Mitochondrion 73 (2023) 51–61

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Complete mitochondrial DNA profile in stroke: A geographical matched case-control study in Spanish population

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ARTICLE INFO

Keywords:
Stroke
Mitochondrial DNA
Mitochondrial haplogroups
Mitochondrial DNA copy number

ABSTRACT

Introduction: Stroke, the second leading cause of death worldwide, is a complex disease influenced by many risk factors among which we can find reactive oxygen species (ROS). Since mitochondria are the main producers of cellular ROS, nowadays studies are trying to elucidate the role of these organelles and its DNA (mtDNA) variation in stroke risk. The aim of the present study was to perform a comprehensive evaluation of the association between mtDNA mutations and mtDNA content and stroke risk.

Material and methods: Homoplasmic and heteroplasmic mutations of the mtDNA were analysed in a case-controls study using 110 S cases and their corresponding control individuals. Mitochondrial DNA copy number (mtDNA-CN) was analysed in 73 of those case-control pairs.

Results: Our results suggest that haplogroup V, specifically variants m.72C > T, m.4580G > A, m.15904C > T and m.16298 T > C have a protective role in relation to stroke risk. On the contrary, variants m.73A > G, m.11719G > A and m.14766C > T appear to be genetic risk factors for stroke. In this study, we found no statistically significant association between stroke risk and mitochondrial DNA copy number.

Conclusions: These results demonstrate the possible role of mtDNA genetics on the pathogenesis of stroke, probably through alterations in mitochondrial ROS production.

1. Introduction

Nowadays, stroke is the second leading cause of death worldwide, accounting for approximately 6.5 million deaths annually, and the first cause of acquired disability in adults in some countries (W. Chen et al., 2020; Donkor, 2018; Feigin et al., 2022). There are 12.2 million new stroke cases per year around the world, which equals one stroke happening every 3 s. Furthermore, 101 million people are living with stroke aftermath and over 143 million years of healthy life are lost each year due to stroke-related death and disability (Feigin et al., 2022). Despite stable incidence rates and declining mortality rates over the past two decades, the public health burden of stroke is set to rise over future decades due to demographic transitions of populations, particularly in developing countries (Donkor, 2018; Hankey, 2017).

There are stroke risk factors such as hypertension, diabetes mellitus, high blood cholesterol, sedentary lifestyle, atrial fibrillation, smoking, and alcohol consumption that can be modified through a change in lifestyle or medication. Alternatively, there are also non-modifiable risk factors that include age, sex, and genetics (Donkor, 2018). Related to genetics, family studies have shown that a family history of stroke increases the risk of ischemic stroke by approximately 75% (Floßmann et al., 2004), and genome-wide association studies have estimated heritability for stroke near 40% (Malik & Dichgans, 2018). From these risk factors, age is the strongest determinant of stroke, followed by hypertension (Donkor, 2018).

Despite the previously referred risk factors, in the last decade,

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https://doi.org/10.1016/j.mito.2023.10.001
Received 17 January 2023; Received in revised form 28 August 2023; Accepted 1 October 2023
Available online 2 October 2023

different studies have been highlighting the importance of reactive oxygen species (ROS) in stroke initiation, as ROS are involved in the pathogenesis of atherosclerosis (Chang et al., 2010; Peluso et al., 2012). The most recent studies are focusing on the role of mitochondria in stroke risk (Yan et al., 2022) since it is widely known that they are responsible for approximately 90 % of cellular ROS production (Balaban et al., 2005).

Mitochondria are cytoplasmatic organelles that supply energy for all cellular metabolic activities via ATP synthesis. Structurally, each mitochondrion is bounded by a porous outer membrane and an inner membrane that contains multi-enzyme complexes responsible for ATP synthesis through oxidative phosphorylation (OXPHOS) (Bhagavan & Ha, 2011; Hatefi, 1985). OXPHOS requires the coordinated action of four respiratory complexes (complexes I-IV), that make up the electron transport chain (ETC), and an ATP synthase (complex V). However, this process is not perfectly coupled, since some of the energy present in the proton gradient (Δp) dissipates as heat (thermogenic role), through pathways independent of ATP synthase (proton leakage) (Cadenas, 2018).

Oxidative Phosphorylation is also the major endogenous source of ROS. These reactive oxygen species are generated when electrons leak directly to oxygen (O_2) to form superoxide (O^-), which can be converted to hydrogen peroxide (H_2O_2). This electron leakage comes mainly from ETC complexes I (NADH dehydrogenase) and III (cytochrome *c* reductase)(Addabbo et al., 2009; Andalib et al., 2017; Chen & Zweier, 2014; Krzywanski et al., 2011). The accumulation of high levels of ROS causes an intracellular REDOX imbalance, creating oxidative stress that leads to mitochondrial dysfunctions and contributes to the development and/or progression of cardiovascular diseases such as atherosclerosis, which is involved in stroke initiation. These processes, which may eventually modulate stroke susceptibility, are variable characteristics among the population that depend on different individual traits (Andalib et al., 2017; Krzywanski et al., 2011).

Mitochondrial ROS generation is mainly modulated by OXPHOS coupling efficiency, which is defined as the percentage of mitochondrial respiration linked to ATP synthesis (Gnaiger et al., 2015). When OXPHOS is highly coupled, Δp is higher since only a low percentage of it dissipates as heat. In those cases, respiration slows down increasing oxygen availability and the steady-state concentration of reduced-state ETC complexes (higher electron availability), which favours superoxide formation by electron leakage. However, mild uncoupling of OXPHOS dissipates Δp attenuating ROS production (Brand, 2000; Cadenas, 2018; Krzywanski et al., 2011; Munro & Treberg, 2017).

Apart from OXPHOS coupling efficiency, Chen and Zweier (2014) proposed that ROS generation could also be modulated by individual variations in OXPHOS rate and that a decrease in the rate of oxidative phosphorylation could increase ROS production. Some OXPHOS complexes are partially encoded by the mitochondrial DNA (mtDNA). Thus, mtDNA variation could be involved in ROS regulation and, therefore, in stroke susceptibility.

The mitochondrial genome is a circular double-stranded and maternally inherited DNA molecule of 16.6 kb present in numerous copies within the mitochondrial matrix. Concretely, mtDNA coding region (577–16,023 bp) encodes 13 essential polypeptides of the OXPHOS system (complexes I, III, IV and V) and the necessary RNA machinery for their translation within the mitochondria (Giles et al., 1980; Robin & Wong, 1988). The mtDNA also has a non-coding region (NCR) spanning approximately 1.1 kb (16,024–16,569; 1–576 bp) that comprises three hypervariable regions (namely HVR1, HVR2, and HVR3), the point of origin of heavy strand replication (OH) and the transcription promoters of both strand (HSP and LSP respectively) (Nicholls & Minczuk, 2014).

The number of molecules of mitochondrial DNA, also known as mtDNA copy number (mtDNA-CN), is variable in cells and tissues. Since mtDNA expression is a prerequisite for OXPHOS functionality, mtDNA-CN must be adjusted according to metabolic needs (Filograna et al., 2021). The mtDNA-CN, while not a direct measure of mtDNA damage, is associated with mitochondrial enzyme activity and adenosine triphosphate production and can therefore serve as a biomarker of mitochondrial function (Ashar et al., 2017; Yue et al., 2018).

The multiple-copy nature of the mitochondrial genome implies that when the mtDNA undergoes a new mutational change, the new variant coexists with other mtDNA molecules with the original sequence within a cell or mitochondria of an individual. The coexistence of different genomes gives rise to mitochondrial heteroplasmy.

Mitochondrial variants accumulated through time differ between population groups that diverged thousands of years ago. In consequence, the related mitochondrial haplotypes (Ht) are grouped into haplogroups (Hg) which allow the matrilineal inheritance of human beings to be traced from their origins in Africa to the geographical distribution of current ethnic groups (Tranah et al., 2011). Mishmar et al. (2003) proposed that the geographic distribution of human mtDNA haplogroups had resulted due to selection mainly driven by adaptation to climate. According to this hypothesis, in colder climates, natural selection would have favoured mtDNA variants that reduced OXPHOS coupling efficiency to increase heat production by proton leakage (Chinnery & Gomez-Duran, 2018; Mishmar et al., 2003). Following this hypothesis and the idea that OXPHOS coupling also alters ROS production and thus stroke susceptibility, some studies have been focused on the association between haplogroups and stroke susceptibility. Some studies based on this idea have obtained significant associations of some haplogroups, such as K, H1 or U, with stroke risk in different European populations (review in Andalib et al., 2017) while others did not (Umbria et al., 2019). Nonetheless this hypothesis has been refuted by several groups (Moilanen, J et al., 2003; Sun, C et al., 2007; Jeedigunta, S.P. et al., 2021).

In recent years, the focus of mtDNA studies has expanded, and a new hypothesis has been gaining interest. This complementary approach, known as the mutational load hypothesis, refers to the synergistic effect of several variants, not looking for associations between cardiovascular disease (CVD) risk and a specific variant but to the accumulative effect of several variants (Piotrowska-Nowak et al., 2019a; Venter et al., 2018).

Based on the evidence that ROS are mainly generated when electrons leak from ETC complexes, we suspect that ROS formation could also be modified by individual variations in ETC complexes. Therefore, variation in genes that encode these complexes may play an important role in ROS regulation, and thus in stroke susceptibility. Furthermore, based on the evidence that mitochondrial dysfunction conditions the cells' energy homeostasis, variation in mtDNA-CN and genetic variation in the NCR, which could alter both replication and transcription of the mtDNA genome, may also play a role in CVD risk.

Given the aforementioned premises, the main goal of this study is to perform a comprehensive evaluation of the association between stroke risk and mitochondrial genetics in a Spanish population from Castilla y León. The more specific objectives are: i) to evaluate the relation between mtDNA-CN and stroke risk, ii) to determine the mitochondrial haplotype and haplogroup of individuals with stroke history and investigate their role concerning stroke risk and iii), to analyse possible differences in the distribution of mitochondrial mutations between stroke cases and controls, and examine the possible accumulative effect of common variants in stroke risk.

2. Material and methods

2.1. Sample collection and DNA extraction

A case-control study was conducted using 110 S patients and 110 corresponding controls taken from the Cardiovascular Disease Risk Study of Castilla y León (Vega Alonso et al., 2007). Cases and controls were matched according to nonmodifiable risk factors, namely, age, sex and geographic origin.

The study of Vega Alonso et al. (2007) was a cross-sectional study initiated in 2004, and followed up in 2009 and 2014, in which 4013

subjects (older than 15 years of age) were randomly selected from Castilla y León population and underwent health examinations in relation to cardiovascular risk factors. From these subjects, blood samples were also obtained, after informed consent, and following the Helsinki protocol (World Medical Association, 2013). Biological samples included in this study were centralized, processed and provided by the Spanish National DNA Bank Carlos III of the University of Salamanca (biobank ID B.0000716 and ISCII-EU grant number PT20/00085), integrated in the "ISCIII Platform Biobanks and Biomodels". The DNA of these samples was extracted using an automated process on an Autopure LS (Gentra System, Minneapolis, MN) and stored at −20 °C.

For each subject, information about age (categories ≤ 44, 45–49, 50–54, 55–59, 60–64 and ≥ 65 years), sex, geographic origin (North, Central and South regions of Castile and Leon), history of hypertension (≥140/90 mmHg), history of diabetes, history of hypercholesterolemia (greater than200 mg/dl), cigarette consumption (smokers, former smokers and non-smokers), presence of overweight or obesity (body mass index ≥ 25 kg/m^2), presence of abdominal perimeter in risk range (risk: ≥ 80 cm for women and ≥ 94 cm for men) and presence of high levels of triglycerides (≥170 mg/dl), was available from the databases of the original study (Vega Alonso et al., 2007).

2.2. Mitochondrial DNA amplification and sequencing

For each sample, the whole mitochondrial genome was amplified in two overlapping fragments of approximately 9 kb and 11 Kb using two distinct sets of primers (SET1 and SET2) previously described (Mikkelsen et al., 2009). This primer design allows to rule out the possibility of NUMT co-amplification due to their location and the target amplicon size, as 77,8% of NUMTs are<500bps (Wei, W. et al., 2022). PCR mix for each reaction consisted of 1 μL (10 pmol/μL) of each primer (Forward and Reverse), 1 μL (10 μM) of dNTPs, 5 μL of 10 X reaction buffer, 2 μL (50 mM) of MgSO4, 0.2 μL (2 U/μL) of Platinum® Taq High Fidelity DNA Polymerase and 1 μL (approximately 50 ng) of DNA in a final volume of 50 μL. The PCR reactions were performed in a Bio-Rad S1000™ Thermal Cycler (Bio-Rad, Hercules, CA) and the amplification program consisted of an initial denaturation step of 2 min at 94 °C, followed by 35 cycles of PCR (30 s at 94 °C, 30 s at annealing temperature – 60 °C for SET1 and 59 °C for SET2 – and 10 s at 68 °C) and a final extension step of 7 min at 72 °C. The PCR amplification results were visualized through 1% agarose gel electrophoresis to further validate the specificity of the PCR.

PCR products were purified using DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA) and quantified using Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA), according to the manufacturer's specifications. Once quantified, for each sample, SET1 and SET2 PCR products were diluted to 0.4 ng/μL and equally mixed to avoid over- representation of any of the fragments. Finally, to prepare the Next Generation Sequencing (NGS) library, 2.5 μL of each individual equimolar mix was placed in a single well of a 96-well plate.

Dual indexed libraries were generated using Nextera® XT DNA Sample Preparation Kit, according to the manufacturer's protocol (Illumina, San Diego, CA). The library was sequenced with paired-end, dual-indexing 2x250 bp cycles per read in the Servei de Genòmica (Universitat Autònoma de Barcelona, http://sct.uab.cat/genomica-bioinformatica/es). Two FASTQ files were generated per sample.

2.3. Mitochondrial DNA copy number (mtDNA-CN) quantification

To a subset of 146 samples, 73 cases and 73 controls, the mtDNA-CN was also evaluated. The relative mtDNA-CN, defined as the copy number ratio of a mitochondrial gene (*Humanin, HN1)* to a single-copy nuclear gene (*Presenilin 2, PSEN2)*, was assayed in duplicate by a quantitative real-time PCR (qPCR) method using the Rotor-gene 6000 System (Qiagen) and the Rotor-Gene SYBR Green PCR kit (Qiagen). Specific primers used to amplify the mt-*HN* gene and the nuclear *PSEN2* gene were as follows: *HN*: F 5′- AGT ACC TAA CAA ACC CAC AGG −3′, R 5′ - TTGGATCAATTGAGTATAGTAGT − 3′; *PSEN2:* F 5′ - CTG TGC ACG CCT CTT CAG T − 3′, R 5′ - CTG TGA GCC TTG GTC TCA A − 3′. Primers were designed to assure HN1-specidicity, carrying out primer blast (NCBI; https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and also alignment using clustal Omega - Multiple Sequence Alignement. EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/) to check specificity against the MT-RNR2 like pseudogenes 1–13 and thus ruling out any possibility of cross amplification with nuclear genes. qPCR conditions consisted of an initial denaturing step of 15 min at 95 °C followed by 35–40 cycles of 15 sec of denaturation at 95 °C, 25 sec of annealing at 57–60 °C and 25 sec of extension at 72 °C. The Cycle Threshold (Ct) difference between replicates was<0.5. Any sample crossing this threshold was repeated to minimize the error in the assay and discarded if the difference persisted.

The mtDNA-CN results were calculated using the comparative-cycle threshold (ΔΔCt) (Livak & Schmittgen, 2001) and Fold Change (Fold change = $2^{-\Delta\Delta Ct}$).

2.4. Data analysis

2.4.1. Analysis of mtDNA sequencing data

FASTQ files from each sample were processed with FASTQC program to perform quality control analyses (Babraham Institute Enterprise, BIE). After FASTQC quality control, data for each sample was imported into mtDNA-Server 1.0.7 (Weissensteiner et al., 2016), which is a free mtDNA NGS analysis pipeline, to identify the sample's haplogroup, homoplasmic and heteroplasmic variants in the sequence and possible contamination. Finally, mtDNA-Server output data was processed with an ad-hoc R application based on Shiny to facilitate downstream analyses. The script is available on the GitHub repository https://github.com/marta-coronado/mtDNA-analysis.

2.4.2. Mutational load calculations and adjustments using MutPred pathogenicity scores

MutPred pathogenicity scores were used to derive "mutational loads", which are useful to test the possibility of a cumulative effect of mildly deleterious (rare) variants in cases and control groups (Elson et al., 2006). The MutPred algorithm assigns a pathogenicity score between 0 and 1, with 0 being a benign substitution. Pathogenic scores above 0.5 can be considered "actionable hypotheses", while scores above 0.75 can be considered "confident hypotheses".

For mutational load calculations, the MutPred scores above 0.5 generated for each of the non-synonymous homoplasmic variants on an individual's mtDNA were summed. Variants with a pathogenicity score below 0.5, which tend to be the most common population variants, were excluded to highlight the impact of rare variants while reducing the effect of population stratification (Venter et al., 2017). Then, the mutational load was adjusted by dividing it by the number of non-synonymous variants presented in each individual. In this way, the effect of the position of an individual in the phylogeny was reduced (Venter et al., 2017).

2.4.3. Statistical comparison between cases and controls

McNemar's test (dichotomous variables) a marginal mean test (variables with more than two categories) and a conditional logistic regression were used to compare the frequency of biochemical and clinical variables (cigarette consumption, hypertension, diabetes, hypercholesterolemia, overweight or obesity, abdominal perimeter and triglycerides) between cases and controls to determine potential confounding variables (stroke risk factors).

A *t*-test for paired samples was used to determine if there were differences in mtDNA Copy number between stroke cases and controls. Haplogroups frequencies were compared using McNemar's test and Odds Ratios (and 95% confidence interval, CI) were calculated.

To compare the mtDNA profile between cases and controls, homoplasmic and heteroplasmic mtDNA variants were compiled into different

matrixes. Concerning heteroplasmies, only reliable point heteroplasmies with an individual frequency of the minor variant of at least 3% and 1000x depth were compiled into the matrix (González et al., 2020). Region 309 – 315 was excluded from the analyses due to difficulties in the alignment of repetitive regions.

To assess a possible imbalance in the distribution of homoplasmic mutations throughout the mitochondrial genome, the number of homoplasmic variants was compared between cases and controls with a Wilcoxon test. Comparisons include the whole mtDNA molecule, dividing the molecule into coding (CR) and non-coding regions (NCR), and stratifying the coding region in groups of genes that configure the ETC complexes and RNAs and the NCR in the three hypervariable regions. Identical analyses were made in the case of point heteroplasmic variants. Adjusted mutational loads (AML) were compared between cases and controls using the one-tailed Wilcoxon test to determine if differences in the number of homoplasmic variants were related to a pathogenic effect. This comparison was performed for both, the whole coding region and complex of the ETC. P-values were corrected for multiple testing (False Discovery Rate, FDR < 5%).

Finally, each mtDNA individual variant - homoplasmic or in heteroplasmy - detected, in at least 5% of cases and/or controls, was individually tested with a McNemar's test to determine if associations with stroke were present. Odds Ratios (and 95% confidence interval, CI) were also calculated.

Statistical analyses were performed using IBM SPSS Statistics 23 (IBM Corp., 2015) and JAMOVI 2.3.21 (Jamovi, 2022). Differences were considered significant at P-values equal or below 0.05.

2.4.4. Possible significance of variants associated with stroke

2.4.4.1. Hits in the phylogeny, population database and nucleotide conservation index (NCI). For variants that appear to be associate with stroke, the number of hits or the number of occurrences in the phylogeny was compiled from the updated mtDNA phylogeny - mtDNA tree Build 17 (van Oven & Kayser, 2009) and from Soares et al., (2009). To calculate the frequency of each variant in the population an online population database with 54,594 human mitochondrial DNA sequences (with size greater than 15.4 kb) was used (Lott et al., 2013).

The nucleotide conservation index (NCI) was estimated for variants associated with stroke across reference sequences of different primate species. A total of 48 sequences were used (for list of species and accession numbers see Supplementary Table S1). Sequences were aligned using Clustal W (Thompson et al., 1994).

2.4.4.2. RNA structure prediction. Secondary structures were performed to understand the structural impact of the different variants. The Secondary structure was generated using the RNAfold web server (http://rnna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi, last accession November 2022) with default parameters established (Gruber et al., 2008). The sequence introduced was either the complete gene or the hypervariable region containing the variant. The minimum free energy, base-pair probabilities and positional entropy of predicted structures were used to estimate the implications in the RNA molecule.

3. Results

3.1. Classical risk factors

The percentage of individuals (cases – controles) included in risk categories for different non-genetic risk factors is shown in Table 1. Sociodemographic variables (age, sex and geographic origin) used to match stroke cases with their corresponding controls can be found in Supplementary Table S2. The most notorious difference is found in hypercholesterolemia, being more frequent in stroke cases (McNemar p-value 0.082). However, the comparison between cases and controls showed no statistically significant differences for any of the risk factors.

Table 1
Sociodemographic, biochemical and clinical characteristics of stroke cases and controls.

	Cases	Controls	p-value	CLR p-value
	n (%)	n (%)		
Cigarette consumption				
Non-smoker	60 (54.5)	62 (56.4)	0.431[b]	0.786
Former smoker	36 (32.7)	38 (34.5)		
Smoker	14 (12.7)	10 (9.1)		
Hypertension (≥140/90 mmHg)	89 (80.9)	88 (80)	1[a]	0.738
Diabetes	19 (17.3)	15 (13.6)	0.556[a]	0.884
Hypercholesterolemia (>200 mg/dL)	58 (52.7)	44 (40)	0.082[a]	0.109
Overweight or obesity (≥25 Kg/m^2)	76 (69.1)	81 (73.6)	0.568[a]	0.842
High abdominal perimeter (>80 cm or > 94 cm)	79 (71.8)	84 (76.4)	0.542[a]	0.539
Triglycerides (≥170 mg/dL)	13 (11.8)	7 (6.4)	0.238[a]	0.206

[a] p-value of McNemar (used for dichotomous variables);
[b] p-value of marginal homogeneity test (used for variables with more than two categories).

Given that these biochemical and clinical characteristics could be influencing each other a conditional logistic regression was performed including those variables that weren't matched between cases and controls. No risk factor presented a statistically significant association with stroke risk (Table 1).

Since these variables do not present differences between cases and controls, they will not be included in further analyses.

3.2. Mitochondrial DNA copy number

The mitochondrial DNA copy number was similar in stroke cases (mean ΔCt = -5.86) in relation to the controls (mean ΔCt = -5.78) (Fold change = 1.35) and the difference was not statistically significant (*t*-test p-value = 0.430).

3.3. Haplogroup frequencies

A summary of haplogroup frequencies for stroke cases and controls is

Table 2
MtDNA macrohaplogroup and haplogroup frequencies in stroke cases and controls.

	Cases	Controls	McNemar p-value	Odd Ratio (CI 95%)
	n (%)	n (%)		
R0	55 (50)	71 (64.55)	0.027	0.5493 (0.312 – 0.943)
H	48 (43.64)	57 (51.82)	0.253	0.720 (0.423 – 1.224)
HV	4 (3.64)	3 (2.73)	1	1.346 (0.294 – 6.159)
V	2 (1.82)	10 (9.09)	0.039	0.185 (0.040 – 0.866)
R0	1 (0.91)	1 (0.91)	1	1 (0.0618 – 16.192)
JT	20 (18.18)	14 (12.73)	0.327	1.524 (0.726 – 3.197)
J	12 (10.91)	7 (6.36)	0.332	1.802 (0.681 – 4.764)
T	8 (7.27)	7 (6.36)	1	1.154 (0.404 – 3.300)
U	28 (25.45)	18 (16.36)	0.144	1.745 (0.900 – 3.386)
U	19 (17.27)	12 (10.91)	0.248	1.705 (0.784 – 3.709)
K	9 (8.18)	6 (5.45)	0.581	1.545 (0.531 – 4.497)
No-R	7 (6.36)	7 (6.36)	1	1 (0.339 – 2.953)
I	2 (1.82)	1 (0.91)	1	2.019 (0.180 – 22.591)
L	0 (0)	2 (1.82)	0.5	0.196 (0.009 – 4.138)
M	1 (0.91)	0 (0)	1	3.027 (0.122 – 75.133)
W	3 (2.73)	2 (1.82)	1	1.514 (0.248 – 9.243)
X	1 (0.91)	2 (1.82)	1	0.495 (0.044 – 5.545)

shown in Table 2. Macrohaplogroup R0 (including haplogroups H, HV, V and R0) was significantly overrepresented in stroke controls (64.55% of controls *vs* 50% of cases; McNemar's test: P = 0.027). When macrohaplogroup R0 was closely examined, haplogroup V appeared overrepresented in stroke controls (9.09% of controls *vs* 1.82% of cases, McNemar's: P = 0.039). In this sense, our results showed that individuals from macrohaplogroup R0 and hg V were 1.82 and 5.41 (inversed odds ratios) times less likely to have a stroke, respectively. This association is not statistically significant after multiple testing correction (FDR < 5%).

3.4. Mutation profile and mutation load

Individual mtDNA profile is reported in Supplementary Table 3 and the number of homoplasmic variants and heteroplasmic positions in cases and controls stratified by mtDNA regions is shown in Table 3. A comparison between the number homoplasmies and heteroplasmies between cases and controls was performed to assess a possible imbalance in their distribution throughout the mtDNA.The analysis of the whole mtDNA molecule (Table 3) showed that the number of homoplasmic mutations was significantly higher in cases (cases (C) = 2525 *vs* controls (CL) = 2176; Wilcoxon test: P = 0.02). Similar results were obtained when the non-coding region was studied separately (Wilcoxon test: P = 0.006). Within the NCR, a significant increase in homoplasmic mutations in the HVR1 (C = 256 *vs* CL = 179; Wilcoxon test P = 0.002) was found. This tendency is also observed in HVR2 and HVR3, although differences were not significant in these cases.

Variants inside the coding region were analysed complex by complex and gene-by-gene. Complex I (NADH dehydrogenase) showed a significant increase in homoplasmic variants in stroke cases in relation to controls (C = 658 *vs* CL = 556, P = 0.041). This can also be observed for genes ND1, ND3, ND4L, ND4 and ND5, with differences in genes ND4 and ND5 being statistically significant. On contrary, ND2 and ND6 show a decrease in homoplasmies in stroke cases. Complex III (cytochrome *b*) also presented a significant increase in homoplasmies in stroke cases in relation to controls (C = 287 *vs* CL = 230, P = 0.002). Homoplasmic variants in Complex IV (Cytochrome *c* oxidase) were similar between cases and controls (C = 176 *vs* CL = 171, P = 0.771), a characteristic observed in all 3 genes (CO1, CO2 and CO3). Similar results were obtained for Complex V (ATP synthase, C = 155 *vs* CL = 160, P = 0.554) and ATP6, ATP8 and the overlapping region between these genes.

Regarding RNA genes, our results showed an increase in the number of homoplasmies in cases in relation to controls in both rRNAs (C = 476 *vs* CL = 429, P = 0.022) and tRNAs (C = 81 *vs* CL = 66, P = 0.21). Differences in rRNA genes were due to differences in RNR1 and RNR2 as no mutation was found in RNR3.

The number of heteroplasmies did not show significant differences between stroke cases and controls in any of the analyses except for the gene CO2, in which heteroplasmic positions were significantly more prevalent in cases than in controls (C = 4 *vs* CL = 0; Wilcoxon test P = 0.046).

In addition, mutational loads of cases and controls were compared to determine if differences in the number of homoplasmic variants were related to a pathogenic effect. Table 4 reports adjusted mutation loads by mtDNA complexes and genes. A higher adjusted mutational load was observed in stroke cases ($\bar{x}$ = 0.364) in comparison to controls ($\bar{x}$ = 0.296), although this difference was not statistically significant (Wilcoxon test: P = 0.109). Analysing by complex and genes, a higher adjusted mutational load in cases was seen in Complex IV and ATP6 with values at the limit of statistical significance (Wilcoxon test: P = 0.05).

3.5. Individual variation association

Each mtDNA variant – in homoplasmy or in heteroplasmy - was individually analyzed to determine if they were associated with stroke risk. The comparison of each between cases and controls allowed to

Table 3
Number of homoplasmic variants and heteroplasmic positions in stroke cases and controls stratified by mtDNA regions.

Region	Number of Homoplasmic Variants			Number of Heteroplasmic Positions		
	Cases	Controls	Wilcoxon p-value	Cases	Controls	Wilcoxon p-value
	Σ (%)	Σ (%)		Σ (%)	Σ (%)	
NC Region	685 (27.13)	557 (25.60)	0.006[b]	45 (34.09)	51 (32.69)	0.665
HVR1	256 (10.14)	179 (8.23)	0.002[b]	17 (12.88)	15 (9.62)	0.713
HVR2	300 (11.88)	258 (11.86)	0.076	19 (14.39)	22 (14.10)	0.729
HVR3	44 (1.74)	36 (1.65)	0.393	9 (6.82)	14 (8.97)	0.445
Outside[a]	85 (3.37)	84 (3.86)	0.912	0 (0)	0 (0)	1
Coding Region	1840 (72.87)	1619 (74.40)	0.056	87 (65.91)	105 (67.31)	0.296
Complex I	658 (26.06)	556 (25.55)	0.041	35 (26.52)	41 (26.28)	0.792
ND1	61 (2.42)	55 (2.53)	0.469	6 (4.55)	4 (2.56)	0.564
ND2	166 (6.57)	173 (7.95)	0.665	3 (2.27)	4 (2.56)	0.705
ND3	33 (1.31)	23 (1.06)	0.210	0 (0)	2 (1.28)	0.157
ND4L	26 (1.03)	16 (0.74)	0.085	1 (0.76)	1 (0.64)	1
ND4	172 (6.81)	125 (5.74)	0.022	10 (7.58)	15 (9.62)	0.614
ND5	167 (6.61)	120 (5.51)	0.017	12 (9.09)	11 (7.05)	0.835
ND6	33 (1.31)	44 (2.02)	0.209	3 (2.27)	4 (2.56)	0.705
Complex III (CYB)	287 (11.37)	230 (10.57)	0.002[b]	8 (6.06)	6 (3.85)	0.593
Complex IV	176 (6.97)	171 (7.86)	0.771	15 (11.36)	14 (8.97)	0.847
CO1	101 (4)	109 (5.01)	0.480	6 (4.55)	5 (3.21)	0.705
CO2	26 (1.03)	25 (1.15)	0.894	4 (3.03)	0 (0)	0.046
CO3	49 (1.94)	37 (1.70)	0.188	5 (3.79)	9 (5.77)	0.285
Complex V	155 (6.14)	160 (7.35)	0.554	7 (5.30)	12 (7.69)	0.251
ATP8	12 (0.48)	10 (0.46)	0.655	0 (0)	1 (0.64)	0.317
ATP8/6	3 (0.12)	1 (0.05)	0.317	0 (0)	0 (0)	1
ATP6	140 (5.54)	149 (6.85)	0.232	7 (5.30)	11 (7.05)	0.346
rRNA	476 (18.85)	429 (19.72)	0.022	19 (14.39)	27 (17.31)	0.179
RNR1	254 (10.06)	233 (10.71)	0.020	7 (5.30)	11 (7.05)	0.346
RNR2	222 (8.79)	196 (9.01)	0.130	12 (9.09)	16 (10.26)	0.375
RNR3	0 (0)	0 (0)	1	0 (0)	0 (0)	1
tRNA	81 (3.21)	66 (3.03)	0.210	3 (2.27)	5 (3.21)	0.480
Whole molecule	2525 (100)	2176 (100)	0.028[b]	132 (100)	156 (100)	0.239

[a] Outside Hypervariable regions; [b] Results that remain statistically significant after FDR < 5% correction.

Table 4
Mean adjusted mutational load in homoplasmic variants in stroke cases and controls.

	Cases	Controls	Wilcoxon p-value
	$\bar{x}$ (CI)	$\bar{x}$ (CI)	
Complex I	0.250 (-0.049—0.550)	0.168 (-0.104—0.440)	0.098
ND1	0.15 (-0.121—0.421)	0.085 (-0.13—0.3)	0.091
ND2	0.117 (-0.113—0.347)	0.06 (-0.112—0.232)	0.086
ND3	0 (0—0)	0.005 (-0.05—0.06)	0.317
ND4	0.017 (-0.084—0.118)	0.01 (-0.064—0.084)	0.225
ND4L	0.007 (-0.062—0.076)	0 (0—0)	0.317
ND5	0.038 (-0.109—0.185)	0.039 (-0.113—0.191)	0.850
ND6	0.007 (-0.065—0.079)	0.034 (-0.111—0.179)	0.176
Complex III (CYB)	0.120 (-0.123 – 0.364)	0.129 (-0.118—0.376)	0.927
Complex IV	0.064 (-0.133 – 0.261)	0.026 (-0.094—0.146)	0.050
CO1	0.022 (-0.09—0.134)	0.005 (-0.051—0.061)	0.343
CO2	0.016 (-0.084—0.116)	0.01 (-0.061—0.081)	0.480
CO3	0.031 (-0.116—0.178)	0.011 (-0.068—0.09)	0.128
Complex V	0.027 (-0.097—0.151)	0.057 (-0.126—0.239)	0.112
ATP6	0.017 (-0.083—0.117)	0.048 (-0.123—0.219)	0.050
ATP6/8	0.006 (-0.053—0.065)	0 (0—0)	0.317
ATP8	0.01 (-0.062—0.082)	0.005 (-0.044—0.054)	0.414
Global	0.364 (0.067—0.661)	0.296 (-0.004—0.596)	0.109

detect three variants (m.73G, m.11719A and m.14766G)- all of them in homoplasmy - that were significantly overrepresented and four variants (m.72C, m.4580A, m.15904 T and m.16298C) – also homoplasmic - that were underrepresented in stroke cases (Table 5).

Variants m.72C, m.4580A, m.15904 T and m.16298C were more prevalent in controls and individuals with these variants were between 3.06 and 5.99 times (inversed odd ratios) less likely to have a stroke. These variants may be related to the protective role of haplogroup V, as they are descriptive of the Hg (Supplementary Fig. 1). In contrast, variants m.73G, m.11719A and m.14766G are extremely common across linages and are not generally associated with a particular haplogroup. Individuals with either one of these three risk variants were approximately 2 times more likely to have a stroke. As variants m.72C, m.4580A, m.15904 T and m.16298C define Hg V, their association with stroke risk was evaluated altogether using a conditional logistic regression (Cox regression). None of the variants reached statistical significance (Supplementary Table 4).

Stability analyses were performed to predict the impact of these mutations. Several measures as the number of hits in the mtDNA phylogeny, the frequency in the population database and the conservation index (NCI) at the nucleotide level were calculated, and results are presented in Table 6.

Positions 4580, 11719, 14,766 and 15,904 appear to be fairly stable as they present 4 or fewer hits in the phylogeny while positions 72, 73 and 16,298 present a higher number and could be considered non-stable positions. Variants in positions 73, 11,719 and 14,766 are highly prevalent in the database population (frequency greater than 75%), whereas variants in positions 72, 4580, 14,766 and 15,904 have a low frequency (<2%).

Using the previously proposed method (RNAFold web server) to predict the impact of these mutations on the stability of the secondary structure of the mtDNA, we studied the 7 positions that had a statistically significant association with stroke risk. A summary of these results is presented in Table 7. Variants m.73A > G, m.4580G > A, m.11719G > A imply a conformational rearrangement of the fragments (HVR2, *ND2* and *CYB* respectively). While the minimum free energy (MFE) of variants m.72 T > C and m.73A > G decreases compared to the wild-type molecule, the MFE of variants m.4580G > A and m.11719G > A increases. Variants m.14766C > T, m.15904C > T and m.16298 T > C led to a folded structure with the same MFE as the wild-type structure. Although variant m.15904 T does no change the MFE of the folded structure, changes in the entropy of nucleotides surrownding this position can be seen in Fig 1. Similar graphical representations for each variant can be foun in Supplementary Table 5.

4. Discussion

Stroke, the second leading cause of death worldwide, is a complex disease influenced by different modifiable and nonmodifiable risk factors (Donkor, 2018). Among these, reactive oxygen species (ROS) are gaining importance and since mitochondria are their main producers, the latest stroke-associated research is trying to elucidate the role of these organelles in stroke risk (Balaban et al., 2005; Chang et al., 2010). In this line, some studies are focusing on the mitochondrial genome, which encodes for OXPHOS complexes related to ROS production, to find significant associations with stroke risk (Andalib et al., 2017). To date, most of these studies have been working with mitochondrial haplogroups; however, few of them have evaluated the importance of the mtDNA variation of the whole molecule. In the present study, we aimed to determine the role of whole mtDNA variation in stroke.

The characterization of biochemical and clinical variables showed no statistically significant differences between cases (C) and controls (CL) (Table 1). This result was unexpected as available literature considers hypertension and hypercholesterolemia as the strongest determinants of stroke risk after age (Donkor, 2018; Umbria et al., 2017). On one hand, the fact that hypertension was not significantly more frequent in cases could be due to the high number of control subjects that suffered from hypertension (80%) in our sample. This could be explained because of the advanced age of the subjects since more than 85% of them were

Table 5
Possible stroke-associated variants.

Variant	Location	Cases	Controls	McNemar p-value	Odds Ratio (95% CI)	Putative role
		n (%)	n (%)			
m.72 T > C	HVR2	2 (1.82)	11 (10.00)	0.012	0.167 (0.036 – 0.771)	Protective
m.73A > G	HVR2	57 (51.82)	38 (34.55)	0.012	2.038 (1.185 – 3.506)	Risk
m.4580G > A	ND2	2 (1.82)	11 (10.00)	0.022	0.167 (0.036 – 0.771)	Protective
m.11719G > A	ND4	55 (50.00)	37 (33.64)	0.014	1.973 (1.145 – 3.340)	Risk
m.14766C > T	CYB	56 (50.91)	38 (34.55)	0.014	1.965 (1.142 – 3.380)	Risk
m.15904C > T	TT	2 (1.82)	11 (10.00)	0.022	0.167 (0.036 – 0.771)	Protective
m.16298 T > C	HVR1	5 (4.55)	14 (12.73)	0.049	0.327 (0.113 – 0.941)	Protective

Table 6
Complete results of stroke homoplasmic mutations analysed.rCRS = revised Cambridge reference sequence.

Variant	Cases: n (%)/ Controls: n (%)	McNemar's p-value	OR (95% CI)	Stability analysis				
				rCRS	Distribution in population database (%)	N. Hits phylogeny (PhyloTree.org)	N. Hits Soares et al.	Nucleotide Conservation Index (%)
m.72 T > C	2 (1.82) / 11 (10)	0.012	0.167 (0.036 – 0.771)	T	T: 98.12; C: 1.79; A: 0.005; G: 0.08; Del: 0.002	9	6	GAP: 87.5; T: 12.5
m.73A > G	57 (51.82) / 38 (34.55)	0.012	2.038 (1.185 – 3.506)	C	G: 75.91; A: 24.08; C: 0.005; Del: 0.002	12	11	GAP: 87.5; A: 2.1; C: 6.3; G: 4.2
m.4580G > A	2 (1.82) / 11 (10)	0.022	0.167 (0.036 – 0.771)	G	G: 98.31; A: 1.69	2	2	A:85.4; G:14.6
m.11719G > A	55 (50) / 37 (33.64)	0.014	1.973 (1.145 – 3.340)	G	A: 77.23; G: 22.77	4	7	A: 60.4; T: 4.2; C: 25; G:10.4
m.14766C > T	56 (50.91) / 38 (34.55)	0.014	1.965 (1.142 – 3.380)	C	T: 76.71; C: 23.29	4	10	A: 25; T: 8.3; C: 66.7
m.15904C > T	2 (1.82) / 11 (10)	0.022	0.167 (0.036 – 0.771)	C	C: 98.40; T: 1.59; A: 0.002	1	1	GAP: 4.2; A: 2.1; T: 25; C: 66.7; G:2.1
m.16298 T > C	5 (4.55) / 14 (12.73)	0.049	0.327 (0.113 – 0.941)	T	T: 93.37; C: 6.61; A: 0.002; G: 0.007; Del: 0.012	18	13	A: 29.2; C: 8.3; T:37.5; G:2.1; GAP: 22.9

above 60 years old, and it is known that hypertension prevalence significantly increases with age, especially from 60 years old (Fryar et al., 2017). On the other hand, hypercholesterolemia differences among groups (C: 52.7% and CL:40%) were higher than with hypertension, triglycerides and the percentage of smokers, and the absence of significant differences could be due to sample size. However, finding no differences in these possible risk factors in our sample strengthens the reliability of the results produced in this study given that there was not a possible bias induced by these variables.

The association between mtDNA-CN and cardiovascular disease risk, although commonly accepted, has yet to be thoroughly investigated. Lower mitochondrial DNA content has been associated with an increased risk of cardiovascular disease (Wei et al., 2021; Yue et al., 2018). Nonetheless, this association has been observed to vary in magnitude depending on the specific CVD studied, and even between studies. In our study, we found no association between mitochondrial DNA copy number and stroke risk. MtDNA-CN varies depending on many factors, many of which are matched between cases and controls in our sample (age, sex and geographical origin) or present no differences (cigarette consumption, hypertension, hypercholesterolemia, etc). This fact increases the value of our results, as we have controlled many factors that could contribute to mtDNA-CN changes, testing the association between mtDNA-CN and stroke risk with more reliability. Even so, some remarks should be considered: a) the sample size, as it could be considered small; b) the fact that we perform a relative quantification instead of an absolute one; and c) the non-differentiation between cellular and cell free mtDNA in our analyses.

Regarding haplogroup distribution between cases and controls, our analyses showed that haplogroup V was the only haplogroup significantly overrepresented in controls (Table 2). This result, which marks V as a potential genetic protective factor for stroke has not been reported before in any stroke-associated study. However, this association loses statistic significance after multiple testing correction. Hg V has been linked with Diabetes mellitus. While Piotrowska-Nowak et al. (2019b) found a lower than expected prevalence of Hg V in Type 2 Diabetes mellitus when compared with the control group (in a Polish population), Soini et al. (2012) found a 3-fold higher frequency of mtDNA Hg V in patients with matrilineal diabetes (in Finnish population). Haplogroup V is most frequent in the Sami population (33–68%), inhabiting the Northern Shield - north of finland, Norway, Sweden and the Koala Peninsula of Russia- (Ingman & Gyllensten, 2007). As previously stated, in colder climates, OXPHOS coupling efficiency decreases to increase heat production (Chinnery & Gomez-Duran, 2018) and, although there are no studies about hg V coupling efficiency, we could hipotetize that a sligh uncoupling might exists reducing ROS production, and mtDNA damage in these individuals. Our study did not replicate other associations previously found in other studies. Mitochondrial Hgs K (Chinnery et al., 2010) and H1 (Rosa et al., 2008) have been reported as protective factors for stroke in European population and haplogroup J with a lower presence of ischemic cardiomyopathy (Fernández-Caggiano et al., 2012).

Concerning the mtDNA profile, we observed a significant increase of homoplasmic variants in stroke cases in comparison to controls (Table 3), this increase was especially important in the genes corresponding to complexes I and III, and in rRNAs genes. Moreover, our results showed a similar distribution of point heteroplasmies between stroke cases and controls. In general, it is accepted that heteroplasmy is associated with many clinical presentations (Wallace & Chalkia, 2013). Nevertheless, our results can be explained by the proven fact that heteroplasmy also occurs with appreciable frequency in the general population (Floros et al., 2018; Ramos et al., 2013) and may also been acquiered tue to aging and be unrelated to cardiovascular health status. Regarding homoplasmic variant results, together with the fact that mitochondrial ROS is mainly generated when electrons leak from ETC complexes I and III (Chen & Zweier, 2014), supports the idea that individual variations in these complexes can modify electron leakage predisposition, and thus ROS formation and stroke risk (Chang et al., 2010). The increase in mutations in the control region observed in our results could be influencing mitochondrial DNA regulation (replication and transcription). However, it is risky to obtain a definitive conclusion as we do not know the character - neutral or pathogenic - of the different variants (Sun & Yu, 2019) and we did not observe differences in mtDNA-CN that could indicate that the increase in mutations in this region is affecting the mitochondrial genome replication.

The mutational load analysis presented in this study focuses on homoplasmic population variants with a score above 0.5 that are predicted to be mildly deleterious to protein function, and thus tend to be rare variants that are less likely to be impacted by population stratification (Pereira et al., 2011; Venter et al., 2017). We observed a higher global mutational load (adjusted) in stroke cases, although the difference is not statistically significant (Table 4). An association between a higher mutational load and atherosclerosis was found by Piotrowska-Nowak et al. (2019a), in contrast Venter et al. (2017) could not identify a relationship between the mutational load and CVD phenotypes. Interestingly, the adjusted mutational load of Complex IV is significantly higher in stroke cases than controls ($p = 0.05$). The enzyme cytochrome *c* oxidase (CO, Complex IV) is the last enzyme in the OXPHOS located in the membrane and although CO is encoded by both the mitochondrial and the nuclear genomes, its catalytic centres are encoded exclusively by two mitochondrial genes, CO1 and CO2 (encoding CO subunits I and II, respectively). Mutations in CO genes have been linked to Leigh Syndrome (Bruno et al., 1999; Campos et al., 2001; Clark et al., 1999) and MELAS syndrome (Wang et al., 2021). And the majority of polymorphism associated with stroke risk reported by Rosa et al. (2008)

Table 7
Summary of the results obtained with the secondary structure prediction of the previously described variants. WT = wild type variant, Mut = mutated variant.

	m.72 T > C		m.73A > G		m.4580G > A		m.11719G > A		m.14766C > T		m.15904C > T		m.16298 T > C	
	WT	Mut	WT	Mut	WT	Mut	WT	Mut	WT	Mut	WT	Mut	WT	Mut
Region of analysis	HVR2		HVR2		ND2		ND4		CYB		TT		HVR1	
Minimum free energy (kcal/mol)	−67,4	−69,4	−67,4	−71,2	−117,68	−114,88	−181,64	−180,54	−180,7	−180,7	−14	−14	−54.30	−54.30
Free energy of the thermodynamic ensemble (kcal/mol)	−72,97	−74,31	−72,97	−75,7	−131,12	−128,37	−200,52	−198,93	−198,84	−199,15	−15,09	−14,8	−60.42	−60.50
Freq of the MFE structure in the ensemble (%)	0,01	0,03	0,01	0,07	0	0	0	0	0	0	16,97	27,12	0.00	0.00
Ensamble diversity	43,93	37,99	43,93	32,79	105,5	109,85	219,45	213,82	210,67	215,66	9,14	5,24	71.28	70.84
Centroid Minimum free energy (kcal/mol)	48,2	−62,9	48,2	−67,5	−103,5	−100,7	−145	−145,6	−132,85	−132,85	−13,8	−13,8	−47.10	−44.40
Secondary structure differences	No		Yes (slight)		Yes		Yes		No (or slight)		No (or slight)		No	

were localized in complexes I and IV, whose deficiencies are the most frequently observed abnormalities of the OXPHOS system. Considering this information, our results are consistent with an increase of mutational load in patients with stroke, as an accumulation of mildly deleterious mutations in these genes that may alter CO function and in turn mitochondrial function and ROS generation, both known risk modifiers of stroke.

In the individual analysis of mitochondrial mutations, variants m.72C, m.4580A, m.15904 T and m.16298C were found to be overrepresented in controls and have a protective role against stroke risk (Table 6). Variant m.72C > T is located in the control region, more precisely in the HVR2. Umbria et al. (2021) described it as a protective variant against myocardial infarction, which is aligned with our results, as seems to also be a protective factor against stroke. M.4580G > A is a synonymous SNP in the ND2 gene and has not been associated with any condition. The ND2 gene shares a similar structure to sodium and potassium antiporters and may be involved in proton pumping. Variant m.15904C > T is located in the mitochondrial threonine tRNA gene, in the D-loop of the secondary structure, not modifying the codon-anticodon pairing. This variant has been described as benign in cases of juvenile myopathy, encephalopathy, lactic acidosis and stroke (Wong et al., 2020) and has been classified as likely benign (MitoTIP score = 1.7). Although the functional impact of the MFE changes in D-loop variants and even non-tRNA gene variants is unclear, we could hypostatize that the decrease in entropy observed in this tRNA gene variant results in an increased stability and therefore increased availability and better protein translation. M.16298C is located in HVR1 and has not been described before as a variant that modifies risk for disease. Nonetheless, these four variants may not play a role in stroke risk independently and could be reflecting the protective role of haplogroup V.

In contrast, variants m.73G, m.11719A and m.14766G were found to be overrepresented in cases and increase the risk of stroke. Variant m.73A > G, also located in HVR2, has been linked to an increased risk of obesity (Eaaswarkhanth et al., 2019), a known risk factor for CVD. In contrast, Umbria et al., (2020), found this polymorphism to play a protective role against myocardial infarction. It had been hypothesized that this variant could alter the replication and/or transcription of the mtDNA (Umbria et al., 2020). With the results of the mtDNA-CN analysis performed in this study, we can infer that these variants do not alter significantly the replication of the mitochondrial genome. M.11719G > A is a synonymous SNP in the ND4 gene. ND4 gene product is a subunit of the respiratory complex I which accepts electrons from NADH, transfers them to ubiquinone and uses the energy released to pump protons across the mitochondrial inner membrane. A mutation in ND4 (m.11778G > A) has been related to over 50% and 90% of all LHON cases among Caucasians and Asians, respectively. In concordance with our results, Rosa et al.(2008) described m.11719G as a protective variant for ischemic stroke and proposed that the proximity of this variant to the known pathogenic polymorphism m.11778G > A suggests that it is located close or in an important functional domain and could potentially alter ND4′s function. Furthermore, 11719G > A has been found to be more prevalent in multiple sclerosis patients (Al-Kafaji et al., 2022). Finally, variant m.14766C > T is a non-synonymous variant located in the CYB gene. This mutation results in the amino-acid substitution Thr7Ile. This change is involved in electron flow and proton pumping and could lead to a decrease in the efficiency of the complex III Q cycle and an increase in the production of ROS (Beckstead et al., 2009).

Our stroke-associated case-control study based on mtDNA next-generation sequencing has allowed us to suggest several genetic risk modifiers for stroke.

Several studies have analysed changes in the distribution of mtDNA variants mostly in relation to cancer (Kazdal et al., 2017; Pérez-Amado et al., 2020), Down Syndrome (Hefti et al., 2017) and Leber Hereditary Optic Neuropathy (LOHN) (Piotrowska-Nowak et al., 2019b). However, to date, no study of this kind related to stroke has been published, being

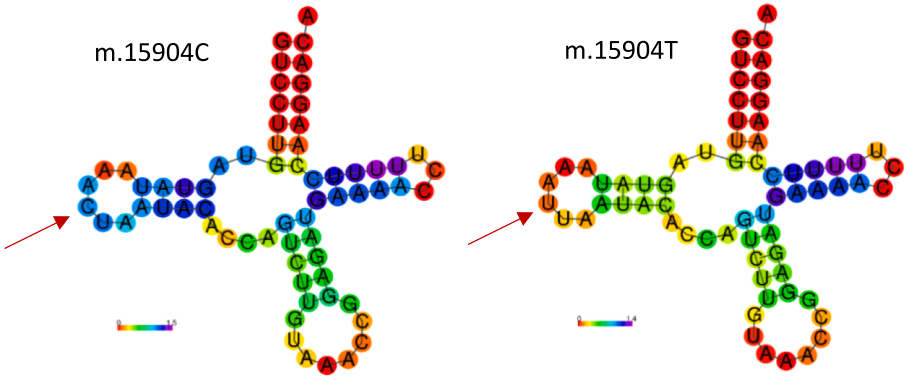

Fig. 1. Entropy changes in tRNA Thr with variant m.15904C > T. A decrease in entropy can be observed in the nucleotides surrounding the variant m.15904 T.

the present the first study of this type. In this regard, we observed an increase in homoplasmic variants in complex III further supporting the implication of this complex in ROS production and thus in stroke risk.

The study of the mutational load proved the cumulative effect of homoplasmic mtDNA complex IV variants with pathogenicity scores above 0.5 in stroke risk and finally, the analyses of the different mtDNA variants exposed variants m.73A > G, m.11719G > A and m.14766C > T as genetic risk factors.

In our study, we did not find an association between mtDNA-CN and stroke risk. This lack of association is quite robust as it is not affected by the impact of other variables also associated with changes in mtDNA-CN.

In conclusion, our findings support the hypothesis that mtDNA variation involved in OXPHOS coupling efficiency and in other mechanisms that modulate ROS production, influences stroke risk but these risk does not appear to be dependent on mtDNA copy number.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work is supported by Ministerio de Economía y Competitividad (project: CGL2014-53781-R) and by Generalitat de Catalunya (Ref. 2017 SGR 1630). We thank Dr. Pere Puig Casado for his advice regarding the statistical analyses.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2023.10.001.

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