Oxidants and antioxidants as targets for cardiovascular disease prevention: evidence from observational and causal inference studies

Martens, L.G.

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4

THE ASSOCIATION BETWEEN MITOCHONDRIAL DNA ABUNDANCE AND STROKE: A COMBINATION OF MULTIVARIABLE-ADJUSTED SURVIVAL AND MENDELIAN RANDOMIZATION ANALYSES.

Leon G Martens
Jiao Luo
Marieke J H Wermer
Ko Willems van Dijk
Sara Hägg
Felix Grassmann
Raymond Noordam
Diana van Heemst

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ABSTRACT

Background and aims

Mitochondrial dysfunction is associated with increased Reactive Oxygen Species that are thought to drive disease risk, including stroke. We investigated the association between mtDNA abundance, as a proxy measure of mitochondrial function, and incident stroke using multivariable-adjusted survival and Mendelian Randomization (MR) analyses.

Methods

Cox-proportional hazard model analyses were conducted to assess the association between mtDNA abundance, and incident ischemic and hemorrhagic stroke over a maximum of 14-years follow-up in European-ancestry participants from UK Biobank. MR was conducted using independent (R^2<0.001) lead variants for mtDNA abundance (p < 5x10-8) as instrumental variables. Single-Nucleotide Polymorphism (SNP)-ischemic stroke associations were derived from three published open source European-ancestry results databases (cases/controls): MEGASTROKE (60,341/454,450), UK Biobank (2,404/368,771) and FinnGen (10,551/202,223). MR was performed per study, and results were subsequently meta-analyzed.

Results

In total, 288,572 unrelated participants (46% men) with mean (SD) age of 57 (8) years were included in the Cox-proportional hazard analyses. After correction for considered confounders (BMI, hypertension, cholesterol, T2D), no association was found between low versus high mtDNA abundance and ischemic (HR: 1.06 [95% CI: 0.95, 1.18]) or hemorrhagic (HR: 0.97 [95% CI: 0.82, 1.15]) stroke. However, in the MR analyses after removal of platelet count-associated SNPs, we found evidence for an association between genetically-influenced mtDNA abundance and ischemic stroke (odds ratio, 1.17; confidence interval, 1.03, 1.32).

Conclusions

Although the results from both multivariable-adjusted prospective and basis MR analyses did not show an association between low mtDNA and increased risk of ischemic stroke, in-depth MR sensitivity analyses may suggest evidence for a causal relationship.

INTRODUCTION

Stroke is the second leading cause of death and loss of disability-adjusted life years worldwide [1]. Oxidative stress has been hypothesized to play an important role in the pathophysiology of stroke by aggravating secondary damage and increases reperfusion injury after ischemic stroke [2-4]. As a result of direct or indirect Reactive Oxygen Species (ROS)-induced damage to the (cerebral) vascular wall, multiple aspects of the vascular system are affected including platelet aggregation, endothelial function, vascular permeability and vasodilation [3]. These local vessel changes induced by oxidative stress can also gradually develop before stroke onset, and therefore may also lead to an increased risk of stroke incidence [5].

Mitochondria are a major source of ROS production [6]. Mitochondrial dysfunction leads to an increase in ROS production due to a change in redox homeostasis [7]. Additionally, impaired mitochondrial function, frequently proxied by the mitochondrial copy number (mtDNA-CN) [8, 9], has been associated with diseases such as diabetes, heart failure, and neurological defects [10]. mtDNA-CN can be assessed relatively easy in large populations by estimating the mtDNA abundance from the intensities of genotyping probes representing mitochondrial DNA on genotyping arrays [9, 11, 12]. Increased ROS production drives mitochondrial dysfunction causing increased defects in mitochondrial fusion, fission, and mitophagy activation [13], which subsequently lead to subsequent excessive ROS production [13].

Although a relatively small study was not able to provide evidence of an association between low mtDNA-CN and increased stroke risk [14], we hypothesized that leukocyte mtDNA might affect brain pathologies, given the available biological data. Based on the combination of the postulated detrimental biological effect of blood oxidative stress on the (cerebro)vascular endothelial system and its role in secondary damage after stroke occurrence, we investigated the prospective association between mtDNA abundance and incident ischemic and hemorrhagic stroke in a large cohort of European-ancestry participants from the UK Biobank. In addition, we applied Mendelian Randomization (MR) to provide evidence for possible causality [15, 16] as a way to triangulate the results from the prospective analyses by obtaining results from two analysis methods, both with different assumptions and limitations [15].

MATERIALS AND METHODS

Population description

The UK Biobank cohort is a prospective general population cohort with 502,628 participants between the age of 40 and 70 years recruited from the general population between 2006 and 2010 [17] (more information can be found online https://www.

4

ukbiobank.ac.uk). Blood samples were collected for genotyping. Access for information to invite participants was approved by the Patient Information Advisory Group (PIAG) from England and Wales. All participants in the UK Biobank provided a written informed consent and local research ethics committees and institutional review boards approved the study. The present study was accepted under project number 56340.

In the present study, genotyped European-ancestry participants were followed (N = 488 377). Exclusion criteria included: 1) non-European ancestry; 2) participants who failed genotyping quality control and/or with low call rate; 3) related individuals defined by principal components (PCs); 4) participants with high SD of autosomal probes; 5) history of any stroke at baseline; 6) missingness on covariates. After the exclusions, the final analyses were performed in 288,572 participants.

Mitochondrial DNA abundance

We used somatic mtDNA abundance as a proxy measure of mtDNA-CN, as the exposure, which is determined from the intensities of genotyping probes on the mitochondrial chromosome on the Affymetrix Array. The method for computing mtDNA abundance has been described previously [11]. In brief, the relative amount of mtDNA hybridized to the array at each probe was the log2 transformed ratio (L2R) of the observed genotyping probe intensity divided by the intensity at the same probe observed in a set of reference samples. We used the median L2R values across all 265 variants passing quality control on the MT chromosome as an initial raw measure of mtDNA abundance. To correct for confounding induced by poorly performing probes, we weighted L2R values of each probe by multiplying the weight of the probe that are generated from a multivariate linear regression model in which those intensities statistically significantly predicted normalized mitochondrial coverage from exome sequencing data, resulting in a single mtDNA abundance estimate for each individual. To eliminate the plate effect, we subsequently normalized the abundance to mean of zero and SD of one within each genotyping plate consisting of 96 wells [9].

Covariates

In addition to age and sex, we took into account data based on self-reported questionnaires (smoking, alcohol consumption, disease status, medication use), blood cell counts (white blood cell counts and platelet counts), body mass index (BMI) in kg/m^2, serum lipid levels (total and LDL cholesterol) in mmol/L, and systolic and diastolic blood pressure in mmHg.

Outcome

The outcome in the analysis was ischemic and hemorrhagic stroke separately, as well as combined, in the time period August 2006 up to January 2021. Stroke incidence was obtained via hospital admission data and national health register data and used to identify the date of the first stroke or stroke-related death after baseline assessment. The primary outcomes were any stroke incidence and further specified ischemic and hemorrhagic stroke incidence. Incident disease diagnoses are coded according to the International Classification of Diseases edition 10 (ICD-10); Ischemic stroke was defined as I63 and hemorrhagic stroke as I61. Any stroke was defined as the combination of I63 and I61. Follow-up time is computed from the baseline visit to the diagnosis of incident disease, loss-to-follow-up or death, or the end of the study period, whichever came first.

Data required for the Mendelian Randomization analyses

For the MR, genetic variants of mtDNA abundance were used as instrument variables. In a previous study, 129 independent Single-Nucleotide Polymorphisms (SNPs) as genetic variants were found to be independently associated with mtDNA abundance at a genome-wide significance threshold ($p < 5x10^{-8}$); SNPs were additionally pruned to an LD $R^2 < 0.0001$ [18]. The study was performed in a total of 465,809 individuals using a combined population of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium and the UK Biobank.

Mendelian Randomization outcome datasets

For the extraction of summary statistics on the associations of the mtDNA abundance related SNPs with ischemic stroke, which was defined as any ischemic stroke (I63), three large studies were used: the MEGASTROKE consortium, the UK Biobank, and the FinnGen study [17, 19]. Both UK Biobank and FinnGen were not part of the main analyses of the MEGASTROKE consortium preventing inclusion of overlapping samples in the analyses. In the three studies insufficient data on hemorrhagic stroke was available.

The trans-ancestry meta-analysis from the MEGASTROKE consortium was used to retrieve the ischemic stroke SNP-outcome data and was based on 60,341 cases and 454,450 controls collected from 29 studies of predominantly the European ancestry (86%) [19].

For the MR analyses in UK Biobank, cases developed before and after enrolment were considered. Follow-up information that included ischemic stroke occurrence was retrieved through the routinely available NHS database. In the European-ancestry dataset with full genomics data available, we had data on 2,404 cases of ischemic stroke and 368,771 controls. We performed new genome-wide association analyses using linear mixed models to assess the associations between genetic instruments and ischemic stroke, adjusted for age, sex and 10 principal components, and corrected for familial relationships using BOLT_LMM (v2.3.2).

Data from FinnGen (Freeze 5; https://www.finngen.fi/en/), which is an ongoing cohort study launched in 2017, and analyses were based on 10,551 cases of ischemic stroke, and 202,223 controls.

Although with lower numbers, we additionally performed MR on subtypes of

ischemic stroke (Cardio Embolic Stroke: 7,193 cases, 406,111 controls, Large Artery Atherosclerosis: 4,373 cases, 406,111 controls, Small Vessel Stroke: 5,386 cases, 192,662 controls) using data from MEGASTROKE and hemorrhagic stroke (1,687cases, 201,146 controls) from FinnGen.

Statistical analysis

Multivariable-adjusted analyses

For the analyses, and for presentation purposes, we divided the study population in 5 equally-sized groups based on the mtDNA abundance, with the first quintile containing the group with the lowest levels of mtDNA abundance and the fifth quintile containing the highest levels (used as reference).

Baseline characteristics of the study population were presented separately per quintile of mtDNA abundance, as mean (SD) for continuous variables if they followed a normal distribution, or as median (interquartile range) otherwise, and frequency (proportion) for categoric variables.

The cumulative incidence for competing risk (CICR) was used to plot the cumulative incidence of ischemic and hemorrhagic stroke against follow-up time separately using a Kaplan-Meier survival curve by mtDNA abundance quintiles, where death was accounted for as a competing event. For any, ischemic, and hemorrhagic stroke, a Cox proportional hazards model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) presented as stroke incidence, comparing the lowest 20% mtDNA abundance with the highest 20%. Analyses were additionally done stratified by sex. Two multivariate regression models were fitted, where for model 2 covariates were first added individually:

- Model 1: age, sex, batch, the first 10 genetic principal components, white blood cell counts, platelet count
- Model 2: Model 1 + BMI, smoking, alcohol consumption, total cholesterol, hypertension, diabetes, cholesterol lowering medication, blood pressure lowering medication

Covariates were included in the regression models given their known relation with both exposure and outcome (age, sex, smoking, alcohol consumption, total cholesterol, disease status, medication status), or were included as a technical correction due to the measurement composition (batch, white blood cell count, platelet count). Participants were censored in the event of loss-to-follow-up or death. In order to check whether the proportional hazards assumption was fulfilled, a Cox proportional hazard assumption test (“*cox.zph*” from R package “Survival”) was performed. Additionally, mtDNA-CN was assessed continuously as a one-SD lower mtDNA-CN on stroke incidence. Analyses were performed using the “Survival” (cran.r-project.org/web/packages/survival) package in R (v4.1.0)

Mendelian Randomization

All the analyses were done using R (v4.1.0) statistical software (The R Foundation for Statistical Computing, Vienna, Austria). MR analyses were performed using the R-based package “TwoSampleMR” (https://mrcieu.github.io/TwoSampleMR/) [20].

For our primary MR analysis, Inverse-Variance weighted (IVW) regression analyses were performed [16]. Estimates were calculated for each genetic instrument using the Wald ratio (SNP – outcome association divided by the SNP – exposure association) and subsequently meta-analyzed using the inverse-weighted meta-analyses weighted on the standard error of the SNP-outcome association (assuming no measurement error [NOME] in the exposure) [21]. The calculated estimates were expressed as odds ratios (OR) on ischemic stroke per SD (obtained from the exposure data) difference in mtDNA abundance.

To ensure that the results obtained from the IVW analyses were not biased due to directional pleiotropy, we performed MR-Egger regression analysis and Weighted-Median Estimator [21]. Although MR-Egger is considered as a relatively inefficient approach (e.g., large confidence intervals), this method does not force the regression line to go through the intercept. The intercept depicts the estimated average pleiotropic effect across the genetic variants, and a value that differs from zero indicates that the IVW estimate is biased. [22] The Weighted-Median estimator analysis can provide a consistent valid estimate if at least half of the instrumental variables are valid [23]. In addition, MR-PRESSO (MR Pleiotropy RESidual Sum and Outlier) was applied to detect and correct for horizontal pleiotropy through removing outlying causal estimates based on individual instruments [24], as implemented in the R-based package “MR-PRESSO” (https://github.com/rondolab/MR-PRESSO). The Cochran’s Q statistic was performed in order to test the heterogeneity between the estimated Wald ratios from different genetic variants [25]. Additionally, a Steiger directionality test was performed to ensure consistent causal direction-of-effect. A power calculation was performed with mRnd (https://shiny.cnsgenomics.com/mRnd/) [26]. With power = 0.80, minimal effect size (OR) was 1.076.

Recent research has proven that two-sample MR methods can safely be used for one-sample MR in large databases [27]. This allows us to use the UK Biobank database in our sample set despite also being used as our exposure dataset. As a limitation to this method, results of MR-Egger analyses are to be interpreted with caution when used to check for pleiotropy [27].

The main MR analyses were performed in the individual datasets, and subsequently meta-analyzed to derive the pooled estimates for the exposure on the risk of ischemic stroke using a fixed-effect model. Heterogeneity testing of the estimates across three datasets was performed by I^2, and corresponding p-value was obtained from the Cochran’s Q test. All meta-analyses were performed in the R-based “meta” package (https://cran.r-project.org/web/packages/meta/index.html).

Sensitivity analysis after stratification of genetic instruments

SNPs identified in relation to mtDNA-CN have been found in relation to platelet activation and megakaryocyte proliferation [18], which both could affect stroke risk and could potentially lead to biased results. We first examined the associations between the SNPs and platelet count in our study sample (adjusted for age, sex, and the first 10 genetic principal components); all SNPs with P<(0.05/123) in its association with platelet count were excluded from further MR sensitivity analyses.

RESULTS

Baseline characteristics of the study population

A total of 288,572 participants were included in the final study sample (see full procedure in **Supplementary Figure 1**) for multivariable-adjusted survival analyses. Participants excluded were due to unavailable genetic data (N = 14,251), not used to compute the genetic principal components (N = 81,623), or having unrealistic SD of autosomal probes (N = 9,440) were excluded according to standard UK Biobank quality control recommendations. Subsequently, we excluded related participants (N = 38,642), and with a non-white British ancestry (N = 65,498). Finally, 4,602 participants were excluded due to a history of stroke before study enrollment. Participants in the lower mtDNA abundance quintile (**Table 1**) had a mean age of 57.5 versus 56.1 year in the highest quintile, a mean BMI of 27.7 versus 27.0 kg/m^2, T2D prevalence of 2.8% versus 2.0%, and 11.3% were current smokers compared with 8.4% in the highest quintile.

Table 1. Baseline characteristics of the study participants stratified by quintiles of mtDNA abundance

	Q1	Q2	Q3	Q4	Q5
N	57 715	57 714	57 714	57 714	57 715
mtDNA abundance (normalized)	-1.4 (0.5)	-0.5 (0.2)	0.0 (0.1)	0.5 (0.2)	1.4 (0.5)
Age (years)	57.5 (8.0)	57.1 (8.0)	56.8 (8.0)	56.5 (8.0)	56.1 (8.0)
Sex (female %)	52.0	53.2	54.0	54.3	55.7
BMI (kg/m^2)	27.7 (5.0)	27.5 (4.8)	27.4 (4.7)	27.2 (4.6)	27.0 (4.5)
Diastolic blood pressure (mmHg)	82.6 (10.2)	82.4 (10.0)	82.3 (10.0)	82.1 (10.1)	81.7 (10.1)
Systolic blood pressure (mmHg)	139.5 (18.8)	138.6 (18.7)	138.2 (18.6)	137.6 (18.3)	136.7 (18.3)
White Blood Cell count (10^9 cells/L)	7.4 (1.8)	7.1 (1.7)	6.9 (1.7)	6.6 (1.7)	6.4 (2.7)
Platelet count (10^9 cells/L)	245.5 (58.0)	250.8 (57.7)	253.4 (58.4)	256.3 (59.2)	259.5 (63.8)
Blood pressure-lowering medication %					
Yes	19.8	18.4	17.5	16.6	15.6
No	80.2	81.6	(82.5	83.4	84.4
Cholesterol (mmol/L)	5.8 (1.2)	5.7 (1.1)	5.7 (1.1)	5.7 (1.1)	5.7 (1.1)
Cholesterol lowering medication %					
Yes	13.9	13.5	13.1	12.5	12.2
No	86.1	86.5	86.9	87.5	87.8
Alcohol consumption %					
Less than once per week	29.1	28.2	27.8	27.3	26.6
Once or twice per week	25.6	26.4	26.2	26.4	26.6
More than four times per week	45.3	45.2	45.9	46.3	46.7
Smoking %					
Never	53.3	54.1	55.0	55.6	56.5
Past	35.0	35.2	34.8	34.9	34.9
Current	11.3	10.4	9.9	9.4	8.4
Type 2 Diabetes %					
Yes	2.8	2.5	2.3	2.2	2.0
No	97.2	97.5	97.7	97.8	98.0

Data are mean (SD) for continuous variables or percentages for dichotomous variables. mtDNA abundance is presented as normalized in unit standard deviations. Abbreviations: BMI, Body Mass Index.

Multivariable-adjusted survival analyses mtDNA abundance and stroke

A total of 6,218 of the 288,572 participants (2.15%) had a stroke incidence, of which 3,994 (1.38%) were ischemic and 1,883 (0.65%) hemorrhagic over a median (IQR) follow-up of 11.8 (11.1 – 12.5) years. The incidence of ischemic stroke was higher in the lower mtDNA-CN quintiles than in the higher quintiles (**Figure 1A**), while hemorrhagic stroke incidence was similar in all mtDNA-CN quintiles (**Figure 1B**); in both cases the analyses fulfilled the proportional hazard assumption (*p-value:* 0.84 & 0.88).

After stratification based on mtDNA-CN (**Table 2**), in model 1, mtDNA abundance was associated with any stroke and ischemic stroke incidence, when comparing the first quintile with the highest 20% mtDNA abundance (any stroke: hazard ratio (HR), 1.11; 95% confidence interval (CI): 1.02 to 1.20; ischemic stroke: HR, 1.15; 95% CI: 1.04 to 1.27). Similarly, a one-SD increase in mtDNA abundance was associated with lower risk of incident ischemic stroke (HR, 0.96; 95% CI: 0.93 to 0.99). No association was found between mtDNA abundance and incident hemorrhagic stroke.

After correcting for other confounders, the associations with stroke and ischemic stroke attenuated (any stroke: HR, 1.06; 95% CI: 0.97 to 1.16; ischemic stroke: HR, 1.07; 95% CI: 0.95 to 1.19), as did the continuous model on ischemic stroke (HR, 0.98; 95% CI: 0.94 to 1.01).

Mendelian Randomization on mtDNA abundance and ischemic stroke

Main analyses

We did not observe evidence favoring an association between genetically-influenced lower mtDNA-CN and ischemic stroke (**Figure 2**). The odds ratios per 1 SD less mtDNA-CN were 1.07 (95%CI: 0.95, 1.20) in MEGASTROKE, 1.04 (95%CI: 0.79, 1.37) in the UK Biobank, and 0.99 (95%CI: 0.82, 1.20) in FinnGen. After meta-analysis, in a combined sample size of 1,098,740 (of which 73,296 cases), the pooled odds ratio was 1.04 (95%CI:

0.95 to 1.15) per 1-SD decrease in genetically-influenced mtDNA abundance.

The exact set of variants, their corresponding coefficients, standard errors, and p-values are presented in **Supplementary Table 1**. Variance explained (R^2) was 2.0% and calculated based on the derived summary statistics. The MR-Egger intercept indicated no pleiotropy ($p > 0.05$). Although several outliers were identified with MR-PRESSO in MEGASTROKE and FinnGen, results remained similar after removal of these outlying SNPs. The Steiger test of directionality showed a correct causal direction, indicating that there is no evidence for reverse causation, and no different results were observed with MR-sensitivity analyses MR-Egger and weighted median (**Supplementary Table 2.**).

Sub-analyses performed with separate outcomes cardioembolic, large artery atherosclerosis, small-vessel, and hemorrhagic stroke (**Supplementary Figure 2 & 3**) showed no evidence favoring a different result.

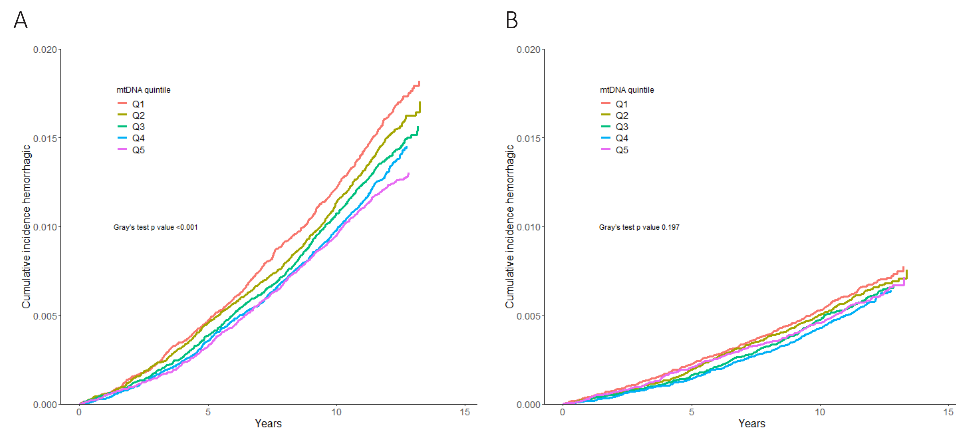

Figure 1. Cumulative incidence of ischemic (A) and hemorrhagic (B) stroke by quintiles of mtDNA abundance
We calculated the Cumulative incidence for ischemic and hemorrhagic stroke, accounting for death as a competing event. Differences in cumulative incidence between groups were assessed using Gray's test.

Additional sensitivity analyses

A total of 61 SNPS were associated with platelet count, which were subsequently excluded from additional sensitivity analyses. In the full sample, a 1-SD genetically-determined lower mtDNA abundance was associated with a higher risk of ischemic stroke (OR: 1.165; 95% CI: 1.026 to 1.323), although results from FinnGen did not align with those obtained in UK Biobank and MEGASTROKE (**Supplementary Figure 4**).

Table 2. The multivariable-adjusted association between mtDNA abundance and incident stroke in European-ancestry participants from UK Biobank.

		Continuous	Q1	Q2	Q3	Q4	Q5
Stroke incidence		**HR (95%CI)**	**HR (95%CI)**	**HR (95%CI)**	**HR (95%CI)**	**HR (95%CI)**	**HR (95%CI)**
Any	Model 1	0.97 (0.95, 1.00)	1.11 (1.02, 1.20)	1.07 (0.98, 1.16)	1.03 (0.95, 1.12)	1.03 (0.95, 1.12)	1.00 (ref)
	Model 2	0.99 (0.96, 1.02)	1.06 (0.97, 1.16)	1.05 (0.96, 1.15)	1.02 (0.94, 1.12)	1.01 (0.92, 1.10)	1.00 (ref)
Ischemic	Model 1	0.96 (0.93, 0.99)	1.15 (1.04, 1.27)	1.11 (1.00, 1.23)	1.07 (0.96, 1.18)	1.05 (0.95, 1.16)	1.00 (ref)
	Model 2	0.98 (0.94, 1.01)	1.07 (0.96, 1.19)	1.08 (0.97, 1.21)	1.03 (0.92, 1.15)	1.00 (0.89, 1.12)	1.00 (ref)
Hemorrhagic	Model 1	1.02 (0.97, 1.07)	0.97 (0.84, 1.13)	0.98 (0.84, 1.13)	0.93 (0.80, 1.09)	0.92 (0.79, 1.07)	1.00 (ref)
	Model 2	1.02 (0.97, 1.07)	0.98 (0.83, 1.15)	0.99 (0.84, 1.17)	0.97 (0.82, 1.14)	0.93 (0.79, 1.10)	1.00 (ref)

Estimated hazard ratios per-SD increase in mtDNA abundance (continuous), or for the 1st to the 4th quintile compared with the 5th (reference) quintile (categorical) on any, ischemic, and hemorrhagic stroke. Model 1 includes age, sex, batch, PCs, white blood cell count, platelet. Model 2 includes model 1, BMI, smoking, total cholesterol, hypertension, diabetes, cholesterol lowering medication, blood pressure lowering medication. Abbreviations: CI, confidence interval; HR, hazard ratio.

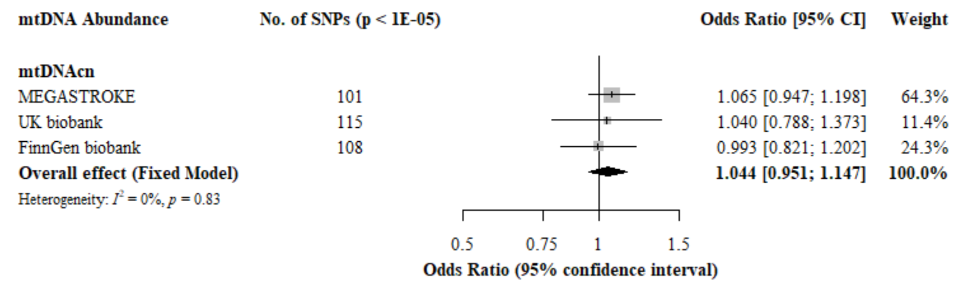

Figure 2. Causal association between mtDNA abundance and ischemic stroke occurrence.
Estimated ORs represent the effect per SD decrease in mtDNA copy number on ischemic stroke. Results were obtained using a Mendelian Randomization inverse-variance weighted method, analyzed per outcome database and combined over the three databases using fixed-effect meta-analyses.

DISCUSSION

In the UK Biobank cohort, consisting of 288,572 participants after exclusion, an initial association was found between mtDNA abundance and incident ischemic stroke, which attenuated after adjustment for confounders. Consistent with the prospective analyses, MR analyses, using a total sample size of 73,296 cases and 1,025,444 controls, showed no evidence for an association between genetically-predicted mtDNA abundance and ischemic stroke. However, some in-depth sensitivity analyses in which SNPs associated with platelet count were excluded, did provide some preliminary evident for low mtDNA-CN as possible causal driver for ischemic stroke.

Although of specific interest, caution of these results is warranted given that the results were mainly driven by results derived from MEGASTROKE, and to a lesser extent by UK Biobank. Furthermore, results from these additional MR sensitivity analyses deviated significantly from those observed in the prospective multivariable-adjusted analyses, and therefore do not meet the requirements for triangulation [15]. Collectively, our results indicate that there is only weak evidence for a causal association between mtDNA abundance and ischemic stroke, and more studies are required to elucidate the nature of the pleiotropy identified in our study, which goes beyond the current scope.

Previously, an association between low mtDNA-CN and increased risk of incident stroke in 20,162 participants who were followed over a 13.5-year period during which 1584 stroke events occurred [28], and therefore deviate from our study done in a larger sample of 288,752 participants with 6,218 stroke cases. Difference in baseline health characteristics are possible reasons explaining the observed differences in results.

Recent studies showed that mtDNA-CN could be a marker of stroke prognosis after hospitalization [29, 30]. By analyzing mtDNA-CN, and consequently oxidative stress, our findings did provide some, albeit circumstantial, evidence for a relationship between oxidative stress and stroke occurrence, although this association attenuated after adjustment for confounders. In the Mendelian Randomization analysis, after excluding SNPs associated with platelet count, we also found an association between genetically determined mtDNA abundance and ischemic stroke risk. In contrast to ischemic stroke, we did not find an association between mtDNA abundance and hemorrhagic stroke in the univariate or MR analyses. This difference might be explained because hemorrhagic stroke, in contrast to ischemic stroke, is also often caused by non-classic cardiovascular mechanisms such as vascular amyloid deposition in cerebral amyloid angiopathy [31].

Our data on mtDNA abundance was obtained from leukocytes. Although some of the leukocytes may be directly involved in the pathology of stroke, additional cell types such as endothelial and smooth muscle cells, that we did not query for mitochondrial abundance, are clearly more directly involved. This could potentially explain our overall null findings. Studies on the differences in mitochondrial function within an individual between cell groups are largely non-existent. However, mtDNA-CN measured in blood has been associated with gene expression in other tissues, which suggests mtDNA-CN derived from leukocytes can reflect metabolic health across multiple tissues [32]. Thus, the evidence so far indicates that mitochondrial dysfunction, as measured with leukocyte mtDNA-CN, is systemic. Of interest, using similar methodology as in our study, low genetically-influenced mtDNA has recently been associated with increased dementia risk [12]. This would further indicate that lower mtDNA-CN, although measured in leukocytes, can reflect processes of a systemic increase in disease risk.

A key strength of this study is the statistical power of the analyses of the association between stroke and mitochondrial abundance (288,572 participants for the multivariable survival analysis and 1,098,740 for the MR, respectively). Additionally, we adopted the triangulation of causal inference [15]. By using two different approaches in observational research to study the association between low mtDNA abundance and (ischemic) stroke risk, we increased the credibility of our results. Although results from both our used approaches were not exactly similar, they were directionally consistent.

Some limitations are to be considered. First, mtDNA abundance was determined from intensities of genotyping probes on the mitochondrial DNA, whereas the assessment with whole-exome sequencing is generally considered to result in more reliable mtDNA abundance estimates [33, 34]. Although Hägg et al showed a moderate correlation between mtDNA based on SNP array intensities and exome sequencing of 0.33 [11], analyses still indicated the measurements of SNP array intensities reflect underlying biology of mtDNA abundance. For this reason, the increased variance is most likely the result of nondifferential measurement error, and therefore considered to be mainly cause a reduction in statistical power. As a main consequence, the true associations, particularly those from the multivariable-adjusted prospective analyses are most likely larger than as observed. Second, our study population consists of predominantly Caucasian participants, limiting the generalizability of the results to other ancestry groups. Third, Mendelian Randomization functions on several assumptions. However,

by using several sensitivity analyses such as MR-Egger and MR-PRESSO, we can establish with some conviction that these are fulfilled. To add, although in a one-sample MR (as conducted in the UK Biobank) the assumption of independence does not hold up, previous studies have shown that two-sample MR methods can be used reliably with large enough biobanks [27]. Last, despite a large sample size in the multivariable adjusted analysis, stroke and especially hemorrhagic stroke incidences were relatively few. However, as an association was found before correction, we think our analyses had enough power to detect a difference between groups. In addition, we used one of the larger data sets available.

In conclusion, despite a large sample size our prospective study did not find evidence for an association between mtDNA abundance and ischemic or hemorrhagic stroke. After exclusion of pleiotropic SNPs associated with platelet count, we found some preliminary evidence for an association between genetically determined lower mtDNA-CN and ischemic stroke risk using MR. However, further studies are required for validation and to examine the nature of this type of pleiotropy.

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LGM, JL, RN and DvH designed research; LGM and JL conducted research; LGM and JL performed statistical analysis; LGM, JL, RN, and MJHW wrote paper; LGM had primary responsibility for final content. DvH, KWvD, SH, FG and MJHW contributed to the data interpretation and commented on initial versions of the manuscript; All authors read and approved the final manuscript.

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Disclosures

None

Conflicts of interest statement

The authors declare to have no conflict of interest.

Supplemental Material

Table S1-2

Figure S1-4

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4