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High number of mitochondrial DNA alterations in postmortem brain tissue of patients with schizophrenia compared to healthy controls

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ABSTRACT

Previous studies have shown mitochondrial dysfunction in schizophrenia (SZ) patients, which may be caused by mitochondrial DNA (mtDNA) alterations. However, there are few studies in SZ that have analyzed mtDNA in brain samples by next-generation sequencing (NGS). To address this gap, we used mtDNA-targeted NGS and qPCR to characterize mtDNA alterations in brain samples from patients with SZ ($n = 40$) and healthy controls (HC) ($n = 40$). 35 % of SZ patients showed mtDNA alterations, a significantly higher prevalence compared to 10 % of HC. Specifically, SZ patients had a significantly higher frequency of deletions (35 vs. 5 in HC), with a mean number of deletions of 3.8 in SZ vs. 1.0 in HC. Likely pathogenic missense variants were also significantly more frequent in patients with SZ than in HC (10 vs. three HC), encompassing 14 variants in patients and three in HC. The pathogenic tRNA variant m.3243A>*G* was identified in one SZ patient with a high heteroplasmy level of 32.2 %. While no significant differences in mtDNA copy number (mtDNA-CN) were observed between SZ and HC, antipsychotic users had significantly higher mtDNA-CN than non-users. These findings suggest a potential role for mtDNA alterations in the pathophysiology of SZ that require further validation and functional studies.

1. Introduction

Schizophrenia (SZ) is a complex spectrum disorder that affects approximately 1 % of the global population and represents a significant burden in terms of morbidity and mortality (Birnbaum and Weinberger, 2017). It manifests as a combination of positive symptoms, such as hallucinations and delusions; negative symptoms, including avolition and withdrawal; and cognitive deficits in multiple domains, including attention, working memory, verbal learning and memory, and executive function (APA, 2013; Kahn et al., 2015). The largest twin study of SZ to date reported a heritability of 79 % (Hilker et al., 2018), but the interplay between an individual's genetic makeup and environmental factors has been identified as critical to the development of SZ (Trifu et al., 2020). Genetic risk factors are thought to include thousands of common genetic variants (single nucleotide polymorphisms, SNPs) that have a small effect on an individual's risk, and a plethora of rare genetic variants (copy number variants (CNVs) and single nucleotide variants (SNVs)) that have a larger individual effect on risk (Liu et al., 2023; Trubetskoy et al., 2022). Manly, their biological effects are concentrated in the brain, and many of the same variants also increase the risk of other psychiatric disorders, such as bipolar disorder, autism, and other neurodevelopmental disorders (Tandon et al., 2024). However, despite the identification of SNPs, SNVs, and CNVs involved in SZ, the specific set of genetic factors underlying the pathophysiological mechanisms remains unknown (Wahbeh and Avramopoulos, 2021).

Mitochondria are cellular organelles responsible for the production of adenosine triphosphate (ATP), which serves as the source of cellular energy. These organelles contain their own genome, the mitochondrial

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DNA (mtDNA), which consists of a double circular molecule of 16,569 base pairs (bp) encoding 37 genes, including 13 polypeptides, 22 tRNAs, and 2 rRNA subunits (Garcia et al., 2017). The mtDNA also contains a 1124 bp triple-stranded non-coding region called the d-loop, which acts as a promoter for mtDNA-encoded genes and houses critical transcription and replication elements (Nicholls and Minczuk, 2014). Essential subunits of the electron transport chain (ETC), required for oxidative phosphorylation and ultimately ATP production, are encoded in genes between the heavy strand replication origin O_H (nucleotides 110–441) and the light strand replication origin O_L (nucleotides 5721–5798), a region known as the major arc. The number of mitochondria and mtDNA molecules in a cell varies depending on the cell type and its energy requirements, with cells with higher energy requirements, such as muscle cells or neurons, containing more mitochondria. On average, a human cell can contain from a few hundred to several thousand mitochondria (Chapman et al., 2020). The number of mtDNA molecules per mitochondrion also varies (typically, between 2 and 10), resulting in approximately 1000 mtDNA molecules in a human cell (Valiente-Pallejà et al., 2022), although human oocytes contain between 100,000 and 200,000 mtDNA copies (Otten and Smeets, 2015), and some recent estimates suggest that a single neuron can reach up to two million mitochondria (Misgeld and Schwarz, 2017). Given the high energy demands of brain tissue and the resulting high number of mitochondria, the brain is highly susceptible to mitochondrial dysfunction (Nissanka and Moraes, 2018).

Systemic abnormalities associated with SZ, such as inflammation (Müller, 2018), redox dysregulation (Kim et al., 2017), and oxidative stress (Jiang et al., 2019), involve mitochondria, and several lines of evidence suggest that mitochondrial abnormalities may contribute to impaired brain function (Konradi and Öngür, 2017), including altered mitochondrial metabolism (Prabakaran et al., 2004; Rosenfeld et al., 2011; Valiente-Pallejà et al., 2020) and a reduction in the number of mitochondria in several brain regions (Das et al., 2022; Roberts, 2017). Mitochondrial dysfunction can be caused by mutations in the mitochondrial DNA (mtDNA), which can be maternally inherited and associated with various syndromes, or acquired during life, and associated with complex traits, including aging and neurodegenerative diseases (Schon et al., 2012; Valiente-Pallejà et al., 2022). Mitochondrial function depends on several characteristics of the mtDNA such as polyploidy and mutation rate. Polyploidy refers to the presence of multiple copies of mtDNA in a cell, allowing for mutations to occur only within in a subset of these mtDNA copies, a state called heteroplasmy (hp), or in all mtDNA copies, a state called homoplasmy. The mutation rate refers to the rate at which mutations are introduced and it is estimated to be about ten times higher in the mtDNA than in the nuclear DNA (Sharma et al., 2005). mtDNA mutations include single nucleotide variants (SNVs) and large rearrangements (deletions and duplications). The 1124 bp d-loop region is a hotspot for SNVs and deletion events are typically centered in the mitochondrial major arc between positions 8469–13,447 (Bua et al., 2006). In addition, changes in mtDNA copy number (mtDNA-CN) can cause mitochondrial dysfunction and have been suggested as a predisposing factor for neurodegeneration (Venkatesan et al., 2023). To date, more than 300 SNVs and rearrangements in mtDNA have been identified as contributing to primary mitochondrial disorders (Li et al., 2019), although only 95 have been confirmed as pathogenic (Gusic and Prokisch, 2021). In this study, we aimed to investigate mtDNA SNVs, rearrangements, and mtDNA-CN by analyzing postmortem brain samples from individuals diagnosed with SZ and healthy controls (HC).

2. Materials and methods

2.1. Human brain samples

Post-mortem brain samples were collected during autopsies performed at the Basque Institute of Legal Medicine (Bilbao, Spain) between 2010 and 2018, according to the guidelines of the research and ethics committees for post-mortem brain studies, specifically Law 14/2007 and RD 1716/2011. The study protocols were reviewed and approved by the Ethics Committee of the Institut d'Investigació Sanitària Pere Virgili (approval number 147/2018, date 09/27/2018).

Only samples with a postmortem interval (PMI) $<$ 24 h were included. Gray matter samples from the dorsolateral prefrontal cortex (DLPFC, approximating Brodmann area (BA) 9) were carefully dissected, avoiding the white matter, and immediately stored at −80 °C until DNA extraction. This study included samples from 40 individuals diagnosed with SZ and 40 healthy controls (HC). A retrospective search of the subjects' medical records was performed for antemortem diagnoses of SZ meeting DSM-IV or ICD-10 criteria. Clinical diagnoses of SZ were all made by a board-certified psychiatrist from the Basque Health System (Osakidetza). Cases with additional psychiatric or neurological diagnoses, including a history of substance abuse, were excluded. Each SZ case was paired with a matched HC subject, matched for sex, age, and PMI of the case, and with no evidence of psychiatric or neurological conditions, according to available antemortem medical records. The demographic characteristics of all HC and SZ subjects are summarized in Table 1. Total DNA was extracted from postmortem tissues using Gentra Puregene® reagents (Qiagen, Germany) according to established procedures (Torrell et al., 2013).

2. 2 mtDNA-targeted next-generation sequencing (NGS)

2.2.1. Enrichment of mtDNA

A 50 ng aliquot of total DNA served as the template for amplification of mtDNA in two overlapping fragments of 8329 and 8605 bp using previously described primers (Fendt et al., 2009). Long-range PCR amplification was performed using TaKaRa LA Taq® Hot Start Version (Takara-Bio, Code No. RR042Q, Japan) under the conditions described in Supplementary Table 1. The DNA sample of one HC could not be successfully amplified and was therefore removed from subsequent NGS analyses.

2.2.2. Library preparation and NGS

Purified amplicons were quantified using a Qubit 4 fluorometer (Fisher Scientific, Invitrogen™ Q33238, Madrid, Spain). Equimolar amounts of the two fragments were pooled (100–250 ng of DNA per subject). DNA libraries were prepared using the Illumina DNA Prep Kit with Tagmentation (Illumina Inc., Ref. 20,018,705, Madrid, Spain) according to the manufacturer's instructions. Final libraries were quantified and qualified using the Qubit 4 Fluorometer and 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA), respectively. All libraries were pooled at 750pM with 1 % Phix and sequenced on an Illumina NextSeq 2000 instrument (Illumina Inc., CA, USA) using a 2 × 150 pb paired-end protocol.

Table 1
Characteristics of the human postmortem samples analyzed in the study.

	HC	SZ
Sex		
Female/Male, *n* (%)	7 (17.5) / 33 (82.5)	7 (17.5) / 33 (82.5)
Age, mean ± *S*.D. (y)	48.4 ± 15.2	48.6 ± 15.4
PMI, mean ± *S*.D. (h)	19.1 ± 2.2	19.5 ± 2.6
Cause of death, *n* (%)		
Accident	28 (70)	2 (5)
Natural	12 (30)	16 (40)
Suicide	0	22 (55)
Antidepressant use, *n* (%)	0	3 (7.5)
Antipsychotic use, *n* (%)	0	20 (50)
Atypical antipsychotics	0	17 (85)
Typical antipsychotics	0	3 (15)

h: hour; HC: healthy controls; *n*: number of individuals; PMI: postmortem interval; S.D.: standard deviation; SZ: schizophrenia; y: year.

2.2.3. Quality control of NGS data

Quality control was performed on FASTQ files using FastQC v0.11.9 (Andrews, 2010) and MultiQC v.1.14 (Ewels et al., 2016). Cutadapt (v.3.4) (Martin, 2011) was used to trim 15 bases from the beginning of reads, remove bases with Phred quality scores less than 20 (sequencing error rate greater than 1 %) from the 3′ and 5′ ends, and then filter out reads shorter than 115 bases. SAMtools (v.1.13) was used to calculate the read depth at each position, and the mean coverage was determined (Li et al., 2009). For the classification of potentially pathological alterations, the hp threshold was set at 5 %.

2.2.4. Analysis of mtDNA rearrangements

MitoSAlt (v.1.1.1) was used to detect and quantify mtDNA rearrangements (Basu et al., 2020). We used default parameters and set the maximum size of tolerated deletions to 16,055 bp, as this is the maximum reported mtDNA rearrangement (Damas et al., 2014). Deletions larger than 16,055 bp were automatically reclassified as potential reverse duplications in the reverse orientation based on the tool's algorithm (Basu et al., 2020). Rearrangements smaller than 100 bp and with both breakpoints within the d-loop were excluded.

2.2.5. Analysis of mtDNA SNVs

The Mitoverse platform (https://mitoverse.i-med.ac.at), which uses mtDNA-Server v2 and Mutserve for variant calling, was used for SNV identification (Weissensteiner et al., 2021, 2016). Nonsynonymous variants with a MutPred score of 0.5 or greater, indicating potentially deleterious effects, were included in the analysis. APOGEE 2, a mitochondrial-centered ensemble method, was used to predict the pathogenicity of missense variants (Bianco et al., 2023). This tool combines information from various predictors and region-wise assessments of genome fragility, as well as mechanistic analyses of specific amino acids that have pronounced long-range effects on protein structure (Bianco et al., 2023; Piotrowska-Nowak et al., 2019). tRNA and rRNA gene variants with a MITOTIP score of likely pathogenic (LP), possible pathogenic, or confirmed pathogenic according to MITOMAP (https://www.mitomap.org) were evaluated (Lott et al., 2013). Conservation scores for the variants were also obtained from MITOMAP. For missense variants, conservation scores or predicted conserved elements were additionally obtained using the PhastCons (Pollard et al., 2010) or phyloP (Siepel et al., 2005) tools implemented in MitImpact 3 (http://mitimpact.css-mendel.it) (Castellana et al., 2021). Finally, mtDNA haplogroups were determined using HaploGrep 3 (https://haplogrep.i-med.ac.at), a reliable and innovative algorithm for automatic classification of mitochondrial DNA haplogroups (Schönherr et al., 2023).

2.3. Quantification of mtDNA-CN

The mtDNA-CN was quantified using a quantitative PCR (qPCR) assay adapted from Rosa et al. 2021, using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Cat. No. A2574, Bedford, MA, USA) (Rosa and Malik, 2021). We targeted a mitochondrial fragment in the d-loop (hMito) and a single-copy region of the nuclear gene, the beta-2-microglobulin (*B2M*). Stock standards for hMito and *B2M* PCR products were prepared at a concentration of 1 × 10^9 copies/μL. Subsequently, 10-fold dilution standards (2–8 logs) were prepared for each PCR product. qPCR reactions were performed in triplicate on an ABI PRISM 7900 HT system (Applied Biosystems, Madrid, Spain), and values deviating more than 0.5 standard deviation (SD) from the mean were excluded. Absolute quantification, which determines the absolute number of mtDNA copies, was performed by dividing the single hMito copy by two copies of *B2M,* as previously described (Rosa and Malik, 2021).

2.4. Statistical analysis

Normality of continuous variables was assessed using the Shapiro-Wilk test. For normally distributed variables, *t*-tests were used to compare groups, while the Mann-Whitney U test was used in cases of non-normal distribution. Discrete data were evaluated by using the Pearson chi-squared test when less than 20 % of cells had less than 5 observations, otherwise Fisher's exact test was used (Kim, 2017). Spearman's Rho was used to understand the strength of the relationship between age and PMI and with the mtDNA-CN. The significance level was set at $p < 0.05$. All descriptive and statistical analyses were performed using jamovi 2.3.28 statistical software (The jamovi, 2022).

3. Results

3.1. Large-scale mtDNA rearrangements

We obtained mtDNA sequence data from 40 patients with SZ and 39 HC. For all the included samples, we achieved 100 % coverage with an average depth of 10,102 reads per base.

In our data set, 44 rearrangements were detected (median size=3888 bp; median hp=9.2 %). 23 % of the individuals in the SZ group and 13 % of the HC group carried mtDNA rearrangements (Table 2). The characteristics of the mtDNA rearrangements were then examined (Supplementary Table 3). The total number of deletions was significantly higher in the SZ group than in the HC group (34 vs. 5, respectively). The number of mtDNA deletions per participant was also higher in the SZ group than in the HC group ($t = 2.502$, $p = 0.03$). Despite no significant difference in deletion size and hp level was observed between groups, SZ patients had larger deletions and higher hp levels (size=3931 bp; hp=9.3 %) compared to HC (size=3640 bp; hp=6.2 %) ($U = 84$, $p = 0.98$ for size and $U = 65$, $p = 0.42$ for hp level, respectively). Notably, no duplications with hp ≥5 % were detected in the SZ group, whereas HC had 5 duplications with a median size of 1504 and a median hp level of 9.3 %. No association was found between the presence of mtDNA rearrangements and age, sex, or PMI (Supplementary Table 2). Similarly, the number of deletions, mean size of deletions, and hp levels did not differ between patients who died of suicide or natural causes ($t = 0.3007$, $p = 0.77$; $t = -0.072$, $p = 0.945$; $U = 4$, $p = 0.19$, respectively). In addition, no association was found between the number of deletions and the cause of death (between violent deaths (accidents or violent suicides) and nonviolent deaths (natural causes or nonviolent suicides) ($U = 6$, $p = 0.10$).

Regarding the location of the mtDNA rearrangements, 76 % of the deletion breakpoints started in *MT-ND4* and 65 % of them ended in *MT-CYB*. 94 % of the deletions involved the *MT-ND5* and *MT-ND6* genes. In HC, most of the deletion breakpoints also started in *MT-ND4* (60 %) and ended in *MT-CYB* (40 %) and *MT-RNR2* (40 %). Most of the duplication breakpoints detected in HC started in *MT-CYB* (60 %) and ended in d-loop (40 %). Similarly, 80 % of the deletions detected in HC involved

Table 2
mtDNA rearrangements identified in the 40 patients with SZ and 39 HC.

	HC	SZ	Statistics	P value
Individuals presenting rearrangements, *n* (%)	5 (13 %)	9 (23 %)	$\chi^2 = 1.27$	$p = 0.26$
N of deletions	5	34		
***n* of deletions/carriers**, mean ± *S.D.*	1.0 ± 0.9	3.8 ± 2.3	$t = 2.502$	$p = 0.03$*
hp level (%), median	6.2	9.3	$U = 65$	$p = 0.42$
size (bp), median	3640	3931	$U = 84$	$p = 0.98$
N of duplications	5	0	–	–
***n* of duplications/carriers**, mean ± *S.D.*	1.0 ± 1.1	0		
hp level (%), median	9.3	–		
size (bp), median	1504	–		

bp: base pairs; HC: healthy control; hp: heteroplasmy; *n*: number; S.D.: standard deviation; SZ: schizophrenia.

MT-ND4, MT-ND5, but also *MT-CYB* (Fig. 1).

Two patients (SZ-4 and SZ-14) and one HC (HC-69) carried the same 3234 bp deletion involving genes between *MT-ND4* and *MT-CYB* and with hp levels of 6 %, 14% hp, and 5 %, respectively. In addition, SZ-4 and SZ-14 shared three additional deletions affecting the region between *MT-ND4L* and *MT-CYB* (size=5122 bp, mean hp=8 %), between *MT-ND4* and *MT-RNR2* (size=7961 bp, mean hp=14.7 %), and between *MT-ND4L* and *MT-CYB* (size=3888 bp, hp=11.8 %).

3.2. Single nucleotide variants (SNVs)

Each participant was assigned to a specific mtDNA haplogroup, with 94 % belonging to common European haplogroups, 5 % to African/African American haplogroup L, and 1 % to Latino/Admixed American haplogroup A (Supplementary Table 4). After excluding haplogroup markers, we used two different approaches to analyze nonsynonymous variants and rRNA/tRNA variants. For nonsynonymous variants, we set the MutPred threshold to 0.5. According to this criterion, the number of identified nonsynonymous variants (Supplementary Table 5) was significantly higher in the SZ group than in the HC group ($U = 651$ $p = 0.036$). In particular, we identified 17 LP variants according to APOGEE 2, of which 14 variants were observed in 10 SZ patients and only three variants were observed in three HC (Table 3, Fig. 2). The number of LP variants was also significantly higher in the SZ patients compared to the HC (χ^2=4.3; $p = 0.038$), and eight of the 17 variants have not been previously reported. The three HC individuals carried one LP variant, whereas two SZ patients (SZ-21 and SZ-42) carried two variants and one patient (SZ-3) carried three LP variants. The average hp level among the variants was 12 %, and the highest hp level (26.1 %) was observed in patient SZ-41, in a non-reported variant, m.13268G>*A*, located in the *MT-ND5* gene. *MT-ND5* was the most affected gene, with six different variants identified, five in SZ patients and one in HC. The second most affected gene was *MT-ND4*, with three variants observed only in SZ patients. Four variants were found in the *MT-CYB* gene, three of which were observed in SZ patients and one in HC. LP variants were also found in the *MT-ND1, MT-ND2, MT-ND3,* and *MT-ATP6* genes.

We identified 17 tRNA and 30 rRNA variants in the ribosomal and transfer genes. Eleven tRNA variants (65 %) and 15 rRNA variants (50 %) were previously reported in MITOMAP, although only five tRNA variants and one rRNA variant were associated with clinical conditions. Thus, information on pathogenicity scores was obtained from the reported variants. One pathogenic, m.3243 A > G (hp=32.2 %), and three possibly pathogenic variants, m.3294 T > C (hp=15.9 %), m.5702 A > G (hp=7.9 %), m.7545 T > C (hp=6.7 %), were detected in the SZ group, whereas two LP variants, m.16002 T > C (hp=7.2 %) and m.10026 T > C (hp=5.8 %), were reported in the HCs. Among the clinically reported tRNA variants, four tRNA variants were detected in the SZ group: m.636 A > G (hp=99.7 %), m.4317 A > G (hp=97.6 %), and m.5819 T > C (hp=5.6 %), while only one tRNA variant was reported in one HC. Interestingly, the m.3243 A > G in *MT-TL1*, one of the most common pathogenic mtDNA variants, was identified in an SZ patient with a relatively high hp level of 32.2 %. Finally, we identified 12 rRNA variants in the SZ group and three previously reported rRNA variants in HCs, although only one present in an HC was associated with a clinical condition (increased risk of nonsyndromic deafness), m.792 C > T in *MT-RNR1* (hp 99 %, Supplementary Table 6). No significant difference in the total number of tRNA and rRNA variants was observed between the groups ($U = 695$; $p = 0.139$; $U = 697$; $p = 0.098$, respectively).

The number of variants did not differ between patients who died by suicide or natural causes ($U = 175$, $p = 0.495$), or between violent and nonviolent deaths ($U = 151$, $p = 0.158$).

3.3. mtDNA-CN

Three SZ patients and eight HC were excluded from the analysis due to the inability to amplify target regions or significant deviations observed in the triplicates. Therefore, our final set included 37 individuals with SZ (7 females, 30 males; mean age 47.6 years) and 32 HC (7 females, 25 males; mean age 47.7 years). No significant differences in mtDNA-CN were found between the groups ($U = 554$, $p = 0.654$) (Fig. 3A). Furthermore, no significant effects of age (ρ=0.057, $p = 0.639$), sex ($U = 353$, $p = 0.638$) and PMI (ρ=0.014, $p = 0.909$) on mtDNA-CN were observed. Interestingly, antipsychotic users had significantly higher mtDNA-CN compared to non-users ($U = 275$, $p = 0.004$) (Fig. 3B). Among the antipsychotic users, 17 SZ patients were on atypical antipsychotics, while three SZ patients were on typical antipsychotics; however, mtDNA-CN did not differ between these two groups ($U = 12$, $p = 0.179$, Fig. 3B). No significant difference in mtDNA-CN was observed regarding the use of antidepressants ($U = 85$, $p = 0.691$). Similarly, no significant difference was observed between benzodiazepine users ($n = 19$) and non-users ($n = 50$) ($U = 450$, $p = 0.742$). Finally, there were no significant differences in mtDNA-CN between individuals with ($n = 11$) and without ($n = 57$) mtDNA rearrangements ($U = 271$, $p = 0.484$). Finally, the mtDNA-CN did not differ between patients who died by suicide or natural causes ($U = 159$, $p = 0.752$), or between violent deaths and nonviolent deaths ($U = 136$, $p = 0.311$).

4. Discussion

In this study, we analyzed several mitochondrial genomic alterations, including rearrangements, SNVs, and mtDNA CN changes, in postmortem brain samples from SZ patients and HC individuals. We identified a higher number of mtDNA alterations in SZ than in HC. Fourteen of 40 patients (35 %) with SZ carried an mtDNA alteration, whereas only four of 39 (10 %) HC were carriers. Case-control analyses based on SNPs or haplogroups were not performed due to the small sample size of the study.

For the analysis of mtDNA rearrangements, we used an hp cutoff of 5 % to detect deletions or duplications that may impact mitochondrial function, discarding variants with lower hp levels that may occur in the general population (Guo et al., 2013). We identified more deletions in the SZ group than in the HC group (35 vs. 5), and the mean number of deletions per participant was also significantly higher in the SZ patients than in the HC (3.8 vs. 1.0). This finding suggests that oxidative stress, drug exposure, or defects in the mtDNA repair system or mtDNA replication may be involved in certain SZ patients, supporting previous evidence that mtDNA deletions may play a role in psychiatric disorders (Das et al., 2022; Hjelm et al., 2019). Most of the deletions in our study covered the major arc of mtDNA, including a region between the *MT-ND4* and *MT-CYB* genes. Deletions in the major arc are common because the H strand, located between the two origins of replication in the major arc, allows the two repeats downstream of the O_H to bind and generate mtDNA deletions via slipped mispairing replication (Chen et al., 2011; Yusoff et al., 2019). Furthermore, it has been demonstrated in human cells that stalled mtDNA replication, which is the initial stage in the formation of mtDNA deletions, occurs in the major arc. This finding supports the notion that deletions are more prevalent in this region (Doimo et al. 2023). This region contains critical mtDNA genes, including *MT-ND4, MT-ND5, and MT-ND6* in complex I and *MT-CYB* in complex III, which are subunits of the mitochondrial respiratory chain involved in the oxidative phosphorylation system. Thus, the loss of large segments of these genes would result in a significant disruption of mitochondrially encoded ETC components (Sanchez-Contreras and Kennedy, 2021). A recent study correlated the increased mtDNA deletion burden in the DLPFC of SZ with the presence of anxiety and depression rather than with a direct pathological etiology of SZ (Das et al., 2022), but we did not have access to data on the symptoms of the SZ patients and could not validate these findings. In contrast to the results obtained for deletions, our study did not find evidence that duplications play a role in SZ, as no duplications were observed in any of the patients, whereas they were present in HC. However, current

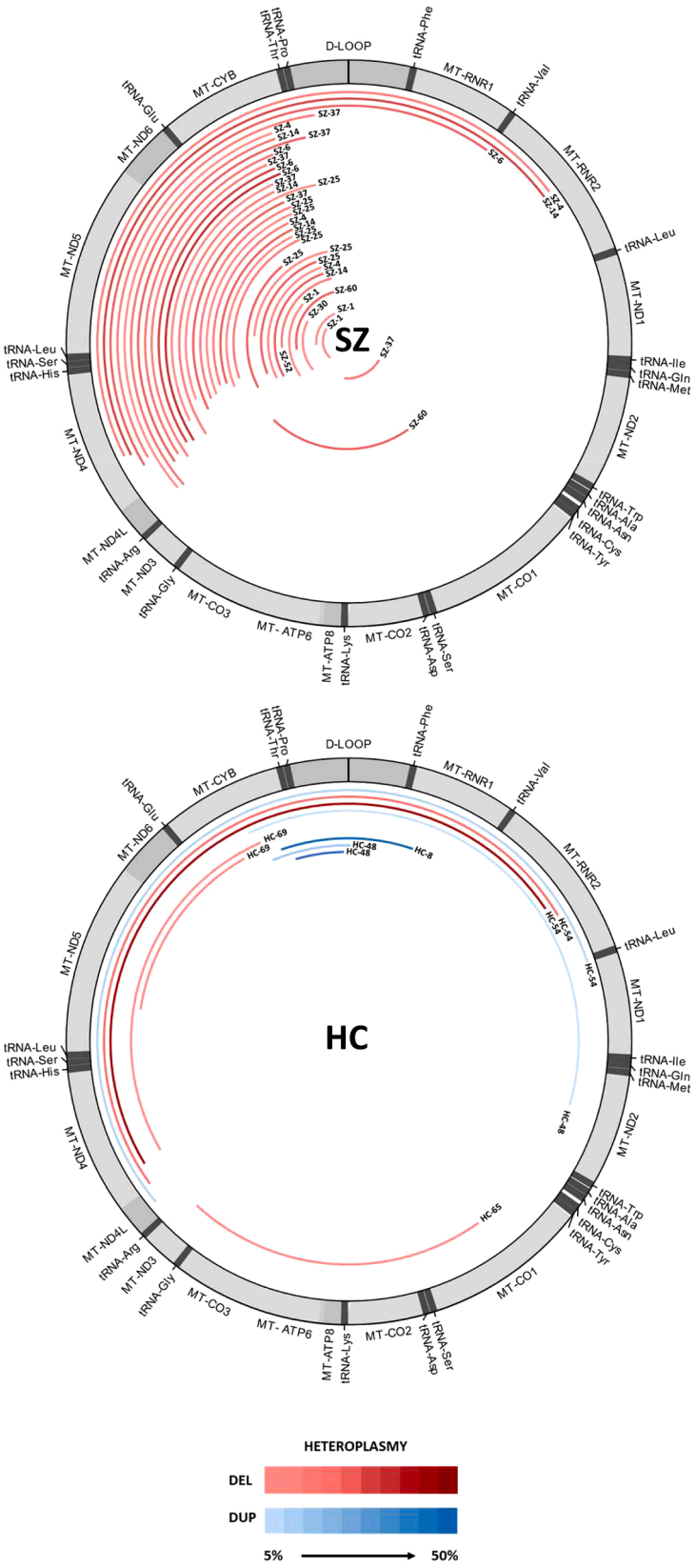

Fig. 1. Distribution of all rearrangements identified in SZ patients and HC on the mtDNA map. The circular plot above shows the five deletions and five duplications detected in HC. The circular plot below shows the 34 deletions detected in SZ patients. Deletions are shown as red arcs and duplications are shown as blue arcs. Hp levels are represented by the intensity of the colors from light (low hp level) to dark (high hp level). HC: healthy control; SZ: schizophrenia.

Table 3
Likely pathogenic (LP) nonsynonymous variants detected in patients with SZ and HC according to APOGEE.

Variant	Group	ID	Amino acid change	PhyloP 100v	PhastCons 100v	Conservation (%)	Gene	Hp (%)	Population frequency
m.3791 T > C	HC	10	Leu162Pro	7.423	0.961	95.56	*MT-ND1*	7.5	<0.00001
m.4852 T > C	SZ	14	Leu128Pro	2.829	0.299	68.89	*MT-ND2*	9.2	<0.00001
m.8786 *T* > *A*	SZ	14	Leu87His	5.880	0.906	93.33	*MT-ATP6*	5.9	0.00003
m.10228 T > C	SZ	21	Leu57Ser	5.898	0.913	100.00	*MT-ND3*	7.1	0
m.11232 T > C	SZ	42	Leu158Pro	7.531	0.961	93.33		5.2	<0.00001
m.11385 T > C	SZ	21	Leu209Pro	7.400	0.961	100.00	*MT-ND4*	9.6	0
m.11404 *A* > *T*	SZ	42	Trp215Cys	5.775	1	100.00		5.1	0
m.12584 *A* > *G*	HC	54	Asp83Gly	6.580	1	100.00		5.9	0
m.13076 T > C	SZ	6	Leu247Pro	5.656	1	100.00		5.0	0
m.13268 *G* > *A*	SZ	41	Gly311Glu	7.274	1	100.00	*MT-ND5*	26.1	0
m.13339 T > C	SZ	32	Phe335Leu	7.449	1	100.00		7.6	<0.00001
m.13360 T > C	SZ	14	Cys342Arg	7.449	1	91.67		7.3	0
m.13486 C > T	SZ	30	Pro384Ser	5.603	1	100.00		6.2	0
m.14849 T > C	SZ	37	Ser35Pro	5.585	1	100.00		7.9	<0.00001
m.15129 T > C	HC	69	Phe128Ser	5.585	1	100.00	*MT-CYB*	17.2	<0.00001
m.15171 *G* > *A*	SZ	60	Gly142Ala	5.124	1	100.00		12.6	0.00002
m.15239 T > C	SZ	52	Trp165Arg	5.585	1	100.00		19.0	<0.00001

Arg: arginine; Asn: asparagine; Asp: aspartic acid; ATP: ATP synthase; CYB: cytochrome b; Cys: cysteine; Glu: glutamic acid; Gly: glycine; HC: healthy control; His: histidine; Hp: heteroplasmy; Ile: isoleucine; Leu: leucine; LP: likely pathogenic; *MT-*: mitochondrially encoded; ND: NADH dehydrogenase; Phe: phenylalanine; Pro: proline; Ser: serine; SZ: schizophrenia; Thr: threonine; Trp: tryptophan.
PhyloP 100v score range (min −20, max 10); predicted accelerated evolution: score ≤ 0; conserved: score > 0.
PhastCons 100v score range (min 0, max 1); non-conserved: score ≤ 0.7; conserved: score > 0.7.
Conservation: percentage of residues that match that of the rCRS among 45 selected species (mammals and non-mammals).

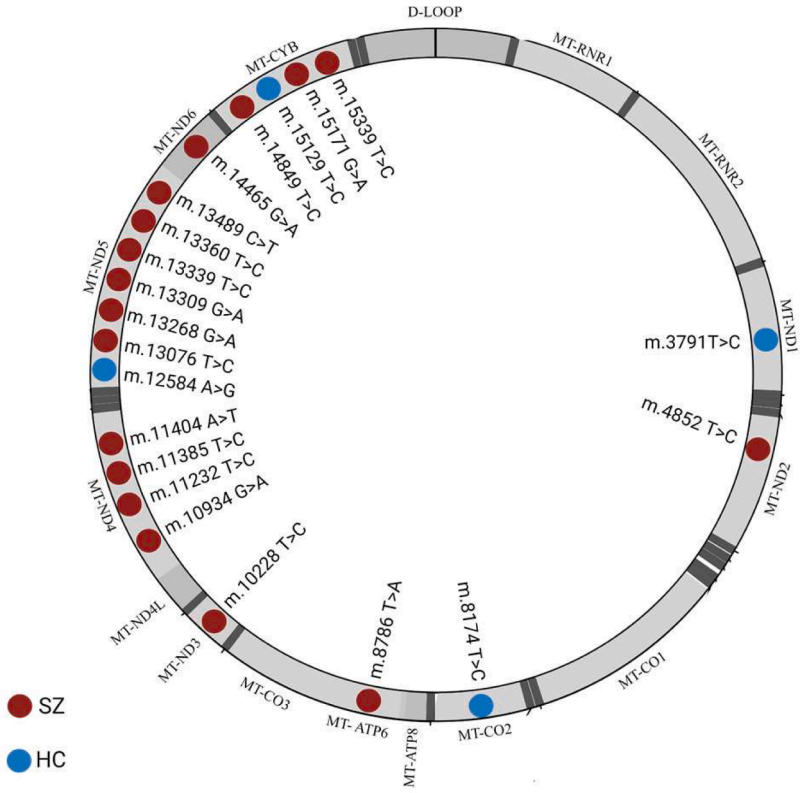

Fig. 2. Distribution of pathogenic nonsynonymous variants on the mtDNA map. HC: healthy control; SZ: Schizophrenia.

knowledge about duplications is limited compared to deletions. Aging has been reported to be an important factor in the accumulation of large mtDNA deletions in the brain. These deletions are typically larger than 1000 bases and lead to a loss of respiratory capacity in the cell (Sanchez-Contreras and Kennedy, 2021). We did not find an association between the presence of deletions and the age of the participants, probably because the patients and controls included in this study were middle-aged (mean 48.5 years). Large-scale duplications of mtDNA were first documented in individuals with Kearns-Sayre syndrome (KSS) (Poulton, 1992) and in Pearson syndrome (PS) (Rötig et al., 1990). Subsequently, such duplications were observed in other cases of sporadic mitochondrial disorders (Cormier-Daire et al., 1994). Currently, single large-scale mitochondrial DNA deletion syndromes (SLSMDS) include overlapping clinical phenotypes such as KSS, KSS spectrum, PS, chronic progressive external ophthalmoplegia (CPEO), and CPEO-plus (Goldstein and Falk, 2023). The reported hp level in muscle of

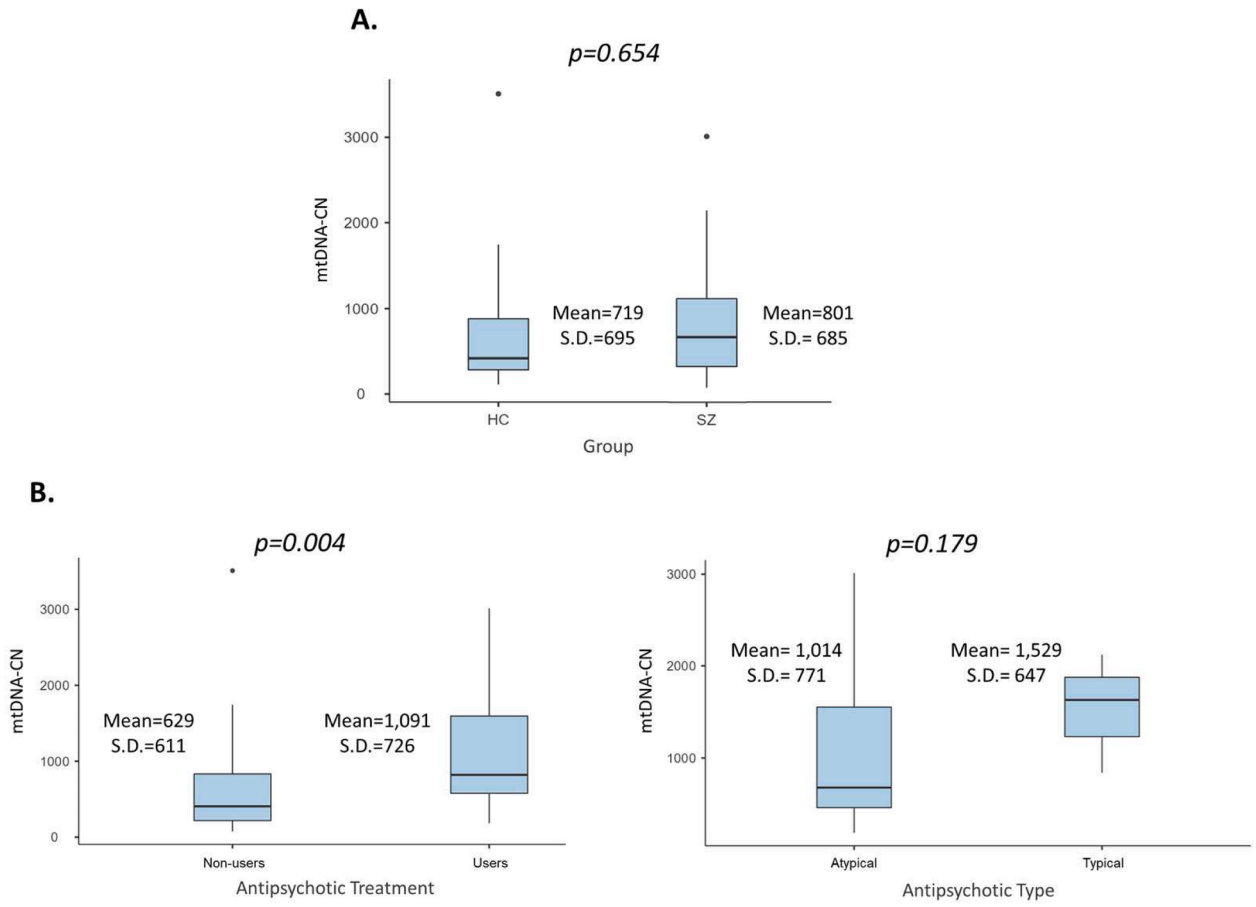

Fig. 3. Comparison of mtDNA-CN: A) between patients with SZ and HC; B) between antipsychotic users and non-users; and C) between patients treated with typical or atypical antipsychotic treatment.

affected individuals is 52.0 % ± 19.1 %; however, low hp levels (25 %–33 % in muscle) have also been reported in symptomatic patients (Broomfield et al., 2015; Mancuso et al., 2015). The percentage of hp levels and the location of the deletion have been reported to have an influence on the phenotype and differ between KKK and CPEO (López-Gallardo et al., 2009). Notably, in addition to neurological, ocular, cardiac, endocrine, and hematological alterations, some studies have reported psychiatric manifestations in 69 % of patients with SLSMDS, highlighting a possible association between SLSMDS and psychiatric disorders (Broomfield et al., 2015; Reynolds et al., 2021). Regarding the duplications we observed in HC, 44 duplications out of 1356 rearrangements have been reported in Mitobreak, all involving the heavy chain origin of replication, and primarily associated with mitochondrial disorders and aging (Damas et al., 2014). MtDNA duplications have been reported in patients with SLSMDS and correlated with the onset of symptoms; however, a predominant reduction of complex III in patients without mtDNA duplications compared to those with duplications suggests that mtDNA duplications do not cause oxidative impairment (Odoardi et al., 2003). We could not exclude that the duplications reported in the HC may have an impact on the phenotype of these individuals; however, our results do not support a role for mtDNA duplications in SZ.

In the analysis of nonsynonymous variants, we identified 17 LP variants, 9 variants that were previously reported and 8 variants that have not been previously reported. LP variants were significantly more frequent in patients with SZ (10 out of 40 individuals) than in HC (3 out of 39 individuals), but no significant difference in the hp level was observed. The variants identified in the patients were located in *MT-ND2, MT-ND3, MT-ND4, MT-ND5, MT-CYB*, and *MT-ND6*, affecting complex I, complex III, and complex V. The highest number of LP variants (6 variants) was observed in the *NT-ND5* gene, as well as the highest hp level (26.1 %), which was identified in m.13268 $G > A$, which has not been previously reported. Mutations in these genes may negatively affect the function of complex I and complex III of the mitochondrial respiratory chain, and the ATP synthase (complex V). Predicting the pathogenicity of mtDNA variants is challenging due to the overall hp landscape, but several in silico tools are available and predict changes in protein/RNA structure and therefore pathogenicity, contributing to the genetic diagnosis of novel mutations and elucidating the involvement of mtDNA in mitochondrial disorders (Bacalhau et al., 2017). We detected hp values below 30 %, and the hp threshold for biochemical changes for many pathogenic nonsynonymous mtDNA variants in the brain is suggested to be 60 % (Rossignol et al., 2003); however, it would be interesting to evaluate the impact of the LP variants at lower hp levels on mitochondrial function. Unfortunately, this was beyond the scope of the present study. A case-control study conducted in a Swedish sample identified a higher incidence of low-frequency variants in patients with SZ than in HC, in agreement with the present study (Gonçalves et al., 2018). For tRNA and rRNA variants, available pathogenicity prediction tools are less common than for the nonsynonymous variants, and data on unreported variants are currently unavailable. We identified five tRNA and one rRNA variants previously associated with clinical conditions that were more frequent in SZ patients (5 variants) than in HC individuals (1 variant). Interestingly, we identified one of the most common mutations in mitochondrial DNA, m.3243 $A > G$ in the *MT-TL1* gene, which is associated with many mitochondrial dysfunctions (Cai et al., 2022; Esterhuizen et al., 2021), with a relatively high hp level (32.2 %) in an SZ patient. This variant has been reported in healthy individuals with hp levels <1 %, suggesting that levels >1 % may be important for disease (Matsumoto et al., 2023).

Of note, m.3243A>*G* hp levels of 10–30 % have previously been associated with autism (Pons et al., 2004). Finally, the only rRNA variant identified in this study, *MT-RNR1* m.792 C > T, was observed in one HC and at a 99% hp level. This variant, which has a 100 % conservation index, was previously reported in one patient from a cohort of 1642 hearing impaired pediatric subjects and was absent in 449 controls (Lu et al., 2010). Unfortunately, we did not have phenotypic data on this HC to confirm this finding.

Changes in mtDNA-CN have been associated with mitochondrial function and several conditions have been correlated with either an increase or decrease in mtDNA-CN (Malik and Czajka, 2013; Valiente-Pallejà et al., 2022). However, we did not find significant differences in mtDNA-CN between SZ and HC, in agreement with previous negative studies (Sabunciyan et al., 2007; Torrell et al., 2013). However, some studies evaluating mtDNA-CN in postmortem brain tissue from SZ patients reported differences in mtDNA-CN depending on the brain region (Das et al., 2022), and antipsychotic drug exposure (Kumar et al., 2018). We found significant differences in mtDNA-CN only between patients who received antipsychotic treatment and those who did not, with antipsychotic-treated patients having higher levels. This finding is inconsistent with a previous study showing that mtDNA-CN was reduced with the use of clozapine and risperidone (Kumar et al., 2018). However, it is important to note that our study had a small sample size, and the total duration of antipsychotic drug treatment was not available in our sample.

In blood samples, mtDNA-CN was reported to be significantly higher in adolescent patients with major depressive disorder who had a history of suicide attempt or serious suicidal behavior in the two weeks prior to the study than in the HC group (Ochi et al., 2023). Similarly, significant increases in mtDNA-CN and mitochondrial DNA gene expression have been associated with increased agonal duration (Vawter et al., 2006). However, we found no association between the number or characteristics of the mtDNA deletions, mtDNA variants, or mtDNA-CN and suicide or agonal death.

Several limitations must be considered when interpreting these findings. First, the study sample size was underpowered for case-control comparisons. Second, our study was based on analysis of the DLPFC only, and some studies have shown that the effect of mtDNA alterations on mitochondria varies depending on factors such as the specific brain region, cell type, and subcellular location (Roberts, 2021). Thus, a comprehensive study of the effects of mtDNA alterations, including analysis of mtDNA from different brain regions, would be necessary. Finally, our study used state-of-the-art molecular and bioinformatic tools, but there is currently no consensus on the standardized use of these sensitive pipelines. However, the high read depth we obtained lends strength to the low levels of hp that we observed.

This study was a comprehensive investigation of mtDNA alterations in postmortem brain tissue from individuals diagnosed with SZ and HC. Our findings reveal an increased frequency of mtDNA alterations in SZ patients compared to HC, suggesting a potential involvement of mtDNA in the pathophysiology of SZ and opening a new window for personalized medicine targeting mitochondria. Further investigations are imperative to validate the increased prevalence of pathogenic variants in postmortem brain tissue of SZ individuals and to elucidate the implications of the identified heteroplasmy levels on mitochondrial function.

CRediT authorship contribution statement

Bengisu K. Bulduk: Writing – original draft, Investigation, Formal analysis, Data curation. **Juan Tortajada:** Methodology, Investigation, Data curation. **Alba Valiente-Pallejà:** Writing – review & editing, Validation, Supervision, Validation, Resources, Methodology. **Luís F. Callado:** Validation, Resources, Methodology. **Helena Torrell:** Validation, Resources, Methodology. **Elisabet Vilella:** Writing – review & editing, Validation, Methodology. **J. Javier Meana:** Resources, Methodology, Funding acquisition. **Gerard Muntané:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Lourdes Martorell:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Ethical statement

We, the authors, confirm that this manuscript has been prepared in accordance with the highest ethical standards in the publication of scientific content for peer-reviewed publications.

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Author statement

All procedures were performed in accordance with relevant laws and institutional guidelines and were approved by the Ethics Committee of the Institut d'Investigació Sanitària Pere Virgili (approval number 147/2018, date 09/27/2018), as described in the Materials and Methods section.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2024.115928.

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