IMPLICATIONS OF MITOCHONDRIAL DNA ALTERATIONS IN BRAIN DISORDERS

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UNIVERSITAT ROVIRA i VIRGILI

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IMPLICATIONS OF MITOCHONDRIAL DNA ALTERATIONS IN BRAIN DISORDERS

Doctoral Thesis

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I STATE that the present study, entitled "**Implications of mitochondrial DNA alterations in brain disorders**" presented by Bengisu Kevser Bulduk for the award of the degree of Doctor, has been carried out under my supervision at the Department of Medicine and Surgery of this university.

Reus, 30 January 2023.

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Implications of mitochondrial DNA alterations in brain disorders

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"The most genuine guide in life is science."

Mustafa Kemal Atatürk

(Founder of modern Turkey, 1881-193∞)

I. RATIONALE

Over the past two decades, human genetics and genomics research has made significant advances, particularly following the publication of the human genome draft sequence in 2001. These developments have provided new insights into the genetic basis of brain disorders and disorders, facilitating the emergence of precision neurology and psychiatry. It is important to note that the most of the knowledge regarding the pathogenesis of brain disorders is derived from studies that focus on nuclear DNA, with genome-wide association studies (GWAS) becoming increasingly popular. However, exploration of the mitochondrial genome's role in the pathogenesis of brain disorders and disorders has been comparatively limited. This doctoral thesis focuses on the mitochondrial genome, shedding light on underexplored aspects within the realm of neurogenetics.

Mitochondria play a crucial role in producing ATP, the ultimate energy source for the cell, through the mitochondrial respiratory chain. They contain their own DNA, known as mitochondrial DNA, which is a small circular molecule consisting of only 16.5 kilobases that can exist in hundreds or thousands of copies within a cell, depending on the cell type. It encodes protein subunits that participate in the synthesis of mitochondrial respiratory chain elements, along with ribosomal and transfer RNAs. Therefore, any alteration in this molecule affects the energy production of a cell. Brain and muscle cells are high-energy-demanding cells and are prone to carry more mitochondria and, consequently, more mtDNA molecules. Certain genetic changes in mtDNA are known to cause mitochondrial dysfunction, resulting in dysfunction in brain and muscle tissue.

Previous studies conducted with the Genetics and Environment in Psychiatry Group (GAP), of which I am a part, have identified mitochondrial dysfunction in autism spectrum disorder, intellectual disability, and schizophrenia. Based on clinical, genetic, and metabolic studies, it has been suggested that certain mitochondrial alterations may play an important role in the development of these brain disorders. This dissertation provides a comprehensive summary of mtDNA alterations in the postmortem brain tissue of individuals with brain disorders reported to date. Additionally, it analyses mtDNA alterations in blood samples from patients with intellectual disability and autism spectrum disorder, as well as in postmortem brain tissue from patients with schizophrenia patients and control individuals, which provide valuable resources to gain direct insight into brain-specific changes. The aim is to determine whether mtDNA alterations may have a significant impact on the development of these disorders.

II. ABREVIATION

5′-UTRs : 5′ untranslated regions

8-OXOG : 8-oxoguanine

A

AD : Alzheimer Disease

ADHD : Attention-Deficit/Hyperactivity Disorder

ADP : adenosine diphosphate

ALKBH : alpha-ketoglutarate-dependent dioxygenase

AP sites : apurinic/apyrimidinic sites

APA : American Psychiatric Association

APOE : Apolipoprotein E

APP : amyloid precursor protein

ASD : Autism Spectrum Disorder

ATP : adenosine triphosphate

Aβ : amyloid-beta

B

BD : Bipolar Disorder

BER : base excision repair

bp : base pairs

C

CARS : Childhood Autism Rating Scale

CoQ : coenzyme Q

CPEO : Chronic Progressive External Ophthalmoplegia

CRS : Cambridge Reference Sequence

CSB2 : conserved sequence block 2

CsCl : cesium chloride

D

Da : Dalton

DBS : double-strand break

D-loop : displacement loop

DR : direct reversal

DRP : dynamin-related protein

DS : Down syndrome

DSM-5 : Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

E

EFG : elongation factor G

EFT : elongation factor

ETC : electron transport chain

F

F0 : Filial 0

F1 : Filial 1

FAHD2 : dihydroflavine-adenine dinucleotide

FapyA : 4,6-diamino-5-formamidopyrimidine

FapyG : formamidopyrimidine

FASTK : Fas-activated serine/threonine kinase

FXS : fragile X syndrome

G

G : guanine

GPx : glutathione peroxidase

GTP : guanosine-5'-triphosphate

GTPases : GTP-binding hydrolases

GWAS : Genome-wide association studies

H

H : hydrogen

H+ : proton ion

H_2O : water

HR : homologous recombination repair

HSP : heavy strand promoter

H-strand : heavy strand

hSuv3 : human Suv3 protein

I

ICD : International Classification of Diseases

ID : intellectual disability

IQ : intellectual quotient

K

kb : kilobase

KSS : Kearns-Sayre Syndrome

kya : thousand years ago

L

Leu : leucine

LHON : Leber's Hereditary Optic Neuropathy

LP-BER : Long-Patch Base Excision Repair

LS : Leigh Syndrome

LSP : light strand promoter

L-strand : light strand

M

MAO : monoamine oxidase

MAPT : microtubule-associated protein tau

MDD : Major Depressive Disorder

MELAS : Mitochondrial Encephalomyopathy, Lactic acidosis and Stroke-like Episodes

MERRF : Myoclonic Epilepsy with Ragged Red Fibres

Met : methionine

MGMT : O6-methylguanine DNA methyltransferase

MMR : mismatch repair

MRT : mitochondrial replacement therapy

MT-ATP6 : mitochondrially encoded ATP synthase 6

MT-ATP8 : mitochondrially encoded ATP synthase 8

MT-CO1 : mitochondrially encoded cytochrome c oxidase 1

MT-CO2 : mitochondrially encoded cytochrome c oxidase 2

MT-CO3 : mitochondrially encoded cytochrome c oxidase 3

MT-CYB : mitochondriall y encoded cytochrome B

mtDNA : mitochondrial DNA

MTERF1 : mitochondrial termination factor 1

mtLSU : large subunit Rrna

MT-ND1 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 1

MT-ND2 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 2

MT-ND3 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 3

MT-ND4 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 4

MT-ND5 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 5

MT-ND6 : mitochondrially encoded NADH-ubiquinone oxidoreductase chain 6

mtPAP *:* polyadenylic acid RNA polymerase

mtRF1 : mitochondrial release factor 1

mtRF1a mitochondrial release factor 2

MT-RNR1 : 12S RNA

MT-RNR2 : 16S RNA

mtSSU : mitochondrial small subunit rRNA

MT-TA : mitochondrially encoded tRNA alanine

MT-TF : mitochondrially encoded tRNA phenylalanine

MT-TG : mitochondrially encoded tRNA glycine

MT-TI : mitochondrially encoded tRNA isoleucine

MT-TL1 : mitochondrially encoded tRNA leucine 1 (UUA/G)

MT-TL2 : mitochondrially encoded tRNA leucine 2 (CUN)

MT-TP : mitochondrially encoded tRNA proline

MT-TQ : mitochondrially encoded tRNA glutamine

N

NAD : nicotinamide adenine dinucleotide

NADH : nicotinamide adenine dinucleotide with hydrogen

NARP : neurogenic muscle weakness, ataxia, and retinitis pigmentosa

nDNA : nuclear DNA

NEIL1 : Nei Like DNA Glycosylase 1

NeuD : Neurodevelopmental disorders (or diseases)

NH4+ : ammonia

NHEJ : non-homologous end joining

nm : nanometer

NMDA : N-methyl-D-aspartate

O

O_2 : oxygen

OGG1 : N-Glycosylase/DNA Lyase

O_H : H-strand origin of replication

O_L : L-strand origin of replication

OPA1 : optic atrophy protein 1

OXPHOS : oxidative phosphorylation

P

PD : Parkinson's disease

Pi : inorganic phosphate

PNPase : polynucleotide phosphorylase

POLG : DNA polymerase gamma gene

POLRMT : mitochondrial RNA polymerase

POLγ : DNA polymerase gamma

POLγA : mitochondrial DNA polymerase gamma catalytic subunit

POLγB : mitochondrial DNA polymerase gamma accessory subunit

PS : Pearson syndrome

PSEN1 : presenilin 1

PSEN2 : presenilin 2

P-site : the peptidyl site

pSNV : pathogenic single nucleotide polymorphism

PsyD : Psychiatric disorders (or diseases)

Q

qPCR : quantitative real-time PCR

R

REXO2 : RNA exonuclease protein

ROS : reactive oxygen species

rRNA : ribosomal RNA

S

SNHL : sensorineural hearing loss

SOD : superoxide dismutase

SP-BER : Short-Patch Base Excision Repair

ssDNA : single-stranded DNA

T

T : thymine

TAS : termination-associated sequence

TEFM : transcription elongation factor

TFAM : mitochondrial transcription factor A

SNP : single nucleotide polymorphism

TFB2M : mitochondrial transcription factor B2

TNT : tunnelling nanotube

tRNA : transfer RNA

TWINKLE : mtDNA helicase

U

UCP1 : Uncoupling protein 1

UNG : Uracil-DNA glycosylase

µm : micrometer

III. INTRODUCTION

1. Mitochondria

Mitochondria (singular: mitochondrion) are unique double membrane-bound organelles located in the cytoplasm of almost all eukaryotic cells that have a well-defined nucleus. As the powerhouses of a cell, they play a central role in energy production through cellular respiration, generating adenosine triphosphate (ATP) to fuel all cellular processes; however, mitochondria are involved in several processes.

This section discusses the structure, function, origin, and evolution of mitochondria and their general role in brain function.

1.1. Structure

A classical mitochondrion can be depicted as rigid, elongated bacterial-like cylinder with a diameter of 0.5–1 μm that can occupy up to 35% of the cytoplasmic volume of a eukaryotic cell (Alberts et al., 2022; Anastacio et al., 2013). Indeed, mitochondria are remarkably dynamic and plastic, moving through the cytoplasm and constantly changing shape (Da Silva et al., 2014).

Mitochondria are enveloped by a double membrane system, consisting of inner and outer mitochondrial membranes that serve different purposes. The inner membrane, which envelops the inner mitochondrial matrix compartment, is extensively folded, forming inward folds called cristae (singular: crista). These cristae provide a large surface area for the electron transport chain (ETC) and ATP synthase complexes, allowing for efficient energy conversion. In areas where the inner membrane runs parallel to the outer membrane, between the cristae, it is referred to as the inner limiting membrane. The 20-30 nm gap between the inner boundary membrane and the outer membrane is known as the intermembrane space (Kühlbrandt, 2015) (Figure 1).

Similar to the bacterial outer membrane, the mitochondrial outer membrane allows the free passage of ions and small molecules, even those as large as 5000 daltons (Da) (Kühlbrandt, 2015). This permeability is primarily due to the presence of a channel protein called porin, which belongs to the β-barrel membrane proteins and creates water-filled pores in the membrane (Alberts et al., 2022). As a result, the intermembrane space between the outer and inner mitochondrial membranes maintains a pH and ionic composition that closely resembles that of the cell cytoplasm, and there is no development of an electrochemical gradient across the outer mitochondrial membrane (Alberts et al., 2022).

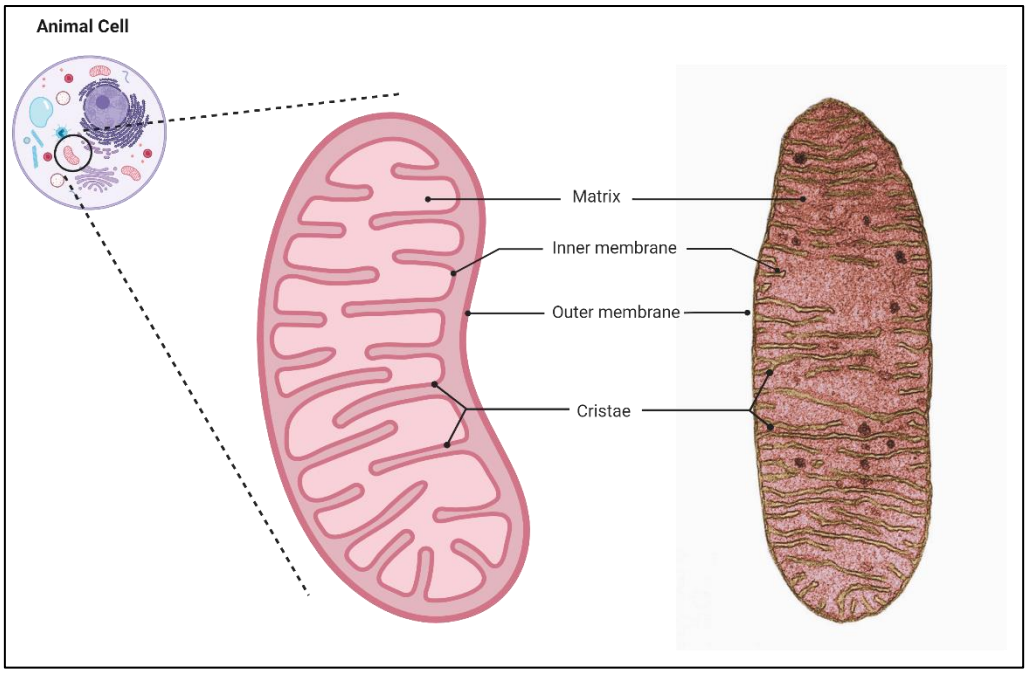

Figure 1. The structure of mitochondria and a transmission electron microscope (TEM) image of a longitudinal section of a mitochondrion in a pancreatic cell are shown.
Created using BioRender. TEM photo credit: Keith R. Porter via Science Source.

The inner mitochondrial membrane acts as a diffusion barrier for ions and small molecules, just like the inner membrane of a bacterium. However, selected ions, especially protons and phosphate, as well as essential metabolites such as ATP, adenosine diphosphate (ADP), and pyruvate, can cross this barrier with the help of specific transport proteins (Murphy et al., 2016).

1.2. Function

Mitochondria play a vital role in the production of ATP, the universal energy currency of cells. This process, known as oxidative phosphorylation (OXPHOS), relies on a series of respiratory complexes in addition to the ATP synthase, embedded in the inner mitochondrial membrane.

During cellular respiration, glucose, fatty acids, and amino acids are broken down in the Krebs cycle (the citric acid cycle). This results in the production of high-energy electron carriers, NADH (nicotinamide adenine dinucleotide (NAD) + hydrogen (H)) and FADH2 (dihydroflavine-adenine dinucleotide). These molecules carry electrons to ETC, a series of protein complexes and other molecules that transfer electrons from electron donors to electron acceptors via redox reactions (both reduction and oxidation occurring simultaneously) and couples this electron transfer with the transfer of protons across a membrane (Figure 2):

- Complex I (NADH-coenzyme Q (CoQ) reductase): This complex receives electrons from NADH and transfer them to ubiquinone (CoQ). In the process, it pumps protons across the inner mitochondrial membrane into the intermembrane space.
- Complex II (Succinate-CoQ reductase): Complex II receives electrons from $FADH_2$, which is produced in the Krebs cycle when succinate is oxidised. It also transfers electrons to CoQ.
- Complex III ($CoQH_2$ cytochrome c reductase): This complex transfers electrons from CoQ to cytochrome c while pumping more protons into the intermembrane space.

- Complex IV (cytochrome c oxidase): Complex IV is the last complex in the ETC. It transfers electrons to oxygen (O_2), which combines with protons to form water (H_2O). This complex also pumps protons into the intermembrane space.

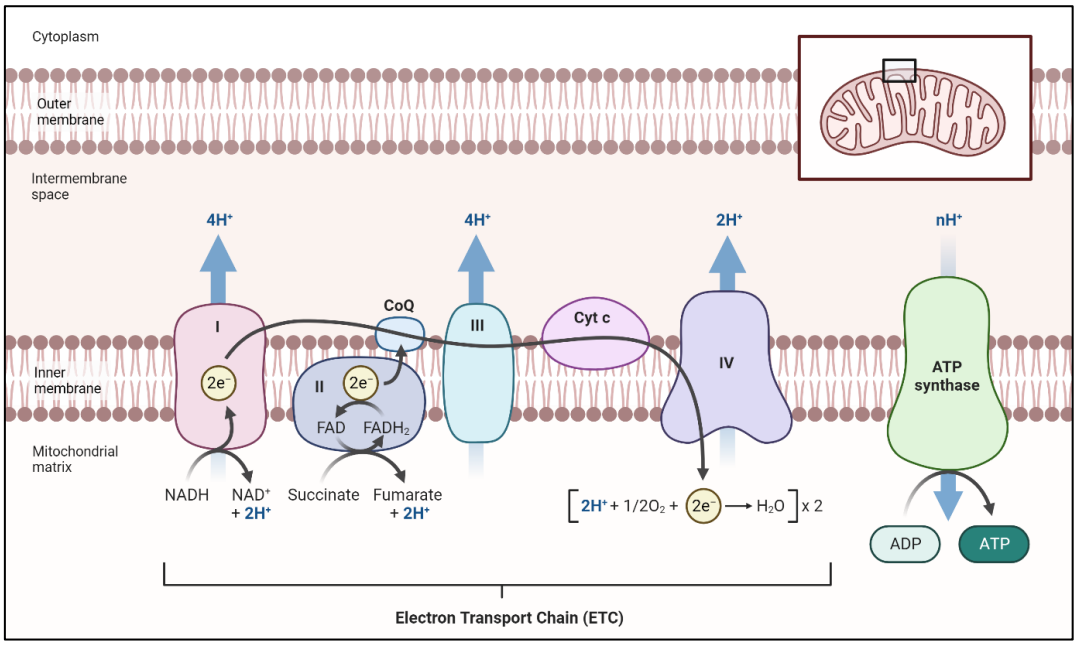

Figure 2. Elements of the electron transport chain and the ATP synthase.
Reprinted from "Electron Transport Chain" by BioRender.com.

As electrons move through the complexes of the ETC, they pump proton ions (H+) from the mitochondrial matrix into the intermembrane space. This creates a proton gradient across the inner mitochondrial membrane that has high potential energy due to the difference in proton concentration and electrical potential compared to the mitochondrial matrix (Brand et al., 2013). Protons flow back into the mitochondrial matrix through a protein complex called ATP synthase. This proton flux drives the rotational action of ATP synthase, which catalyses the synthesis of ATP from ADP and inorganic phosphate (Pi) (Fontanesi, 2015). This proton motive force generates reactive oxygen species (ROS) as by-products of ATP production, which can have a signalling role in some cellular processes. Mitochondria also help regulate the pH and ion balance within the cell and intermembrane space by actively transporting protons (Wisnovsky et al., 2016). This

regulation is critical for the proper functioning of cellular processes, including enzymatic reactions and the maintenance of membrane potential.

Although mitochondria are primarily known for their role in energy production, they also have crucial roles in several metabolic pathways, biosynthesis of lipids and amino acids, cell signalling and apoptosis (Brand et al., 2013). For instance, the liver cell mitochondria play a crucial role in the key steps of the urea cycle. This cycle converts ammonia (NH_4+), a byproduct of the breakdown of nitrogen-containing compounds such as amino acids, into urea, which is excreted in the urine (Alberts et al., 2022). In addition to their role in metabolic pathways, mitochondria play a crucial role in the synthesis of haem and iron-sulphur clusters (Stehling & Lill, 2013). The canonical synthesis of haem occurs in the mitochondrial matrix, with succinyl-CoA, a precursor molecule derived from the Krebs cycle, serving as a key starting point (Piel et al., 2019). Furthermore, the sulphur for iron-sulphur cluster synthesis is derived from cysteine, and the assembly process occurs on scaffold proteins within the mitochondria (Read et al., 2021). In certain tissues, such as brown adipose tissue, mitochondria express mitochondrial uncoupling protein 1 (UCP1), which allows protons to leak back into the mitochondrial matrix without generating ATP (Xue et al., 2022). This uncoupling of proton flow from ATP production results in heat production and serves as a thermoregulatory mechanism in response to cold environments or in hibernating mammals (D. G. Nicholls, 2021). Mitochondria play a multifaceted role within the cell, not only regulating intracellular calcium levels by storing and releasing calcium ions, which impact various cellular processes including muscle contraction and cell signalling, but also serving a central role in the initiation of apoptosis, a controlled process of programmed cell death (Osellame et al., 2012). During apoptosis, cytochrome c, located in the mitochondrial intermembrane, are released, setting off a cascade of events that ultimately lead to cell self-destruction (Wang & Youle, 2009).

1.3. Organisation and distribution

Mitochondria are dynamic organelles, and their distribution within eukaryotic cells is influenced by energy requirements. Mitochondria are often found close to the nucleus and constantly move along cytoskeletal tracks within the cell to respond to changing energy demands (Westermann, 2010). Interestingly, mitochondria can continuously fuse and divide. Mitochondrial fusion involves the merging of multiple mitochondria into larger networks, promoting the exchange of genetic material and components, and enhancing mitochondrial function. The essential fusion machinery is formed by two widely conserved large guanosine-5'-triphosphate (GTP)-binding hydrolases (GTPases): mitofusins, located in the outer membrane, and optic atrophy protein 1 (OPA1),

located in the inner membrane (Westermann, 2010). Mitochondrial fission, on the other hand, leads to the division of mitochondria into smaller organelles, allowing them to be distributed to different regions of the cell and allowing quality control by segregating damaged mitochondria for removal. Mitochondrial fission is facilitated by dynamin-related proteins (DRPs) and essential cofactors that assist in the assembly of DRP rings and spirals on the mitochondrial surface (Westermann, 2010). These finely balanced processes are essential for maintaining mitochondrial health, energy production, and cellular homeostasis.

Mitochondria can also be transferred from one cell to another. The intercellular transfer of mitochondria is facilitated by structures such as tunnelling nanotubes (TNTs) and extracellular vesicles (Puhm et al., 2019; Rustom et al., 2004). This process allows healthy cells to rescue damaged or stressed neighbouring cells by providing functional mitochondria, contributing to tissue repair, regeneration, and immune responses (Paliwal et al., 2018; Spees et al., 2006). The transfer of mitochondria from one cell to another plays a role in maintaining overall cell and tissue function, contributing to cellular homeostasis and adaptability.

1.4. Origin and evolution

The origin of mitochondria is thought to be the result of an evolutionary process known as endosymbiosis. Lynn Margulis's paper entitled "On the Origin of Mitosing Cells", provided substantial evidence and popularised the endosymbiotic theory, leading to its widespread acceptance in the scientific community. The widely accepted endosymbiotic theory is that mitochondria originated from a symbiotic relationship between an ancestral eukaryotic cell (an archaeon) and a free-living bacterium. This bacterium was probably an alpha-proteobacterium, a type of bacterium capable of aerobic respiration (Roger et al., 2017).

According to the theory of endosymbiosis, the host cell and the engulfed bacterium developed a mutually beneficial association over time, with the host cell providing protection and resources to the bacterium, while the bacterium contributed its energy-producing capabilities through aerobic respiration, producing ATP (W. F. Martin et al., 2015). This collaboration allowed the host cell to thrive in oxygen-rich environments and efficiently use the energy produced by the bacterium (Roger et al., 2017). As the relationship continued, the engulfed bacterium gradually evolved into the mitochondria, becoming an integral part of the host cell. This transformation included the development of a double membrane structure like that of free-living bacteria and the presence of circular DNA, similar to bacterial DNA (Roger et al., 2017).

The endosymbiotic theory is supported by substantial evidence, including the structural and genetic similarities between mitochondria and free-living bacteria, and the unique presence of mitochondrial DNA (mtDNA). This theory explains how mitochondria, with their vital role in energy production, became a fundamental part of eukaryotic cells and ultimately contributed to the evolution of complex life forms.

1.5. Role of mitochondria in brain function

The brain accounts for approximately 2% of the total body weight of an average adult (Raichle & Gusnard, 2002). Surprisingly, despite its relatively modest size, the brain is responsible for approximately 20% of the body's oxygen consumption (Maldonado & Alsayouri, 2020). This elevated metabolic rate remains relatively constant regardless of significant fluctuations in mental and physical activity levels. This high energy demand makes the brain highly dependent on mitochondria. Neurons have a high metabolic rate and rely on ATP to maintain their resting membrane potential, which is crucial for the transmission of electrical signals. ATP is also required for synaptic transmission, the process by which neurons in the nervous system communicate with each other. When a neuron generates an electrical signal, called an action potential, it releases chemical messengers called neurotransmitters into the synaptic cleft, a small gap between the presynaptic neuron and the receiving (postsynaptic) neuron. These neurotransmitters bind to receptors on the membrane of the receiver, either exciting or inhibiting it. This binding causes a change in the electrical potential of the receiving neuron, potentially triggering a new electrical signal. After transmission, neurotransmitters are removed to terminate the signal. This energy-intensive communication is essential for brain functioning, including sensory perception, motor control, and cognitive processes such as learning and memory (Maldonado & Alsayouri, 2020).

Mitochondria are also involved in the synthesis of certain neurotransmitters. For example, the synthesis of dopamine, which is important for mood regulation and motivation, involves monoamine oxidase (MAO) in the outer mitochondrial membrane (Meiser et al., 2013). MAO is present in neurons, and non-neuronal cells such as microglia cells and astrocytes in the central nervous system (Levitt et al., 1982; Meiser et al., 2013). Similarly, serotonin synthesis, which is important for mood and cognitive function, relies on the conversion of tryptophan to serotonin, some of which occurs in mitochondria (Boccuto et al., 2013).

Mitochondria play an important role in maintaining proper calcium levels in neurons. Calcium is a key player in neuronal signalling and synaptic plasticity (Walters & Usachev, 2023). Mitochondria sequester excess calcium, preventing its accumulation in the cytoplasm, which could lead to

overstimulation and even cell damage (Duchen, 2000; Walters & Usachev, 2023). This calcium buffering function is critical for maintaining the delicate balance of excitatory and inhibitory signalling in the brain (Matuz-Mares et al., 2022).

The brain is particularly vulnerable to oxidative stress due to its high oxygen consumption and high lipid content, which is susceptible to oxidation (Salim, 2017). While mitochondria are a source of ROS, they also contain antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which help to neutralise ROS and protect neurons from oxidative damage (Ekoue et al., 2017). The failure of this defence mechanism may contribute to neurodegenerative disorders in which oxidative stress plays a significant role.

Mitochondria are key players in apoptosis, a tightly regulated process of programmed cell death. In neurons, apoptosis is critical for eliminating damaged or non-functional cells, maintaining proper cell numbers, and shaping the developing brain. Mitochondria release apoptotic factors, including cytochrome c, which initiate apoptosis when necessary. Dysfunctional regulation of apoptosis can lead to neurodegenerative disorders, as excessive cell death or insufficient removal of damaged neurons can disrupt brain function (Naoi et al., 2019).

Brain mitochondria are adaptable and can switch between different metabolic pathways depending on energy requirements and available resources. They can use oxidative phosphorylation when oxygen and glucose are abundant, or switch to glycolysis under conditions of low oxygen or glucose availability (X. Zhang et al., 2021). This metabolic flexibility helps neurons to meet their changing energy needs and ensures that they can function effectively under different conditions (X. Zhang et al., 2021). Mitochondria are transported along the axons to provide ATP precisely where it is needed. Proper axonal transport of mitochondria is essential for maintaining neuronal function. Disruptions in this transport process can lead to energy deficits in specific neuronal regions, affecting neurotransmission and overall brain function (Mandal & Drerup, 2019).

In summary, mitochondria are multifunctional powerhouses within brain cells that play a critical role in maintaining energy balance, supporting neurotransmission, defending against oxidative stress, regulating calcium levels, and ensuring the proper development and maintenance of the nervous system. Their dysfunction can have profound effects on brain health and is implicated in several neurological disorders (NeuD).

2. Mitochondrial DNA

Mitochondria have their own DNA, known as mtDNA, which is similar to the genetic material found in bacteria (see section 1.4).

mtDNA was first discovered and isolated by Margit M. K. Nass and Sylvan Nass in 1963 (Nass & Nass, 1963). Nass and Nass used an electron microscope to detect DNA in chick embryos and developed further isolation and staining experiments to confirm its presence and structure (Nass, 1966). In the 1970s, scientists noticed sub-mitochondrial structures near the inner membrane, later called nucleoids, where the mtDNA was located (Barchiesi & Vascotto, 2019). However, it took eighteen years before the full sequence of the first mtDNA was published and officially established as the mtDNA Cambridge Reference Sequence (CRS) in 1981 (Anderson et al., 1981), The CRS was eventually upgraded to the revised version (rCRS) in 1999 (R. M. Andrews et al., 1999)and there was a proposal to replace the rCRS with the so-called Reconstructed Sapiens Reference Sequence (RSRS) (Dunlow & Duff, 1990).

2.1. Structure

The structure of mtDNA is unique and different from the nuclear DNA (nDNA) found in the cell nucleus. mtDNA is a small, circular, double-stranded molecule. It typically consists of 16,569 base pairs (bp) in humans and encodes a relatively limited number of genes, 37, that are essential for mitochondrial function, including energy production and the maintenance of the organelle. Of these 37 genes, 13 encode protein subunits, 22 encode transfer RNAs (tRNAs) essential for protein translation, and 2 are ribosomal RNAs (rRNAs) required for mitochondrial protein synthesis (Wallace et al., 1992) (Figure 3).

Unlike nuclear DNA, which is organised into linear chromosomes, mtDNA forms a compact, closed loop without introns. This circular DNA consists of two strands, the heavy (H) and light (L) strands, which differ in their nucleotide composition. The H strand is characterised by a higher percentage of guanine (G) and thymine (T) residues, resulting in its greater mass (Alexeyev, 2020). The 2 rRNAs and all structural proteins except *MT-ND6* are encoded by the H strand. mtDNA contains a 1.1 kilobase (kb) non-coding region located between *MT-TP* and *MT-TF*, also known as the control region. A significant portion of the control region often accommodates an additional linear third DNA strand of DNA of approximately 650 nucleotides in length, resulting in the formation of a stable displacement loop (D-loop) structure (Menger et al., 2021). That's why the control region is often referred to as the D-loop region. The control region also contains critical components for transcription and replication such as the H-strand promoter (HSP), the L-strand

promoter (LSP), three conserved sequence boxes, the H-strand origin of replication (O_H) and the termination-associated sequence (TAS).

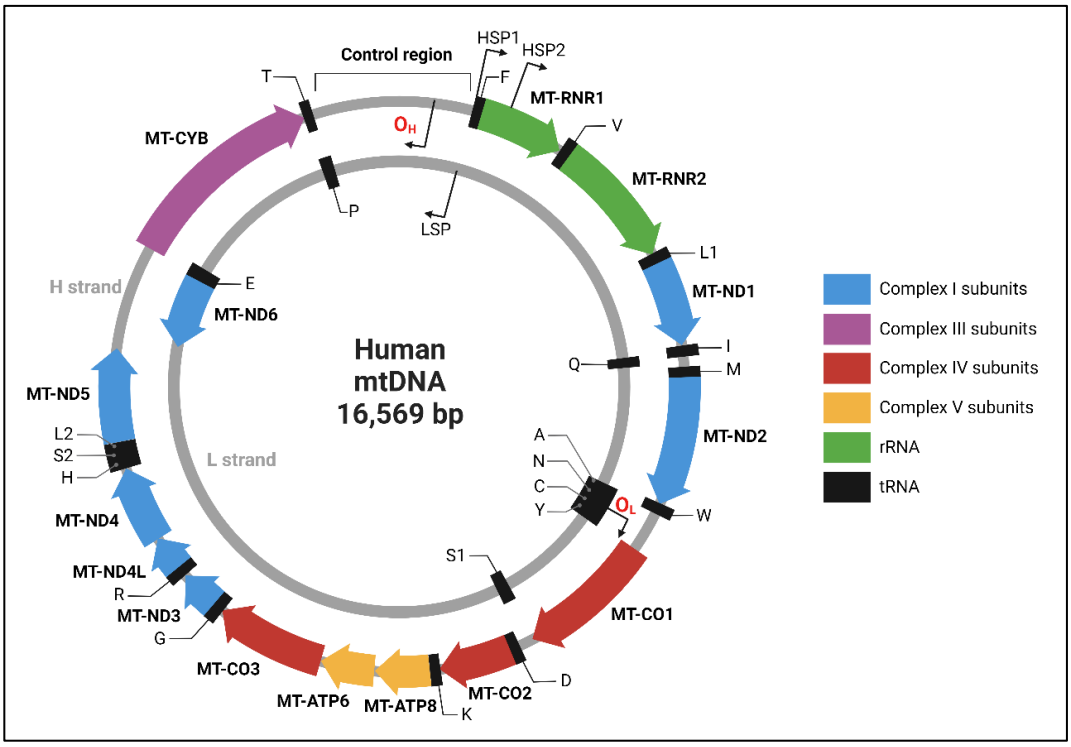

Figure 3. Schematic representation of human mtDNA.
The mtDNA contains genes encoding seven subunits of complex I (NADH ubiquinone oxidoreductase chain 1-6, ND1-ND6) (blue), one subunit of complex III (cytochrome B, CYB) (purple), three subunits of complex IV (cyclooxygenase, CO1-CO3) (red) and two subunits of complex V (ATP synthase 6 and 8, ATP6 & ATP8) (yellow). In addition to these protein-coding genes, the mtDNA encodes two rRNAs (RNR1 encoding 12S RNA; and RNR2 encoding 16S RNA) (green) and 22 tRNAs for specific amino acids (marked by black boxes with letters for specific amino acids). The heavy (H) and light (L) strand promoter regions are labelled HSP (1-2) and LSP, respectively. The H- and L-strand replication origins are represented by O_H and O_L, respectively.
Adapted from "Human mtDNA Sequence Map" by BioRender.com.

This circular structure has several consequences, including a lack of histones for structural support and a higher mutation rate than in nDNA (~10-fold higher), making mtDNA more susceptible to genetic changes over time (Alexeyev, 2020). These changes in mtDNA can occur at the single nucleotide level or involve rearrangements, such as deletions and duplications.

2.2. Replication and inheritance

mtDNA replication involves different proteins compared to nuclear DNA replication, some of which share similarities with bacteriophages (Falkenberg, 2018). This multi-protein molecular machinery for the replication is known as the replisome. The minimal mitochondrial replisome is

composed of a set of three nuclear genome-encoded proteins: the replicative mtDNA helicase Twinkle, DNA polymerase γ (Pol γ), and the mitochondrial single-stranded DNA binding protein (mtSSB). The primary replicative polymerase for mtDNA replication is a DNA polymerase gamma (POLγ), which in human cells consists of one catalytic subunit (POLγA) and two accessory subunits (POLγB). POLγA has a 3′-5′ exonuclease domain for proofreading, making it highly accurate with a low rate of misincorporation (Longley et al., 2001). POLγB enhances the interaction of POLγA with the DNA template, catalytic activity and processivity (Carrodeguas et al., 2002). mtDNA replication also requires a DNA helicase, TWINKLE, which unwinds the double-stranded DNA template during replication. TWINKLE travels ahead of POLγ, and mtSSB protects the single-stranded DNA, enhancing the helicase activity of TWINKLE and increasing the processivity of POLγ (Falkenberg, 2018). Together, these components contribute to efficient and accurate mtDNA replication.

Historically, mammalian mtDNA replication was first studied using mtDNA purified with caesium chloride (CsCl) and visualized by electron microscopy, and this research contributed to a widely accepted model of mtDNA replication, known as **the strand-displacement model**, which has been maintained for more than 35 years **(Bogenhagen & Clayton, 2003)**. In the strand-displacement model, H-strand synthesis begins within the D-loop region at O_H and proceeds continuously (Figure 4). As the H-strand synthesis progresses, replication intermediates accumulate an increasingly larger displaced parental H-strand, which is maintained in a single-stranded form. mtSSB covers the displaced parental H-strand to prevent the mitochondrial RNA polymerase (POLRMT) from initiating random RNA synthesis on the exposed strand (Wanrooij et al., 2008). As the replication fork progresses about two-thirds of the way around the mitochondrial genome, it encounters the second origin of replication, O_L. When O_L is exposed in its single-stranded conformation, the parental H-strand at O_L forms a stem-loop structure. This stem-loop efficiently blocks mtSSB from binding, leaving a short segment of single-stranded DNA accessible in the loop region. This accessibility allows POLRMT to initiate RNA synthesis. Since POLRMT is not a processive enzyme on single-stranded DNA templates, it is replaced by POLγ after about 25 nucleotides. This transition marks the start of L-strand DNA synthesis. From this point, both H- and L-strand synthesis proceed continuously until both strands have completed a full cycle of replication. These two strand syntheses are interlinked, as H-strand synthesis is a prerequisite for the initiation of L-strand synthesis (Falkenberg, 2018).

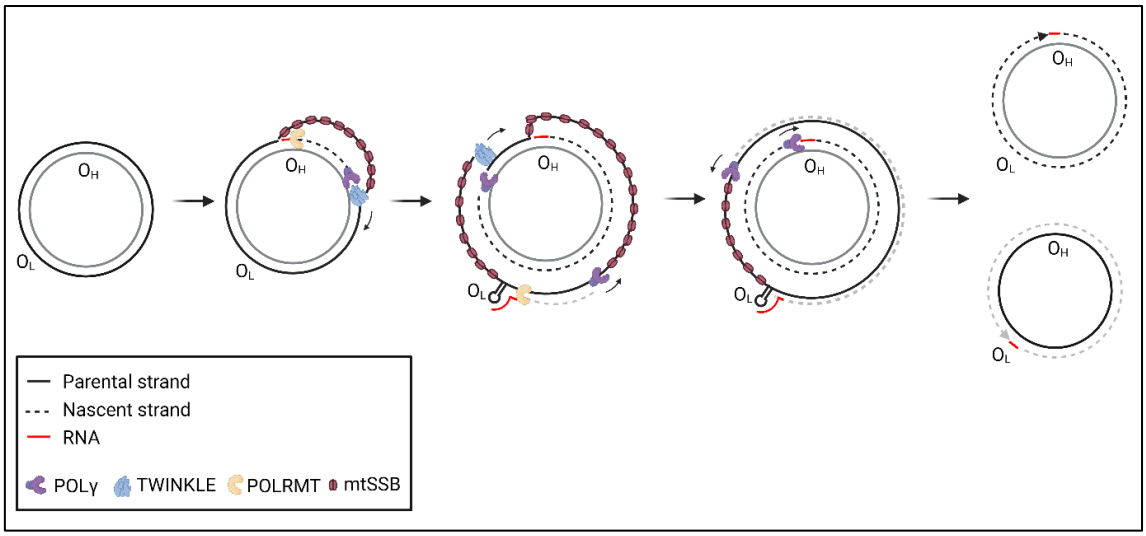

Figure 4. Replication of the human mitochondrial genome.
Replication starts at the O_H and proceeds in a single direction, generating the complete nascent H-strand. mtSSB attaches to and protects the exposed parental H-strand. As the replisome moves beyond the O_L, a stem-loop structure forms, preventing mtSSB binding and providing a single-stranded loop region for POLRMT to initiate primer synthesis. The switch to L-strand DNA synthesis occurs after approximately 25 nucleotides, with POLγ taking over from POLRMT at the 3' end of the primer. Both strands are then continuously synthesised until two complete double-stranded DNA molecules are produced. mtSSB: mitochondrial single-stranded DNA binding protein; O_H: H-strand origin of replication; O_L: L-strand origin of replication; POLγ: DNA polymerase gamma; POLRMT: mitochondrial RNA polymerase.
Adapted from Falkenberg, M. (2018). Mitochondrial DNA replication in mammalian cells: Overview of the pathway. In Essays in Biochemistry (Vol. 62, Issue 3). https://doi.org/10.1042/EBC20170100.

Some studies have proposed an alternative view in which processed RNA molecules form hybrids with the single-stranded H-strand and serve as a temporary lagging strand that is subsequently replaced by DNA during later stages of mtDNA replication (Yasukawa et al., 2006). This alternative view, known as ribonucleotide incorporation throughout the lagging strand, **the RITOLS model**, suggests that processed transcripts (rRNA, tRNA, and mRNA with poly A tails) progressively hybridise to the paternal H-strand as the replication fork advances (Reyes et al., 2013; Yasukawa et al., 2006). However, this model fails to identify any molecular machinery that could explain how these processed transcripts are threaded onto the displaced parental H-strand during mtDNA replication **(Falkenberg & Gustafsson, 2020)**. Furthermore, the presence of RNase H1 in mammalian mitochondria, an enzyme responsible for actively degrading RNA molecules hybridised to single-stranded DNA (ssDNA), provides evidence against the use of RNA to stabilise ssDNA regions **(Falkenberg & Gustafsson, 2020)**. Moreover, the abundance of mtSSB in mammalian mitochondria negates the need for alternative methods of ssDNA protection, unlike what is observed in many other biological systems. In this respect, the RITOLS model remains an unproven hypothesis until further biochemical evidence is provided.

A third model for mtDNA replication, known as **the strand-coupled model**, has been proposed to account for observations in certain cell types and conditions (Holt et al., 2000). This model proposes that L-strand DNA synthesis is initiated at multiple sites on the parental H-strand, resulting in the synthesis of shorter fragments that are later ligated to form a continuous strand, similar to conventional DNA replication in other systems (Holt et al., 2000). Unlike the strand-displacement model, the strand-coupled model does not suggest a direct physical link between the DNA polymerases operating on the H and L strands **(Falkenberg & Gustafsson, 2020)**. Okazaki-like replication intermediates observed in certain cell types support this model (Miralles Fusté et al., 2014). POLRMT can initiate primer synthesis with only a short T-stretch on ssDNA, typically at the O_L, which is regulated by mtSSB. However, reduced mtSSB levels of disruptions in the replication machinery could allow POLRMT to initiate DNA synthesis from sites outside the O_L **(Falkenberg & Gustafsson, 2020)**. Evidence from several studies suggests alternative origins of L-strand mtDNA synthesis, possibly related to loss of mtSSB function due to identified disease-causing mutations in the mtSSB gene **(Falkenberg & Gustafsson, 2020)**. As with the RITOLS model, further analysis of replication intermediates is needed to understand priming outside the O_L region.

2.3. Genetic code, transcription and translation

Several differences have emerged between mtDNA and nDNA since the original symbiotic event. In vertebrate mtDNA, the codons AUA and AUG both code for methionine, UGA codes for tryptophan rather than a stop codon as in nDNA, and AGA and AGG are read as stop codons rather than arginine as in nDNA **(Stewart & Chinnery, 2015)**.

Mitochondrial transcription and translation are essential for the assembly and function of oxidative phosphorylation complexes. Mitochondrial gene expression is unique in that its components have dual sources, originating from both the mitochondria (all RNAs) and the nucleus (all protein factors). Transcription of mtDNA starts in LSP and HSP located in the D-Loop region. The LSP is responsible for the transcription of eight tRNAs and the *MT-ND6* gene. On the H-strand, a two-promoter system has been proposed, with HSP1 initiating transcription for a transcript containing $tRNA^{Phe}$, $tRNA^{Val}$ and both rRNAs (12S and 16S), while HSP2 generates a transcript spanning almost the entire genome (Montoya et al., 1983). However, recent evidence from animal models and in vitro experiments suggests that heavy strand transcription may be regulated by a single promoter on the H-strand **(D'Souza & Minczuk, 2018)**.

The initiation of transcription, driven by POLRMT, requires the cooperation of mitochondrial transcription factors A (TFAM) and B2 (TFB2M) (Figure 5) (D'Souza & Minczuk, 2018). Recent evidence suggests that in the transcription initiation complex at both the HSP and LSP, DNA-bound TFAM recruits POLRMT to the promoter through its N-terminal extension, while TFB2M modifies the structure of POLRMT, facilitating promoter opening (D'Souza & Minczuk, 2018; Kanki et al., 2004; Ramachandran et al., 2017).

POLRMT requires an additional transcription elongation factor (TEFM) for the elongation phase (Figure 5). The LSP transcript is often prematurely terminated around the conserved sequence block 2 (CSB2) of the D-loop (D'Souza & Minczuk, 2018). The short RNA molecule generated is thought to play a pivotal role in priming DNA replication, as multiple RNA-to-DNA transition sites are clustered around CSB2. TEFM's ability to enhance the processivity of POLRMT prevents the formation of G-quadruplex structures that hinder the progress of the elongation complex at CSB2. This enhancement is proposed to act as a switch, transitioning from replication to transcription of the primary LSP-derived transcript. Recent structural studies have shown that TEFM contains a pseudonuclease core that forms a "sliding clamp" around the mtDNA downstream of the transcribing POLRMT, interacting with POLRMT via its C-terminal domain (D'Souza & Minczuk, 2018).

The mechanism of HSP transcription termination remains unclear. A previous hypothesis suggested that mitochondrial termination factor 1 (MTERF1) causes a bend in the mtDNA, connecting the HSP1 promoter site to its apparent $tRNA^{Leu(UUR)}$ termination site (Figure 5). This bend would lead to transcription termination via base flipping and DNA unwinding, which was initially proposed to explain the higher abundance of mitochondrial rRNAs (D'Souza & Minczuk, 2018). However, recent evidence contradicts this notion. A study in MTERF1 knockout mice reported that there is no effect on steady-state rRNA levels, suggesting that the increased abundance is likely due to increased stability rather than a different promoter (Terzioglu et al., 2013). In addition, MTERF1 was observed to prematurely terminate transcription from the LSP at the 3′ end of the rRNA coding sequence. Binding of MTERF1 to this site stops the progression of the replication fork into the mt-rRNA genes during transcription and prevents the transcription of the antisense rRNA sequence (D'Souza & Minczuk, 2018).

Transcription from the HSP and LSP produces long polycistronic transcripts, which are then processed by various enzymes to release the mRNAs and rRNAs (Figure 5). The processing of mt-tRNAs from the primary transcript is performed by RNase P and RNase Z at the 5′ and 3′ ends, respectively. Fas-activated serine/threonine kinase (FASTK) proteins are required for mtRNA stability. Polyadenylation of mitochondrial transcripts is performed by homodimeric polyadenylic

acid RNA polymerase (mtPAP). Leucine-rich pentatricopeptide rich domain containing protein (LRPPRC) regulates the stability of HSP-derived mitochondrial transcripts. A complex of polynucleotide phosphorylase (PNPase) and human Suv3 protein (hSuv3) mediates human mitochondrial RNA degradation with an RNA exonuclease protein called REXO2 **(Barchiesi & Vascotto, 2019)**. The mt-tRNAs undergo post-transcriptional maturation, including chemical nucleotide modifications and CCA addition at the 3′ end **(D'Souza & Minczuk, 2018)**.

The initiation phase of mitochondrial translation is dependent on nuclear-encoded factors, mtIF2 and mtIF3. mtIF3 ensures proper mRNA initiation codon positioning at the mitochondrial small subunit rRNA (mtSSU) peptidyl site and prevents premature association with the mitochondrial large subunit rRNA (mtLSU). **(Haque & Spremulli, 2008)**. Although initiation starts with methionine, mitochondria use a single $tRNA^{Met}$ for both initiation and elongation, distinguished by the formylation of methionine. Due to the absence of 5′ untranslated regions (5′-UTRs) and other regulatory elements on mRNAs in mammalian mitochondria, some protein factors bind directly to the mitochondrial transcripts to regulate gene expression, specifically affecting the translation of the complex IV subunit CO1 **(D'Souza & Minczuk, 2018)**.

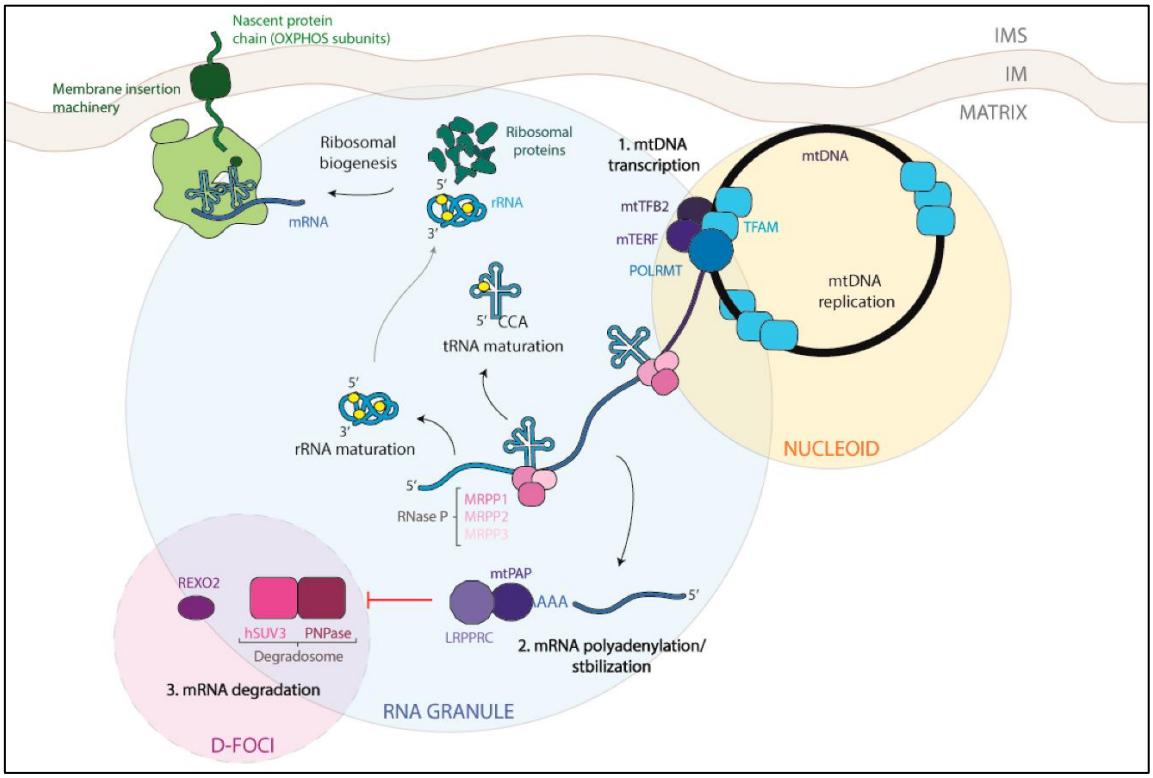

Figure 5. Schematic view of mtDNA transcription, RNA processing and degradation.
TFAM, together with POLMRT, TFBM1, and TFBM2, plays a crucial role in transcription initiation. Following the transcription of polycistronic molecules, the RNA is immediate processed by RNase P and Z, resulting in the release of tRNAs. Once tRNAs, mRNAs, and rRNAs are released, the translation process begins. The degradation of mRNA takes place in the degradation foci (D-foci), which are located near the mitochondrial RNA granules (MRGs). The degradosome, consisting of PNPase and hSuv3, is involved in this process. In

addition, REXO2 is located in this compartment and contributes to the degradation of small RNA oligonucleotides. Black arrows indicate transitions, while the red arrow indicates inhibition. IM: Inner membrane; IMS: Intermembrane space.
Adapted from Barchiesi, A., & Vascotto, C. (2019). Transcription, processing, and decay of mitochondrial RNA in health and disease. In International Journal of Molecular Sciences (Vol. 20, Issue 9). https://doi.org/10.3390/ijms20092221.

Factors such as EFTu, EFTs, and EFGM are involved in the elongation of mitochondrial translation (Hammarsund et al., 2001). EFTu forms a complex with GTP and aminoacyl tRNA, guiding the tRNA to the acceptor site and facilitating peptide bond formation. EFGM plays a role in releasing deacetylated tRNA from the P site, translocating peptidyl tRNAs, and advancing the mRNA by one codon. Termination occurs upon encountering a stop codon at the A site, with human mitochondrial release factors, including mtRF1, mtRF1a, C12orf65, and ICT1, being recognised by a GGQ motif that triggers peptidyl tRNA hydrolysis. However, the role of ICT1 in terminating the translation of specific mRNAs, such as *MT-CO1* and *MT-ND6,* is unclear due to their unique termination sequences. Subsequently, mtRRF and EFG2 recycle ribosomal components, mRNAs, and deacetylated tRNAs during polypeptide release (D'Souza & Minczuk, 2018).

2.4. Repair

mtDNA repair mechanisms play a critical role in maintaining the integrity of the mitochondrial genome. Several pathways for mtDNA repair have been identified, including base excision repair (BER), mismatch repair (MMR), direct reversal (DR), and double-strand break (DBS) repair.

BER is a fundamental and highly conserved process responsible for the repair of non-bulky DNA lesions, primarily caused by factors such as oxidation, alkylation, deamination, and methylation (Rong et al., 2021) (Figure 6). This repair pathway involves four key steps: 1) recognition and removal of modified DNA bases, 2) formation of apurinic/apyrimidinic (AP) sites, 3) correction of nucleotide synthesis, and 4) rejoining of DNA strands. BER uses two main approaches: Short-Patch Base Excision Repair (SP-BER) for single mutated bases and Long-Patch Base Excision Repair (LP-BER) for larger mutated DNA fragments (Liao et al., 2022). DNA glycosylases are critical for initiating BER by recognising and eliminating damaged DNA. Monofunctional glycosylases recognise mismatched bases and cleave the N-glycosidic bond, leading to the formation of AP sites, while bifunctional glycosylases have AP lyase activity that helps to break the DNA backbone (Liao et al., 2022; Rong et al., 2021). Several mitochondrial glycosylases have been identified, including UNG, OGG1, and NEIL1. OGG1 is a nuclear-encoded DNA glycosidase with dual functionality, capable of recognising and repairing 8-OXOG lesions, while NEIL1 is predominantly localised to mitochondria and can remove FapyG and FapyA lesions. After the initiation step, the

POLγ, the apurinic/apyrimidinic endodeoxyribonuclease, and ligase 1 work together to repair damaged sites within the mtDNA, ensuring the maintenance of mitochondrial genomic integrity.

MMR is responsible for correcting errors in DNA replication that result in mismatches between base pairs (Figure 6). Unlike the nuclear MMR system, in mitochondria there is no evidence that this process relies on established nuclear MMR proteins such as MSH and MLH (Liao et al., 2022). However, some studies show that it may depend on the Y-box binding protein YB-1 (de Souza-Pinto et al., 2009; Lyabin et al., 2014). These observations are supported by the results of YB-1 depletion, which leads to a reduction in MMR activity in mitochondrial extracts and an increase in mtDNA mutations in YB-1-deficient cells (de Souza-Pinto et al., 2009; Lyabin et al., 2014). While these findings strongly suggest the existence of a YB-1-dependent MMR in mitochondria, further investigations are required to elucidate its precise mechanism, in particular the mitochondrial YB-1-associated cofactors involved in this process.

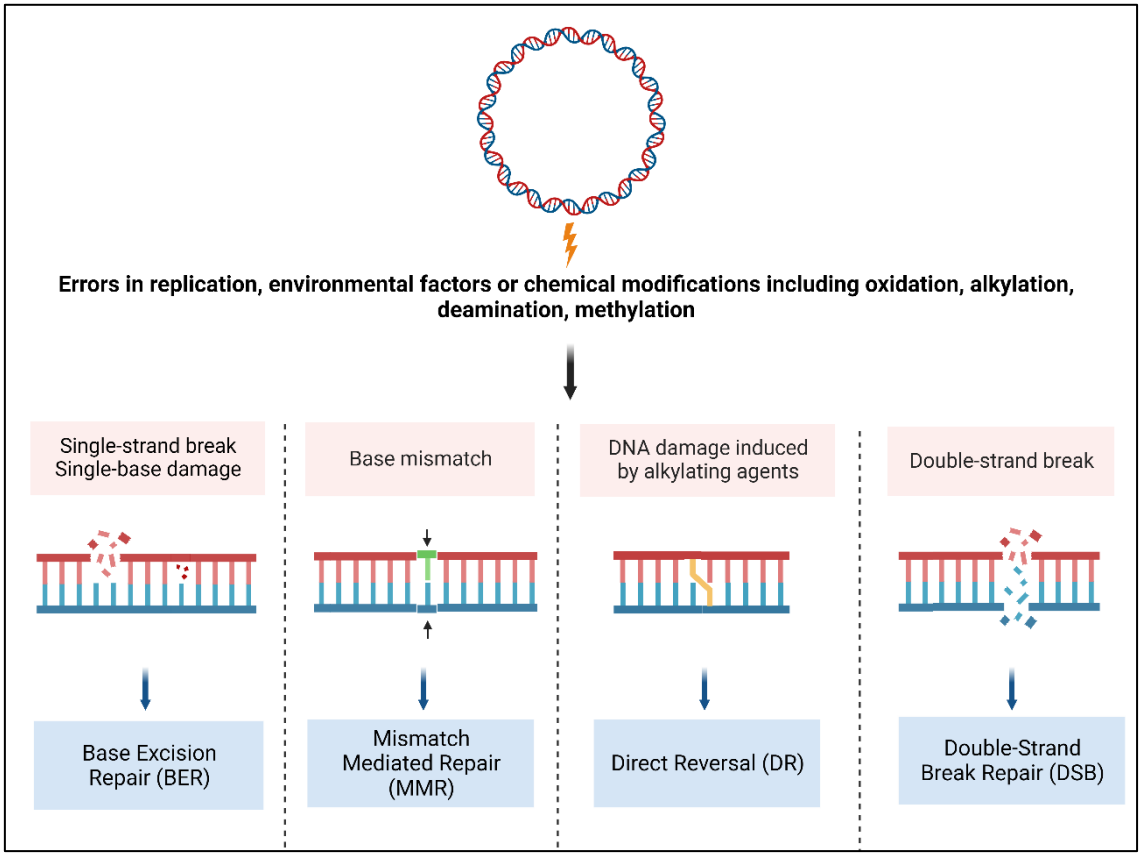

Figure 6. mtDNA repair mechanisms.
Created with BioRender.com.

The DR mechanism focuses on the repair of specific types of DNA lesions caused by environmental factors or chemical modifications induced by alkylating agents (Liao et al., 2022) (Figure 6). Unlike other repair pathways, DR does not involve the excision, synthesis or ligation of DNA. In

mammalian cells, DR is primarily carried out in the cell nucleus by proteins such as O6-methylguanine DNA methyltransferase (MGMT) and alpha-ketoglutarate-dependent dioxygenase (ALKBH) proteins. MGMT functions by removing methyl groups from O6-methylguanine lesions, whereas ALKBH is involved in the repair of specific N-alkyl lesions through dealkylation (Ahmad et al., 2015; Ragg et al., 2000). The mitochondrial localisation of ALKBH has not been definitively established, and there are conflicting reports regarding the presence of a protein with a molecular weight similar to MGMT in mammalian mitochondria (Liao et al., 2022). Therefore, the existence of DR in mitochondria remains an area that requires further investigation and clarification.

DSB occurs when both strands of mtDNA strands are broken, which is a severe form of damage (Figure 6). This pathway involves the rejoining of the broken DNA strands, preventing the loss of genetic information and maintaining the structural stability of the mtDNA. DSB repair involves two primary mechanisms: homologous recombination repair (HR) and non-homologous end joining (NHEJ) repair (Liao et al., 2022). In HR, DSB repair is primarily facilitated by RAD51 and its homologs, while the presence and detailed mechanisms of HR and NHEJ in mitochondria require further investigation (Mishra et al., 2018). The specific components, signals, and protective mechanisms associated with DSB repair for mtDNA have not been comprehensively characterised, highlighting the need for further research to unravel the intricate molecular processes involved in different mtDNA damage repair pathways.

2.5. Human mitochondrial genetics

One of the most interesting features of human mitochondrial genetics is maternal inheritance. Unlike nDNA, which is inherited from both parents, mtDNA is inherited from the mother. Several factors contribute to the maternal inheritance of mtDNA. The egg contains a significantly higher number of mitochondria (hundreds of thousands) compared to the sperm (~100 copies) (Stewart & Chinnery, 2015); this increases the likelihood that mtDNA will be passed from the mother to the offspring. There is also selective degradation of mitochondria during fertilisation (Patel, 2017). This maternal inheritance pattern makes mitochondrial genetics a powerful tool for tracing maternal ancestry and understanding population genetics. However, some studies have reported that there are exceptional cases where paternal mtDNA can be passed on to the offspring (Luo et al., 2018). mtDNA undergoes continuous destruction and replication, independent of the cell cycle, known as relaxed replication. Despite the presence of the mtDNA repair machinery, mtDNA is susceptible to inherited or acquired mutations that accumulate with age. For example, a human post-mitotic neuron with approximately 10,000 mtDNA molecules, can persist for 80 years.

Remarkably, during its lifespan, this neuron undergoes approximately 3,000 complete replacements of its entire mtDNA pool, all without concurrent replication of nDNA (Stewart & Chinnery, 2020a; Yasukawa & Kang, 2018). This distinctive pattern contributes to the susceptibility of mtDNA to mutation accumulation.

An average human cell contains several hundred to 1,000 mitochondria, and each of these mitochondria contains between 2 and 10 copies of mtDNA (Y. Zhang et al., 2015). Mutated mtDNA molecules often coexist with wild-type mtDNA in the same cell, a condition known as heteroplasmy. Heteroplasmic molecules can change their proportions during both mitotic and meiotic cell division, resulting in a continuous range of bioenergetic defects -a phenomenon known as replicative segregation (Figure 7). As the proportion of mutant mtDNA increases, the associated bioenergetic impairment becomes progressively more severe. Since different tissues have different bioenergetic thresholds, a decline in an individual's bioenergetic capacity will eventually fall below the critical threshold for a particular tissue, leading to the onset of mitochondrial defects (Figure 7). Given that the tissues and organs with the highest bioenergetic demands are often the primary targets in common metabolic and degenerative disorders, this suggests that mitochondrial dysfunction may contribute significantly to the complexity of these disorders (Stewart & Chinnery, 2020a).

Females carrying deleterious heteroplasmic mutations are more likely to have affected offspring, with the nature and severity of the specific phenotype depending on both the type of mtDNA mutation and the heteroplasmy level (Figure 7). This phenomenon is explained by the 'mitochondrial bottleneck' hypothesis, whereby only a small proportion of mtDNA is passed from mother to offspring (Wallace & Chalkia, 2013). Over time, cells and individuals can accumulate a wide range of mtDNA mutations that collectively compromise the energetic capacity of the cell. These mutations play an important role in processes such as ageing and cancer. Despite the considerable explanatory potential of heteroplasmic mtDNA mutations, surprisingly little is understood about their origin, genetic characteristics, and the phenotypic effects they induce (Stewart & Chinnery, 2015, 2020b).

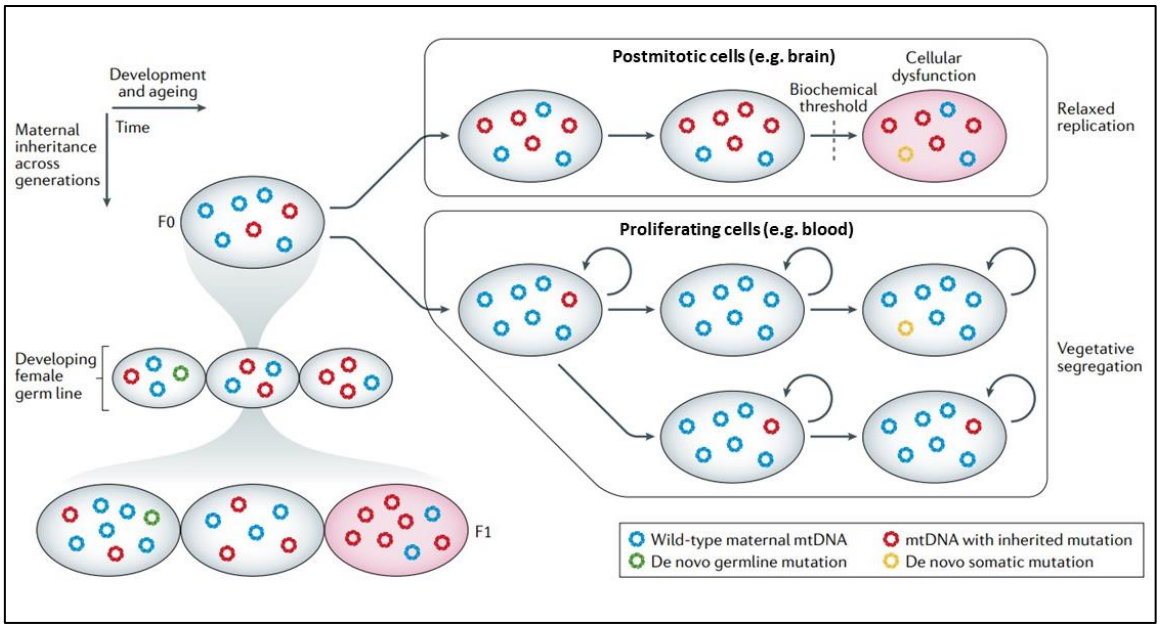

Figure 7. Fluctuations in mtDNA heteroplasmy levels over time.
Levels of mtDNA heteroplasmy can fluctuate throughout the lifespan of an individual, primarily due to vegetative segregation during cell division and relaxed replication in both dividing and non-dividing cells. In post-mitotic cells, the process of relaxed replication can lead to increased levels of mutant mtDNA over time. If this exceeds a critical threshold, it can lead to a biochemical dysfunction in oxidative phosphorylation. The genetic bottleneck that occurs in the germline accelerates the process of segregation between generations, causing significant shifts in heteroplasmy levels. In both scenarios, the presence of high levels of a pathogenic mutation can induce a cellular biochemical impairment in ATP synthesis. In addition, heteroplasmy can be introduced by de novo mutations in both somatic and germline cells. F0: Filial 0 (the first generation); F1: Filial 1 (The first generation of progeny).
Adapted from Stewart, J. B., & Chinnery, P. F. (2021). Extreme heterogeneity of human mitochondrial DNA from organelles to populations. Nature Reviews. Genetics, 22(2), 106–118. https://doi.org/10.1038/s41576-020-00284-x.

2.6. Human mitochondrial variation

The human mitochondrial genome undergoes rapid evolution. Early attempts to calibrate the mitochondrial molecular clock in primates estimated 2 × 10−8 substitutions per bp per year, which is 5- to 10-fold higher than the nDNA rate (PMID: 109836). Changes in the mtDNA sequence have also been linked to environmental adaptation in recent human evolution; therefore, selective forces must be considered when analysing mitochondrial diversity between populations (Hernández, 2023).

Mitochondrial DNA polymorphisms have been extensively used to reconstruct human evolutionary history due to its maternal inheritance, lack of recombination, and high mutation rate compared to nuclear DNA. MtDNA has been instrumental in deepening our understanding of our own past, leading to a scientific consensus on the common African genetic origin of all present-day human populations around 200 thousand years ago (kya)(Pakendorf & Stoneking, 2005).

The term “haplogroup” in mitochondrial studies, originally defined as a “group of related haplotypes”, is represented by terms such as ‘lineage’, ‘clade’ or ‘cluster’, denoting groups of sequences defined by shared mutations and which tend to show regional specificity (Torroni et al., 1993). In this sense, mtDNA variation, as a haploid marker, is studied from a phylogeographic perspective, examining the connection between genealogy and geography (Hickerson et al., 2010).

The first mtDNA haplogroups (A, B, C, D) were identified in Native American populations, followed by the definition of lineages H, I, J and K in American individuals of European ancestry. Subsequent characterisation of haplogroups T, U, V, W, and X described most of the extra-African human mitochondrial diversity. All of the above lineages are grouped into the macro-haplogroup L3, which represents the out-of-Africa dispersion (Hernández, 2023). It is noteworthy that most of the mtDNA haplogroup markers are SNP.

MtDNA has been used extensively in several fields, including human population genetics, forensics, paleogenomics, and biomedicine. As mitochondria are integral to essential biological functions such as cellular energy production, cell signalling, homeostasis, growth, and inflammation, mtDNA plays a key role in several physiological processes. Variants in mtDNA are likely to have significant consequences for human biology and can be studied from multiple perspectives (PMID: 26406369). Each haplogroup, with its unique set of SNPs, may have distinct bioenergetic and biochemical properties. In addition to SNP, mutational events in the mtDNA sequence are a major cause of inherited human disease(P. Chinnery, 2022) . Pathogenic variants causing mitochondrial disorders (MitD) can be single nucleotide variants (SNV or pSNV) or large scale mtDNA rearrangements. Several tools, including the Mitoverse platform (https://mitoverse.i-med.ac.at), which uses mtDNA-Server v2 and Mutserve for variant calling (Weissensteiner et al., 2016, 2021) and MToolBox (Calabrese et al., 2014), have been developed for calling heteroplasmic and homoplasmic SNP and SNV, while MitoSAlt (S. Basu et al., 2020) and eKLIPse (Goudenège et al., 2019) have been developed for identifying mtDNA rearrangements.

2.7. Primary mitochondrial disorders (MitD)

Primary MitD are a clinically heterogeneous group of diseases that result from dysfunction of the mitochondrial respiratory chain. Many genetic and non-genetic disorders involve mitochondrial mechanisms as a secondary feature. However, primary MitD refers specifically to known or suspected genetic disorders caused by pathogenic variants in genes encoding the mitochondrial respiratory chain and associated proteins (P. Chinnery, 2022).

The inner mitochondrial membrane brings together over 70 different polypeptides to form the respiratory chain. Inside the mitochondrion, the mtDNA carries the genes for thirteen key subunits, as well as ribosomal and transfer RNAs necessary for protein synthesis and mtDNA replication. The remaining respiratory chain polypeptides, together with proteins essential for respiratory chain assembly, maintenance of mitochondrial structure and management of the mtDNA, are controlled by the nDNA. Consequently, primary MitD result from mutations in either nDNA or mtDNA that disrupt critical processes that ultimately affect energy production (P. Chinnery, 2022).

The clinical manifestations of primary MitD can vary widely, even among individuals with the same genetic variation, making diagnosis and treatment complex. Neurological symptoms are common and can include seizures, developmental delay, and progressive cognitive decline. Muscular symptoms such as weakness and fatigue are also common, highlighting the impact on metabolically demanding tissues (P. Chinnery, 2022). Diagnosis often involves a combination of clinical assessment, genetic testing and, in some cases, muscle biopsy. Approximately 1 in 5,000 people are affected by a mitochondrial disease (P. F. Chinnery & Horvath, 2020). Misdiagnosis is common in these cases due to the variety of symptoms and organ systems involved, which may lead to an underestimation of the prevalence.

Unfortunately, there is currently no effective treatment for mitochondrial diseases. Treatment strategies focus mainly on improving quality of life. Many treatments focus on managing specific symptoms associated with mitochondrial diseases, such as muscle weakness, seizures, and organ dysfunction. This may involve the use of medications, physiotherapy and other supportive measures tailored to individual needs (Pfeffer et al., 2012). Maintaining a balanced diet and addressing specific nutritional deficiencies are also important in the management of mitochondrial disease. Certain supplements, such as coenzyme Q10, carnitine, and vitamins, have been recommended to support mitochondrial function in patients (Pfeffer et al., 2012; Virmani & Cirulli, 2022). There are some experimental approaches, including mitochondrial replacement therapy (MRT), which involves replacing or manipulating defective mitochondria, such as mitochondrial donation or transfer to replace damaged mitochondria with healthy ones from a donor egg or cell. While MRT holds great promise for preventing the transmission of mitochondrial diseases, addressing its limitations requires ongoing research, ethical scrutiny, and careful consideration of societal perspectives (Sharma et al., 2020).

2.7.1. MitD caused by mtDNA alterations

The mitochondrial genome is clearly a focus of attention in biomedicine with regard to the relevant clinical outcomes of mtDNA mutations and the mitochondrial influence on human aging. The role of mitochondrial mutations in disease is complex, and MitD very in severity and clinical expression. Thus, there is a wide range of situations from high-penetrance variants to low-penetrance risk mutations that, together with a specific population profile or certain environmental conditions, could lead to disease (Herrnstadt & Howell, 2004). MitD caused by pathogenic variants in mtDNA usually follow maternal inheritance. These pathogenic variants include SNV and large rearrangements (mostly deletions).

The syndromes caused by single large mtDNA deletions include a number of overlapping clinical phenotypes, including **Kearns-Sayre syndrome (KSS)**, **Pearson syndrome (PS)** and **chronic progressive external ophthalmoplegia (CPEO)** (Figure 8). The main features of KSS are pigmentary retinopathy, CPEO, and cardiac conduction abnormalities. Cerebellar ataxia, intellectual disability (ID) or cognitive decline, dementia, sensorineural hearing loss (SNHL), exercise intolerance, muscle weakness, and endocrinopathies are also considered additional features of KSS (Goldstein & Falk, 2023). PS is characterised by a reduction in the number of almost all blood cells (pancytopenia), sideroblastic anaemia of childhood, exocrine pancreatic dysfunction, poor weight gain, and lactic acidosis. Renal tubular acidosis, short stature, and elevated liver enzymes are also secondary features of PS (P. Chinnery, 2022; Goldstein & Falk, 2023). CPEO is characterised by upper eyelid drooping (ptosis), ophthalmoplegia, oropharyngeal weakness, variable proximal limb weakness and/or exercise intolerance (Goldstein & Falk, 2023).

MitD caused by pSNV include **Leigh syndrome (LS)**, **neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP)**, **mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERFF)** and **Leber's hereditary optic neuropathy (LHON).** LS is a severe neurological disorder that usually presents in the first year of life. It is characterised by progressive loss of mental and motor skills (psychomotor regression) and usually results in death from respiratory failure within two to three years. It is primarily caused by pSNV in complex I (almost one third of cases) and complex IV (about 15% of cases) (Bakare et al., 2021). There are at least 3 major causes of LS, each transmitted by a different mode of inheritance: X-linked recessive, mitochondrial, and autosomal recessive. Thus, LS may also be associated with pSNV in nDNA. The most common pathological mtDNA variant in LS affects the *MT-ATP6* gene and blocks ATP production via complex V deficiency (Bakare et al., 2021; P. Chinnery, 2022) . **NARP** is a rare progressive neurodegenerative disease that classically presents with the features of its name and other neurological findings, including cognitive impairment and

seizures. It is primarily caused by a thymine to guanine point mutation at nucleotide 8993 of the MT-ATP6 gene (m.8993T>G). Other less common variants of NARP have been described, including a thymine to cytosine substitution at the same site (m.8993T>C) and a guanine to adenine substitution at nucleotide 14459 of the MT-ND6 gene (m.14459G>A) (P. Chinnery, 2022; Ng et al., 2019). MELAS is a genetically heterogeneous MitD with a variable clinical phenotype. Affected individuals typically develop signs and symptoms between the ages of two and 40 years, including stroke-like episodes, encephalopathy with seizures and/or dementia, muscle weakness and exercise intolerance, recurrent headaches or vomiting, hearing impairment, peripheral neuropathy, learning disability, and short stature (El-Hattab et al., 2015). More than 80% of MELAS cases have the m.3243A>G pSNV variants in *MT-TL1*, but other pSNV in *MT-ND1, MT-ND5, MT-TH,* and *MT-TV* have been reported (El-Hattab et al., 2015; Ikeda et al., 2018). MERRF is a multisystem mitochondrial syndrome characterised by progressive myoclonus and seizures. Other features associated with MERRF include cerebellar ataxia, myopathy, cardiac arrhythmias, sensorineural hearing loss, optic atrophy, and dementia **(Hameed & Tadi, 2021)**. In the *MT-TK* gene, pSNV are the most common cause of MERRF, although variants in *MT-TL1, MT-TH* and *MT-TS1* have also been reported (DiMauro, 2004). LHON is characterised by a sudden or gradual loss of vision in young adults, with a higher incidence in males (P. Chinnery, 2022). While about a dozen different SNVs in mtDNA structural genes have been associated with LHON, it has been established that three primary pSNV in *MT-ND1* (m.3460 G>A), *MT-ND4* (m.11778 G>A) and *MT-ND6* (m.14484 T>C) are found in 90% of affected families, (DiMauro, 2004).

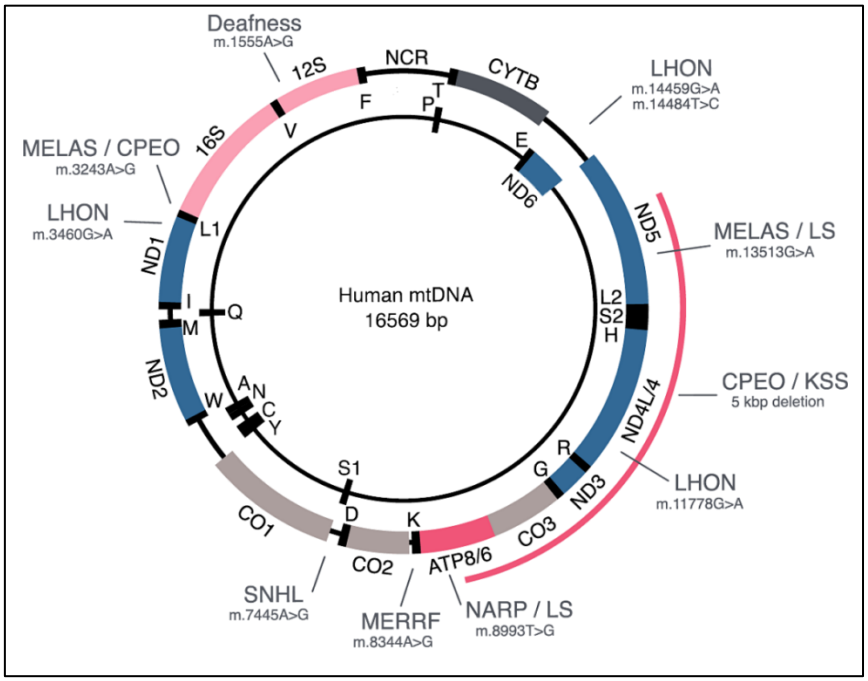

Figure 8. Morbidity map of the human mitochondrial genome.
Taken from Blueprint Genetics. (n.d) *Mitochondrial Disorders. https://blueprintgenetics.com/tests /panels/ mitochondrial-disorders/*

2.7.2. MitD caused by nDNA alterations

MitD caused by mutations in the nDNA result from defects in genes critical for mitochondrial structure and function. The nuclear genome encodes the majority of proteins essential for mitochondrial processes. Patterns of inheritance vary, with autosomal recessive and dominant modes predominant. The clinical spectrum includes a wide range of manifestations affecting tissues with high energy demands. Neurological symptoms, myopathies, and multi-organ dysfunction are common. Clinical variability is also frequent, and individuals affected by these conditions often defy simple classification into distinct categories. For example, pathogenic variants in *POLG*, an nDNA-encoded gene, are the major contributors to nDNA MitD (Wong et al., 2008). The impact of POLG variations is notable because it shows a spectrum of overlapping disease phenotypes, highlighting the complexity of clinical manifestations. In addition, variations in nDNA genes encoding structural subunits, assembly factors and translation factors cause LS with complex I deficiency or complex II deficiency, cardiomyopathy, myopathy, encephalopathy, optic atrophy, ataxia, hypokalemia and lactic acidosis (P. Chinnery, 2022; Wong et al., 2008). Some nDNA MitD, including Alpers-Huttenlocher syndrome, autosomal progressive external ophthalmoplegia, and mitochondrial neurogastrointestinal encephalomyopathy, are also associated with multiple mtDNA deletions or mtDNA depletion (El-Hattab et al., 1993).

3. Role of mitochondria in brain disorders and ageing

Brain disorders include a wide range of conditions that affect the structure and function of the brain, resulting in cognitive, behavioural and neurological impairment. These disorders can be caused by a variety of factors, including genetic predisposition, environmental exposures, or a combination of both. Neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), involve a progressive loss of neuronal function and are often associated with ageing. Neurodevelopmental disorders, such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD) and ID, result from disruptions in early brain development and affect cognition and behaviour and therefore social, academic, and occupational functioning. ID can co-occur with some of these disorders, highlighting the complexity of their interrelationships. Traumatic brain injury is caused by external forces and can result in immediate or delayed neurological dysfunction. Psychiatric disorders (PsyD), including major depressive disorder (MDD), schizophrenia (SZ), and bipolar disorder (BD), involve disturbances in mood, thought processes, and perception. Finally, brain dysfunction and ageing are interrelated

phenomena characterised by structural and functional changes in neural tissue. As people age, there is a natural decline in cognitive abilities, such as memory, processing speed, and executive function. Age-related brain dysfunction involves changes in neurotransmitter systems, synaptic plasticity, and the accumulation of cellular damage. Common manifestations include cognitive decline, increased susceptibility to neurodegenerative disorders, and changes in neuronal connectivity.

Advances in neuroimaging, molecular biology and genetics have contributed to a deeper understanding of the underlying mechanisms of these disorders. Genetic studies have greatly advanced our understanding of brain disorders, revealing the complex interplay between genetic factors and neurological conditions. Genome-wide association studies (GWAS) have identified numerous risk loci associated with disorders such as AD (S. J. Andrews et al., 2020; Stoccoro et al., 2017) and SZ (Ripke et al., 2013; Trubetskoy et al., 2022). Exome sequencing has revealed rare variants associated with neurodevelopmental disorders such as ASD (Iossifov et al., 2014; Zhou et al., 2022) and SZ (Liu et al., 2023). Much of genetic research has focused on the study of the nuclear genome and little effort has been devoted to the study of mitochondrial DNA (mtDNA). However, the presence of mtDNA alterations, whether inherited or acquired, has emerged as an important factor in the aetiology and progression of several brain disorders. Understanding the impact of mtDNA changes on brain function provides critical insights into the molecular mechanisms underlying these conditions, offers potential avenues for therapeutic intervention and advances our understanding of the complex interplay between mitochondrial integrity and brain health.

3.1. Neurodevelopmental disorders

Neurodevelopmental disorders refer to a range of different conditions characterised by delays or impairments in the attainment of skills across multiple developmental domains, including motor skills, social interactions, language acquisition, and cognitive functions (Thapar et al., 2017). There is a wide range of neurodevelopmental disorders, with some individuals experiencing specific deficits, such as difficulties in maintaining concentration, that do not affect their ability to lead an independent and fulfilling lives. On the other hand, some may require ongoing support with basic life skills, such as walking and feeding themselves, throughout their lives (Thapar et al., 2017). The causes of neurodevelopmental disorders vary, and while many cases are of unknown origin, several factors can affect normal brain development. These include genetic factors, such as mutations and inborn errors of metabolism, prenatal factors such as nutritional deficiencies and maternal infections, perinatal factors resulting from complications during labour (e.g., hypoxia or

lack of oxygen), and postnatal factors such as traumatic brain injury, infections such as meningitis, or exposure to environmental toxins after birth (Dubovický, 2010).

Neurodevelopmental disorders are classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (APA, 2013) as ID, ASD, ADHD, communication disorders, specific learning disorder, motor disorders and other neurodevelopmental disorders (Figure 9). In this section, we will expand on the information about ID and ASD, as these are the phenotypes analysed in the present work.

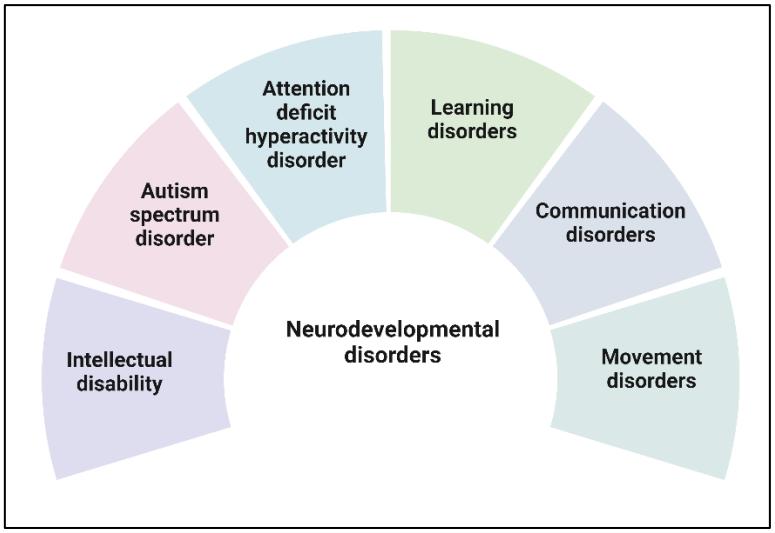

Figure 9. Classification of neurodevelopmental disorders according to DSM-5.

3.1.1. Intellectual disability (ID)

ID is characterised by significant limitations in both intellectual functioning and adaptive behaviour that affect every day social and practical skills. These limitations manifest themselves during development and lead to impairments in various areas such as communication, self-care, and interpersonal relationships. Research published over the last decade on the global prevalence of ID has generally provided a descriptive range of rates, typically estimated to be between 1% and 3% (Nair et al., 2022).

The severity of ID is categorised on the basis of levels of adaptive functioning and intellectual quotient (IQ) scores (APA, 2013). In this framework, individuals with a mild ID, who account for approximately 85% of cases, typically have an IQ range of 50-69 and can live independently with minimal support. Individuals with a moderate ID, who account for 10% of cases and have an approximate IQ range of 36-49, can achieve independent living with moderate support, such as in group homes. Severe ID, which accounts for 3.5% of cases with an approximate IQ range of 20-35, requires daily assistance with self-care activities and safety supervision. Profound ID, which

accounts for 1.5% of cases with an IQ of less than 20, requires 24-hour care, with extensive support needed for all aspects of daily living (APA, 2013; Boat & Wu, 2015b).

Environmental factors, including prenatal exposure to hazardous substances such as alcohol and lead, nutritional deficiencies such as prenatal iodine deficiency, brain irradiation, childhood brain infections, traumatic brain injury, and maternal infections such as rubella and cytomegalovirus, may contribute to the development of ID (Bartoshesky & Wright, 2021). In addition, prenatal and postnatal complications, such as prematurity-related problems such as hypoxaemia and periventricular haemorrhage, have the potential to cause brain damage leading to ID (Boat & Wu, 2015b).

Genetic factors play a central role in the manifestation of ID, and several genetic causes have been identified. The two most common genetic causes of ID are Down syndrome (DS) and fragile X syndrome (FXS). DS, also known as trisomy 21, is the most common genetic cause of ID in the United States, occurring in approximately once in every 700 live births (Parker et al., 2010), while the estimated incidence worldwide is between 1 in 1,000 and 1 in 1,100 live births (https://www.un.org/en/observances/down-syndrome-day) (Coffee et al., 2009). The exact number of people with FXS is unknown, but a review of research studies estimated that about 1 in 7,000 males and about 1 in 11,000 females have been diagnosed with FXS (Hunter et al., 2014)..

The brain's increased energy needs result in exceptionally high OXPHOS activity, leading to a corresponding increase in electron leakage. In addition, the brain is rich in unsaturated fatty acids in the myelin sheath and long-chain fatty acids in cell membranes, making it highly susceptible to peroxidation (Massaad & Klann, 2011). Furthermore, the brain lacks robust antioxidant enzyme defences (Kovacic & Somanathan, 2012; Natelson, 2013). In this context, there is compelling evidence that deficiencies in mitochondrial function may be a central aetiological mechanism contributing to ID. It is known that most individuals diagnosed with primary suspiciousness diseases commonly manifest delayed developmental milestones, seizures, and ID (Ortiz-González, 2021; Valiente-Pallejà et al., 2018). A significant number of these individuals also progress to show clear neurodegenerative features, as observed in conditions such as mitochondrial depletion syndrome associated with POLG variants, or the progressive ataxia, neuropathy, and dementia in Kearns-Sayre syndrome (Ortiz-González, 2021).

3.1.2. Autism spectrum disorder (ASD)

Autism or ASD is an early-onset neurodevelopmental disorder that was first defined by Leo Kanner in 1943. It is characterised by challenges in social interaction and communication, coupled

with repetitive or stereotyped patterns of behaviour and often restricted interests (Boat & Wu, 2015a). Approximately 75 million people are affected by ASD, representing 1% of the global population, and 1 in 100 children will be diagnosed with an ASD in 2021 (Centers for Disease Control and Prevention, 2020). The median percentage of autism cases with concurrent ID is 33.0% (Zeidan et al., 2022).

A commonly used assessment tool for identifying and diagnosing ASD is the Childhood Autism Rating Scale (CARS), developed by Schopler and colleagues in 1980 and later revised in 1988 (Chlebowski et al., 2010). The CARS includes 14 domains that assess behaviours associated with autism, and a 15th domain that provides an overall impression of autism (Schopler et al., 2010). Each domain is scored on a scale of one to four, with higher scores indicating a greater degree of impairment. Total scores on the CARS can range from a minimum of 15 to a maximum of 60. Scores below 30 indicate a non-autistic range, scores between 30 and 36.5 indicate mild to moderate autism, and scores between 37 and 60 indicate severe autism (Schopler et al., 2010).

Despite the availability of assessment tools, assessing the severity of impairment in autism remains complex due to changes in the expression of the syndrome with age, particularly in early childhood and adolescence (Boat & Wu, 2015a). During these stages, some individuals may make significant progress, while others may experience regression in skills. Furthermore, there is no universally accepted convention for categorising differences in skill levels or the severity of impairment. Terms such as “high functioning” or “low functioning” are commonly used, but these refer primarily to cognitive ability or IQ (Kenny et al., 2016). In reality, individuals with a high IQ may have severe impairments in adaptive functioning, such as having social skills equivalent to a 4-year-old child (Klin et al., 2007). As a practical consideration, the severity of impairment across multiple domains is the primary determinant of disability, regardless of IQ.

Genetic factors are estimated to account for 40-80% of the risk of ASD. Several genes associated with ASD play crucial roles in brain development, such as the production, growth and organisation of neurons (Lim et al., 2022). Some genes are involved in the formation or functionality of synaptic connections between neurons, where intercellular communication takes place (Lim et al., 2022). In addition, certain genes are involved in the development of dendrites, which are responsible for transmitting signals received at synapses to the neuron (Martínez-Cerdeño, 2017).

In 2010, a pivotal study found that 80% of children with ASD in their study had blood test results indicating mitochondrial dysfunction (Giulivi et al., 2010). Furthermore, a meta-analysis of case reports on children with both ASD and mitochondrial disease confirmed a higher prevalence of neurodevelopmental regression and gastrointestinal complaints in this subgroup compared with

the general ASD population and some associations between mitochondrial disease in ASD and seizures/epilepsy and motor delay (Frye, 2020a). Subsequent research studies have provided additional support for this finding, demonstrating biochemical evidence of mitochondrial dysfunction in post-mortem brain tissue, markers on brain magnetic resonance imaging, and mitochondrial and immune abnormalities in individuals with ASD (Chauhan et al., 2011; Goh et al., 2014; Tang et al., 2013). The accumulating evidence strongly suggests that mitochondrial dysfunction is prevalent in a significant proportion of individuals with ASD, implying its potential role as a contributing factor to the symptoms associated with ASD.

3.2. Neurological disorders (NeuD)

NeuD are diseases of the central and peripheral nervous systems. In other words, the brain, spinal cord, cranial nerves, peripheral nerves, nerve roots, autonomic nervous system, neuromuscular junction, and muscles. These disorders include epilepsy, AD and other dementias, cerebrovascular disease including stroke, migraine and other headache disorders, multiple sclerosis, PD, neuroinfections, brain tumours, traumatic disorders of the nervous system due to head trauma, and NeuD due to malnutrition. This section addresses the two most prevalent diseases of our era, AD and PD.

3.2.1. Alzheimer's disease (AD)

Dementia, a decline in cognitive function that impairs daily activities, includes several disorders. AD is the most common form of dementia, accounting for more than two-thirds of cases in people aged 65 years and older (A. Kumar et al., 2022). As a neurodegenerative disorder, AD manifests with a gradual onset and progressive impairment of behavioural and cognitive functions, and is the seventh leading cause of death worldwide (Sie et al., 2023). Early onset before 65 is rare, affecting less than 10% of people with AD (A. Kumar et al., 2022). The primary risk factor for AD is increasing age. Other factors that have been identified as increasing risk include traumatic head injury, depression, cardiovascular and cerebrovascular disease, older parental age, smoking, a family history of dementia and elevated homocysteine levels (A. Kumar et al., 2022).

The decline in cognitive function in AD is linked to the accumulation of abnormal neuritic plaques (tiny spherical lesions) called amyloid-beta (Aβ) and neurofibrillary tangles, fibrillary intracytoplasmic structures in neurons formed by a protein called tau (A. Kumar et al., 2022). Tau, derived from alternative splicing of the microtubule-associated protein tau (MAPT) gene is abundant in the axons of neurons where it stabilizes microtubule bundles (Abubakar et al., 2022;

Sie et al., 2023). Aβ results from the sequential cleavage of amyloid precursor protein (APP) by beta-secretase and gamma-secretase, and aggregation of extracellular Aβ leads to hyperphosphorylation of tau, resulting in the formation of tau aggregates. Tau aggregates form twisted, paired helical filaments known as neurofibrillary tangles. (Figure 10) (Abubakar et al., 2022). The autosomal dominant form of AD is associated with pathogenic variants in three genes: APP, presenilin1 (*PSEN1*), and presenilin 2 (*PSEN2*). Apolipoprotein E (APOE) in the e4 form is the strongest known genetic risk factor for sporadic AD, which is primarily produced by astrocytes (Rosenthal et al., 2018). Mutations in the *SORT1* gene, which is essential for APP transport, have been identified in familial and sporadic AD cases (Nicolas et al., 2018).

Mitochondrial dysfunction has a significant impact on AD by promoting Aβ formation and accumulation, which affects ATP levels (Bhatia et al., 2021). Tau protein, associated with mitochondrial dysfunction, disrupts mitochondrial transport and distribution; conversely, mitochondrial dysfunction can cause tau hyperphosphorylation, contributing to neurofibrillary tangles pathology (Su et al., 2010). APOE e4 exacerbates mitochondrial dysfunction, reducing respiratory complex levels and glucose metabolism, emphasising its role as a major risk factor in AD (Orr et al., 2019).

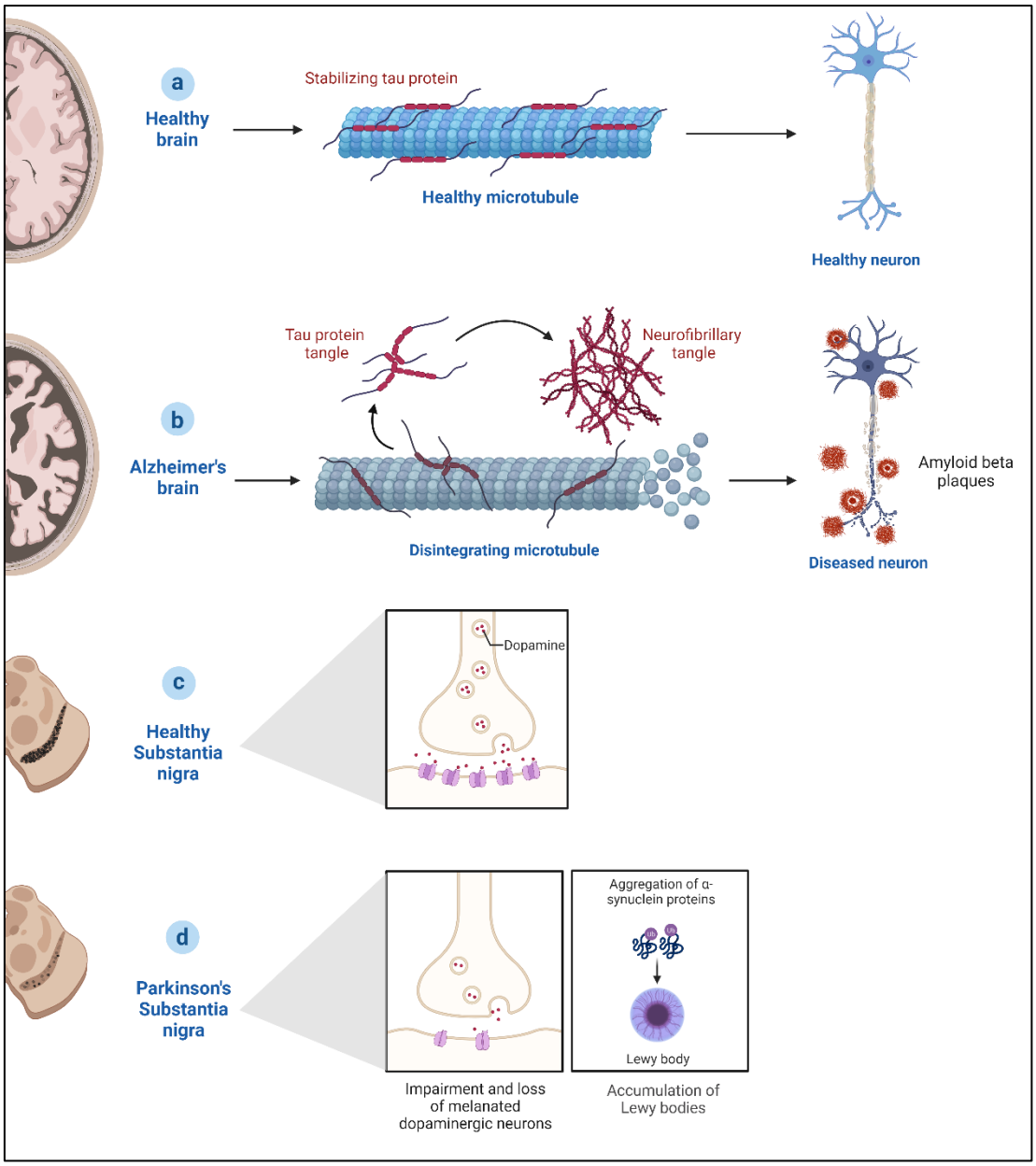

Figure 10. Differences between brain tissue from individuals with Alzheimer's disease, Parkinson's disease and healthy individuals. **a.** In the healthy brain, the tau-microtubule pair maintains the architecture and integrity of axons. **b.** In the Alzheimer's disease, aggregation of extracellular beta-amyloid leads to hyperphosphorylation of tau, resulting in the formation of tau aggregates and neurofibrillary tangles. **c.** Healthy substantia nigra typically shows a normal complement of dopaminergic neurons, absence of Lewy bodies and a balance in neurotransmitter levels that supports smooth motor function. **d.** In Parkinson's disease, there is a progressive loss of dopaminergic neurons and the presence of abnormal protein aggregates called Lewy bodies in the substantia nigra. Created using BioRender.

3.2.2. Parkinson's disease (PD)

PD is a progressive disorder that affects motor and cognitive function and is characterised by the degeneration of neurons in the part of the brain that controls movement, called the substantia nigra (Kouli et al., 2018). PD is the second most common neurodegenerative disease after AD. Its prevalence is approximately 0.5–1% in individuals aged 65–69, increasing to 1–3% in those aged

80 years and older (Kouli et al., 2018). The global prevalence of PD has been steadily increasing over the past three decades, with a 155% increase from 1990 to 2019 (Zhong & Zhu, 2022).

The loss of dopaminergic neurons, nerve cells that produce and release dopamine, is the key feature of PD (Figure 10). As dopaminergic neurons weaken or die, individuals may experience symptoms such as tremors, muscle stiffness, slowed movement, and impaired balance (Fröhlich, 2016; Kouli et al., 2018). Beyond motor symptoms, people with PD may face various challenges, including difficulty swallowing, speech problems, urinary problems, skin problems, sleep disturbances, muscle spasms, pain, fatigue, emotional changes, cognitive decline, hallucinations, and psychotic symptoms (Bloem et al., 2021; Fröhlich, 2016; Kouli et al., 2018).

PD is primarily considered idiopathic, with approximately 10–15% of cases reporting a family history and approximately 5% showing Mendelian inheritance (Deng et al., 2018). The 23 identified PARK genes, genetic variations that are associated with PD show either autosomal dominant (e.g., *SNCA*, *LRRK2*, *VPS32*) or autosomal recessive inheritance (e.g., *PRKN*, *PINK1*, *DJ-1*) (Kouli et al., 2018). Interestingly, the proteins PINK1 and Parkin, encoded by the PRKN, are involved in the same mitochondrial quality control pathway, with PINK1 recruiting Parkin to malfunctioning mitochondria, thereby initiating the process of mitophagy, a selective degradation of mitochondria by autophagy (Pickrell & Youle, 2015). This link is the clearest evidence linking mitochondrial dysfunction to PD. In addition, recent evidence suggests that dopamine and its oxidation products may directly inhibit mitochondrial respiratory chain proteins, particularly complexes I and IV (Keane et al., 2011). This link between mitochondrial dysfunction and dopamine is further supported by studies showing that PD neurotoxins, such as MPTP and rotenone, increase dopamine oxidation and turnover, which may explain why dopaminergic neurons are more susceptible to toxin- or mutation-mediated mitochondrial dysfunction in PD (Keane et al., 2011).

3.3. Psychiatric disorders (PysD)

PsyD, also known as mental disorders or mental illnesses, are conditions that affect a person's thoughts, feelings, behaviour or a combination of these. These disorders can cause distress, interfere with daily functioning and often require clinical intervention for diagnosis and treatment. PsyD include a wide range of conditions, such as mood disorders (e.g., major depressive disorder and bipolar disorder), anxiety disorders, psychotic disorders (e.g., SZ), substance use disorders, neurodevelopmental disorders (e.g., ASD and ID) and others.

PsyD are usually diagnosed on the basis of specific criteria outlined in diagnostic manuals such as the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), the 2013 update of the DSM, the taxonomic and diagnostic tool published by the American Psychiatric Association (APA), or the International Classification of Diseases (ICD), which was adopted by the 72nd World Health Assembly in 2019 and will entered into effect on 1st January 2022. The introduction of quantitative behavioural genetic methods, such as twin studies, to psychiatry has led to a consensus that the aetiology of PsyD is multifaceted, involving a combination of genetic and environmental factors (Assary et al., 2018). There is also increasing experimental evidence that epigenetic signals play a critical role in neuronal development, differentiation and communication, as well as synaptic plasticity in general. This suggests that epigenetic regulation is essential for neural and brain function, and that putative epimutations may play a role in the pathogenesis of complex PsyD (Keverne & Binder, 2020; Ptak & Petronis, 2010; Srancikova et al., 2021). Research into their causes has also highlighted the importance of environmental stressors, including childhood maltreatment. Heritability estimates for PsyD vary widely, with major depression having a heritability estimate of 37% (Sullivan et al., 2000), SZ having a range of 70%-80% (Akingbuwa et al., 2022) and bipolar disorder having a range of 60%-85% (Barnett & Smoller, 2009).

3.3.1. Major depressive disorder (MDD)

Depressive disorders are characterised by persistent feelings of sadness, hopelessness, and a lack of interest or pleasure in activities. The World Health Organization reports that approximately 350 million people worldwide are affected by depressive disorders (Shadrina et al., 2018). According to the DSM-IV, one of the main forms of depressive disorder is MDD (APA, 2013). Its global prevalence is approximately 12%, with higher rates in developed countries (Kessler et al., 2011). Genetic predisposition is evident through familial aggregation and substantial heritability estimates. The pathophysiology of depression is linked to biological factors, including dysregulation of neurotransmitter systems such as serotonin, norepinephrine and dopamine (Dean & Keshavan, 2017). In addition, alterations in mitochondrial functions such as OXPHOS and membrane polarity can lead to increased oxidative stress and apoptosis, which may precede the development of depressive symptoms (Khan et al., 2023).

3.3.2. Bipolar disorder (BD)

BD is characterised by cyclic fluctuations between depressive and manic or hypomanic episodes. BD has a high familial aggregation. Heritability estimates from twin studies, which compare disease concordance between monozygotic and dizygotic twins, range from 60% to 90% (O'Connell & Coombes, 2021). Genetic studies suggest a polygenic inheritance with multiple susceptibility loci (O'Connell & Coombes, 2021). Neurobiological factors contributing to the pathophysiology of the disease include alterations in neurotransmitter systems, particularly dopamine and glutamate pathways. (Grunze et al., 2021; Guglielmo & Hasler, 2022). Dopamine is a key neurotransmitter in reward and movement regulation in the brain and glutamate is the major excitatory neurotransmitter of the central nervous system, with a particular role in memory, cognition, and mood regulation (McCutcheon, Krystal, et al., 2020). Mitochondrial dysfunction has also been identified as a common pathway in the pathophysiology of BD, triggered by mechanisms such as impaired oxidative phosphorylation, a shift to glycolytic energy production, an overall decrease in energy, and abnormalities in mitochondrial morphology and intracellular distribution (Scaini et al., 2020).

3.3.3. Anxiety disorders

Anxiety disorders are the most common mental disorders, often appearing in early adulthood or earlier. They are characterised by excessive fear and anxiety or avoidance of perceived threats that are persistent and harmful. Anxiety disorders account for 3.3% of the global burden of disease and have a significant impact on individuals and communities (Penninx et al., 2021). In 30 European countries, the cost of anxiety disorders is estimated to be around €74 billion (Gustavsson et al., 2011). The development of anxiety disorders is influenced by a combination of genetic and environmental factors and their epigenetic interactions. Emerging evidence from both animal and human studies also suggests that mitochondria play a critical role in modulating anxiety-related behaviour (Filiou & Sandi, 2019). There is a bidirectional relationship whereby alterations in mitochondrial energy metabolism and oxidative stress are observed in high anxiety, while alterations in mitochondrial function may contribute to increased anxiety (Filiou & Sandi, 2019).

3.3.4. Schizophrenia (SZ)

SZ is a functional psychotic disorder characterised by delusions, hallucinations, and disturbances in thought, perception, and behaviour. The term was first coined by Eugen Bleuler in 1908 and is derived from the Greek words 'schizo' meaning 'split' and 'phren' meaning 'mind' (Marder &

Cannon, 2019). SZ symptoms are traditionally divided into two main categories: positive and negative. Positive symptoms refer to changes in behaviour or thoughts, such as hallucinations, suspiciousness and delusions, while negative symptoms manifest as withdrawal from the world, a lack of interest in social interactions, and an emotionless and flat appearance (Figure 11) (McCutcheon, Reis Marques, et al., 2020). These categories encompass a range of manifestations, including formal thought disorder, anhedonia, poverty of speech, and lack of motivation (Correll & Schooler, 2020).

SZ affects approximately 1% of the world's population and is one of the 10 leading causes of disability worldwide (Velligan & Rao, 2023). In the population of the European Union, the incidence of SZ is 15.2 per 100,000 people (Fasseeh et al., 2018). Patients with SZ use a considerable amount of healthcare services, resulting in a significant economic burden for patients, their families, and society as a whole (Fasseeh et al., 2018). The significant costs are due to its typical onset in early adulthood and long-term impairments in social and occupational functioning (Kovács et al., 2018). SZ is also associated with reduced life expectancy, with people with the disorder living an average of 15 years less than that of the general population (McCutcheon, Reis Marques, et al., 2020). In addition, people with the disorder have a 5-10% lifetime risk of suicide (McCutcheon, Reis Marques, et al., 2020).

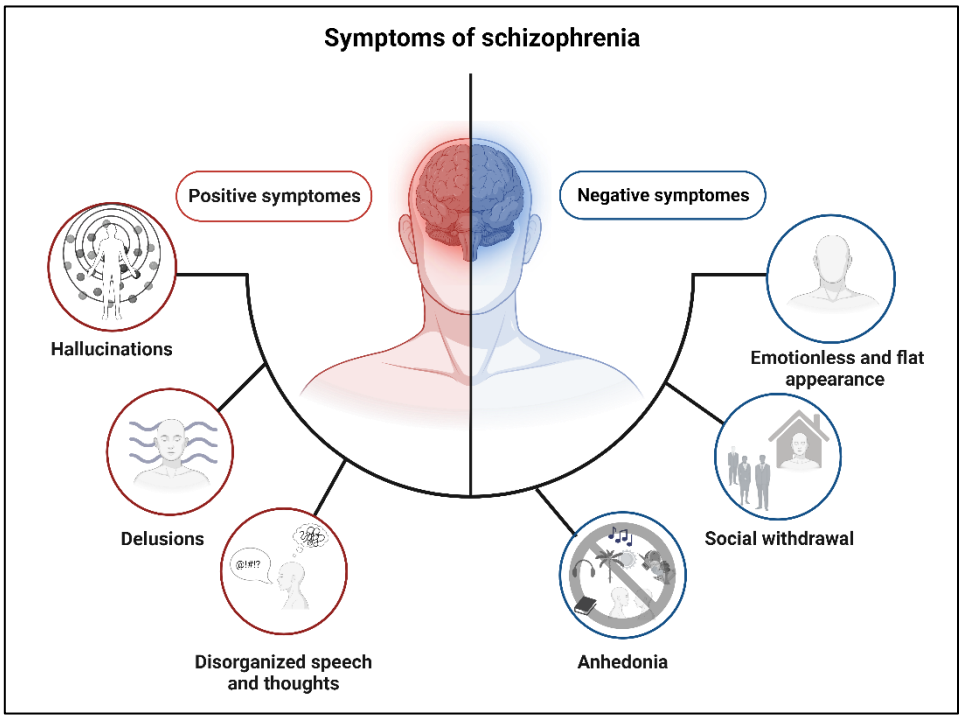

Figure 11. Positive and negative symptoms of schizophrenia.
Created using BioRender.

About 80% of the risk of SZ in a population is thought to be explained by heritable factors, based on studies of twins and families. However, only a small fraction of this heritability has been linked to common disease-associated single nucleotide polymorphisms (SNP), each of which has a modest effect on risk, or to rare variants, each of which may have a more significant effect on risk (Marder & Cannon, 2019). Genome-wide association, exome sequencing and gene expression profiling studies have identified a number of genes suggesting involvement in pathways related to the immune system (Radhakrishnan et al., 2017), cytoskeletal development, and synaptic plasticity and function (Trubetskoy et al., 2022), which are conserved across different human populations (Liu et al., 2023). Environmental factors, such as obstetric complications, early life adversity, and living in urban areas during childhood, are thought to interact with genetic risk to contribute to susceptibility to SZ (Marder & Cannon, 2019).

Understanding of SZ is currently based on the dopaminergic hypothesis, which suggests changes in dopamine neurotransmission in the mesolimbic system, responsible for positive symptoms, and in the mesocortical pathway, which is responsible for negative symptoms (McCutcheon, Krystal, et al., 2020; Stępnicki et al., 2018). This is complemented by the glutamatergic hypothesis, which proposes changes in prefrontal neuronal connectivity involving glutamatergic neurotransmission at a receptor of glutamate called the N-methyl-D-aspartate (NMDA)(Stępnicki et al., 2018). Some structural brain changes have been reported in SZ. reduced grey matter volume on magnetic resonance imaging in patients compared to age-matched controls has been reported, and some postmortem studies have found fewer dendrites and dendritic spines detected in patients compared to controls (Glausier & Lewis, 2013; Marder & Cannon, 2019). Increased levels of immunological markers, such as tumour necrosis factor alpha, are associated with increased rates of grey matter loss, also suggesting a potential role for cytokine-mediated microglial activation in SZ (Cannon et al., 2015). These changes are thought to affect physiological activity and functional connectivity between the prefrontal cortex, temporal cortex, thalamus, hippocampus, and cerebellum (Van Den Heuvel et al., 2013).

Mitochondrial dysfunction, including oxidative stress, energy metabolism, and synaptic efficiency, also plays an important role among the many brain abnormalities associated with SZ (Hjelm et al., 2015; Martins-De-Souza et al., 2011; Maurer et al., 2001; Roberts, 2021). SZ is characterised by reduced activity of complexes I and IV in the electron transport chain, as well as abnormal levels of individual subunits (Roberts, 2021). Ultrastructural studies have shown specific reductions in mitochondria across layers, inputs, and cells. In the cortex, fewer mitochondria are found in axon terminals, neuronal somata, and oligodendrocytes in both grey and white matter (Roberts et al., 2015; Uranova et al., 2020). Astrocytic mitochondria are reduced in SZ, whereas mitochondria in

the nucleus accumbens and substantia nigra remain similar in density, size, and structural integrity compared to controls (Roberts, 2021). Dysfunctional mitochondrial processes could lead to reduced metabolism and defective synaptic activity, which may impact synaptic strength (Roberts, 2021). The impact of mitochondrial dysfunction in SZ varies by brain region, cell type, subcellular location, treatment status, treatment response, and predominant symptoms. Therefore, mitochondrial pathology is a common finding in SZ, as demonstrated by various techniques in patients, postmortem samples, cell lines, and animal models (Roberts, 2021); however, non replication is common in the literature, which is a common problem in SZ research. Differences between studies in techniques, brain areas, and patient characteristics contribute to the challenge of reconciling the literature on mitochondria in SZ.

3.4. Aging

Aging is a natural and complex biological process characterised by a series of molecular, cellular, and tissue changes that contribute to the overall deterioration of an organism's structure and function (J. Guo et al., 2022). The ageing process is influenced by several factors, including genetics, environment, lifestyle choices, and the cumulative effects of cellular damage and stress over time. Key features of ageing include genomic instability, telomere shortening, epigenetic changes, loss of proteostasis, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín et al., 2023).

Ageing is a major risk factor for the development of several age-related diseases. As individuals age, they become more susceptible to conditions such as cardiovascular disease, neurodegenerative diseases, cancer, diabetes, and other chronic diseases (J. Guo et al., 2022). Aging processes may contribute to the increased incidence and severity of these diseases, and understanding the mechanisms underlying ageing is crucial for developing strategies to prevent or delay age-related diseases.

Mitochondria play a crucial role in the ageing process, and their functionality is closely linked to different aspects of cellular ageing. As cells age, mitochondria undergo changes that contribute to the overall decline in cellular function. Mitochondrial dysfunction is associated with an accumulation of oxidative damage, reduced energy production, and impaired regulation of cell death pathways (Sun et al., 2016). The decrease in mitochondrial quality control mechanisms, such as mitophagy, can lead to the accumulation of damaged mitochondria in cells (Doblado et al., 2021). These dysfunctional mitochondria can release ROS and trigger inflammatory responses,

further exacerbating cellular damage. The relationship between mitochondrial function and ageing is complex; however, it is important to understand the molecular mechanisms underlying mitochondrial ageing in order to gain insights into promoting healthy ageing and reducing age-related diseases.

IV. HYPOTHESIS

General Hypothesis

Mitochondrial dysfunction can be caused by alterations in mtDNA, which can be inherited maternally or acquired during life. The general hypothesis of this thesis is that mtDNA alterations are higher in brain disorders than in the control population.

Aim of the first article (a systematic review):

Alterations in mtDNA cause or are associated with a number of MitD and are also thought to play a role in brain disorders and ageing. The collection of mtDNA alterations reported in human brain tissue, including SNVs, mtDNA rearrangements, and mtDNA copy number changes (mtDNA-CN), will advance current knowledge of the underlying mechanisms of brain disorders.

Hypothesis of the second article:

MtDNA rearrangements, deletions and duplications, can be detected in blood samples and are more frequent in patients with ID than in healthy controls (HC).

Hypothesis of the third article:

MtDNA alterations, SNVs and mtDNA rearrangements, are more frequent in postmortem brain samples from individuals diagnosed with SZ than in HC. Conversely, the mtDNA copy number will be lower in patients than in controls.

V. OBJECTIVES

The aim of this thesis is to identify mtDNA alterations that may play a role in the pathophysiology of brain disorders. The specific objectives were:

1st Article:

1- To identify and summarise the specific mtDNA alterations in MitD, NeuD, PsyD and aging reported to date by performing a PubMed and Embase search for articles published before 10 June 2021.

2nd Article:

1- To analyse the mtDNA of 135 adult individuals with ID, including 59 with comorbid ASD, and 32 HC using mtDNA-targeted next-generation sequencing and the MitoSAlt (Mitochondrial Structural Alterations) high-throughput computational pipeline to identify mtDNA rearrangements.
2- To compare the frequency of mtDNA rearrangements between ID and ID-ASD and HC.
3- To investigate whether the presence of mtDNA rearrangements is associated with clinical features of ID and ASD.

3rd Article:

1- To identify mtDNA alterations in post-mortem brain samples from individuals diagnosed with SZ (40) and HC (40) using mtDNA-targeted next-generation sequencing.
2- To compare the mtDNA copy number (mtDNA-CN) in post-mortem brain tissue from patients with SZ and HC using quantitative real-time PCR (qPCR).
3- To investigate the relationship between mtDNA alterations in brain and sex, age, medication, and postmortem interval.

VI. RESULTS

Comprehensive summary of mitochondrial DNA alterations in the postmortem human brain: A systematic review. Alba Valiente-Pallejà, Juan Tortajada, Bengisu K. Bulduk, Elisabet Vilella, Glòria Garrabou, Gerard Muntané, and Lourdes Martorell. eBioMedicine 2022;76: 103815.

Article 1. Comprehensive summary of mitochondrial DNA alterations in the postmortem human brain: A systematic review.

In this systematic review, we have summarised the existing knowledge on mtDNA alterations reported in postmortem human brain tissue in a comprehensive and structured manner, shedding light on their pathophysiological relevance and possible implications for future research in the context of brain function. We structured the reported alterations according to mutation type and phenotypic or diagnostic category. For mutation type, 3 categories were established: nucleotide changes (both SNP or pSNV) or INDELS (small insertions or deletions), mtDNA rearrangements (deletions or insertions) and mtDNA-CN. And as for phenotypic categories, they were grouped into 6: MitD, NeuD, PsyD, a miscellaneous group containing control population data and other clinical features, aging, and technical issues.

We searched the PubMed and Embase databases for different terms related to mtDNA and postmortem human brain up to 10 June 2021. We reported 158 out of 637 studies that met the inclusion criteria, including 48 studies in MitD, 55 studies in NeuD, 15 studies in PsyD, 5 studies in the miscellaneous group, 20 studies in aging, 5 studies in technical issues, and 10 studies clustered that could be ascribed to more than one group.

We noted that pSNVs have been commonly reported in MitD with varying levels of heteroplasmy between brain regions, whereas they have been reported less frequently in NeuD, PsyD, and aging. For instance, the m.3243A>G variant in the *MT-TL1* gene is most widely found in MELAS patients, often with mutation loads above 70% in the brain and similar levels in other tissues, although some discrepancies were observed. Most of the included studies indicated a lower mtDNA CN in these diseases, although some exceptions were noted. For instance, one of the studies found that mtDNA CN was significantly higher in patients with Leigh syndrome than in controls.

In the NeuD studies, mtDNA deletions were the most commonly investigated alterations, followed by SNVs and mtDNA CN changes. The studies revealed varying results regarding mtDNA variants and their association with NeuD. Overall, most studies agree that the mtDNA CN levels are lower in patients with AD than in controls, and conflicting results have been reported regarding mtDNA CN in specific brain regions of patients with PD. In general, patients with PD showed higher levels of mtDNA deletions in the substantia nigra than in other brain regions.

Studies examining mtDNA alterations in PsyD have revealed the presence of rare mtDNA variants. Some patients with SZ or BD had specific mtDNA deletions, with varying percentages. Most studies

reported no significant changes in mtDNA CN between study groups with some discrepancies. The accumulation of deletions was higher in specific brain regions in SZ and BD, and an age-related association was observed for the common deletion in BD. Finally, in MDD, specific mtDNA variants and high-impact deletions were identified, suggesting the involvement of complex mtDNA rearrangements in mood disorders.

In the context of ageing, variants in the mitochondrial D-loop were associated with age, with an overall higher level of heteroplasmy in older individuals. Most studies reported a decrease in mtDNA CN in the frontal cortex with age, but the substantia nigra showed an increase in mtDNA CN as a potential compensatory mechanism. The common deletion of mtDNA, a focus of aging research, increased with age in several brain regions.

In conclusion, we provide a clearer relationship between mtDNA alterations and MitD, while most of the mtDNA alterations reported in NeuD and PsyD showed a great heterogeneity with some contradictory results. Heteroplasmy levels, threshold effects, affected brain regions, and mitotic segregation patterns of mtDNA alterations may be involved in the complex inheritance of these diseases, so future research directions should include mtDNA analyses in larger samples and concurrent analyses of all types of mtDNA alterations and in multiple brain regions.

Comprehensive summary of mitochondrial DNA alterations in the postmortem human brain: A systematic review

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Summary

Background Mitochondrial DNA (mtDNA) encodes 37 genes necessary for synthesizing 13 essential subunits of the oxidative phosphorylation system. mtDNA alterations are known to cause mitochondrial disease (MitD), a clinically heterogeneous group of disorders that often present with neuropsychiatric symptoms. Understanding the nature and frequency of mtDNA alterations in health and disease could be a cornerstone in disentangling the relationship between biochemical findings and clinical symptoms of brain disorders. This systematic review aimed to summarize the mtDNA alterations in human brain tissue reported to date that have implications for further research on the pathophysiological significance of mtDNA alterations in brain functioning.

Methods We searched the PubMed and Embase databases using distinct terms related to postmortem human brain and mtDNA up to June 10, 2021. Reports were eligible if they were empirical studies analysing mtDNA in postmortem human brains.

Findings A total of 158 of 637 studies fulfilled the inclusion criteria and were clustered into the following groups: MitD (48 entries), neurological diseases (NeuD, 55 entries), psychiatric diseases (PsyD, 15 entries), a miscellaneous group with controls and other clinical diseases (5 entries), ageing (20 entries), and technical issues (5 entries). Ten entries were ascribed to more than one group. Pathogenic single nucleotide variants (pSNVs), both homo- or heteroplasmic variants, have been widely reported in MitD, with heteroplasmy levels varying among brain regions; however, pSNVs are rarer in NeuD, PsyD and ageing. A lower mtDNA copy number (CN) in disease was described in most, but not all, of the identified studies. mtDNA deletions were identified in individuals in the four clinical categories and ageing. Notably, brain samples showed significantly more mtDNA deletions and at higher heteroplasmy percentages than blood samples, and several of the deletions present in the brain were not detected in the blood. Finally, mtDNA heteroplasmy, mtDNA CN and the deletion levels varied depending on the brain region studied.

Interpretation mtDNA alterations are well known to affect human tissues, including the brain. In general, we found that studies of MitD, NeuD, PsyD, and ageing were highly variable in terms of the type of disease or ageing process investigated, number of screened individuals, studied brain regions and technology used. In NeuD and PsyD, no particular type of mtDNA alteration could be unequivocally assigned to any specific disease or diagnostic group. However, the presence of mtDNA deletions and mtDNA CN variation imply a role for mtDNA in NeuD and PsyD. Heteroplasmy levels and threshold effects, affected brain regions, and mitotic segregation patterns of mtDNA alterations may be involved in the complex inheritance of NeuD and PsyD and in the ageing process. Therefore, more

Abbreviations: mtDNA, Mitochondrial DNA; MitD, Mitochondrial disease/s; NeuD, Neurological disease/s; PsyD, Psychiatric disease/s; pSNV, Pathogenic single nucleotide variant; CN, copy number; DEL, deletion

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information is needed regarding the type of mtDNA alteration, the affected brain regions, the heteroplasmy levels, and their relationship with clinical phenotypes and the ageing process.

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Keywords: Mitochondrial DNA; Mitochondrial diseases; Neurological diseases; Psychiatric diseases; Ageing; Postmortem

Research in context

Evidence before this study

The human mitochondrial genome consists of a 16,569 bp molecule present in almost all cell types with some exceptions, the most significant being erythrocytes. On average, there are approximately 1,000 mtDNA molecules in a human cell, but the specific mtDNA CN depends on the energy requirements of each cell. Brain tissue has a high energy requirement, which leads to a large number of mitochondria in the brain.[1] It is known that alterations in mtDNA cause MitD, a clinically heterogeneous group of disorders that arise as a consequence of mitochondrial respiratory chain dysfunction. The clinical characteristics of MitD show enormous variability, and although they can affect a single organ, most of them involve multiple organ systems often presenting with neurological disturbances. Moreover, there is increasing evidence of mitochondrial dysfunction in neurodegeneration,[2] in the development of psychiatric symptoms,[3] and in the brain ageing process.[4,5] The aims of this study were a) to determine the specific diagnoses or health conditions in which the presence of mtDNA alterations has been assessed in the human postmortem brain and b) to identify and summarize the specific mtDNA defects reported. For these purposes, we conducted a PubMed and Embase search for articles published before June 10, 2021, using several search strings and keywords, including postmortem, brain, neuron, glia, mtDNA, variant, mutation, and deletion.

Added value of this study

Our systematic review identified that mtDNA alterations have been investigated in human postmortem brain samples associated with ageing and disease, mostly in individuals with MitD, neurological, or psychiatric diagnoses. We report a comprehensive summary of the identified mtDNA alterations organized into three clinical categories (MitD, NeuD and PsyD) plus a miscellaneous clinical group and two further categories, including ageing and technical issues. pSNVs, alterations in mtDNA CN and mtDNA deletions have been reported in MitD. In NeuD, most of the studies investigated the presence of mtDNA deletions or differences in mtDNA CN between affected and nonaffected individuals, with conflicting results, while few studies evaluated mtDNA pSNVs. Similarly, pSNVs and altered mtDNA CNs were not consistently evaluated in PsyD; in contrast, several studies identified mtDNA deletions in some patients. Finally, mtDNA deletions have been recurrently associated with ageing. We also identified some studies that have explored mtDNA gene expression, oxidation and methylation.

Implications of all the available evidence

Among the three types of well-known mtDNA alterations (pSNVs, mtDNA CN and mtDNA rearrangements) that are associated with mitochondrial dysfunction, only one or two of them were investigated in most studies. Most studies reported mtDNA alterations, demonstrating their presence in the postmortem brain of patients with MitD, NeuD and PsyD and the ageing process. With the currently available molecular techniques and bioinformatic tools, it is crucial to further investigate the presence of all types of mtDNA alterations in postmortem brain samples of patients with MitD, NeuD and PsyD in all age groups and in healthy individuals to shed light on the role of mtDNA in brain function, disease development and the ageing process. These studies should be conducted with current validated techniques to obtain unambiguous data regarding mtDNA alterations and associated heteroplasmy levels and are particularly relevant when measuring mtDNA CN. Because mitochondria can be acknowledged as a therapeutic target for ameliorating brain function, it is crucial to decipher the role of all types of mtDNA variations in health and disease.

Introduction

Mitochondrial DNA (mtDNA)

Mitochondria are membrane-bound organelles that generate most of the chemical energy needed to power the cell's biochemical reactions; this energy is stored as adenosine triphosphate (ATP) molecules. Two distinct bilayer membranes separate the matrix of the mitochondria from the cytosol—the smooth outer membrane and the highly folded inner membrane—forming invaginated structures called cristae. In these cristae, ATP

synthesis takes place by the oxidative phosphorylation system (OXPHOS) through oxidoreductase complexes I-IV of the electron transport chain (ETC) and the ATP synthase enzyme of complex V. Mitochondria act as a signalling hub regulating cellular processes relevant to cell differentiation, cell proliferation, apoptosis and the immune response.[6 10] The mitochondrial proteome is estimated to contain 1,500 proteins, most of which are under nuclear genome control.

Human mtDNA consists of a 16,569 bp circular DNA molecule that is maternally inherited and whose sequence and gene organization were published in 1981.[11,12] It encodes 37 genes, including 13 protein subunits of the respiratory chain, 2 ribosomal units (rRNAs 12S and 16S) and 22 transfer (tRNA) genes. mtDNA also contains a noncoding control region of approximately 1,200 bp in length and is known as the displacement loop (D-loop) region, which regulates mtDNA transcription and replication. The 13 resulting proteins are crucial for the proper function of the respiratory chain even though they represent only a small fraction of the ~100 subunits that constitute complexes I-V.[13] Another peculiar feature of mtDNA is polyploidy. A uniform collection of mtDNA copies—either completely normal mtDNA or completely mutant mtDNA—is known as homoplasmy, while heteroplasmy refers to different proportions of normal and mutant mtDNA in a mitochondrion, cell, organ or tissue. The amount of mtDNA in a cell, known as the mtDNA content or mtDNA CN, usually varies from hundreds to thousands[14] and depends on the cell function and the cell response to endogenous and exogenous agents.[15] Tissues with high energy requirements contain large amounts of mitochondria in their cells and, accordingly, a high mtDNA CN. The central nervous system, cardiac and skeletal muscles, endocrine system, and liver and renal systems are among those with the highest energy requirements.[10]

mtDNA is composed of double-stranded DNA, comprising heavy strands (H) and light strands (L). The H-strand, which is guanine-rich, encodes 28 genes, while the L-strand, which is cytosine-rich, encodes the remaining 9 genes. Several characteristics differ between the nuclear and mitochondrial genomes: mtDNA is circular, small, has no introns, is not enveloped with proteins and is maternally inherited; in contrast, nuclear DNA (nDNA) is linear, has a large number of nucleotides (~3.3 billion bp), has introns, is packaged into chromatin, undergoes recombination and is biparentally inherited. Both mtDNA and nDNA use the same deoxynucleotide triphosphates (dNTPs) for DNA replication, and although mtDNA follows the universal codon usage rules when coding sequences are translated into proteins, there are some specific deviations: UGA codes for tryptophan instead of a stop codon, AGA and AGG are also stop codons, and AUA codes for methionine. Additionally, some nucleotide bases exhibit functional overlap between two genes, as they are the last base of one gene and the first base of the next gene.[16] In the mitochondrial matrix, mtDNA forms nucleoids with mitochondrial transcription factor A, which acts to provide structure to the mtDNA genome.

Finally, the mtDNA mutation rate (the speed at which mutations are introduced) is much higher than that of nDNA. In animals, it is estimated that the mutation rate in mtDNA is ~25-fold higher than that in nDNA.[17] In humans, based on the appearance of de novo mtDNA variants in human pedigree studies, an ~10-fold higher rate in mtDNA than in nDNA has been suggested.[18] The molecular damage to mtDNA is thought to be due to the high levels of reactive oxygen species present in the mitochondrion, the high mtDNA replication levels, and the high coding rate of mtDNA, which is ~93%.[19] Additionally, this higher mutation rate suggests that many mtDNA variants may be subjected to poor selection, which can occur at the germline level or at the somatic level throughout life, implying that even though a specific variant is not detected in blood, a tissue commonly used in genetic testing, it cannot be ruled out that the variant is not present in other tissues or organs. Correspondingly, mtDNA alterations can lead to cellular energy impairment that might cause a disease or be implicated in the physiopathology of age-associated diseases or the ageing process itself.[20]

mtDNA and human ageing

The decline of mitochondrial functioning has been largely implicated in the ageing process and is characterized by a reduced density of mitochondria and reduced mitogenesis.[21] In fact, the ageing process is strongly linked to noninherited mtDNA changes, mainly point variants and large deletions, that increase in frequency with age.[22 24] Such changes, which originate as replication errors, accumulate in postmitotic tissues during ageing, leading to increased proportions of impaired mitochondria that may differ between cells and tissues.[25] In the ageing brain, dysfunctional synaptic mitochondria leading to impaired neurotransmission and cognitive failure[26] have been amply demonstrated,[27,28] and mtDNA deletions correlate with mitochondrial respiratory chain malfunction.[27,28] Thus, elucidating the temporal and spatial distribution of mutated mtDNA in the brain might resolve important questions regarding the importance of mtDNA changes in the ageing process.

mtDNA and disease

Given the nature of the genetic material and the dual genomic control of mitochondria, alterations occurring either in the nDNA or mtDNA sequence can potentially cause mitochondrial functional defects. Indeed, some are known to cause very heterogeneous diseases that together are called primary mitochondrial disease

(MitD). More than 300 nuclear genes are known to cause MitD, although most adult patients exhibit variants in mtDNA. The mechanisms involved in these nuclear genes involve assembly factors, mitochondrial structure, coenzyme Q biosynthesis, protein synthesis, and mtDNA maintenance.[29,30] However, this review focuses on MitDs associated with mtDNA pathogenic variants that include pathogenic single nucleotide variants (pSNVs), mtDNA rearrangements (mostly deletions), and altered mtDNA CNs. They can be either maternally inherited or occur de novo, and their pathogenic role can be established by taking advantage of the association between each variant and a specific phenotype.[31] This was investigated by a recent analysis of 265 mtDNA SNVs in 483,626 individuals from the United Kingdom (UK) biobank, which has allowed the identification of 260 new mtDNA-phenotype associations, including type 2 diabetes, multiple sclerosis, adult height, and liver and renal biomarkers. Notably, this study identified a key role for mtDNA common and rare SNV variation (only homoplasmic) in many quantitative human traits and disease risks, with a particular emphasis on cardiometabolic and neurodegenerative diseases.[32] mtDNA pSNVs can be observed in homoplasmy or heteroplasmy, while mtDNA deletions are always heteroplasmic, since within the deleted region, mtDNA consistently contain one or more tRNAs indispensable for the translation of the protein-associated mtDNA genes, which are essential for life. Known mtDNA pSNVs lead to syndromes such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF), neuropathy, ataxia and retinitis pigmentosa (NARP), and Leigh syndrome (LS).[33–36] mtDNA rearrangements are responsible for Kearns–Sayre syndrome (KSS), progressive external ophthalmoplegia (PEO), and Pearson's syndrome.[37] These are considered the most typical MitDs associated with mtDNA alterations; however, there are many other diseases and human characteristics related to mtDNA variation, such as diabetes and hypertension, as well as ageing.[31] In fact, it has been estimated that 1 out of 3,500–6,000 individuals are affected by or are at risk of developing a MitD.[38,39] Human phenotypes associated with mtDNA alterations include extremely severe diseases that can be present from infancy to adulthood and can affect a single organ or multiple tissues, and most of them are included as rare diseases (ORPHANET, https://www.orpha.net/). In June 2021, the Online Mendelian Inheritance in Man (https://omim.org) catalogue[40] included 33 phenotypic descriptions (Supplementary Table 1) in which the molecular basis is known to be associated with mtDNA alterations. The catalogue also included the 37 mtDNA genes (Supplementary Table 2) containing several allelic variants associated with human conditions. In addition, the human mitochondrial genome database (https://www.mitomap.org[41]) includes a large and growing number of variants, some of which are related to a wider constellation of phenotypes. Among the reported mtDNA base substitution disease variants, 431 entries are located in rRNA or tRNA genes, and 481 are located in coding regions or the noncoding (D-loop) control region. These include 52 rRNA/tRNA and 43 coding/D-loop variants that have been confirmed as pathogenic (on March 2021).[41–44] However, it is worth mentioning that most of the variants seem to have no effect and have been widely used as haplotype markers in evolutionary anthropology and population history, genetic genealogy, and forensic science in addition to medical genetics.[45] Moreover, a group of phenotypes known as mtDNA maintenance defects or mitochondrial depletion syndromes must be noted; these are characterized by mtDNA depletion and/or the presence of multiple mtDNA deletions, resulting in inadequate energy production.[46] These mtDNA defects are caused by pathogenic variants located in one of the 20 nuclear-encoded genes that are involved in mtDNA maintenance.[47–49] The involvement of nuclear gene defects causing mitochondrial depletion syndromes is beyond the scope of this review and is discussed elsewhere.[50,51] Another aspect that must be considered is that an altered mtDNA CN is associated with mitochondrial function and dysfunction, and consequently, several conditions have been associated with either increases or decreases in the mtDNA content.[52]

MitD refers to a heterogeneous group of phenotypes; the common clinical features include ptosis, external ophthalmoplegia, proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, and diabetes mellitus. Regarding the central nervous system, phenotypes include fluctuating encephalopathy, seizures, dementia, migraine, stroke-like episodes, ataxia, and spasticity. Some MitD types affect only a single organ, while many others involve multiple organ systems,[36,37] leading to clinical heterogeneity, a hallmark of MitD. The organs/tissues most often affected in MitD are the brain and skeletal muscle, but the heart, liver, peripheral nerves, gastrointestinal tract and endocrine system can also be involved. Therefore, it is relevant to identify the mtDNA defects present in the brain, their nature and prevalence, and the correlation between the genetic defects and the postmortem neuropathologic features to advance our understanding of the underlying mechanisms of mitochondrial function in disease. Within this systematic review, we aim to summarize the main mtDNA alterations reported in postmortem human brain samples and the related phenotypes.

Methods

Search strategy and selection criteria

We examined PubMed and Embase for English language articles published from inception to June 10, 2021 using two search strings combining the following keywords: postmortem/post-mortem, brain, mitochondrial DNA/mtDNA, mutation, variant, deletion, neuron and glia, according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.[53] The specific search strings in both databases are shown in Figure 1. We examined the retrieved titles and abstracts and selected empirical studies based on the eligibility criteria. The literature search strategy, data collection, data extraction and appraisal were conducted independently by three authors (AV-P, JT and BKB). When there was no agreement, a fourth author (LM) contributed to gain consensus. The abstraction and summary of the main results of the studies were first performed independently by one of the three main authors and revised by the remaining authors.

Inclusion and exclusion criteria. Studies were included only if they reported results of mtDNA analyses in postmortem human brain tissue and were written in English. The exclusion criteria were as follows: i) studies that did not report the results of mtDNA analyses in postmortem human brain tissue, ii) cell models, iii) animal models; iv) review studies; v) studies focused on brain tumours or cancer, vi) studies that reported duplicated data; or vii) forensic studies. No other restrictions were applied.

Review process

The combined search yielded 637 potentially eligible studies. Abstracts or full articles (if the eligibility criteria were not clearly stated in the abstract) were screened to decipher eligibility. Figure 1 shows the PRISMA flow chart depicting the specific information at the different stages of the systematic review, and Supplementary Table 3 provides the PRISMA checklist of items to include when reporting a systematic review.[53] We discarded 479 reports, and a detailed examination was performed on the 158 remaining records and on others obtained from hand-searching references.

Data extraction

From eligible articles, we recorded the name of the first author, PMID number, publication year, number of patients and controls analysed, age, sex, disease or condition, brain region, technique used, main results

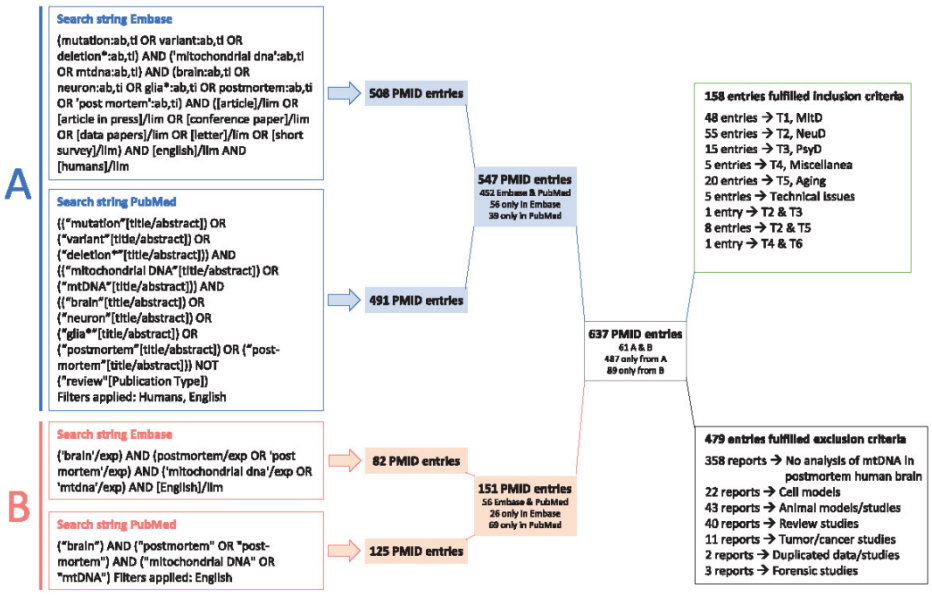

Figure 1. PRISMA flow diagram for selecting published articles for review. The two search strategies used, the number of articles with PMID numbers obtained for each of them, and the result of combining them are shown. Those that met the inclusion criteria and those that were excluded and the groups to which they were assigned are indicated. The final number of articles included in each group after manual inclusion of references is indicated in brackets.

regarding mtDNA variants, mtDNA CN and/or mtDNA rearrangements reported, and additional information.

Pathogenicity assignment of mtDNA variants

Pathogenicity status was collected based on the information present in the Mitomap and ClinVar databases when available.

Role of the funding source

The funders had no role in the study design, in the collection and interpretation of data, in the report writing, or in the decision to submit the manuscript for publication.

Results

Characteristics of the examined studies

The manuscripts fulfilling the inclusion criteria could be ascribed to the following 6 categories: 1) mitochondrial diseases (MitD), 2) neurological diseases (NeuD), 3) psychiatric diseases (PsyD), 4) other clinical conditions included in a miscellaneous group, 5) ageing, and 6) technical issues. Some reports could be ascribed to two groups, as they presented results from more than one category, and others were manually included after reading specific references of the included studies. A summary of the relevant data, number of evaluated subjects, age, sex, brain region/s studied, techniques used, and mtDNA alterations reported are presented in Tables 1–5, while the technical issues are summarized at the end of this section. We screened the variants for putative pathogenic characteristics, and a selection of presumably pathogenic mtDNA variants is shown in Table 6, with pathogenicity information obtained from public databases.

mtDNA analysis in MitD

We included 50 reports referring to MitD (Table 1). Figure 2 shows the variants reported in a varied number of phenotypes, with the most reported being MELAS (13 reports); MERRF (8 reports); LS (7 reports); KSS (5 reports); mitochondrial encephalomyopathy (ME) (6 reports) and PEO (4 reports); in addition to optic neuropathy, sensorineural hearing loss and diabetes mellitus type I; Leber hereditary optic neuropathy (LHON); early-onset cataracts, ataxia and progressive paraparesis; mitochondrial depletion syndrome; neuropathy, ataxia, retinitis pigmentosa and maternally inherited LS; sideroblastic anaemia; and Alpers-Huttenlocher syndrome (AHS). Among the 50 reports, eight reported variants in the nDNA that ultimately produced mtDNA alterations. Most of the studies were case reports; only 11 were carried out after 2010, and only one study analysed the three different types of mtDNA alterations: pSNV, mtDNA CN variations, and deletions. Seven studies analysed two types of alterations, and 41 investigated just one, mostly pSNV.

mtDNA analyses in MELAS. The m.3243A>G variant in the *MT-TL1* gene coding for tRNA-Leu is the most reported variant in postmortem brain samples of patients with MELAS,[54 64] although one study also identified the presence of m.13513G>A, p. Asp393Asn, located in the *MT-ND5* gene.[65] All the studies except one[60] reported mutation loads greater than 70% in the brain and similar mutation loads in other evaluated tissues.[54,55,57 59,61 66] However, some discrepancies were also observed—while some studies reported that mutation loads did not vary between brain regions,[55] others reported high mutation load variability between different cells of the same region.[57] None of the studies on MELAS analysed mtDNA CN or the presence of mtDNA deletions in the brain. The most frequent m.3243A>G variant associated with MELAS was also present in the brain of a 4.5-year-old child with a lethal MitD, with a Barth syndrome-like presentation. This child showed the m.3243A>G variant in all of the analysed tissues, including blood, skeletal muscle, cardiac muscle, and liver. Additionally, in the peripheral blood mtDNA of the mother, as well as in four of the 5 siblings, heteroplasmy percentages were not reported.[67] m.3243A>G was also detected in a patient with optic neuropathy, sensorineural hearing loss and diabetes mellitus type 1, but not MELAS, with a mutation load greater than 75% in white and grey matter, putamen, caudate, pons, visual cortex, among other brain areas and 60% in the biceps muscle.[68]

mtDNA analyses in MERRF. The m.8344A>G variant in the *MT-TK* gene coding for tRNA-Lys was reported in all eight studies evaluating postmortem brain samples of patients with MERRF.[55,56,69 74] Moreover, one of these studies also reported m.8603T>C, p.Phe26Ser, in the *MT-ATP6* gene and m.3257A>G in *MT-TL1*.[72] Interestingly, two distinct reports from 1995 and 2010 using distinct molecular techniques that respectively evaluated an 18- and a 16-year-old patient with MERRF syndrome found similar percentages of the m.8344A>G variant that ranged between 93% and 97% across different brain regions. Both patients also showed similar heteroplasmy percentages in other tissues.[69,71] Notably, one of the studies reporting the m.8344A>G variant also described a 3.7-fold increased mtDNA CN in brain-affected tissues compared to non-affected tissues.[69]

mtDNA analyses in LS. Nine studies reported postmortem brain mtDNA data in LS.[56,75 82] The m.8993T>G, p.Leu156Arg in the *MT-ATP6* gene was reported in four LS studies with mutation load percentages in the brain

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Laine-Menéndez et al. 2021 (34070501)	1/1	43/37	F/F	MM (*TK2*, c.323C>T, p. Thr108Met)	FCtx, TCtx, OCtx, Hi, Amg, Th, Hy, Cb, pons, spinal	qPCR	NR	No differences in mtDNA CN between P and C in brain tissue	NR	Low mtDNA content was observed in skeletal muscle, liver, kidney, small intestine, and particularly in the diaphragm. Heart and brain tissue did not show differences. mtDNA deletions were observed in skeletal muscle and diaphragm
Scholle et al. 2020 (32085658)	1	46	F	Optic neuropathy, sensorineural hearing loss and diabetes mellitus type I. Not MELAS	Pu, Cd, Mb, Th, Hy, Glc, GP, visual Ctx, pons, medulla oblongata, Ver, spinal, WM	RFLP, qPCR	m.3243A>G, *MT-TL1* Mutation load: >75%	Higher heteroplasmy levels significantly correlated with lower mtDNA CN	NR	60% level of heteroplasmy in the biceps brachii muscle
Geffroy et al. 2018 (29454073)	1	32	F	MELAS	NA	Fluorescence RFLP	m.3243A>G, *MT-TL1* Mutation load: 92.1%	NR	NR	-
Gramegna et al. 2018 (29348134)	1/3	36/ Age-matched	1/NA	MNGIE (*TYMP*, c.457G>A, p. G153S)	FCtx, SN	NA	NR	mtDNA depletion in frontal GM and WM, and SN	NR	Severe mtDNA depletion in the P, also in smooth muscle and endothelial cells
Lax et al. 2016 (25786813)	5	45	4M, 8F	MELAS MERRF	FCtx, TCtx, OCtx	Pyrosequencing	MELAS, m.3243A>G, *MT-TL1* MERRF, m.8344A>G, *MT-TK* Mutation load range (%): MELAS 79–94; MERRF 87–93	NR	NR	Mutation load did not vary between brain regions
Tzoulis et al. 2014 (24841123)	8/15	Infantile and adult/ age-matched C	NA	ME (*POLG*)	SN, SNC	qPCR, Long-range PCR, NGS	The overall burden of mtDNA point mutations present at a frequency >0.2% was higher in P than in C in *MT-HV2* and *MT-CO3* and	In homogenate tissue, apparent 20–30% mtDNA depletion was present in infantile P. Microdissected SN neurons showed a 40% lower mtDNA CN	Neurons of both P and C harbored mtDNA DELs in the homogenate. In microdissected neurons mtDNA DELs were more prominent in P with longer disease duration	mtDNA analyses conducted in laser microdissected neurons and homogenate tissue

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
							associated with disease duration	than the neurons of age-matched C		
Giordano et al. 2014 (24369379)	4/8	71/68	NA	LHON	Optic nerve	NA	m.11778G>A, p. Arg340His, *MT-ND1*, present in affected P and unaffected carriers	Unaffected female carries had a higher mtDNA CN than two C	NR	Unaffected mutation carriers showed higher mitochondrial DNA CN than their affected relatives and C
Lax et al. 2013 (23334599)	1	45	F	Early-onset cataracts, ataxia and progressive paraparesis	Optic nerve, basal ganglia, Cb, medulla oblongata, pons	Sequencing, pyrosequencing	m.14685G>A, *MT-TE*. Mutation load range (%): medulla oblongata 82, basal ganglia 68, Cb 58, pons 51, optic nerve 44	NR	NR	-
Tzoulis et al. 2013 (23625061)	2/4	34/58	NA	ME (*POLG*)	Mesencephalon pons, Th, Str	Long-range PCR, nested PCR, qPCR	NR	P neurons contained 50–60% lower mtDNA CNs than age-matched C neurons	DELs were detectable in P and C, and appeared to be more prominent in P	-
Lax et al. 2012 (22491194)	3/1	53/-	M/-	KSS and ME (*POLG*, p. A467T, p. K1240Q; *POLG*, p. G848S, p. W748S)	Dt WM and GM	Long-range PCR, sequencing, qPCR	NR	NR	The P with KSS showed the single m.1167_15636del (3978 bp) and heteroplasmy levels in WM were higher than the 60% threshold, thus considered pathogenic. In this P, heteroplasmy levels in WM where higher than in GM. The P with *POLG* mutations showed multiple mtDNA DELs, and heteroplasmy levels were lower than in the P with KSS	mtDNA analyses conducted in laser microdissected regions. The P with KSS showed decreased immunoreactivity of complex I and COX compared with complex II
Lax et al. 2012 (22249460)	14/-	42/-	5M/9F	MELAS (7), MERRF (1), MELAS/LS (1), KSS (1), Ataxia-retinopathy, aiPEO (3)	Dt, CbCtx, IO	qPCR, pyrosequencing	m.3243A>G; m.8344A>G; m.14709T>C; m.13094T>C. Mutation load range % IO 44–93, CbCtx 47–95, Dt 51–95	NR	Single and multiple large-scale mtDNA DELs. Mutation load % IO 68, CbCtx 20–88, Dt 25–44	Neuronal cell loss occurred independently of the level of mutated mtDNA present within surviving neurons
Brinckmann et al. 2010 (20976001)	1/4	16/16-32	F/NA	MERRF	Mcer, Ox, Mb, Th, Cd, GP, Pu, Ic, Hy, Hi,	qPCR, pyrosequencing	m.8344A>G, *MT-TK*. Mutation load (%) Mcer 98, Ox 97,	mtDNA CN increased between 3–7-fold in affected brain	NR	Mutation load (%): Skeletal muscles 97–98,

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
					sensomotor Ctx, visual Ctx, WM, Chpx, Ver, folia of Cb, medulla of Cb, pons, SN		Mb 94, Th 89, Cd 95, GP 94, Pu 95, Ic 95, Hy 94, Hi 94, sensomotor Ctx 96, visual Ctx 96, white matter 95, Chpx 100, Ver 97, folia of Cb 97, medulla of Cb 93, pons 95, SN 91	tissues compared to nonaffected tissues		diaphragm 97, external ocular muscles 95, heart 93, renal cortex 99, renal medulla 95, adrenal cortex 100, lung 98, liver 67, pancreas 73, spleen 98, stomach 98, ileum 97, bladder 100, uterus 100, ovary 100, adipose tissue 99, skin 100
Sanaker et al. 2010 (19744136)	1	64	M	IoME	OCtx, TCtx, CbCtx	Sequencing, PCR-RFLP	m.5556G>C, *MT-TW*. Mutation load (%): Octx 34, TCtx 32, CbCtx 12	NR	NR	Mutation load (%): skeletal muscle 70, heart muscle 27, kidney 26, liver 27, adrenal cortex 5, pancreas 8, lung 2, spleen 4
Zsurka et al. 2008 (18716558)	1/30	17/28	M/NA	ME (*POLG*)	NA	Long-range PCR, qPCR	NR	Progressive decrease of mtDNA CN in the disease course in P but not significantly different from that in C	Presence of the m.8342_14004del (7662 bp)	-
Götz et al. 2008 (18819985)	2/2	NA	NA	MDS (Patient 1: *TK2*, c.739C>T, p. R172W Patient 2: TK2, c.898C>T, p. R225W)	Ctx, Cb, Basal ganglia	qPCR	NR	Severe brain mtDNA depletion in patients with R172W but not with R225W mutation. Higher mtDNA content in the Cb of the patient with R2 25W mutation	NR	mtDNA depletion present in muscle and liver in all patients
Rojo et al. 2006 (16525806)	1	64/-	M	NARP-MILS	Pu, brain stem, Th, Ctx	Southern blot, PCR-RFLP	m.8993T>G, p. Leu156Arg, *MT-ATP6* Mutation load (%): brain stem and Ctx 89, Pu and Cb 90, Th 91	NR	NR	Mutation load (%): blood 75, muscle 87
Betts et al. 2006 (16866982)	2	47	F	MELAS	Chpx, Hi, CbCtx, Dt, GPI, OCtx, FCtx, GPI	PCR-RFLP using radiolabeled nucleotides	MELAS, m.3243A>G, *MT-TL1* Mutation load in patient 1 and 2,	NR	NR	Mutation load was higher in COX-deficient than in COX-positive

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
							respectively (%): chpx 81 and NA, Hi 79 and 92, CbCtx 54 and 96, Di 75 and NA, GPI 70 and 93, Octx 89 and 93, FCtx NA and 92			microdissected cells from Hi, chpx and skeletal muscle. Mutation load varied considerably between different cells of the same region
Matthes et al. 2006 (16856911)	1	19	M	Sideroblastic anemia	NA	Long-range PCR, sequencing, qPCR	NR	NR	Presence of the m.5853_9468del (3614 bp). Mutation load (%): 70	Mutation load (%): Liver 90, skeletal muscle 75, pancreas 85, peripheral blood 80
Ferrari et al. 2005 (15689359)	1/2	19/age-matched	M/-	AHS (*POLG1*, c.1399G>A, p.A467T)	NA	NA	NR	30% mtDNA content reduction	NR	-
Pistilli et al. 2003 (14608542)	1	36/-	F	KSS	FCtx, PCtx, TCtx, OCtx, Cb, basal ganglia, brain stem	Sequencing, Southern blot, competitive PCR using radiolabeled nucleotides	NR	NR	Presence of the m.8631_13580del (4949 bp). DEL load (%): FCtx 54; Octx 54; TCtx 58; PCtx 59; basal ganglia 75; Cb 88	-
Uusimaa et al. 2003 (12612282)	1	7/-	F	AHS-like disease	NA	CSGE + sequencing, PCR-RFLP using radiolabeled nucleotides	m.7706G>A, p. Ala41Thr, *MT-CO2*. Mutation load: 91%	NR	NR	Mutation load (%): blood 87, heart 87
Kirby et al. 2003 (14520659)	3	31	2M, 1F	LS	Medulla oblongata, pons, Cb, basal ganglia, Th, midbrain, Hi, FCtx, Chpx	PCR-RFLP with radiolabeled nucleotides	m.13513G>A, p. Asp393Asn, *MT-ND5* Mutation load (%): 73	NR	NR	-
Jiang et al. 2002 (12174968)	1	-	-	LS	NA	NA	m.8993T>G, Leu156Arg, *MT-ATP6* Mutation load (%): 89	NR	NR	Mutation load (%): muscles 85, lymphocytes 72
De Kremer et al. 2001 (11241464)	1/20	4/NA	1/NA	Barth syndrome-like disorder	NA	PCR-RFLP sequencing	m.3243A>G, *MT-TL1*. The mutation was heteroplasmic in brain but not quantified	NR	NR	The mutation was also heteroplasmic in all the tissues analyzed: blood, skeletal muscle, cardiac muscle, and liver
	1	61	F	PEO		Long-range PCR, Southern blot	NR	NR	Multiple DELs (25), most of them were less than	DELs present in all brain regions.

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Moslemi et al. 1999 (10408540)					WM, FCtx, Th, Pu Cd, SN, CbCtx				8 kb. The CbCtx showed the lowest mutation load %. The common DEL was found in all the specimens	skeletal muscle samples and myocardium
Nagashima et al. 1999 (10208283)	1	43	F	LS	FCtx	PCR-RFLP	m.8993T>G, p. Leu156Arg, *MT-ATP6*. Mutation load (%): 95	NR	NR	Mutation load (%): liver 98, muscle 90, heart 91, kidney 99, pancreas 93
Santorelli et al. 1998 (9851442)	1	12	F	MERRF	NA	Sequencing with radiolabeled nucleotides	m.8344A>G, *MT-TK*, mutation load (%): 92%. m.8603T>C, Phe26Ser, *MT-ATP6*, m.3257A>G *MT-TL1*	NR	NR	-
Di Trapani et al. 1997 (9266144)	1	27/-	M	MELAS	NA	PCR-RFLP	m.3243A>G, *MT-TL1*. Mutation load: 84%	NR	NR	Mutation load (%): liver 79, kidney 86, skeletal muscle 83, cardiac muscle 83
Zhou et al. 1997 (9315896)	1	14	F	MERRF	CbCtx, Dt, IO, FLV	PCR-RFLP	m.8344A>G, *MT-TK*. Mutation load (%): between 81 and 98. Similar % in soma, neuropil, glia and homogenate tissue.	NR	NR	Analyses of homogenate issue and individual neurons
Suomalainen et al. 1997 (9153451)	2	67	F	adPEO	FCtx, TCtx, Cd, Cb	Southern blot	NR	NR	Multiple DELs ranging from 0.5 kb to 10.0 kb DEL load (%): FCtx 50, TCtx 40, Cd 60, Cb 10	Mutation load (%): Skeletal muscle 50, kidney 10, liver 10
Santorelli et al. 1997a (9299505)	1	45	M	MELAS	NA	PCR-RFLP	m.13513G>A, p. Asp393Asn, *MT-ND5*. Mutation load (%): 73	NR	NR	Mutation load (%): muscle 68
Santorelli et al. 1997b (9266739)	1	1.4	M	MM, LS	Basal ganglia, SN, brain stem	Southern blot PCR-RFLP sequencing	m.5537_insT *MT-TW*. Mutation load (%): ≥98	NR	NR	Mutation load (%) in other tissues: >95
Kaido et al. 1996 (8870835)	1	53/-	F	MELAS	FCtx, Pu, GP	Southern blot	m.3243A>G, *MT-TL1*. Mutation load (%): FCtx 89, Pu 86, GP 80	NR	NR	-
	1	14/-	F	MERRF	CbCtx, FCtx, Cd, Pu		m.8344A>G, *MT-TK*. Mutation load (%):	NR	NR	Mutation load (%): blood 75, muscle

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Sanger et al. 1996 (8652018)						PCR-RFLP using radiolabeled-nucleotides	CbCtx 97, FCtx 88, Cd 88, Pu 88			86–93, spinal cord 83, medulla 87
Melberg et al. 1996 (8937533)	1	20	M	MELAS-like syndrome	FCtx	Southern blot for variants m.3243A>G and m.8344A>G	No presence of these two variants	NR	NR	-
Houshmand et al. 1996 (8786060)	1	14	F	MM, lactic acidosis and complex I deficiency	NA	PCR-RFLP	m.3251A>G *MT-TL*. Mutation load (%): 90	NR	NR	Mutation load (%): muscle 94, fibroblast 93, heart 79, liver 80
Oldfors et al. 1995 (8525809)	1	18/-	M	MERRF	FCtx, PCtx, TCtx, OCtx, frontal WM, Pu, pallidium, Th, pons, IO, CbCtx, Dt	PCR-RFLP using radiolabeled-primer	m.8344A>G, *MT-TK*. Mutation load (%): FCtx 95, PCtx 95, TCtx 95, Octx 95, Pu 95, pallidum 96, Th 94, pons 94, IO 94, low. Brain stem 94, CbCtx 95, Dt 93	NR	NR	Mutation load (%): skeletal muscle 97, myocardium 97, aorta 97, subcutaneous adipose tissue 95, liver 95, pancreas 91, spleen 95, lymph node 93, bone marrow 94, testis 99, adrenal gland 97, thyroid gland 98
Nelson et al. 1995 (7695240)	1	53/-	M	ME	Ctx, Cb	Southern blot	m.5549G>A, *MT-TW* Mutation load (%): Ctx 87, Cb 88	NR	NR	Mutation load (%): blood 40, muscle 83–86, myocardium 93, kidney 93, lung 79, liver 77, optic nerve 51
Brockington et al. 1995 (7561952)	1	41	M	KSS	TCtx, OCtx, Cb	Southern blot	NR	NR	Presence of the common DEL. DEL load (%): TCtx 62, OCtx 70, Cb 17	Mutation load (%): quadriceps 91, psoas 72, diaphragm 75, cardiac muscle 25, kidney 21, liver 84, lung 18, spleen 0, testis 0, blood 0
Sweeney et al. 1994 (8133313)	1	18	M	LS	Cb, CbCtx	PCR	m.8993T>G, p. Leu156Arg, *MT-ATP6* Mutation load (%): Cb 97, CbCtx 97	NR	NR	Mutation load (%): blood 81, quadriceps muscle 99, extraocular muscle 97, cardiac muscle 97, liver 99, kidney 98, blood 72
	2	24	F	MELAS		PCR-RFLP		NR	NR	

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
MacMillan et al. 1993 (8351017)					PCtx GM, Octx GM, PCtx WM, Cd, Cb, pons.		m.3243A>G, *MT-TL1*. Mutation load (%): PCtx GM 79, Octx GM 80, PCtx WM 62, Cd 73, Cb 72, pons 70			Mutation load (%): psoas muscle 82, oculomotor muscle 59, cardiac atrium 86, myocardium 67, oesophagus 84
Love et al. 1993 (8226463)	2	23, 16	F	MELAS	FCtx, TCtx, pons, PCtx, OCtx	PCR-RFLP, sequencing with radiolabeled primers	Case 1: m.3243A>G, *MT-TL1*. Mutation load (%): FCtx 46, TCtx 40, PCtx 33, pons 30. Not detected in OCtx. Case 2: neither m.3243A>G nor m.3271T>C, *MT-TL1*, was present.	NR	NR	Mutation load (%) in case 1: liver 78, thenar muscle 77, myocardium 73. Poor correlation between mutation load and distribution of histological lesions
Tanno et al. 1993 (8170566)	2	29, 30	M, F	MERRF	FCtx, TCtx, CbCtx	PCR-RFLP	8344A>G, *MT-TK*. Mutation load (%): FCtx 97, TCtx 98, CbCtx 97	NR	NR	Mutation load (%): heart 96, kidney 96, adrenal gland 93, liver 94, muscle 96–99, leukocytes 93
Shiraiwa et al. 1993 (8138807)	1	27	F	MELAS	FCtx, TCtx, PCtx, OCtx, Cd, Pu, pallidum, Th, frontal WM, Pit, CbCtx, Dt	PCR	m.3243A>G, *MT-TL1*. Mutation load (%): Pit 95; FCtx, TCtx, PCtx and Octx 85–88; Cd, Pu, pallidum and WM 73–79	NR	NR	-
Tatuch et al. 1992 (1550128)	1	0.6	F	LS	NA	Southern blot PCR-RFLP	m.8993T>G, p. Leu156Arg, *MT-ATP6*. Mutation load (%): >95	NR	NR	Mutation load (%): >95 in fibroblasts, kidney and liver
Suomalainen et al. 1992 (1634620)	1	60	F	PEO and MDD	FCtx, basal ganglia	Southern blot PCR	NR	NR	Presence of several DELs. Most of the DEL breakpoints were between ~m.11,900 and m.12,600. DEL sizes between ~2.0 to 10 kb	Mutation load (%): kidney 10, liver 20, extraocular muscle 40, heart 40, vastus lateralis muscle 60. No DELs in blood
Lombes et al. 1991 (1849240)	3	5, 2, 0.5	M	LS and COX deficiency	NA	Southern blot Northern blot	NR	mtDNA/nDNA content was higher in P than in C (in P3, 4.6 times higher)	No mtDNA DEL detected	mRNA levels of the mtDNA encoded COX subunits was decreased compared to the

Table 1 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex in P/C	Disease (nuclear gene)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
										nDNA encoded subunits
Ciafaloni et al. 1991 (1922812)	1	26	M	MELAS	NA	Southern blot PCR-RFLP	m.3243A>G, *MT-TL1* Mutation load (%): 84	NR	NR	Mutation load (%): muscle 83, liver 79, heart 83, kidney 86
Enter et al. 1991 (1684568)	1	12	F	MELAS	NA	Southern blot PCR-RFLP	m.3243A>G, *MT-TL1* Mutation load (%): 80	NR	NR	Mutation load (%): cardiac muscle 70, skeletal muscle 30, liver 70, diaphragm 60
Bordarier et al. 1990 (2359483)	1	17	F	KSS	NA	Southern blot	NR	NR	DEL located between positions ~ m.8,200 and m.13,000 (±400 bp)	The DEL was also found in muscle and spinal cord

Table 1: **Studies reporting the results of mtDNA analyses in postmortem brain samples of patients with mitochondrial diseases (MitD).**

C: control; **DEL**: deletion; **F**: female; **GM**: gray matter; **M**: male; **mtDNA CN**: mitochondrial DNA copy number; **N**: number of subjects; **NA**: information not available; **NR**: not reported; **P**: patient; **y**: years; **WM**: white matter.
ad/arPEO: autosomal dominant/autosomal recessive progressive external ophthalmoplegia; **AHS**: Alpers-Huttenlocher syndrome; **KSS**: Kearns-Sayre syndrome; **LHON**: Leber hereditary optic neuropathy; **LoME**: late onset mitochondrial encephalomyopathy; **LS**: Leigh's syndrome; **MDD**: major depressive disorder; **MDS**: mitochondrial depletion syndrome; **ME**: mitochondrial encephalomyopathy; **MELAS**: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; **MERRF**: myoclonic epilepsy with ragged-red fibers; **MILS**: maternally inherited LS; **MM**: mitochondrial myopathy; **MNGIE**: mitochondrial neurogastrointestinal encephalopathy; **NARP**: neuropathy, ataxia, retinitis pigmentosa; **POLG**: DNA polymerase subunit gamma. **TK2**: thymidine kinase 2.
Amg: amigdala; **Clx**: cerebellum; **CbCtx**: cerebellar cortex; **Cd**: caudate nucleus; **Chpx**: choroid plexus; **Ctx**: cortex; **Dt**: dentate nucleus; **FCtx**: frontal cortex; **FLV**: frontal horn of the lateral ventricle; **Gic**: internal capsula, genu; **GP**: globus pallidus; **GPI**: internal globus pallidus; **Hi**: hippocampus; **Hy**: hypothalamus; **Ic**: internal capsule; **IO**: inferior olive; **Mb**: mammillary bodies; **Mcer**: middle cerebral artery; **OCtx**: occipital cortex; **Ox**: optic chiasm; **PCtx**: parietal cortex; **Pit**: pituitary gland; **Pu**: putamen; **SN**: substantia nigra; **SNC**: substantia nigra, pars compacta; **Spinal**: spinal cord; **Str**: striatum; **TCtx**: temporal cortex; **Th**: thalamus; **Ver**: vermis of cerebellum.
CSGE: conformation-sensitive gel electrophoresis; **NGS**: next-generation sequencing; **qPCR**: quantitative real-time polymerase chain reaction; **RFLP**: restriction fragment length polymorphism.
ATP6: ATP synthase subunit 6; *COx*: cytochrome c oxidase II; *COy*: cytochrome c oxidase III; *HVs*: hypervariable segment s; *MTx*: mitochondrially encoded gene; *NDn*: NADH-ubiquinone oxidoreductase subunit n; *TE*: tRNA-Glu; *TK*: tRNA-Lys; *TLx*: tRNA-Leu x; *TW*: tRNA-Trp.

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration		Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant	mtDNA CN		
Castora et al. 2020 (31561357)	40/40	NA	NA	AD (40)	PCtx, TCtx, Cd, Hi	PCR-RFLP	6 AD P showed the m.9861T>C variant compared to none in C. Mutation load %: between 11 and 95	NR	NR	m.9861T>C was found in multiple regions, with the highest mutation load % occurring in PCtx and TCtx
Chen et al. 2020 (31689514)	20/15	49–81/75	21/14	MD (12) PD (8) C (15)	SN	qPCR	NR	High mtDNA CN in healthy aged TH-positive neurons of C, despite showing similar DEL levels as P with PD. mtDNA CN was significantly lower in neurons of P with MD showing mtDNA point mutations or multiple deletions and in P with PD. Data showed evidence of mtDNA depletion in PD neurons, with large individual diversity in cases.	DELs in *MT-ND4* were detected in healthy TH-positive neurons of aged C. Comparable DEL levels in *MT-ND4* were found in the cases with multiple mtDNA DELs and in P with PD. Prominent *MT-ND1* DELs were detected in neurons from cases with multiple DELs. They were also present in older C and P with PD, however, with lower levels.	-
Kim et al. 2020 (32005289)	34/25	NA	NA	ALS (34) AD (10) C (15)	Ctx	Aldehyde reactive probe-based assay, ELISA	NR	NR	NR	Apurinic/apyrimidinic sites (abasic sites) did not differ between ALS and C. Levels of oxidized mtDNA did not differ between ALS and C. mtDNA analyses were conducted in laser-microdissected neurons
Thubron et al. 2019 (31388037)	44/30	79/77	36/38	AD (34) MCI (10) NCI (30)	PCtx, FCtx, Cb	qPCR	NR	48% mtDNA CN reduction in PCtx of nondiabetic AD vs. nondiabetic NCI. No reduction observed in diabetic AD compared to diabetic NCI	NR	The lowest mtDNA CN level was observed in Cb, the highest in PCtx, both in NCI. Higher mtDNA CN was observed in all brain regions of diabetic vs. non-diabetic cases, irrespective of cognitive status
Alvarez-Mora et al. 2019 (30887649)	2/3	93.97/73-83	NA	FXTAS (1) no-FXTAS (1) C (3)	Ver, Dt, PCtx, TCtx, Th, Cd, Hi	ddPCR	NR	mtDNA CN in Ver, Dt, PCtx and TCtx was descreased in FMR1 premutation carriers with FXTAS than in C but no differences were detected in Th, Cd and Hi. P with FXTAS showed lower mtDNA CN than Cs	NR	-
Soltys et al. 2019 (30359878)	10/20	82/80	11/19	AD (10) HpC (10) C (10)	Cb, TCtx	RMC assay, ddPCR	mtDNA mutation frequencies were similar in all three groups in both brain regions	mtDNA CN depletion observed in TCtx of AD P vs. HpC or C. No mtDNA CN variation in Cb between all three groups	NR	-
Strobel et al. 2019 (30475765)	22/10	75/70	16/16	AD (22) C (10)	Hi, Cb	qPCR	NR	NR	In C, higher rates of mtDNA DELs were observed in astrocytes and microglia of the Hi compared to brain stem and Cb	-
	6/6	76/84	Ratio 5:1		PPN	qPCR	NR			

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex (M/F)	Disease (N)	Brain region	Technique	mtDNA alteration Variant	mtDNA CN	Rearrangements	Additional findings
Bury et al. 2017 (29149768)				PD (6) C (6)				Significant mtDNA CN increase in PPN cholinergic neurons from PD P (10.7%) vs. those from C (7.0%)	mtDNA DEL levels were significantly increased in PPN cholinergic neurons from PD P (21.6%) vs. those from C (17.2%)	mtDNA DELs correlated with Braak staging in PD
Wei et al. 2017 (28153946)	902/461	69/59	765/598	AD (282) CJD (181) DLB-PD (89) FTD-ALS (236) Other (114) C (461)	Cb, Cbx, other	Exome sequencing	No evidence of disease association with homoplasmic or heteroplasmic rare variants	mtDNA CN was significantly lower in AD and CJD P. Positive correlation between age and mtDNA CN in CJD P	NR	No correlation between mean levels of heteroplasmy, total number of heteroplasmic variants or variant pathogenicity score with age or disease group
Nido et al. 2018 (29257976)	2/NA	NA	NA	PD (2)	SN	NGS	Sequence heteroplasmy was significantly different between deleted and nondeleted mtDNA populations for 4/55 of the DELs 11.3% of the analysed positions had a heteroplasmic frequency significantly different between deleted and nondeleted mtDNA populations	NR	The 20 microdissected neurons showed 373 unique DELs (only 31 previously annotated). Each neuron contained on average 38.2 ± 29.6 distinct DELs. Mean size deletion was 5080 ± 2267 bp. The common DEL was detected in 15/17 neurons and was the most prevalent DEL in 6 neurons	20 single laser-microdissected neurons were evaluated
Flones et al. 2017 (29270838)	18/11	79/74	17/12	PD (18) C (11)	FCtx, SN, Cb, Hi, Pu	qPCR	NR	There was no significant difference between C and PD for mtDNA CN in Pu or Hi	Only dopaminergic neurons from the SN harboured significantly higher mtDNA DEL levels	1189 single laser-microdissected neurons were evaluated. Neuronal complex I deficiency did not correlate with mtDNA damage
Dölle et al. 2016 (27874000)	10/22	82/56	21/11	PD (10) C (22)	SN, FCtx, CbCtx	qPCR, NGS (4,767 bp)	The mean load of heteroplasmic SNVs was 33.14%/1,000bp per neuron, and most of these clustered in the low-frequency spectrum in both PD and C. The overall burden of heteroplasmic SNVs was similar in PD and C. The proportion of G:C to T:A transversions was also similar in the two groups	mtDNA CN was similar in PD and C. In SN, however, the subset of neurons with high mtDNA depletion (<10,000 copies/cell) was 14% in PD and 2.7% in C (p=0.01). No differences were observed in FCtx or CbCtx	SN neurons from PD contained significantly higher mtDNA DEL levels than C. The proportion of neurons with DEL levels exceeding 60%, was 21.4% in PD and 10.8% in C. mtDNA DEL levels were generally low in frontal neurons and Cb of both PD and C	871 single laser-microdissected neurons were evaluated. In SN but not in FCtx or CbCtx, DELs and mtDNA CN showed a positive correlation with age, DEL was a predictor of mtDNA CN
Chen et al. 2016 (27599301)	13/12	81/82	NA	AD (13) C (12)	FCtx	NGS, qPCR	Similar heteroplasmy levels were observed in some mtDNA positions in AD P and C	NR	DELs were increased in AD P (9%) vs. C (2%). Rearrangement rate was higher in AD P (18%) than in C (7%). The common DEL was detected in most samples but at low % (1-5%)	Different numbers and types of mtDNA rearrangement fragments were detected depending on the sequencing coverage depth
	81/33	79/80	67/47		PFCtx	qPCR	NR	mtDNA CN was significantly reduced (18%) in PDD vs. C	NR	Although mtDNA CN was reduced in PDD,

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration		Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant	mtDNA CN	Rearrangements	
Gatt et al. 2016 (26853899)				PD (41) PDD (40) C (33)				No significant decrease was observed in PD		mitochondrial biogenesis was unaffected, as the expression of mitochondrial proteins in PDD and C was similar
Blanch et al. 2016 (26776077)	26/18	NA	23/21	AD (16) PD (10) C (18)	Ent, SN	PiQ, qPCR	NR	NR	NR	Increased 5-methylcytosine levels observed in the D-loop in Ent of AD P vs. C. Lower 5-methylcytosine levels observed in the D-loop in SN of PD P vs. C
Cowhead et al. 2016 (26639157)	180/40	78/77	128/92	IPD (180) C (40)	SNC, FCtx	NGS	The mean heteroplasmic variant burden differed between PD P and C in both SNC and FCtx	NR	NR	No significant correlation of heteroplasmy with age. Increased heteroplasmic variation was observed in COX genes
Grunewald et al. 2016 (26605748)	10/10	76/75	5/5	IPD (10) C (10)	SN	qPCR	NR	mtDNA CN was reduced (73.1%) in IPD P vs. C. 75 D-loop mtDNA CN was reduced (91%) in IPD P vs. C	mtDNA DEL prevalence did not differ between IPD P and C	-
Rice et al. 2014 (24448779)	10/9	79/59	12/22	AD (10)	Hi	qPCR	NR	mtDNA CN was significantly reduced in PyNs from AD. CN in other neuronal cells was not significantly different between P/C	Deletion levels were not significant	-
Azaldi et al. 2014 (23872536)	1/0	36	0/1F	MTLE+HS (1)	Hi (6 regions)	Pyrosequencing	The number of heteroplasmic variants was higher in the *CA2* region and accumulated in *MT-ND2*, *MT-ND3* and *MT-ND5* genes: m.3563insC, p. Trp96>S was suggested to be studied in other MTLE+HS patients	NR	NR	Hi contained more heteroplasmic variants than blood
Müller et al. 2013 (23566333)	14/14	78/73	NA	PD (7) AD (7) C (14)	SN, Hi	qPCR	NR	In PD, mtDNA CN did not differ significantly between LB+ and LB- neurons. In AD, mtDNA CN did not differ significantly between tau protein+ and tau protein- neurons	In SN, DEL levels differed between P with LB+ neurons (40.5%), LB- neurons (31.8%) and C (25.6%), p<0.005. In Hi, DEL levels did not differ between groups, independent of disease status and cell type (tau protein + or -)	2–6 single laser-microdissected neurons were evaluated per patient/control
Krishnan et al. 2012 (21925769)	10/6	76/76	NA	AD (10) C (6)	Hi	qPCR, Long-range PCR + sequencing	NR	NR	mtDNA DEL levels were higher in COX-deficient neurons (57%) than in COX-normal neurons (9%) in AD P and in C (48% and 24%, respectively). No differences were observed in COX-deficient	-

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration	mtDNA CN	Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant			
									neurons from AD P and C. mtDNA DELs ranged in size from 3670 to 6088 bp. mtDNA DELs accumulated with age	
Keeney et al. 2010 (20504367)	10/7	NA	NA	ALS (10) AD (1) C (6)	Cb, Cbx	qPCR	NR	There were no differences in ND2 CN between P and C	There was an approximate doubling of DELs for ND4 and a consistent increase for CO3. Two P showed greater abundance in ND4 DELs with higher CN levels. Purkinje neurons showed low levels of DELs	Single laser-microdissected neurons from cervical spinal cord, anterior gray matter and Cb were evaluated
Naydenov et al. 2010 (20740286)	32/31	75/71	NA	PD (32)	Pu, Cb	qPCR	NR	Dopamine led to a 25% down-regulation of mtDNA levels and L-Dopa caused an increase in mtDNA levels. In dyskinetic-PD, lower mtDNA levels in early disease processes were found. Pu showed a 50% reduction and a significant negative correlation with L-DOPA/year	Pu from dyskinetic-PD showed a higher % of deletions and a correlation with mtDNA levels (↓mtDNA levels↑mtDNA deletions in PD and dyskinetic-PD)	-
Coskun et al. 2010 (20463402)	38/25	NA	NA	AD (13) DS (11) DSAD (14) C (25)	FCtx	PNA clamping PCR, qPCR, sequencing-(1,115 bp)	The frequency of mtDNA control region mutations was significantly higher in AD than in C m.4141T>G/ was found in 69% of AD but not in C, and in 57% of DSAD but not in DS. Other heteroplasmic mutations common in AD reported: m.68G>A, m.70G>A, m.72T>C, m.185G>A, m.207G>A, m.228G>A, m.309delC, m.309insC, m.408T>C, m.4141>C, m.418C>T, m.466-2insCC	mtDNA CN of AD brains was significantly lower than age-matched C. Additionally, mtDNA CN of DSAD was significantly lower than age-matched C mtDNA CN declined with age in C	NR	AD-like neuropathology was present in AD and DSAD but not in DS. The frequency of somatic mutations in the regulatory control region increased with age in the normal brain (p=0.0029). The *MT-ND6* to *MT-ND2* transcript ratio did not change with age but was significantly lower in AD, DSAD and DS compared to C. This; reduced mtDNA L-strand transcription level was associated with intellectual disability and dementia. β-Secretase activity was associated with some mtDNA alterations
13/10	55/74	NA	MS (13) C (10)	Cbx	Long-range PCR, qPCR,	sequencing	NR	NR	DELs were evident in 66% of normal appearing gray matter regions and in 33% of lesioned regions of MS P and in 16% of C. Multiple DELs were observed t in respiratory-deficient laser	Single laser-microdissected neurons from WM and GM were evaluated. No differences in DEL heteroplasmy levels between WM and GM. DELs were supposed to be

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics		Disease (N)	Brain region	Technique	mtDNA alteration		Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)			Variant	mtDNA CN		
								microdissected pooled neurons when compared to neurons with intact complex IV activity. Heteroplasmy DEL levels were also increased in respiratory-deficient neurons compared to neurons with intact complex IV activity	pathogenic because they contained MT-CO1 and MT-CO2 catalytic subunits
Arthur et al. 2009 (19775436)	8/10	78/67	12/6	FCtx and SN	Surveyor nuclease assay, qPCR	Non significant variation in heteroplasmy levels between sPD P and C	No differences in mtDNA CN between sPD P and C	NR	-
Alievet al. 2008 (18827923)	NA	NA	NA	AD	Cytological in situ hybridization	NR	NR	5 kb mtDNA DEL was localised in lysosomes of P but not in neuronal cell bodies. The main location of these DELs was in lysosomes, but not in other neuronal cell compartments	DEL detection was achieved by electron microscopy ultrastructural visualisation of immune-positive gold particle clusters
Bender et al. 2008 (18604467)	9/8	76/71	10/7	AD (9) C (10)	Pu, FCtx, SN	qPCR	NR	DEL levels were higher in AD SN (32%) than in FCtx (13%) and Pu (14%) but did not differ from that of C (SN 35%, FCtx 14% and Pu 14%)	1530 single laser-microdissected neurons were evaluated. There was no difference in mtDNA DEL levels per brain region between groups
Hakonen et al. 2008 (18775955)	4/9	23/44	9/6	IOSCA (4) C (9) (EFTR, c.1523A>G, p. Y508C)	Ctx, Cb	Long-range PCR, qPCR, Southern blot, sequencing	IOSCA P did not show increased mtDNA point mutation load in affected tissues	mtDNA depletion was present in Ctx and Cb of IOSCA P	No mtDNA DEL was detected in IOSCA P	mtDNA CN decreased by 5–20% in brain and 10–70% in liver of IOSCA P compared to C but similar amounts were observed in skeletal muscle
Reeve et al. 2008 (18179904)	6/5	77/78	NA	PD (5) PEO (1) C (5)	SN	Long-range PCR	NR	NR	Various DELs were found in P and C, there was no difference in the distribution nor in the types of DEL breakpoints detected between groups	Single laser-microdissected neurons from SN were evaluated
Blokhin et al. 2008a (18566918)	5/9	38-53/ 34-80	8/6	MS (5) C (9)	FCtx, PCtx, OCtx	qPCR	NR	No differences in mtDNA CN between COX+ neurons, COX- neurons and neurons of chronic active plaques in MS. mtDNA CN in WM did not differ between MS P and C. In MS, mtDNA CN in neurons of GM was higher than in cells of other regions	NR	mtDNA CN decreased with age in both MS P and C
Blokhin et al. 2008b (18286391)	5/12	38-53/ 34-80	8/6	MS (5) C (12)	FCtx, PCtx, OCtx	qPCR	NR	NR	No pathology-related accumulation of mtDNA DELs was observed when comparing distinct brain specimens from MS or when comparing MS and C. The rate of mtDNA DELs	Proportion of mtDNA DELs correlated with age

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration		Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant	mtDNA CN		
										was higher in COX- than in COX+ cells
Bortwick et al. 2006 (16358336)	1/0	73	M	MND (1)	FCtx, Pons, spinal cord	Sequencing, PCR-RFLP with radiolabeled nucleotides	m.4274T>C in *MT-TI*. Mutation load %: FCtx 45%, Pons 37%.	NR	NR	Single laser-microdissected motor neurons were evaluated. Mutation levels were significantly higher in all COX-deficient than in COX-positive motor neurons
Bender et al. 2006 (16604074)	15/8+8	76/77	NA	PD (15) C (8+8)	Hi, SN	Long-range qPCR, sequencing	Nonpathogenic m.189A>G, m.16184T>C and m.96337>C variants were detected	NR	In SN, mtDNA DEL levels did not differ between PD (52.3%) and aged C (43.3%), however, they differed in the Hi (17.8% in PD and 14.3% in C), p=0.0002. DEL levels correlated with age	50 single laser-microdissected neurons were evaluated. The level of mtDNA DELs was significantly greater in COX-deficient neurons
Wang et al. 2006 (16405502)	8/6	90/81	NA	MCI (8) C (6)	FCtx, TCtx, PCtx, Cb	GC/MS-SIMA	NR	NR	NR	Levels of multiple oxidised bases were significantly higher in FCtx, TCtx and PCtx of MCI P than in C
Wang et al. 2005 (15857398)	8/8	85/84	8/8	AD (8) C (8)	FCtx, TCtx, PCtx, Cb	GC/MS-SIMA	NR	NR	NR	Levels of multiple oxidised bases were significantly higher in FCtx, TCtx and PCtx of AD P than in C. mtDNA had ~10-fold higher levels of oxidised bases than nDNA
Aliyev et al. 2005 (15766652)	NA	NA	NA	AD	Hi	Cytological in situ hybridization	NR	NR	5 kb mtDNA DELs were mostly localised in lysosomal structures but not in neuronal cell bodies. There was a 3-fold increase of mtDNA DEL in AD compared to C	DEL detection was achieved in neurons by electron microscopy ultrastructural visualization of immune-positive gold particle clusters
Coskun et al. 2004 (15247418)	23/40	NA	NA	AD (23) C (40)	FCtx	PNA-clamped PCR, RT-qPCR, sequencing- (669 bp)	Frequency of heteroplasmic variants in the mtDNA control region showed a 63% increase in P with AD compared to C ($p<0.01$). In P 80 years and older this increase was 130%. m.4141>G proved to be specific for AD brains: m.1467>C, m.1951>C and m.4777>C showed heteroplasmic levels up to 70–80% in P with AD aged between 74 and 83 years	There was a significant 50% mtDNA CN reduction in AD compared to C	NR	Paper focused on studying the mtDNA control region. Variants identified in brains of P with AD were preferentially located in known functional transcription and replication elements and were also frequently present at exceptionally high proportions. P with AD showed reduced *MT-ND6* mRNA expression
	4/4	61/57	3/5			qPCR	NR	NR		

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration		mtDNA CN	Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant				
Mavrin et al. 2004 (15036587)				AD (1) PD (1) FTD-MND (1) DLB (1) C (4)	FCtx, OCtx, TCtx, Hi, SN, Cb, basal ganglia, brain stem					In FTD-MND, high levels of the common DEL were found in the brain stem, FCtx and TCtx. In DLB, they were observed in OCtx. In PD and AD, no increase of the common DEL was observed. In PD, high levels of common DEL were observed in SN after normalisation for the mtDNA deletion in Cb. The lowest levels of the common DEL were found in the Cb	Levels of the common DEL were markedly raised with increasing age
Mavrin et al. 2003 (12924443)	7/3	NA	NA	ALS (7) C (3)	Cbx, brain stem	PCR	NR		NR	Common DEL levels did not differ between ALS and C. Common DEL levels increased with age	Single laser-microdissected neurons were evaluated
Aliev et al. 2003 (14503922)	NA	57-93/54-85	NA	AD	Hi, TCtx, Cb, FCtx	In situ hybridization	NR		NR	mtDNA DEL showed a 3-fold increase in AD compared to C. The majority of these DELs were found in mitochondria-derived lysosomes	DEL detection was achieved by electron microscopy ultrastructural visualisation of immune-positive gold particle clusters
Gu et al. 2002 (12125742)	24/4	77/81	17/11	PD (8) AD (6) DLB (6) MSA (4) C (4)	SN, Ctx, Hi, Cb (in 5 PD) Hi (in 5 AD)	Long-range PCR + FIGE Southern blot	NR		NR	The number of mtDNA DEL/rearrangements in SN of P with PD was significantly higher than that in other groups. In PD, the number of mtDNA DEL/rearrangements did not differ between the other brain regions studied. No significant increase was observed in the total number of DEL/rearrangements in the Hi of AD P	The average number of rearranged forms in patients with PD, AD, MSA, DLB and C was 10.8, 5.7, 4.8, 4.8 and 6.0, respectively
Zhang et al. 2002 (12039426)	26/21	NA	NA	PD (7) MSP-A (4) PSP (4) DLB (4) AD (7) C (21)	SN, other midbrain regions	ISH	NR		NR	Common DEL accumulated in neurons but not glia in both the SN and other midbrain regions with no differences between P and age-matched controls	-
Simon et al. 2001 (11352572)	38/44	NA	NA	AD (8) PD (27) MSA (4) C (44)	Cbx, FCbx, OCbx, TCbx, PCbx	PCR-RFLP, sequencing	No presence of the m.4141>G variant. mtDNA variants reported in AD: m.267T>C, m.347G>A, m.389G>A, m.409T>C,		NR	NR	No further investigation in the pathogenicity of these mutations was made

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration	mtDNA CN	Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant			
							m.4161>C, m.4336C>T, m.4456C>T, m.511C>T, m.523delAC, m.555A>G, m.566C>T and m.6448>G			
de la Monte et al. 2000 (10950123)	37/25	76/78	NA	AD (37) C (25)	TCtx	PCR with radiolabeled nucleotides	NR	mtDNA CN levels were significantly lower in AD P than in C although several AD cases showed high levels of mtDNA CN	NR	-
Chang et al. 2000 (10872587)	20/20	82/71	NA	AD (20) C (20)	PCtx, Hi, Cb	PCR with radiolabeled nucleotides, PCR-RFLP	Point mutation frequencies were 2 to 3-fold higher in PCtx, Hi and Cb of AD P than in C. Point mutation frequencies did not differ between brain regions	NR	Common DEL levels of AD did not differ from the C. Common DEL frequency was 15 to 25-fold lower in Cb than Ctx in both AD and C	In blood, point mutation frequencies were not elevated in AD. No correlation was found between age and common DEL frequency in AD and C
Dhaliwal et al. 2000 (10943712)	6/4	72/83	NA	ALS (6) C (4)	FCtx, TCx	PCR	NR	NR	Common DEL levels were higher in FCtx than in TCtx in both ALS and C. The relative difference in the two brain regions was >>11-fold higher in ALS than in C	In ALS, there was no correlation between duration of disease and common DEL levels in the two brain regions
Ito et al. 1999 (10051601)	1/NA	81/NA	1M/NA	AD (1)	SN, GP	PCR	NR	NR	The common DEL was detected in SN and GP	Percentage load of the m.3243A>G *MT-TL1* was 0.44% and 0.05% in cybrids obtained from SN and GP, respectively. m.3243A>G, *MT-TL1* and the common DEL was not detected in blood
Hatanpaa et al. 1998 (9729244)	5/4	83/72	2M/2M	AD (5) C (4)	Motor Ctx, midtemporal Ctx	Northern blot	NR	NR	NR	COXII mRNA expression was lower in midtemporal Ctx of AD P than in C
Hafeiankarp et al. 1998 (9561330)	2/0	54/0	0/2	Disseminated neocortical and subcortical encephalopathy	Brain stem	PCR	Both patients revealed the same homoplasmic variants at positions m.13708G>A and m.15257G>A. Patient 2 also carried an homoplasmic variant at position m.15812G>A	NR	NR	-
Ozawa et al. 1997 (9196054)	1/1	65/65	1F/1F	PD (1) ALS (1)	Str	PCR	NR	NR	Presence of 134 DEL in the P with PD and 98 DEL in the P with ALS	The ALS patient was considered a C individual in this study
Hamblet & Castora. 1997 (9357554)	9/9	68/66	NA	AD (9) C (9)	TCtx	PCR Southern blot	NR	NR	Mean % of the Common DEL in AD P and C was 0.059 and 0.069, respectively, a 6.5-fold significant change	-

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics			Disease (N)	Brain region	Technique	mtDNA alteration		Rearrangements	Additional findings
	N P/C	Age (y) in P/C	Sex (M/F)				Variant	mtDNA CN		
Kösel et al. 1997 (9380043)	4/4	74/73	7/1	PD (4) C (4)	SN, CCx, CbCtx, Pu, Cb	PCR, PCR-RFLP	m.4336A>G homoplasmic in 1 PD. m.5466G>A heteroplasmic (95% mutation load) in 1 C	NR	DEL levels in CbCtx were 50 to 200-fold lower compared to SN, and 12 to 23-fold greater in Pu than in Cb	-
Hutchin et al. 1997 (9425253)	65/76	76/71	NA	AD (65) C (76)	NA	PCR-RFLP	Frequencies of the analysed variants: m.3196G>A, m.3397A>G and m.8021A>G were not detected in P or in C. m.4336A>G was only present in 1.7% of the C. m.5466G>A was present in 3.1% of P and 2.6% of C. m.5705T>C was present in 1.5% of AD P but not in C	NR	NR	-
Janetzky et al. 1996 (8738945)	48/19	75/71	NA	AD (48) C (19)	FCtx, Ent, Hi	Nested, allele-specific PCR; sequencing	m.5466G>A, p.Ala331Thr, *MT-ND2* was present in 6 AD cases (4 homo- and 2 heteroplasmic, with a mutation load of 5%). Not present in C	NR	NR	-
Schnopp et al. 1996 (8937782)	2/2	NA	NA	PD (2) C (2)	FCtx, PCtx, TCtx, OcCtx, Hi, Pu, Th, Cd, CbCtx, CC, SN, among others	PCR-RFLP	Ratios of the m.5466G>A, varied between 44% and 98% in the brain regions studied. No differences were observed when comparing MM and SM, or between PD P and C	NR	NR	-
Kösel et al. 1996 (8723226)	21/77	74/72	45/53	PD (21) C (77)	SN, CCx, Cb, Pu	PCR-RFLP	m.5466G>A heteroplasmic variant was found in 4/21 PD P and in 5/77 C. m.4336A>G homoplasmic variant was present in 1 PD P	NR	NR	-
Chen et al. 1995 (7599213)	3/3	27-42/ 27-42	3/3	HD (3) C (3)	Pu, OCtx, Cd	Competitive PCR	NR	NR	Similar levels of the common DEL in the three regions when comparing HD P and C. Lower levels of the common DEL in Pu and OCtx	-
Cavelier et al. 1995 (8530074)	33/9	80/72	20/22	AD (33) C (9)	FG, Cd, FCtx, OCtx, PCtx	Competitive PCR	NR	NR	Similar % of the common DEL in AD P (0.01—7.9) and C (0.003—2.0). Levels of the common DEL	No correlation between COX activity and the common DEL levels

Table 2 (Continued)

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex (M/F)	Disease (N)	Brain region	Technique	mtDNA alteration Variant	mtDNA CN	Rearrangements	Additional findings
									were higher in Cd than in FG	
Mecocci et al. 1994 (7979220)	13/12	71/75	1S/10	AD (13) C (12)	FCtx, TCtx, PCtx, Cb	PCR	NR	NR	NR	The amount of oxidised mtDNA showed a threefold increase in PCtx of AD P vs. C
Reichmann et al. 1993 (8393064)	7/7	77/77	8/6	AD (7) C (7)	PCtx, Ent	PCR	NR	NR	DELs larger than 500 bp were discarded	-
DiDonato et al. 1993 (8232940)	1/1	72/62	1M/1M	PD (1) C (1)	SN, Hi, OCtx, Th, FCtx, Pu, GP, CbCtx, R, LC	qPCR	NR	NR	The SN showed the highest proportion of the common DEL (3.16%), while CbCtx showed the lowest (0.02%)	-
Blanchard et al. 1993 (8347829)	6/6	80/64	7/5	AD (6) C (6)	FCtx	PCR	NR	NR	Similar mtDNA DEL levels were observed in AD (0.14%) and C (0.12%)	-
Lestienne et al. 1991 (2013767)	1/1	NA	NA	PD (1) C (1)	Pu, SN, Ctx	PCR	NR	NR	The common DEL was present in PD P and in C	-
Lestienne et al. 1990 (2120388)	1S/S	60–85/NA	NA	PD (15) C (S)	Pu, SN, FCtx	Southern blot	NR	NR	No DELs were identified	-
Schapira et al. 1990 (1979656)	6/6	NA	NA	PD (6) C (6)	SN	RFLP hybridization using a radiolabeled probe	NR	NR	No DELs were identified	-
Kebe et al. 1990 (2390073)	5/6	68/55	6/5	PD (5) C (6)	FCtx, Str	PCR	NR	NR	Proportion of deleted mtDNA to normal mtDNA was lower in FCtx than in the Str of both PD and C	-

***Table 2*: Studies reporting results of the mtDNA analyses in postmortem brain samples of patients with neurological diseases (NeuD).**
C: control; **DEL**: deletion; **F**: female; **GM**: gray matter; **M**: male; **mtDNA CN**: mitochondrial DNA copy number; **N**: number of subjects; **NA**: information not available; **NR**: not reported; **P**: patient; **TH**: tyrosine hydroxylase; **y**: years; **WM**: white matter.
AD: Alzheimer's disease; **ALS**: amyotrophic lateral sclerosis; **CJD**: Creutzfeldt-Jakob disease; **DLB**: dementia with Lewy bodies; **DS**: Down's syndrome; **DSAD**: Down's syndrome and dementia; **FTD**: frontotemporal dementia; **HD**: Huntington disease; **HpC**: high-pathology control subjects, individuals who meet criteria for high AD neuropathologic changes but remain cognitively normal; **IOSCA**: infantile onset spinocerebelar ataxia; **IPD**: idiopathic Parkinson's disease; **LB**: Lewy bodies; **MCI**: mild-cognitively impaired; **MD**: mitochondrial disease; **MND**: motor neuron disease; **MS**: multiple sclerosis; **MSA**: multiple system atrophy; MSA-P: MSA-parkinsonian type; **MTLE-HS**: mesial temporal lobe epilepsy-hippocampal sclerosis; **NCI**: noncognitively impaired controls; **NFT**: neurofibrillary tangles; PD: Parkinson's disease; **PDD**: PD with dementia; **sPD**: sporadic PD; **PEO**: progressive external ophthalmoplegia; PSP: progressive supranuclear palsy.
Cb: cerebellum; **CbCtx**: cerebellar cortex; **CC**: corpus callosum; **Cd**: caudate nucleus; **Ctx**: cortex; **Ent**: entorhinal cortex; **FCtx**: frontal cortex; **FG**: frontal gyrus; **GP**: globus pallidus; **Hi**: hippocampus; **IO**: inferior olive; **LC**: locus coeruleus; **PCtx**: parietal cortex; **PFCtx**: prefrontal cortex; **PPN**: pedunculopontine nucleus; **SN**: substantia nigra; **SNC**: substantia nigra pars compacta; **OCtx**: occipital cortex; **Pu**: putamen; **R**: red nucleus; **Str**: striatum; TCtx: temporal cortex; Th: thalamus; Ver: vermis of cerebellum.
ddPCR: digital-droppled PCR; **ELISA**: enzyme-linked immunosorbent assay; **FIGE**: field inversion gel electrophoresis; **GC/MS-SIMA**: gas chromatography/mass spectrometry with selective ion monitoring analysis; **NGS**: next-generation sequencing; **PQ**: pyrosequencing; **qPCR**: quantitative real-time polymerase chain reaction; **RMC**: random mutation capture; **RFLP**: restriction fragment length polymorphism; **RT-qPCR**: reverse transcription qPCR.
COX: cytochrome c oxidase; **D-loop**: displacement loop; *MT-*: mitochondrially encoded gene; *ND1*: NADH-ubiquinone oxidoreductase subunit 1; *TL1*: tRNA-Leu 1.

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Disease (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Fries et al. 2019 (31746071)	32/32	45/47	P: 15/17 C: 19/13	BD (32)	HI	qPCR	NR	Less mtDNA CN in BD P vs. C Significant correlations between telomere length and mtDNA CN	NR	mtDNA CN was not correlated with chronological age
Hjelm et al. 2019 (30869147)	39/2	46	33/8	SZ (12) BD (10) MDD (9) ADO (8) C (2)	ACCx, DLPFCx, HI, Pu, Cd	Long-range PCR and NGS, exome sequencing, splice-break pipeline, qPCR, Sanger sequencing	NR	NR	The study identified 4489 DELs: 513 with size range 7–8 kb and 127 with size of approximately 50 bp, 346 unique; 12 out of the 30 most frequent DELs were previously described. 13 high-impact DELs (read rate ≥ 5%) were present in 14 brain tissue samples of 9 subjects with psychiatric diagnoses The group of subjects with SZ or BD had a higher DLPFCx/ACCx DEL ratio than the group of C, MDD or ADO subjects. The highest DEL burdens occurred in two subjects with MDD. The common DEL was neither the most frequent nor the most abundant	Brain samples contained significantly more DELs and higher cumulative read percentages than blood samples. The 13 high-impact DELs detected in the brain were not detected in the blood. Many individual DELs had significant positive correlations with age. The highest DEL burdens were observed in MDD P, at higher levels than INS muscle
Bodenstein et al. 2019 (31797868)	66/37	HI C. 60 BD. 59 SZ. 62 BA24 and Cb C. 71 BD. 72 SZ.69 PFCx C. 72 BD. 65 SZ. 71	NA	SZ (35) BD (31) C (37)	HI, BA24, Cb, PFCx	qPCR	NR	BD group had significantly higher mtDNA content for *MT-ND4* and *MT-ND5* in HI tissue compared to C. Cb of patients with BD or SZ also had higher mtDNA CN than BA24 region of the same patients	No significant alteration in the accumulation of the common mtDNA DEL across the brain regions and groups	BA24 and Cb tissues came from the same patients
Otsuka et al. 2017 (28600518)	20/25	SV: 52 C. 58	SV:11/9 C. 19/6	SV (20) C (25)	DLPFCx	qPCR	NR	Significantly lower mtDNA CN in the DLPFC of SV compared to C ($p = 0.0044$)	NR	In the DLPFCx of SV, significantly shorter telomere length was reported ($p = 0.0014$)
Rollins et al. 2018 (29594135)	53/41	BD. 46 SZ. 41 C. 58	BD. 13/13 SZ. 24/3 C. 35/6	BD (26) SZ (27) C (41)	PFCx	qPCR with SYBR green and TaqMan probes	NR	BD and SZ groups displayed a significant increase in mtDNA CN	No significant decrease in mtDNA common DEL in PFCx of patients with SZ.	Complex I ELISA data indicated that the brains of SZ and BD had fewer functional

Table 3 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Disease (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
								(p=0.028 and p=0.025, respectively)	Female subjects displayed 60% increase in the accumulation of the common DEL	mitochondria with larger amounts of mtDNA compared to controls
Mamdani et al. 2014 (25270547)	30/10	MDD: 48 BD: 52 SZ: 47 C: 46	MDD: 3/7 BD: 5/5 SZ: 5/5 C: 7/3	MDD (10) BD (10) SZ (10) C (10)	ACCtx, Amg, Cd, DLPFCtx, Hi, Ac, OFCtx, Pu, SN, and Th	qPCR, Sanger sequencing	NR	NR	Common DELs in SZ were significantly decreased, mostly in dopaminergic regions, compared to MDD, BD and C.	Possible impacts of antipsychotic and antidepressant medications were not quantified.
Torrell et al. 2013 (23355257)	45/15	SZ: 45 BD: 43 MDD: 47 C: 48	SZ: 9/6 BD: 9/6 MDD: 9/6 C: 9/6	SZ (15) BD (15) MDD (15) C (15)	OCtx	qPCR	NR	No differences in mtDNA CN were observed between study groups	NR	*MT-MD1* gene expression was increased in BD P vs. C
Gu et al. 2013 (24002085)	14/12	ASD: 11 C: 11	ASD: 11/3 C: 9/3	ASD (14) C (12)	FCtx	RT-PCR	NR	A significant increase of mtDNA CN in ASD subjects compared with C.	*MT-ND4* deletion was found in 44% of the ASD group, and 33% of them also had *Cyt B* deletion.	
Tang et al. 2013 (23333625)	20/25	ASD: 2–67 C: 2–60	ASD: 18/3 C: 22/2	ASD (20) C (25)	TL	MitoChip assay, qPCR	m.7064T>C, Phe387, *MT-CO1*	No changes in mtDNA CN (studied in 8 ASD P and 8 C)	No presence of large-scale DELs or duplications.	
Sequeira et al. 2012 (22723804)	3 cohorts Co 1: 4/7 Co 2: 29/6 Co 3: 6/17	Co 1: SZ: 53 C: 57 Co 2: SZ: 44 BD: 50 MDD: 51 C: 53 Co 3: SZ: 41 BD: 57 MDD: 41 C: 52	Co 1: SZ: 2/3 C: 2/4 Co 2: SZ: 11/3 BD: 9/3 MDD: 11/4 C: 60/16 Co 3: SZ: 2/2 BD: 3/1 MDD: 4/1 C: 16/7	Co 1: SZ (5) C (6) Co 2: SZ (14) BD (11) MDD (15) C (76) Co 3: SZ (4) BD (4) MDD (5) C (10)	Co 1: ACCtx, Amg, Cd, DLPFCtx, Ac, OFCtx, Pu, SN, Th Co 2: DLPFCtx Co 3: DLPFCtx	NGS; Affymetrix 6.0 SNP chip; qPCR	149 homoplasmic novel or rare variants: 7 not previously reported (5 synonymous variants, 1 in the D-loop, 1 in a tRNA); 88% transitions and 11% transversions; Higher number of transitions and transversions in MDD vs. C and vs. SZ or BD. The m.1951C>T, *MT-RNR2* and m.16519T>C, D-Loop were underrepresented in pooled SZ and BD vs. C.	NR	In the DLPFCtx, the common DEL levels were significantly increased in P with BD, marginally increased in P with MDD and not increased in P with SZ vs. C. Higher levels of the common DEL in SN and Cd. The common DEL levels were correlated with age	Certain brain regions accumulated somatic mutations at higher levels than the blood.
Ichikawa et al. 2012 (22030097)	28	63.1	20/8	SZ	FCtx	Sanger sequencing, allele-specific PCR	Homoplasmic substitutions m.8881T>C and m.9699 A>G were detected exclusively in SZ patients. Unregistered m.6617 C>T and m.9590 C>T substitutions with 50% heteroplasmy were found in a brain sample from a single patient. m.9956 A>G was also found as a homoplasmic variant in a brain sample of an SZ patient, while it	NR	NR	m.7196C>A was detected in SZ patients' brain tissue in both a homoplasmic and heteroplasmic state; it was also detected in blood samples of SZ patients and in blood samples of controls as a homoplasmic variant.

Table 3 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Disease (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
							was heteroplasmic in controls.			
Rollins et al. 2009 (19290959)	41/36	BD: 50 SZ:45 MDD: 51 C: 53	SZ: 11/3 BD: 9/3 MDD: 11/4 C: 31/5	SZ (14) BD (12) MDD (15) C (36)	DLPFCtx	Affymetrix mtDNA resequencing array; SNaPshot and allele-specific RT- PCR	The rate of synonymous base pair substitutions in the coding regions of the mtDNA was 22% higher in P with SZ vs. C. One MDD P carried a homoplasmic mutation in DLPFC at m.10652T>C (Ile61 *MT-ND4L* and two P with SZ showed less than 1% heteroplasmy. Low levels of heteroplasmic nonsynonymous mutations were found in the brain. Several mtDNA variants were significantly associated with specific psychiatric disorders: m.1141>C, m.195C>T, m.10652T>C and m.16309G>A with BD; m.750A>G, 14338A>G and 4769A>G with SZ, and 10652C>T, 14668T>C and 15043A>G with MDD.	NR	NR	Brain pH was significantly associated with super haplogroup U, K and UK
Fuke et al. 2008 (18514404)	99/48	NA	NA	SZ (50) BD (49) C (48)	FCtx	RT-qPCR with SYBR Green	NR	NR	Age- and sex-dependent accumulation of the common DEL, independent of the diagnosis. One P with SZ showed high levels of the common DEL	
Sahuncioyan et al. 2007 (17195919)	100/44	≤ 68	NA	SZ (45) BD (40) MDD (15) C (44)	PFCtx	qPCR, RT-PCR with TaqMan and SYBR Green	NR	No significant difference in mtDNA CN level between C and P	No significant difference in the amount of the common DEL between C and P with SZ or BD	Female BD patients had significantly less common DEL ($p=0.03$) compared with male patients.
Vawter et al. 2006 (16636682)	20/20	BD: 54 MDD: 51 C: 53	P: 14/6 C: 14/6	BD (9) MDD (11) C (20)	ACCtx, DLPFCtx, Cb	qPCR	NR	NR	NR	mtDNA gene expression increased with agonal duration
Munakata et al. 2005 (15737668)	43/14	SZ: 44 BD: 42 MDD: 47 C:49	SZ: 7/6 BD: 9/6 MDD: 9/6 C: 9/5	SZ (13) BD (15) MDD (15) C (14)	PFCtx	PNA-clamped PCR-RFLP	m.3243A>G, *MT-TL1*, detected in two BD (mutation load range: 0.90–1.00%) and one SZ (0.60%).	NR	NR	*LARS2* was upregulated in cybrids carrying the m.3243A>G, *MT-TL1*

Table 3 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Disease (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Marchbanks et al. 2003 (14623372)	15/9	70/69	SZ: 10/5 C: 5/4	SZ (15)	NA	PCR-RFLP	m.12027T>C, Ile4237Thr, *MT-ND4* mutation load of 54% in SZ P vs 58% in C	NR	NR	NR
Kato et al. 1997 (9359971)	16/9	BD: 45 SV: 39 C: 40	BD: 3/4 SV: 4/5 C: 4/5	BD (7) SV (9) C (9)	CbCtx	qPCR	NR	NR	The ratio of the common DEL was significantly higher in BD (0.22) compared with that in age-matched C (0.06, $p < 0.05$).	No significant difference in the common DEL ratio between SVs and controls. Antidepressant was found in the blood of 5 SVs.
Caveller et al. 1995 (8530074)	13/9	80/73	SZ: 7/6 C: 5/4	SZ (13)	FG, Cd	Competitive PCR	NR	NR	No accumulation of the common DEL with age in Cd and a decrease in FG. Lack of age-related accumulation of the DEL. Higher DEL levels in Cd compared to FG.	No correlation between COX activity and levels of common DEL

***Table 3*: Studies reporting the results of mtDNA analyses in postmortem brain samples of patients with psychiatric diseases (PsyD).**

C: control; **Co**: cohort; **DEL**: deletion; **F**: female; **M**: male; **mtDNA CN**: mitochondrial DNA copy number; **N**: number of subjects; **NA**: information not available; **NR**: not reported; **P**: patient; **y**: years.
ADO: alcohol/drug abuse/other psychiatric symptoms; **ASD**: autism spectrum disorder; **BD**: bipolar disorder; **MDD**: major depressive disorder; **SV**: suicide victims; **SZ**: schizophrenia; **KSS**: Kerns-Sayre syndrome.
Ac: nucleus accumbens; **ACCtx**: anterior cingulate cortex; **Amg**: amigdala; **BA24**: Brodmann area 24; **Cb**: cerebellum; **CbCtx**: cerebellar cortex; **Cd**: caudate nucleus; **DLPFCtx**: dorsolateral prefrontal cortex; **FCtx**: frontal cortex; **FG**: frontal gyrus; **Hi**: hippocampus; **OCtx**: occipital cortex; **OFCtx**: orbitofrontal cortex; **PFCtx**: prefrontal cortex; **Pu**: putamen; **SN**: substantia nigra; **Th**: thalamus; **TL**: temporal lobe.
NGS: next-generation sequencing; **PNA**: peptide nucleic acid; **qPCR**: quantitative real-time polymerase chain reaction; **RFLP**: restriction fragment length polymorphism; **RT-qPCR**: reverse transcription qPCR.
COx: cytochrome c oxidase 1; **COX**: cytochrome c oxidase; ***D-loop***: displacement loop; ***HVs***: hypervariable segment 2; ***LARS2***: Leucyl-tRNA synthetase 2; ***MT***: mitochondrially encoded gene; ***ND1***: NADH-ubiquinone oxidoreductase subunit 1; ***ND5***: NADH-ubiquinone oxidoreductase subunit 4; ***ND4L***: NADH-ubiquinone oxidoreductase subunit 4L; ***TL1***: tRNA-Leu 1.

Study reference (PMID)	Patient/control characteristics N P/C	Age (y) in P/C	Sex (M/F)	Disease or condition (N)	Brain region	Technique	mtDNA alteration Variant	mtDNA CN	Rearrangements	Additional findings
Wnek et al. 2016 (27457581)	3/5	64/76	5/3	HSE (3) C (5)	FCtx, Amg, Hi, CiG and ICtx	Microarray, qPCR	NR	*MT-CO1* exhibited lower abundance in CiG, Amg and FCtx in P than C	NR	Greater decline in P than C in mtDNA-encoded compared to nDNA-encoded transcripts.
Var et al. 2016 (26807965)	27/30	48/51	57/0	HIV+METH- (16) HIV+METH- (11) C (30)	Cbc Brodmann areas 7, 8, 9, 46	ddPCR	NR	HIV+METH- group had higher mtDNA CN compared with HIV +METH- and HIV-METH- (WM from area 8)	Higher abundance of the common DEL was associated with increasing age.	A higher proportion of the common DEL was associated with lower neurocognitive function in HIV+/METH but higher in HIV+/METH-
Nave et al. 2014 (25526677)	0/98	52	67/31	C (100)	NA	qPCR, sequencing, minisequencing, NGS	Heteroplasmies were observed in 37% of the individuals (47 observations). 13 of the 98 samples showed 1 bp deletion between positions 66 and 71	NR	NR	The highest relative numbers of heteroplasmies was detected in muscle and liver (79%, 69%), followed by brain, hair, and heart (36.7%–30.2%). Bone (19.8%), blood (18%), lung (17%), and buccal cells (16.2%) showed a comparatively low number of heteroplasmies
Lynn et al. 2003 (12627331)	1/0	46	1/0	Diabetes and recurrent stroke-like episodes, seizures and cognitive decline	Cb, OCtx	Hot last cycle PCR, radioactive PCR	m.3243A>G mutation load %: OCtx 78, Cb 66	NR	NR	Mutation load %: skeletal muscle 60, liver 60, pancreas 31, kidney 75, myocardium 58, blood 8
Nádasi et al. 2003 (14711030)	15/8	<4m/d/66	13/10	Deceased neonates, newborns and infants (15), adults (8)	FCtx, TCtx, Cb,Cd, Th, Hi	PCR	NR	NR	The common DEL was present in all brain samples from all individuals. The ratio of the common DEL/wild-type mtDNA was lowes in the infant group than in adults	The ratio of the common DEL/wild-type mtDNA was lower in blood than in brain

***Table 4*: Studies reporting the results of the mtDNA analyses in postmortem brain samples of individuals with a diagnosis not included in Tables 1–3.**
C: control; **DEL**: deletion; **F**: female; **M**: male; **mtDNA CN**: mitochondrial DNA copy number; **mth**: month; **N**: number of subjects; **NR**: not reported; **P**: patients; **y**: year; **WM**: white matter.
HSE: Herpes simplex virus type-1 encephalitis; **HIV**: human immunodeficiency virus infection; **METH**: methamphetamine use.
Amg: amigdala; **Cb**: cerebellum; **Cd**: caudate nucleus; **CiG**: cingulate gyrus; **Ctx**: cortex; **FCtx**: frontal cortex; **Hi**: hippocampus; **ICtx**: insular cortex; **OCtx**: occipital cortex; **TCtx**: temporal cortex; **Th**: thalamus.
ddPCR: digital-droplet PCR; **NGS**: next-generation sequencing; **qPCR**: quantitative real-time polymerase chain reaction.
MT-CO1: mitochondrially encoded cytochrome c oxidase I gene.

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Condition (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Roca-Bayerri et al. 2020 (32722761)	52/40	NA	NA	PLWH (52) HIV-negative C (40)	FCtx, FL, GM	qPCR, long-range PCR, NGS	Mutations accumulated in the mtDNA non-coding D-loop were significantly associated with age	The mtDNA CN in FCtx decreased with age	An increase of the mutation load of the common DEL was associated with increasing age	The observed effects of HIV were calculated as equal to approximately 32 years for mtDNA CN, and approximately 12 years for the common DEL in advancing age
Dölle et al. 2016 (27874000)	21	11–87	13/8	C with no neurological disease	SN, FCtx, Cb	dPCR, qPCR, NGS	NR	Total mtDNA CN increased with age (p=0.0004)	Major arc DEL showed a significant positive correlation with age in SN neurons	In FCtx neurons and Purkinje cells, the level of the common DEL was generally low and did not increase with age. Ageing upregulated mtDNA CN in SN neurons, and this correlated with the levels of somatic mtDNA DELs in cells
Taylor et al. 2014 (23911137)	21	15–89	NA	NA	NA	3D, ddPCR, NGS	NR	NR	The deletion load increased with age, while the number and diversity of unique deletions remained constant	The analysis was based on over 8 billion mitochondrial genomes
Kennedy et al. 2013 (24086148)	10	Yi: <1 Ai: 79–90	NA	Yi (5) and Ai (5) individuals without known brain pathology	PFCtx	qPCR, duplex sequencing	A significant (5-fold) increase in mutation rate was reported in Ai. 78.3% of mutations were nonsynonymous and predicted to be more deleterious. Most of them accumulated in the D-loop during ageing.	NR	NR	No significant increase in G>T mutations, considered the hallmark of oxidative damage to DNA with age
Coskun et al. 2018 (29463402)	38/25	DSAD: 40–62 DS: Newborn–45 AD: 55–90 C: Newborn–95	M:F ratio equal in both groups	DSAD (14) DS (11) AD (13) C (25)	FCtx	PNA-clamp PCR, sequencing& qPCR, RT-qPCR	Mutations in the regulatory coding region of mtDNA increased with age in C and were significantly elevated in AD and DSAD relative to age-matched C and DS	mtDNA CN declined in C brains after age 65 in parallel with the increased mtDNA mutation rate	NR	*MT-ND6* (L-strand)/*MT-ND2* (H-strand) mRNA ratio did not change significantly with age in C, while it was significantly lower in both AD and DSAD
Meissner et al. 2008 (18439778)	92	0.2–102	NA	Acute or peracute cause of death with no known brain pathology	SN, Cd	PCR-CE	NR	NR	A positive correlation between the common DEL amount and ageing, and a strong interindividual variability were detected. Abundance of the common DEL varied with tissue type: SN>Cd>Put>FL>Cb	The common DEL was detectable in individuals as young as 10 and 12 years
	15/16		NA		SN, HI	Long-range PCR, qPCR	NR	NR	High levels of mtDNA DELs were observed	The level of mtDNA DELs accumulated in HI

Table 5 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Condition (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Bender et al. 2006 (16604074)	5 Yi 5 Ai	PD: 76 C: 77 Ai:19–51		PD (15) C (8) Ai (8)					(52.3% ± 9.3% in SN of individuals with PD, and 42.3% ± 9.3% in Ai, p = 0.06). The level of mtDNA DELs increased linearly with age. These DELs were characterised as the common DEL and the m.7499_13687 DEL	neurons was significantly lower than in SN neurons in both groups
Kraytsberg et al. 2005 (16604072)	1/8	AD: 80 C: 31–102	NA	AD (1) C (8)	SN	Single molecule PCR	NR	NR	The number of mtDNA DELs was significantly different between old and young tissues. The was a very high absolute prevalence of mtDNA DELs in aged SN	mtDNA DELs were directly involved in the development of COX defects in aged SN
Frahm et al. 2005 (16099018)	50	0.2–93	NA	Acute or peracute cause of death with no neurological disease	Cd, FCtx, CbCtx,	qPCR	NR	mtDNA CN in three age groups (0–30, 31–59 and >60 years) revealed no significant age-dependent increase	NR	In skeletal muscle and heart muscle tissue samples, mtDNA CN did not show any significant change
Cantuti-Castelvetri et al 2005 (16243605)	6	40–69	4/2	C without neurological disease	SN	Single-cell, allele-specific PCR, cloning-sequencing	Somatic mtDNA point mutations were distributed throughout tRNAs for Thr and Pro and in portions of the *MT-CYB* and *D-loop* in both glia and neurons. Higher mutation levels were detected in aged neurons	NR	NR	Mean number of somatic point mutations per mitochondrial genome was 3.3 for single neurons and 2.2 for single glia
Mawrin et al. 2004 (15036587)	4/4	FTD-MND: 33 AD: 84 DLB: 74 PD: 54 C: 35–75	FTD-MND: M AD: F DLB: F PD: F C: 2/2	FTD-MND (1) AD (1) DLB (1) PD (1)	FL, TL, OL, Hi, SN, Cb	qPCR	NR	NR	The common DEL ratio increased with age. In the basal ganglia, it reached the highest level	The lowest common DEL levels in individual cases were reported in Cb, with no age-related increase
Simon et al. 2004 (14675733)	16/32	C Group 1: 1–4 C Group 2: 12–24 C Group 3: 65–91 PD: 71–86	NA	C Group 1 (5) C Group 2 (10) C Group 3 (17) PD (16)	FCtx, SN	PCR, sequencing	Accumulation of G>C to T>A and T>A to G>C transversions and all point mutations increased with age in FCtx. No significant differences in somatic mutation level between PD patients and age-matched controls	NR	NR	SN of young individuals had similar accumulation of point mutations as in the SN and FCtx of older individuals
	5 Yi 5 Ai	Yi: 16–25 Ai: 80–91	Yi: 4/1 Ai: 1/4		FL	Single-cell dPCR	NR	NR	Significant differences in the relative	The frequency of the common DEL in Yi

Table 5 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Condition (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Storm et al. 2002 (12594408)				Diverse causes of death with no neuropathology					distribution of the common DEL levels were not identified, neither between astrocytes and neurons, nor between healthy Yi and AI	was 15.4% in astrocytes and 21.6% in neurons. In AI, 21.6% of the astrocytes and 17.8% of the neurons carried the common DEL
Murdock et al. 2000 (11068135)	16	23–93	7/9	NA	Cb,CdEC, FCtx, TCtx, SN, Pu	Competitive PCR, PNA-directed PCR clamping	m.3243A>G, m.8344A>G and m.4147>S mutations did not accumulate with age to levels >1/1000 in brain	NR	NR	The accumulation of m.4147>S mutation was identified in muscle samples of aged individuals
Chang et al. 2000 (10873587)	20/20	82–71	NA	AD (20)	Cb, PG, HI	PCR, RFLP	No significant correlation with age for the frequency of m.16390 G>A in both AD and C groups	NR	No significant increase in the common DEL levels was detected with age	-
Lezza et al. 1999 (10336891)	7/6	AD: 51–79 C: 63–86	AD: 4/3 C: 3/3	AD (7)	FCtx, PCtx	Kinetics PCR	NR	NR	The common DEL levels increased with age in C. The common DEL % in AD was 3-fold lower than in C. DEL levels were much lower in younger AD than in older AD	HPLC-EC revealed a positive correlation between the common DEL and OH^{8}dG levels in aging human brain. Lower levels of common DEL in the presence of a higher content of OH^{8}dG in AD compared with that of C
McDonald et al. 1999 (10501524)	67/43	STS-CI: 50 LTS-HI:56 C:50	NA	STS-CI (53) LTS-HI (14)	TL, HI	PCR, RFLP	NR	NR	The common DEL was found in 54% of C, 57% of STS, and 21% of LTS. Additionally, the common DEL was more prevalent in older individuals among C. A trend toward increased prevalence of the m.8649_16084del (7436-bp DEL) in older patients in all groups was observed	A strong correlation was found in the presence of the common DEL and 7436 bp DEL within individual cases
Melov et al. 1999 (10638530)	5 Y 6 E	Y: 23—44 E: 51—79	NA	No histopathological abnormalities. No history of neurodegenerative disease	Cb, FCtx, Pu, ECtx, SN, Cd, TCtx	Long-range PCR, sequencing	NR	NR	An increase in the number and the variety of mtDNA rearrangements in aged brains was detected. In the FCtx of 79-year-old subjects, a unique m.1999_14366del (12 kb) was reported	-
	4/4	57–87/55–84	3/1 4/0	PD (4)	SN, CDCtx		NR	NR		-

Table 5 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) in P/C	Sex M/F	Condition (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
Kösel et al. 1997 (9380043)						Competitive PCR, PNA-directed PCR clamping			The common DEL levels increased with age in C	
Kapral et al. 1996 (8994125)	2/2	C: 83–75 PD: 74–75	C: 1/1 PD: 2/0	PD (2)	SN	PCR, sequencing	23 missense, 2 tRNA and one nonsense polymorphism were detected. Eight missense polymorphisms caused nonconservative amino acid replacements at evolutionary constrained sites	NR	Several multiple DELs (4.5–7.1 kb) were detected in the SN of both aged P and C groups	-
Merril et al. 1996 (8579367)	41/22	34–73/ (Age-comparable individuals)	NR	SZ (13) Neuroleptic Suicides (14) ADO (5) C (9)	FG, Pu	PCR	NR	NR	mtDNA DELs were associated with chronic hypoxia conditions rather than ageing	Neuroleptic drugs had little or no effect on the observed levels of deleted mtDNA
Jazin et al. 1996 (8901590)	3/4	SZ: 99 EAD: 72 LAD: 95 C: 60	NA	SZ (1) EAD (2) LAD (1) C (4)	FG, Cd	PCR, sequencing	The overall heteroplasmy level in D-loop was 2.2-fold higher in two aged individuals (96 and 99) compared with a 28-year-old individual	NR	A 7.7-fold increase of small insertions and deletions was detected in aged individuals	Substitutions did not show any significant increase with age
Corral-Debrinski et al. 1992 (1303288)	7	24–94	NA	No history of neurodegenerative disease	FCbx, TCbx, OL, Pu, Cb	Dilution-PCR, PCR-RFLP	NR	NR	A significant increase in the common DEL ratio was detected in Cbx and Pu but not in Cb. In Cbx it ranged from 0.00023 to 0.012 in 67–77-year-olds and up to 0.034 in those over 80. In Pu 0.0016 to 0.010 in 66–77-year-olds and up to 0.12 in those over 80. Age-related accumulation of the 7436 bp DEL was also detected with similar changes	Some adult subjects presented neurofibrillary tangles and amyloid plaques consistent with their age
Mann et al. 1992 (1544498)	6/6	NA	NA	PD (6)	SN	Southern blot	NR	NR	No significant difference in % of the common DEL among patients and age-matched controls (0.01–0.02%)	No correlation between complex I activity and the common DEL levels
Soong et al. 1992 (1303287)	7	0.3–82	4/3	No neuropathology.	Cd, Pu, SN, GP, Th, Cbx GM and WM, Cb GM	PCR, qPCR with radiolabelled primers	NR	NR	In neonatal brain regions the common DEL level was 0.0004%. An age-related significant increase in DEL levels among adults was reported with	Adult Cd, Pu and SN had an average of 355, 204 and 121 times higher DEL level, respectively. GP, Th, neocortical GM and WM showed ratios

Table 5 (Continued)

Study reference (PMID)	Patient/Control characteristics N P/C	Age (y) In P/C	Sex M/F	Condition (N)	Brain region	Technique	mtDNA alteration in brain Variant	mtDNA CN	Rearrangements	Additional information
									0·94 correlation efficiency in SN	10–83 times higher than in Cb GM
Zhang et al. 1992 (1551433)	1	69	F	Patients with primary carcinoma of splenic flexure of bowel	NA	PCR, sequencing	NR	NR	The common DEL and the 7436 bp DEL were detected in brain tissue	Heart and skeletal muscle samples were examined, and multiple DELs were also detected in these tissues, suggesting that accumulation of multiple DELs is a general phenomenon during normal ageing
Lestienne et al. 1991 (2013767)	1/1	60–85	NA	PD (1) C (1)	SN	PCR shift assay	NR	NR	Low levels of the common DEL were detected in SN of the aged control without neurological or psychiatric antecedents and the PD P	
Cortopassi et al. 1990 (2263455)	22	Adults: 27–104, Stillborn: 32, 40 weeks Spontaneous abortions: 22, 29 weeks Newborn: 4d	10/3 NA (9)	No neuropathology	Cb	PCR, RFLP, nested PCR, dilution PCR	NR	NR	The common DEL was detected in the brain of older individuals while it was not observed in foetal brains. DELs in foetal tissues were estimated to be 1/100 to 1/1 00,000 times less than in adults	The heart tissue of 7 adults and 5 foetuses were also examined, and the common DEL was only found in aged adults
Ikebe et al. 1990 (2390073)	5/6	51–77/38–73	2/3 4/2	PD (5)	Str, FCtx	PCR, sequencing	NR	NR	Accumulation of the common DEL was reported in both PD and aged C. The DEL load % was higher in Str than in FCtx in both PD and aged C	The common DEL seemed to selectively accumulate in the nigrostriatal pathway

Table 5: **Studies reporting the results of mtDNA analyses in postmortem brain samples in ageing.**

AI: aged individuals; **C:** control; **DEL:** deletion; **E:** elderly; **F:** female; **M:** male; **mtDNA CN:** mitochondrial DNA copy number; **N:** number of subjects; **NA:** information not available; **NR:** not reported; **P:** patient; **y:** years; **YI:** young individuals.

ADO: alcohol/drug abuse/other psychiatric symptoms; **AD:** Alzheimer's disease; **DS:** Down's syndrome; **DSAD:** Down's syndrome and dementia; **DLB:** dementia with Lewy bodies; **EAD:** early-onset Alzheimer's disease; **FTD-MND:** frontotemporal dementia with motor neuron disease-like inclusions; **LoAD:** late-onset Alzheimer's disease; **LTS-HI:** long-term survivor of head injury; **PD:** Parkinson's disease; **PLWH:** people living with HIV; **STS-CI:** short-term survivor of cerebral ischaemia; **SZ:** schizophrenia.

Cb: cerebellum; **CbCtx:** cerebellar cortex; **Cd:** caudate nucleus; **Ctx:** cortex; **ECtx:** entorhinal cortex; **FCtx:** frontal cortex **FG:** frontal gyrus; **FL:** frontal lobe; **GM:** gray matter; **GP:** globus pallidus; **Hi:** hippocampus; **OCtx:** occipital cortex; **OL:** occipital lobe; **PFCtx:** prefrontal cortex; **PG:** parietal gyrus; **Pu:** putamen; **SN:** substantia nigra; **Str:** striatum; **TCtx:** temporal cortex; **Th:** thalamus; **TL:** temporal lobe; **WM:** white matter

3D: digital deletion detection; **dPCR:** duplex PCR; **ddPCR:** digital-droplet PCR; **HPLC-EC:** high-performance liquid chromatography with electrochemical detection; **NGS:** next-generation sequencing; **PNA:** peptide nucleic acid; **qPCR:** quantitative real-time polymerase chain reaction; **RFLP:** restriction fragment length polymorphism; **RT-qPCR:** reverse transcription qPCR.

COX: cytochrome c oxidase; ***CYB:*** cytochrome B; **D-loop:** displacement loop; ***MT-:*** mitochondrially encoded gene; ***ND6:*** NADH-ubiquinone oxidoreductase core subunit 6; ***ND2:*** ADH-ubiquinone oxidoreductase core subunit 2; **OH^8dG:** 8'-hydroxy-2'-deoxyguanosine.

Locus	Nucleotide position	Nucleotide change	Variant type	Pathogenicity status/TOOLS	GB Freq (%)	Reported phenotype (Homo-/heteroplasmy)	Cell or tissue type of reported mtDNA somatic variant (Homo-/heteroplasmy)	Database	Related clinical features in this systematic review
MT-HV2, MT-ATT, MT-CR, MT-7S	68	G>A	Noncoding	NR	0.021	NR	NR (NA)	MITOMAP	*AD*
	70	G>A	Noncoding	NR	0.073	NR	NR (NA)	MITOMAP	*AD*
	72	T>C	Noncoding	NR/NA	1.792	NR	Aging brains, POLG/PEO & control muscle, normal tissues (-/+)	MITOMAP	*AD*
MT-HV2, MT-OHR, MT-ATT, MT-CR, MT-7S	114	C>T	Noncoding	R/NA	0.442	BD-associated (+/-)	POLG/PEO muscle, bladder tumour back-mutation (-/+)	MITOMAP	*BD*
	146	T>C	Noncoding	R/NA	19.510	Absence of endometriosis (+/-)	Elderly fibroblasts, elderly/AD brains, POLG/PEO & control muscle, various tumours (+/+)	MITOMAP	*AD*
	185	G>A	Noncoding	R/NA	3.999	Low VO_2 max response (+/-)	POLG/PEO muscle, thyroid tumour, glioblastoma (+/+)	MITOMAP	*AD*
	189	A>G	Noncoding	NR	5.436	NR	Elderly muscle & brains, myocyte, POLG/PEO muscle & fibroblasts, various tumours (-/+)	MITOMAP	*PD*
MT-HV2, MT-OHR, MT-ATT, MT-CR	195	T>C	Noncoding	R/NA	19.228	BD-associated/melanoma (+/+)	Elderly fibroblasts, elderly/AD brains, tumours: lung, thyroid, ovarian, prostate, glioblastoma (+/+)	MITOMAP	*AD/BD*
	207	G>A	Noncoding	NR	4.645	NR	Oral, prostate & thyroid tumours/OPA1 defect (+/+)	MITOMAP	*AD*
MT-HV2, MT-OHR, MT-CSB1, MT-ATT, MT-CR	224	T>C	Noncoding	NR	0.012	NR	NR	MITOMAP	*BD/MDD*
	228	G>A	Noncoding	R/NA	2.579	Low VO_2 max response (+/-)	NR	MITOMAP	*AD*
MT-HV2, MT-OHR, MT-ATT, MT-CR	267	T>C	Noncoding	NR	0.027	NR	NR	MITOMAP	*AD*
MT-HV2, MT-OHR, MT-CSB2, MT-ATT, MT-CR	309	delC	Noncoding	NR	0.000	NR	buccal cell, colonic crypt (-/+)	MITOMAP	*AD*
		insC	Noncoding	R/NA	1.142	AD-weakly associated (NR)	NR	MITOMAP	*AD*
MT-HV2, MT-OHR, MT-CSB3, MT-ATT, MT-CR	347	G>A	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*
MT-OHR, MT-ATT, MT-CR	380	G>A	Noncoding	NR	0.004	NR	NR	MITOMAP	*AD*
	405	T>C	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*

Table 6 (Continued)

Locus	Nucleo-tide position	Nucleo-tide change	Variant type	Pathogenicity status/TOOLS	GB Freq (%)	Reported phenotype (Homo-/ heteroplasmy)	Cell or tissue type of reported mtDNA somatic variant (Homo-/ heteroplasmy)	Database	Related clinical features in this systematic review
MT-OHR, MT-LSP, MT-ATT, MT-CR	408	T>C	Noncoding	NA	0.004	NR	NR	MITOMAP	*AD*
	414	T>C	Noncoding	NR/NA	0.002	NR	AD brains/POLG OPA1 and control samples (-/+)	MITOMAP	*AD*
	414	T>G	Noncoding	NR/NA	0.029	NR	Elderly fibroblasts, elderly muscle, POLG/PEO, DS, AD brains, oocytes, normal tissues (-/+)	MITOMAP	*AD*
	416	T>C	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*
MT-OHR, MT-LSP, MT-TFL, MT-ATT, MT-CR	418	C>T	Noncoding	NR	0.114	NR	NR	MITOMAP	*AD*
MT-OHR, MT-TFL, MT-ATT, MT-CR	436	C>T	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*
MT-HV3, MT-ATT, MT-CR	456	C>T	Noncoding	NR/NA	2.450	NR	Thyroid tumour (+/-)	MITOMAP	*AD*
	466	2insCC	Noncoding	NR	NA	NR	NR	MITOMAP	*AD*
	477	T>C	Noncoding	NR/NA	0.938	NR	AD brains, ovarian tumour (-/+)	MITOMAP	*AD*
MT-HV3, MT-CR	511	C>T	Noncoding	NR	0.143	NR	NR	MITOMAP	*AD*
MT-HV3, MT-TFH, MT-CR	523	delAC	Noncoding	NR	0.019	NR	NR	MITOMAP	*AD*
MT-HV3, MT-HSP1, MT-CR	555	A>G	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*
	566	C>T	Noncoding	NR	0.000	NR	NR	MITOMAP	*AD*
MT-TF	644	A>G	(tRNA)	NR/Likely benign	0.050	NR	NR	MITOMAP	*AD*
				Benign	NA	Juvenile MELAS	NR	ClinVar	
MT-RNR1	750	A>G	(rRNA)	R/NA	98.277	NR	NR	MITOMAP	*SZ*
				R/NA	NR	Not provided	NR	ClinVar	
	1438	A>G	(rRNA)	NR/NA	94.853	NR	NR	MITOMAP	*SZ*
				Benign	NR	Not provided, not specified	NR	ClinVar	
MT-RNR2	3196	G>A	(rRNA)	R/NA	0.025	ADPD (+/+)	NR	MITOMAP	*AD*
MT-TER, MT-TL1	3243	A>G	(tRNA)	Confirmed pathogenic	0.019	MELAS/Leigh syndrome/DMDF/ MIDD/SNHL/ CPEO/MM/FSGS/ ASD/cardiac multi-organ dysfunction (-/+)	NR	MITOMAP	*MELAS/BS-LD/ALD/PD/ BD/SZ*
				Pathogenic	NR	BD- and SZ-associated	NR	ClinVar	
	3251	A>G	(tRNA)	R/Possibly benign	0.000	MM/MELAS with chorea-ballism (-/+)	NR	MITOMAP	*MM/LAoCID*
				Pathogenic	NR		NR	ClinVar	

Table 6 (Continued)

Locus	Nucleo-tide position	Nucleo-tide change	Variant type	Pathogenicity status/TOOLS	GB Freq (%)	Reported phenotype (Homo-/ heteroplasmy)	Cell or tissue type of reported mtDNA somatic variant (Homo-/ heteroplasmy)	Database	Related clinical features in this systematic review
						Juvenile MELAS/PEO, proximal myopa-thy, and sudden death			
	3257	A>G	(tRNA)	NR	NA	NR	NR	MITOMAP	*MERRF*
MT-ND1	3397	A>G	Met31Val	R/Likely pathogenic	0.305	ADPD/possibly LVNC cardiomyopathy-associated/resis-tance to high alti-tude pulmonary oedema (+/-)	NR	MITOMAP	*AD*
				Benign	NR	PD, LoLS, AD	NR	ClinVar	
MT-TI	4274	T>C	(tRNA)	R/Likely pathogenic	0.000	CPEO/motor neuron disease (-/+)	NR	MITOMAP	*Motor neuron disease*
MT-TQ	4336	A>G	(tRNA)	Unclear/Possibly benign	0.839	ADPD/hearing loss & migraine/ASD/ID (+/+)	NR	MITOMAP	*PD*
				Conflicting reports	NA	Sensorineural deaf-ness and migraine/Juvenile MELAS	NR	ClinVar	
MT-ND2	4769	A>G	Met100	R/NA	97.604	NR	NR	MITOMAP	*SZ*
				R/NA	NR	Not provided	NR	ClinVar	
	5460	G>A	Ala331Thr	Conflicting reports/ Possibly benign	6.904	AD/PD/LHON (+/+)	NR	MITOMAP	*AD/PD*
				Benign	NR	LS	NR	ClinVar	
MT-TW	5537	insT	(tRNA)	R/NA	0.000	LS (-/+)	NR	MITOMAP	*LS*
				Pathogenic	NR	LS/ME	NR	ClinVar	
	5549	G>A	(tRNA)	R/Likely pathogenic	0.000	Dementia and cho-rea (-/+)	NR	MITOMAP	*ME*
				Pathogenic	NR	ME	NR	ClinVar	
	5556	G>C	(tRNA)	R/Possibly benign	0.000	Encephalomyopathy (-/+)	NR	MITOMAP	*LoME*
MT-TN	5705	T>C	(tRNA)	NR	0.017	NR	NR	MITOMAP	*AD*
MT-CO1	6617	C>T	Phe238	NR/NA	0.015	NR	NR	MITOMAP	*SZ*
	7064	T>C	Phe387	NR/NA	0.062	NR	NR	MITOMAP	*ASD*
MT-CO2	7716	G>A	Ala41Thr	R/Possibly benign	0.015	AHS-like (+/+)	NR	MITOMAP	*AHS-like disease*
	7834	C>T	Ile83	NR/NA	0.004	NR	NR	MITOMAP	*SZ*
MT-TK	8344	A>G	(tRNA)	Confirmed pathogenic	0.008	MERRF; Other-LD/ DMD/leukoence-phalopathy/HICM (-/+)	Bone marrow, elderly muscle (-/+)	MITOMAP	*MERRF*
				Pathogenic	NR	LS/MERRF/PD/juve-nile MELAS	NR	ClinVar	
MT-ATP6	8603	T>C	Phe26Ser	NR/Possibly benign	0.336	NR	NR	MITOMAP	*MERRF*

Table 6 *(Continued)*

Locus	Nucleotide position	Nucleotide change	Variant type	Pathogenicity status/TOOLS	GB Freq (%)	Reported phenotype (Homo-/heteroplasmy)	Cell or tissue type of reported mtDNA somatic variant (Homo-/heteroplasmy)	Database	Related clinical features in this systematic review
				Benign	NR	LS	NR	ClinVar	
	8881	T>C	Ser119Pro	R/Possibly benign	0.002	Patient with suspected mitochondrial disease (NR/NR)	NR	MITOMAP	*SZ*
	8993	T>G	Leu156Arg	Confirmed/Likely pathogenic	0.012	NARP/LS/MILS/other (+/+)	NR	MITOMAP	*LS/NARP/MILS*
				Pathogenic	NR	Mitochondrial complex V (ATP synthase) deficiency, mitochondrial type 1/LS/NARP/other	NR	ClinVar	
MT-CO3	9500	C>T	Phe98	NR/NA	0.008	NR	NR	MITOMAP	*SZ*
	9633	T>C	Ser143Pro	R/Possibly benign	0.000	NR	NR	MITOMAP	*PD*
				Likely benign	NR	NR	NR	ClinVar	
	9699	A>G	Ile165Val	NR/Possibly benign	0.008	NR	NR	MITOMAP	*SZ*
				Uncertain significance	NR	LS	NR	ClinVar	
	9861	T>C	Phe219Leu	R/Possibly benign	0.220	AD (+/-)	NR	MITOMAP	*AD*
				Benign/Likely benign	NR	LS	NR	ClinVar	
	9956	A>G	Leu250	NR/NA	0.006	NR	NR	MITOMAP	*SZ*
MT-ND4L	10652	T>C	Ile61	R/NA	0.104	BD/MDD-associated (-/+)	NR	MITOMAP	*BD/MDD*
MT-ND4	10858	T>C	Ile33	NR/NA	0.033	NR	NR	MITOMAP	*BD*
	11778	G>A	Arg340His	Confirmed/Possibly pathogenic	0.357	LHON/progressive dystonia (+/+)	NR	MITOMAP	*LHON*
				Pathogenic	NR	LHON	NR	ClinVar	
	12027	T>C	Ile423Thr	R/Possibly benign	0.004	SZ-associated (NR/NR)	NR	MITOMAP	*SZ*
MT-ND5	13094	T>C	Val253Ala	Confirmed/Likely pathogenic	0.002	Ataxia + PEO/MELAS, LD, LHON, myoclonus, fatigue (+/+)	NR	MITOMAP	*MELAS*
				Pathogenic	NR	Juvenile MELAS	NR	ClinVar	
	13513	G>A	Asp393Asn	Confirmed/Likely pathogenic	0.002	LS/MELAS/LHON-MELAS overlap syndrome/negative association with carotid atherosclerosis (-/+)	NR	MITOMAP	*MELAS/LS*
				Pathogenic	NR	Mitochondrial diseases/LS/LS due to CID/Juvenile MELAS	NR	ClinVar	

Table 6 (Continued)

Locus	Nucleo-tide position	Nucleo-tide change	Variant type	Pathogenicity status/TOOLS	GB Freq (%)	Reported phenotype (Homo-/ heteroplasmy)	Cell or tissue type of reported mtDNA somatic variant (Homo-/ heteroplasmy)	Database	Related clinical features in this systematic review
MT-ND6	14668	C>T	Met2	R/NA	3.951	Depressive disorder-associated (+/-)	NR	MITOMAP	*MDD*
MT-TE	14685	G>A	(tRNA)	R/Likely pathogenic	0.000	Cataracs with spastic paraparesis & ataxia (-/+)	NR	MITOMAP	*ECOAPP*
	14709	T>C	(tRNA)	Confirmed pathogenic	0.000	MM+DMDF/encephalomyopathy/ dementia + diabetes + ophthalmoplegia (+/+)	NR	MITOMAP	*Ataxia*
				Pathogenic/Likely pathogenic	NR	Mitochondrial diseases/MI DMDF/ Juvenile MELAS/ MM	NR	ClinVar	
MT-CYB	15043	G>A	Gly99	R/NA	23.640	MDD-associated/ possible role in high-altitude sickness (+/-)	NR	MITOMAP	*MDD*
				Likely pathogenic	NR	Familial breast cancer	NR	ClinVar	
MT-HV1, MT-ATT, MT-CR, MT-TS	16184 16300	C>T A>G	Noncoding Noncoding	NR/NA R/NA	0.735 0.536	NR BD-associated (+/-)	Colonic mucosa (-/+) Head/neck tumour (+/-)	MITOMAP MITOMAP	*PD* *BD*

***Table 6*: mtDNA disease-related variants with pathogenicity information retrieved from public databases.**
NA: not available; **NR**: not reported; **R**: reported.
AD: Alzheimer's disease; **ADPD**: Alzheimer's disease and Parkinson's; **AHS**: Alpers-Huttenlocher syndrome; **ASD**: autism spectrum disorder; **BD**: bipolar disorder; **BS-LD**: Barth syndrome-like disorder; **CPEO**: chronic progressive external ophthalmoplegia; **CID**: combined immunodeficiency; **DMD**: depressive mood disorder; **DMDF**: diabetes mellitus + deafness; **ECOAPP**: early-onset cataracts, ataxia and progressive paraparesis; **DS**: Down's syndrome; **FSGS**: focal segmental glomerulosclerosis; **HiCM**: histioovtoid cardiomyopathy; **LAaCID**: lactic acidosis and complex I deficiency; **LD**: learning disabilities; **LHON**: Leber's hereditary optic neuropathy; **LoLS**: late-onset Leigh syndrome; **LoME**: late-onset mitochondrial encephalomyopathy; **LVNC**: left ventricular non-compaction; **ME**: mitochondrial encephalopathy; **MELAS**: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; **MERRF**: myoclonic epilepsy with ragged red fibres; **MDD**: major depressive disorder; **MIDD**: maternally inherited diabetes and deafness; **MILS**: maternally inherited Leigh syndrome; **MM**: mitochondrial myopathy; **NARP**: neuropathy, ataxia, and retinitis pigmentosa; **PEO**: progressive external ophthalmoplegia; **PD**: Parkinson's disease; **SNHL**: sensorineural hearing loss; **SZ**: schizophrenia; VO_2 **max**: maximum rate of oxygen consumption.
TOOLS: If available, predictive data of pathogenicity are obtained from the tools MitoTIP, HmtVar and/or APOGEE (https://www.mitomap.org/foswiki/bin/view///Main/SearchAllele); del: deletion; GB Freq: The frequency data derived from 51836 GenBank sequences with sizes greater than 15.4 kbp.
The frequency data and disease-associated phenotypes were retrieved from the MITOMAP and ClinVar databases in June 2021.

Articles

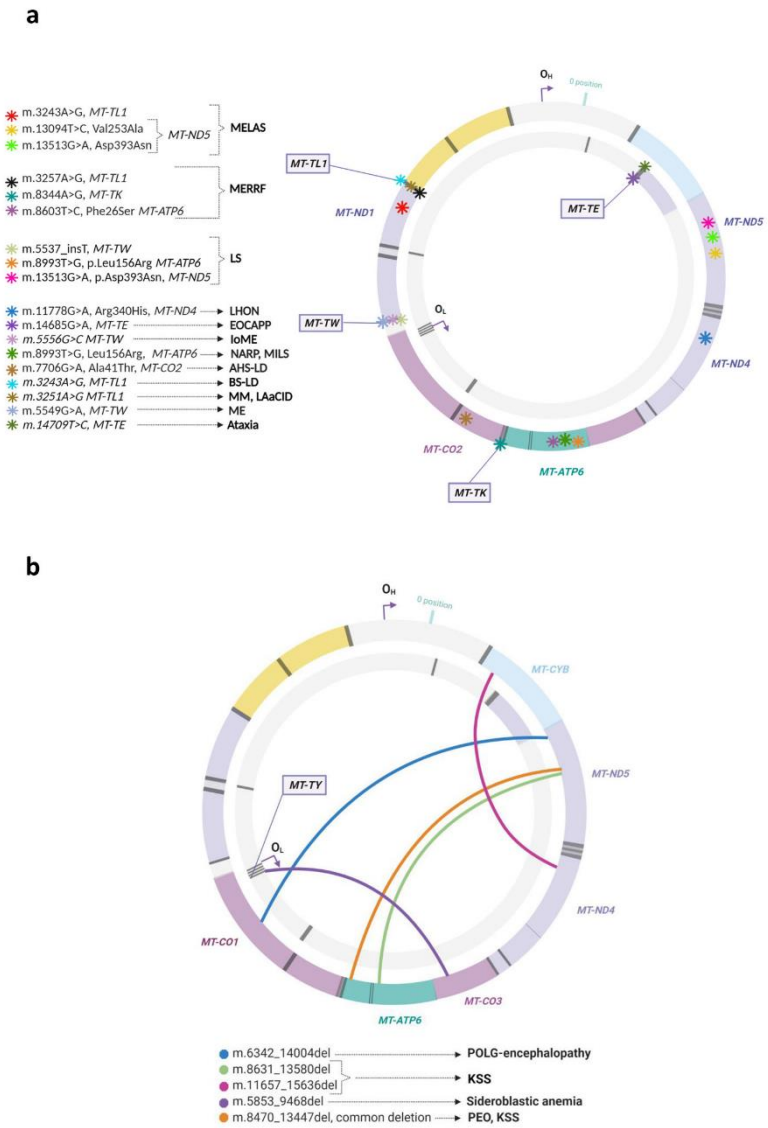

Figure 2. Map of human mtDNA with variants (**a**) and deletions (**b**) identified in postmortem brain samples of patients with MitD. mtDNA replication initiates within the D-loop region and proceeds from the origin of heavy-strand replication (O_H) until the origin of light-strand replication (O_L). The positions of variants are represented by asterisks, while deletions are represented by circles. MitD diagnoses are indicated in boldface. MELAS: mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; ME: mitochondrial encephalomyopathy; AHS: Alpers-Huttenlocher syndrome; MERRF: myoclonic epilepsy with ragged-red fibres; NARP: neuropathy with ataxia and retinitis pigmentosa; MILS: maternally inherited Leigh's syndrome; LHON: Leber hereditary optic neuropathy; EOCAPP: early-onset cataracts, ataxia and progressive paraparesis; *POLG*: DNA polymerase gamma gene; KSS: Kearns-Sayre syndrome. *MT-*: mitochondrially encoded gene; *TL1*: tRNA-Leu 1; *TW*: tRNA-Trp; *CO2*: cytochrome c oxidase II; *TK*: tRNA-Lys; *ATP6*: ATP synthase subunit 6; *ND4*: NADH-ubiquinone oxidoreductase subunit 4; *TE*: tRNA-Glu; *CO1*: cytochrome c oxidase I.

between 89% and 97%, and similar percentages in other tissues were lower than those in blood (72% and 81%).[77,78,80,81] In addition, m.13513G>A, p.Asp393Asn, *MT-ND5* was also reported in LS.[76] Only one study analysed the mtDNA CN, identifying that it was 4.6 times higher in patients with LS than in controls.[82]

mtDNA analysis in NeuD

Table 2 includes 67 reports referring to NeuD, and Figure 2 shows the variants reported in neurodegenerative conditions, with the most reported variants being found in Alzheimer's disease (AD, 33 reports) and Parkinson's disease (PD, 27 reports). Other reported phenotypes were amyotrophic lateral sclerosis, mild cognitive impairment, Creutzfeldt-Jakob disease, dementia with Lewy bodies, frontotemporal dementia, mesial temporal lobe epilepsy-hippocampal sclerosis, Down syndrome, Down syndrome and dementia; multiple sclerosis, infantile onset spinocerebellar ataxia, motor neuron disease, multiple system atrophy, disseminated neocortical and subcortical encephalopathy, and Huntington disease. Taking all NeuD studies into account, the alterations most frequently investigated were mtDNA deletions (40 reports), followed by the presence of mtDNA variants (22 reports) and mtDNA CN (19 reports) (Figure 3).

mtDNA analyses in AD. Among the AD studies reviewed, deletions were the most frequent mtDNA alterations analysed (17/33), followed by mtDNA variants (10/33) and mtDNA CN (9/33). The most frequently assessed brain tissues were the frontal cortex, hippocampus, cerebellum, temporal cortex and parietal cortex.

The results in relation to the variants are diverse; some studies agreed that the frequency of variants is similar between AD patients and controls regarding their levels of heteroplasmy,[83 85] while others reported higher levels of heteroplasmy and a higher frequency of variants in the parietal cortex, hippocampus and cerebellum in AD patients.[86,87] Two studies reported the m.5460G>A variant, which produces the p.Ala331Thr amino acid change in *MT-ND2*; this is described in MitoMap with conflicting reports regarding its pathogenicity for AD, PD and LHON. This amino acid change was reported in AD and control individuals.[88] Additionally, in six patients with AD, four showed homoplasmy and two showed heteroplasmy, with a mutation load percentage of 5%. In this last study, the variant was not present in the control group.[89] Similarly, the m.3243A>G variant involved in MELAS was identified in a patient with AD, although with a very low percentage (<0.05%).[90] More recent studies, some of them conducted with a large number of individuals and using novel techniques, did not identify that mtDNA variation had a role in AD.[83 85]

Overall, most of the studies agree that the mtDNA CN levels are lower in AD patients than in controls,[83,84,86,91 94] although some specific hallmarks should be mentioned: 1) no difference was identified in the mtDNA CN levels between tau-positive and tau-negative neurons;[95] 2) focusing on brain regions, the hippocampus and the temporal cortex showed a significant mtDNA CN reduction in pyramidal neurons compared to other neuronal cells[93] but not in the cerebellum,[83] although a study analysing a large number of samples (282 patients and 461 control subjects) mostly obtained from the cerebellum (87.3%) was able to identify a significant reduction in the mtDNA CN levels in AD;[84] 3) when considering the clinical characteristics of the patients, one study observed that the mtDNA CN was reduced by 48% in nondiabetic patients with AD compared to that in nondiabetic noncognitive-impaired individuals, and this effect occurred in the parietal cortex but not in the frontal cortex or cerebellum; however, compared with nondiabetic patients, diabetic patients showed higher mtDNA CNs in the frontal cortex, parietal cortex and cerebellum;[91] and 4) although a reduced mtDNA CN was reported by most of the studies, some authors highlighted that some patients with AD exhibited a high mtDNA CN.[92]

Regarding mtDNA rearrangements, the first studies carried out in the nineties focused on the analysis of the 4977 bp common deletion (m.8470_13477del). Similar percentages of the deletion have been reported in individuals with AD and in control individuals.[87,96,97] However, the deletion was found to be more abundant in the temporal cortex of individuals with AD than in controls, although in both cases the percentage was low (<0.059%).[98] Regarding the mutation load of the common deletion in the distinct brain regions, there are some aspects to note. First, higher percentages were present in the nucleus caudate than in the gyrus frontalis.[96] Second, in a more recent study, a 1.5% mutation load was reported in the frontal cortex samples of patients with AD and in control individuals and, although the percentage was still low, it was higher than previously reported.[85] Third, the common deletion was also present in the substantia nigra and the globus pallidus of a single individual with AD but was not detected in the blood.[90] Finally, the mutation load was found to be 15 to 25-fold lower in the cerebellum than in cortices of AD and control individuals. More recent studies have investigated the presence of other rearrangements in addition to the common deletion, with controversial results. Some agree that the number of deletions is higher in AD than in controls,[85,99] but they differ depending on the region studied. For instance, one of the studies did not find any significant increase in the total number of deletions in the hippocampus of patients with AD,[100] while others observed higher

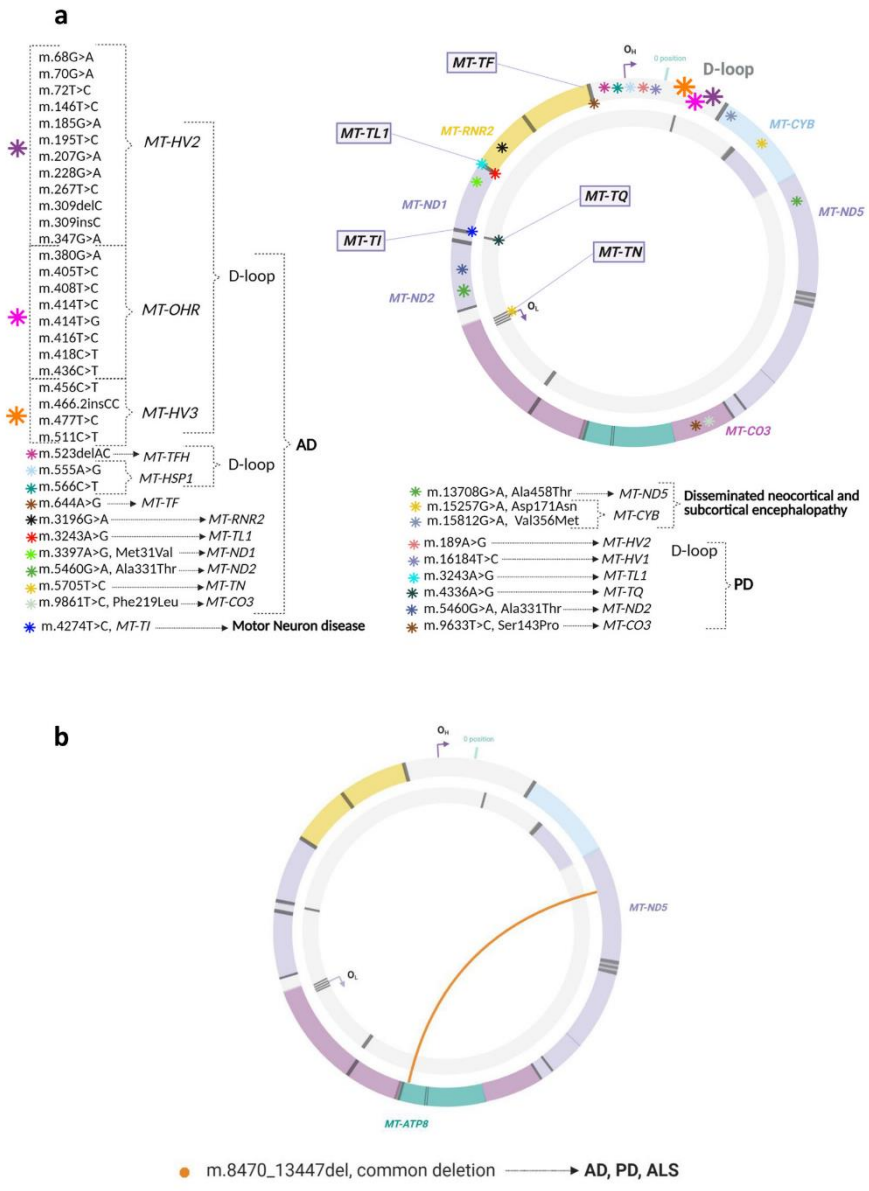

Figure 3. Map of human mtDNA with variants (**a**) and deletions (**b**) identified in postmortem brain samples of patients with NeuD. mtDNA replication initiates within the D-loop region and proceeds from the origin of heavy-strand replication (O_H) until the origin of light-strand replication (O_L). The positions of variants are represented by asterisks, while deletions are represented by circles. NeuD diagnoses are indicated in boldface. AD: Alzheimer's disease; PD: Parkinson's disease. *MT-*: mitochondrially encoded gene; *TL1*: tRNA-Leu 1; *ND2*: NADH-ubiquinone oxidoreductase subunit 2; *ND5*: NADH-ubiquinone oxidoreductase subunit 5; *ATP8*: ATP synthase subunit 8.

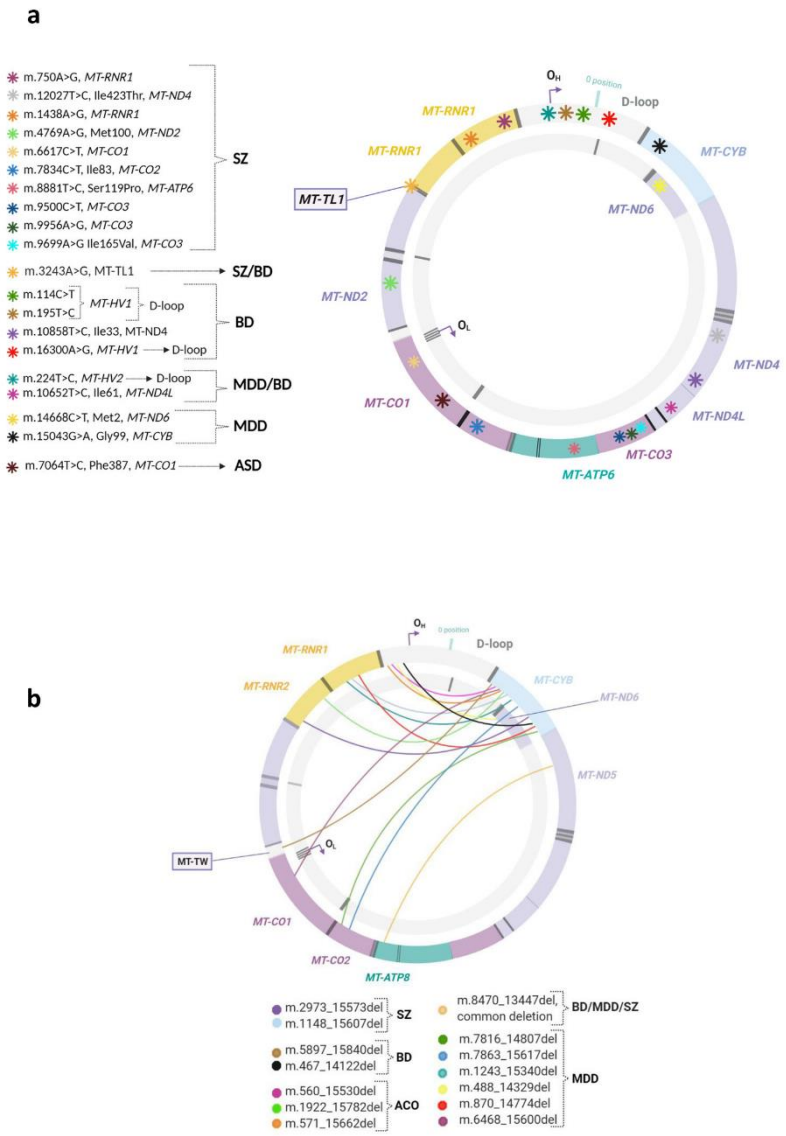

Figure 4. Map of human mtDNA with variants (**a**) and deletions (**b**) identified in postmortem brain samples of patients with PsyD. mtDNA replication initiates within the D-loop region and proceeds from the origin of heavy-strand replication (O_H) until the origin of light-strand replication (O_L). The positions of variants are represented by asterisks, while deletions are represented by circles. PsyD diagnoses are indicated in boldface. SZ: schizophrenia; ASD: autism spectrum disorder; MDD: major depressive disorder; BD: bipolar disorder; ADO: alcohol/drug abuse and other psychiatric symptoms. *MT-*: mitochondrially encoded gene; *RNR1*: 12S rRNA; *RNR2*: 16S rRNA; *ND4L*: NADH-ubiquinone oxidoreductase subunit 4L; *D-loop*: displacement loop; *ND4*: NADH-ubiquinone oxidoreductase subunit 4; *ND6*: NADH-ubiquinone oxidoreductase subunit 6; *CYB*: cytochrome b; *CO1*: cytochrome c oxidase I; *TL1*: tRNA-Leu 1; *ND2*: NADH-ubiquinone oxidoreductase subunit 2; *CO2*: cytochrome c oxidase II; HSP1: major H-strand promoter 1; *ATP8*: ATP synthase subunit 8.

deletion rates in both astrocytes and microglia of the hippocampus compared with the brainstem and cerebellum in control individuals.[101] Additionally, a high number of deletions in cytochrome oxidase (COX)-deficient neurons of the hippocampus[102] and in frontal cortices of individuals with AD[85] have been reported, but there were no differences between tau-positive and tau-negative neurons.[95] Reportedly, the average mtDNA deletion size is 5080±2367 bp,[103] the deletion size ranges from 3670 to 6088 bp, accumulation occurs with age,[102] and the number and characteristics of the rearrangements depend on the sequence coverage depth.[85]

mtDNA analyses in PD. mtDNA deletions were the most investigated mtDNA alterations (19/27), followed by mtDNA variants (10/27) and the CN (10/27). The brain tissue most frequently assessed was the substantia nigra, followed by the frontal cortex, cerebellum, putamen and hippocampus. mtDNA variants and heteroplasmy levels have been widely discussed regarding PD. The first study investigating mtDNA variants in PD reported no differences between variant heteroplasmy levels in PD and control individuals;[104] this was also found by two additional recent studies.[84,105] Another recent study investigating a large number of samples and taking advantage of next-generation sequencing found that the mtDNA mutation load in *MT-CO1*, *MT-CO2* and *MT-CYB* in the substantia nigra pars compacta and in *MT-CYB* in the frontal cortices of patients with PD was increased compared to those in control individuals.[106] A significant increase in the frequency of heteroplasmy levels in neurons with a higher number of deletions has also been reported.[103] Conversely, no association was found between mtDNA variants, either homoplasmic or heteroplasmic, and PD.[84]

Conflicting results have also been reported regarding mtDNA CN in PD, such as 1) decreased levels in the prefrontal cortices of patients with PD and dementia and in neurons of the substantia nigra in patients with idiopathic PD;[107 109] 2) increased levels in cholinergic neurons of the pedunculopontine nucleus in PD patients;[110] and 3) no significant differences between patients and controls.[84,95,104,105,108,111]

In general, patients with PD showed higher mtDNA deletion levels in the substantia nigra than in other brain regions,[100,105,111,112] although some studies found a significant increase in other regions, such as the putamen or hippocampus, but not in the substantia nigra.[113,114] Additionally, although most reports showed a trend or reported increased mtDNA deletion levels in PD, others did not.[109,115] Notably, mtDNA deletion levels have been reported to be significantly lower in the cerebellum than in other brain regions.[112,116] One of the most recent studies found that patients with PD tended to exhibit deletion levels greater than 60% in the substantia nigra, while markedly lower levels were found in the frontal cortex, cerebellum and putamen. The authors also reported that in the substantia nigra, deletion levels were a good predictor of mtDNA CN variation.[105] This is in line with a reported correlation between the percentage of deletions and mtDNA CN in the putamen; the lower the CN, the higher the deletion levels.[113] Some specificities regarding mtDNA deletions in PD should also be mentioned. First, increased mtDNA deletion levels were found to correlate with Braak staging,[108] although these changes were not explored in other studies.[109,117] Second, a few studies using laser microdissection techniques to focus on specific cell types identified 1) a significant increase in deletion levels in cholinergic neurons of the pedunculopontine nucleus;[110] 2) increased deletions in the dopaminergic neurons of the substantia nigra;[111] 3) increased deletions in the Lewy-positive neurons of the substantia nigra compared with those in Lewy-negative neurons or neurons from control individuals;[95] and 4); the common deletion was also identified as the most frequent deletion in the substantia nigra of microdissected neurons.[103]

mtDNA analysis in PsyD

Table 3 includes 19 reports referring to PsyD, and Figure 4 shows the variants reported in various phenotypes, with the most reported being schizophrenia (SZ, N=13) and bipolar disorder (BD, N=13), followed by major depressive disorder (MDD, N=8). Other phenotypes assessed included subjects with alcohol/drug abuse and other psychiatric symptoms (ADO), suicide victims, and autism spectrum disorder (ASD). The most recurrent brain region explored was the dorsolateral prefrontal cortex (DLPFC), but many others were included in some studies. Eleven studies investigated the presence of mtDNA rearrangements, eight studies examined mtDNA CN and seven studies focused on mtDNA variants.

mtDNA analyses in SZ. A study that focused on mtDNA variants identified a total of 142 rare variants, with a minor allele frequency less than 1% based on mtDB[118] and PhyloTree,[45] but a relevant role in SZ was not identified.[119] Similarly, in the DLPFC, a 22% higher rate of synonymous variants and an increased number of mtDNA substitutions were found in SZ patients compared with control individuals, but again, there was no major involvement in the diagnosis.[120] Other rare variants (frequency <0.02%), such as m.6617C>T p.Phe238 (*MT-CO1*), m.8881T>C p.Ser119Pro (*MT-ATP6*), and m.9500C>T p.Phe98, m.9699A>G p.Ile165Val and m.9956A>G p.Leu250 (*MT-CO3*) were identified only in patients with SZ.[121] Finally, two

studies reported results regarding specific variants. The m.3243A>G variant was detected in one patient with SZ with a 60% mutation load,[122] and the m.12027T>C, p.Ile423Thr (*MT-ND4*) was found to have no prominent effect on SZ.[123]

mtDNA deletions were detected in two patients with SZ, m.2973_15573del (*MT-RNR2–MT-CYB*) and m.1148_15607del (*MT-RNR1–MT-CYB*), with deletion read percentages of 31.8% and 16.8%, respectively.[124] Regarding the common deletion, most studies did not observe differences between SZ patients and control individuals,[96,119,125] but it has also been reported that the deletion levels highly differed among the 11 brain regions analysed, increased with age, and showed little change in blood samples.[119] Additionally, one study showed a significant decrease in the accumulation of the common deletion in patients with SZ compared to MDD, BD and control subjects, mostly in dopaminergic regions.[126]

mtDNA analyses in BD. The synonymous variant m.10858T>C in the *MT-ND4* gene was identified in a patient with BD,[119] and the m.3243A>G variant was present in two patients with a low variant frequency of 0.90%-1%.[122]

The mtDNA CN was lower in 32 patients with BD than in 32 control individuals, and no differences were observed between subjects who died from suicide and those who did not,[127] which was not observed in a previous study.[128] On the other hand, high mtDNA CNs in postmortem hippocampal tissue from 47 BD patients have been described.[125]

Two of 10 patients showed m.5897_15840del (*MT-TY/MT-CO1–MT-CYB*) and m.467_14122del (D-LOOP–*MT-ND5*) with deletion percentages of 23.0% and 5.0%, respectively. The authors reported that patients with SZ and BD had a higher cumulative deletion ratio in the DLPFC/anterior cingulate cortex (ACCtx) than those in the MDD and ADO diagnostic groups.[124] The ratio of the common deletion was significantly higher in patients with BD than in control individuals in the DLPFC[119] and in the cerebellum,[129] with a significant association between increased levels and advanced age.[119] This age association was previously observed but was unrelated to the BD diagnosis.[130] On the other hand, no significant accumulation of the common mtDNA deletion across distinct brain regions of patients with BD compared to control subjects has been reported.[125]

mtDNA analyses in MDD. The m.224T>C variant in the H-strand replication origin (O_H) located in the D-loop region was present in one of five evaluated patients with MDD, and the m.10652T>C homoplasmic variant (which does not alter the amino acid Ile61) in the *MT-ND4L* gene was previously reported in another patient.[120] Finally, the m.3243A>G MELAS variant identified in patients with BD was not present in 15 patients with MDD.[122]

Interestingly, the m.1243_15340del (*MT-RNR1–MT-CYB*) deletion with a high mutation load of 90.1% in the DLPFC and 85% in the ACCtx was suggested to considerably impact a 75-year-old male who had MDD and diabetes mellitus.[124] Furthermore, another 46-year-old patient with MDD who committed suicide also exhibited four high-impact deletions: m.7863_15617del (*MT-CO2–MT-CYB*), m.7816_14807del (*MT-CO2–MT-CYB*), m.870_14774del (*MT-RNR1-MT-CYB*), and m.6468_15600del (*MT-CO1–MT-CYB*), with read percentages of 52.4%, 26.5%, 10.2% and 8.5%, respectively. Even though deletion mutation loads were lower than those of the former patient, the cumulative mutation load was very high. In the latter patient, five brain regions and blood samples were explored, and deletions were detected only in the caudate nucleus. Interestingly, some deletions were clonally expanded to some brain regions, while they were absent in others.[124] Additionally, this study 1) did not find significant differences in the cumulative mtDNA deletion mutation load in the DLPFC or ACCtx across disorders; 2) stated that only 12 of the 30 most frequent deletions identified were previously described, 14 of which were detected only in the brain and not in other tissues from the same subjects; and 3) found that the brain contained significantly more deletions than the blood.[124]

mtDNA in other clinical conditions

Table 4 shows the mtDNA alterations explored in a few studies regarding other phenotypes: 1) herpes simplex virus type-1 encephalitis, 2) human immunodeficiency virus infection with or without methamphetamine use, 3) diabetes and recurrent stroke-like episodes, seizures and cognitive decline and 4) deceased neonates, newborns and infants and 5) control individuals. The most notable finding is that the common deletion was present in brain samples from stillborn individuals.[131]

mtDNA analyses in ageing

Table 5 presents 29 studies that analysed mtDNA in postmortem brain samples; these studies support mtDNA involvement in ageing.

mtDNA variations. Variants in the D-loop have been significantly associated with age, although when they were weighted by their heteroplasmic levels, the association was lost.[132] Additionally, the overall heteroplasmy level in the D-loop was found to be higher in older individuals than in younger individuals.[133] It has been reported that 78% of the accumulated variants were nonsynonymous and more deleterious in older

individuals.[24] In addition, a high aggregation of somatic point variants in the tRNAs for Thr and Pro, portions of the *MT-CYB* gene, and the D-loop region were detected in neurons of the elderly.[134] The ageing process is inextricably associated with neurodegeneration. In this sense, variants in the regulatory control region were found to be increased with age in AD and Down syndrome and dementia.[86] Similarly, 23 missense variants (8 of them causing nonconservative amino acid replacements at evolutionarily constrained sites), 2 tRNAs and one nonsense polymorphism were detected in the substantia nigra of elderly nonparkinsonian and idiopathic PD patients.[135] Moreover, the accumulation of G>C to T>A and T>A to G>C transversions was found to increase with age in the frontal cortex of patients with PD.[136]

mtDNA CN. Most of the studies found that the mtDNA content in the frontal cortex decreased with age.[86,132] However, in the substantia nigra, it was recently reported to be increased with age, allegedly to maintain the pool of wild-type mtDNA despite accumulating deletions.[105] Additionally, no significant age-dependent increase in mtDNA CN among three age groups (0–30, 31–59 and >60 years) was identified.[137]

mtDNA rearrangements. Most of the studies on ageing focused on the common deletion in the brain, and they reported that the accumulation of the common deletion was associated with increasing age.[108,116,132,138 142] According to Cortopassi et al., deletions in foetal tissues were estimated to be 1/100 to 1/100,000 times less than those in adults,[143] while Soong et al. reported that the common deletion level was detected in neonatal brain regions as 4/10,000.[144] The distribution of the common deletion varies among different parts of the brain, with the highest and the lowest levels reported in the substantia nigra and cerebellum, respectively.[138] Similarly, the common deletion ratio has been reported to reach the highest levels in the basal ganglia with age and the lowest levels in the cerebellum without any age-related association.[139] Additionally, a significant increase in the common deletion ratio was detected in the cortex and putamen with increasing age.[22] Regarding clinical conditions associated with age, some PD studies also showed that the accumulation of the common deletion was more likely to be an age-related phenomenon rather than the pathogenic condition,[116,117,135,139,145,146] and in AD, the common deletion levels were much lower in younger patients than in older patients.[147] Similarly, small insertions and deletions were found to be significantly increased in aged individuals among controls, early- and late-onset AD patients, and SZ patients.[133] On the other hand, no significant increase in common deletion with age in AD has been reported.[87] Major arc deletions showed a significant positive correlation with age in nigral neurons, while the level of mtDNA deletions was commonly detected at low levels and did not increase with age in frontal neurons and Purkinje cells.[105]

Other mtDNA deletions have been investigated in relation to age. The accumulation of the 7436 bp deletion[22,140,142] and a unique 12 kb deletion (m.1989_14366del) were also age-related in brain samples.[148] In addition, several multiple deletions (4.5–7.1 kb),[135] including m.7409_13687del,[145] have also been reported in the substantia nigra of both aged patients with PD and controls. Apart from these findings, one study showed that mtDNA deletions were associated with chronic hypoxia conditions rather than ageing in the samples of patients with PsyD.[149]

mtDNA technical issues

Historically, studies exploring mtDNA variants often used radiolabelled nucleotides, primers or probes for PCR, sequencing or Southern blot techniques, while most recent studies have used exome or genome sequencing. The mtDNA CN can be assessed by quantitative real-time PCR (qPCR). Some studies explored just one region, while others investigated mtDNA alterations in distinct brain regions, in homogenate tissues, or in laser-captured single cells. Although most of the studies used molecular techniques focused on mtDNA sequences, others were based on obtaining mtDNA. In this case, the first and crucial step of mtDNA analysis is effective extraction. Phenol-chloroform DNA extraction, which isolates both nDNA and mtDNA, is a widely used method. Devall et al. performed the first systematic comparison of the effectiveness of five different mtDNA isolation protocols from frozen postmortem brain tissue.[150] They reported that linear DNA digestion that leaves circular DNA (mtDNA) intact gave the lowest purity (mtDNA/nDNA), while the magnetic isolation of mitochondria using anti-human TOM22-labeled microbeads to isolate mitochondria gave the highest mtDNA enrichment.[150]

Although PCR-based technologies have accelerated the analysis of mtDNA deletions, their effectiveness can vary.[151] Distinct results were obtained when comparing the serial dilution PCR method (in which total DNA is diluted and amplified by primers spanning the common deletion) and the kinetic PCR method (based on removing reaction tubes from 10 to 20 cycles for undeleted mtDNA and stopping reactions from 22 to 32 cycles for deleted mtDNA to obtain the ratio of deleted mtDNA to normal mtDNA). According to their serial dilution PCR results, the caudate had 10 times more deleted mtDNA than the parietal cortex, while kinetic PCR resulted in a lower difference.[151] Taylor et al. used a digital deletion detection (3D) assay for absolute quantification and

characterization of rare mtDNA deletions in aged human brain samples.[23] This technique involves an enrichment step for deleted molecules by wild-type targeted endonucleolytic digestion, the amplification of intact mutant molecules by target-specific TaqMan probes in water-in-oil droplets, and, finally, a quantification step for chambers carrying many droplets representing thousands of single-molecule reactions.[23]

As an alternative to standard PCR techniques, Marquis et al. developed a novel sensitive mtDNA assay that used rolling circle amplification and sequencing (MitoRS) to detect mtDNA variants and their heteroplasmy level with high accuracy.[152] In the first step, they used Phi29 polymerase (with a low error rate and strong strand displacement activity) to generate several individual mtDNA copies (mtDNA enrichment) that were not species-specific and were insensitive to nuclear mtDNA sequences and to mtDNA polymorphism priming events. Combined with high-throughput tagmentation-based library generation for next-generation sequencing (NGS), they could quantify mtDNA SNVs at the minimum 1% frequency level.[152]

Some additional difficulties have been reported in mtDNA analyses. The chromogen 3,3′-diaminobenzidine, a standard stain for COX activity, has a strong inhibitory effect on qPCR, thus causing significant bias in the estimation of mtDNA CN and deletion levels between COX-positive and COX-negative neurons.[153] Regarding methylation, Devall et al. developed an assay to identify differentially methylated regions in mtDNA among different regions of the cortex and cerebellum by using pre-existing methylated DNA immunoprecipitation sequencing data. Interestingly, they identified 74 nominally differentially methylated regions in the mtDNA and 8 differentially methylated regions between the total cortex and cerebellum.[154]

Discussion

Biological processes are defined not only by cell structures but also by energy status. The brain represents between 2 and 3% of the weight of our body while consuming 20% of the total energy, which is mainly generated in the mitochondria. Unlike muscle, the brain is always highly metabolically active and thus is highly sensitive to mitochondrial functioning.[36,155] For this reason, the mitochondria operate under several control mechanisms, such as mitochondrial fusion and fission, the removal of damaged proteins, and mitophagy.[5] Recently, it has been suggested that if the available energy is limited, remaining below the bioenergetic threshold, neurological symptoms may appear; however, if the bioenergetic defect is subtler, the lack of energy can lead to the appearance of psychiatric symptoms.[16]

We collected mtDNA variants, CN and/or deletions reported in postmortem human brain samples. mtDNA variants and deletions can be inherited or occur at the germline or somatic level and have been associated with clinical and nonclinical conditions, while mtDNA CN is a proxy measure for mitochondrial function that has been associated with ageing-related diseases.[156] Multiple mtDNA deletions and duplications can arise due to the accumulation of multiple errors in postmitotic tissues, often with clonal expansion of one particular mtDNA form, or be attributed to variants in nuclear genes involved in mtDNA maintenance and repair.[36] Some MitD syndromes arise as a result of a sporadic large-scale single deletion that is the only deletion present. This single deletion can be of any size but there is a common deletion of 4.9kb. Notably, none of the studies in this review reported mtDNA duplications.

This review identified that many pSNVs are present in high heteroplasmy levels (generally >80%) in the postmortem brains of patients with MitD, although with variable heteroplasmy levels across brain regions and nonbrain tissues. However, the clinical condition characterized by early-onset cataracts, ataxia and progressive paraparesis showed mutation loads less than 60% in the affected tissues.[66] Some pSNVs may impact the mtDNA CN in the brain, either decreasing[68] or increasing[69] its levels, but few studies have analysed both mtDNA alterations. A low mtDNA CN was reported in most of the studies,[68,157–159] but not all,[160] and mtDNA deletions have only been investigated in KSS and DNA polymerase gamma gene (POLG) encephalopathy.[157,158,160,161] Only one study investigated the three types of mtDNA alterations in patients affected by POLG encephalomyopathy, and interestingly, the three types were present.[157] All-encompassing mtDNA analyses were not performed in most of the reviewed studies and should be encouraged in prospective studies.

The current data from postmortem human brain samples indicate that pSNVs do not have a prominent role in NeuD or PsyD, and a reduced mtDNA CN has been extensively observed in NeuD but not PsyD. Conversely, mtDNA deletions may have a more prominent role in PsyD. This would be in accordance with the hypothesis that MitD may be underdiagnosed in some patients with PsyD[162] and that bioenergetic defects can lead to the appearance of psychiatric symptoms.[3,16] Furthermore, NeuD and PsyD can be accompanied by metabolic disorders that are often not considered because they are not the target of the studies. Regarding this issue, the parietal cortices of diabetic individuals showed a 48% reduction in the mtDNA CN compared with those of nondiabetic individuals.[91] In nondiabetic AD subjects, the loss of mtDNA could lead to the loss of mitochondrial mass and bioenergetic capacity, whereas in diabetic AD subjects, an increased nutrient supply

due to insulin resistance and hyperglycaemia could result in reduced oxidative phosphorylation and increased glycolysis, which would also lead to an energy deficit. In line with this idea, in one of the evaluated studies, a high-impact deletion was present in a patient with MDD who also had diabetes.[124] Future studies should include all the phenotypic characteristics of the assessed individuals to shed light on the role of mtDNA alterations in other comorbid traits. Notably, in NeuD and PsyD, many of the early studies on human post-mortem brain samples were based on the comparison of frequencies and heteroplasmy levels of specific SNV between patients and controls, and even though some reported significant differences, these results have not been confirmed in a more recent and larger dataset, which advocates that mtDNA CN rather than mtDNA SNV would have more clinical relevance in NeuD.[84] However, the association of homoplasmic common and rare SNV on longevity and NeuD such as multiple sclerosis has been recently confirmed by analysing a large number of blood samples.[32] An interesting study that also investigated blood samples demonstrates the involvement of the nuclear female genome in the evolution of human mtDNA variation and also suggests the different values of heteroplasmy that an individual cell, tissue or organism may exhibit during embryonic development of human germ cells.[163] The different expansion of heteroplasmic variants during the ageing process could have consequences for age-associated diseases such as NeuD but also PsyD, which present in many cases within certain age ranges. However, the biological processes associated with ageing have mostly been studied in relation to NeuD.[5]

Different methodological issues may have interfered with the results reported in this systematic review. These include, among others, the origin of the samples and the way the DNA was obtained and stored, as some reagents and degraded samples considerably limit the PCR/qPCR technique.[164] In addition, PCR-based techniques may favour the amplification of short (deleted) versus long (nondeleted) fragments resulting in inadequate detection of mtDNA fragments. NGS methodology based on previous long-range amplification is the most powerful tool to detect mtDNA alterations. It provides high and uniform coverage of each of the 16,569 bases, allowing the detection of nucleotide changes, as well as heteroplasmy levels. Moreover, it also allows the detection of small insertions/deletions (indels) and large deletions and the mapping of exact deletion breakpoints.[165] Several bioinformatic tools allow a reliable analysis of large mtDNA sequences with high coverage levels for detecting mtDNA alterations.[122,166] [169] Additionally, current genome and exome sequencing techniques can detect heteroplasmy levels at less than 5%, and it has been reported that heteroplasmy levels depend on the coverage and the number of sequence reads. Moreover, these techniques have also been reported to be more accurate for mtDNA CN quantification than the gold standard qPCR technique.[169] In any case, even with the NGS technique, it is necessary to take into account the initial enrichment strategy used and the reference used in the downstream bioinformatic analysis, as both may influence the accurate detection and quantification of mtDNA heteroplasmy levels.[170]

A broad understanding of brain regional variation regarding heteroplasmy levels of pSNVs, mtDNA CN and mtDNA deletions is necessary for understanding the metabolic requirements of different regions of the brain, which can improve our understanding of region-specific cell type changes and/or vulnerability to metabolic insults and related neuropathological processes. Highly automated and sensitive tools to evaluate mtDNA alterations from data obtained through exome or genome sequencing are currently available,[124,166,167,171,172] and larger datasets should be assessed for mtDNA analyses, along with a full phenotypic characterization to better associate molecular mtDNA defects or variations with biochemical, metabolic and clinical or health aspects. This is supported by the recent success in identifying associations between a large number of phenotypes and homoplasmic mtDNA variants by analysing a large number of blood samples from the UK Biobank.[32] In summary, most studies that explored mtDNA alterations in neurological diseases, mental illnesses and the natural ageing process have reported conflicting results. Some reasons for this are that different studies have investigated different mtDNA alterations, different diagnoses and/or different brain regions. This has made it difficult to draw conclusions. In addition, methodological issues mainly related to the techniques used but also to sample collection have led to difficulties in data interpretation. More studies are needed to identify the specific mtDNA alterations associated with health and disease. Nevertheless, the findings discussed in this systematic review argue for the involvement of mtDNA in brain disorders.

Limitations

Many of the sample sizes used to study mtDNA CN were underpowered. As an example, differences in mtDNA CN between patients with AD and control individuals were not often identified in the cerebellum[58,59] until a larger number of patients and controls were screened.[62] The techniques used in early mitochondrial genetics studies are not comparable to more recent technologies. It would therefore be interesting if some of the phenotypes that were analysed at the time of identifying the first mtDNA alterations could be analysed with the new technologies, especially to confirm the levels of heteroplasmy that were indicated at the time.

Conclusions

This study provides a comprehensive summary of the mtDNA alterations reported in human brain samples. The results identified in relation to MitD provide a clear idea of which genetic alteration is involved in each disorder, despite the great heterogeneity of the alterations described. Unfortunately, this is not the picture for NeuD and PsyD, where the findings are often contradictory. While mtDNA alterations have pathological implications in MitD, for most of the alterations identified in NeuD and PsyD an association has been suggested, but the pathological consequences are not yet proven. Low mtDNA CN is the most reported mtDNA alteration in NeuD, and specific mtDNA deletions may have prominent consequences for PsyD. There is also strong and abundant evidence that the ageing process is related to neurodegeneration and the loss of mtDNA integrity. Future research directions should include mtDNA analyses in larger samples and concurrent analyses of all types of mtDNA alterations and in several brain regions. Additionally, genotype-phenotype correlation studies will help to advance our understanding of the underlying molecular mechanisms and the implications of mtDNA variability in health and disease.

Declaration of interests

The authors declare that they have no conflicts of interest.

Contributors

Conceptualization and design, L.M.; Methodology, writing and editing, A.V.-P., J.T., B.K.B., and L.M.; Writing and editing, E.V., G.G., and G.M.; Acquisition and verification of reported data, A.V.-P., J.T., B. K.B. and L.M. All authors contributed to the interpretation of the findings, read and approved the final version of the manuscript, had full access to all the data and final responsibility for the decision to submit for publication.

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Data sharing statement

The data collected for this study can be provided upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103815.

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High frequency of mitochondrial DNA rearrangements in the peripheral blood of adults with intellectual disability. Bengisu K. Bulduk, Juan Tortajada, Leire Torres-Egurrola, Alba Valiente-Pallejà, Rafael Martínez-Leal, Elisabet Vilella, Helena Torrell, Gerard Muntané, and Lourdes Martorell. International Journal of Molecular Sciences 2023; XXXX

Article 2. High frequency of mitochondrial DNA rearrangements in the peripheral blood of adults with intellectual disability.

In the previous systematic review (article 1), it was shown that most of the mtDNA rearrangements reported in human postmortem brain tissues were also detected in blood although at lower heteroplasmy levels. In this study, we hypothesised that mtDNA rearrangements, in addition to the SNVs we previously identified, might be present in blood samples of patients with ID. Their detection in blood could indicate their presence in the brain and potentially their involvement in the complex genetic mechanisms underlying this heterogeneous neurodevelopmental condition.

To address this hypothesis, we conducted a study using mtDNA-targeted next-generation sequencing and the high-throughput computational pipeline MitoSAlt (Mitochondrial Structural Alterations). Our study focused on peripheral blood samples from a group of 135 adult individuals with ID, including 59 with comorbid ASD, and 32 HC.

In this study, we identified a total of twelve unique mtDNA rearrangements. Ten individuals with ID (13.2%), one with ID-ASD (1.7%), and one HC (3.1%) had large rearrangements in their mtDNA. The median size of all detected mtDNA alterations was 8332 bp, with a median heteroplasmy level of 2.8%. Among these rearrangements, six were classified as deletions with a median size of 6937 bp and a median heteroplasmy level of 2.3%, while the other six were classified as duplications with a median size of 10455 bp and a median heteroplasmy level of 1.9%. Additionally, a 29 bp duplication was reported in four individuals with ID. Moreover, more than one alteration in two individuals with ID and one ID-ASD were reported. We found a higher frequency of mtDNA alterations in patients with ID (13.2%) than in HCs (3.1%), however this difference was not statistically significant (U=3.4; p=0.465). Note that the frequency of mtDNA rearrangements in the ID-ASD group was low (1.7%), even lower than in the controls.

The most commonly affected genes in individuals with ID were reported to be *MT-ATP6, MT-CO3, MT-TT, MT-ND3, MT-TW, MT-ND4L,* and *MT-ND4*, which may cause possible defects in ATP generation in the cell. In parallel with these results, we found some notable clinical features associated with MitD in patients with ID and ID-ASD carrying mtDNA alterations; however, no significant differences were observed between carriers and noncarriers of mtDNA alterations.

This study has some limitations that should be mentioned being the most relevant the specimens we used and the sample size of the studied groups. We used blood samples, a self-renewing tissue that tend to lose mtDNA deletion variants, instead of brain samples where they may be

present at higher heteroplasmy levels as brain samples were not available; however, obtaining blood it is a noninvasive procedure and the presence of mtDNA rearrangements in blood could be indicative of their presence in postmitotic tissues such as the brain. Regarding the relatively small sample size, it may have reduced the statistical power in identifying significant differences between the diagnostic groups and in the association between the presence of mtDNA alterations and specific clinical features.

As far as we know, this is the first study analysing low levels of mtDNA rearrangements in ID. The results of this study suggest that mtDNA rearrangements are prevalent in individuals with ID, particularly when compared to individuals with ID-ASD, although further studies with a larger sample size are needed. These findings suggest a potential role for mtDNA rearrangements in ID, possibly due to their presence at higher levels of heteroplasmy in the brain, contributing to the observed mitochondrial dysfunction in this neurodevelopmental condition.

High frequency of mitochondrial DNA rearrangements in the peripheral blood of adults with intellectual disability

Abstract

Background Mitochondrial DNA (mtDNA) rearrangements are recognised factors in mitochondrial disorders and ageing, but their involvement in intellectual disability (ID) remains poorly understood.

Method We used mtDNA-targeted next-generation sequencing and the MitoSAlt high-throughput computational pipeline to investigate the presence of mtDNA rearrangements in peripheral blood samples from 135 adults with ID, 59 of whom had comorbid ASD (ID-ASD), and 32 healthy controls (HCs).

Results The study revealed a high frequency of mtDNA rearrangements in patients with ID (13.2%), which was lower in patients with ID-ASD (1.7%) and in HCs (3.1%). We identified six deletions (median size 6937 bp, median heteroplasmy level 2.3%) and six duplications (median size 10455 bp, median heteroplasmy level 1.9%).

Conclusions Our results show that mtDNA rearrangements are common in patients with ID. This finding needs to be confirmed in a larger sample; however, it suggests that mtDNA rearrangements may contribute to ID.

Keywords autism spectrum disorder, deletion, duplication, intellectual disability, mitochondrial DNA, MitoSAlt

Introduction

Mitochondria, the major cellular organelles responsible for ATP production, vary in number from hundreds to thousands per cell depending on the energy needs of the tissue (Nissanka & Moraes, 2020; Verge et al., 2011). Mitochondria have their own genome called mitochondrial DNA (mtDNA). mtDNA is a circular, 16,569 bp double-stranded DNA molecule that contains 37 genes encoding 13 essential polypeptides for the oxidative phosphorylation system (OXPHOS), 22 tRNAs and two rRNAs involved in mtDNA transcription and protein synthesis. mtDNA also contains a non-coding region called the displacement loop (D-loop). The D-loop plays a critical role in mtDNA replication and transcription by containing the origin of replication for the heavy strand (OH), the light strand promoter (LSP) and the H-strand promoters (HSP1 and HSP2) (U. Basu et al., 2020; Nissanka & Moraes, 2020; Verge et al., 2011). Notably, mtDNA has a mutation rate 10 to 20 times higher than nuclear DNA (nDNA) due to its less efficient DNA repair capacity, lack of protective histone proteins, and exposure to elevated levels of reactive oxygen species (ROS) (Chen et al., 2022; Nissanka & Moraes, 2020; Verge et al., 2011). mtDNA alterations include single nucleotide variants (SNVs), short insertions and deletions (indels), and large rearrangements of mtDNA fragments. While SNVs are commonly inherited (Chen et al., 2022; El-Hattab & Scaglia, 2016; Verge et al., 2011), large mtDNA rearrangements (mostly deletions) tend to occur sporadically and are associated with many mitochondrial disorders. They also accumulate in post-mitotic tissues during ageing (Taylor et al., 2014). The mechanisms behind mtDNA deletions remain unknown; however, approximately 90% of clinically relevant deletions are flanked by repeat sequences generated by copy-choice recombination during active synthesis of the mtDNA light (L) strand (Persson et al., 2019). In addition, the polyploid nature of mtDNA allows for the coexistence of different mtDNA species within a cell, a phenomenon known as heteroplasmy. The level of heteroplasmy can vary between tissues within an individual and is typically characterised by the proportion of mutant mtDNA compared to normal mtDNA. In contrast, homoplasmy is a state in which all mtDNA molecules are identical (Nissanka & Moraes, 2020).

Intellectual disability (ID) and autism spectrum disorder (ASD) are etiologically heterogeneous neurodevelopmental disorders caused by the interaction of multiple genetic and environmental factors, with a combined prevalence of approximately 3% worldwide (Lai et al., 2014; Srour & Shevell, 2014). Individuals with ID have difficulties with cognitive and adaptive skills that can affect their ability to function independently in daily life, while individuals with ASD have difficulties with social communication and interaction, as well as restricted and repetitive patterns of behaviour, interests, or activities (Thurm et al., 2019). Approximately 4% to 40% of individuals with ID are

thought to have comorbid ASD, while 50% to 70% of individuals with ASD also have comorbid ID (Goldin et al., 2014).

Mitochondrial dysfunction has been reported in individuals with ID (Valenti et al., 2014; Wilson et al., 2020) and in patients with ID-ASD (Scuderi et al., 2023; Valiente-Pallejà et al., 2018; Varga et al., 2018). In addition, conditions commonly associated with mitochondrial disorders, such as migraine, peripheral neurovascular disorders, gastrointestinal dysmotility, neurological disorders, cardiac abnormalities, skeletal muscle disorders, endocrine disorders, and constitutional disorders, are frequently observed in ID and ASD, suggesting that systemic mitochondrial defects may play a potential role in the development of these disorders (Frye, 2020b; Valiente-Pallejà et al., 2018). Many nuclear variants have been associated with ID and ASD; however, while defects in mtDNA may also lead to or be involved in these disorders, there are currently very few studies in this area (Citrigno et al., 2020; Pei & Wallace, 2018). Whole-genome sequencing (WGS) and whole-exome sequencing (WES) are crucial techniques in the current identification of the underlying genetic defects in ID and ASD; however, mtDNA is usually not specifically targeted, with a few exceptions (de Boer et al., 2021; Trost et al., 2022). These studies examined mtDNA SNVs but not mtDNA rearrangements, although 1312 deletions and 44 duplications have been reported in the MitoBreak database, a comprehensive online resource of curated mtDNA rearrangement datasets (Damas, Carneiro, et al., 2014).

In a recent systematic review, we found that most of the mtDNA rearrangements reported in the brain are also present in the blood, albeit at lower cumulative read percentages (Valiente-Pallejà et al., 2022). We therefore hypothesised that mtDNA rearrangements may be present in blood samples from patients with ID and may be involved in the complex genetic mechanisms underlying this heterogeneous neurodevelopmental disorder. We therefore used mtDNA-targeted next-generation sequencing technology combined with the MitoSAlt (Mitochondrial Structural Alterations) pipeline (Basu, Xie, X. et al. 2020) to investigate the presence of mtDNA rearrangements (duplications and deletions) in peripheral blood samples from adult patients with ID and in HCs.

Methods

Participants

The study was carried out in 76 patients with ID (mean age 52.5 years; standard deviation (SD) 10.2) and 59 patients with ID-ASD (mean age 41.3 years; SD 8.7) institutionalised in the Villablanca Care Services, and in 32 healthy controls (HC) (mean age 42.4 years; SD 12.4) from the same geographical area. All subjects were adult Caucasians. The Institut Pere Mata manages the Villablanca Care Services as a reference centre in Catalonia for the care of people with severe and profound ID. The study was approved by the Ethics Committee of the Hospital Universitari Sant Joan de Reus, and data were coded for anonymity in accordance with current Spanish legislation on biomedical research. Informed written consent was obtained from the patient's relatives or other legal guardians and the HCs. The characteristics of the participants are shown in Table 1.

The patients' clinical records, diagnoses and physical examination results were obtained by a trained psychiatrist, as previously described (Valiente-Pallejà et al., 2018). In addition, patients underwent a comprehensive laboratory workup, including standard karyotyping, subtelomeric and targeted multiplex ligation probe amplification (MLPA) screening for chromosomal abnormalities, fragile X molecular testing and array comparative genomic hybridisation (aCGH), as previously described) (Cuscó et al., 2009).

HCs were assessed by an experienced psychiatrist using the Spanish adaptation of the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (Vázquez-Barquero et al., 1994) to exclude a past or current history of psychiatric disorders. Information on clinical conditions commonly associated with mitochondrial disorders was also obtained from the controls.

mtDNA targeted next-generation sequencing and prediction of mtDNA alterations

Total DNA was obtained from the participants' peripheral blood leukocytes, and total mtDNA was amplified in two or three overlapping fragments and then sequenced using an Ion Torrent Personal Genome Machine (PGM, Fisher Scientific, Madrid, Spain), as previously described (Valiente-Pallejà et al., 2018).

Read quality was assessed using the FastQC (S. Andrews, 2010) and MultiQC (Ewels et al., 2016) tools. Cutadapt (v.3.4) (M. Martin, 2011) was used to remove 10 bases from the start of reads and bases with Phred quality scores less than 20 (sequencing error greater than 1%) from the 3' and 5' ends, and to crop reads at 200 base pairs (bps) (Cortes-Figueiredo et al., 2021;

Harvey et al., 2019). Read depth at each position and mean coverage of sequences were calculated using SAMtools (v.1.13) (Li et al., 2009). Sequences with coverage greater than 99% and mean read depth greater than 100x were included in the analysis, as recommended (Harvey et al., 2019; Yao et al., 2018).

The MitoSAlt (v.1.1.1) pipeline (Basu, Xie, X. et al. 2020) was then used to detect and quantify mtDNA rearrangements. This tool allows the detection of mtDNA alterations even at extremely low levels of heteroplasmy, as is common in blood samples (Hjelm et al., 2019a; Taylor et al., 2014). MitoSAlt is available on SourceForge at https://sourceforge.net/projects/mitosalt to identify, quantify and visualise both deletions and duplications of mtDNA, as it predicts rearrangements based on split alignments to the linear mitochondrial genome (Basu, Xie, X. et al. 2020). The MitoSAlt parameters used in this study are listed in Table S1. The MitoSAlt plots of patients carrying more than one mtDNA alteration are also shown in Figure S1. All identified mtDNA breakpoints were adjusted for the presence of direct repeats by using the MitoBreak Classifier tool (Damas, Carneiro, et al., 2014), and we checked whether they had been previously reported in the MitoBreak database (Damas, Carneiro, et al., 2014) and the Splice-Break dataset (Hjelm et al., 2019a).

Statistical analyses

Statistical tests were chosen according to the nature of the variables. Normality of continuous variables was tested using the Shapiro-Wilk test. In cases where the data were normally distributed, t-tests were used to compare groups, and in cases of non-normal distribution, the Mann-Whitney U test was used. Discrete data were evaluated by using the Pearson chi-squared test when less than 20% of cells had fewer than five observations. Otherwise, Fisher's exact test was used (H.-Y. Kim, 2017). The significance level was set at $p < 0.05$. All data were processed using jamovi 2.3.24 (The jamovi, 2022).

Results

mtDNA alterations

We obtained mtDNA sequences from 195 patients with ID, 98 of whom had comorbid ASD (ID-ASD), and 39 HC subjects. After quality control, we excluded four ID and one ID-ASD sample

because we did not obtain a complete mtDNA sequence (less than 99% coverage of the mitochondrial genome). In addition, 17 ID, 38 ID-ASD, and 7 HC samples were excluded due to low mean read depth (<100x). Therefore, the dataset consisted of 76 mtDNA sequences from patients with ID, 59 from patients with ID-ASD and 32 from HC, with a mean read depth of 196 (range 101-1662) (**Figure 1**).

We identified a total of twelve unique mtDNA alterations in ten individuals with ID (13.2%), one individual with ID-ASD (1.7%) and one HC (3.1%). Although we found a higher frequency of mtDNA alterations in patients (ID and ID-ASD) than in HCs, this difference was not statistically significant (U=3.4; p=0.465). The median size of the detected mtDNA alterations was 8332 bp (range 29-13521 bp), with a median heteroplasmy level of 2.8% (range 1.1-6.7%). Six mtDNA alterations (50%) were identified as deletions with a median size of 6444 bp and a median heteroplasmy level of 2.6%, and another six alterations (50%) were identified as duplications with a median size of 10455 bp and a median heteroplasmy level of 3.0% (**Table 2**). A 29 bp duplication, m.8765-8793dup, was present in four individuals with ID. In addition, two individuals with ID (ID-101 and ID-104) and one with comorbid ASD (ID-ASD-152) carried two mtDNA alterations. The highest levels of heteroplasmy were observed in the 29 bp duplication carried by patients ID-101 (6.7%) and ID-112 (5.3%). Also of note are m.9422-15606del, a 6185 bp deletion with a heteroplasmy level of 5.2% present in patient ID-036, and m.61-13581dup, a 13521 bp duplication with a heteroplasmy level of 4.6% present in patient ID-008.

Regions involved in mtDNA alterations

In seven of the eleven patients (63.6%), mtDNA alterations involved at least one of the mtDNA replication origins: four patients (57.2%) had a duplication in the OH, while three patients (42.8%) had a deletion in the OH. Two of them had duplications in both the OH and the OL. In addition, ID-104 carried two different duplications in the OH (**Figure 2**). The deletion present in the HC did not involve any of the replication origins or promoter regions.

The genes *MT-ATP6, MT-CO3, MT-TT, MT-ND3, MT-TW, MT-ND4L*, and *MT-ND4* were affected in eight patients with ID. Indeed, these genes were deleted in four subjects with an average heteroplasmy level of 2.5% and duplicated in another four subjects with an average heteroplasmy level of 2.2%. In addition, the 29 bp duplication, m.8765-8793dup, present in four patients with an average heteroplasmy level of 4.1%, involved the *MT-ATP6* gene.

One HC subject had a deletion between the *MT-ATP6* and *MT-CYB* genes with a heteroplasmy level of 2.8%, affecting eight protein genes and six tRNA genes; however, the replication and transcription capacity was not altered as it did not involve the replication and transcription origins of the mtDNA.

Demographic and clinical characteristics between mtDNA alteration carriers and non-carriers

The mean age of the ID group was significantly higher than that of the ID-ASD group ($p < 0.001$) and the HCs ($p<0.001$). Similarly, subjects with ID had a significantly higher BMI than the ID-ASD and HC groups ($p=0.007$ and $p< 0.001$, respectively). However, we did not observe significant differences between carriers and non-carriers of mtDNA alterations in age ($U=754$, $p=0.276$), sex ($\chi^2=3.31e\text{-}4$, $p=0.985$), BMI ($U=915$, $p=0.958$) or tobacco use ($\chi^2=0.0253$, $p=0.874$).

We observed that mtDNA carriers have a significantly lower CARS score ($U=392$, $p=0.028$) and lower ID severity ($p=0.036$) compared to non-carriers. In addition, we did not observe any relationship between CARS scores or ID severity and the presence of mtDNA alterations in replication or transcription origins.

Clinical characteristics of the participants with mtDNA alterations

Some notable clinical features were present in patients with mtDNA alterations (**Figure 3**). None of these patients, with the exception of ID-083, had nuclear DNA alterations. ID-008 was a 50-year-old woman with severe ID who carried a 13521 bp duplication and had a diagnosis of a psychotic disorder not otherwise specified, Hashimoto's thyroiditis, high cholesterol, obesity (BMI 31.6) and constipation. ID-036, a 39-year-old woman with profound ID, carried a 6185 bp deletion and had comorbid obesity (BMI 33.1), constipation, seizures, hypertension and nocturnal enuresis. ID-074, a 48-year-old overweight man (BMI 27.8) with profound ID, carried an 8186 bp deletion and had comorbid conditions including psychotic disorder with onset in infancy, cerebral palsy, congenital mitral stenosis and arthritis. ID-075 was a 53-year-old man with severe ID who carried a 10096 bp duplication and had a history of acute hepatitis B, anaemia and immune thrombocytopenia. ID-083 was a 48-year-old man with profound ID and a diagnosis of cri-du-chat syndrome (5p14 deletion), who carried a 11044 bp duplication and was also diagnosed with situs inversus, congenital cataract and optic atrophy. This individual also had congenital cardiopathy due to the persistence of the patent ductus arteriosus and a heart murmur. He also showed symptoms of multiple depressive episodes and had a history of suicide attempts. ID-101 was a

50-year-old woman with severe ID carrying both an 8480 bp deletion and a 29 bp duplication. She was reportedly obese (BMI 45.8) and had comorbid diagnoses of epilepsy with generalised seizures, hirsutism, constipation, and lymphatic filariasis. ID-104 was a 53-year-old woman with severe ID carrying two duplications of 8689 and 10813 bp. She had a grade II/IV systolic heart murmur, spinal osteoarthritis, constipation and obesity (BMI 30.6). ID-ASD-152, the only individual diagnosed with severe ID and ASD, was a 21-year-old male carrier of two duplications (5886 bp and 6370 bp) who had comorbid constipation, thrombocytopenia and Becker nevus syndrome. Thus, among the eleven patients with mtDNA alterations, constipation was present in six patients, while seizures and uncorrected visual impairment were present in three patients each.

Three males and a one female with severe ID carried the 29 bp duplication. Within this group, ID-011 also had inactive hepatitis B and hypertension; ID-019 had amblyopia, pleural thickening, and pes cavus; and ID-112 had chronic hepatitis B, hypertension, diabetes mellitus and a cataract.

Finally, the HC carrying a 6517 bp deletion was a 50-year-old woman diagnosed with migraine.

Because of the large number of clinical conditions associated with mitochondrial disorders observed in patients with ID (**Figure 3**), we investigated whether these conditions were more frequent in carriers of mtDNA alterations than in non-carriers; however, no significant differences were observed. For example, constipation was reported in 54.5% of the mtDNA alteration carriers and 67.7% of non-carriers (p=0.505), and seizures were reported in 51.6% of the mtDNA alteration carriers and 27.3% of non-carriers (p=0.207).

Discussion

Despite the predominant focus on nuclear DNA in studies of neurodevelopmental disorders, the observed mitochondrial dysfunction in ID and ID comorbid with ASD (ID-ASD) highlights the importance of assessing mtDNA in individuals with these clinical conditions (Noda, 2022; Valiente-Pallejà et al., 2018; Varga et al., 2018). Two recent informatics tools, eKLIPse and MitoSAlt, analyse major mtDNA rearrangements. Among them, MitoSAlt stands out as the most accurate and sensitive tool to detect, quantify, and visualise mtDNA deletions and duplications (Goudenège et al. 2019, Basu, Xie, X. et al. 2020). To date, the mechanism underlying mtDNA deletions and their potential impact on mitochondrial dysfunction has been more extensively studied than the mechanism underlying duplication formation **(Fontana & Gahlon, 2020)**. For

example, in MitoBreak, only 44 mtDNA duplications between 150 bp and 13.5 kb in length were reported out of a total of 1356 mtDNA alterations (Damas, Carneiro, et al., 2014).

Here, we used MitoSAlt to identify mtDNA rearrangements in patients with ID, ID-ASD and HCs. Our results showed that mtDNA rearrangements were present in 13.2% of individuals with ID, 1.7% of individuals with ID-ASD, and 3.1% of HCs. This result highlights that patients with ID are more likely to carry mtDNA rearrangements in their blood than HCs; however, statistical significance was not achieved, probably due to the small sample size in our study. We report twelve new mtDNA rearrangements, six deletions and six duplications. Deletions were identified in the range of 5.9-8.4 kb, which is similar to previous results showing mtDNA deletions ranging from 2.4 to 7.9 kb in children with ASD (Varga et al., 2018) and in postmortem brain tissue and blood samples from individuals with psychiatric disorders (Valiente-Pallejà et al., 2022; Varga et al., 2018). Blood is a self-renewing tissue and tends to lose mtDNA species with partial deletions (Filograna et al., 2021; Hjelm et al., 2015; Taylor et al., 2014), which is consistent with the low levels of heteroplasmy observed in our study. However, the fact that we detected mtDNA rearrangements in the blood suggests that they are also present in other tissues, such as the brain, where higher levels of heteroplasmy can be observed (Damas, Samuels, et al., 2014; Valiente-Pallejà et al., 2022). It is important to note that mtDNA heteroplasmy is quite common in humans, typically occurring at lower levels ranging from 0.5% to 1.5%. In contrast, the levels reported in this study are notably higher, reaching up to 6.7%. However, it is important to note that these findings relate to mtDNA SNVs, and that mtDNA rearrangements have not yet been investigated (Parakatselaki & Ladoukakis, 2021; Payne et al., 2013). In terms of duplications, we reported a large duplication of 13521 bp in length, encompassing both replication origins, the entire *MT-ND4* gene and part of the *MT-ND5* gene. Similarly, MitoBreak reported a duplication of 13491 bp that also included both replication origins and, in this case, the entire *MT-ND5* gene and part of the *MT-ND4* gene. Importantly, three duplication carriers in our study showed optic atrophy, amblyopia, and cataracts, and the majority of single duplications reported in MitoBreak were identified in patients with progressive external ophthalmoplegia (Damas, Carneiro, et al., 2014).

In terms of genes involved in mtDNA rearrangements, *MT-ND4*, which plays a critical role in complex I assembly (Giachin et al., 2016), was deleted (or partially deleted) in most of the deleted mtDNA molecules we identified. In a previous study, *MT-ND4* gene deletion was also found in the frontal cortex of 44% of ASD patients examined by quantitative real-time PCR (Gu et al., 2013). Similarly, we found that the *MT-ATP6* gene was deleted in four patients, which could lead to defects in complex V (ATP synthase) activity. The human *MT-ATP6* transcript, m.8527-9207,

consists of 681 bp, and the resulting polypeptide subunit has 226 residues. The four duplication carriers we identified had a transcript length of 710 bp, and although the results of the duplication do not change the first 22 amino acids, 2 new amino acids (K and S) and a stop codon are introduced afterwards, probably resulting in a non-functional ATP6 subunit of 24 residues. *MT-ND3, MT-ND4, MT-ND4L, MT-ND5*, and *MT-ND6* (complex I, NADH dehydrogenase) were deleted in five patients who may therefore have defects in ATP generation. This finding also supports the results of a meta-analysis of 300 studies reporting 730 mtDNA deletions and 37 mtDNA duplications, which showed that the mtDNA fragment between the *MT-ATP8/MT-ATP6* and *MT-ND5* genes was absent in more than 70% of reported clinical features associated with mitochondrial defects (Damas, Samuels, et al., 2014). Finally, the OH deficiency found in three patients in our study is not commonly observed in wild-type mtDNA molecules, as it results in limited replication capacity (Lujan et al., 2020). As expected, no individual in this study was missing both replication origins.

We examined the socio-demographic and clinical characteristics of the patients with mtDNA rearrangements. No differences in age or sex were observed between carriers and non-carriers of mtDNA rearrangements. Patients carrying mtDNA rearrangements had significantly lower CARS scores and lower ID severity than non-carriers; however, it is likely that these significant differences are due to differences between the two clinical groups, ID and ID-ASD, and not to the presence or absence of mtDNA rearrangements, since CARS scores were lower and ID severity was higher in the ID-ASD group than in the ID group, and only one patient out of 59 (1.7%) in the ID-ASD group had an mtDNA rearrangement.

People with ID have higher prevalence rates of poor social determinants of health, behavioural risk factors, depression, diabetes, and suboptimal health status compared with adults without ID (Krahn & Fox, 2014). People with ID have a higher risk of weight gain than the general population (Koritsas & Iacono, 2016; Skelly et al., 2021), and obesity is a major factor in the development of metabolic disorders, such as hypertension, high cholesterol and hyperglycaemia, which are associated with mitochondrial dysfunction (Todosenko N et al., 2023). Consistently, six of the eleven patients carrying an mtDNA alteration in this study had a BMI in the obese or overweight range. Six patients had constipation, which is a prominent gastrointestinal manifestation in mitochondrial disorders (Finsterer & Frank, 2017). Psychotic disorders and schizophrenia, which were present in two patients with mtDNA alterations in this study, are also common psychiatric manifestations in mitochondrial diseases (Anglin et al., 2012). Three patients had seizures, which have been reported to occur in 35-60% of people with mitochondrial disease (Rahman, 2012). We have previously reported mitochondrial dysfunction and low mtDNA copy number in a family

with psychosis and chronic fatigue syndrome (Torrell et al., 2017). In addition, two mtDNA deletions between the *MT-RNR1* and *MT-CYTB* gene regions have been reported in brain samples from patients with schizophrenia (Hjelm et al., 2015), which is consistent with the mtDNA alteration present in the individual with schizophrenia in this study. Optic neuropathy is also a hallmark of mitochondrial disorders and is associated with the male sex (Carelli et al., 2023). In this study, we found optic atrophy and a congenital cataract in a male individual carrying a 5.5 kb deletion, which is similar to the 6.7 kb mtDNA deletion found in a male subject with a neuromuscular disease who presented with congenital cataract as the first symptom (Bene et al., 2003). In addition, one of the patients carrying the 29 bp duplication in the *MT-ATP6* gene also showed amblyopia, which is common in Leber hereditary optic neuropathy (Yu-Wai-Man et al., 2011). Three patients with mtDNA alterations in the present study had congenital heart disease or a heart murmur. Cardiac involvement in mitochondrial diseases is common through the effect of metabolism in the heart [40]. Similarly, some studies have reported that patients with ID and mitochondrial disorders also have epilepsy (Guevara-Campos et al., 2015; Ortiz-González, 2021). Finally, epileptic seizures have been reported in 5% of patients with large mtDNA deletion-associated syndromes with deletions of 2 to 9 kb (Björkman et al., 2023). In our study, we found one subject with an 8 kb deletion who presented with epilepsy with generalised seizures.

To our knowledge, this is the first study to investigate low levels of heteroplasmy in the blood (range 1.1% to 6.7%) using these new tools, next-generation sequencing and the MitoSAlt pipeline. We observed a high frequency of mtDNA rearrangements in blood samples from patients with ID. Due to the limitations of the previous sequencing technologies, studies have generally examined mtDNA deletions with a high heteroplasmy threshold, usually above 10% (Y. Guo et al., 2013). Therefore, mtDNA rearrangements with low levels of heteroplasmy have not been extensively studied. However, in the coming years, with the decreasing cost of high-throughput sequencing technologies and the development of bioinformatics tools such as MitoSAlt, these new methods will provide a powerful tool to study the involvement of low levels of heteroplasmy in diseases in which mitochondrial dysfunction is present.

We are aware that our study has some limitations. First, we analysed blood samples, a self-renewing tissue that tends to lose mtDNA deletion variants. However, obtaining blood is a non-invasive procedure and the presence of mtDNA rearrangements in blood could be indicative of their presence in post-mitotic tissues such as the brain, where they may be present at higher levels of heteroplasmy (Valiente-Pallejà et al., 2022). Second, although Ion Torrent technology is widely used, it has some shortcomings that require additional manual curation of the data (Harvey et al., 2019; Seneca et al., 2015), which we addressed by applying several quality control

criteria. Third, this study was conducted in individuals with a mean age of approximately 47 years, and although we did not find age differences between carriers and non-carriers of mtDNA alterations, there are no younger ages in our dataset. Fourth, although some genetic testing was performed to identify the presence of mutations in the nuclear genome, no exome (or genome) sequencing was performed and it cannot be excluded that the clinical features of the patients have a nuclear genetic cause. However, the contribution of both genomes to the observed features cannot be excluded either. Finally, our study had a relatively small sample size, especially in the HC group, which reduced the statistical power. Therefore, we were not able to detect statistically significant differences between the groups or to assess whether the presence of an mtDNA alteration was associated with a specific clinical feature.

Conclusions

mtDNA rearrangements with low levels of heteroplasmy were identified in blood samples from individuals with severe or profound ID, suggesting that these alterations may be present with higher levels of heteroplasmy in other postmitotic tissues, such as the brain. This is the first study to investigate the presence of mtDNA rearrangements in blood samples from patients with ID. Future studies are needed to confirm the present findings and to determine whether these alterations are a contributing factor to ID or a downstream effect of the neurodevelopmental process. Identifying the presence of mtDNA alterations in individuals with neurodevelopmental disorders is likely to have therapeutic relevance, as disorders with different aetiological causes may respond differently to specific treatments. In the present study, we found that mtDNA-targeted next-generation sequencing and the high-throughput MitoSAlt computational pipeline are very sensitive tools for detecting mtDNA rearrangements in blood and low levels of heteroplasmy.

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Table 1 Characteristics of the participants

	ID N = 76	ID-ASD N=59	HC N= 32	Compared groups	Statistics	P value
Sex, N (%)						
Male	48 (63)	32 (54)	17 (53)	ID *vs.* ID-ASD	$\chi^2 = 0.95$	0.295
Female	28 (37)	27 (46)	15 (47)	ID *vs.* HC	$\chi^2 = 0.95$	0.331
				ID-ASD *vs.* HC	$\chi^2 = 0.01$	0.919
Age, years	52.5 ± 10.2	41.3 ± 8.7	42.4 ± 12.4	ID *vs.* ID-ASD	t = 6.75	**< 0.001**
				ID *vs.* HC	U = 650	**< 0.001**
				ID-ASD *vs.* HC	t =-0.487	0.627
BMI, kg/m2	26.8 (17.6 - 45.8)	23.4 (17.1 - 40.8)	23.6 (17.5-31-1)	ID *vs.* ID-ASD	U = 1604	**0.007**
				ID *vs.* HC	U = 581	**< 0.001**
				ID-ASD *vs.* HC	U = 753	0.141
Tobacco use, N (%)						
No	69(92)	58 (98)	23 (72)	ID *vs.* ID-ASD	FET	0.134
Yes	6 (8)	1 (2)	9 (28)	ID *vs.* HC	$\chi^2 = 7.54$	**0.006**
				ID-ASD *vs.* HC	FET	**< 0.001**
ID severity, N (%)						
Moderate	1 (1.3)	0	-			
Severe	45 (59.2)	12 (20.7)	-	ID *vs.* ID-ASD	$\chi^2 = 21.4$	**< 0.001**
Profound	30 (39.5)	46 (79.3)	-			
CARS score	21 (14.5 - 39.5)	41 (28.0 - 55.0)	-	ID *vs.* ID-ASD	U =78.5	< 0.001
Genetic alterations, N (%)						
nDNA	7 (9)	39 (66)	-	ID *vs.* ID-ASD	$\chi^2 = 47.9$	**< 0.001**
				ID *vs.* ID-ASD		**0.023**
mtDNA	10 (13.2)	1 (1.7)	1 (3.1)	ID *vs.* HC	FET	0.169
				ID-ASD *vs.* HC		1

ASD: autism spectrum disorder; BMI: body mass index; CARS: Childhood Autism Rating Scale; FET: Fisher's Exact Test; ID: intellectual disability; mtDNA: mitochondrial DNA; N: number of cases; nDNA: nuclear DNA. Data are shown as the N (%), mean ± standard deviation, or median (range). Significant differences are indicated in bold.

Table 2 Characteristics of the mtDNA rearrangements identified in the participants of the study

Alteration N	Subject ID	Mean read depth	5' bkp	3' bkp	Hp % (range)	Alteration size	Final event
1	ID-008	190	61[§]	13581[*†,‡]	4.6	13521	dup
2	ID-011, ID-019,	190	8765[§]	8793[§†]	4.1	29	dup
	ID-101, ID-112				(2.0-6.7)		
3	ID-036	255	9422	15606[†,‡]	5.2	6185	del
4	ID-074	284	9003[§‡]	621	3.1	8186	del
5	ID-075	241	7112[†]	638	1.7	10096	dup
6	ID-083	378	8348[§]	11391[§]	1.3	11044	dup
7	**ID-101**	180	8895[§]	805[§‡]	1.4	8478	del
8	**ID-104**	176	8764	892[‡]	1.1	8698	dup
9			8580[§†]	2823[§‡]	2.0	10813	dup
10	**ID-ASD-152**	284	6075[†,‡]	11962[‡]	1.1	5886	del
11			1317[‡]	1117	1.7	6370	del
12	HC-019	161	9090	15606	2.8	6517	del

ASD: autism spectrum disorder; bp: base pair; bkp: breakpoint; del: deletion; dup: duplication; hp: heteroplasmy level; ID: intellectual disability; n.d.: not detected. Patients carrying more than one mtDNA alteration are highlighted in bold.

§: corrected breakpoint according to MitoBreak Classifier; †: breakpoint reported in the MitoBreak database; ‡: breakpoint reported in the Splice-Break dataset.

Figure 1 Study workflow including the initial number of participants and those included (in blue)/excluded (in red) after each quality control step. Created with Biorender.com.

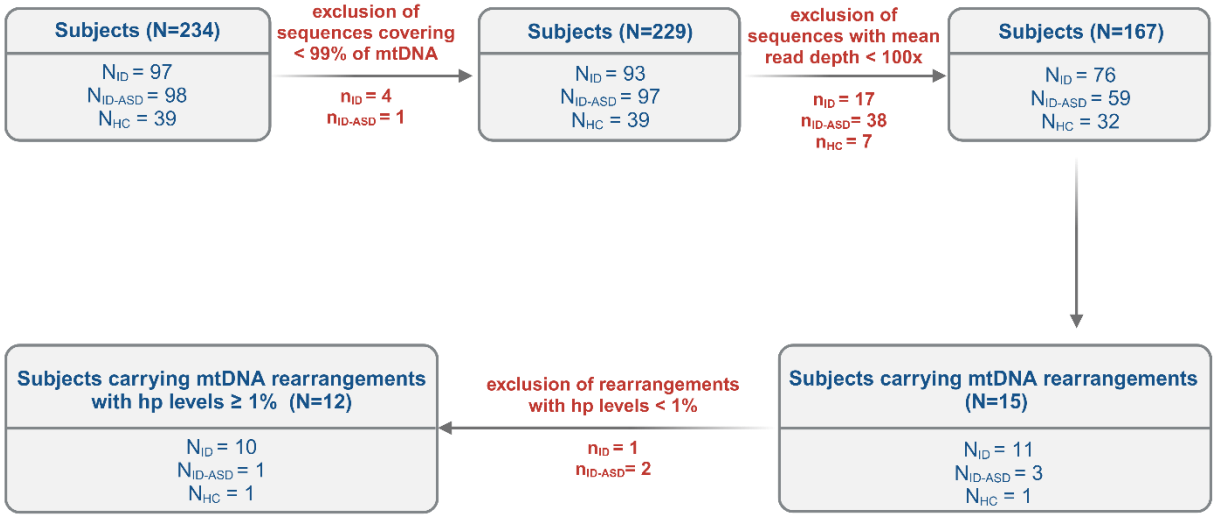


Figure 2. Illustrative plots showing the location of the mtDNA duplications (left) and deletions (right) detected by MitoSAlt. The 4 IDs represent ID-011, ID-019, ID-101, and ID-112, which carried the 29 bp duplication. Created with BioRender.com.

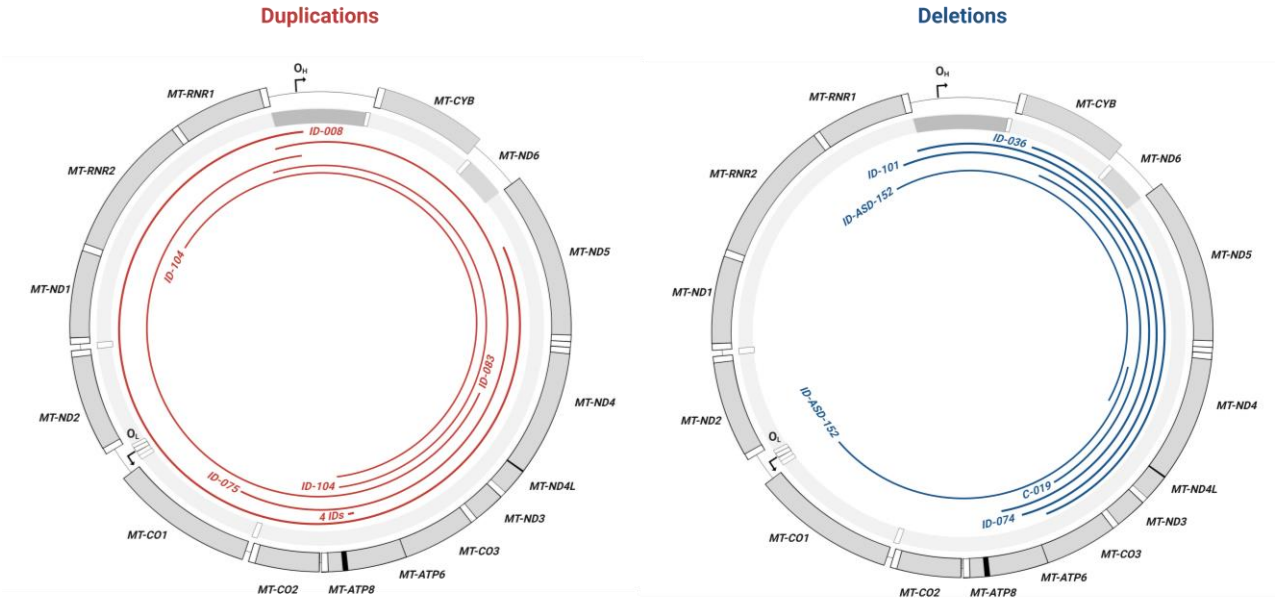
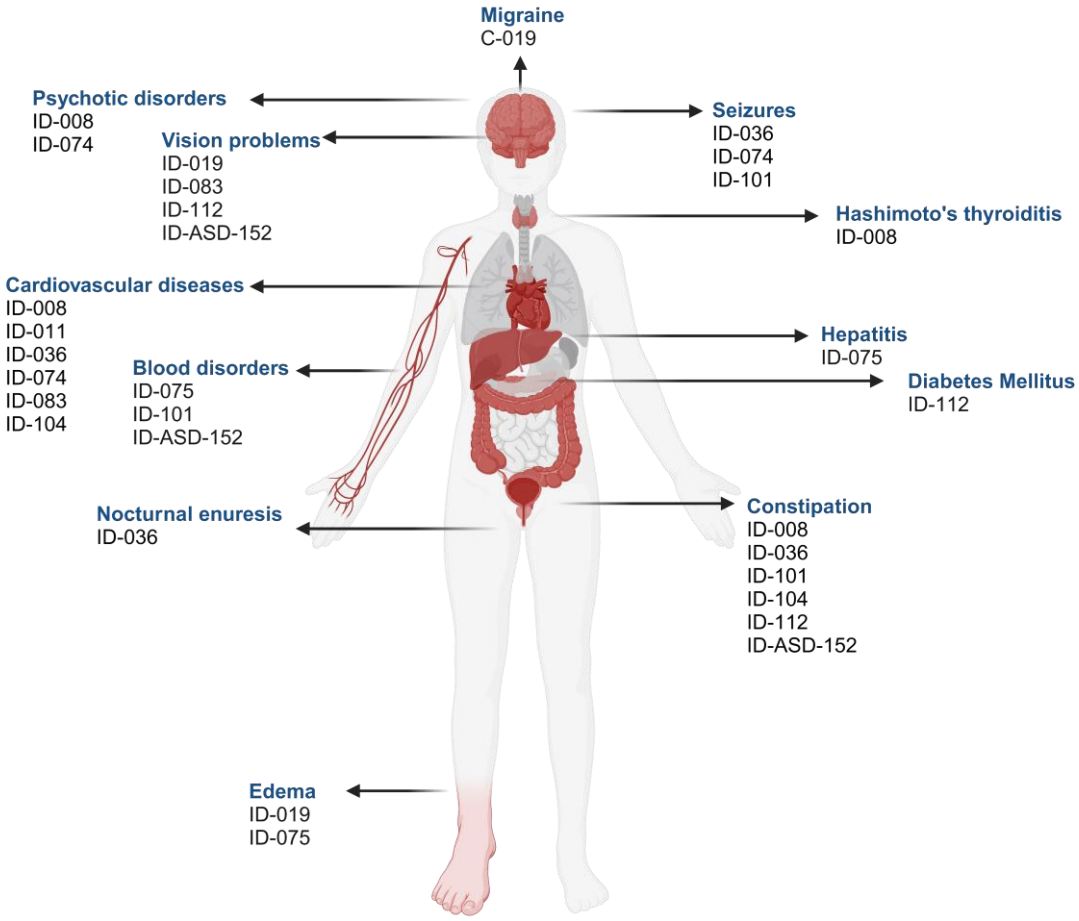


Figure 3. Clinical conditions associated with mitochondrial disorders present in individuals carrying mtDNA alterations. ASD: autism spectrum disorder; ID: intellectual disability. Created with BioRender.com.



Comparative analysis of mitochondrial DNA in postmortem brain tissue of patients with schizophrenia and controls. Bengisu K. Bulduk, Juan Tortajada, Alba Valiente-Pallejà, Luís Felipe Callado, Helena Torrell, Elisabet Vilella, José Javier Meana, Gerard Muntané and Lourdes Martorell

Article 3. Comparative analysis of mitochondrial DNA in postmortem brain tissue of patients with schizophrenia and controls

In the previous systematic review (article 1), rare mtDNA variants and specific mtDNA deletions with varying percentages were identified in the post-mortem brain tissue of patients with SZ. However, a substantial amount of data included in the systematic review originated from studies that used low-throughput technologies. As a result, there was a lack of comprehensive insight into the frequency and impact of mtDNA alterations on the pathogenesis of SZ.

To address the hypothesis that mtDNA alterations involved the pathogenesis of SZ, we conducted a comprehensive study to investigate mtDNA rearrangements, SNVs, and mtDNA-CN changes in the post-mortem brain samples of SZs and HC. The mitochondrial genome of the dorsolateral prefrontal cortex (DLPFC) was analysed using mtDNA-targeted next-generation sequencing (NGS) in 40 individuals diagnosed with SZ and 40 HCs. We identified mtDNA SNVs and rearrangements using the Mitoverse platform and the MitoSAlt pipeline, respectively. Additionally, we reported mtDNA-CN alterations using a quantitative PCR assay.

The analysis of mtDNA rearrangements identified 35 deletions in the SZ group and only 5 deletions in the HC group. As expected, the number of mtDNA deletions per participant was higher in the SZ group than in the HC group. Although there was no significant difference in deletion size and hp level between the groups, SZ patients had larger deletions and higher hp levels (median size = 3931 bp; median hp = 9.3%) compared to HC (median size = 3640 bp; median hp = 6.2%). No duplications with hp ≥5% were detected in the SZ group, whereas HC had 5 duplications. MtDNA deletions in individuals with SZ were predominantly initiated at *MT-ND4* and concluded at *MT-CYB* whereas deletions in HCs included these regions as well as *MT-RNR2*. Duplications in HC mostly begin at *MT-CYB* and end at the D-loop. The number of likely pathogenic variants was significantly higher in the SZ patients (n=14) compared to the HC (n=3). *MT-ND5* gene was the most affected gene with five different variants observed in SZ group, and one variant in a HC. Additionally, the pathogenic m.3243 A>G variant in *MT-TL1* was reported in one SZ patient, with a high heteroplasmy level of 32.2%. Previously reported rRNA variant, m.792 C>T in *MT-RNR1*, was identified in a HC. In mtDNA-CN analysis, no significant difference in mtDNA-CN was found between SZ and HC groups, although mtDNA-CN was significantly higher in antipsychotic users compared to non-users.

The main limitation of our study was the small sample size for adequately powered case-control comparison. Secondly, our study was based on analysis of only one region of brain, DLPFC, and our first systematic review have already shown that mtDNA alterations varies depending on

factors such as the specific brain region, cell type, and subcellular location. Finally, our study employed state-of-the-art molecular and bioinformatic tools in which no consensus on the standardized use of these sensitive pipelines.

Our study is the first to conduct a comprehensive analysis of mtDNA alterations, involving deletions and duplications, SNVs and mtDNA-CN in postmortem brain tissue from both SZ patients and HC individuals. Further studies in different type of brain regions are required to confirm the excess of pathogenic variants in SZ in postmortem brain tissue and to determine the role of the identified hp levels in mitochondrial function.

Comparative analysis of mitochondrial DNA in postmortem brain tissue of patients with schizophrenia and controls

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Abstract

Background: Extensive evidence supports the presence of mitochondrial dysfunction in schizophrenia (SZ), implicating potential involvement of mitochondrial DNA (mtDNA) alterations. This study aimed to examine the occurrence of mtDNA alterations, including mtDNA rearrangements, single nucleotide variants (SNV), and copy number changes (mtDNA-CN), through comprehensive analysis of postmortem brain samples obtained from individuals diagnosed with SZ and healthy controls (HC).

Methods: MtDNA extracted from the dorsolateral prefrontal cortex of 40 SZ individuals and 40 HC subjects underwent examination using mtDNA-targeted next-generation sequencing (NGS) and quantitative real-time polymerase chain reaction (qPCR). The MitoSAlt pipeline allowed the identification of mtDNA rearrangements, while the Mitoverse platform, together with APOGEE 2 and MITOMAP, was employed for the detection and pathogenicity assessment of mtDNA SNV. Absolute quantification following qPCR analysis determined mtDNA-CN.

Results: Our analysis revealed that 35% of SZ patients exhibited mtDNA alterations, in contrast to only 10% of HC individuals. Notably, the SZ group displayed a significantly higher number of deletions compared to the HC group (35 vs. 5, respectively), with patients exhibiting a greater mean count than controls (3.8 vs. 1.0, respectively; $t = 2.502$, $p=0.03$). The prevalence of likely pathogenic missense variants was also elevated in SZ compared to HC (10 patients vs. three HC; $\chi^2= 4.3$; $p=0.038$), encompassing 14 variants in patients and three in HC. The pathogenic tRNA variant, m.3243 A>G, was identified in one patient, exhibiting a high heteroplasmy level of 32.2%. Mean heteroplasmy levels for mtDNA rearrangements and SNV were 7.8% and 12%, respectively. While no significant disparities in mtDNA-CN were observed between SZ and HC, antipsychotic users exhibited significantly higher mtDNA-CN compared to non-users.

Conclusions: Our findings propose a potential role of mtDNA alterations, particularly involving rearrangements, likely pathogenic missense variants, and the m.3243A>G variant, in the pathophysiology of SZ. However, further functional studies are imperative to validate the impact of the identified heteroplasmy levels on mitochondrial function.

Keywords: Mitochondrial DNA, schizophrenia, postmortem, brain, DLPFC, next-generation sequencing

1.Introduction

Schizophrenia (SZ) is a complex spectrum disorder that affects approximately 1% of the world's population and represents a significant burden in terms of morbidity and mortality (Birnbaum & 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 (American Psychiatric Association, 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 critical in the development of SZ (Trifu et al., 2020). Genetic risk factors are thought to include thousands of common genetic variants (single nucleotide polymorphisms, SNPs), each having a small effect on an individual's risk, and a plethora of rare gene variants (copy number variants, CNVs), which have a larger individual effect on risk. 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., 2023). However, despite the identification of SNPs and CNVs involved in SZ, the specific set of genetic factors underlying the pathophysiological mechanisms remains unknown (Wahbeh & 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 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 1,124 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 (T. J. Nicholls & 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 1,000 mtDNA molecules in a human cell (Valiente-Pallejà et al., 2022) even

though human oocytes contain between 100,000 and 200,000 mtDNA copies (Otten & Smeets, 2015), and some recent estimates indicate that a single neuron may reach two million mitochondria (Misgeld & Schwarz, 2017). Given the high energy requirements of brain tissue and therefore the high number of mitochondria, the brain is highly susceptible to mitochondrial dysfunction (Nissanka & Moraes, 2018).

Systemic abnormalities associated with SZ, such as inflammation (Müller, 2018), redox dysregulation (S. Y. Kim et al., 2017) and oxidative stress (Jiang et al., 2019) involve mitochondria with several lines of evidence suggesting that mitochondrial abnormalities could contribute to the disrupted brain functioning (Konradi & 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 (somatic) during lifetime, 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 including polyploidy and mutation rate. Polyploidy refers to the presence of multiple copies of mtDNA in a cell, allowing for mutations to occur only within 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 nDNA (Sharma et al., 2005). mtDNA mutations include single nucleotide variants (SNVs) and large rearrangements (deletions and duplications), with a hot spot for mtDNA SNVs being the D-loop region and deletion events typically centered in the mitochondrial major arc (Bua et al., 2006). In addition, due to the polyploidy nature of the mtDNA, changes in mtDNA copy number (mtDNA-CN) can cause mitochondrial dysfunction and has been suggested as a predisposing factor for neurodegeneration (Venkatesan et al., 2023). To date, more than 300 SNVs and rearrangements have been identified in the mtDNA as contributing to primary mitochondrial disorders (H. Li et al., 2019), although only 95 have been confirmed as pathogenic based on robust evidence (Gusic & Prokisch, 2021). This highlights the difficulty in interpreting the impact of mtDNA variants in heterogeneous complex diseases such as SZ. 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 specimens were collected during autopsies performed between2010 and 2018 at the Basque Institute of Legal Medicine (Bilbao, Spain), in accordance with the guidelines of 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 postmortem interval (PMI) < 24 h were included. Grey matter specimens 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). Retrospective search into subjects' medical records was conducted for antemortem diagnoses of SZ meeting DSM-IV or ICD-10 criteria. Clinical diagnoses of SZ were all performed by a board-certified psychiatrist of the Basque Healthcare System (Osakidetza). Cases with additional psychiatric or neurologic diagnoses, including a history of substance abuse, were excluded. Samples from 40 SZ cases meeting the above criteria were assayed in the present study. Each SZ case was paired to a matched HC subject, matching case's sex, age, and PMI, and with no evidence of psychiatric or neurological conditions, according to available antemortem medical records. Demographic characteristics of all HC and SZ subjects are summarized in **Table 1**. Total DNA was extracted from post-mortem tissues using Gentra Puregene ® reagents (Qiagen, Germany) according to established procedures (Torrell et al., 2013).

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

	HC	**SZ**
Sex	40	40
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 (%)		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 mtDNA-targeted next-generation sequencing (NGS)

2.2.1 Enrichment of mtDNA

A 50-ng aliquot of total DNA served as the template for amplifying 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 outlined in Supplementary Table 1.

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 participant). DNA libraries were prepared using the Illumina DNA Prep Kit with Tagmentation (Illumina Inc., Ref. 20018705, Madrid, Spain) according to the manufacturer's instructions. Integrated DNA Technologies (IDT®) for Illumina® DNA/RNA Unique Dual Indexes were used (Illumina Inc., CA, USA). 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 2x150 pb paired-end protocol.

2.2.3 Quality control of NGS data

Quality control was performed on FASTQ files using FastQC v0.11.9 (S. Andrews, 2010) and MultiQC v.1.14 (Ewels et al., 2016). Cutadapt (v.3.4) (M. 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 (H. Li et al., 2009).

2.2.4 Analysis of mtDNA rearrangements

MitoSAlt (v.1.1.1) was used to detect and quantify mtDNA rearrangements (S. Basu et al., 2020). We used default parameters and the maximum size of tolerated deletions was set to 16055 bp as this is the maximum reported mtDNA rearrangement (Damas, Carneiro, et al., 2014). Deletions beyond 16055 bp were automatically reclassified as potential duplications in the reverse orientation based on the tool's algorithm (S. Basu et al., 2020). For the classification of potentially pathologic alterations, the heteroplasmy (hp) threshold was set at 5%. 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., 2016, 2021). Nonsynonymous variants with a MutPred score of 0.5 or greater, indicating potential deleterious effects, were included in the analysis. APOGEE 2, a mitochondrially-centered ensemble method, was used to predict the pathogenicity of missense variants (Bianco et al., 2023a). 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 noticeable 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). 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 & 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*). The D-loop region was amplified using the forward primer 5'- CACTTTCCACACAGACATCA and the reverse primer 5'- TGGTTAGGCTGGTGTTAGGG; the B2M region was amplified using the forward primer 5'- TGTTCCTGCTGGGTAGCTCT and the reverse primer 5'- CCTCCATGATGCTGCTTACA. 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 & 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-square test when less than 20% of cells had less than 5 observations, otherwise Fisher's exact test was used (H.-Y. 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 the statistical software jamovi 2.3.28 (The jamovi, 2022).

3. Results

3.1 Large-scale mtDNA rearrangements

We obtained mtDNA sequence data from 40 individuals with SZ and 39 HC. One HC was excluded due to the inability to amplify their mtDNA. For the 79 included samples, we achieved complete coverage of all bases (100%) with a mean depth of 10,102 reads per base.

In our dataset, 44 rearrangements were detected (median size = 3888 bp; median hp = 9.2%). 23% of the individuals in the SZ group and only 13% of HC showed mtDNA rearrangements (**Table 2**) (Supplementary Table 3).

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.

The characteristics of the mtDNA rearrangements were then examined. The 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). Although no significant difference in deletion size and hp level was observed between groups, SZ patients had larger deletions and higher hp levels (median size = 3931 bp; median hp = 9.3%) compared to HC (median size = 3640 bp; median hp = 6.2%) (U=84, p=0.98 for size and U=65, p=0.42 for hp level). 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 relationship was observed between the presence of mtDNA rearrangements and age, sex, or PMI (Supplementary Table 2). Regarding the location of the mtDNA rearrangements, as mentioned above, individuals with SZ did not carry a duplication and 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, the majority of 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 involve *MT-ND4, MT-ND5*, but also *MT-CYB*.

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. Moreover, SZ-4 and SZ-14 shared three additional deletions affecting the loci between *MT-ND4L* and *MT-CYB* (size = 5122, mean hp= 8%), between *MT-ND4* and *MT-RNR2* (size= 7961, mean hp= 14.7%), and between *MT-ND4L* and *MT-CYB* (size= 3888, mean hp= 11.8%).

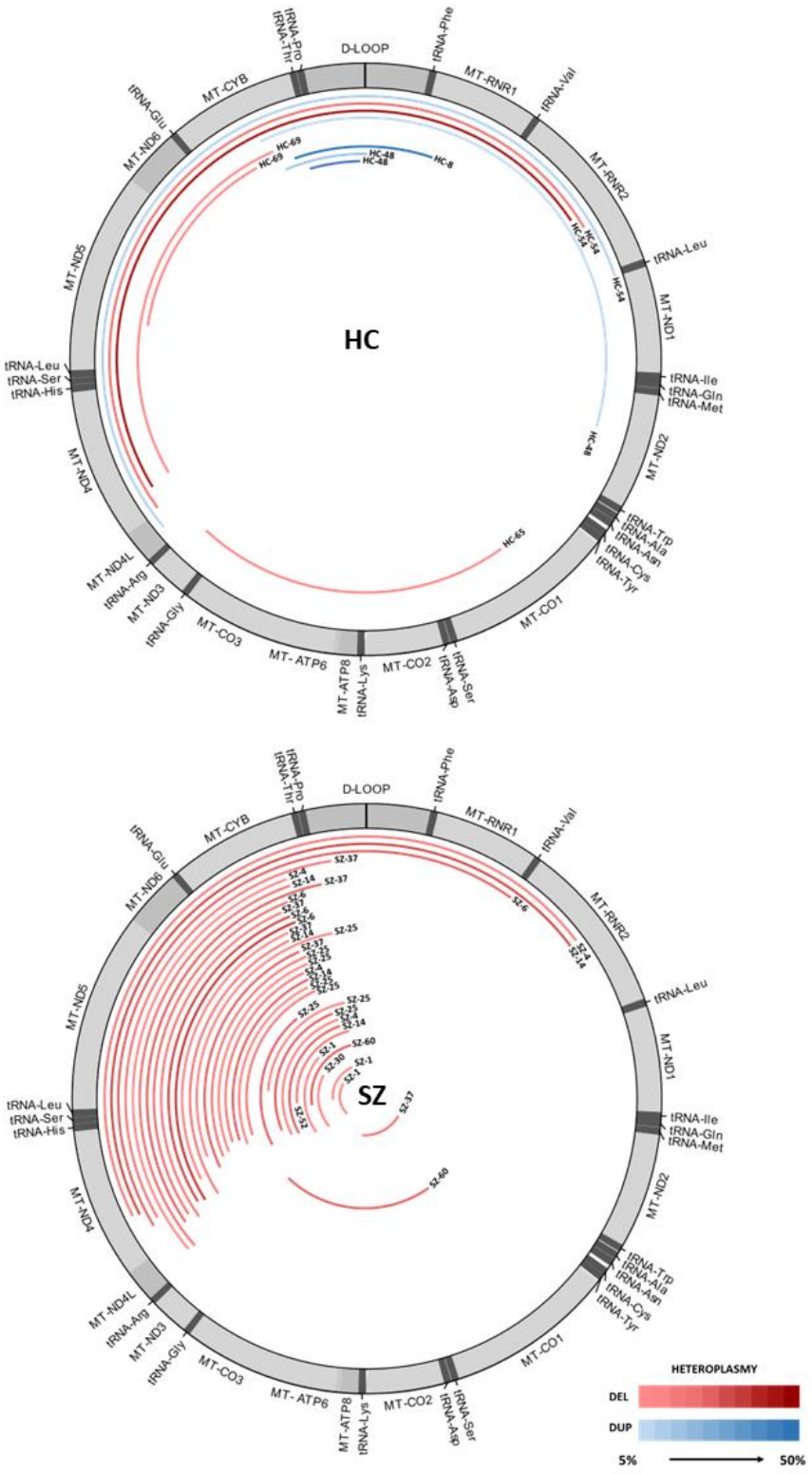

Figure 1. Distribution of all rearrangements identified in SZ patients and HC on the mtDNA map. The circular plot above shows the five deletions and five duplications detected in HC. The circular plot below shows the 34 deletions detected in SZ patients. Deletions are shown as red arcs and duplications 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.

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 and the hp≥5% threshold. Following these criteria, 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, Figure 2**). Eight of the 17 variants were not previously reported. The number of LP variants was significantly higher in the SZ patients compared to the HC (χ^2= 4.3; p=0.038). 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, which was the most affected gene, with six different variants identified, five variants observed in SZ patients and one variant in a 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 a HC. Additionally, LP variants were observed in the *MT-ND1, MT-ND2, MT-ND3, and MT-ATP6* genes.

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

Variant	Group	ID	Amino acid change	Gene	Hp (%)	Population frequency
m.3791 T>C	HC	10	Leu162Pro	*MT-ND1*	7.5	<0.00001
m.4852 T>C	SZ	14	Leu128Pro	*MT-ND2*	9.2	<0.00001
m.8786 T>A	SZ	14	Leu87His	*MT-ATP6*	5.9	0,00003
m.10228 T>C	SZ	21	Leu57Ser	*MT-ND3*	7.1	0
m.11232 T>C	SZ	42	Leu158Pro	*MT-ND4*	5.2	<0.00001
m.11385 T>C	SZ	21	Leu209Pro		9.6	0
m.11404 A>T	SZ	42	Trp215Cys		5.1	0
m.12584 A>G	HC	54	Asp83Gly	*MT-ND5*	5.9	0
m.13076 T>C	SZ	6	Leu247Pro		5	0
m.13268 G>A	SZ	41	Gly311Glu		26.1	0
m.13339 T>C	SZ	32	Phe335Leu		7.6	<0.00001
m.13360 T>C	SZ	14	Cys342Arg		7.3	0
m.13486 C>T	SZ	30	Pro384Ser		6.2	0
m.14849 T>C	SZ	37	Ser35Pro	*MT-CYB*	7.9	<0.00001
m.15129 T>C	HC	69	Phe128Ser		17.2	<0.00001
m.15171 G>A	SZ	60	Gly142Ala		12.6	0.00002
m.15239 T>C	SZ	52	Trp165Arg		19	<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.

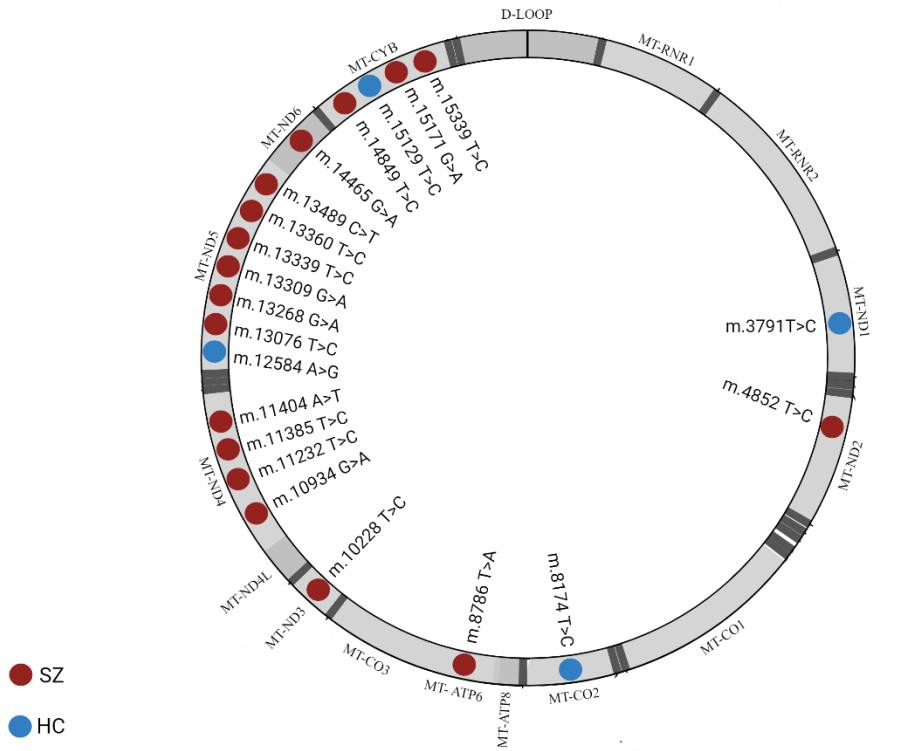

Figure 2. Distribution of pathogenic nonsynonymous variants on the mtDNA map. HC: healthy control; SZ: Schizophrenia

Excluding haplogroup markers, we identified 30 tRNA and 17 rRNA variants in the ribosomal and transfer genes. Only five tRNA variants (29%) and one rRNA variant (3%) were previously reported in MITOMAP, and information about pathogenicity scores could only be obtained from these variants and not from the non-reported variants. Regarding the reported variants, four tRNA variants were detected in the SZ group: m.636 A>G (hp 99.7%), m.3243 A>G (hp 32.2%), m.4317 A>G (hp 97.6%), and m.5819 T>C (hp 5.6%), while only one tRNA variant, m.16002 T>C (hp 7.2%), was reported in a 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%. Therefore, one tRNA variant was confirmed as pathogenic and the remaining four variants had a “reported” status in MITOMAP, which means that the variant could be considered

as possibly pathogenic. Finally, we detected only one reported rRNA variant, m.792 C>T in *MT-RNR1*, (hp 99%) in a HC (Supplementary Table 5).

3.3 mtDNA-CN

DNA samples from 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 subjects with SZ and HC (*U*=554, p=0.654) (Figure 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) (Figure 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, Figure 3B). No significant difference in mtDNA-CN was observed regarding the use of antidepressants (U=85, p=0.691) (Figure 3C). Similarly, no significant difference was observed between benzodiazepine users (n=19) and non-users (n=50) (U=450, p=0.742). In addition, there were no significant differences in mtDNA-CN between individuals with (n=11) and without (n=57) mtDNA rearrangements (U=271, p=0.484).

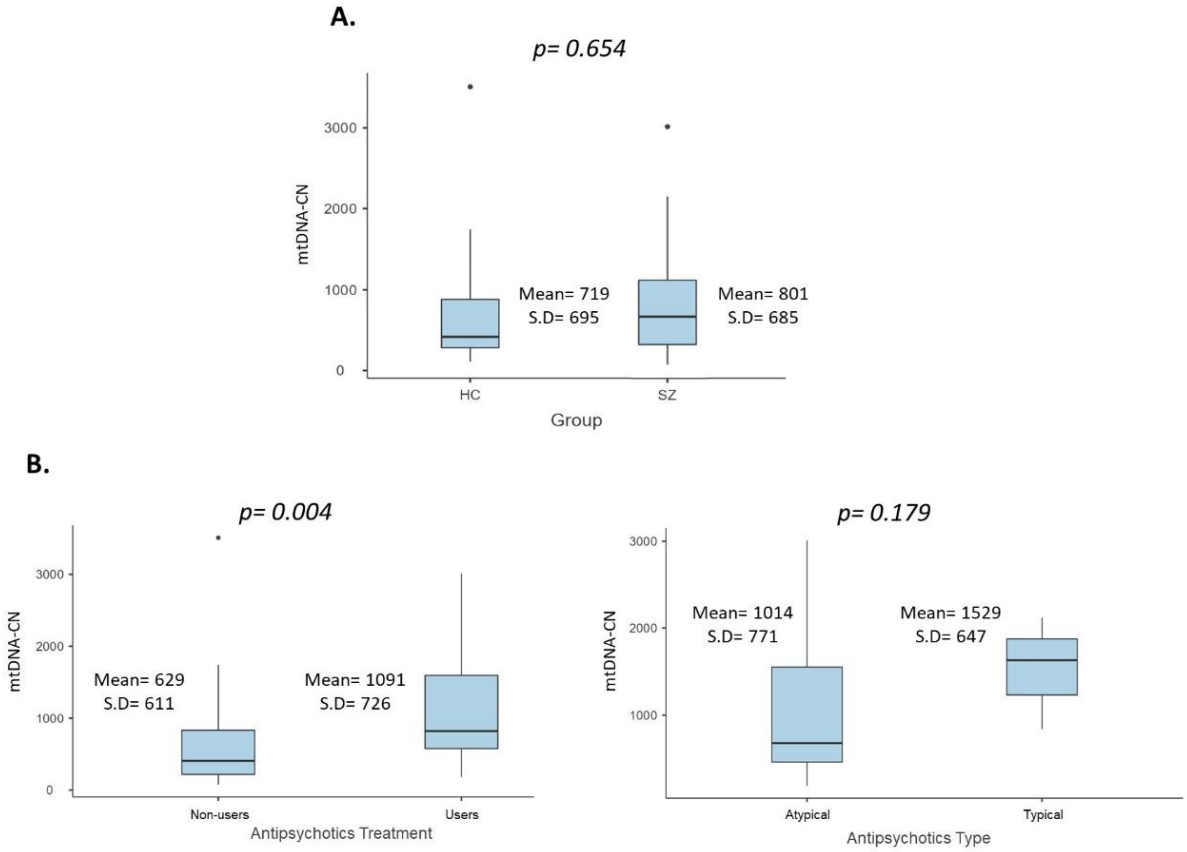

Figure 3. Comparison of mtDNA-CN: A) Between patients with SZ and HC; **B)** Among antipsychotic users and non-users; and **C**) Among individuals treated with typical or atypical antipsychotic treatment.

4. Discussion

In this study, we analyzed all possible 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.

In the analysis of mtDNA rearrangements, we decided to use a hp cutoff of 5% to detect deletions or duplications that may have an impact on mitochondrial function, and to discard variants with hp levels below 5% because very low levels of hp occur in the general population (Y. Guo et al., 2013). We identified a higher number of 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 result suggests that some mechanism related to mtDNA replication may be involved in specific SZ patients supporting previous evidence that mtDNA deletions may play a role in psychiatric disorders (Das et al., 2022; Hjelm et al., 2019b).

The majority of deletions covered the major arc of mtDNA, including a region between the *MT-ND4* and *MT-CYB* genes. This region contains crucial 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 due to the loss of protein-coding genes along with tRNA and mRNA transcripts (Sanchez-Contreras & 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 a direct pathological etiology of SZ (Das et al., 2022). We did not have data on the symptoms of the SZ patients and could not confirm these previous findings. In contrast to the results obtained with regard to deletions, the present study did not find evidence that duplications play a role in SZ, since no duplication was observed in any of the patients, whereas they were present in HC. In any case, it is worth mentioning that current knowledge about duplications is limited compared to deletions. Aging has been reported to be a significant 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 & 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 of middle age (mean 48.5 years). Large-scale duplications of mtDNA were first documented in individuals with Kearns-Sayre syndrome (KSS) in 1989 (Poulton et al., 1989) and in Pearson syndrome (PS) in 1990 (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 & Falk, 1993). The reported hp level in muscle of affected individuals are 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 hematologic 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, MitoBreak, the mtDNA breakpoint database, reported only 44 duplications out of 1356 rearrangements, all involving the origin of replication in the heavy chain, and primarily associated with these mitochondrial disorders and aging (Damas, Carneiro, et al., 2014). mtDNA

duplications have been reported in patients with SLSMDS and correlated with onset of symptoms before the age of 15 years; 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.

For the analysis of nonsynonymous variants, we focused only on variants that are classified as LP according to the latest prediction models (Bianco et al., 2023a). We identified 17 LP variants, 9 variants that were previously reported and 8 variants that have not been previously reported. Our study identified that 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 three HC individuals each 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 variants identified in the patients were located in 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 and also the highest hp level (26.1%), which was identified in m.13268 G>A, with the amino acid change Gly311Glu, 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 to contribute to the genetic diagnosis of novel mutations and to elucidate the mtDNA 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 and focused on the analysis of common and rare variants (with minor allele frequency (MAF) < 1%) identified a higher frequency in patients with SZ than in HC, in agreement with the present study (Gonçalves et al., 2018). According to tRNA and rRNA variants, available pathogenicity prediction tools are less common than for the nonsynonymous variants, and data on unreported variants are currently not available. According to the reported variants, we identified five tRNA and one rRNA variants in the data set that were more frequent in SZ patients (5 variants) than in HC individual (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 (SZ-15). This variant has been reported in healthy individuals with hp levels <1%, suggesting that levels >1% may be diagnostically significant (Matsumoto et al., 2023). Notably, m.3243A>G hp levels of 10–30% have been associated with autism (Pons et al., 2004). Finally, the only rRNA identified in this study, *MT-RNR1* m.792 C>T, was observed in a 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.

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 & Czajka, 2013; Valiente-Pallejà et al., 2022). However, in our study, no significant differences in mtDNA-CN were found between SZ and HC adding further evidence to previous studies (Sabunciyan et al., 2007; Torrell et al., 2013). Previous 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 the age of the antipsychotic treatment received (P. 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 indicated that mtDNA-CN was reduced with the use of clozapine and risperidone (P. Kumar et al., 2019). 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.

Several limitations need to be considered when interpreting these results. First, the study sample size was insufficient for adequately powered case-control comparisons. Second, our study was based on analysis of the dorsolateral prefrontal cortex 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. For example, the occipital cortex plays a role in the interpretation of visual images, and may be associated with visual hallucinations in SZ, and should not be overlooked in the further studies (Torrell et al., 2013). 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 of reads that we obtained lends strength to the low levels of hp that we observed.

This study conducted an exhaustive examination of mtDNA alterations in postmortem brain tissue from individuals diagnosed with SZ and HC. In conclusion, our findings reveal a notable increase in the frequency of mtDNA alterations in SZ patients contrasted with HC, suggesting a potential involvement of mtDNA in the pathophysiology of SZ. Subsequent investigations are imperative to validate the increased prevalence of pathogenic variants in postmortem brain tissue of SZ individuals and to elucidate the functional implications of the identified heteroplasmy levels in mitochondrial function.

Supplementary figure of this article is available at https://doi.org/10.34810/data1078.

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VII. DISCUSSION

The brain is the most complex organ in the human body, accounting for 2-3% of body weight. It consumes up to 20% of our oxygen and 25% of our glucose (Pei & Wallace, 2018). The brain is primarily powered by mitochondria and maintains a high metabolic activity, making it particularly sensitive to mitochondrial dysfunction. Alterations (mutations) in the mitochondrial genome, such as point mutations or large-scale deletions, can affect the function of the proteins encoded by the mtDNA. This can compromise the efficiency of the electron transport chain and ATP production. Genetic variations and rearrangements can be inherited or occur at the germline or somatic level (Nissanka & Moraes, 2018). The presence of multiple deletions and duplications in mtDNA can result from the accumulation of multiple errors in postmitotic tissues (P. Chinnery, 2022). Symptoms associated with OXPHOS failure typically occur when pathogenic SNVs reach approximately 80% of the heteroplasmy level, or when deleted or duplicated mtDNA molecules exceed a heteroplasmy threshold of 60% (Zeviani & Di Donato, 2004). Meanwhile, mtDNA-CN can vary among individuals and within different tissues of the same individual. Several factors contribute to these differences, including cell type, cellular activity and metabolic state, environmental factors, genetic factors and disease conditions such as mtDNA depletion syndromes (Castellani et al., 2020) and ageing (Filograna et al., 2021). Inevitably, these alterations in mtDNA can lead to a variety of brain disorders, from neurodevelopmental to psychiatric diseases.

The first article of this thesis provided a comprehensive summary of the identified mtDNA alterations in postmortem brain samples from individuals with MitD, NeuD, PsyD, and ageing. Our results showed that the majority of pathogenic SNVs reported in postmortem tissues of patients with MitD typically have higher heteroplasmy levels than the threshold for biochemical manifestations, which was set to be higher than 80% (Tranah et al., 2018). The most important finding about these pathogenic SNVs in MitD is the different heteroplasmy levels in different post-mitotic tissues and different brain regions, probably due to differences in the metabolic activity rates of the cells. In the same study, we found no evidence for a significant role of pathogenic SNVs in NeuD, PsyD and ageing. However, recent studies have reported that not only SNVs in mtDNA but also mitochondrial haplogroups may be involved in the pathomechanisms of these disorders affecting the cognitive progression in AD and PD (G. Liu et al., 2023; Smullen et al., 2023). Some rare variants with a low heteroplasmy level were detected in the brain of PsyD patients, whereas a well-defined m.3423A>G MELAS variant was detected in one SZ and two BD patients with a low heteroplasmy level of 0.6%-1% (Munakata et al., 2005). In our third study, we also identified the m.3243 A>G variant, but with a relatively high heteroplasmy level (32.2%) in an SZ patient. m.3243 A>G variants is a *MT-TL1* variant that is associated with many mitochondrial

dysfunctions (Cai et al., 2022; Esterhuizen et al., 2021). In addition, m.3243A>G has also been reported in ASD with heteroplasmy levels of 10-30% (Pons et al., 2004), and another study found that individuals with the m.3243A>G variant often have PsyD with a prevalence of 37% (Shen & Du, 2021). We also identified five tRNA and one rRNA variants in the dataset that were more common in SZ patients (5 variants) than in HC individuals (1 variant). This variant has also been reported in healthy individuals, but at low heteroplasmy levels (<1%) (Matsumoto et al., 2023), suggesting that m.3243A>G may have an impact on brain disorders if it exceeds 1% heteroplasmy levels, and supportting the hypothesis that some individuals with MitD may experience psychiatric symptoms before being diagnosed or affected (Kasahara & Kato, 2018).

Many MitD studies have reported single or multiple mtDNA deletions in diseases such as KSS and POLG-related syndromes (Jacoby et al., 2022). A higher number of mtDNA deletions at elevated heteroplasmy levels were found in brain samples compared to blood, resulting in impaired energy production (El-Hattab et al., 2017). Furthermore, some deletions identified in the brain were not present in the blood. Due to the continuous replacement of blood cells throughout life, damaged mitochondria are removed, leading to a gradual decrease in mtDNA deletions over time (Palozzi et al., 2018, Ahmadi et al., 2019). Studies of NeuD have reported variation in mtDNA deletion load across brain regions, supporting the hypothesis that brain regions with high levels of dopamine metabolism, such as the substantia nigra, have a greater number of mtDNA deletions than other regions (Grünewald et al., 2019). In NeuD, the deletions detected may also be associated with age-related mitochondrial decline, which is characterized by the accumulation of somatic mtDNA deletions in post-mitotic tissues (Hjelm et al., 2022). Our findings in the first study also suggested that bioenergetic defects due to mtDNA deletions may lead to the manifestation of psychiatric symptoms. However, although an initial study in 1997 reported an increased frequency of the common mitochondrial deletion in the cerebral cortex of people with BD compared with age-matched controls (Kato et al., 1997), subsequent studies with larger sample sizes failed not confirm this association in individuals with schizophrenia and bipolar disorder (Sabunciyan et al., 2007).

In contrast to deletions, we did not find any studies reporting mtDNA duplications in our systematic review. This may be due to the fact that the majority of the studies reported in our systematic review used low-throughout genetic analysis, such as southern blot or PCR, to identify of mtDNA rearrangements. Our understanding of mtDNA duplications is limited because many current bioinformatic tools predict deletions through gapped alignments but overlook that each event may indicate either a deletion or a duplication due to the circular nature of mtDNA (S. Basu et al., 2020). Some studies have suggested that mtDNA duplications may play a pathogenic role

in generating mtDNA deletions through homologous recombination and determining the clinical expression of syndromes such as KSS and PEO (Odoardi et al., 2003; Poulton, 1992). There is a notable lack of mtDNA rearrangements in the literature, especially those dealing with duplications, using sensitive and accurate tools (Damas, Carneiro, et al., 2014; Fontana & Gahlon, 2020). This gap motivated our next study, in which we aimed to investigate both mtDNA deletions and duplications, in the blood samples of adults with ID, ID-ASD and HC using a highly sensitive and accurate pipeline for both deletions and duplications, MitoSAlt (S. Basu et al., 2020).

We reported that patients with ID are more likely to carry mtDNA rearrangements in their blood than HCs, although statistical significance was not reached, probably due to the small sample size of the study. In the study, we reported novel six deletions and six duplications in 13.2% of individuals with ID, 1.7% of individuals with ID-ASD, and 3.1% of HCs. The deletions were identified in the range of 5.9-8.4 kb, similar to previous findings of 2.4-7.9 kb mtDNA deletions identified in children with ASD (Varga et al., 2018). *MT-ND4,* which plays a critical role in complex I assembly (Giachin et al., 2016), was deleted (or partially deleted) in most of the deleted mtDNA molecules we identified. This finding is also consistent with the postmortem brain study reported in the first study of the thesis, which showed that 44% of ASDs had deleted *MT-ND4* (Gu et al., 2013). The majority of deletions were reported in the *MT-ATP6*, *MT-ND3, MT-ND4, MT-ND4L, MT-ND5*, and *MT-ND6* genes, which could lead to defects in ATP generation. We also identified a significant 13.5 kb duplication that includes both replication origins, the entire *MT-ND4* gene, and part of the *MT-ND5* gene. The largest duplication reported in MitoBreak, a comprehensive online resource with curated datasets of mtDNA rearrangements (Damas et al., 2014), is also a 13.4 kb duplication that includes the portion of the *MT-ND5* gene and a portion of the *MT-ND4* gene, which has been reported in both muscle and blood of KSS patients (Wong et al., 2003). Our study also identified a duplication in four patients with ID that could also cause a shorter *MT-ATP6* transcript, probably resulting in a non-functional ATP6 subunit. These duplications showed a low level of heteroplasmy (range 1.1% to 6.7%) in the blood, and they may not have a major effect on the blood, but they could present with higher levels of heteroplasmy in the brain. Our results also showed that 55% of carriers with mtDNA rearrangements had a BMI in the overweight or obese range, suggesting a possible role in metabolic symptoms. We also reported that ID patients with mtDNA rearrangements also had congenital heart disease or a heart murmur, congenital cataracts, which have been reported to occur in the majority of patients with mitochondrial disease (Lopriore et al., 2022), and psychotic symptoms and seizures, which are common psychiatric manifestations in mitochondrial disease (Klein et al., 2021).

In this thesis we assessed mtDNA deletions and duplications in post-mortem brain samples from SZ and HC using the same techniques as in the previous study. We reported that the number of mtDNA deletions per participant was significantly higher in the SZ group than in the HC group. Although there was no significant difference in deletion size and hp level between the groups, SZ patients had larger deletions and higher hp compared to HC. Contrary to what we observed in blood samples from patients with ID, no duplications were detected in brain samples from SZ patients, whereas they were present in five HC. This finding suggests that a mechanism related to mtDNA replication may be involved in certain individuals with SZ, supporting previous evidence that mtDNA deletions may play a role in PsyD (Das et al., 2022; Hjelm et al., 2019b). The major arc of mtDNA containing *MT-ND4, MT-ND5*, and *MT-ND6* for complex I and *MT-CYB* for complex III, was largely deleted. This deletion could significantly disrupt mitochondrially encoded ETC components (Sanchez-Contreras & Kennedy, 2021). A recent study found a correlation between the increased mtDNA deletion burden in the DLPFC of SZ and the presence of anxiety and depression, rather than a direct pathological aetiology of SZ. (Das et al., 2022). However, we could not confirm this association as we did not have the clinical symptoms of the patients.

Regarding mtDNA-CN, we did not report consistent results in NeuD and PsyD, although low mtDNA-CN has been frequently reported in in studies of NeuD, particularly in AD (Gramegna et al., 2018; Scholle et al., 2020; Tzoulis et al., 2013, 2014). The decrease in mtDNA-CN in AD may be associated with worse cognitive performance (S. J. Andrews & Goate, 2020; Gao & Ma, 2022; Y. Zhang et al., 2023), suggesting that mitochondrial function plays a role in the pathogenesis of AD. In addition, we have reported that metabolic disorders may coexist with NeuD, but have often been overlooked in studies. In contrast, one study found that diabetic individuals had increased mtDNA-CN in all brain regions (Thubron et al., 2019). In non-diabetic AD subjects, the loss of mtDNA could lead to a reduction in mitochondrial mass and bioenergetic capacity. Conversely, in diabetic AD subjects, increased nutrient supply due to insulin resistance and hyperglycaemia could lead to decreased oxidative phosphorylation and increased glycolysis, resulting in an energy deficit (Galizzi & Di Carlo, 2022). This decrease in mtDNA-CN may also be associated with natural ageing (Hjelm et al., 2022). Most studies in MitD have also reported low mtDNA-CN, but this varies depending on diagnosis and brain region. (Brinckmann et al., 2010; Götz et al., 2008). An increase in mtDNA-CN may be associated with an attempt to restore and normalise the overall copy number (Nissanka & Moraes, 2020). Although some studies have suggested a possible effect of SNVs on mtDNA-CN levels, it is not possible to draw a definitive conclusion (Brinckmann et al., 2010; Scholle et al., 2020).

Age-related mitochondrial decline is characterised by reduced mitochondrial content and mitogenesis, as well as the accumulation of non-inherited mtDNA deletions in postmitotic tissues (Hjelm et al., 2022). Investigating the temporal and spatial distribution of mutated mtDNA is important for undersatnding the aging process, as dysfunctional mitochondria are associated with impaired neurotransmission and cognitive decline (Sharma et al., 2021). We propose that differential expansion of heteroplasmic variants during the aging process may have implications for age-related diseases, such as NeuD and PsyD. However, most studies investigating the role of mtDNA in the biological processes of ageing have focused on NeuD, mostly AD.

We have reported inconsistencies in the detection of the mtDNA-CN in PsyD. Recent studies have shown that the association between copy number and diagnosis in BD patients is type-dependent, with BD type 1 (BD1) associated with lower blood mtDNA CN, whereas BD type 2 (BD2) is associated with higher mtDNA CN (Calarco et al., 2024; Chung et al., 2022). Although mtDNA levels in peripheral blood cells have limitations in reflecting brain function or brain pathology, mtDNA-CN in brain tissue may be associated with pathophysiological differences between BD1 and BD2. With this in mind, we also analyzed the mtNDA-CN levels in the post-mortem tissue from SZ patients and HC. We reported no significant differences in mtDNA-CN between SZ and HC adding further evidence to the previously mentioned studies (Sabunciyan et al., 2007; Torrell et al., 2013). Furthermore, previous studies assessing mtDNA-CN in postmortem brain tissue from SZ patients reported that mtDNA-CN varied between different brain regions (Das et al., 2022), and between the types of antipsychotic treatment received (Kumar et al., 2018). We found significantly higher mtDNA-CN in patients who received antipsychotic treatment than those who did not, which is inconsistent with this 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.

There are several limitations that need to be considered when interpreting the results of this thesis. The common limitation of the original studies is the small sample size, which reduces the statistical power. The situation seemed to be different in the systematic review where 158 studies were included. However, it is worth noting that most of the studies had small sample sizes, which resulted in underpowered analyses. In addition, the results of the studies using low-throughput genetic techniques are not directly comparable with the results of the more recent studies using high-throughput techniques. In terms of the techniques, the second study used the Ion Torrent technology, while we used Illumina technology for the third study. Ion Torrent has been reported to introduce insertion/deletion (indel) error types (Bragg et al., 2013) and has some shortcomings

that require additional manual curation of the data (Harvey et al., 2019; Seneca et al., 2015), which we addressed by applying several quality control criteria to avoid. Another limitation was the age of the participants. The second study was conducted with participants who had an average age of approximately 47 years. Although there were no age differences between carriers and non-carriers of mtDNA alterations, our dataset lacks representation of younger age groups. Finally, although genetic testing was performed to detect copy number variants and specific mutations associated with ID in the nuclear genome, no exome or genome sequencing was performed. This leaves open the possibility that either the ID, ASD and the clinical features of the patients may have a nuclear genetic origin. However, it is also plausible that the observed features result from contributions from both genomes. The main limitation of the third study is that mtDNA alterations were only analysed in brain tissue from a single region. As noted in the first systematic review, alterations in mtDNA vary depending on the specific brain region, cell type, and subcellular location. Therefore, a thorough investigation of the effects of mtDNA alterations is needed, including an examination of mtDNA from different regions of the brain. For example, the occipital cortex is involved in the interpretation of visual images and may be associated with visual hallucinations in individuals with SZ (Torrell et al., 2013). Another limitation of the third study is the use of state-of-the-art molecular and bioinformatic tools that are still in development, as there is currently no consensus on the standardised use of these sensitive pipelines and tools. However, the high read depth of the reads obtained gives strength to the low level of heteroplasmy that we observed.

Overall, the aim of this work was to comprehensively analyse mtDNA changes in a wide range of brain disorders. Many mtDNA changes that may cause or be associated with mitochondrial dysfunction were identified in both patients with ID and patients with SZ. However, larger samples are needed to confirm our findings. In addition, the interpretation of the pathogenicity of the mtDNA alterations was based on previous reports and bioinformatic tools designed to unravel putative pathogenicity. In the future, functional analyses are needed to confirm the role of the identified variants. Functional analysis of mitochondrial DNA (mtDNA) variants to unravel their pathogenicity often involves a combination of molecular, cellular, and biochemical assays. These may include measurement of respiratory chain enzyme activities (DiMauro & Schon, 2003), measurement of mitochondrial membrane potential (D. G. Nicholls & Ward, 2000)ROS production (Indo et al., 2007), and the use of cybrids, which have provided valuable insights into the field of mitochondrial genetics and has contributed to our understanding of mitochondrial genetics.

VIII. CONCLUSIONS

- To date, the study of mtDNA alterations in human postmortem brain samples has been investigated in the context of ageing and disease, focusing primarily on individuals with MitD, NeuD, and PsyD.

- MtDNA deletions, pSNVs, and mtDNA-CN changes have been reported in postmortem brain tissue from patients with MitD, with pSNVs showing heteroplasmy levels that vary between brain regions and are generally present in blood.

- In NeuD, most studies have investigated the presence of mtDNA deletions or differences in mtDNA-CN between affected and unaffected individuals, while few studies have evaluated mtDNA pSNVs. Low mtDNA-CN is the most commonly reported mtDNA alteration in NeuD.

- In PsyD, pSNVs and mtDNA-CN have not been consistently evaluated; however, specific mtDNA deletions have been identified.

- MtDNA deletions have been recurrently associated with ageing, demonstrating that ageing is strongly associated with loss of mtDNA integrity.

- MtDNA rearrangements with low levels of heteroplasmy have been identified in blood samples from individuals with severe or profound ID, suggesting that these alterations may be present with higher levels of heteroplasmy in other postmitotic tissues, such as the brain.

- The low frequency of mtDNA rearrangements in patients with ID-ASD suggests that they may not be involved in this phenotype.

- MtDNA alterations are more frequent in patients with SZ than HC. Specially, mtDNA rearrangements, likely pathogenic missense variants, and the pathogenic m.3243A>G variant. However, further functional studies are needed to validate the impact of the identified heteroplasmy levels on mitochondrial function.

- A comprehensive understanding of the potential involvement of mtDNA alterations in the pathophysiology of brain disorders will require larger case-control studies and functional investigations.

IX. REFERENCES

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