Mitochondrial DNA Copy Number as a Marker and Mediator of Stroke Prognosis: Observational and Mendelian Randomization Analyses

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Abstract

Background: Low buffy coat mitochondrial DNA copy number (mtDNA-CN) is associated with incident risk of stroke and post-stroke mortality; however, its prognostic utility has not been extensively explored.

Objective: To investigate whether low buffy coat mtDNA-CN is a marker and causal determinant of post-stroke outcomes using epidemiological and genetic studies.

Methods: First, we performed association testing between baseline buffy coat mtDNA-CN measurements and 1-month post-stroke outcomes in 3498 acute, first stroke cases from 25 countries from the international, multicenter case-control study, “Importance of Conventional and Emerging Risk Factors of Stroke in Different Regions and Ethnic Groups of the World” (INTERSTROKE). Then, we performed two-sample Mendelian Randomization analyses to evaluate potential causative effects of low mtDNA-CN on 3-month modified Rankin Scale (mRS). Genetic variants associated with mtDNA-CN levels were derived from the UKBiobank study (N=383476), and corresponding effects on 3-month mRS were ascertained from the Genetics of Ischemic Stroke funCtional Outcome study (GISCOME; N=6021).

Results: A 1-standard deviation (SD) lower mtDNA-CN at baseline was associated with stroke severity (baseline mRS; OR=1.27; 95% CI, 1.19-1.36; $P=4.7x10^{-12}$). Independent of baseline stroke severity, lower mtDNA-CN was associated with increased odds of greater 1-month disability (ordinal mRS; OR=1.16; 95% CI, 1.08-1.24; $P=4.4x10^{-5}$), poor functional outcome status (mRS 3-6 vs. 0-2; OR=1.21; 95% CI, 1.08-1.34; $P=6.9x10^{-4}$), and mortality (OR=1.35; 95% CI, 1.14-1.59; $P=3.9x10^{-4}$). Subgroup analyses

demonstrated consistent effects across stroke type, sex, age, country income level, and education level. In addition, mtDNA-CN significantly improved reclassification of poor functional outcome status (Net Reclassification Index (NRI)=0.16; 95% CI, 0.08-0.23; $P=3.6x10^{-5}$) and mortality (NRI=0.31; 95% CI, 0.19-0.43; $P=1.7x10^{-7}$) beyond known prognosticators. Using independent datasets, Mendelian Randomization revealed that a 1 SD decrease in genetically determined mtDNA-CN was associated with increased odds of greater 3-month disability quantified by ordinal mRS (OR=2.35; 95% CI, 1.13-4.90; P=0.02) and poor functional outcome status (OR=2.68; 95% CI, 1.05-6.86; P=0.04).

Conclusions: Buffy coat mtDNA-CN is a novel and robust marker of post-stroke prognosis that may also be a causal determinant of post-stroke outcomes.

Classification of Evidence: This study provides class II evidence that low buffy coat mtDNA-CN (>1-standard deviation) was associated with worse baseline severity and 1-month outcomes in patients with ischemic or hemorrhagic stroke.

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Introduction

Stroke patients from low and middle-income countries bear a disproportionate burden of post-stroke complications[1–3]. As such, identifying cost-effective and highly predictive biomarkers that mediate post-stroke recovery will allow for better risk stratification and novel targets for acute stroke treatment[4].

Mitochondrial health has an important role in both stroke pathogenesis and recovery[5,6], and mitochondrial DNA copy number (mtDNA-CN) is an emerging biomarker that has recently garnered interest due to its inexpensiveness, accessibility,

and association with chronic diseases including stroke[7]. mtDNA-CN reflects the ratio of mitochondrial to nuclear DNA copies and acts as a rough surrogate for the number of mitochondria[8,9]. Rare genetic disorders characterized by severe loss of mtDNA-CN, formally referred to as “mtDNA depletion” syndromes, can cause migraine, leukoencephalopathy, and stroke-like episodes[10,11]. Conversely, higher mtDNA-CN levels have been reported in patients with primary mitochondrial disorders and some cancers[12,13]. In the broader population, perturbations of leukocyte mtDNA-CN have been proposed to reflect general mitochondrial dysfunction, oxidative stress, impaired oxidative phosphorylation, and inflammation[8,9], which are key pathophysiological determinants of stroke injury and prognosis. Indeed, low leukocyte mtDNA-CN is associated with increased risks of secondary hospitalization and mortality in patients with atherosclerotic and chronic kidney disease[14–16]. To our knowledge, only one study has investigated the association between mtDNA-CN and post-stroke outcomes, wherein a prospective cohort study of 1484 Chinese stroke patients reported an association between mtDNA-CN and mortality[17]. While these findings suggest a potential role for mtDNA-CN as a risk factor for post-stroke outcomes, several important questions remain to be addressed regarding: (i) the robustness of associations across stroke type and other clinically relevant subgroups, (ii) whether associations are independent of baseline stroke severity, (iii) if mtDNA-CN is associated with the degree of functional disability among stroke survivors, and (iv) if mtDNA-CN is a causal determinant of post-stroke outcomes.

To answer these questions, we investigated the relationships between both measured and genetically predicted mtDNA-CN levels with post-stroke outcomes using large-scale

datasets. First, we evaluated the association between buffy coat mtDNA-CN levels measured within one week of stroke symptom onset and 1-month outcomes in 3498 stroke patients from the "Importance of Conventional and Emerging Risk Factors of Stroke in Different Regions and Ethnic Groups of the World" (INTERSTROKE) study[18]. Second, whereas epidemiological analyses may suggest that low mtDNA-CN is a marker of stroke injury (reverse causation), there is also a possibility that low mtDNA-CN captures an underlying susceptibility to brain ischemia engendering worse stroke outcome (causation). Therefore, to assess whether lower mtDNA-CN levels may be a causal risk factor for poor outcomes at 3-months after stroke, we conducted two-sample Mendelian Randomization (MR) analyses using genetic effects derived from the UKBiobank (N=383476)[19] and the Genetics of Ischemic Stroke funCtional Outcome (GISCOME; N=6165)[20]. Overall, to address the primary research question of defining the extent to which low buffy coat mtDNA-CN is associated with post-stroke outcomes, we evaluated whether low mtDNA-CN represents a marker and causal driver of poor post-stroke outcomes.

Methods

INTERSTROKE

INTERSTROKE is a large international case-control study encompassing 32 countries across Asia, North America, South America, Europe, Australia, and Africa[18]. The study design has been described in detail previously[21]. In brief, participants were enrolled between January 11, 2007 and August 8, 2015. Cases consisted of patients with acute, first stroke (ischemic or hemorrhagic) presenting within 5 days of symptom onset and 72 hours of hospital admission. Strokes were defined according to the World

Health Organization definition, and subtypes were confirmed by neuroimaging (CT or MRI). Demographic characteristics, medical history, and risk factor data were collected through standardized questionnaires and physical examination. For patients who could not communicate, a proxy respondent was used (spouse or first-degree relative living in the same household aware of the patient's medical history and current treatments). The modified-Rankin scale (mRS)[22] was used as a marker of stroke severity and was measured at baseline and at 1-month follow-up. The presence of hemorrhagic transformation after ischemic stroke was assessed through neuroimaging (either CT or MRI) and adjudicated locally by a site investigator. The present analyses were performed on a subset of 3498 INTERSTROKE cases with qPCR mtDNA-CN measurements.

MtDNA-CN Measurement and Quality Control

At each recruitment centre, non-fasting peripheral blood samples were collected in EDTA tubes from stroke patients within one week of symptom onset (and within 72 hours of hospital admission). Blood samples were shipped from all regions (except South Asia and China due to sample transport restrictions) to the Clinical Research Laboratory and Biobank (Hamilton, Ontario, Canada). DNA was extracted from the buffy coat layer of centrifuged samples using the QIAGEN QIAsymphony DNA Midi (96.7%), DNA Mini (2.7%) or DSP DNA Midi (0.6%) kits. mtDNA-CN was assayed by the Genetic and Molecular Epidemiology Lab (Hamilton, Ontario, Canada) using a plasmid-normalized quantitative Polymerase Chain Reaction (qPCR) method developed by Fazzini *et al.* (2018)[23]. Samples were run in duplicate and those with a high coefficient of variation (>5%) were removed. Upon visual inspection of the distribution of mtDNA-

CN values, a single sample with an extreme outlying value was removed. Additional outliers beyond 3 standard deviations (SD) of the mean were winsorized to the 99.7th percentile. MtDNA-CN values were normalized for known confounders by taking the residuals from a linear regression model for mtDNA-CN (dependent variable) versus age, sex, ethnicity, and qPCR batch (independent variables). The resulting numerical representation of mtDNA-CN was standardized to a mean of 0 and SD of 1 for subsequent analyses.

Statistical Analysis

All statistical analyses were performed using the statistical programming language 'R' (version 3.6.0). Plots were generated using a combination of the "ggplot2", "viridis", "dplyr", "grid", and "gridExtra" R packages. In INTERSTROKE, association testing was conducted to assess the relationship between low mtDNA-CN at baseline (continuous variable or discretized into quartiles) and stroke markers at two timepoints: 1) markers collected at the time of the stroke event (hereafter referred to as 'baseline' severity markers) and 2) markers collected 1-month after the stroke event. The primary marker of baseline stroke severity was ordinal mRS. Secondary markers included level of consciousness and hemorrhagic transformation after ischemic stroke. The primary stroke outcome at 1-month follow-up was ordinal mRS. Secondary outcomes at 1-month follow-up included other formulations of mRS, specifically, poor functional outcome status (dichotomized mRS 3-6 vs. 0-2) and mortality status. Ordinal regression was used for analysis between ordinal mRS and consciousness ("polr" R package). The proportional odds assumption was evaluated using the Brant test ("Brant" R package). Logistic regression analysis was conducted for dichotomous variables including

hemorrhagic transformation at baseline and 1-month post-stroke outcomes (poor functional outcome and mortality statuses). All regression models were adjusted for age, sex, region, education level (none or primary school vs. high school, trade school, college, or university), 2018 World Bank country income stratum (high, upper-middle, and lower-middle or low income), household income (adjusted for country), primary stroke type (ischemic vs. hemorrhagic stroke) and ischemic stroke Oxfordshire Community Stroke Project (OCSP) classification, pre-stroke dependency (pre-stroke mRS 3-5 vs. 0-2), Charlson comorbidity index, and stroke risk factors (hypertension, diabetes, hypercholesterolemia, atrial fibrillation or flutter, current smoker status, and waist to hip ratio) as defined previously[18]. In addition to these covariates, baseline stroke severity (baseline mRS) was additionally included in models for 1-month post-stroke outcomes. For analysis of dichotomous outcomes, additional subgroup analyses were performed stratifying by primary stroke type, baseline stroke severity, sex, age, country income level, and education level. The Net Reclassification Index (NRI) was used to assess model reclassification improvement of 1-month post-stroke outcomes upon addition of mtDNA-CN to a baseline model including the same set of covariates as aforementioned ("Hmisc" R package). Statistical analyses were adjusted for multiple hypotheses testing of six outcomes (three markers of baseline stroke severity and three 1-month mRS formulations), corresponding to a Bonferroni-corrected P-value threshold of 0.008 ($P<0.05/6=0.008$).

Mendelian Randomization

Mendelian Randomization (MR) is a statistical genetics framework that leverages the random assortment of genetic alleles (Mendel's second law of independent

assortment) to perform causal inference between an exposure and an outcome [24–26]. The use of randomized, genetic alleles as instrumental variables for an exposure endows several advantages including robustness to traditional confounding factors and reverse causation. Indeed, evidence from animal models suggests that stroke induces changes in mtDNA-CN levels, and therefore reverse causality is a relevant concern that is addressed by MR[27,28]. To evaluate the potential causal relationship between low mtDNA-CN (exposure) and stroke prognosis (outcome), we performed "two-sample" MR analyses incorporating summary-level GWAS data from two independent studies. Genetic variants associated with mtDNA-CN levels were identified from a previous genome-wide association study (GWAS) we conducted in 383476 Caucasian participants from the UKBiobank study[29]. UKBiobank is a prospective cohort study including UK residents (ages 40-69 years) recruited from 2006-2010[30]. Eligibility criteria included Caucasian participants with suitable genetic microarray data who had non-outlying blood cell count and array intensity values[29]. UKBiobank mtDNA-CN estimates were derived using AutoMitoC, a computational pipeline that leverages array-based data to estimate mtDNA-CN[29]. Corresponding genetic effects on 3-month mRS were obtained from the Genetics of Ischaemic Stroke Functional Outcome (GISCOME) GWAS. GISCOME included 6021 Caucasian ischemic stroke patients from 12 studies across Europe, the United States, and Australia[20]. Two formulations of 3-month mRS were tested in the present study: ordinal mRS and poor functional outcome status (mRS 3-6 vs. 0-2). In GISCOME, 2280 (63%) participants suffered poor functional outcome. There is no sample overlap between UKBiobank and GISCOME datasets.

As previously described[29], an independent set of 26 genome-wide significant variants associated with mtDNA-CN located nearby or within genes expressed in the mitochondria were selected as instruments to genetically approximate mtDNA-CN levels (eAppendix; eTables 1 & 2). Collectively, these variants had an F-statistic of 100 which is sufficient (F>10) for the purposes of identifying a causal effect.

Two-sample MR analyses were executed using the "TwoSampleMR" (version 0.5.5) and "MRPRESSO" (version 1.0) R packages[25,31]. Three MR methods were employed including the inverse variance weighted, weighted median, and MR-Egger methods. MR-PRESSO was used to detect global heterogeneity with P-values derived based on 1000 simulations. The Egger intercept test was used to assess directional pleiotropy. Causal effects were expressed as odds of a higher mRS category (or of poor functional outcome) per 1 standard deviation decrease in genetically determined mtDNA-CN. MR analyses tested a similar set of hypotheses as epidemiological analyses and were therefore viewed as confirmatory. Accordingly, a nominal P-value threshold of 0.05 was considered statistically significant. To assess the potential for bidirectional effects (i.e. susceptibility to worse stroke outcome influencing mtDNA-CN), reverse MR analyses were also performed using suggestive loci ($P<5x10^{-6}$) from GISCOME as genetic instruments.

Beyond commonly employed tests, we also performed additional sensitivity analyses to address sources of confounding specific to mtDNA-CN and the prognostic nature of analyses. First, phenotypic mtDNA-CN measurements may reflect differences in immune cell proportions[32,33], so we examined the relationship between genetically determined blood cell traits and 3-month stroke outcomes. Blood cell traits entailed

neutrophil, lymphocyte, white blood cell, and platelet counts, as well as the neutrophil to lymphocyte ratio. Genetic variants associated with blood cell counts were ascertained from a large European GWAS by the Blood Cell Consortium X (2021) comprising over half of a million individuals[34]. Genetic variants associated with neutrophil to lymphocyte ratio were derived from a UKBiobank GWAS we conducted in 340002 British participants (unpublished data; eAppendix)[35]. Causal effect estimates were expressed per 1 standard deviation increase in genetically determined blood cell traits. Second, as mtDNA-CN instruments were derived from a generally healthy population, the transferability of genetic effects to stroke patients is unknown. Accordingly, we consolidated the mtDNA-CN instruments derived from the UKBiobank to calculate weighted polygenic scores for INTERSTROKE cases with genotyping data to test the association between genetically predicted and qPCR-measured mtDNA-CN levels in stroke patients (eAppendix). Third, a potential challenge of evaluating prognostic factors among disease patients is vulnerability to “index event bias”, a form of selection bias that occurs when studying risk factors for subsequent events among disease cases that can induce dependence among originally independent risk factors and lead to spurious associations[36]. Accordingly, we repeated MR analyses using stroke outcome genetic effects corrected for this bias using the method by Dudbridge *et al.* (2019) (eAppendix)[20,37,38].

Standard Protocol Approvals, Registrations, and Patient Consents

Research was approved by the Hamilton Integrated Research Ethics Board under project # 06-331. All INTERSTROKE participants (or their proxies) provided

written informed consent. INTERSTROKE analyses were reported following STREGA guidelines, and MR analyses were reported according to STROBE-MR guidelines.

Data Availability

The primary datasets in this study include INTERSTROKE, UKBiobank, and GISCOME. Anonymized INTERSTROKE data may be made available by request from any qualified investigator upon approval of collaboration with study PI, Martin O'Donnell. UKBiobank individual-level data can be acquired upon application[39]. UKBiobank mtDNA-CN GWAS summary statistics will be posted on the GWAS catalogue[40]. GISCOME summary statistics are freely available to download from the Cerebrovascular Disease Knowledge Portal[41].

Results

Baseline Characteristics of INTERSTROKE cases

A subset of 3498 stroke patients consented to genetic analysis, had peripheral blood specimen collected within one week of symptom onset, and had DNA samples that were successfully assayed for buffy coat mtDNA-CN (Figure 1). The stroke patients analyzed in this study spanned 25 countries and 98 enrollment sites across Western Europe (26.6%), Eastern / Central Europe (11.8%), South America (28.1%), Africa (13.3%), South East Asia (6.8%), the Western Asia (6.8%), and North America and Australia (6.5%) (Table 1). The average age of stroke patients was 64.6 years (SD=14.4 years) and 1482 (42.4%) individuals were female. The sample comprised 677 (19.4%), 1259 (36.0%), and 1562 (44.6%) individuals from lower-middle / low income, upper-middle income, and high-income countries, respectively. Primary stroke types consisted of 592 (16.9%) hemorrhagic, 2889 (82.6%) ischemic, and 17 (0.5%) undefined cases.

Among the 2889 patients with ischemic stroke, 54 (1.9%) had hemorrhagic transformation of their infarct. At baseline, 2010 (57.5%) participants were functionally dependent on others to perform basic activities of daily living (mRS 3-5). The level of consciousness was reduced (drowsy or unconscious) in 1129 (29.9%) patients.

Notably, the characteristics of this INTERSTROKE subsample in this study differed from the whole sample because blood specimen from South Asia and China were precluded from genetic analyses (eTable 3). For example, cases included in mtDNA-CN analyses were more likely to live in a high-income country, have at least a high-school education, and have certain risk factors (hypercholesterolemia and atrial fibrillation), whereas those not included in the present analyses were more likely to be current smokers and to have hemorrhagic stroke.

Lower mtDNA-CN is associated with greater stroke severity at baseline

At baseline, a 1-SD lower mtDNA-CN was significantly associated with increased odds of having a more severe stroke (ordinal mRS; OR=1.27; 95% CI, 1.19-1.36; $P=4.7x10^{-12}$) and reduced consciousness (OR=1.34; 95% CI, 1.21-1.48; $P=1.8x10^{-8}$) (eFigure 1). Among ischemic stroke patients, the association with hemorrhagic transformation was non-significant (OR=1.33; 95% CI, 0.92-1.93; P=0.13). Stratifying stroke patients by mtDNA-CN quartile, there was a stepwise increase in the proportion of individuals with higher stroke severity as mtDNA-CN decreased (eTable 4; Figure 2A). Stroke patients in the lowest mtDNA-CN quartile were at greatest risk of having a more severe stroke (OR=2.00; 95% CI,1.65-2.44; $P=2.9x10^{-12}$) and reduced consciousness (OR=2.42; 95% CI, 1.84-3.17; $P=1.6x10^{-10}$) compared to those in the highest mtDNA-CN quartile (eTable 4; Figure 2B). These associations were step-wise

and graded, and there was no significant evidence suggesting that the proportional odds assumption had been violated in any ordinal analysis (Brant P > 0.05; eTable 4). Time from symptom onset to blood draw was not significantly associated with mtDNA-CN levels (P=0.11).

Lower mtDNA-CN is associated with poor stroke prognosis at 1-month

Of the 3498 stroke patients, mRS was recorded at follow-up for 3470 (99.2%) individuals. At 1-month follow-up, 1354 (39.0%) patients had poor functional outcome (mRS 3-6) including 337 (9.7%) patients who died. Adjusting for baseline stroke severity in addition to previous covariates, a 1-SD lower mtDNA-CN was significantly associated with higher 1-month mRS (OR=1.16; 95% CI, 1.08-1.24; $P=4.4x10^{-5}$), poor functional outcome (OR=1.21; 95% CI, 1.08-1.34; $P=6.9x10^{-4}$), and mortality (OR=1.35; 95% CI, 1.14-1.59; $P=3.9x10^{-4}$) (eFigure 2; eTable 5). The magnitude of effect for mtDNA-CN on mortality risk was comparable to age, an established predictor of stroke outcomes (eFigure 3). Conversely, the effect of mtDNA-CN on post-stroke disability (mRS category and poor functional outcome status) was weaker than age (eFigure 3). There was no significant evidence suggesting that the proportional odds assumption had been violated in any ordinal analysis (Brant P > 0.05; eTable 5). Stratification by mtDNA-CN quartile revealed a consistent relationship between lower mtDNA-CN quartile and higher risk of adverse stroke outcomes (Figure 3). Stroke patients in the lowest quartile had greater odds of being classified in a higher mRS stratum (OR=1.40; 95% CI, 1.15-1.71; P=0.001), having poor functional outcome (OR=1.51; 95% CI, 1.11-2.04; P=0.01), and mortality (OR=2.09; 95% CI, 1.34-3.25; P=0.001) compared to stroke patients in the highest quartile (eTable 6; Figure 3).

To further assess the robustness of mtDNA-CN-outcome associations, we performed subgroup analyses stratifying by primary stroke type, baseline stroke severity, sex, age, country income level, and education level. Directionally consistent associations were observed across all subgroups for both poor functional outcome and mortality statuses with no significant heterogeneity between subgroups detected (Cochran Q Heterogeneity P > 0.10; Figure 4; eTable 7).

Lastly, we assessed whether incorporation of mtDNA-CN improved prediction of post-stroke outcomes beyond known prognosticators, risk factors, and demographic characteristics. Addition of mtDNA-CN led to significant improvements in reclassification of functional outcome status (Net Reclassification index $(NRI)_{overall}$=0.16; 95% CI, 0.08-0.23; P=$3.6x10^{-5}$) and mortality status ($NRI_{overall}$=0.31; 95% CI, 0.19-0.43; P=$1.7x10^{-7}$). For both outcomes, NRI improvement was attributable to better reclassification of events ($NRI_{Poor\ Outcome}$=0.20; 95 % CI, 0.15-0.26; P=$4.3x10^{-12}$; NRI_{Death}=0.33; 95% CI, 0.22-0.44; P=$3.4x10^{-9}$) as opposed to non-events ($NRI_{Favourable\ Outcome}$=-0.05; 95% CI, -0.09 to -0.001; P=0.045; NRI_{Alive}=-0.02; 95% CI, -0.06 to 0.02; P=0.30) (eTable 8).

Low mtDNA-CN is a putative causal risk factor for 3-month stroke outcomes

Using the UKBiobank and GISCOME studies (independent of INTERSTROKE), we found that genetically low mtDNA-CN was significantly associated with worse 3-month outcomes after stroke quantified by the ordinal mRS (OR=2.35 per SD decrease in genetically predicted mtDNA-CN; 95% CI, 1.13-4.90; P=0.02) and poor functional outcome (OR=2.68; 95% CI, 1.05-6.86; P=0.04) (Figure 5; eTables 9-11). For all analyses, there was no significant evidence of directional pleiotropy (MR-Egger intercept P > 0.05), nor global heterogeneity (Cochran Q and MR-PRESSO global test P

> 0.05). Results were also directionally consistent when using other MR methods (weighted median and MR-Egger) and when using stroke outcome effects adjusted for index event bias (eTable 9). As buffy coat mtDNA-CN is known to be correlated with immune cell counts, we also performed MR analyses for blood cell traits. Despite sufficient instrument strength for neutrophil (F=100), platelet (F=154), lymphocyte (F=108), total white blood cell counts (F=106) and the neutrophil to lymphocyte ratio (F=61), none were significantly associated with 3-month outcomes (Figure 5; eTable 10). Reverse MR analyses did not suggest that susceptibility to worse stroke outcome affected mtDNA-CN levels (P>0.10; eTable 11). Finally, because genetic variants used to approximate genetically determined mtDNA-CN levels were originally derived from a healthy population, we verified that such genetic effects persist in INTERSTROKE stroke cases (beta=0.08 SD increase in mtDNA-CN per 1 SD increase in genetically predicted levels; 95% CI, 0.02-0.05; $P=4.1x10^{-6}$; eTable 12).

This study provides class II evidence that low buffy coat mtDNA-CN (>1-standard deviation) was associated with worse baseline severity and 1-month outcomes in patients with ischemic or hemorrhagic stroke.

Discussion

Our study represents the first international multicenter exploration of buffy coat mtDNA-CN as a potential prognosticator of post-stroke outcomes. First, lower buffy coat mtDNA-CN measured within one week of symptom onset correlated with functional and clinically relevant stroke severity indicators. Second, lower buffy coat mtDNA-CN was associated with greater risk of poor functional outcome and death at 1-month follow-up, which were consistent across primary stroke type, sex, age, country income, and

education strata, as well as, independent of baseline severity. Third, in addition to being a strong predictor of mortality with a magnitude of effect comparable to age, the inclusion of buffy coat mtDNA-CN improved the prediction of functional outcome and death. Fourth, MR analysis provided support for low buffy coat mtDNA-CN as a causal mediator of 3-month mRS and poor functional outcome status. Altogether, our findings confirm the hypothesis that low buffy coat mtDNA-CN is a biomarker and mediator of worse stroke prognosis.

The main clinical implication of our study is that buffy coat mtDNA-CN may represent a useful prognostic marker of post-stroke outcomes. First, buffy coat mtDNA-CN is a blood biomarker of post-stroke outcomes that does not suffer from inter-rater variability and is not influenced by a patient's communication deficit. Second, the mtDNA-CN-outcome associations are consistent across stroke type, sex, age, country income level, education level, and baseline severity, which positions mtDNA-CN to have widespread utility for stroke patients globally. To our knowledge, we provide the first evidence suggesting that low mtDNA-CN may have consistent effects in both ischemic and hemorrhagic stroke patients. This is particularly relevant for health systems in low-income settings, which bear a disproportionate global burden of hemorrhagic stroke[1–3], though further analyses in larger samples of hemorrhagic stroke patients are warranted to confirm. Third, low mtDNA-CN represents a strong risk marker with effects comparable to established prognosticators including older age. Moreover, the observed effect for mtDNA-CN on mortality is also comparable to that of carrying an *APOE* □2 allele, which confers a 1.5-fold increased risk of 3-month mortality in intracerebral hemorrhage patients and is present in approximately 15% of the population[42]. For

comparison, we found that stroke patients in the bottom 15% of mtDNA-CN levels had a 1.6-fold increased risk of 1-month mortality (OR=1.57; 95% CI, 1.13-2.17; P=0.007) relative to the remaining 85% participants with higher levels. Fourth, mtDNA-CN is an easily accessible biomarker as (i) it can be measured from peripheral blood after stroke, (ii) the assay necessitates only basic molecular laboratory techniques (qPCR), and (iii) the cost per sample is low (< $5 USD). Logistic and operational convenience combined with evidence for robust, objective, and strong prognostic utility raises the prospect of implementing mtDNA-CN clinically; however, replication of such findings in a prospective analysis and formal economic analyses is warranted.

Findings from MR analyses suggest that proper mtDNA regulation may be imperative for stroke protection and recovery, which aligns with animal model experiments demonstrating an important role for mtDNA-CN regulators in mediating protection against ischemia reperfusion injury. For example, reoxygenation of rodents with acute kidney injury induces the formation of excessive mitochondrial reactive oxygen species, accompanied by a sharp decline in mtDNA-CN levels[28]. In addition, genetic upregulation of the mtDNA replication initiation factor, TFAM, is sufficient to rescue this acute drop in mtDNA-CN thereby attenuating ischemia reperfusion injury. In the context of stroke models, mice with transient middle cerebral artery occlusion exhibit excessive cleavage of OPA1, another important mtDNA regulator, and treatment with either a cleavage-resistant form of OPA1 or mild overexpression of OPA1 markedly reduces infarct volume and neuronal apoptosis[27,43]. In conjunction with prior mechanistic studies, our epidemiological and genetic findings contribute to the mounting evidence that maintaining adequate mtDNA-CN may mediate cellular resilience to

ischemic insults. Consistent epidemiological associations in hemorrhagic stroke patients suggest that mtDNA-CN may protect against stroke injury through general mechanisms pertinent to both etiologies, such as the maintenance of blood-brain-barrier integrity or anti-inflammatory effects[44]. Indeed, circulating endothelial progenitor cells transfer their mitochondria to damaged endothelium which restores the integrity of the blood-brain-barrier, and this mitochondrial transfer may be mtDNA-dependent as has been shown for cancer cells[45,46]. Also, Castellani *et al.* (2020) proposed that low blood mtDNA-CN may indicate a shift from anti-inflammatory to pro-inflammatory macrophage subtypes[9]. Beyond potential mechanisms involving circulating leukocytes, it is plausible that genetic perturbation of blood mtDNA-CN reflects differences in mtDNA-CN levels in other cell and tissue types. Altogether, future experiments are required to decipher the underlying mechanisms and contributing cell types mediating this association, and additional MR analyses are necessary to assess potential causative effects in the post-hemorrhage setting.

Our study had several limitations. First, as INTERSTROKE was an international multicenter study, measures of baseline severity (NIHSS) and outcome (3-month mRS) that are common in smaller studies were substituted with baseline mRS and 1-month mRS for feasibility, respectively, as was done in Langhorne *et al.* (2018). The interchangeability of such measures has been validated in previous studies showing high correlation between baseline NIHSS and mRS (r=0.69) and between 1-month and 3-month mRS (r=0.87; weighted kappa agreement = 0.86)[47,48]. Granted, future studies are warranted to assess whether mtDNA-CN provides added utility to established measures of clinical and neuroanatomical severity. Second, complete blood cell counts

were not measured in INTERSTROKE; thus, we cannot directly evaluate to what extent blood cell counts influence observational associations with post-stroke outcomes. This is particularly important given that post-stroke infection increases neutrophils which have low mtDNA-CN[49]. However, most (>95%) blood samples were collected within 4 days of symptom onset thereby mitigating confounding from infections occurring after this period. Moreover, our genetic analyses suggest that mtDNA-CN may have a direct role in stroke prognosis independent of changes in blood cell counts since (i) mtDNA-CN GWAS effects had already been adjusted for major cell count determinants of mtDNA-CN levels (neutrophil, white blood cell, and platelet counts) and (ii) no significant association was observed for genetically determined immune cell counts *per se*. Nonetheless, the genetic determinants of post-stroke immune cell changes may differ from those influencing variation in cell counts within the general population as suggested by Torres-Aguila *et al.* (2019)[50]. Third, although associations were corrected for a crude surrogate of infarct volume (OCSP classification), direct measurements of infarct and hematoma volumes were not available. Fourth, survivorship bias may have led to conservative effect estimates as INTERSTROKE cases included patients surviving to hospital admission, and consequently, patients with severe, early fatal strokes were not represented. Finally, MR analyses were limited by the following considerations: (i) causal effect estimates were imprecise albeit consistent in direction-of-effect with epidemiological associations, (ii) although sensitivity analyses did not show significant evidence of heterogeneity, directional pleiotropy, or outlying effects, it is impossible to completely exclude bias due to pleiotropy or index event bias, (iii) mortality and hemorrhagic stroke outcomes could not be evaluated directly for lack of

available GWAS summary statistics, and (iv) analyses were solely based on Europeans, and while we found that genetically predicted mtDNA-CN was significantly associated with measured mtDNA-CN in an ethnically diverse sample of stroke patients, future stroke outcome GWAS in non-Europeans are necessary to enable analyses addressing whether this causative relationship extends to other populations.

Conclusions

Low buffy coat mtDNA-CN measured within one week of symptom onset represents an accessible and robust biomarker of both stroke severity and prognosis. MR findings suggest that low mtDNA-CN may mediate post-stroke outcomes. Additional investigations are warranted to replicate such findings in additional populations, to establish the temporal profile of post-stroke mtDNA-CN changes in more detail, and to assess whether compounds that maintain mtDNA-CN levels after cerebral insult hold promise as a novel therapeutic strategy.

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Tables

Table 1. **Demographic characteristics, comorbidities, and stroke characteristics for 3498 INTERSTROKE cases included in this study.**

Demographic Characteristics (N=3498)	
Age, years (SD)	64.6 (14.4)
Sex, N (%)	-
Female	1482 (42.4)
Male	2016 (57.6)
Region, N (%)	-
Western Europe	931 (26.6)
Eastern / Central Europe	413 (11.8)
South America	984 (28.1)
Africa	466 (13.3)
South East Asia	239 (6.8)
Western Asia	238 (6.8)
North America / Australia	227 (6.5)
Country income category, N (%)	-
Lower-middle or low income	677 (19.4)
Upper-middle income	1259 (36.0)
High income	1562 (44.6)
Ethnicity, N (%)	-
European	1562 (44.7)
Latin American	958 (27.4)
African	395 (11.3)
South East Asian	259 (7.4)
South Asian	108 (3.1)
Arab	105 (3.0)
Persian	103 (2.9)
Other	8 (0.2)
Education, N (%)	-
None	246 (7.0)
Primary school	870 (24.9)
High school or trade school	1545 (44.1)
College or university	527 (15.1)
Unknown	310 (8.9)
Comorbidity Burden and Risk Factors	
Charlson Comorbidity Index, N (%)	-
None	832 (23.8)
One or more comorbidities	2665 (76.2)
Unknown	1 (< 0.1)
Risk Factors, N (%)	-
Hypertension	2200 (62.9)
Diabetes Mellitus	683 (19.5)
Hypercholesterolemia	925 (26.4)
Atrial Fibrillation or Flutter	576 (16.5)
Current Smoker	776 (22.2)
Waist-to-hip Ratio, mean (SD)	0.95 (0.09)
Baseline Stroke Characteristics	
Stroke type, N (%)	-
Hemorrhagic Stroke	592 (16.9)
Intracerebral Hemorrhage	587 (16.9)
Subarachnoid Hemorrhage	5 (0.1)
Ischemic Stroke	2889 (82.6)
Total anterior circulation infarct	252 (7.2)
Partial anterior circulation infarct	1333 (38.1)
Posterior circulation infarct	439 (12.6)
Lacunar infarct	628 (17.9)
Other infarct	237 (6.8)
Unknown	17 (0.5)
Hemorrhagic Transformation, N (%)	-
Present	54 (1.9*)
Absent	2835 (98.2)
Stroke severity, N (%)	-
No symptoms (mRS 0)	151 (4.3)
Symptomatic but no disability (mRS 1)	573 (16.4)

Slight disability (mRS 2)	760 (21.7)
Moderate disability (mRS 3)	954 (27.3)
Moderately severe disability (mRS 4)	711 (20.3)
Severe disability (mRS 5)	345 (9.9)
Unknown	4 (0.1)
Level of consciousness, N (%)	-
Alert	2487 (71.0)
Drowsy	768 (22.0)
Unconscious	237 (6.8)
Unknown	6 (0.2)

* Percentage of ischemic stroke patients, not total number of participants

Figure Titles and Captions

Figure 1. Participant flow chart for the INTERSTROKE mtDNA-CN substudy.

Of 26919 research participants enrolled in the INTERSTROKE study, 11707 (43%) consented to genetic analysis and had blood specimen collected at baseline. Previous DNA extraction yielded 10146 (87%) samples with sufficient DNA for genotyping experiments (not included in the present investigation), of which, 9727 (96%) successfully genotyped samples passed quality control. Due to repeated genotyping and sequencing experiments performed in the same set of 9727 extracted DNA samples, sufficient DNA remained for only 8062 (83%) to be run on the mtDNA-CN qPCR assay. Quality control of mtDNA-CN measurements led to 7633 (95%) samples with suitable mtDNA-CN measurements. In the present analyses, we focus on a final subset of 3498 participants with acute stroke.

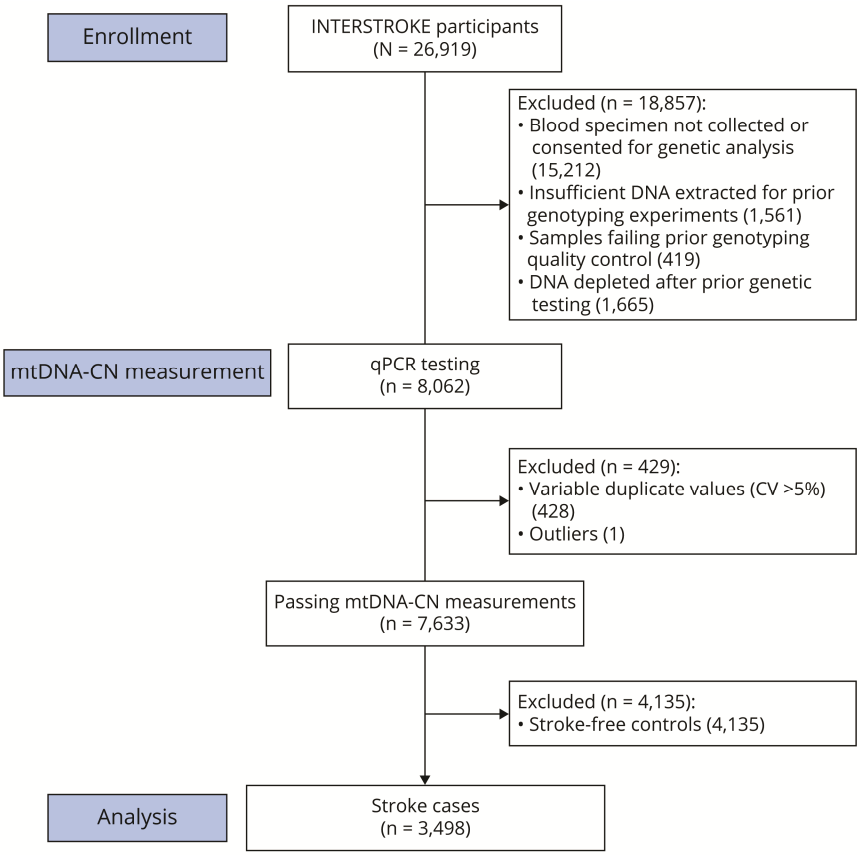

Enrollment
INTERSTROKE participants
(N = 26,919)
Excluded (n = 18,857):
• Blood specimen not collected or consented for genetic analysis (15,212)
• Insufficient DNA extracted for prior genotyping experiments (1,561)
• Samples failing prior genotyping quality control (419)
• DNA depleted after prior genetic testing (1,665)
mtDNA-CN measurement
qPCR testing
(n = 8,062)
Excluded (n = 429):
• Variable duplicate values (CV >5%) (428)
• Outliers (1)
Passing mtDNA-CN measurements
(n = 7,633)
Excluded (n = 4,135):
• Stroke-free controls (4,135)
Analysis
Stroke cases
(n = 3,498)

Figure 2.

Association between mtDNA-CN and baseline stroke severity.

(A) Stacked bar plots illustrate the proportion of each (a) ordinal mRS and (b) consciousness level category per mtDNA-CN quartile. (B) Forest plots illustrate the association between mtDNA-CN quartile and risk of having (a) more severe strokes as indicated by ordinal mRS and (b) reduced consciousness. The highest (4th) mtDNA-CN quartile was used as the reference group.

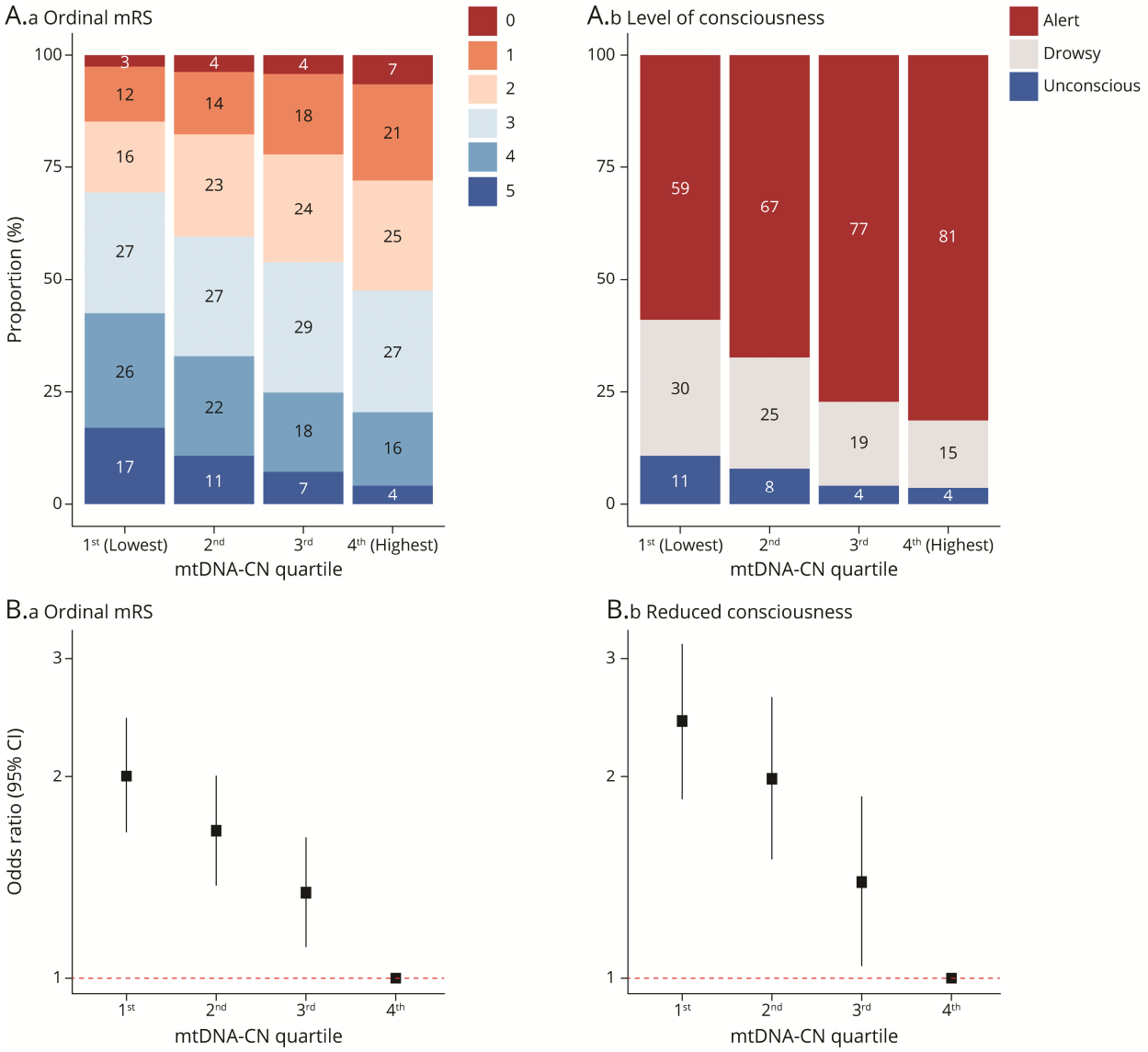

A.a Ordinal mRS
Proportion (%)
100
75
50
25
0
3
12
16
27
26
17
4
14
23
27
22
11
4
18
24
29
18
7
7
21
25
27
16
4
0
1
2
3
4
5
1st (Lowest)
2nd
3rd
4th (Highest)
mtDNA-CN quartile
A.b Level of consciousness
59
30
11
67
25
8
77
19
4
81
15
4
Alert
Drowsy
Unconscious
1st (Lowest)
2nd
3rd
4th (Highest)
mtDNA-CN quartile
B.a Ordinal mRS
Odds ratio (95% CI)
3
2
1
1st
2nd
3rd
4th
mtDNA-CN quartile
B.b Reduced consciousness
3
2
1
1st
2nd
3rd
4th
mtDNA-CN quartile

Figure 3. Association between mtDNA-CN and 1-month post-stroke prognosis.

(A) Stacked bar plots illustrate the proportion of individuals belonging to (a) ordinal mRS,(b) functional outcome status, and (c) mortality categories per mtDNA-CN quartile. (B) Forest plots convey the association between mtDNA-CN quartile and post-stroke outcomes with the fourth quartile as the reference for comparison.

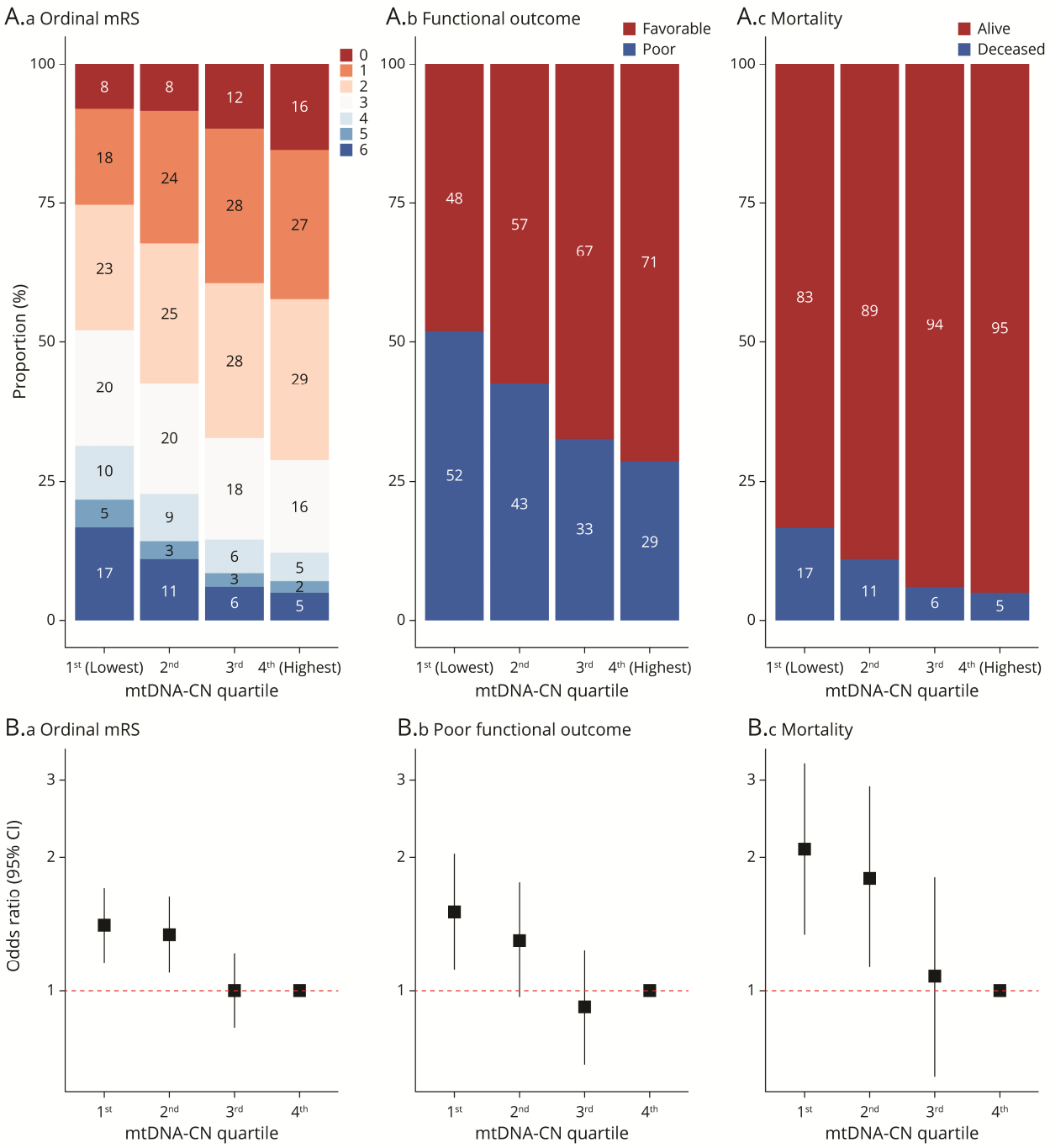
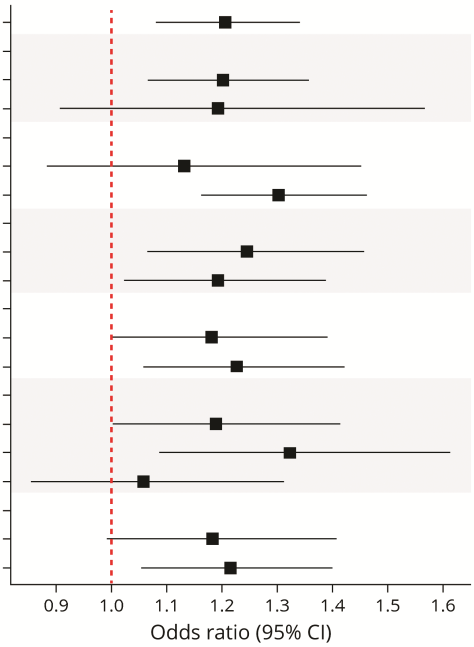

A.a Ordinal mRS
Proportion (%)
100
75
50
25
0
0
1
2
3
4
5
6
8
18
23
20
10
5
17
8
24
25
20
9
3
11
12
28
28
18
6
3
6
16
27
29
16
5
2
5
1st (Lowest)
2nd
3rd
4th (Highest)
mtDNA-CN quartile
A.b Functional outcome
Favorable
Poor
48
52
57
43
67
33
71
29
mtDNA-CN quartile
A.c Mortality
Alive
Deceased
83
17
89
11
94
6
95
5
mtDNA-CN quartile
B.a Ordinal mRS
Odds ratio (95% CI)
3
2
1
1st
2nd
3rd
4th
mtDNA-CN quartile
B.b Poor functional outcome
mtDNA-CN quartile
B.c Mortality
mtDNA-CN quartile

Figure 4. Association between mtDNA-CN and 1-month post-stroke prognosis stratified by subgroup.

Forest plots show the association between low mtDNA-CN (per SD decrease) on (A) poor functional outcome (mRS 3-6) and (B) mortality status across various strata. Except for the subgroup variable used to stratify, regression models were adjusted for age, sex, region, education level, country income level, household income level, primary stroke type and OCSP classification, Charlson comorbidity index, cardiovascular risk factors, pre-stroke disability, and baseline mRS.

A

Subgroup	N (outcome)	N (nonoutcome)	OR (95% CI)	Het *p*
All	1,354	2,116	1.21 (1.08–1.34)	
Primary stroke type				
Ischemic stroke	916	1,701	1.2 (1.07–1.36)	
Hemorrhagic stroke	303	245	1.19 (0.91–1.57)	0.96
Baseline mRS				
mRS 0–2	111	1,367	1.13 (0.88–1.45)	
mRS 3–5	749	1,239	1.3 (1.16–1.46)	0.32
Primary stroke type				
Female	638	828	1.25 (1.06–1.46)	
Male	712	1,288	1.19 (1.02–1.39)	0.69
Age				
≤65 years	549	1,183	1.18 (1–1.39)	
>65 years	801	933	1.23 (1.06–1.42)	0.74
Country income level				
Lower-middle/low	326	339	1.19 (1–1.41)	
Upper-middle	633	615	1.32 (1.09–1.61)	
High	391	1,162	1.06 (0.86–1.31)	0.33
Education level				
None or primary	531	572	1.18 (0.99–1.41)	
High school or more	692	1,375	1.22 (1.05–1.4)	0.81



B

Subgroup	N (outcome)	N (nonoutcome)	OR (95% CI)	Het *p*
All	337	3,133	1.35 (1.14–1.59)	
Primary stroke type				
Ischemic stroke	189	2,428	1.33 (1.08–1.63)	
Hemorrhagic stroke	117	431	1.33 (1.00–1.77)	1.00
Baseline mRS				
mRS 0–2	20	1,458	1.88 (0.93–3.77)	
mRS 3–5	313	1,675	1.4 (1.19–1.66)	0.43
Primary stroke type				
Female	161	1,305	1.41 (1.11–1.79)	
Male	172	1,828	1.32 (1.04–1.68)	0.71
Age				
≤65 years	143	1,589	1.56 (1.2–2.01)	
>65 years	190	1,544	1.2 (0.97–1.49)	0.12
Country income level				
Lower-middle/low	125	540	1.23 (0.99–1.53)	
Upper-middle	175	1,073	1.45 (1.09–1.93)	
High	33	1,520	1.74 (0.81–3.72)	0.51
Education level				
None or primary	159	944	1.39 (1.08–1.78)	
High school or more	151	1,915	1.33 (1.06–1.67)	0.79

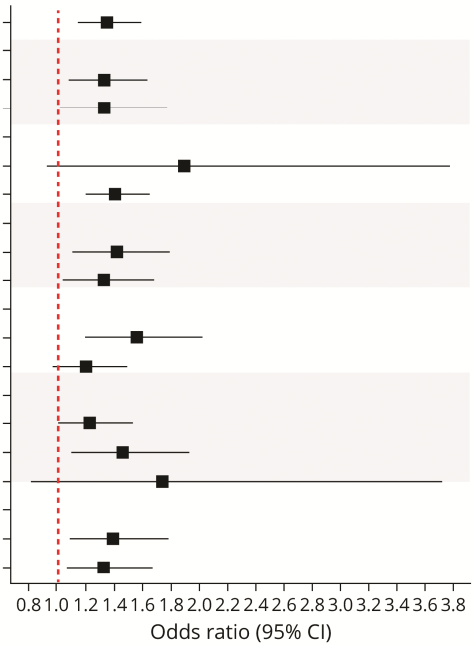


Figure 5. MR analyses assessing the effects of low mtDNA-CN (and blood cell traits) on 3-month post-ischemic stroke prognosis.

Genetic predisposition to low mtDNA-CN, but not blood cell counts, is associated with higher risk of 3-month outcomes after stroke. Effect estimates for mtDNA-CN are expressed per 1 SD decrease in genetically predicted mtDNA-CN, whereas those for blood cell traits were expressed per 1 SD increase in genetically predicted blood cell counts (or neutrophil to lymphocyte ratio). Causal effect estimates obtained by the inverse variance weighted method are displayed as there was no significant heterogeneity or directional pleiotropy detected for any analysis (eTables 9 & 10).