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Association of mitochondrial DNA copy number with cardiometabolic diseases in a large cross-sectional study of multiple ancestries

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1 # Abstract

2 Aims

3 We tested the hypothesis that mitochondrial DNA copy number (CN) is
4 associated with cardiometabolic disease (CMD) traits.

5 Methods and results

6 We determined the cross-sectional association of mtDNA CN measured in
7 whole blood with several CMD traits in 65,996 individuals (mean age 60, 54%
8 women, and 79% European descent). Cohort- and ancestry/ethnicity-specific
9 association analysis was performed adjusting for trait- and cohort-specific
10 covariates. Age was slightly positively associated with age (0.03 s.d. / 10 years
11 (95% CI=0.01, 0.05)) before 65 years, while every 10 years older age was
12 associated with 0.14 s.d. lower level of mtDNA CN after 65 years (95% CI=
13 -0.18, -0.10). In meta-analysis without adjustment for white blood cell (WBC)
14 and differential count in participants of European descent (N=52,491), low
15 mtDNA CN was associated with increased odds of obesity (OR with 95%
16 CI=1.13 (1.11, 1.16), P=3.3e-30) and hypertension (OR=1.05 (1.03, 1.08),
17 P=4.0e-07). Further adjusting for WBC and differential count in the same
18 participants of European descent (N=44,035), associations became
19 non-significant (P>0.05) for hypertension, attenuated for obesity ($OR_{without\ cell}$
20 $_{count}$=1.15 (1.12, 1.18) versus $OR_{cell\ count}$=1.06 (1.03, 1.08)) but strengthened for
21 hyperlipidemia ($OR_{without\ cell\ counts}$ =1.03 (1.00, 1.06) versus $OR_{cell\ counts}$ =1.06

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1 (1.03, 1.09)). The magnitude and directionality of most associations were
2 consistent between participants of European descent and other
3 ethnicity/ancestry origins.
4 Conclusion:
5 Low levels of mtDNA CN in peripheral blood were associated with an
6 increased risk of CMD diseases.
7

8 Key words: mitochondrial DNA copy number, cardiometabolic disease, whole
9 genome sequencing

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1 # Translational Perspective

2 The mitochondrial genome (mtDNA) is represented at variable copy number
3 (CN) in human cells and plays essential roles in cellular metabolism. We
4 determined the cross-sectional association of mtDNA CN measured in whole
5 blood with several cardiometabolic traits in 65,996 individuals (mean age 60,
6 54% women, and 79% participants of European descent). Low mtDNA CN
7 levels were significantly associated with an increased risk of obesity and
8 hyperlipidemia after accounting for clinical covariates and blood cell counts.
9 The magnitude and directionality of associations were consistent between
10 participants of European descent and other ancestries/ethnicities.
11 Understanding the role of mtDNA CN in cardiometabolic will provide insight
12 into the pathobiology underlying cardiometabolic diseases.
13

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1 # Introduction

2 Mitochondria convert dietary calories to molecular energy through oxidative
3 phosphorylation (OXPHOS).[1] In addition, mitochondria play essential roles in
4 cellular differentiation, proliferation, reprogramming, and aging.[2-7] Mitochondria
5 contain their own genome (mtDNA) which is a circular, double-stranded DNA
6 molecule of 16.6 kb. mtDNA encodes 13 key OXPHOS proteins, 22 transfer
7 RNAs (tRNAs), and two ribosomal RNAs (rRNAs)[1]. Multiple copies of mtDNA
8 are present per mitochondrion, and cells contain up to 7000 mitochondria per
9 cell.[8] The mtDNA copy number (mtDNA CN) correlates with cellular ATP
10 generating capacity and metabolic status,[9] and therefore, varies greatly across
11 tissue and cell types depending on cellular energy demand.[1,10,11]

12 Several previous studies have demonstrated that mtDNA CN is lower in
13 older individuals and this decrease is associated with a general decline in
14 health.[12-14] Low mtDNA content was also associated with higher fasting blood
15 glucose (FBG), hemoglobin A1c (HbA1c), and lipid levels including high
16 density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRIG),
17 and total cholesterol (TC) in both diabetic patients and controls.[15] In addition,
18 mtDNA content was inversely related to BMI and fat accumulation in 94
19 healthy young individuals (mean age 30 years).[16] A more recent study in two
20 independent cohorts of women of European origin (n=2,278, mean age 30
21 years; and n=2,872, mean age 69 years), however, failed to detect significant

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1 associations between mtDNA CN and CMD risk factors including systolic
2 blood pressure (SBP), diastolic blood pressure (DBP), HDL, LDL, TRIG, and
3 glucose levels.[17]

4 Given inconsistent findings in previous studies and the central role of
5 mtDNA in metabolism, we set out to investigate the association between
6 mtDNA CN and several CMD risk factors in eight US cohorts representing
7 participants of different ethnicity/ancestry origins with whole genome
8 sequencing (WGS) and extensive cardiometabolic phenotyping. We also
9 included individuals with Whole Exome Sequencing (WES) from the UK
10 Biobank for validation. We performed cohort- and ancestry-specific association
11 analysis between mtDNA CN and several CMD phenotypes. We also
12 performed meta-analyses separately in participants of European descent and
13 African Americans, and also combining all ancestry groups.

14 Methods

15 Study participants

16 Several cohorts from the NHLBI's Trans-Omics for Precision Medicine
17 (TOPMed) program contained a small number of duplicated participants. We
18 removed one copy of the duplicated participants and this study included
19 26,890 individuals with WGS in the TOPMed program (67.4% women; age
20 range of 20-100 years; 45.4% European Americans, 32.6% African Americans,
21 19.6% Hispanic/Latino Americans and 2.4% Chinese Americans)

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(**Supplemental Table 1**). Additionally, we included 39,106 participants of White British from the UK Biobank with WES (54% women; 40-75 years) for validation (**Supplemental Table 1**). All study participants provided written informed consent for genetic studies. The protocols for WGS and WES were approved by the institutional review boards (IRB) of the participating institutions (**Supplemental Materials**).

mtDNA copy number estimation

mtDNA CN estimation in WGS: whole blood derived DNA was used for WGS through TOPMed sequencing centers. The average coverage was ~39x across samples. The program *fastMitoCalc* of the software package *mitoAnalyzer* was used to estimate mtDNA copy number across TOPMed participants.[13] The average mtDNA CN per cell was estimated as twice the ratio of average coverage of mtDNA to average coverage of the nuclear DNA (nDNA). The coverage was defined as the number of reads that were mapped to a given nucleotide in the reconstructed sequence.[13]

mtDNA CN estimation in UK BioBank: whole blood derived DNA was used for WES in UK BioBank. mtDNA CN estimates were generated by customized regression with specific terms in Perl and R software using Exome SPB CRAM files (version Jul 2018) downloaded from the UK BioBank data repository (**Supplemental Materials**).

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1 *mtDNA CN estimation in ARIC using other methods:* mtDNA CN estimation
2 from low-pass WGS was calculated as the ratio of mitochondrial reads to the
3 number of total aligned reads (**Supplemental Materials**). mtDNA CN
4 estimated from the Affymetrix Genome-Wide Human SNP Array 6.0 was
5 calculated using Genvisis 15 software package (**Supplemental Materials**).
6 The participants whose mtDNA CN were estimated from low-pass WGS and
7 Affymetrix Genome-Wide Human SNP Array 6.0 were independent to each
8 other and were independent to those with TOPMed WGS in the ARIC cohort,
9 as previously described[18] (**Supplemental Materials**).

10 **Cardiometabolic disease phenotypes**

11 Metabolic disease phenotypes were mapped to the examinations when blood
12 was drawn for DNA extraction for mtDNA CN estimates. Our primary analysis
13 focused on four CMD phenotypes, obesity, hypertension (HTN), type 2
14 diabetes (T2D), and hyperlipidemia. Obesity was defined as body mass index
15 (BMI) ≥30 (kg/m^2). T2D was defined as fasting blood glucose ≥126 mg/dL or
16 currently receiving glucose-lowering or diabetes medication (s). Hypertension
17 (HTN) was defined as systolic blood pressure (SBP) ≥140 mmHg, or diastolic
18 blood pressure (DBP) ≥90 mmHg, or use of antihypertensive medication(s) for
19 blood pressure control. Hyperlipidemia was defined as fasting total cholesterol
20 (TC) ≥200 mg/dL or triglyceride (TRIG) ≥150 mg/dL, or use of any lipid-lowering
21 medication.

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We also analyzed the association of mtDNA CN with continuous cardiometabolic traits: BMI, SBP, DBP, FBG, HDL cholesterol, LDL cholesterol, and TRIG levels. In the analysis of FBG, we excluded individuals with diabetes, defined as glucose value ≥126 mg/dL and/or taking glucose-lowering or diabetes medications.[19] SBP and DBP values (mmHg) were derived from the averages of two measurements. We added 15 mmHg and 10 mmHg to SBP and DBP, respectively, for individuals taking any BP lowering medications.[20] The total cholesterol (TC) measurements were divided by 0.8 for individuals using lipid treatment medications.[21] LDL (mg/dL) was calculated as (TC - HDL - TRIG/5) in individuals with TRIG <400 mg/dL using imputed TC values.[21] In analyses of FBG and lipid levels, we excluded individuals whose fasting status was not established. TRIG, LDL and HDL values were log-transformed to approximate normality. Other continuous outcome variables were not transformed.

Metabolic syndrome is a collection of risk factors that increase the risk for cardiovascular disease (CVD).[22] We analyzed the presence of metabolic syndrome variable in relation to mtDNA CN. An individual was classified as having metabolic syndrome (0/1) if he/she had three of the five following conditions: [22] 1) obesity – waist circumference >40 inches in men and >35 inches in women; 2) hyperglycemia – fasting glucose ≥100 mg/dL or currently receiving glucose-lowering or diabetes medication; 3) dyslipidemia –

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triglyceride ≥150 mg/dL or on lipid-lowering treatment; 4) dyslipidemia – High density lipoprotein cholesterol <40 mg/dL in men or <50 mg/dL in women or on lipid-lowering treatment; and 5) hypertension – 130 mmHg systolic or >85 mmHg diastolic or the current use of antihypertensive medication (s). Of note, the thresholds in defining metabolic syndrome are different from those for individual disease phenotypes in our primary analysis. Waist circumference was not measured in approximately a third of the FHS participants. Because BMI is the most common measure of overall obesity,[23] to increase the sample size we used BMI ≥30 to define obesity in FHS participants with missing waist circumference values.

Statistical analyses

In all analyses, we used mtDNA CN as the primary independent variable. To identify confounders and covariates in association analyses, we first examined whether mtDNA CN levels were associated with 'blood collection year' (the year when blood was drawn, as a surrogate of batch effects for blood-derived DNA samples) in all participating cohorts. White blood cell (WBC) count and blood differential count were previously reported to be associated with mtDNA CN.[18,24] Therefore, we investigated whether mtDNA CN was associated with total WBC count, blood differential count, and platelet count in cohorts that measured or imputed[25,26] these variables (**Supplemental Table 2**). We further

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examined mtDNA CN in relation to age and sex after adjusting for blood collection year (**Supplemental Figure 1**).

Based on observing significant associations of mtDNA CN in relation to 'blood collection year', age and sex, we generated mtDNA CN residuals for downstream analyses by regressing mtDNA CN on age, age squared, sex and blood collection year (as a factored variable) in each cohort. The residuals were standardized to a mean of zero and standard deviation (s.d.) of one, and used as the main predictor in all regression models. In the primary analysis, we used logistic regression (for unrelated individuals) and mixed effects logistic regression model (related individuals) to analyze binary outcomes (i.e., obesity, HTN) in relation to mtDNA CN residuals. Because age, sex and BMI are important confounders or covariates for cardiometabolic traits, we further adjusted for sex and age as covariates in the analysis of obesity, and adjusted for sex, age, age-squared (only for HTN) and BMI as covariates in the analysis of T2D, hyperlipidemia, and HTN. We used linear effects models to analyze continuous outcome variables, adjusting for the same set of covariates as for the respective binary outcomes. For cohorts with family structure, we accounted for maternal lineage as random effects in linear or logistic mixed models. A maternal lineage was defined to include a founder woman with all of her children, and all grandchildren from daughters of the founder woman.[27]

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1 We performed the discovery meta-analysis in European American
2 participants in TOPMed with fixed effects (P_Q>0.01) or random effects
3 (P_Q≤0.01) inverse variance method and performed validation analyses using
4 participants of White British in UK Biobank. We further compared
5 meta-analysis results in participants of European descent to those from other
6 racial/ethnic origins in TOPMed cohorts. Finally, we performed
7 inverse-variance meta-analysis to combine results from TOPMed and UK
8 Biobank. The primary results included associations of mtDNA CN with the four
9 disease outcomes. We used p=0.01 for significance to account for multiple
10 testing for primary results, and used p=0.05/9~0.006 for significance in
11 analysis of continuous outcomes.

12 Measured and/or imputed WBC variables were available in a subset
13 participants in TOPMed and in all participants in UK Biobank. We compared
14 associations between mtDNA CN and individual outcomes in the same
15 participants with and without WBC count, differential count and platelet count
16 as additional covariates.

17 We further investigated whether sex or age modified the association
18 between mtDNA CN and outcome variables, adjusting for the same set of
19 covariates described in primary analyses. We included an interaction term
20 between mtDNA CN and sex/age in association analyses. We also performed
21 stratified analyses between mtDNA CN and CMD traits in participants less

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1 than 65 years old and participants 65 years or above (see **Supplemental**
2 **methods** for details). The statistical software R (version 3.6.0) was used for all
3 statistical analyses.

4 # Results

5 **Characteristics of Study Participants**

6 The current study included 13,385 European Americans, 8,012 African
7 Americans, 601 Chinese Americans, and 4,892 Hispanic/Latino Americans
8 from the TOPMed program as well as 39,106 White British from the UK
9 Biobank. On average, 55% of study participants were women, and the
10 participants' mean age was 60 years (range 20 to 100 year; **Supplemental**
11 **Table 1**). We observed moderate to high heterogeneity in distributions of age,
12 sex, and cardiometabolic phenotypes across cohorts and ancestries. For
13 example, the age range was 20 to 100 years in the Framingham Heart Study
14 (FHS) (mean age 60 years, 40% pariticipants≥65 years). In contrast, all
15 participants in the Cardiovascular Health Study (CHS) were older than 65 year
16 of age (mean age 74). HTN, obesity, T2D, and hyperlipidemia were more
17 prevalent in African Americans than participants of other ethnic and racial
18 groups (**Supplemental Table 1**)

19 **A threshold effect between age and mtDNA CN**

20 Because low mtDNA CN was associated with older age and reported to be
21 associated with increased cardiometabolic disease risk,[15-17,28] we reported

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1 beta estimates as the change in an outcome variable in response to 1 s.d.
2 lower mtDNA CN in all of analyses. We observed that, on average, age was
3 associated with a slightly higher level of mtDNA (0.032 s.d. / 10 years (95%
4 CI= 0.013, 0.052), P=0.0014) of mtDNA CN from 20s to 65 years. We
5 observed a threshold effect of age on mtDNA CN, and every 10 years older
6 age was associated with 0.14 s.d. lower level of mtDNA CN after 65 years (95%
7 CI= -0.18, -0.10), P=1.82e-13) (**Figure 2**). The relationship between mtDNA
8 CN and age appeared to be similar in men and women. Women had higher
9 mtDNA CN than men (beta=0.23, 95% CI= (0.20, 0.26), P=7.4e-60) as noted
10 previously.[13,24] The threshold effect between age and mtDNA CN was slightly
11 attenuated after adjusting for WBC counts (**Supplemental Figure 2**).

12 **Discovery meta-analysis in European Americans participants**

13 We performed the discovery meta-analysis in European American participants
14 in TOPMed (N =13,385). We found that 1 s.d. decrease in mtDNA CN was
15 significantly associated with 1.08-fold odds of obesity (95% CI= (1.03, 1.13),
16 P=5.0e-04), 1.07-fold odds of hypertension (95% CI= (1.03, 1.12), P=1.4e-03),
17 1.16-fold odds of metabolic syndrome (95% CI= (1.06, 1.27), P=1.8e-03). We
18 also found that 1 s.d. decrease in mtDNA CN was nominally (0.01<P<0.05)
19 associated with 1.18-fold odds of diabetes (95%= (1.01, 1.37), P=0.041). For
20 continuous traits, 1 s.d. decrease in mtDNA CN was significantly associated
21 with 0.033 unit (95% CI= (0.023, 0.041), P=1.7e-04) increase in TRIG and

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nominally associated with 0.38 mmHg (95% CI= (0.021, 0.73), P=0.038) increase in SBP and 0.22 kg/m^2 (95% CI= (0.074, 0.25), P=0.028) increase in BMI. mtDNA CN was not significantly associated with DBP, HDL, LDL, or fasting glucose (P>0.05) in the discovery meta-analysis (**Table 1, Figures 3 and 4**)

Bidirectional validation and meta-analysis with participants of European descent between TOPMed and UK Biobank

Most of the significant associations in meta-analysis of the discovery phase were validated in the UK Biobank (**Table 1**). Compared to those in the discovery meta-analysis, the UK Biobank data yielded larger effect sizes for associations of several traits (**Supplemental Figure 3**). For example, 1 s.d. decrease in mtDNA CN was associated with 1.15-fold (95% CI= (1.12, 1.18)) odds of obesity in the UK Biobank versus 1.08-fold (95% CI= 1.03-1.13) in the TOPMed EA-specific meta-analysis. Additionally, 1 s.d. unit decrease in mtDNA CN was associated with 0.32 kg/m^2 increase in BMI in UK Biobank (p = 9.1e-42) compared to 0.22 kg/m^2 increase in BMI (p=0.028) in the meta-analysis of TOPMed EA participants. DBP and FBG were not significant in the discovery TOPMed EA meta-analysis (beta=0.02, 95% CI= (-0.37, 0.41), P= 0.92 for DBP; beta=0.067, 95% CI= (-0.11, 0.25), P=0.47 for FBG) while significant in UK Biobank (beta=0.19 , 95% CI= (0.086, 0.30) with P=0.00038 for DBP; beta=0.20, 95% CI = (0.093, 0.30) with P=2.0e-4 for FBG) (**Table 1**).

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1 We performed meta-analysis of all participants of European descent in
2 TOPMed and UK Biobank (n=52,491) using fixed effects inverse-variance
3 method to combine results (**Table 1**). One s.d. increase in mtDNA CN was
4 significantly associated with an increase of odds of obesity (OR=1.13, 95% CI=
5 (1.11, 1.16), P=3.3e-30), hypertension (OR=1.05, 95% CI= (1.03, 1.08),
6 P=4.0e-07), metabolic syndrome (OR=1.13, 95% CI= (1.11, 1.16), P=9.7e-27),
7 and nominally associated with diabetes (OR=1.05, 95% CI= (1.00, 1.10),
8 P=0.051), hyperlipidemia (OR=1.03, 95% CI= (1.00, 1.05), P=0.023). For
9 continuous phenotypes, 1 s.d. decrease in mtDNA CN was significantly
10 associated with 0.15 mmHg increase in DBP (95% CI=(0.056, 0.25),
11 P=1.9e-03), 0.42 mmHg increase in SBP (95% CI= (0.26, 0.59), P=6.0e-07),
12 0.32 kg/m^2 increase in BMI (95% CI=(0.27, 0.36), P=2.5e-41), 0.16 mg/dL
13 increase in fasting glucose (95% CI= (0.069, 0.25), P=5.4E-04), and 0.025 unit
14 increase in TRIG (95% CI= (0.021, 0.029), P=8.3e-30).

15 mtDNA CN estimated from low-pass WGS and Affymetrix Genome-Wide
16 Human SNP Array 6.0 gave rise to consistent associations for most of the
17 CMD traits compared to that from WGS (**Supplemental Table 7**).

18 **Comparison of results from the European descent group with those from**
19 **other racial/ethnic groups**

20 The directionality of associations of mtDNA CN with CMD traits was consistent
21 in African Americans (N=8,012), Hispanic/Latino Americans (N=4,892) and

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1 Asian Americans (N=601) compared to that in participants of European
2 descent for most of the phenotypes (**Table 1**, **Figure 5** and **Supplemental**
3 **Table 3, 4, Supplemental Figure 4, 5**). In the meta-analysis of AA participants,
4 1 s.d. decrease in mtDNA CN was significantly associated with 1.14-fold odds
5 of diabetes (95%CI= (1.06, 1.22), P=2.5e-04) and a 0.68 mmHg increase in
6 SBP (95% CI= (0.21, 1.15), P=4.0e-03). In Asian-only TOPMed participants
7 (n=601), 1 s.d. decrease in mtDNA CN was significantly associated with
8 1.43-fold odds of hyperlipidemia (95% CI= (1.19, 1.72), P=0.00014). In
9 Hispanic-only TOPMed participants (n=4,900), One s.d. decrease in mtDNA
10 CN was significantly associated with an increase of odds of diabetes (OR=1.16,
11 95%CI= (1.06, 1.26), P=0.0012), and significantly associated with 0.019 unit
12 decrease in LDL (95 % CI= (-0.029, -0.0079), P=6.6e-04) and 0.028 unit
13 increase in TRIG (95% CI= (0.010, 0.046), P=0.0020). (**Supplemental Table**
14 **4**). Results of meta-analysis in pooled-samples are included in **Supplemental**
15 **Table 5**.

16 **Accounting for WBC and blood differential count as additional covariates**

17 WBC count and blood differential count were available in a subset of
18 participants in TOPMed (n=12,345) and in participants in the UK Biobank
19 (n=39,021). mtDNA CN was inversely associated with the total WBC count and
20 two blood cell differentials, neutrophil and monocyte. In contrast, mtDNA CN
21 was positively associated with lymphocyte and platelet (**Supplemental Table**

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1 **2**). Because the WBC count is associated with mtDNA CN and it is an indicator
2 of a systemic subclinical inflammation state that accompanies CMD risk
3 factors,[29-33 34-36] we compared results between models with and without the
4 total WBC count and blood differentials as additional covariates in the same
5 participants. Directionality remained the same for all associations except for
6 HDL after adjusting for WBC count and blood differential count in participants
7 of European descent (**Supplemental Table 6, Supplemental Figure 6, 7**).
8 Comparison of regression coefficients with and without adjusting for WBC
9 count and differential in participants of other ethnicity and ancestry origins
10 were available in **Supplemental Figure 8, 9**. Non-lipid CMD traits and TRIG
11 attenuated their associations with mtDNA CN after adjusting for WBC cell
12 counts and differentials. For example, the association of mtDNA CN with
13 obesity was attenuated: $OR_{without\ cell\ counts}$=1.15 (1.12, 1.18), P=7.7e-31 versus
14 $OR_{cell\ counts}$=1.06 (1.03, 1.08), P=2.5e-06. In contrast, hyperlipidemia and LDL
15 were not significantly associated with mtDNA CN before adjusting for cell
16 counts (P>0.05) and became significant after adjusting for cell counts and
17 blood differential count (OR=1.06 (1.03, 1.09), P=3.9e-05 for hyperlipidemia
18 and beta=0.012 (0.0096, 0.015), P=7.8e-18 for LDL) (**Supplemental Table 6**).

19 **Interaction analyses**

20 We did not find statistically significant interactions between sex and mtDNA
21 CN, or between age and mtDNA CN with respect to the phenotypes tested

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1 when including interaction terms in the models (**Supplemental Table 8**). Due
2 to the threshold effect between age and mtDNA CN, we further performed
3 association analyses in younger (<65 years) and older (≥65 years) participants.
4 It appeared that the effect sizes were larger in younger individuals compared
5 to older individuals for a number of traits although the directionality remained
6 to be the same for these traits (**Supplemental Materials**, **Supplemental**
7 **Table 9** and **Supplemental Figure 10**).

8 **Discussion**

9 We demonstrated that low levels of mtDNA CN in peripheral blood were
10 associated with an increased risk of CMD diseases in 65,996 individuals
11 representing multiple ethnicity and ancestry origins. Consistent effect
12 estimates were obtained from the discovery meta-analysis and the validation
13 analysis in UK Biobank participants with adjustment for traditional clinical
14 covariates as well as adjustment for WBC and blood differential count,
15 demonstrating the robustness of our findings. These findings further suggests
16 that altered levels of mitochondrial energy production may be involved in the
17 development of a cluster of conditions that increase the risk of CVD.

18 A longitudinal study reported that a 1 s.d. decrease in mtDNA CN was
19 associated with 1.29-fold and 1.11-fold increased risk of developing CHD and
20 stroke, respectively.[37] Cardiometabolic factors are known risk factors for the
21 development of CVD. This study, however, discovered only modest

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1 associations of mtDNA CN with several cardiometabolic (CMD) traits (odds
2 ratio=1.04 to 1.16 across individual CMD traits and the composite metabolic
3 syndrome), indicating that factors other than CMD may drive or mediate
4 associations between mtDNA CN and cardiovascular outcomes. The
5 mechanisms underlying mitochondrial dysfunction and CVD are largely
6 unknown. One mechanism by which mtDNA CN may influence CVD is through
7 the regulation of nuclear DNA methylation and gene expression. A mouse
8 hybrid nuclear DNA and mtDNA system was previously developed, i.e., a
9 hybrid mouse containing nuclear DNA from control mouse strain A and a
10 specific mtDNA from control mouse strain B.[38,39] Using this model, two studies
11 provided direct evidence that different mtDNA background influences DNA
12 methylation, gene expression, and cellular adaptive response.[38,39] We recently
13 assessed the relationship between mtDNA-CN and nuclear DNA methylation
14 in up to 5,000 African American and European American study participants.
15 Several validated mtDNA-associated CpGs were also known to be associated
16 with coronary heart disease and CVD.[40] Functional assessment demonstrated
17 that modification of mtDNA CN levels resulted in changes in DNA methylation
18 at specific CpGs and expression levels of nearby genes through knockout of
19 the mitochondrial transcription factor, the protein that regulates mtDNA
20 replication in CRISPR-Cas9.[40] These results provided evidence that mtDNA
21 CN may impact cardiovascular health through DNA methylation and differential
22 expression of specific genes that may impact cell signaling. Further studies are

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1 needed to investigate whether DNA methylation and gene expression mediate
2 the effects of mtDNA CN on cardiovascular risks.

3 WBC count is a blood biomarker of systemic inflammation. It has been
4 increasingly recognized that chronic low-grade inflammatory state
5 accompanies CMD risk.[29] Previous studies reported that WBC count is
6 associated with increasing risk of obesity,[30,31] diabetes,[29,32,33]
7 hypertension,[34-36] while heterogeneous relationships with lipid levels.[41] This
8 study and previous studies also showed that a high WBC count was
9 associated with low mtDNA CN.[42-47] Therefore, the connections between
10 mtDNA CN, inflammation, and metabolic disease phenotypes are complex.
11 These findings indicates that mtDNA CN and WBC count may interplay for
12 CMD risk. Further studies are warranted to investigate underlying molecular
13 mechanisms to establish a potential causal pathway among mtDNA CN,
14 inflammation and CMD risk.

15 Most previous studies reported a tendency toward low mtDNA CN with
16 advancing age,[12,13,18,24,48,49] The current cross-sectional study, using a large
17 sample size with a wide age range, demonstrated that mtDNA CN increases
18 slightly from young adult (age 20 to 29) to late middle age (65 years old), and
19 decreases in individuals older than 65 years. Most of the previous studies
20 included participants with a limited age range, that is, the majority of study
21 participants were young, middle-aged, or older individuals.[12,49,50] In addition,

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1 post of these previous studies only investigated the linear relationship between
2 mtDNA CN and age.[12,13,18] Using a large sample size, this study discovered a
3 threshold effect of age on mtDNA CN. However, this study only investigated
4 age-mtDNA CN association cross-sectionally. The intra-individual mtDNA CN
5 variability with advancing age needs to be explored in a longitudinal setting in
6 future studies.

7 **Strengths and Limitations**

8 This study includes a large sample size of men and women of multiple ethnicity
9 and ancestry origins across a wide age range, thus enables us to investigate
10 the relationships of mtDNA CN with CMD phenotypes throughout the entire
11 adult life span. In addition, we performed careful phenotype harmonization and
12 examined several potential confounding variables of mtDNA CN in association
13 analysis with metabolic traits. Furthermore, we included association analyses
14 of CMD traits with mtDNA CN estimated from several technologies further
15 demonstrated the utility of mtDNA CN estimated from these different
16 technologies in association analysis. Despite the multiple strengths in this
17 study, several limitations should be noted. mtDNA CN was estimated using
18 DNA derived from whole blood, which is not necessarily the relevant tissues
19 with respect to cardiometabolic (e.g., cardiac muscle, skeletal muscle, adipose
20 tissue) and aging-related (e.g., brain) disease phenotypes. Nevertheless,
21 peripheral blood is easily accessible, changes in mtDNA in whole blood should
22 reflect metabolic health across multiple systems. A recently study found that

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1 blood-derived mtDNA CN is associated with gene expression across multiple
2 tissues and is predictive for incident neurodegenerative disease, which
3 provides evidence supporting the hypothesis that changes in mtDNA in whole
4 blood may reflect metabolic health across multiple systems.[51] Second, though
5 we accounted for confounders and known batch effects in mtDNA CN and
6 harmonized metabolic traits, we still observed moderate to high heterogeneity
7 in the association coefficients in meta-analysis of most of the phenotypes in
8 both ancestry-specific analyses and across ancestries. Different distributions
9 of age, sex, and phenotypes across study cohorts may partially explain the
10 heterogeneity in these associations. Unobserved confounding factors, such as
11 experiment conditions for blood drawing, DNA extraction, and storage may
12 also have contributed to heterogeneity. Finally, we were not able to determine
13 causal relationships between mtDNA CN and metabolic traits due to the
14 cross-sectional nature of the study. Future studies with mtDNA CN and CMD
15 traits at two time points will provide further insight into associations of
16 aging-related mtDNA CN change with CMD traits.
17

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21 Atherosclerosis Risk in Communities study, Cardiovascular Health Study,

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Coronary Artery Risk Development in Young Adults Study, Framingham Heart Study, Jackson Heart Study, Genetic Epidemiology Network of Arteriopathy Study, Hispanic Community Health Study/Study of Latinos, Multi-Ethnic Study of Atherosclerosis Study, and UK Biobank for their important contributions. We gratefully acknowledge the studies and participants who provided biological samples and data for TOPMed.

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Table 1. Cross-sectional association and meta-analysis of mtDNA CN with metabolic disease phenotypes.

	Traits	Discovery (European Americans) (N=13,385)			Replication (White British) (N=39,106)			Meta-analysis (N=52,491)		
		OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
Binary outcomes	Obese	1.08	1.03-1.13	5.0e-04	1.15	1.12-1.18	1.6e-30	1.13	1.11-1.16	3.3e-30
	HTN	1.07	1.03-1.12	1.4e-03	1.04	1.02-1.07	0.00023	1.05	1.03-1.08	4.0e-07
	Diabetes	1.18	1.01-1.37	0.041	1.04	0.99-1.10	0.10	1.05	1.00-1.10	0.051
	Hyperlipids	1.04	0.99-1.08	0.11	1.03	1.00-1.06	0.024	1.03	1.00-1.05	0.023
	MetS	1.16	1.06-1.27	1.8e-03	1.13	1.10-1.15	1.1e-24	1.13	1.11-1.16	9.7e-27
		Beta	SE	P-value	Beta	SE	P-value	Beta	SE	P-value
Continuous outcomes	BMI	0.22*	0.10*	0.028*	0.32	0.023	9.1e-42	0.31	0.023	2.5e-41
	DBP	0.02*	0.20*	0.92*	0.19	0.054	0.00038	0.15	0.049	1.9e-03
	SBP	0.38	0.19	0.038	0.42	0.094	7.2e-06	0.42	0.085	6.0e-07
	FBG	0.067	0.092	0.47	0.20	0.052	2.0e-04	0.16	0.046	5.4e-04
	HDL	-0.0097*	0.0099*	0.33*	-0.099	0.069	0.16	-0.012	0.0098	0.21
	LDL	-0.008*	0.0073*	0.27*	-0.0040	0.0014	0.0045	-0.0027	0.0014	0.059
	TRIG	0.033	0.0088	1.7e-04	0.023	0.0025	8.6e-21	0.025	0.0022	8.3e-30

The beta estimates are in units of metabolic traits corresponding to one s.d. lower mtDNA-CN. Association analysis of mtDNA CN with metabolic traits was performed in cohorts of European Americans in TOPMed and also in White British participants in UK Biobank. Meta-analysis with fixed or random effects inverse variance method was used to summarize the results in European descent in TOPMed and White British in UK Biobank. DBP, diastolic blood pressure; SBP, systolic blood pressure; BMI, body mass index; FBG, fasting blood glucose; HDL, high density lipoprotein; Trig, triglyceride. Obese, obesity; HTN, hypertension; Diabetes, Diabetes; MetS, metabolic syndrome.

Asterisk (*) denotes inverse variance random effect meta-analysis.

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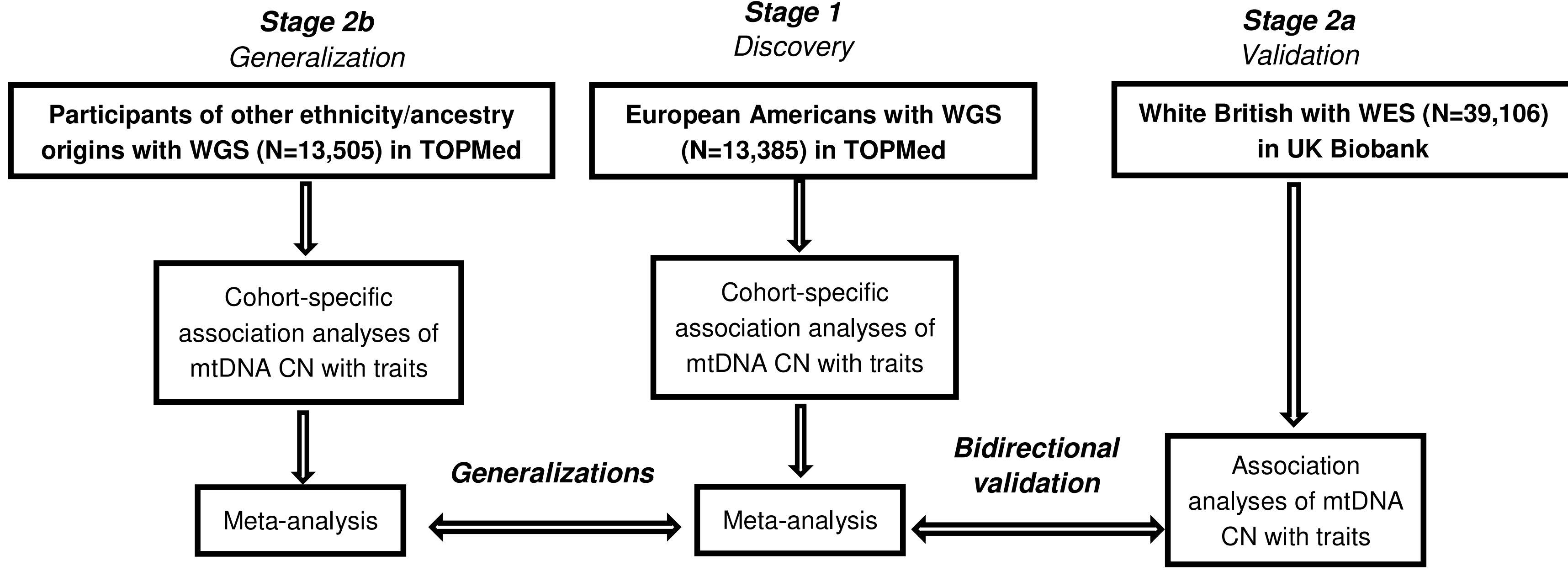

Figure 1. Study design. Association analysis of mtDNA CN with metabolic traits was performed in cohorts of European Americans (N=13,385), African Americans (N=8,012), Chinese Americans (N=601) and Hispanic and Latino Americans (N=4,892) in TOPMed and in White British participants of the UK Biobank (N=39,106). Meta-analysis using fixed or random effects inverse variance method was used to summarize the results in European Americans and African Americans in TOPMed. Primary analysis included age, age^2 (blood pressure traits), sex, BMI (not in BMI traits) and batch effects. Second analysis included covariates in primary analysis and imputed/measured white blood cell count and blood differentials. Interaction analysis was performed to investigate whether age and sex modified the relationship between mtDNA CN and cardiometabolic disease traits.

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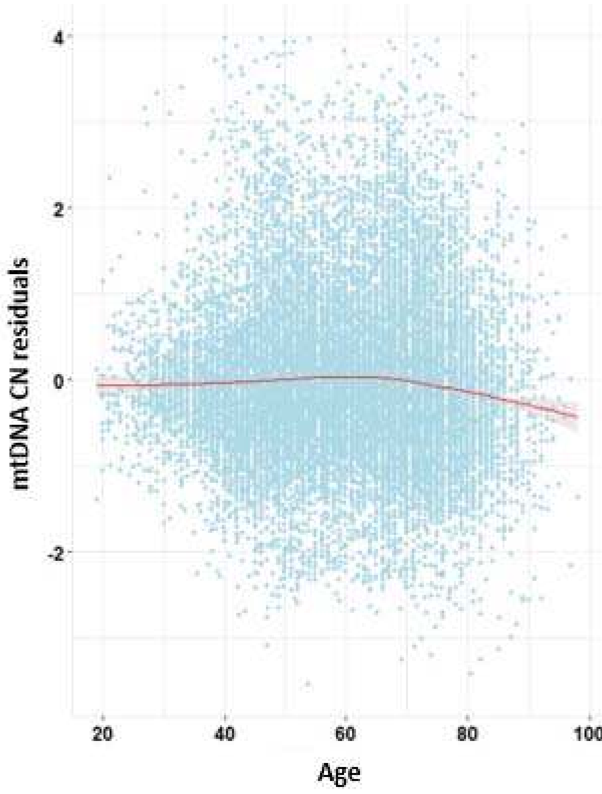

Figure 2. The relationship of mtDNA CN with age in TOPMed participants. Scatterplots of mtDNA CN residuals versus age. Cohort specific mtDNA CN residuals were obtained by regressing mtDNA CN on batch information.

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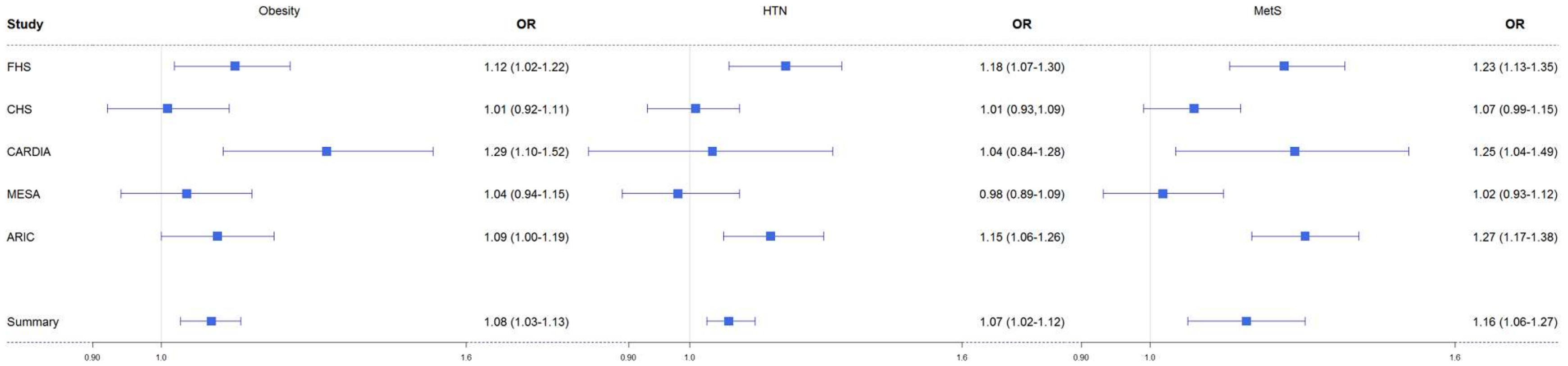

Figure 3. Association and meta-analyses of mtDNA CN with obesity and hypertension (HTN) and metabolic syndrome (MetS) in European Americans (N=13,385) in TOPMed. Fixed effects inverse variance method was used to summarize the results. The odds ratio (OR) corresponds to one s.d. decrease in mtDNA-CN. Covariates included age, sex, BMI (for HTN and MetS) and batch variables.

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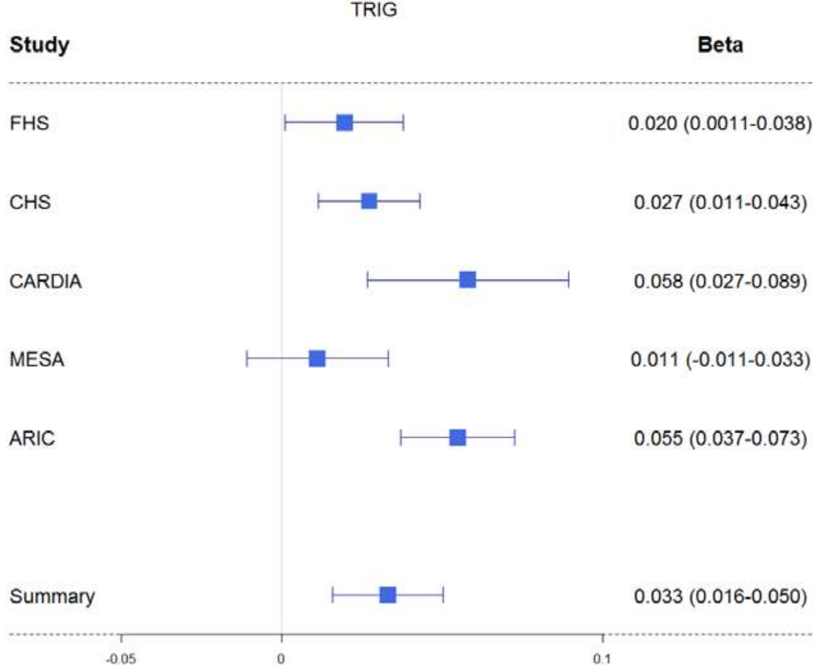

Figure 4. Association and meta-analyses of mtDNA CN with triglyceride in European Americans (N=13,385) in TOPMed. Fixed effects inverse variance method was used to summarize the results. The effect size estimates are in units of metabolic traits corresponding to one s.d. decrease in mtDNA-CN. TRIG, triglyceride. Covariates included age, sex, BMI (for HTN and MetS) and batch variables.

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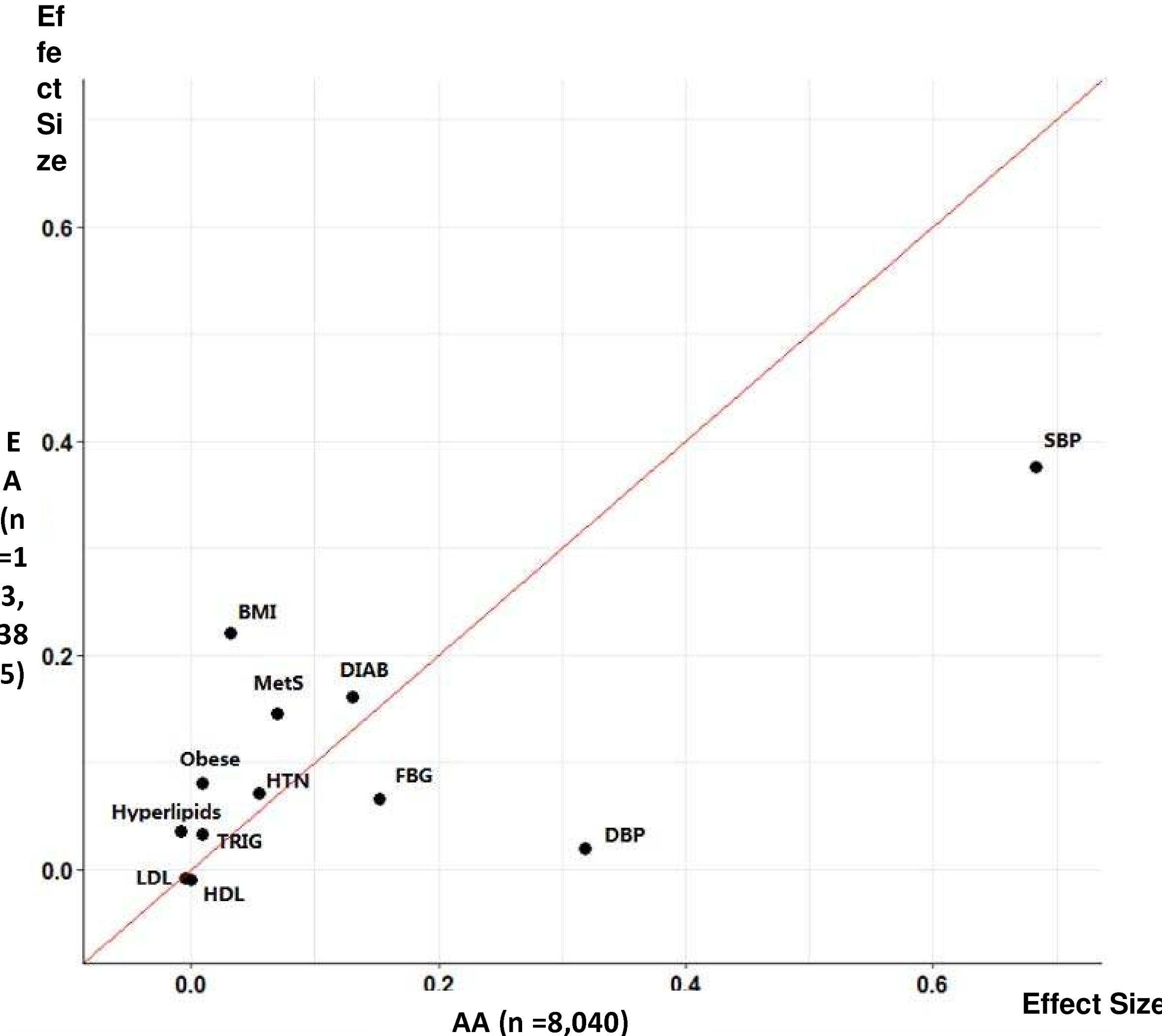

Figure 5. Comparison of beta of metabolic traits in the European Americans and American Americans in TOPMed. DBP, diastolic blood pressure; SBP, systolic blood pressure; BMI, body mass index; FBG, fasting blood glucose; HDL, high density lipoprotein; LDL, low density lipoprotein, TRIG, triglyceride. TC, total cholesterol; Obese, obesity; HTN, hypertension; Diabetes, Diabetes; Hyperlipids, hyperlipidemia; MetS, metabolic syndrome.

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