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ORIGINAL RESEARCH

Association Between Whole Blood–Derived Mitochondrial DNA Copy Number, Low-Density Lipoprotein Cholesterol, and Cardiovascular Disease Risk

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BACKGROUND: The relationship between mitochondrial DNA copy number (mtDNA CN) and cardiovascular disease remains elusive.

METHODS AND RESULTS: We performed cross-sectional and prospective association analyses of blood-derived mtDNA CN and cardiovascular disease outcomes in 27 316 participants in 8 cohorts of multiple racial and ethnic groups with whole-genome sequencing. We also performed Mendelian randomization to explore causal relationships of mtDNA CN with coronary heart disease (CHD) and cardiometabolic risk factors (obesity, diabetes, hypertension, and hyperlipidemia). $P<0.01$ was used for significance. We validated most of the previously reported associations between mtDNA CN and cardiovascular disease outcomes. For example, 1-SD unit lower level of mtDNA CN was associated with 1.08 (95% CI, 1.04–1.12; $P<0.001$) times the hazard for developing incident CHD, adjusting for covariates. Mendelian randomization analyses showed no causal effect from a lower level of mtDNA CN to a higher CHD risk ($\beta=0.091$; $P=0.11$) or in the reverse direction ($\beta=-0.012$; $P=0.076$). Additional bidirectional Mendelian randomization analyses revealed that low-density lipoprotein cholesterol had a causal effect on mtDNA CN ($\beta=-0.084$; $P<0.001$), but the reverse direction was not significant ($P=0.059$). No causal associations were observed between mtDNA CN and obesity, diabetes, and hypertension, in either direction. Multivariable Mendelian randomization analyses showed no causal effect of CHD on mtDNA CN, controlling for low-density lipoprotein cholesterol level ($P=0.52$), whereas there was a strong direct causal effect of higher low-density lipoprotein cholesterol on lower mtDNA CN, adjusting for CHD status ($\beta=-0.092$; $P<0.001$).

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CONCLUSIONS: Our findings indicate that high low-density lipoprotein cholesterol may underlie the complex relationships between mtDNA CN and vascular atherosclerosis.

Key Words: cardiometabolic risk factors ■ cardiovascular disease ■ low-density lipoprotein cholesterol ■ Mendelian randomization ■ mitochondrial DNA copy number ■ vascular atherosclerosis

CLINICAL PERSPECTIVE

What Is New?

- Although we validated the previously reported associations between mitochondrial DNA copy number (mtDNA CN) and cardiovascular disease outcomes, univariable and multivariable Mendelian randomization analyses found no causal associations between mtDNA CN and coronary heart disease status in either direction.
- A causal association was observed between low-density lipoprotein cholesterol and mtDNA CN, controlling for coronary heart disease status, indicating that high low-density lipoprotein cholesterol may underlie the complex relationships between mtDNA CN and vascular atherosclerosis.

What Are the Clinical Implications?

- The strong direct causal effect of higher low-density lipoprotein cholesterol on lower mtDNA CN, independent of coronary heart disease status, underscores the significance of optimizing lipid profiles to preserve mitochondrial function, in addition to mitigating cardiovascular disease risk.

Nonstandard Abbreviations and Acronyms

CMD	cardiometabolic disease
CN	copy number
IVW	inverse variance weighted
LD	linkage disequilibrium
MR	Mendelian randomization
mtDNA	mitochondrial DNA
MVMR	multivariable Mendelian randomization
WGS	whole-genome sequencing

Cardiovascular diseases (CVDs) are the leading cause of death globally.[1] A large proportion of CVDs results from atherosclerosis, an inflammatory process involving the endothelium and vascular wall.[2,3] Mitochondria are primary sites for oxidative phosphorylation that generates energy via adenosine triphosphate (ATP) production.[4] Mitochondria have their own DNA (mitochondrial DNA [mtDNA]), a circular 16.6-kb molecule encoding essential proteins for ATP production and energy homeostasis.[5] In apolipoprotein E knockout mice, mtDNA damage accompanies the initiation of atherogenesis[6]; during this process, low-density lipoprotein cholesterol (LDL-C) is trapped and accumulates in the subendothelial space of the arterial walls.[3,7,8] The accumulation of LDL-C in the arterial wall makes LDL-C more susceptible to oxidation. Oxidative stress induces mitochondrial fragmentation by inhibiting fusion and enhancing fission, which may cause disruption of mtDNA replication, and thus may reduce mtDNA copy number (CN).[9,10]

mtDNA CN is strictly regulated for energy homeostasis. Each human cell contains hundreds (eg, in a blood cell) or thousands (eg, in a cardiac muscle cell) of mtDNA molecules, depending on the cell's energy requirement. Thus, mtDNA CN may serve as a surrogate marker of mitochondrial function.[11–13] In epidemiologic studies, a lower level of mtDNA CN in blood has been found to be associated with a general decline in health,[14] all-cause mortality,[14–16] and multiple cardiometabolic traits, including a higher level of LDL-C.[17,18] Recent prospective studies have also reported significant associations between lower mtDNA levels and CVD outcomes.[15,19,20] However, the causal relationship between mtDNA CN and CVD remains to be determined.

To that end, this study pursued 2 aims to test the hypothesis that mtDNA CN is casually associated with CVD outcomes. The first aim was to validate the associations of mtDNA CN with CVD outcomes and total mortality using blood-derived mtDNA CN estimated from whole-genome sequencing (WGS) in 8 cohorts of diverse races and ethnicity. Previous studies of mtDNA CN associations with CVD and mortality used mtDNA CN measured by array-based methods or by quantitative polymerase chain reaction in fewer cohorts.[15,19] The second aim was to explore the causal relationship between mtDNA CN and coronary heart disease (CHD) using Mendelian randomization (MR), a method that has been increasingly used to minimize issues of confounding and reverse causation with genetic variants as an instrumental variable (Figure 1).[21]

METHODS

This study was an observational study. All study participants provided written informed consent for genetic

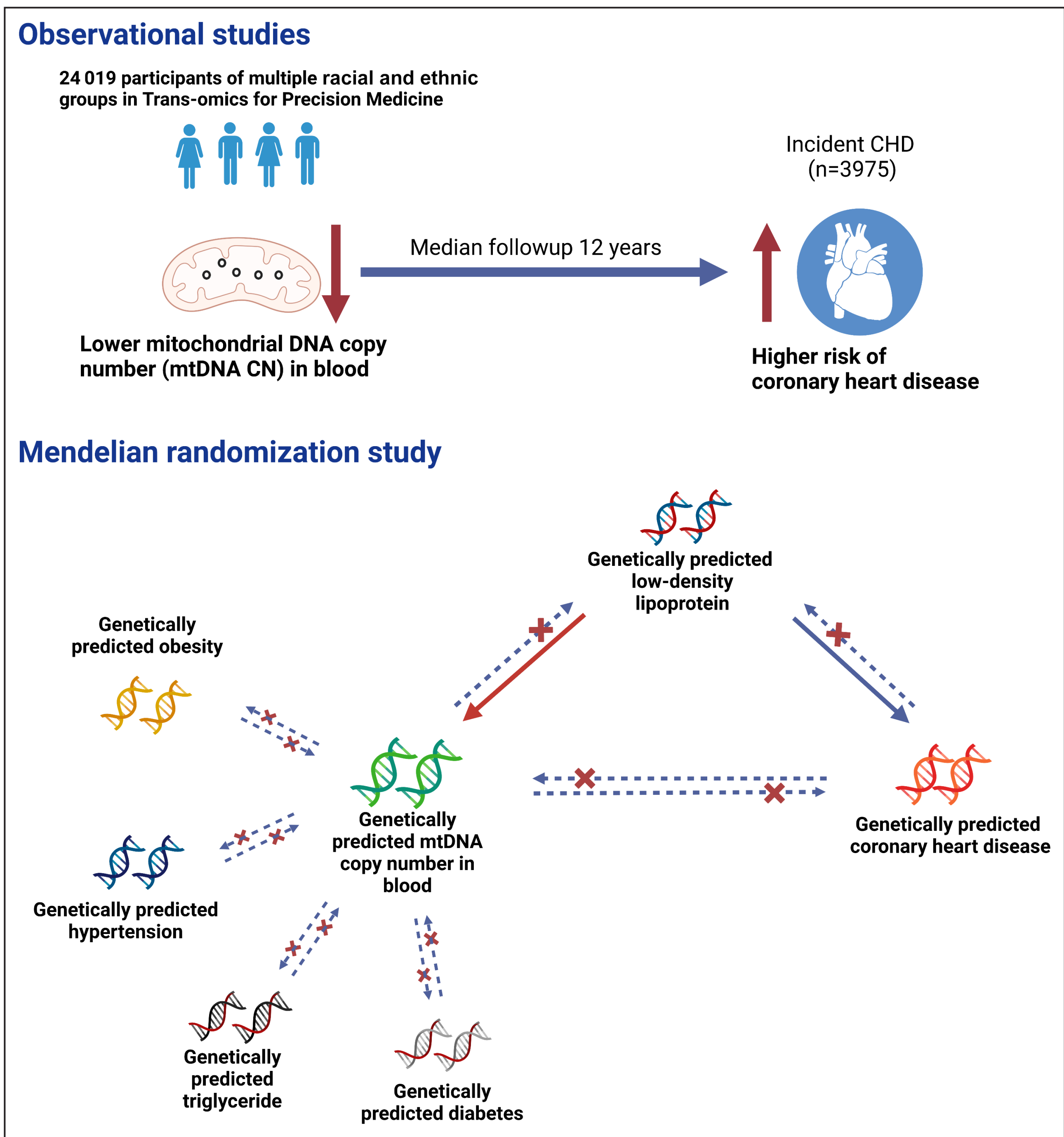

Figure 1. Study design.
Association analyses of mitochondrial DNA copy number (mtDNA CN) with cardiovascular disease traits were performed in 8 cohorts of multiple races and ethnicities (n=27 316). Meta-analysis was performed using the fixed-effects inverse variance method. Bidirectional univariable Mendelian randomization was performed to test for causality between mtDNA CN, coronary heart disease (CHD), and low-density lipoprotein cholesterol (LDL-C). Multivariable Mendelian randomization was performed to test the direct causal effect of LDL-C or CHD on mtDNA CN.

studies. The protocols for WGS were approved by the institutional review boards of the participating institutions, including those involved in the following studies: ARIC (Atherosclerosis Risk in Communities) study, CARDIA (Coronary Artery Risk Development in Young Adults Study), CHS (Cardiovascular Health Study), FHS (Framingham Heart Study), GENOA (Genetic Epidemiology Network of Arteriopathy) study, JHS (Jackson Heart Study), MESA (Multi-Ethnic Study of Atherosclerosis), and WHI (Women's Health Initiative)

study. All data and materials have been made publicly available at the database of genotypes and phenotypes and can be accessed at https://www.ncbi.nlm.nih.gov/gap/. Code used for analysis is available from the corresponding author on reasonable request for collaboration and reproducibility purposes.

Study Sample

This study included participants with WGS from 8 prospective cohort studies of multiple racial and ethnic groups with WGS (Table S1): the ARIC study[22] (n=3585), the CARDIA[23] (n=3473), the CHS[24] (n=3546), the FHS[25–27] (n=4133), the GENOA study[28] (n=1253), the JHS[29] (n=3286), the MESA[30] (n=4596), and the WHI study[31] (n=7197). Except for the WHI study, in which only female participants were included, the other 7 cohorts included both men and women. The CHS only included participants aged ≥65 years (mean age, 74 years), and the other cohorts included mostly middle-aged participants at blood draw for this study (mean age range, 58–69 years). MESA excluded participants with any clinically recognized CVD at the baseline visit,[30] whereas the other cohorts contained prevalent CVD cases at baseline. Several of the cohorts contained a small number of duplicate participants (n=136) because of study design and data collection.[22,28,29] We removed these duplicate participants from subsequent association analyses. We also excluded participants with missing values in the predictor and outcome variables. Participants with missing values in covariates were also removed.

Blood-Derived mtDNA CN Estimation in WGS

WGS was performed by the trans-omics for precision medicine (TOPMed) sequencing centers using blood-derived DNA for all participants in the 8 cohorts included in this study. The average genome-wide coverage was ≈39-fold across samples in the TOPMed.[32] The TOPMed Information Research Center conducted analyses to estimate mtDNA CN across all TOPMed participants using the program *fastMitoCalc* of the software package *mitoAnalyzer*.[33] Because nuclear DNA is diploid, whereas mtDNA is haploid, the average mtDNA CN per cell was estimated as twice the ratio of the average coverage of mtDNA/the average coverage of the nuclear DNA.[33]

CVD Traits and Total Mortality

The 8 longitudinal cohorts in this study have been established to investigate risk factors contributing to CVD, morbidity, and mortality. Each cohort used standardized definitions to adjudicate CVD outcomes. CHD was defined as the first incident myocardial infarction or death attributable to CHD and cardiac procedures (typically revascularization).[34] Stroke was defined as the first nonfatal stroke or death attributable to stroke.[35] Heart failure is a complex clinical syndrome resulting from a structural or functional cardiac disorder that impairs the ability of one or both ventricles to fill with or eject blood sufficiently to meet the needs of the body.[36,37] CVD included CHD, stroke, and heart failure, and death attributable to CHD, stroke, and heart failure. All-cause mortality included deaths of all causes. We analyzed associations of mtDNA CN with prevalent and incident CVD outcomes (CHD, stroke, and CVD) and with all-cause mortality.

Covariates

In the primary analysis, age at blood draw, sex, study center (if applicable), and self-reported racial and ethnic group were adjusted for in the base model. Additional variables included body mass index (BMI; kg/m^2), fasting plasma lipid measures, including total cholesterol (mg/dL) and high-density lipoprotein cholesterol (mg/dL), systolic blood pressure (mm Hg), treatment for high blood pressure or hypertension, current smoking status, and diabetes status. Diabetes was defined as fasting blood glucose level of ≥126 mg/dL or currently receiving medications to lower blood glucose levels to treat diabetes. This study used mtDNA CN calculated from WGS of blood-derived DNA. Different blood cell types (eg, neutrophils and lymphocytes) contain different levels of mtDNA CN.[38,39] To minimize potential confounding, we accounted for white blood cell count, differential components (the proportions of neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and platelet count in association analyses in cohorts in which these cell count variables were available.[17]

Statistical Analyses of mtDNA CN With CVD Outcomes and Total Mortality

For primary analyses, we generated mtDNA CN residuals by regressing mtDNA CN on age, age squared, sex, and blood collection year (as a factor variable to reflect batch effect variable) in each cohort.[17] For age-stratified analysis (<60 and ≥60 years), we generated mtDNA CN residuals by regressing mtDNA on sex and blood collection year in each cohort. For sex-stratified analysis, we generated mtDNA CN residuals by regressing mtDNA on age, age squared, and blood collection year in each cohort. The residuals were standardized to have a mean of 0 and an SD of 1. The standardized residuals were used as the main predictor in all regression models.[17] We removed participants whose mtDNA CN standardized residuals were >4 SDs from the mean.

We performed cohort-specific association analyses between mtDNA CN and outcomes. We used logistic

regression to quantify the associations of mtDNA CN with prevalent CVD outcomes. We used a Cox proportional hazards regression model to quantify the association of mtDNA CN with incident CVD outcomes and total mortality in all cohort-specific analyses. Because of a special study design in selecting participants for WGS in WHI study, we applied a weighted logistic regression for cross-sectional outcomes or a weighted Cox proportional hazards regression for incident outcomes in the WHI study (Data S1). We performed 3 models for association analyses of mtDNA CN with both prevalent and incident outcomes. Model 1 included age, sex, study center (if applicable), and race and ethnicity. In model 2, we additionally adjusted for several traditional covariates, including BMI, total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, treatment for high blood pressure or hypertension, current smoking status, and diabetes, for CVD outcomes. For analyzing total mortality as the outcome, we excluded participants who have prevalent CHD or diabetes and adjusted for BMI, total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, treatment for high blood pressure or hypertension, and current smoking status[15] in model 2. In model 3, white blood cell and differential counts as well as platelet counts were further adjusted in addition to covariates in model 2. We used an inverse variance meta-analysis with a fixed-effects model to summarize cohort-specific association analyses. An odds ratio (OR) or a hazard ratio (HR) was reported corresponding to 1-SD decrease in the mtDNA CN level. We used 0.05/4≈0.01 for significance to account for multiple testing with 4 different outcomes (ie, CHD, stroke, CVD, and total mortality) in association analyses.

In secondary analyses, we performed association analyses between mtDNA CN and outcomes in: (1) male- and female-only samples and (2) in participants who were younger than 60 years and at least aged 60 years at blood draw for WGS. We also performed several sensitivity analyses in FHS to investigate if different cardiometabolic disease status (ie, hypertension, diabetes, and hyperlipidemia) and medication (ie, lipid treatment) may result in different directionalities or effect sizes in associations of mtDNA CN with CVD.

Mendelian Randomization

To evaluate the causal relationship between mtDNA CN and CHD, we first conducted univariable bidirectional 2-sample MR analyses between mtDNA CN and CHD.[40] Because several cardiometabolic traits are leading risk factors for CVD and are associated with mtDNA CN, we conducted additional univariable bidirectional 2-sample MR analyses to evaluate the causal relationships between mtDNA CN and cardiometabolic traits (Figure 1), including BMI, type 2 diabetes, hypertension, triglyceride, and LDL-C, because large genome-wide association studies (GWASs) were available for these cardiometabolic traits (Data S1).[17,40–46] If a cardiometabolic trait displayed a causal relationship with mtDNA CN, we conducted multivariable MR (MVMR)[47–49] to assess the direct effect of an exposure on an outcome, adjusting for another exposure. An MVMR analysis accounts for possible horizontal pleiotropy effect that may result from common single-nucleotide polymorphisms (SNPs) underlying the exposures, which violates the third assumption of MR.[50–52]

In both univariable and multivariable MR analyses, we used independent SNPs (linkage disequilibrium [LD] r^2<0.001 based on the European reference panel) that were significant (P<5e-8) in several large GWASs and meta-analyses (Data S1). We also excluded SNPs with ambiguous allele information (ie, palindromic SNPs)[40] and SNPs that are known to be pleiotropic (ie, the missense mutations rs7412 and rs429358 in apolipoprotein E).[53] We used the inverse variance–weighted (IVW) method to combine the causal effects of independent SNPs in both univariable and multivariable MR analyses. We also performed several MR sensitivity analyses to minimize bias attributable to outliers and pleiotropic SNPs in assessing causal effects in univariable MR analyses. These methods included leave-one-out and Mendelian randomization pleiotropy residual sum and outlier to detect and correct for potential outliers.[54] Furthermore, we conducted MR-Egger regression, Cochran Q statistic, and funnel plots, and obtained median and mode estimates to test the validity of MR estimators.[52,55–58] For multivariable MR analyses, the sensitivity analyses included the extended framework of Mendelian randomization pleiotropy residual sum and outlier to account for possible outliers and the generalized Cochran Q test to assess instrumental variable validity in the 2-sample summary data setting.[47,59]

In secondary analysis to test for possible causality of mtDNA CN to CHD, we conducted MR analysis using selected SNPs identified by Gene Ontology PANTHER analysis. These selected SNPs are directly involved in mitochondrial functions (Table S2).[60] To assess the risk of type 2 error in MR analyses, we conducted power calculations using an online tool (https://shiny.cnsgenomics.com/mRnd/) (Data S1 and Table S3). TwoSampleMR package (version 0.5.0) in R (version 0.5.6) and the MVMR package (version 0.2.0) in R (version 0.5.6) were used for univariable and multivariable MR analyses. To account for multiple testing, we used 0.05/6=0.0083 for significance in both univariable and multivariable MR analyses with 6 traits (CHD, BMI, LDL-C, triglycerides, hypertension, and type 2 diabetes).

RESULTS

Participant Characteristics

This study included up to 27 316 participants (mean age, 62 years; age range, 20–98 years; 68% women) with 16 636 (60.9%) White Americans, 8709 (31.9%) Black Americans, 1229 (4.5%) Hispanic or Latino Americans, and 728 (2.7%) East Asian Americans (Table S1). The prevalence of any CVD outcome was higher in Black individuals (13.4%) than in other ethnic and racial groups (White Americans, 9.4%; Hispanic or Latino Americans, 9.8%; and East Asian Americans, 10.2%). During a median of 6 to 16 years (across the cohorts) of follow-up, the prevalence and incidence rates of CVD outcomes varied across cohorts (Table S1).

Association Analyses of mtDNA CN in Blood With Prevalent CVD Outcomes

In total, 2158 (7.9%) participants had prevalent CHD, 751 (4.2%) had prevalent stroke, and 3394 (12.4%) had prevalent CVD at baseline (Table S1). Meta-analysis showed that 1-SD lower level of mtDNA CN was significantly associated with 1.11 times the odds of CHD (95% CI, 1.07–1.16; P<0.001), 1.13 times the odds of stroke (95% CI, 1.05–1.22; P=0.0020), and 1.14 times the odds of CVD (95% CI, 1.11–1.16; P<0.001), adjusting for age, sex, and race and ethnicity (Figure 2; model 1). The associations were slightly attenuated after further adjusting for traditional CVD risk factors (Figure S1; model 2) and white blood cell count in addition to traditional CVD risk factors (Figure S2; model 3). The association directions were consistent across 6 of the 7 cohorts, with 1 null association for CVD outcomes (Figure 2).

Association Analyses of mtDNA CN in Blood With Incident CVD Outcomes and All-Cause Mortality

A total of 24 019 participants free of CVD at baseline were followed up for a median of 12 years (6–14 median years across cohorts) (Table S1). During the follow-up, 3975 (16.5%) developed incident CHD, 5208 (21.7%) developed incident stroke, and 8590 (35.4%) developed incident CVD. Meta-analysis showed that 1-SD lower in mtDNA CN at the baseline was significantly associated with 1.08 times the hazard for developing incident CHD (95% CI, 1.04–1.12; P<0.001) and 1.07 times the hazard for developing incident CVD (95% CI, 1.03–1.10; P<0.001) when we adjusted for age, sex, and race and ethnicity in association analyses (Figure 3). The associations were slightly attenuated after further adjusting for traditional CVD risk factors in model 2 (incident CHD: HR, 1.05 [95% CI, 1.01–1.09]; P=0.023; incident CVD: HR, 1.05 [95% CI, 1.02–1.09]; P<0.001) (Figure S3). The associations also changed slightly after additionally adjusting for white blood cell count/differential count and platelet count in model 3 (incident CHD: HR, 1.07 [95% CI, 1.02–1.12]; P<0.001; incident CVD: HR, 1.06 [95% CI, 1.03–1.10]; P<0.001; Figure S4). Incident stroke was not significantly associated with mtDNA CN in meta-analyses of the 3 models (Figures S3 and S4).

Examining the individual cohorts, we found that lower mtDNA CN was associated with higher hazards for developing incident CHD and incident CVD in 5 cohorts, with the ARIC study displaying the strongest associations, whereas FHS and WHI study showed weak inverse associations or no association (Figure 3). A sensitivity analysis removing ARIC study showed

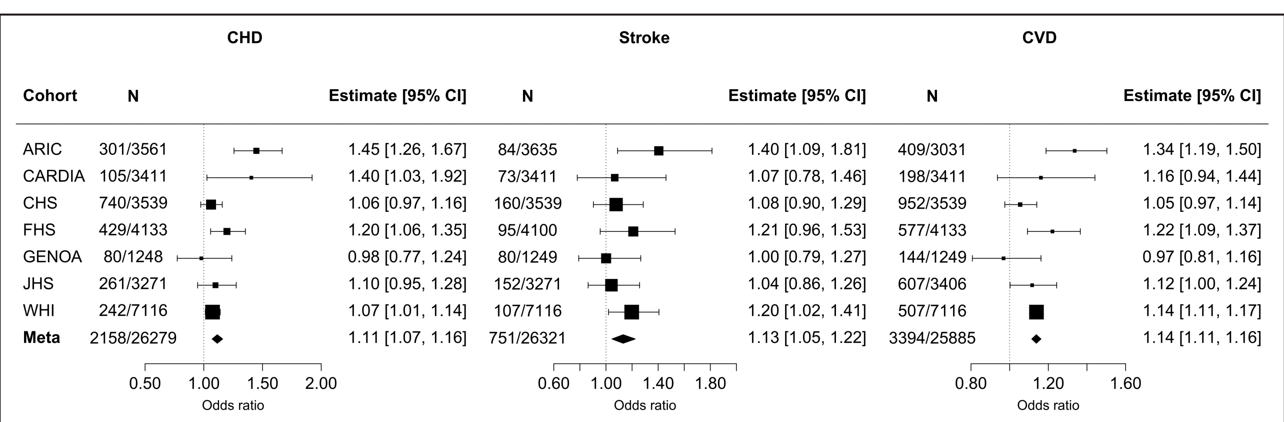

Figure 2. Association and meta-analysis of mitochondrial DNA copy number (mtDNA CN) and prevalent cardiovascular disease (CVD) outcomes.

We performed a logistic regression analysis between each outcome and mtDNA CN residuals as the independent variable, adjusting for age, sex, study center (if applicable), and race and ethnicity. The size of the square represents the weight of each cohort in the meta-analysis. ARIC indicates Atherosclerosis Risk in Communities; CARDIA, Coronary Artery Risk Development in Young Adults Study; CHD, coronary heart disease; CHS, Cardiovascular Health Study; FHS, Framingham Heart Study; GENOA, Genetic Epidemiology Network of Arteriopathy; JHS, Jackson Heart Study; and WHI, Women's Health Initiative.

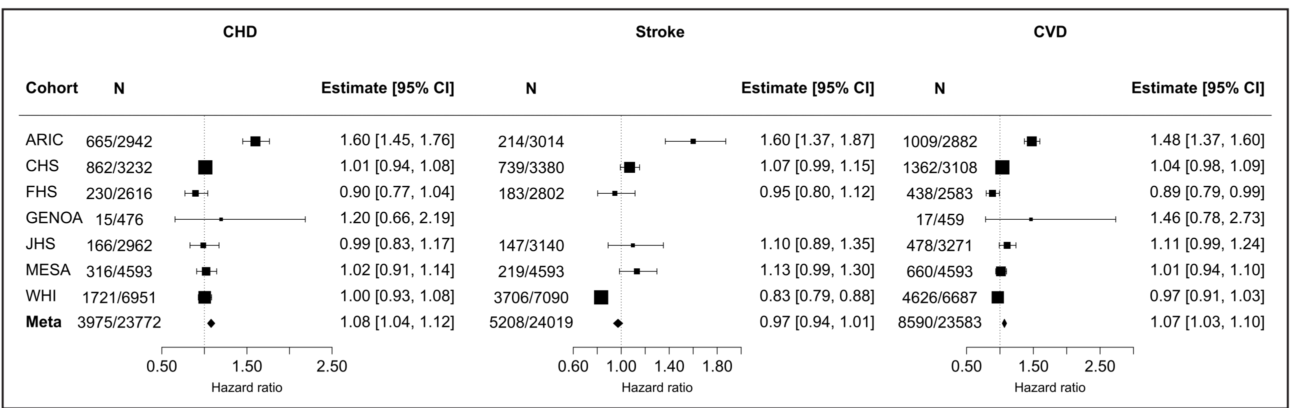

Figure 3. Association and meta-analysis of mitochondrial DNA copy number (mtDNA CN) and incident cardiovascular disease (CVD) outcomes.

We performed a Cox proportional hazards regression between each outcome and mtDNA CN residuals as the independent variable, adjusting for age, sex, study center (if applicable), and race and ethnicity. The size of the square represents the weight of each cohort in the meta-analysis. Because of a limited number of stroke cases, a Cox proportional hazard regression was not performed for stroke in the GENOA (Genetic Epidemiology Network of Arteriopathy) study. ARIC indicates Atherosclerosis Risk in Communities; CHD, coronary heart disease; CHS, Cardiovascular Health Study; FHS, Framingham Heart Study; JHS, Jackson Heart Study; MESA, Multi-Ethnic Study of Atherosclerosis; and WHI, Women's Health Initiative.

nonstatistically significant results (Figure S5). Additional sensitivity analyses demonstrated that several factors, including age, sex, hypertension status, diabetes status, and hyperlipidemia status, were not the cause for the inverse effect or null association observed in FHS compared with other cohorts, although the magnitude of associations seemed different in stratified analyses (Tables S4 and S5). The stratified analyses showed that participants with statin treatment had a much smaller effect size compared with those without statin treatment (Table S4).

A total of 8018 (33.3%) participants died attributable to any cause during a median of 14 years of follow-up (10–19 median years across cohorts) (Table S1). A 1-SD decrease in mtDNA CN was significantly associated with 1.06 times the hazard for all-cause mortality (95% CI, 1.03–1.09; P<0.001), adjusting for age, sex, race and ethnicity. All of the cohorts showed consistent directionality between mtDNA CN and total mortality in model 1 (ie, lower mtDNA CN was associated with higher rates of all-cause mortality) (Figure S6). The associations were similar after further adjusting for multiple clinical covariates in model 2 (HR, 1.05 [95% CI, 1.02–1.08]; P<0.001) and additionally adjusting for cell counts/differential components and platelet count in model 3 (HR, 1.06 [95% CI, 1.02–1.10]; P=0.0011; Figure S6).

MR Analyses to Test Causality

Power Calculations

We conducted power calculations with continuous and binary outcomes in MR analyses at α=0.0083 (0.05/6). For example, for MR analyses using SNPs identified from a large GWAS of mtDNA CN and meta-analysis of a continuous outcome (n=550 000), we had 80% power to detect a significant causal relationship if 1-SD change in mtDNA CN resulted in at least 0.033-SD change in the continuous outcome. Similarly, when n=550 000 and α=0.0083, we had 80% power to detect a significant causal relationship if the per SD change in mtDNA CN resulted in an OR of 1.15 (binary outcome with 5% prevalence; Table S3).

Bidirectional Univariable MR Analyses Between Blood-Derived mtDNA CN and an Outcome

We selected 74 independent SNPs (LD r^2<0.001) from the GWAS of blood-derived mtDNA CN as instrumental variables to infer a possible causal effect of mtDNA CN on CHD[61] (Table S6). The MR IVW analyses yielded insufficient evidence (OR, 1.10 [95% CI, 0.98–1.22]; P=0.11) to support a casual effect of lower mtDNA CN on higher odds of CHD (Table S7 and Figure S7). Sensitivity analyses and the secondary analysis with 18 SNPs that are directly involved in mitochondrial function further showed no causal relationship of mtDNA CN on CHD (Tables S8 and S9 and Figure S8). To test for the reverse causal relationship from CHD to mtDNA CN, we used 142 significant CHD GWAS SNPs (LD r^2<0.001; Table S10).[62] The MR IVW analysis showed that having CHD was associated with a lower level of mtDNA CN; however, this causal association was not significant (β=−0.012 [95% CI, −0.025 to 0.00094]; P=0.076) (Table S7 and Figure S9). The MR-Egger test showed a nominally significant causal effect (P<0.05) of CHD on a lower level of mtDNA CN (β=−0.030 [95% CI, −0.055 to −0.0045]; P=0.029) (Table S7). Additional MR sensitivity analyses showed no statistically significant causal effect of CHD on a lower level of mtDNA CN (Table S7).

Table. Comparison of Causal Inference Between Univariable and Multivariable MR Analyses

Exposure	No. of SNPs	β	SE	*P* value
Univariable MR				
CHD	142	−0.012	0.0066	0.076
LDL-C	345	−0.084	0.011	1.1E-14
Multivariable MR				
CHD	80	0.0057	0.0088	0.52
LDL-C	291	−0.092	0.013	6.0E-13

Univariable MR was performed to infer possible causal effect of CHD or LDL-C on mitochondrial DNA copy number (mtDNA CN). Multivariable MR was performed to evaluate the direct causal effect of CHD or LDL-C on mtDNA CN. β, SE, and *P* values were obtained from inverse variance (univariable) or extended inverse variance (multivariable) weighted MR analyses. Results from additional sensitivity analyses were presented in Tables S7, S17, and S26 and Figures S9 and S15. CHD indicates coronary heart disease; LDL-C, low-density lipoprotein cholesterol; MR, Mendelian randomization; and SNP, single-nucleotide polymorphism.

We performed additional bidirectional MR analyses between blood-derived mtDNA CN and several cardiometabolic traits that are major risk factors for CVD. We used 63 to 75 independent SNPs (LD r^2<0.001) to test the causal relationships of mtDNA CN on cardiometabolic traits (Tables S11–S15). We found no significant causal effects of lower mtDNA CN on a higher level of BMI (MR IVW *P*=0.53), LDL-C (MR IVW *P*=0.059), or triglycerides (MR IVW *P*=0.22) (Tables S16–S18 and Figures S10–S12). Similarly, we found no causal effects of lower mtDNA CN on a higher risk of hypertension (MR IVW *P*=0.22) or type 2 diabetes (MR IVW *P*=0.89) (Tables S19 and S20 and Figures S13 and S14).

In the reverse direction, we selected cardiometabolic trait–associated GWAS SNPs (LD r^2<0.001; 267 for BMI, 345 for LDL-C, 403 for triglycerides, 53 for hypertension, and 181 for type 2 diabetes) to infer possible causal effects of these traits on lower mtDNA CN in MR analyses (Tables S21–S25).[40–46] We observed a significant causal effect of LDL-C on mtDNA CN using the MR IVW and other MR methods (Table S17 and Figure S15). The IVW MR analysis showed a strong causal association of a higher LDL-C level on a lower level of mtDNA CN (β=−0.084 [95% CI, −0.11 to −0.062]; *P*<0.001). All of the additional sensitivity analyses presented a significant causal effect of LDL-C on mtDNA CN (*P*<0.001) (Table S17). We found no causal effects of a higher level of BMI (MR IVW *P*=0.59), a higher level of triglycerides (MR IVW *P*=0.78), a higher risk of hypertension (MR IVW *P*=0.85), and type 2 diabetes (MR IVW *P*=0.45) on lower mtDNA CN (Tables S16 and S18–S20 and Figures S16–S19).

Multivariable MR Analyses of LDL-C and CHD on mtDNA CN

Previous studies found that LDL-C plays a key role in CHD development.[7,8] We observed a significant causal effect of LDL-C on CHD using the univariable MR IVW method (OR: 1.68 [95% CI, 1.54–1.84]; *P*<0.001) with the selected SNPs (Tables S10 and S22). Given the findings from univariable MR analyses, we conducted MVMR to estimate the direct effect of CHD with LDL-C on mtDNA CN, controlling for each other. After excluding SNPs with a pairwise r^2>0.001, 346 SNPs from GWASs of LDL-C and CHD were used in the MVMR analyses. We observed strong evidence for a direct causal effect of LDL-C on mtDNA CN, adjusting for CHD: 1-SD higher genetically predicted LDL-C level was causally associated with a 0.092-SD lower mtDNA CN level (IVW β=−0.092 [95% CI, −0.12 to −0.067]; *P*<0.001) (Table and Figure 4). In contrast, the direct causal effect of CHD on mtDNA CN was not

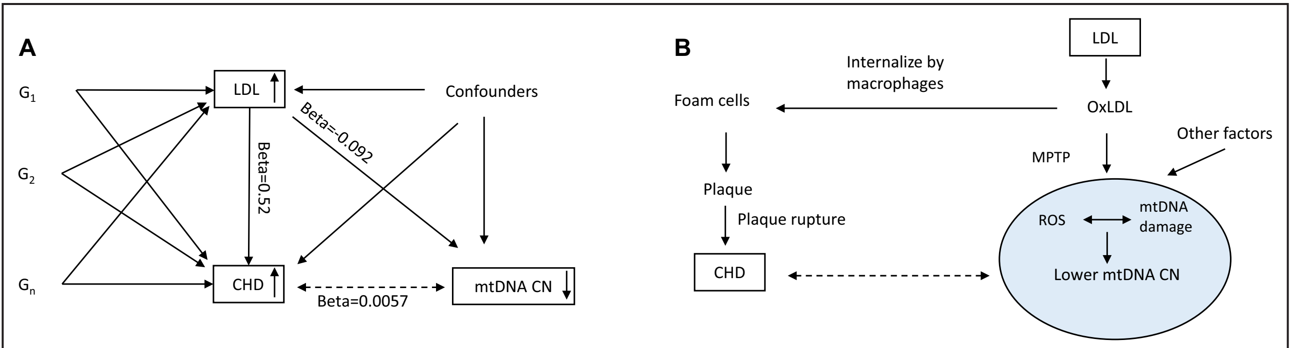

Figure 4. The causal relationships between low-density lipoprotein cholesterol (LDL-C), coronary heart disease (CHD), and mitochondrial DNA copy number (mtDNA CN).
A, Mendelian randomization. **B**, Biological experiment (see study by Lee et al[3]). A 1-SD lower genetically predicted LDL-C was causally associated with 1.68 times (β=0.52) the odds (95% CI, 1.54–1.84) of having CHD. Multivariable Mendelian randomization analysis demonstrated that 1-SD lower genetically predicted LDL-C was causally associated with 0.092-SD lower mtDNA CN (*P*=6.0E-13), adjusting for CHD. However, multivariable Mendelian randomization showed that the direct effect of genetically predicted CHD was not associated with mtDNA CN (β=0.0057; *P*=0.52), adjusting for LDL-C. MPTP indicates mitochondrial permeability transition pore; OxLDL, oxidized LDL-C; and ROS, reactive oxygen species.

significant, controlling for LDL-C level (IVW β=0.0057 [95% CI, −0.012 to 0.023]; *P*=0.52) (Table). The MVMR-Egger test yielded consistent results as those from IVW MVMR analysis to test the relationship of LDL-C and CHD on mtDNA CN. Additional sensitivity analyses by multivariable Mendelian randomization pleiotropy residual sum and outlier yielded consistent results (Table S26).

DISCUSSION

In this study, we validated the association of blood-derived mtDNA CN with prevalent and incident CVD outcomes (except for incident stroke), as well as with all-cause mortality during a median of 12 (for CVD) or 14 (for mortality) years of follow-up in up to 27 316 participants from 8 cohort studies, including self-identified White Americans, Black Americans, Hispanic or Latino Americans, and East Asian Americans. The associations of mtDNA CN with the outcome variable remained statistically significant after further adjustment for traditional clinical variables (ie, total cholesterol and high-density lipoprotein cholesterol) and blood cell counts. More importantly, we performed comprehensive univariable and multivariable MR analyses, using SNPs identified from the latest GWAS for CHD,[62] mtDNA CN,[61] and cardiometabolic disease (CMD) traits[40–46] to explore the causal relationships between mtDNA CN, CMD traits, and CHD. The MR analyses implicate that an elevated LDL-C level in blood is likely the primary driver for the observed significant association of blood-derived mtDNA CN with CHD.

It has always been challenging to assess causality in epidemiologic association analyses. The bidirectional univariable MR analyses in this study found weak evidence that having CHD may be causally associated with lower mtDNA CN level rather than an opposite direction that lower mtDNA CN had a causal effect on CHD. CHD is a multifactorial endpoint disease that is characterized as the reduction of blood flow to the heart muscle attributable to a build-up of atherosclerotic plaque.[3] Our recent study reported that higher levels of CMD traits are associated with lower mtDNA CN in blood.[17] Thus, CMD traits may play a role in the observed association between CHD and mtDNA CN. Bidirectional MR analyses showed no causal relationships between mtDNA CN and the 3 CMD traits (BMI, hypertension, and diabetes) in the forward or reverse directions. However, bidirectional MR analyses displayed that the higher LDL-C levels in plasma displayed a causal effect on lower mtDNA CN, whereas mtDNA CN had no causal effect on LDL-C. Recent advances have found that excess LDL-C levels initiated atherosclerosis, the key factor in the development of CHD.[63] MR analysis has also displayed a causal effect of LDL-C on CHD.[50] Because LDL-C and CHD share common genetic variants, we performed an MVMR analysis to assess the direct causal effect of CHD or LDL-C on mtDNA CN. We observed a significant, direct causal effect of LDL-C on mtDNA CN, adjusting for CHD (Figure 4A), whereas the direct causal effect of CHD on mtDNA CN became nonsignificant, controlling for LDL-C. On the basis of these findings, it is reasonable to speculate that the observed association between mtDNA CN and CVD outcomes (prevalent and incident) may be a manifestation of the causal effect of higher LDL-C levels on lower mtDNA CN and a higher risk for CVD. Our study showed strong evidence for a direct causal effect between higher LDL-C levels and lower mtDNA CN. A future study is needed to investigate whether the statin treatment is associated with a higher-level mtDNA CN in blood.

Our findings and the recent advances in animal models supported each other. Animal models were developed to elucidate the role of oxidative stress and mitochondrial dysfunction in vascular inflammation and atherosclerosis in animal models.[6,64,65] In these animal models, LDL-C in the plasma was the primary molecule that triggered a cascade of inflammation responses. The excess of LDL-C was oxidized into oxidized LDL-C, which attracts immune cells, like monocytes, into the arterial wall, gradually building up atherosclerotic plaques.[6,64,65] CHD occurred when plaques were ruptured to form a large thrombus. On the other hand, the oxidized LDL-C and other factors result in reactive oxygen species production in mitochondria.[66,67] This oxidative stress leads to damages in mtDNA replication enzyme and, in turn, results in lower mtDNA CN.[9] The MR analyses in our study supported that higher LDL-C levels may be the driver for the development of CHD and lower mtDNA CN levels in blood (Figure 4B). Nonetheless, the role of mtDNA CN in the atherosclerotic formation, the pathogenesis of CVD, and inflammation is complex and warrants further investigation.

Limitations of the Study

Heterogeneity was observed in the association estimates of CVD outcomes across cohorts, although we harmonized phenotypes and accounted for confounders and known batch effects in association analyses with mtDNA CN. This observed heterogeneity may be partially attributable to different distributions in age, sex, and CVD phenotypes across study cohorts. Experiment conditions for blood draws, DNA extraction, storage, and other unobserved confounding factors may also have contributed to the heterogeneity.[17] Another limitation was that our study was an epidemiologic study, and it used existing mtDNA CN data derived from WGS in whole blood for association analyses with CVD. Therefore, we were unable

to investigate whether mtDNA assayed in the blood adequately reflects other tissues that were involved in atherosclerosis. A few previous studies have provided indirect evidence that mtDNA CN level in whole blood may reflect the mtDNA CN level in other tissues to some extent. One study found a moderate correlation (r=0.5) between mtDNA CN levels in whole blood and plasma in 18 participants.[68] A more recent study (n=419) found that blood-derived mtDNA CN was associated with gene expression in several tissues (Including heart [left ventricle]).[69] In addition, given our previous findings and the strong component of inflammation in the pathogenesis of CVD, we have recognized that the relationship between cell counts, mtDNA CN, and CVD is complex. We adjusted the cell count variables to minimize confounding in regression models. MR analyses help address the question of causality in the complex relationship. Further studies are warranted to investigate the role of cell counts in the relationship between mtDNA CN and CVD. Although we observed that blood-derived mtDNA CN had no causal effect on CHD, this result should be interpreted with caution. In this study, the total sample size used for 2-sample MR analyses of mtDNA CN with CHD was ≈550 000. Using this sample size, we can only reject the null hypothesis that mtDNA CN had no causal effect on CHD if we observe an OR >1.12. If the OR was <1.12, we were unable to detect the causal relationship. In MR analyses, overlapping samples may be used in both exposure GWASs and outcome GWASs, which may violate the assumption of independent samples in 2-sample MR analyses. A previous study conducted extensive simulations to investigate the effects of overlapping samples in 2-sample MR analyses. They showed that multiple MR methods produced similar causal estimates with overlapping samples compared to independent samples if the total sample size in exposure GWAS and outcome GWAS was large (ie, >300 000), except for the MR-Egger method, in which the results should be interpreted with caution.[70]

Strength of the Study

The main strength of this study is that we adopted bidirectional and multivariable MR analysis to disentangle the complex relationship between mtDNA CN and CHD in cohort studies. Observational epidemiologic studies are susceptible to confounding, and subclinical disease stage may impact observed associations between CVD outcomes and mtDNA CN.[71] Robust genetic variants have been identified in large GWASs with mtDNA CN (n=465 809), CHD (n=547 261), and LDL-C (n=1 166 583).[41,44,45,61] To minimize bias in MR analyses, we removed known pleiotropic SNPs (eg, *APOE* SNPs) that are associated with both mtDNA CN and CVD traits. We performed MR IVW as well as multiple sensitivity analyses, including MR-Egger, median, and mode methods, to provide evidence for the validity of MR estimators. Overall, the multivariable MR results provide evidence that higher LDL-C level is likely the driving factor for the observed association between lower mtDNA CN and CHD. The inclusion of participants of multiple races and ethnicities enhances the generalizability of the study results. An additional strength is that the CVD outcomes and all-cause death data have been regularly adjudicated and collected by physician endpoint review committees in most of the cohorts.[72,73] The joint estimation of mtDNA CN in TOPMed cohorts, well-characterized outcome and predictor variables, and hierarchical association analyses with 3 models were likely to reduce potential confounding.

In summary, this study validated the previously reported association of mtDNA CN with CVD outcomes and all-cause mortality. In addition, we used both univariable and multivariable MR analyses to demonstrate an independent causal effect of LDL-C underlying the relationship between mtDNA CN and CHD. Findings from this study add to an increasing volume of evidence surrounding the harmful effects of high LDL-C in the complex relationships between vascular inflammation, atherosclerosis, and lower mtDNA CN, a biomarker for mitochondrial function. Therefore, the control for LDL-C and inflammation may be a feasible therapeutic strategy to improve mitochondrial function and cardiovascular health.

ARTICLE INFORMATION

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Supplemental Material

Data S1
Tables S1–S26
Figures S1–S19

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