metabolic health status (metabolically healthy/unhealthy normal weight/obesity) using ANOVA adjusted for the same covariates to identify differential methylation patterns associated with the subtypes. Given the significant differences in methylation (31-33) and metabolic and cardiometabolic diseases (34) driven by sex, adjustment may not fully account for these differences. We will conduct sensitivity analyses of the replicated sites stratified by sex in the ARIC cohort to examine the differences in the main effects by sex. In longitudinal analyses, significant sites identified to associate with change in metabolic health status will be examined by individual metabolic parameters to identify the driving metabolic factor.

Limitations

There are some limitations. Given the cross sectional design, we cannot determine any causal association and may be at risk for reverse causality, if methylation is contributing to changes in BMI or metabolic risk factors. Moreover, metabolic risk factors may also be a product of duration of obesity, since several studies have found metabolically healthy obesity to be a transitory state (35-37). However, understanding the methylomic differences in these populations would still be advantageous to identify biological mechanisms that may be driving the differences in outcomes. Another potential limitation includes the potential for confounding by cell composition. Obesity and several of the metabolic health parameters associate with excess inflammation (38). While we will control for cell composition using methods of Houseman et al (27), we may not be able to account for rarer cell types and the identified CpG sites may be a reflection of these differences in cell composition associated with differential inflammatory profiles associated with these disease exposures.

7.a. Will the data be used for non-CVD analysis in this manuscript? __X__ Yes ____ No

b. If Yes, is the author aware that the file ICTDER03 must be used to exclude persons with a value RES_OTH = "CVD Research" for non-DNA analysis, and for DNA analysis RES_DNA = "CVD Research" would be used? _X___ Yes ____ No

(This file ICTDER has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript? _X___ Yes ____ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER03 must be used to exclude those with value RES_DNA = "No use/storage DNA"? __X__ Yes ____ No

9. The lead author of this manuscript proposal has reviewed the list of existing ARIC Study manuscript proposals and has found no overlap between this proposal and previously approved manuscript proposals either published or still in active status. ARIC Investigators have access to the publications lists under the Study Members Area of the web site at: http://www.cscc.unc.edu/aric/mantrack/maintain/search/dtSearch.html

___X_ Yes _____ No

10. What are the most related manuscript proposals in ARIC (authors are encouraged to contact lead authors of these proposals for comments on the new proposal or collaboration)?

MS#2106 Demerath E. W. et al. Epigenome-wide association study of obesity traits in

African American adults: The Atherosclerosis Risk in Communities (ARIC) Study

MS#2635 Demerath E.W. et al. Adiposity-related DNA methylation variants as predictors of type 2 diabetes and coronary heart disease in African-Americans: The ARIC Study

MS#2802 Justice A. et al. Epigenetic factors influencing central adiposity

MS#2399 Bressler J. et al. Genome-wide analysis of DNA methylation and coronary heart disease (CHD): the Atherosclerosis Risk in Communities (ARIC) Study

11.a. Is this manuscript proposal associated with any ARIC ancillary studies or use any ancillary study data? __ __ Yes __X__ No

11.b. If yes, is the proposal

___ A. primarily the result of an ancillary study (list number* _____)

___ B. primarily based on ARIC data with ancillary data playing a minor role (usually control variables; list number(s)* _____ _____ _____)

*ancillary studies are listed by number at https://www2.cscc.unc.edu/aric/approved-ancillary-studies

12a. Manuscript preparation is expected to be completed in one to three years. If a manuscript is not submitted for ARIC review at the end of the 3-years from the date of the approval, the manuscript proposal will expire.

12b. The NIH instituted a Public Access Policy in April, 2008 which ensures that the public has access to the published results of NIH funded research. It is **your responsibility to upload manuscripts to PubMed Central** whenever the journal does not and be in compliance with this policy. Four files about the public access policy from http://publicaccess.nih.gov/ are posted in http://www.cscc.unc.edu/aric/index.php, under Publications, Policies & Forms. http://publicaccess.nih.gov/submit_process_journals.htm shows you which journals automatically upload articles to PubMed central.

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