Stroke

CLINICAL AND POPULATION SCIENCES

Subtype-Specific Association of Mitochondrial DNA Copy Number With Poststroke/TIA Outcomes in 10 241 Patients in China

Yi Jiang, PhD*; Si Cheng, MD*; Yanfeng Shi, PhD; Zhe Xu, PhD; Huihui Wang, MS; Yanran Li, MD; Yang Liu, PhD; Zixiao Li, MD; Yong Jiang, PhD; Xia Meng, MD; Shanshan Cheng, PhD; Hao Li, MD; Chaolong Wang, PhD; Yongjun Wang, MD

BACKGROUND: Mitochondrial DNA copy number (mtDNA-CN) is associated with the severity and mortality in patients with stroke, but the associations in different stroke subtypes remain unexplored.

METHODS: We conducted an observational prospective cohort analysis on patients with ischemic stroke or transient ischemic attack enrolled in the Third China National Stroke Registry. We applied logistic models to assess the association of mtDNA-CN with functional outcome (modified Rankin Scale score, 3–6 versus 0–2) and Cox proportional hazard models to assess the association with stroke recurrence (treating mortality as a competing risk) and mortality during a 12-month follow-up, adjusting for sex, age, physical activity, National Institutes of Health Stroke Scale at admission, history of stroke and peripheral artery disease, small artery occlusion, and interleukin-6. Subgroup analyses stratified by age and stroke subtypes were conducted.

RESULTS: The Third China National Stroke Registry enrolled 15 166 patients, of which 10 241 with whole-genome sequencing data were retained (mean age, 62.2 [SD, 11.2] years; 68.8% men). The associations between mtDNA-CN and poststroke/transient ischemic attack outcomes were specific to patients aged ≤65 years, with lower mtDNA-CN significantly associated with stroke recurrence in 12 months (subdistribution hazard ratio, 1.15 per SD lower mtDNA-CN [95% CI, 1.04–1.27]; P=5.2×10^{-3}) and higher all-cause mortality in 3 months (hazard ratio, 2.19 [95% CI, 1.41–3.39]; P=5.0×10^{-4}). Across subtypes, the associations of mtDNA-CN with stroke recurrence were specific to stroke of undetermined cause (subdistribution hazard ratio, 1.28 [95% CI, 1.11–1.48]; P=6.6×10^{-4}). In particular, lower mtDNA-CN was associated with poorer functional outcomes in stroke of undetermined cause patients diagnosed with embolic stroke of undetermined source (odds ratio, 1.53 [95% CI, 1.20–1.94]; P=5.4×10^{-4}), which remained significant after excluding patients with recurrent stroke (odds ratio, 1.49 [95% CI, 1.14–1.94]; P=3.0×10^{-3}).

CONCLUSIONS: Lower mtDNA-CN is associated with higher stroke recurrence rate and all-cause mortality, as well as poorer functional outcome at follow-up, among stroke of undetermined cause, embolic stroke of undetermined source, and younger patients.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: DNA, mitochondrial ■ follow-up studies ■ ischemic attack, transient ■ ischemic stroke ■ prognosis

Correspondence to: Chaolong Wang, PhD, Department of Epidemiology and Biostatistics, Ministry of Education Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China, Email chaolong@hust.edu.cn; or Yongjun Wang, MD, Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing 100070, China, Email yongjunwang@ncrcnd.org.cn
*Y. Jiang and S. Cheng contributed equally.
Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.123.045069.
For Sources of Funding and Disclosures, see page 1269–1270.

Stroke is available at www.ahajournals.org/journal/str

Nonstandard Abbreviations and Acronyms

CCS	causative classification system
CE	cardioaortic embolism
CNSR-III	Third China National Stroke Registry
CVD	cardiovascular disease
ESUS	embolic stroke of undetermined source
HR	hazard ratio
IL-6	interleukin 6
IS	ischemic stroke
mRS	modified Rankin Scale
mtDNA-CN	mitochondrial DNA copy number
OR	odds ratio
SAO	small artery occlusion
sHR	subdistribution hazard ratio
SUC	stroke of undetermined cause
TIA	transient ischemic attack

Stroke is a major cause of death worldwide, with an alarming average of 80.5 deaths per 100 000 persons.[1] Despite advances in stroke treatment and management, a significant proportion of patients continues to experience poor outcomes, such as recurrent stroke, increased mortality, and long-term functional disabilities.[2] According to a global meta-analysis, about 11% of patients with stroke are at risk of recurrence within a year,[3] which can result in more severe functional disability and higher mortality rates.[4,5] Thus, numerous studies have explored the impact of different genetic, epigenetic, and molecular factors on poststroke outcomes.[6–10] Among these factors, recent research has highlighted mitochondrial DNA copy number (mtDNA-CN) as a promising predictor of poststroke outcome.[9,10]

mtDNA-CN measures the number of mitochondrial genomes per cell and is linked to the activity of mitochondrial respiratory enzymes and the production of ATP.[11] As such, it serves as a biomarker of mitochondrial function. Recent research has shown that mtDNA-CN is significantly associated with both stroke prevalence and incidence, particularly in younger people.[12–16] Moreover, lower mtDNA-CN levels are strongly associated with worse stroke outcomes, such as increased disability and mortality.[9,10] However, it is currently unclear whether the relationship with poor outcomes is driven by stroke recurrence. Additionally, there is a lack of research examining the association between mtDNA-CN and stroke recurrence. Further studies are needed to fully understand the potential of mtDNA-CN as a prognostic indicator for stroke.

Stroke is a heterogeneous disease that can be classified into different subtypes based on distinct causes. The identification of subtype-specific associations is crucial for the development of personalized treatments and prognostic tools. mtDNA-CN is a potential prognostic factor in patients with stroke,[9,10] but its association with poststroke outcomes in different subtypes has not been well explored. In particular, the stroke of undetermined cause (SUC) subtype represents a complex and challenging diagnostic category, and its association with mtDNA-CN has not been fully elucidated. It is crucial to investigate the potential association between mtDNA-CN and poststroke outcome in different subtypes, with a specific focus on the SUC subtype.

In this study, we aimed to investigate the relationship between mtDNA-CN and poststroke/transient ischemic attack (TIA) outcomes over a 12-month follow-up period. The study enrolled 10 241 Chinese individuals of different subtypes based on the Third China National Stroke Registry (CNSR-III).[17] We examined the association between mtDNA-CN and poststroke/TIA outcomes during follow-up, including stroke recurrence, functional outcome, and all-cause mortality. Furthermore, we conducted subgroup analyses stratified by age and stroke subtype to explore potential differences in the relationship between mtDNA-CN and poststroke/TIA outcomes among different patient groups.

METHODS

Data Availability

The access to whole-genome sequencing data was managed by the CNSR-III Data Access Committee. Any additional information supporting the findings in this article is available from the corresponding author upon reasonable request.

Participants

The CNSR-III is a nationwide prospective registry in China. A total of 15 166 patients from 201 hospitals diagnosed with ischemic stroke (IS) or TIA between August 2015 and March 2018 were recruited.[17] We monitored poststroke/TIA outcomes, including stroke recurrence, modified Rankin Scale (mRS), and mortality, for all samples throughout a 12-month follow-up period. The median time from event onset to enrollment was 2 days (interquartile range, 1–4).[17] Blood samples were collected immediately after enrollment. White blood cells of 10 914 patients were subjected to whole-genome sequencing using the BGISEQ-500 platform,[18] from which 10 241 samples passed quality control were used in this study.[19] There were no differences in risk factors between the 10 241 samples and 15 166 samples (Table S1). Among these 10 241 patients, 9553 were diagnosed with IS, and 688 with TIA. This article follows the Strengthening the Reporting of Observational Studies in Epidemiology reporting guideline.

Definition of Poststroke/TIA Outcomes and Clinical Variables

Poststroke/TIA outcomes including stroke recurrence, functional outcome, and all-cause mortality at 3- and 12-month follow-ups were included in this study. Patients were interviewed face-to-face at 3 months and contacted over the telephone by

 Stroke. 2024;55:1261–1270. DOI: 10.1161/STROKEAHA.123.045069

trained research coordinators at 12 months. Stroke recurrence is defined as the confirmation of a new IS or hemorrhagic stroke (including intracerebral and subarachnoid hemorrhage) through Computed Tomography or Magnetic Resonance Imaging. Functional outcome is defined as mRS of 3 to 6 (coded as 1) versus 0 to 2 (coded as 0), where mRS is an ordinal variable ranging from 0 (no symptoms) to 6 (death). All-cause mortality is a combination of cardiovascular disease (CVD, including IS, hemorrhagic stroke, sudden cardiac death, acute myocardial infarction, heart failure, etc) and non-CVD (including cancer, pneumonia, accidental death, unknown causes, etc) mortality.

Smoking is a binary variable for current smokers versus nonsmokers. Heavy drinking means more than 20 g of alcohol per day. Physical activity is a binary variable defined as engaging in agricultural work, employment, household chores, physically demanding activities related to transportation, recreational exercises, or sports, among others.

The causative classification system (CCS) was used for etiological subtyping of patients by trained and certified investigators based on the standardized system.[20] Embolic stroke of undetermined source (ESUS) was diagnosed following the criteria proposed by the Cryptogenic Stroke/ESUS International Working Group.[21]

Quantification of mtDNA-CN

mtDNA-CN was quantified by using the fastMitoCalc program,[22,23] which removed reads with polymerase chain reaction duplicates, mapping quality <20, or secondary alignment. It defined mtDNA-CN as the ratio of mtDNA coverage relative to autosomal coverage, where autosomal coverage was calculated based on randomly selected 3000 sequences (1000 bp for each). As illustrated by Ding et al,[22] the removal of reads with a mapping quality <20 effectively eliminated the majority of nuclear mitochondrial DNA segments, with the remaining segments having minimal impact on the estimation of mtDNA-CN. Given that mtDNA-CN is diverging in different cell types, especially in platelets and neutrophils[24] (Figure S1), we regressed mtDNA-CN on the proportions of platelets and neutrophils and used the residuals plus the mean mtDNA-CN across all samples for subsequent analyses.

Statistical Analysis

Clinical variables with missing rates >20% were discarded, except for fasting plasma glucose, glycated albumin, hemoglobin A1c, and insulin that were only shown in Tables S2 and S3. For the remaining variables, missing values were filled with medians for continuous variables. No missing values were found for any categorical variables. Continuous variables were scaled to have a mean of 0 and an SD of 1. For outcome variables, there were no missing values for stroke recurrence or mortality during the 12-month follow-up. When analyzing functional outcome as the outcome, samples with missing mRS data were removed, which accounted for 114 and 249 samples for 3- and 12-month mRS, respectively.

We classified the 10 241 samples into 4 quartiles according to mtDNA-CN adjusted for platelet and neutrophil proportions. Clinical variables at baseline were tested for associations with mtDNA-CN using linear regression models, with the clinical variables being independent variables and mtDNA-CN being the dependent variable. Fourteen variables with $P<1.5\times10^{-3}$ (Bonferroni correction on 34 variables) were included in a multivariable linear regression model (Table S2). Seven variables with $P<3.6\times10^{-3}$ in the multivariable model (Bonferroni correction on the 14 variables) were selected as covariates in the association analyses between mtDNA-CN and poststroke/TIA outcomes. These variables include sex, age, physical activity, National Institutes of Health Stroke Scale score of 4 to 15 at admission, peripheral artery disease history, diagnosis of small artery occlusion (SAO), and IL-6 (interleukin-6) level.

Cox proportional hazard models were used to estimate the hazard ratios (HRs) and 95% CIs for the associations between mtDNA-CN and poststroke/TIA outcome (stroke recurrence or mortality). We treated mtDNA-CN as an independent variable and the survival time before stroke recurrence or death as the dependent variable. For each analysis, we applied 2 models, with the base model (model 1) adjusting for sex and age and the full model (model 2) additionally adjusting for physical activity, National Institutes of Health Stroke Scale of 4 to 15 at admission, peripheral artery disease history, diagnosis of SAO, IL-6 level, and stroke history. The coxph function from the survival R package[25] was used for all-cause mortality. The crr function from the cmprsk R package[26] was used for stroke recurrence, incorporating death as a competing event. Likewise, we used this function when analyzing CVD or non-CVD mortality as outcomes, accounting for non-CVD or CVD mortality as competing events.

Logistic regression models were used to estimate the odds ratios (ORs) and 95% CIs for the association between mtDNA-CN and functional outcome at follow-up. We treated mtDNA-CN as an independent variable and functional outcome as the dependent variable, adjusting for the same covariates as in the Cox models.

We conducted subgroup analyses based on stratification by age and stroke subtypes. We used CCS to group patients with stroke into 5 subtypes: large artery atherosclerosis, cardioaortic embolism (CE), SAO, stroke of other determined causes, and SUC.[27] We further stratified the patients with SUC according to the diagnosis of ESUS. To be consistent with previous studies,[9,12] we used the age of 65 years as the threshold to stratify younger and older patients.

The Cochran Q test was used for testing the heterogeneity of effect sizes. Forest plots were produced using the forestplot R package.[28] Given the exploratory nature of our study, we considered $P<0.05$ as statistically significant without strict adjustment for multiple testing.[29]

We wrapped the restricted cubic spline function in a Cox model to test the nonlinear association of mtDNA-CN with stroke recurrence. Similarly, we wrapped the restricted cubic spline function in a logistic model to test the nonlinear association of mtDNA-CN with functional outcome. The R package rms was used.[30] We set 3 knots at the 10th, 50th, and 90th percentiles of mtDNA-CN and the maximum as the reference. The significance of nonlinearity was inspected by testing whether the coefficients associated with the nonlinear component were equal to 0.

Standard Protocol Approvals and Patient Consent

The study was approved by the ethics committee of Beijing Tiantan Hospital (institutional review board approval number: KY2015-001–01) and all participating centers. All CNSR-III

participants or their legal representatives provided written informed consent.

RESULTS

Baseline Characteristics of the 10 241 Patients

Among the 10 241 patients (mean age, 62.2 [SD, 11.2] years), 7044 (68.8%) were men. mtDNA-CN was calculated based on whole-genome sequencing data, which had a mean depth of 41.8× on autosomes and 3732.5× on mitochondrial genome. To account for the influence of blood cell composition, we adjusted mtDNA-CN for platelet and neutrophil proportions and used these adjusted values for subsequent analyses. After adjustment, mtDNA-CN had a mean of 178.7 [SD, 61.4] (Figure S2). Consistent with a previous study,[31] mtDNA-CN increased slightly with age before 60 and started to decrease afterward (ANOVA; P=1.0×10^{-8}). Women had higher mtDNA-CN than men (P=5.6×10^{-15}). Across stroke subtypes, patients with the CE subtype had the lowest mtDNA-CN, while those with SAO had the highest (P=2.6×10^{-24}). There was no difference in mtDNA-CN between patients with ESUS and patients without ESUS (P=0.24). It is worth noting that physical activity was positively associated with mtDNA-CN (P=3.8×10^{-12}), and the association remained significant after correcting for other covariates (P=1.6×10^{-3}; Table S2).

Twenty clinical characteristics showed significant differences among CCS subtypes of stroke (P<1.5×10^{-3}; Bonferroni correction for 33 variables; Table S3). Patients with CE were the oldest (mean age, 67.94 years), mostly had a disease history of atrial fibrillation (61.1%), and had the highest inflammation levels as indicated by several inflammation factors. Patients with SUC, on the other hand, had the highest proportion of being diagnosed with ESUS (30.9%). Notably, patients with SUC and SAO tended to have lower clinical severity at admission, as reflected by the National Institutes of Health Stroke Scale, than patients with large artery atherosclerosis and CE (ANOVA; P=1.7×10^{-54}).

Lower mtDNA-CN Was Associated With a Higher Stroke Recurrence Rate

During the 12-month follow-up, 927 (9.1%) recurrent ISs and 92 (0.9%) recurrent hemorrhagic strokes were reported. The stroke recurrence rate increased as mtDNA-CN decreased (Figure 1A). After adjusting for age and sex, lower mtDNA-CN was significantly associated with an increased hazard of stroke recurrence in 12 months (subdistribution HR [sHR], 1.12 [95% CI, 1.04–1.20]; P=3.7×10^{-3}; Figure 2A, model 1). The inclusion of additional CVD risk factors led to a slight reduction in sHR (sHR, 1.11 [95% CI, 1.03–1.20]; P=4.9×10^{-3};

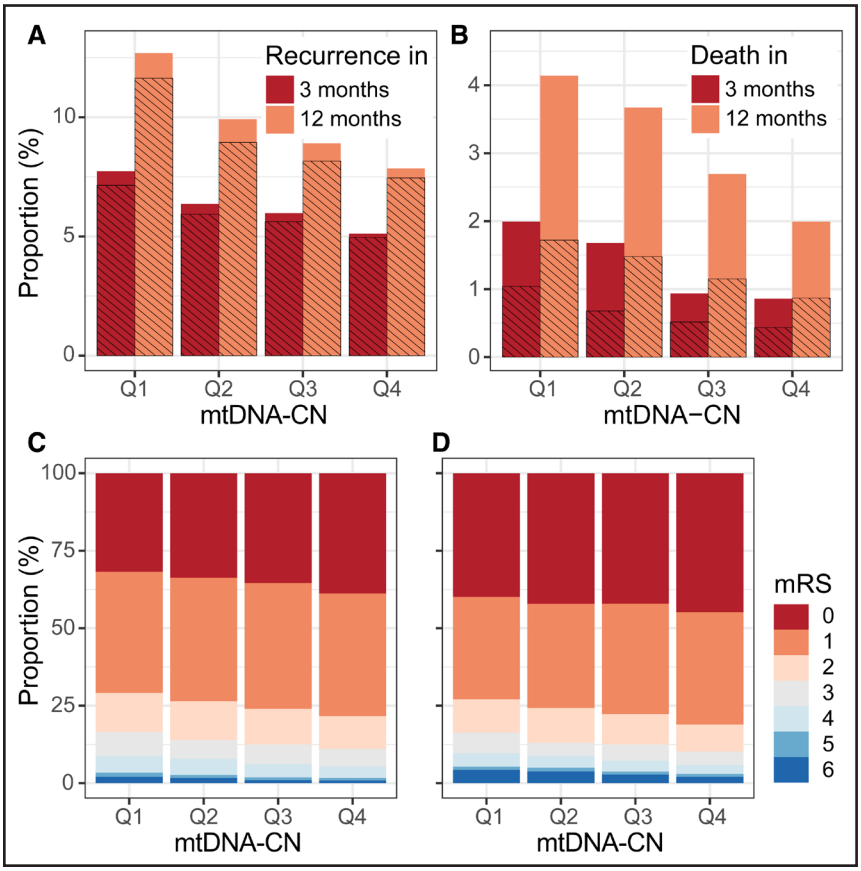
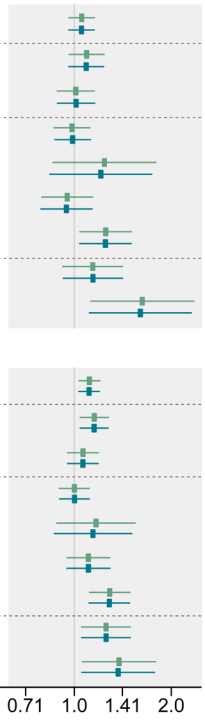


Figure 1. Overall relationship between mitochondrial DNA copy number (mtDNA-CN) and poststroke/transient ischemic attack outcomes. **A**, Proportion of patients with the recurrence of stroke in 3 and 12 mo at different mtDNA-CN quartiles. The shaded areas indicate ischemic stroke recurrence, while the unshaded areas indicate hemorrhagic stroke recurrence. **B**, Proportion of all-cause mortality events in 3 and 12 mo. The shaded areas indicate cardiovascular disease (CVD) mortality, while the unshaded areas indicate non-CVD mortality. **C** and **D**, Distribution of modified Rankin Scale (mRS) at (**C**) 3 and (**D**) 12 mo among patients in different mtDNA-CN quartiles.

A

Group	Sample size	No. of events	Model 1 sHR (95% CI)	Model 1 P	Model 2 sHR (95% CI)	Model 2 P
Stroke recurrence in 3 months:						
All patients	10,241	645	1.05 (0.96–1.15)	0.250	1.05 (0.96–1.15)	0.270
Age ≤ 65	6278	353	1.09 (0.97–1.24)	0.160	1.09 (0.96–1.23)	0.180
Age > 65	3963	292	1.01 (0.89–1.15)	0.880	1.01 (0.89–1.16)	0.860
LAA	2872	245	0.98 (0.87–1.12)	0.790	0.99 (0.87–1.12)	0.830
CE	704	59	1.24 (0.86–1.79)	0.250	1.21 (0.84–1.73)	0.310
SAO	2598	123	0.95 (0.79–1.14)	0.580	0.94 (0.79–1.13)	0.540
SUC	3981	212	1.25 (1.04–1.50)	0.017	1.25 (1.04–1.50)	0.018
Non-ESUS SUC	2572	148	1.14 (0.92–1.41)	0.230	1.14 (0.92–1.41)	0.220
ESUS SUC	1229	64	1.62 (1.12–2.34)	9.6×10^{-3}	1.60 (1.11–2.30)	0.011
Stroke recurrence in 12 months:						
All patients	10,241	1008	1.12 (1.04–1.20)	3.7×10^{-3}	1.11 (1.03–1.20)	4.9×10^{-3}
Age ≤ 65	6278	568	1.16 (1.05–1.28)	4.7×10^{-3}	1.15 (1.04–1.27)	5.2×10^{-3}
Age > 65	3963	440	1.07 (0.96–1.19)	0.250	1.06 (0.95–1.19)	0.270
LAA	2872	362	1.00 (0.90–1.11)	0.970	1.00 (0.90–1.11)	0.980
CE	704	91	1.17 (0.88–1.54)	0.270	1.14 (0.86–1.50)	0.340
SAO	2598	202	1.11 (0.95–1.29)	0.190	1.11 (0.95–1.29)	0.200
SUC	3981	346	1.29 (1.12–1.49)	**5.4×10^{-4}**	1.28 (1.11–1.48)	**6.6×10^{-4}**
Non-ESUS SUC	2572	240	1.26 (1.06–1.49)	0.010	1.25 (1.05–1.49)	0.011
ESUS SUC	1229	106	1.38 (1.06–1.79)	0.016	1.37 (1.06–1.77)	0.018



B

Outcome	Sample size	No. of events	Model 1 sHR (95% CI)	Model 1 P	Model 2 sHR (95% CI)	Model 2 P
All patients						
IS recurrence	10,241	927	1.09 (1.01–1.18)	0.020	1.09 (1.01–1.18)	0.026
HS recurrence	10,241	92	1.33 (1.03–1.72)	0.029	1.33 (1.03–1.72)	0.029
Patients aged ≤ 65						
IS recurrence	6278	515	1.12 (1.01–1.24)	0.035	1.11 (1.01–1.24)	0.040
HS recurrence	6278	60	1.59 (1.17–2.15)	2.8×10^{-3}	1.58 (1.17–2.13)	2.5×10^{-3}
SUC patients						
IS recurrence	3981	324	1.24 (1.07–1.43)	3.8×10^{-3}	1.23 (1.07–1.42)	4.6×10^{-3}
HS recurrence	3981	27	2.23 (1.25–3.98)	6.7×10^{-3}	2.27 (1.26–4.08)	6.2×10^{-3}

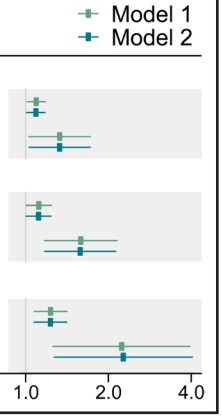


Figure 2. Association between mitochondrial DNA copy number (mtDNA-CN) and stroke recurrence during follow-up. **A**, Association between mtDNA-CN and stroke recurrence in 3 and 12 mo. All patients were stratified by age, causative classification system subtypes (the stroke of other determined causes subtype was not shown because of the small sample size), and embolic stroke of undetermined source (ESUS) diagnosis. **B**, Association of mtDNA-CN with ischemic stroke (IS) and hemorrhagic stroke (HS) recurrence in 12 mo. Stroke recurrence was divided into IS and HS recurrence for the top 3 significant associations in (**A**). Cox models were used to estimate the subdistribution hazard ratio (sHR) of stroke recurrence per SD decrease of mtDNA-CN. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for cardiovascular disease-risk factors (methods). We highlighted *P* values achieving nominal significance ($P<0.05$) in red and those passing the Bonferroni-corrected threshold ($P<9.6\times10^{-4}$ based on 52 tests) in bold. CE indicates cardioaortic embolism; LAA, large artery atherosclerosis; SAO, small artery occlusion; and SUC, stroke of undetermined cause.

model 2). In contrast, no significance was observed when using stroke recurrence in 3 months as the outcome (sHR, 1.05 [95% CI, 0.96–1.15]; $P=0.27$) probably due to a smaller number of recurrence events.

The association between mtDNA-CN and stroke recurrence in 12 months was much more significant in the patients aged ≤65 years (sHR, 1.15 [95% CI, 1.04–1.27]; $P=5.2\times10^{-3}$) than in those aged >65 years (sHR, 1.06 [95% CI, 0.95–1.19]; $P=0.27$; Figure 2A, model 2). Stratifying by CCS subtypes, we found that large artery atherosclerosis and CE had much higher stroke recurrence rates than SAO and SUC (Figure S3A). Nevertheless, the association between mtDNA-CN and stroke recurrence in 12 months was exclusively significant in patients

with SUC (sHR, 1.28 [95% CI, 1.11–1.48]; P=6.6×10^{-4}). When further stratifying the patients with SUC according to the ESUS diagnosis, we found that the patients with ESUS (sHR, 1.37 [95% CI, 1.06–1.77]; P=0.018) had a slightly higher sHR than the patients without ESUS (sHR, 1.25 [95% CI, 1.05–1.49]; P=0.011). While most recurrent events were IS, we found mtDNA-CN showed a stronger association with recurrent hemorrhagic stroke, especially in younger patients (Cochran Q heterogeneity; P=0.030) and patients with SUC (Cochran Q heterogeneity; P=0.047; Figure 2B, model 2).

Lower mtDNA-CN Was Associated With Poorer Functional Outcome

We observed that the fraction of patients with higher mRS at 3 months (ie, poorer functional outcome) increased as mtDNA-CN decreased (Figure 1C), despite that this trend was insignificant (OR, 1.04 per SD decrease in mtDNA-CN [95% CI, 0.97–1.10]; P=0.285; Figure 3, model 2). Comparing across CCS subtypes, large artery atherosclerosis and CE had higher 3-month mRS than SAO and SUC (Figure S3B). Interestingly, we found that mtDNA-CN was associated with functional outcome in SAO (OR, 0.87 [95% CI, 0.77–0.99]; P=0.038) and patients with SUC (OR, 1.16 [95% CI, 1.03–1.31]; P=0.017), especially in patients with SUC and ESUS diagnosis (OR, 1.53 [95% CI, 1.20–1.94]; P=5.4×10^{-4}) and in patients with ESUS SUC aged ≤65 years (OR, 1.63 [95% CI, 1.15–2.32]; P=6.2×10^{-3}; Figure 3, model 2). Both the base model and full model produced similar results, with the ORs from the full model being a little attenuated.

Furthermore, we assessed whether the association between mtDNA-CN and functional outcome at

Group	Sample size	Model 1 OR (95% CI)	Model 1 P	Model 2 OR (95% CI)	Model 2 P
Functional outcome at 3-month:					
All patients	10,127	1.04 (0.98–1.11)	0.220	1.04 (0.97–1.10)	0.285
Age ≤ 65	6204	1.09 (0.99–1.19)	0.081	1.09 (0.99–1.19)	0.083
Age > 65	3923	1.00 (0.91–1.09)	0.946	0.99 (0.90–1.08)	0.810
LAA	2826	1.06 (0.96–1.18)	0.256	1.06 (0.96–1.18)	0.274
CE	696	1.01 (0.80–1.28)	0.921	1.00 (0.79–1.26)	0.967
SAO	2579	0.88 (0.77–1.00)	0.045	0.87 (0.77–0.99)	0.038
SUC	3941	1.17 (1.03–1.32)	0.014	1.16 (1.03–1.31)	0.017
Non-ESUS SUC	2720	1.04 (0.90–1.19)	0.631	1.03 (0.90–1.19)	0.648
ESUS SUC	1221	1.55 (1.22–1.96)	**3.4×10^{-4}**	1.53 (1.20–1.94)	**5.4×10^{-4}**
ESUS SUC aged ≤ 65	790	1.65 (1.16–2.34)	5.2×10^{-3}	1.63 (1.15–2.32)	6.2×10^{-3}
ESUS SUC aged > 65	431	1.49 (1.07–2.07)	0.017	1.46 (1.05–2.04)	0.024
Functional outcome at 12-month:					
All patients	9992	1.04 (0.97–1.11)	0.282	1.03 (0.96–1.10)	0.427
Age ≤ 65	6126	1.07 (0.97–1.18)	0.189	1.06 (0.96–1.18)	0.226
Age > 65	3866	1.01 (0.92–1.10)	0.844	1.00 (0.91–1.09)	0.971
LAA	2776	1.07 (0.96–1.19)	0.236	1.06 (0.95–1.18)	0.284
CE	689	0.95 (0.76–1.19)	0.663	0.93 (0.74–1.17)	0.538
SAO	2546	0.97 (0.84–1.11)	0.625	0.96 (0.83–1.10)	0.532
SUC	3896	1.05 (0.94–1.18)	0.395	1.05 (0.93–1.18)	0.464
Non-ESUS SUC	2690	1.02 (0.89–1.18)	0.772	1.02 (0.88–1.17)	0.807
ESUS SUC	1206	1.13 (0.91–1.39)	0.276	1.11 (0.90–1.37)	0.341
ESUS SUC aged ≤ 65	783	1.06 (0.78–1.45)	0.708	1.04 (0.77–1.42)	0.799
ESUS SUC aged > 65	423	1.19 (0.89–1.60)	0.247	1.19 (0.88–1.60)	0.263

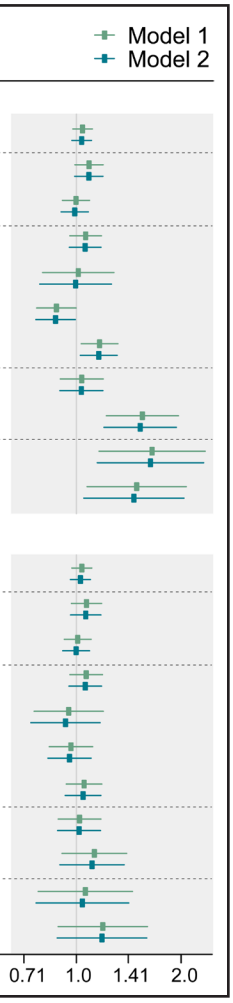


Figure 3. Association between mitochondrial DNA copy number (mtDNA-CN) and functional outcome during follow-up. All patients were stratified by age, causative classification system subtypes (the stroke of other determined causes subtype was not shown because of the small sample size), and embolic stroke of undetermined source (ESUS) diagnosis. Logistic models were used to calculate the odds ratio (OR) of poorer functional outcomes per SD decrease of mtDNA-CN. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for cardiovascular disease-risk factors (methods). We highlighted P values achieving nominal significance (P<0.05) in red and those passing the Bonferroni-corrected threshold (P<9.6×10^{-4} based on 52 tests) in bold. CE indicates cardioaortic embolism; LAA, large artery atherosclerosis; SAO, small artery occlusion; and SUC, stroke of undetermined cause.

3 months was influenced by stroke recurrence. There were 645 patients who experienced recurrent stroke in 3 months, and 188 (29%) of them had higher mRS at 3 months than their baseline. After removing patients with recurrent stroke, we found that the association between mtDNA-CN and functional outcome at 3 months remained significant in patients with ESUS SUC (OR, 1.49 [95% CI, 1.14–1.94]; P=3.0×10^{-3}; Figure S4, model 2).

In contrast to functional outcome at 3 months, we observed no significant association between mtDNA-CN and functional outcome at 12 months (Figure 3) although there was a trend indicating that lower mtDNA-CN tended to associate with higher mRS (Figure 1D).

Lower mtDNA-CN Was Associated With Higher Mortality

During the 12-month follow-up period, 320 (3.1%) cases of all-cause mortality were reported, including 131 (1.3%) cases of CVD mortality. The all-cause mortality of patients with stroke in both the 3- and 12-month follow-up periods was inversely associated with mtDNA-CN (Figure 1B). This trend remained consistent when all-cause mortality was split into CVD and non-CVD mortalities. After adjusting for covariates, the association was significant only in younger patients (Figure 4). Specifically, among patients aged ≤65 years, 1-SD lower mtDNA-CN was associated with a 119% increase (HR, 2.19 [95% CI, 1.41–3.39]; P=5.0×10^{-4}) and 30% increase (HR, 1.30 [95% CI, 1.04–1.64]; P=0.024) in the hazard of all-cause mortality in 3 and 12 months, respectively (model 2). The incorporation of extra CVD risk factors into the full model resulted in a slight decrease in HRs. The effect size was much higher for mortality in a 3-month follow-up compared with that in a 12-month follow-up (Cochran Q heterogeneity; P=0.042). When using CVD mortality as the outcome, the associations were nominally significant in both the 3-month (sHR, 2.38 [95% CI, 1.21–4.69]; P=0.012) and 12-month (sHR, 1.48 [95% CI, 1.04–2.09]; P=0.028) follow-up periods (Figure S5, model 2). In contrast, the association between mtDNA-CN and non-CVD mortality was nominally significant in 3-month follow-up (sHR, 1.85 [95% CI, 1.13–3.02]; P=0.015) but insignificant in 12-month follow-up (sHR, 1.12 [95% CI, 1.84–1.49]; P=0.45).

Nonlinear Associations Between mtDNA-CN and Poststroke/TIA Outcomes

For the most significant linear associations between mtDNA-CN and poststroke/TIA outcomes, we further examined their nonlinear associations by restricted cubic spline regressions, including 12-month stroke recurrence in patients aged ≤65 years (Figure 5A) and patients with SUC (Figure 5B) and 3-month functional outcome in patients with ESUS SUC (Figure 5C) and those aged ≤65 years (Figure 5D). Interestingly, we found significant nonlinear associations in the 2 analyses of 12-month recurrence and the 1 analysis of 3-month functional outcome among all patients with ESUS SUC. The risks of recurrence or poorer functional outcome increased quickly as the mtDNA-CN dropped below 200, while no changes in the risk effects were observed when mtDNA-CN was above 200. The nonlinear association between mtDNA-CN and 3-month functional outcome among patients with ESUS SUC aged ≤65 years was insignificant, probably due to the small sample size. When focusing on samples with mtDNA-CN ≤200, 1-SD lower mtDNA-CN was associated with 36% (sHR, 1.36 [95% CI, 1.13–1.64]; P=1.3×10^{-3}) and 62% (sHR, 1.62 [95% CI, 1.28–2.05]; P=6.0×10^{-5}) increase in the hazard of stroke recurrence within 12 months in younger patients and patients with SUC, respectively. Similarly, 1-SD lower mtDNA-CN was associated with 61% (OR, 1.61 [95% CI, 1.13–2.30]; P=8.1×10^{-3}) and 69% (OR, 1.69 [95%

Group	Sample size	No. of events	Model 1 HR (95% CI)	Model 1 P	Model 2 HR (95% CI)	Model 2 P
All-cause mortality in 3 months:						
All patients	10,241	140	1.13 (0.93–1.37)	0.235	1.11 (0.91–1.35)	0.290
Age ≤ 65	6278	47	2.22 (1.43–3.44)	**3.5×10^{-4}**	2.19 (1.41–3.39)	**5.0×10^{-4}**
Age > 65	3963	93	0.88 (0.71–1.09)	0.255	0.88 (0.71–1.09)	0.229
All-cause mortality in 12 months:						
All patients	10,241	320	1.11 (0.98–1.26)	0.111	1.09 (0.96–1.24)	0.168
Age ≤ 65	6278	109	1.33 (1.06–1.68)	0.014	1.30 (1.04–1.64)	0.024
Age > 65	3963	211	1.01 (0.87–1.17)	0.923	1.00 (0.86–1.17)	0.992

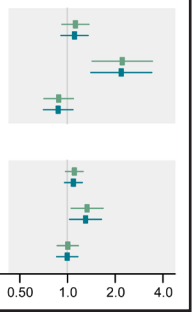


Figure 4. Association between mitochondrial DNA copy number (mtDNA-CN) and all-cause mortality during follow-up.
Cox models were used to estimate the hazard ratio (HR) of all-cause mortality per SD decrease of mtDNA-CN. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for cardiovascular disease-risk factors (methods). We highlighted P values achieving nominal significance (P<0.05) in red and those passing the Bonferroni-corrected threshold (P<9.6×10^{-4} based on 52 tests) in bold.

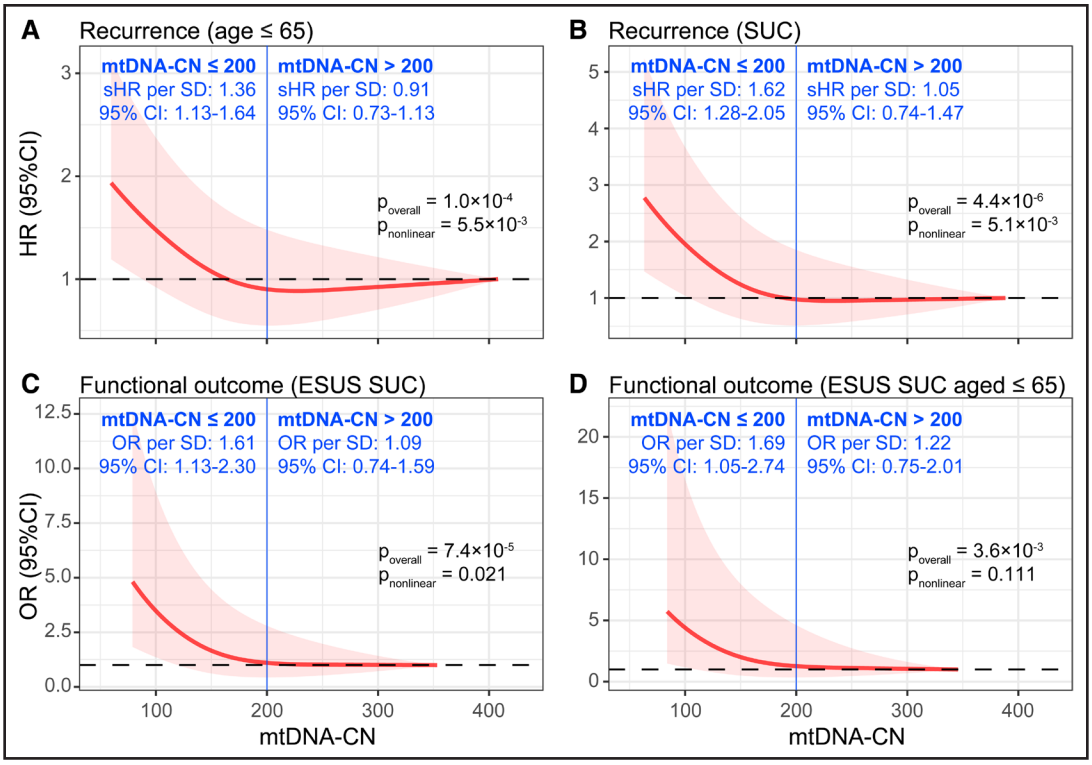


Figure 5. Nonlinear association between mitochondrial DNA copy number (mtDNA-CN) and poststroke/transient ischemic attack outcome.

A, Subdistribution hazard ratio (sHR) of stroke recurrence in 12 mo based on 6278 samples aged ≤65 y. **B**, sHR of stroke recurrence in 12 mo based on 3981 stroke of undetermined cause (SUC) samples. **C**, Odds ratio (OR) of poor functional outcome at 3 mo based on 1221 embolic stroke of undetermined source (ESUS) SUC samples. **D**, OR of poor functional outcome at 3 mo based on 790 ESUS SUC samples aged ≤65 y. Restricted cubic spline functions were wrapped into the Cox (**A** and **B**) or logistic models (**C** and **D**) to test nonlinear associations. Covariates in model 2 were adjusted. Solid red lines represent sHRs or ORs. Shaded areas represent 95% CIs.

CI, 1.05–2.74]; *P*=0.032) increase in the odds of poorer functional outcome at 3 months in patients with ESUS SUC and those aged ≤65 years, respectively. We did not examine the nonlinear associations between mtDNA-CN and mortality because of the small number of death events.

DISCUSSION

Based on comprehensive genetic and clinical data of 10 241 individuals from the prospective CNSR-III study in China, we provide evidence that lower mtDNA-CN was significantly associated with poorer poststroke/TIA outcomes. These findings are consistent with previous studies that have reported the role of mtDNA-CN in the prognosis of patients with stroke.[9,12] Our study goes beyond previous findings as we presented the first association study between mtDNA-CN and stroke recurrence. Moreover, we conducted stratified analyses based on age and stroke subtypes. Our findings suggested that mtDNA-CN can be a useful prognostic biomarker for poststroke/TIA outcome, and the underlying mechanisms of mtDNA-CN in stroke prognosis may differ between age groups and stroke subtypes.

A key strength of this study relies on the comprehensive clinical information of a large sample size, which enabled us to adjust for many potential confounders. For example, we included stroke history and National Institutes of Health Stroke Scale at admission as covariates in our analysis to ensure that the associations between mtDNA-CN and poststroke/TIA outcomes were not confounded by baseline stroke severity. In addition, inflammation factors have been shown to be closely related to poststroke/TIA outcomes, including mortality and stroke recurrence.[32] By adjusting for IL-6 level in our analysis, we demonstrated that the associations between mtDNA-CN and poststroke/TIA outcomes were independent of inflammation, suggesting that mtDNA-CN may be involved in stroke prognosis through pathways beyond the immune response.

We found that the effects of mtDNA-CN on poststroke/TIA outcomes were more evident in younger (≤65 years) patients with stroke. Consistently, Ashar et al[12] also found that the association between mtDNA-CN and incident CVD was only significant in patients aged <65 years. A possible explanation is that older patients often have multiple comorbidities, such as hypertension (63% in this study), diabetes (24%), and heart disease

(14%). These comorbidities may contribute to the overall prognosis of stroke and overshadow the effect of mtDNA-CN.

The significant association between mtDNA-CN and functional outcome at 3-month follow-up was consistent with Chong et al,[9] which also provided causal evidence from Mendelian randomization analysis. In our analysis, we further considered the potential confounding effect introduced by stroke recurrence, which often leads to more severe disability and higher mortality due to vascular embolism.[4,5] We showed that after removing samples with stroke recurrence during follow-up, mtDNA-CN remained significantly associated with 3-month functional outcomes in patients with ESUS SUC. These findings suggested that the association between lower mtDNA-CN and poorer functional outcome was likely due to neuronal injury, rather than vascular embolism, and is independent of stroke recurrence.

Interestingly, we found that the association of mtDNA-CN with stroke recurrence or functional outcome was exclusively significant in the SUC or ESUS SUC subtype. ESUS is a subset of cryptogenic stroke, which consists of mostly the SUC subtype.[21] However, the mechanism of the SUC or ESUS subtype remains understudied. Our findings, for the first time, highlighted a crucial role of mtDNA-CN in the prognosis of the SUC and ESUS subtypes.

Furthermore, the above associations had significant nonlinear components, which aligned with the theory of mitochondrial threshold effects in that a certain decreasing level of mtDNA-CN does not impair mitochondrial function until it falls below a specific threshold.[24,33] Our results showed that this threshold of mtDNA-CN was around 200, above which reduction of mtDNA-CN had minimal effects on poststroke/TIA outcomes.

Maintaining a healthy pool of mitochondria is essential for neuronal homeostasis.[34] Lai et al[35] found that overexpression of the mitochondria regulator optic atrophy 1 in rats increases mtDNA-CN and inhibits neuronal apoptosis, which implies that higher mtDNA-CN is associated with better neuronal function. Considering mtDNA-CN as a biomarker of mitochondria,[11] a substantial decrease in mtDNA-CN may be indicative of aberrant mitochondrial function, potentially leading to neuronal damage and poorer stroke prognosis.

We acknowledge that this study may have several limitations. First, with comprehensive clinical information, we have explored the association between mtDNA-CN and multiple poststroke/TIA outcomes at multiple timepoints and subgroups, totaling 52 statistical tests in the main analyses, which may introduce false positive findings without controlling for multiple testing. We have chosen not to perform multiple testing corrections given the exploratory nature of our study[29] although several of the associations remained significant after Bonferroni correction, which is known to be conservative and will introduce false negative results. Second, we only enrolled patients with IS at baseline. Third, this study enrolled a relatively young patient with a less severe stroke/TIA group, which might limit the generalizability of the findings. Fourth, given the unclear cause and prognosis of SUC and ESUS, the underlying mechanisms of the observed associations remain to be explored. Fifth, the SUC and ESUS subtypes may contain patients with undiagnosed CE or SAO, which could potentially confound the associations between mtDNA-CN and poststroke/TIA outcomes. While the CCS classification system effectively removed patients with quasi-SAO from the SUC subtype,[27] we cannot completely rule out this confounding issue. Future studies with refined classification methods may provide further insights into the relationships between mtDNA-CN and poststroke/TIA outcomes in different stroke subtypes.

CONCLUSIONS

Our findings suggest that lower mtDNA-CN is associated with higher stroke recurrence rate in patients aged ≤65 years or diagnosed with SUC, higher all-cause mortality in patients aged ≤65 years, and poorer functional outcome in patients with ESUS SUC and those aged ≤65 years. We found significant nonlinear associations of mtDNA-CN with poststroke/TIA outcomes, supporting the theory of mitochondrial threshold effects for stroke prognosis.

ARTICLE INFORMATION

Received September 3, 2023; final revision received February 1, 2024; accepted February 12, 2024.

Affiliations

Department of Epidemiology and Biostatistics, Ministry of Education Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (Yi Jiang, H.W., Shanshan Cheng, C.W.). Department of Neurology (Si Cheng, Y.S., Z.X., Y. Li, Y. Liu, Z.L., Yong Jiang, X.M., H.L., Y.W.) and Center of Excellence for Omics Research (Si Cheng, Y.S., Z.X., Y. Li, Y. Liu, H.L., Y.W.), Beijing Tiantan Hospital, Capital Medical University, China. China National Clinical Research Center for Neurological Diseases, Beijing (Si Cheng, Y.S., Z.X., Y. Li, Y. Liu, Z.L., Yong Jiang, X.M., H.L., Y.W.). Changping Laboratory, Beijing, China (Si Cheng, Yong Jiang, Y.W.). Clinical Center for Precision Medicine in Stroke (Si Cheng, Y.W.) and Advanced Innovation Center for Human Brain Protection (Y.W.), Capital Medical University, Beijing, China.

Acknowledgments

The authors acknowledge all the investigators and participants of the Third China National Stroke Registry for their contributions to this research. Yi Jiang and Si Cheng analyzed the data, interpreted the results, and drafted the article. Drs Shi, Xu, and Meng, Y. Li, and Y. Liu performed quality control of clinical and whole-genome sequencing data. H. Wang analyzed the data. Drs Meng, Z. Li, H. Li, Yong Jiang, and Shanshan Cheng interpreted the results and revised the article. Drs C. Wang and Y. Wang conceived and supervised the study, interpreted the results, and cowrote the article.

Sources of Funding

This study was supported by grants from the National Key Research and Development Program of China (2022YFC2502400, 2022YFC2502402, and 2022YFE0209600), the National Natural Science Foundation of China (U20A20358, 82325044, 82101359, and 81973148), the Capital's Funds for

Health Improvement and Research (2020-1-2041), the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2019-I2M-5-029), and the China Postdoctoral Science Foundation (2021M701318).

Disclosures

None.

Supplemental Material

Figures S1–S5
Tables S1–S3

REFERENCES

1. GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018;392:1736–1788. doi: 10.1016/S0140-6736(18)32203-7
2. Hankey GJ, Jamrozik K, Broadhurst RJ, Forbes S, Anderson CS. Long-term disability after first-ever stroke and related prognostic factors in the Perth Community Stroke Study, 1989-1990. *Stroke*. 2002;33:1034–1040. doi: 10.1161/01.str.0000012515.66889.24
3. Mohan KM, Wolfe CD, Rudd AG, Heuschmann PU, Kolominsky-Rabas PL, Grieve AP. Risk and cumulative risk of stroke recurrence: a systematic review and meta-analysis. *Stroke*. 2011;42:1489–1494. doi: 10.1161/STROKEAHA.110.602615
4. Skajaa N, Adelborg K, Horvath-Puho E, Rothman KJ, Henderson VW, Thygesen LC, Sorensen HT. Risks of stroke recurrence and mortality after first and recurrent strokes in Denmark: a nationwide registry study. *Neurology*. 2021;98:E329–E342. doi: 10.1212/WNL.0000000000013118
5. Aarnio K, Haapaniemi E, Melkas S, Kaste M, Tatlisumak T, Putaala J. Long-term mortality after first-ever and recurrent stroke in young adults. *Stroke*. 2014;45:2670–2676. doi: 10.1161/STROKEAHA.114.005648
6. Morris-Blanco KC, Chokkalla AK, Arruri V, Jeong S, Probelsky SM, Vemuganti R. Epigenetic mechanisms and potential therapeutic targets in stroke. *J Cereb Blood Flow Metab*. 2022;42:2000–2016. doi: 10.1177/0271678X221116192
7. Liu X, Wang Q, Zhu R. Association of GWAS-susceptibility loci with ischemic stroke recurrence in a Han Chinese population. *J Gene Med*. 2021;23:e3264. doi: 10.1002/jgm.3264
8. Zhu R, Zhao Y, Tian D, Guo N, Zhang C, Liu X. GWAS-linked hot loci predict short-term functional outcome and recurrence of ischemic stroke in Chinese population. *Am J Transl Res*. 2021;13:4521–4534.
9. Chong MR, Narula S, Morton R, Judge C, Akhabir L, Cawte N, Pathan N, Lali R, Mohammadi-Shemirani P, Shoamanesh A, et al. Mitochondrial DNA copy number as a marker and mediator of stroke prognosis. *Neurology*. 2022;98:e470–e482. doi: 10.1212/WNL.0000000000013165
10. Song L, Liu T, Song Y, Sun Y, Li H, Xiao N, Xu H, Ge J, Bai C, Wen H, et al. mtDNA copy number contributes to all-cause mortality of lacunar infarct in a Chinese prospective stroke population. *J Cardiovasc Transl Res*. 2020;13:783–789. doi: 10.1007/s12265-019-09943-9
11. Jeng JY, Yeh TS, Lee JW, Lin SH, Fong TH, Hsieh RH. Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. *J Cell Biochem*. 2008;103:347–357. doi: 10.1002/jcb.21625
12. Ashar FN, Zhang Y, Longchamps RJ, Lane J, Moes A, Grove ML, Mychaleckyj JC, Taylor KD, Coresh J, Rotter JI, et al. Association of mitochondrial DNA copy number with cardiovascular disease. *JAMA Cardiol*. 2017;2:1247–1255. doi: 10.1001/jamacardio.2017.3683
13. Sundquist K, Sundquist J, Palmer K, Memon AA. Role of mitochondrial DNA copy number in incident cardiovascular diseases and the association between cardiovascular disease and type 2 diabetes: a follow-up study on middle-aged women. *Atherosclerosis*. 2022;341:58–62. doi: 10.1016/j.atherosclerosis.2021.11.020
14. Liu H, Ma Y, Fang F, Zhang Y, Zou L, Yang Y, Zhu S, Wang S, Zheng X, Pei P, et al. Wild-type mitochondrial DNA copy number in urinary cells as a useful marker for diagnosing severity of the mitochondrial diseases. *PLoS One*. 2013;8:e67146. doi: 10.1371/journal.pone.0067146
15. Zhang Z, Yang D, Zhou B, Luan Y, Yao Q, Liu Y, Yang S, Jia J, Xu Y, Bie X, et al. Decrease of MtDNA copy number affects mitochondrial function and involves in the pathological consequences of ischaemic stroke. *J Cell Mol Med*. 2022;26:4157–4168. doi: 10.1111/jcmm.17262
16. Martens LG, Luo J, Wermer MJH, Willems van Dijk K, Hagg S, Grassmann F, Noordam R, van Heemst D. The association between mitochondrial DNA abundance and stroke: a combination of multivariable-adjusted survival and Mendelian randomization analyses. *Atherosclerosis*. 2022;354:1–7. doi: 10.1016/j.atherosclerosis.2022.06.1012
17. Wang Y, Jing J, Meng X, Pan Y, Wang Y, Zhao X, Lin J, Li W, Jiang Y, Li Z, et al. The Third China National Stroke Registry (CNSR-III) for patients with acute ischaemic stroke or transient ischaemic attack: design, rationale and baseline patient characteristics. *Stroke Vasc Neurol*. 2019;4:158–164. doi: 10.1136/svn-2019-000242
18. Cheng S, Xu Z, Liu Y, Lin J, Jiang Y, Wang Y, Meng X, Wang A, Huang X, Wang Z, et al. Whole genome sequencing of 10K patients with acute ischaemic stroke or transient ischaemic attack: design, methods and baseline patient characteristics. *Stroke Vasc Neurol*. 2021;6:291–297. doi: 10.1136/svn-2020-000664
19. Cheng S, Xu Z, Bian S, Chen X, Shi Y, Li Y, Duan Y, Liu Y, Lin J, Jiang Y, et al. The STROMICS genome study: deep whole-genome sequencing and analysis of 10K Chinese patients with ischemic stroke reveal complex genetic and phenotypic interplay. *Cell Discov*. 2023;9:75. doi: 10.1038/s41421-023-00582-8
20. Ay H, Benner T, Murat Arsava E, Furie KL, Singhal AB, Jensen MB, Ayata C, Towfighi A, Smith EE, Chong JY, et al. A computerized algorithm for etiologic classification of ischemic stroke: the Causative Classification of Stroke System. *Stroke*. 2007;38:2979–2984. doi: 10.1161/strokeaha.107.490896
21. Hart RG, Diener HC, Coutts SB, Easton JD, Granger CB, O'Donnell MJ, Sacco RL, Connolly SJ; Cryptogenic Stroke/ESUS International Working Group. Embolic strokes of undetermined source: the case for a new clinical construct. *Lancet Neurol*. 2014;13:429–438. doi: 10.1016/S1474-4422(13)70310-7
22. Ding J, Sidore C, Butler TJ, Wing MK, Qian Y, Meirelles O, Busonero F, Tsoi LC, Maschio A, Angius A, et al. Assessing mitochondrial DNA variation and copy number in lymphocytes of ~2,000 sardinians using tailored sequencing analysis tools. *PLoS Genet*. 2015;11:e1005306. doi: 10.1371/journal.pgen.1005306
23. Qian Y, Butler TJ, Opsahl-Ong K, Giroux NS, Sidore C, Nagaraja R, Cucca F, Ferrucci L, Abecasis GR, Schlessinger D, et al. fastMitoCalc: an ultra-fast program to estimate mitochondrial DNA copy number from whole-genome sequences. *Bioinformatics*. 2017;33:1399–1401. doi: 10.1093/bioinformatics/btw835
24. Picard M. Blood mitochondrial DNA copy number: what are we counting? *Mitochondrion*. 2021;60:1–11. doi: 10.1016/j.mito.2021.06.010
25. Andersen PK, Gill RD. Cox's regression model for counting processes: a large sample study. *Ann Stat*. 1982;10:1100–1120. doi: 10.1214/aos/1176345976
26. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc*. 1999;94:496–509. doi: 10.1080/01621459.1999.10474144
27. Chen PH, Gao S, Wang YJ, Xu AD, Li YS, Wang D. Classifying ischemic stroke, from TOAST to CISS. *CNS Neurosci Ther*. 2012;18:452–456. doi: 10.1111/j.1755-5949.2011.00292.x
28. Gordon M, Lumley T. Forestplot: advanced forest plot using "grid" graphics. 2016. Accessed August 1, 2023. https://cran.r-project.org/web/packages/forestplot/index.html
29. Althouse AD. Adjust for multiple comparisons? It's not that simple. *Ann Thorac Surg*. 2016;101:1644–1645. doi: 10.1016/j.athoracsur.2015.11.024
30. Jr FEH. rms: regression modeling strategies. 2023. Accessed August 1, 2023. https://cran.r-project.org/web/packages/rms/index.html
31. Liu X, Longchamps RJ, Wiggins KL, Raffield LM, Bielak LF, Zhao W, Pitsillides A, Blackwell TW, Yao J, Guo X, et al; TOPMed mtDNA Working Group in NHLBI Trans-Omics for Precision Medicine (TOPMed) Consortium. Association of mitochondrial DNA copy number with cardiometabolic diseases. *Cell Genom*. 2021;1:100006. doi: 10.1016/j.xgen.2021.100006
32. Esenwa CC, Elkind MS. Inflammatory risk factors, biomarkers and associated therapy in ischaemic stroke. *Nat Rev Neurol*. 2016;12:594–604. doi: 10.1038/nrneurol.2016.125
33. Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP, Letellier T. Mitochondrial threshold effects. *Biochem J*. 2003;370:751–762. doi: 10.1042/BJ20021594
34. Mandal A, Drerup CM. Axonal transport and mitochondrial function in neurons. *Front Cell Neurosci*. 2019;13:373. doi: 10.3389/fncel.2019.00373
35. Lai Y, Lin P, Chen M, Zhang Y, Chen J, Zheng M, Liu J, Du H, Chen R, Pan X, et al. Restoration of L-OPA1 alleviates acute ischemic stroke injury in rats via inhibiting neuronal apoptosis and preserving mitochondrial function. *Redox Biol*. 2020;34:101503. doi: 10.1016/j.redox.2020.101503