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THESIS TITLE

Using hiPSC-derived neural cells to explore mitochondrial abnormalities in Leber Hereditary Optic Neuropathy

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SUMMARY

1 General Introduction

1.1 Mitochondria: features, genetics and alterations

Mitochondria are subcellular organelles that play a pivotal role in sustaining life by serving as the primary source of cellular energy, orchestrating critical metabolic pathways, and participating in a variety of cellular processes. Their involvement in cellular functions extends to regulating apoptosis, influencing redox signalling, participating in calcium homeostasis, and playing a role in cell cycle regulation. Mitochondria are remarkable double-membraned organelles with a complex and highly organized structure. Their unique architecture plays a crucial role in their function as the powerhouses of the cell, where adenosine triphosphate (ATP), the cell's primary energy source, is produced. Morphologically, mitochondria exhibit several distinctive features (Fig. 1):

- *Double Membrane Structure:* Mitochondria have a double membrane structure, consisting of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM). These two membranes form two distinct compartments within the organelle, the intermembrane space (IMS) between them, and the matrix enclosed by the IMM.
- *Cristae:* The IMM contains numerous folds known as cristae. These cristae significantly increase the surface area of the inner membrane, and provide more space for chemical reactions involved in energy production. The presence of cristae is a crucial morphological aspect of mitochondria related to their function.
- *Matrix:* The matrix is the innermost compartment of the mitochondrion, enclosed by the IMM. It contains enzymes, mitochondrial DNA, ribosomes, and other factors necessary for the production of ATP through processes such as the citric acid cycle and oxidative phosphorylation.

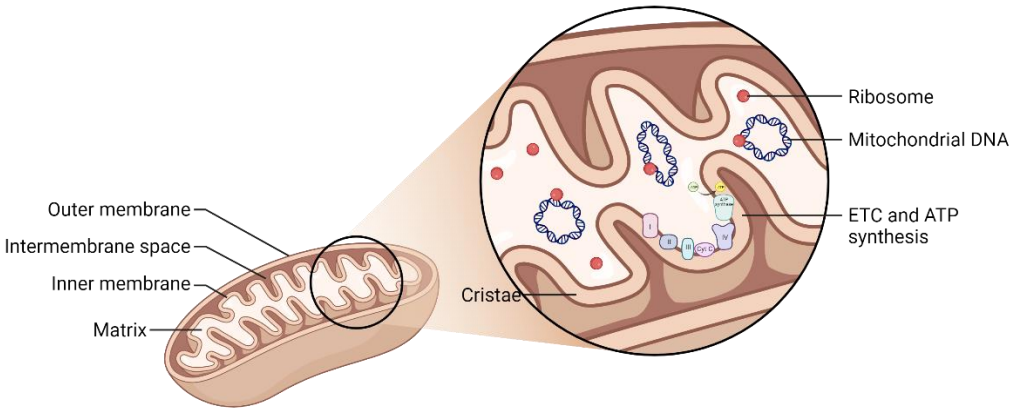

Fig. 1: The structure of a mitochondrion

Every cell has mitochondria, in hundreds or thousands, depending on the cell type. Although they are normally considered as solitary organelles, the process of fusion and fission ensures that a sufficient pool of mitochondria is maintained during cell division and proliferation (Bereiter-Hahn and Vöth, 1994). Based on interactions between proteins on the outer mitochondrial surface and cytoskeletal components such as actin filaments, intermediate filaments, and microtubules, the mitochondria are distributed and function in cells (Scheffler, 2001).

1.1.1 Genetics

Mitochondrial DNA (mtDNA) is the only source of essential cellular proteins outside the eukaryotic nucleus. Most eukaryotes have circular, double-stranded DNA molecules for their mtDNA (Fig.2). The strands' nucleotide content helps distinguish then two strands: the heavy (H-strand) is rich in guanine, while the light (L-strand) is rich in cytosine. The length of the mtDNA is constant in humans, about 16 569 bp. Cells contain between 100 and 10,000 copies of mtDNA, a multi-copy DNA, depending on the energy requirements of the cell. The 37 genes in the mtDNA are divided into 28 on the H-strand and 9 on the L-strand. The mitochondrial respiratory chain (RC), where cellular energy is produced by OXPHOS, consists of polypeptides that are encoded by thirteen genes of the mtDNA. In addition, 22 mitochondrial tRNA molecules, one 16s rRNA (large ribosomal subunit), and one 12s rRNA (small ribosomal subunit) are produced by the other 24 genes (Andrews et al., 1999). Because over 93% of its length consists of coding region, mtDNA is incredibly efficient and free of intronic segments. Most neighbouring genes are usually separated by one or two non-coding base pairs. The displacement loop (D-loop) is the only non-coding region found in mtDNA. The D-loop includes the two transcription promoters of the H-strand (HSP1 and HSP2) as well as the origin of heavy strand synthesis, where mtDNA replication begins (Chinnery and Hudson, 2013).

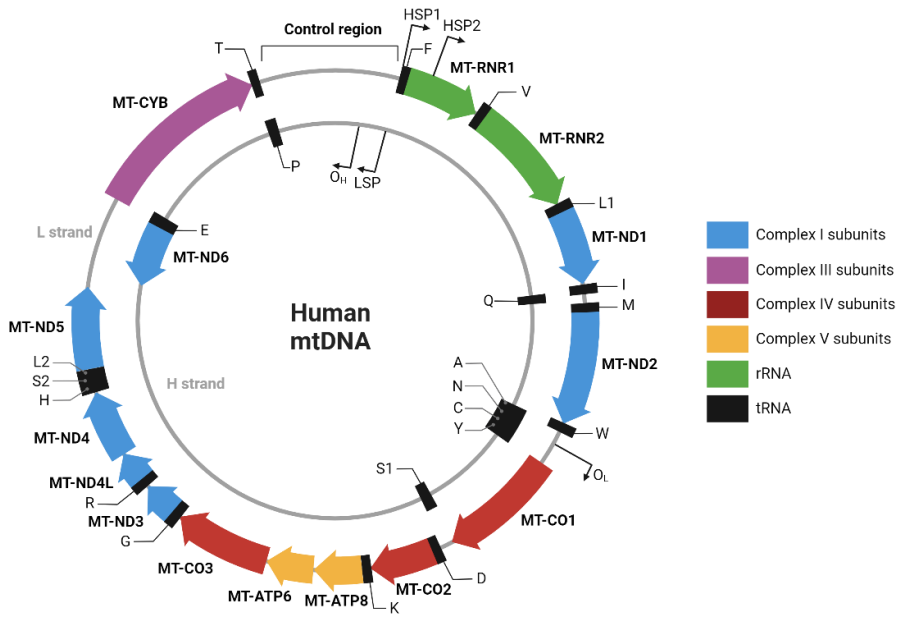

Fig. 2: Human mtDNA. The human mitochondrial DNA consists of 16,568 base pairs. It encodes 22 tRNA, the 12 S and the 16 S rRNA, and 13 peptides forming with the 74 nuclear-encoded polypeptides the five respiratory chain complexes. The displacement loop (Control Region in the image) is the only non-coding region found in mtDNA. The D-loop includes the two transcription promoters of the H-strand (HSP1 and HSP2) and the origin of replication of Purine-rich Heavy strand (OH). Replication is asymmetric, starting with the synthesis of the Heavy strand until the origin of the Light strand (OL) located about 2/3 from OH

The mtDNA genes alone are not sufficient to encode all the proteins required for the OXPHOS system. In fact, over ~1,500 different nuclear genes encode mitochondrial proteins (Gorman et al., 2016) necessary for structural proteins of the OXPHOS system and other enzymes required for replication, repair, transcription, translation, and maintenance. Comparison of the genetic code of mtDNA and nDNA reveals small but significant differences. The only stop codons used by mtDNA are 'AGA' and 'AGG' (in contrast to 'UAA', 'UGA', and 'UAG' in nDNA); 'UGA', on the other hand, encodes tryptophan. UAA codons must be added at the post-transcriptional level to make up for this. Isoleucine, or "AUA" in nDNA, also codes for methionine in mtDNA (Sengupta et al., 2007). In addition, it is assumed that mtDNA is inherited from the mother, according to the prevalent theory. However, recent studies also show the possibility of paternal inheritance (Luo et al., 2018). mtDNA replication is not controlled by the cell cycle and is a continuous process. The replication and maintenance mechanisms are encoded by the nDNA. In eukaryotes, the mtDNA is replicated in a ‘replisome’ by a trimeric protein complex consisting of polymerase gamma encoded by *POLG* and two additional proteins encoded by *POLG2*. To stabilize the mtDNA single strand at the replication forks the replisome also contains the mitochondrial single stranded binding protein (encoded by mtSSB) (Falkenberg, 2018). Twinkle is a 5’-3’ DNA helicase that also functions as an mtDNA by unwinding double-stranded mtDNA and supporting mtDNA synthesis (Korhonen et al., 2003). The mitochondrial topoisomerases 1 (encoded by TOP1mt) and IIIα

(encoded by TOP3a) are among the topoisomerases identified in humans (Pommier et al., 2022). Finally, the relationship between mtDNA copy number and mitochondrial transcription factor A (encoded by TFAM) shows that TFAM can act as an mtDNA chaperone, and protect it from oxidative damage (Ekstrand et al., 2004). Like nDNA, also mtDNA is packaged in a protein-mtDNA complex, called “nucleoid”, localized along the cristae of the inner membrane. Being a site of the replication, the proteins that make up the nucleoids are the same as those that act in the replication mechanisms with the addition of two chaperone proteins suitable for stabilize mtDNA (HSP90-β and HSP70) (Chinnery and Hudson, 2013). MtDNA is transcribed polycistronically. RNA transcription is regulated by mitochondrial transcription factor A (TFAM). Mitochondrial RNA polymerase (POLRMT) elongates transcripts, which is assisted by "mitochondrial transcription elongation factor" (MTEF), and “mitochondrial termination factor 1” (MTERF1) terminates mature transcripts. The 13 protein-coding mtDNA genes are translated in the mitochondria. Mitoribosomes are partially encoded by mtDNA (MTRNR1 and MTRNR2), but 81 additional nDNA proteins are needed.

1.1.2 The Oxidative Phosphorylation

Oxidative phosphorylation, the most important activity of mitochondria, generates the largest and most important amount of energy in the cell. This process is carried out by a series of multi-heteromeric complexes, the mitochondrial respiratory chain (MRC), in the inner mitochondrial membrane by sequential reactions of reduction and oxidation. The name of these reactions together is “cellular respiration”(Fig. 3). Dehydrogenation of the redox equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) causes a flow of electrons that causes the matrix to extrude hydrogen ions into the IMS, where they are used by complexes I, III, and IV to move protons and create a membrane potential of about 180 mV, and by complex IV to produce water from molecule oxygen. The energy generated by these reactions, which corresponds to the proton gradient across the IM, produce energy that is then used by Complex V, or ATP synthetase, to condense inorganic phosphate Pi and adenosine diphosphate (ADP) into ATP, the “energy of the cell”.

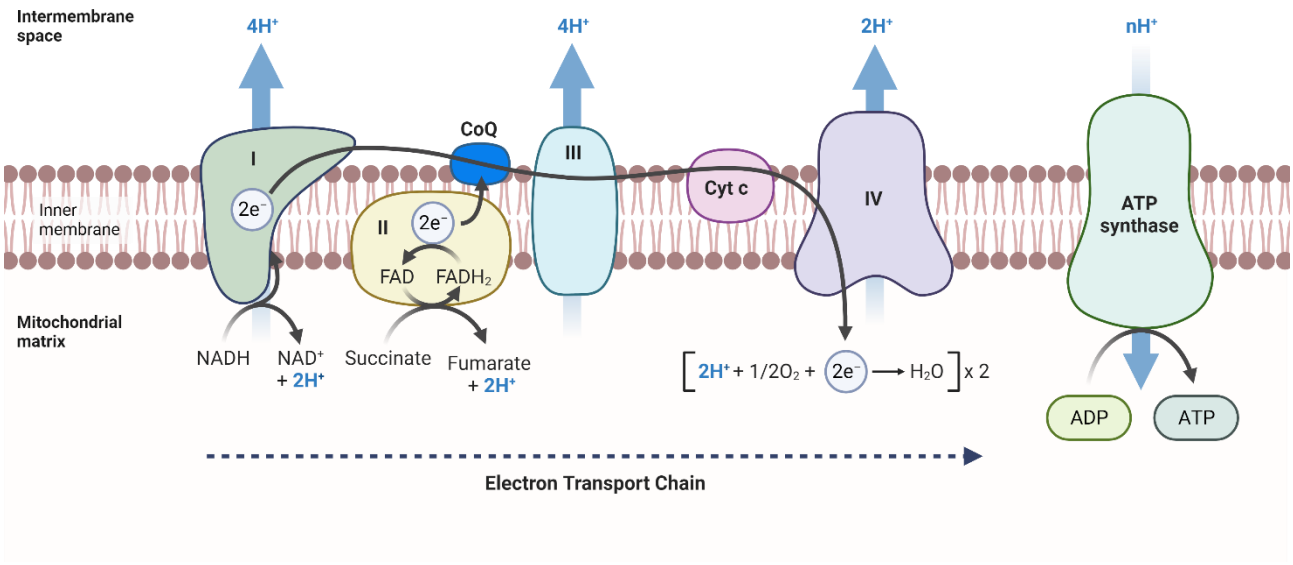

Fig. 3: ***The Oxidative Phosphorylation System (OXPHOS).*** *Complex I or II are reached by electrons produced from cellular metabolism via NADH or FADH2, respectively. Coenzyme Q (ubiquinone), a carrier of electrons from complex I or II to complex III, then transfers these electrons. In the latter, particles switch from cytochrome b to cytochrome c before moving to Complex IV (cytochrome oxidase), where they reduce oxygen. The movement of protons across the intermembrane space is connected with the movement of electrons via the mitochondrial complexes. Complex V produces an electrochemical gradient that it uses to produce ATP.*

In addition to the complexes already mentioned, this process also involves two electron carriers (ubiquinone or CoQ, and cytochrome c), as well as a number of accessory complexes that can provide electrons, such as the Complex II (succinate-ubiquinone reductase), the electron transfer flavoprotein-ubiquinone reductase (ETF-QR), and dihydroorotate dehydrogenase (DHODH). In mammalian mitochondria Complex I (NADH-ubiquinone oxidoreductase) catalyzes the oxidation of NADH, derived from the oxidation of pyruvate, fatty acids, and amino acids, by ubiquinone. Complex I is a macromolecular structure cosisting of ≈45 subunits with a total molecular mass of 1000kDa (Carroll et al., 2006). Seven subunits are encoded by mtDNA, the others by nuclear genes. Complex II (Succinate-ubiquinone reductase) is composed of four subunits all encoded by nuclear genome. It catalyzes the oxidation of succinate to fumarate and transfers electrons to ubiquinone moieties. Complex III (ubiquinol-cytochrome c reductase) is made up of 11 subunits all encoded by nDNA except for the subunit cytochrome b. Human cytochrome c oxidase (COX, complex IV) is composed of thirteen subunits. The three largest ones are encoded by mtDNA genes, while the remaining subunits are encoded by nuclear genes. ATP synthase (complex V) comprises an integral membrane component F0 and a peripheral moiety F1. Only two F0 proteins (ATP6 and 8) are encoded by mtDNA (Boyer, 1993), the remaining subunits are encoded by nDNA. Through glycolysis, fatty acid or amino acid oxidation, metabolic fuels supply reducing equivalents to the respiratory chain. At the crossroads of glycolysis, gluconeogenesis, and OXPHOS, pyruvate is a crucial step in carbohydrate oxidation. It feeds into the TCA cycle via the pyruvate dehydrogenase complex (PDHC) to generate acetyl-CoA. This molecule is also produced by the β-oxidation

of the fatty acids. Reducing equivalents produced by the TCA cycle or during the oxidation of the primary substrate are transported to the OXPHOS as reduced flavins (entering at cII or cIII) or NADH (cI). More than 90% of the energy used by our system comes from the mitochondria. The survival of the cell, and thus, the existence of our entire organism, is considerably endangered if the energy supply fails due to a mitochondrial OXPHOS deficiency. The tissues and organs with the highest energy demand are most frequently affected, such as the heart, the skeletal muscles and the brain.

1.1.3 Mitochondrial Alterations

The pathogenic mutations that occur in mitochondrial DNA can significantly alter the structural components of the respiratory chain and the apparatus responsible for the synthesis of mitochondrial proteins. Unlike nuclear DNA, mitochondrial DNA is more susceptible to damage. Although it has a repair mechanism, the capacity for repair is limited. Mitochondrial diseases are multifaceted and can be attributed to three distinct categories of mtDNA mutations: mtDNA rearrangements, like large-scale mtDNA deletions; mutations in genes important for protein synthesis, including those affecting mitochondrial tRNA or rRNA; and mutations in genes encoding structural proteins (Lax et al., 2011). The polyploid nature of the mitochondrial genome, characterized by the presence of numerous copies within a single cell, ranging into the thousands, leads to a fundamental concept in mitochondrial genetics: homoplasmy and heteroplasmy. Put simply, homoplasmy refers to a scenario in which all copies of the mitochondrial genome are identical, while heteroplasmy refers to the presence of a mixture of two or more mitochondrial genotypes within an individual (Taylor and Turnbull, 2005). The meaning of these terms becomes clear when we consider the variety of mtDNA mutations and their impact on disease. Some mutations affect every copy of the mitochondrial genome, resulting in a uniform genetic change (homoplasmic mutation). In contrast, others manifest in only a subset of copies of the mitochondrial genome's, resulting in a mosaic pattern of genetic variation (heteroplasmic mutation). In the context of heteroplasmy, a critical threshold for mutation proves to be a decisive factor. The threshold for a pathogenic mtDNA mutation is the minimum percentage of mutant mtDNA required for biochemical expression and clinical manifestation of the disease. The threshold of a particular mutation may vary from tissue to tissue, depending on their dependence on oxidative metabolism (Ryytty and Hämäläinen, 2023). Basically, the interplay between homoplasmy and heteroplasmy, and the distribution of mutations within the mitochondrial genome, is a crucial factor in the pathogenesis and clinical manifestation of mitochondrial disorders.

Furthermore, the interplay between mitochondrial and nuclear DNA (nDNA) is crucial, as alterations in over 1500 nDNA-encoded genes responsible for mitochondrial proteins can have pathological consequences. These consequences include a wide spectrum of pathogenic effects, ranging from defects in mtDNA

maintenance and mtDNA translation to disruptions in mitochondrial homeostasis (Gorman et al., 2016). Defects in mtDNA maintenance arise from genetic mutations in genes encoding proteins involved in mtDNA replication, as in the autosomal dominant progressive external ophthalmoplegia (adPEO) with a pathogenic mutation in POLG (polymerase y) (Horvath et al., 2006) and deoxynucleotide triphosphate (dNTP) synthesis, such as mutations in the thymidine kinase 2 gene (Oskoui et al., 2006). These mutations led to secondary aberrations within mtDNA, manifested either by a decrease in mtDNA copy number or the presence of multiple mtDNA deletions. Equally consequential is the potential impairment of mitochondrial function due to mutations in genes encoding translation and release factor proteins, mitochondrial tRNA-modifying proteins, mitochondrial mRNA-processing enzymes, mitochondrial aminoacyl-tRNA synthetases, and mito-ribosomal proteins, all of which can influence mtDNA translation (Chinnery and Hudson, 2013).In addition, other factors contribute to dysfunctional mitochondrial activity. These factors include apoptosis, mitochondrial chaperone proteins, and mitochondrial metabolic processes, among other critical elements. It is imperative to emphasise that the structural integrity of mtDNA is inextricably linked to an extensive ensemble of nuclear-encoded proteins, that are essential for both mtDNA replication and repair. Disruptions in these intricate processes, triggered by mutations in these nuclear-encoded genes, can manifest in two distinct manners: qualitatively, giving rise to a plethora of point mutations or extensive mtDNA deletions that can accumulate over the course of an individual's lifespan, or quantitatively, resulting in near-complete depletion of the mitochondrial genome, a phenomenon commonly referred to as mtDNA depletion. This intricate interplay forms the cornerstone of in-depth research in molecular biology and genetics, and has profound implications for our understanding of mitochondrial dysfunction and its clinical manifestations (Alston et al., 2017; Chinnery and Hudson, 2013; Gorman et al., 2016).

1.2 Mitochondrial Optic Neuropathies (MONs)

MON describe a group of optic neuropathies, that selectively damage retinal ganglion cells (RGCs), with clinical features and similar pathophysiology of mitochondrial dysfunction in the prelaminar area of the optic nerve. This area contains a high number of mitochondria compared to the post-laminar area, indicating a higher energy demand. In addition, the fibres that forms the RGCs' axons before the *lamina cribosa* are unmyelinated, which is necessary for retinal transparency and light penetration into the photoreceptors (Pilz et al., 2017). This peculiarity reduces the efficiency of conduction of the electrical potential, which can not occurs in a saltatory manner as in myelinated fibres, thus increasing energy demand (Purves et al., 2001). In addition, energy conduction is also reduced due to the narrower caliber of the fibers (Carelli et al., 2004). Because of its high energy requirements, limited energy production, and slow axoplasmic transport,

the pre-laminar area is vulnerable to mitochondrial malfunction when it arises in MON (Carelli et al., 2004; Pan et al., 2012). Clinical symptoms include central or cecocentral scotomas, impairments in colour vision, and painless, gradual bilateral loss of visual acuity as a result of the subsequent selective injury and death of RGCs. Although MONs share the same clinical features, some aetiologies are more common than others. There is no gender, ethnic, or age prevalence for acquired MONs. The majority of genetic aetiologies, however, frequently affect children or young adults. Two are the most representative pathologies classified as MONs: Leber hereditary optic neuropathy (LHON) and the autosomal dominant optic atrophy (DOA) (Carelli et al., 2023). First, thanks to (Wallace et al., 1988), the first mutation of the mtDNA associated with LHON was discovered. Autosomal dominant optic atrophy (DOA) was then linked with mutations in the nuclear DNA affecting the OPA1 gene in 2000 (Alexander et al., 2000; Delettre et al., 2000). Both syndromes are characterized by selective degeneration of retinal ganglion cells due to mitochondrial dysfunction. This concentrates on the distinctive clinical manifestations caused by respiratory complex I impairment in LHON and faulty mitochondrial dynamics in OPA1-related DOA (Carelli et al., 2023). The molecular and phenotypic landscape associated with mitochondrial optic neuropathies has rapidly expanded over the last decade, and it is notewhorty that all the genes found so far converge on a small number of common pathways that ultimately disrupt mitochondrial homeostasis and cause irreversible RGC loss. Today, optic nerve involvement, is thought to be a classic sign of mitochondrial disease. A subset of LHON and DOA can sometimes present with extraocular features referred to as LHON “plus” and DOA “plus” (Chen et al., 2023).

1.2.1 Leber hereditary optic neuropathy (LHON)

Leber’s hereditary optic neuropathy (OMIM #535000) is caused by maternally inherited missense point mutations of mitochondrial DNA (Wallace et al., 1988) and is estimated as the most common mitochondrial disease (Chinnery and Turnbull, 2000). This blinding disorder is characterized by selective degeneration of retinal ganglion cells, the retinal neurons projecting their axons, which form the optic nerve to the brain, despite that the homoplasmic mtDNA mutation is present in all tissues. The pathogenic mechanism leading to cell death is therefore highly tissue and cell specific (Carelli et al., 2004; Yu-Wai-Man and Chinnery, 1993). The phenotype of these mutations is characterized by defective ATP synthesis when driven by complex I substrates (Baracca et al., 2005), increased oxidative stress (Floreani et al., 2005), and increased propensity for apoptosis (Ghelli et al., 2003). Extensive loss of RGCs and their axons leads to optic nerve atrophy, with a severe defect of central vision, in most cases leaving the patient legally blind (Carelli et al., 2004) (Fig. 4). More than 50 inherited mitochondrial DNA (mtDNA) variations are associated with LHON (Shamsnajafabadi et al., 2023); however, more than 95% of cases are caused by one of three missense variations (m.11778 G>A, m.3460 G>A, and m.14484 T>C) encoding for subunits ND4, ND1, and ND6 of the respiration complex I,

respectively. Due to the phenomenon of the incomplete penetrance, only some individuals develop the disease, most frequently young males showing a gender prevalence. In fact, approximately 50% of male LHON-carriers and 10% female LHON-carriers lose vision (Stramkauskaitė et al., 2022). Incomplete penetrance in LHON carriers is complicated and influenced by a variety of genetic, hormonal and environmental factors, for many reasons. The incomplete penetrance in LHON may have environmental causes, such as alcohol consumption and smoking. Patients with the m.14484T > C mutation are most likely to have spontaneous recovery of vision, followed by patients with the m.3460G > A mutation and patients with the m.11778G > A mutation (Pilz et al., 2017). A central scotoma may form small islands of vision around it, and colour vision may return as part of the process of vision recovery. In addition, early onset of LHON, moderate progression of visual loss, and a large surface area of the optic nerve increase the likelihood of spontaneous visual recovery. Patients with the LHON+ phenotype have a higher risk of developing other diseases, although LHON primarily damages RGCs. Cardiovascular conduction abnormalities, ataxia, peripheral neuropathy, myopathy, and dystonia are more common in LHON carriers (Pilz et al., 2017).

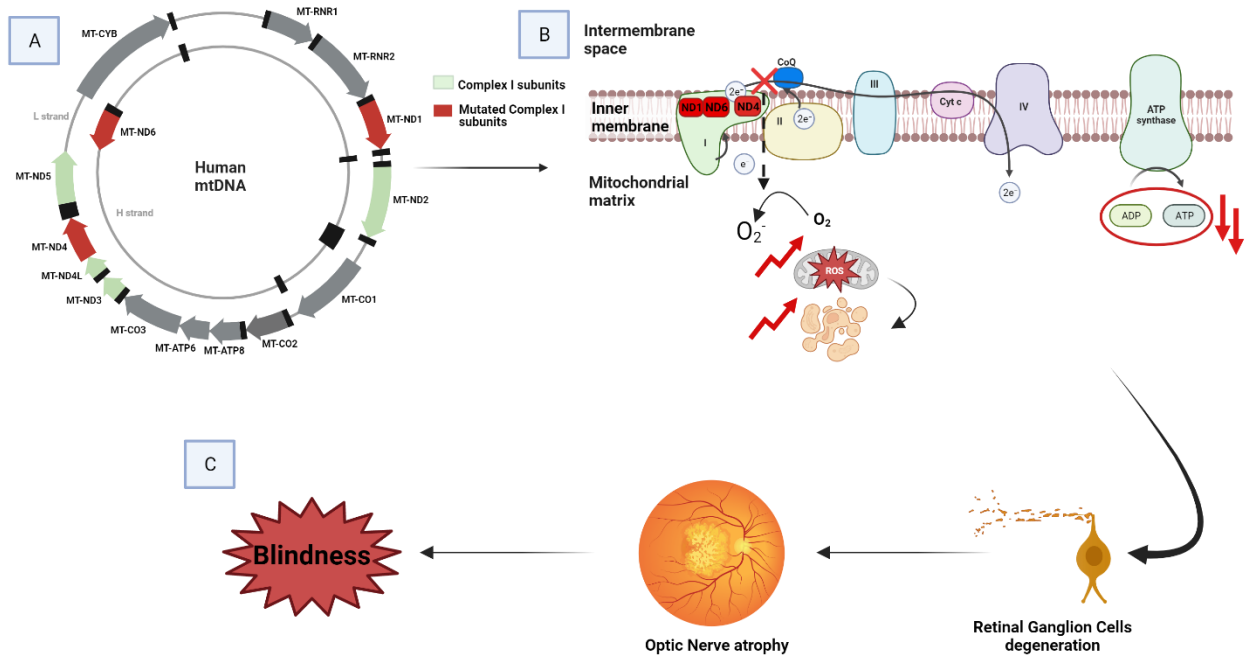

Fig. 4: ***The course of LHON syndrome****: A) LHON syndrome is mainly due to three different point mutations in mitochondrial DNA, each affecting a different subunit of Complex I of the electron transport chain. These subunits are: ND1, ND4 and ND6 respectively affected by the following mutations m.3460G>A; m.11778G>A and m.14484T>C. B) NADH dehydrogenase mutations of Complex I may increase the leaking of electrons from the chain, leading to increased accumulation of ROS, oxidative stress, reduced ATP production and cells death. C) Although the mutation is present in all tissues, the only specific target of LHON syndrome are the Retinal Ganglion Cells (RGCs), which degenerate, resulting in optic nerve atrophy. The result of this is loss of vision, which usually starts in one eye and then within months involves the other eye.*

1.3 Mitochondrial disorders modelling using Human Induced Pluripotent Stem Cells (hiPSCs)

In order to understand the underlying aetiology and pathogenic mechanisms of diseases and to identify new therapeutic options, it is essential to create pathophysiologically relevant experimental models of disease, either in vitro or in vivo or both. Animal models have made the greatest contribution to both basic and pharmaceutical research. However, due to the innate biological and metabolic differences between animals and humans, many pharmacological studies that have been successfully investigated in animal models often fail when translated to humans. Therefore, it is crucial to create appropriate *in vitro* models for human diseases that faithfully represent the associated pathophysiological processes. According to Doss and Sachinidis (2019), hiPSCs are reliable and valid in vitro models for studying diseases of the neurological and cardiovascular systems, as well as other hard-to-access organs. Induced pluripotent stem cells are a new type of pluripotent cells that can be obtained by reprogramming animal and human differentiated cells. In 2006, cellular reprogramming of terminally differentiated mouse cells into ESC-like cells, called induced pluripotent stem cells (hiPSCs) became a reality owing to the ground-breaking work of the Yamanaka's group (Takahashi and Yamanaka, 2006). A year later, the same group generated human induced pluripotent stem cells from human fibroblasts (Takahashi et al., 2007). In the following years, it has enabled the creation of patient-specific hiPSCs, a major step forward in gaining insight into specific diseases and their pathophysiological mechanisms, as well as creating cellular tools for drug screening and designing cell-based therapy. In fact, human hiPSCs can differentiate into almost all cell types, may be obtained using minimally invasive techniques, and have a limitless capacity for proliferation. Their ability to be genetically altered using nuclease-based genome editing approaches is of paramount importance for the development of innovative therapeutic pathways. Using these techniques, hiPSCs can undergo site-specific genetic modifications, such as gene knockouts or gene correction in healthy and diseased cell lines, respectively (Merkert and Martin, 2016). Genome editing allows the creation of human hiPSC lines that are genetically identical to the hiPSCs derived from the patient (isogenic controls) and differ only in the change that was originally present in a particular gene (Grobarczyk et al., 2015). These cell lines are an extremely regulated biological system that makes it easy to link any phenotypic variation to a specific DNA change. Since the nuclear genome is accessible and can be modified using gene editing tools, the development of disease models for pathogenetic research of mitochondrial disorders caused by pathogenic nuclear variants is quite straightforward. In contrast, maternally inherited mitochondrial disorders caused by a pathogenic mitochondrial mutation have proven more difficult to treat despite the latest and innovative technologies with mtDNA editing capabilities (Cho et al., 2022; Silva-Pinheiro et al., 2022)

Due to the lack of model systems, development of novel therapeutic options for mitochondrial diseases caused by maternal inheritance has been delayed. The development of animal and cellular models has been made more difficult by a number of factors: 1) The multicopy nature of the mitochondrial genome, which results in a population of wild-type and mutated mitochondrial genomes mixed together, known as heteroplasmy; 2) An increase in spontaneous mutations over time; 3) Mitochondrial segregation; and 4) The challenge of genetically engineering or transforming the multicopy mitochondrial genome. The cybrid system was one of the most commonly used cellular methods to study the consequences of pathogenic mitochondrial mutations before the discovery of hiPSCs (Cavaliere et al., 2022). Cybrids are cells created when immortalized cell lines depleted of their mitochondrial genome are united with enucleated patient cells carrying their mitochondrial DNA. In this case, the interactions between the nuclear and mitochondrial genomes are lost, which can change how the diseased cells behave. The production of stem cell populations with pathogenic mtDNA mutations appears to be a successful application of the hiPSC technology. In principle, these patient-derived hiPSCs contain both the original mitochondrial and nuclear genetic background as hiPSCs can be produced from peripheral cells taken from patients with a maternally transmitted mitochondrial disease. More importantly, because hiPSCs are pluripotent, they can develop into virtually any type of cell, making them an endless biological resource for studying the function of specific pathogenic mitochondrial mutations while committing to particular cell lineages. Another advantage of hiPSCs is their capacity to produce in vitro models that are specific to the desired disease without the need for laborious and time-consuming gene editing techniques.

1.4 Current therapeutic strategies

Despite the numerous clinical and preclinical studies conducted to date, effective therapies for LHON are still limited. Effective means that a therapy should be able to appreciably alter the natural course of the disease, either by aborting or reversing the catastrophic wave of cell death, or at least by limiting the progression of the disease such that visual function is essentially preserved based on anatomically measurable sparing of RGCs. Several therapeutic strategies for the treatment of LHON are under development, including antioxidant therapies, mitochondrial biogenesis/mitophagy modulation, genetic therapies and mitochondrial replacement therapy. In the treatment of mitochondrial disorders, such as LHON, several antioxidant molecules, some of which have direct effects on mitochondrial respiration, have been tested in patients: idebenone, Coenzyme Q10 (CoQ10), EPI-743, Elamipretide, curcumin (Amore et al., 2021)

To date, idebenone, a synthetic analogue of coenzyme Q10 (CoQ10), is the most widely studied antioxidant drug for the treatment of LHON. Compared to coenzyme Q10 (CoQ10), idebenone has a higher bioavailability due to its less lipophilic tail, which allows it to cross the blood-brain barrier and mitochondrial membrane

(Hargreaves, 2014). There is evidence that idebenone treated cell lines from patients with LHON m.11778A>G mutations can increase ATP production, decrease ROS levels and improve cells growth (Yu-Wai-Man et al., 2017). The efficacy of the treatment is controversial; it is effective in some patients but not in others. Treatment with idebenone has allowed patients at risk of further vision loss to maintain their Tritan colour vision and prevent further colour vision loss (Yu-Wai-Man et al., 2017). In 2015, idebenone (Raxone®) was approved by the European Medicines Agency (EMA) in the European Union for the treatment of LHON. Another small trial confirmed that EPI-743 halted disease progression and reversed vision loss in four patients (Sadun et al., 2012). Elamipretide is another promising candidate. It has been found to reduce reactive oxygen species (ROS) and increase adenosine triphosphate (ATP) levels (Karanjia et al., 2019).

In LHON, mitobiogenesis or mitophagy act as a compensatory strategy for mitochondrial failure (Bianco et al., 2017). In the LHON cybrid (G11778A) and Ndufs4 knockout mouse models, rapamycin, an mTOR inhibitor, causes selective mitophagy of the defective mutant mitochondria and retains the visual function (Dai et al., 2014). In addition, there are other potential strategies, for example, inhibition of miRNA181a/b, which acts on both mitobiogenesis and mitophagy targeting Nrf1. In previous studies, deactivation of MiR181a/b in LHON mouse models improved visual phenotypes. Therefore, this represents a gene-independent therapeutic target for the treatment of LHON (Indrieri et al., 2019).

In addition to pharmacological clinical trials, encouraging results of gene therapy approach for patients carrying the m.11778G > A/ MT -ND4 mutation are reported nowadays. Compared to other human tissues, the RGC layer of the retina is relatively amenable to insertion of corrected genes, so a promising treatment for LHON is to replace the dysfunctional gene with the healthy wild-type gene using gene therapy (Amore et al., 2021). Allotopic gene expression was developed to overcome the natural barrier that is the double membrane for the transport of molecules into the mitochondria. In this method, mitochondrial genes are codified using the nuclear code and inserted into the nucleus. A mitochondrial targeting sequence is added to allow the import of the resulting protein into the mitochondria. Several approaches have been developed in recent years. The first comes in 2002 with the viral vector scAAV2-P1ND4v2 that carries the wild-type ND4 subunit and the MTS sequence from the ATP1 gene. The genetic cargo is first taken up in the nucleus, transcribed into mRNA and then translated by cytosolic ribosome to produce the MTS-containing protein. The protein is then incorporated into complex I of the mitochondrial respiratory chain after being actively transported into mitochondria (Cwerman-Thibault et al., 2015; Koilkonda et al., 2010). In subsequent years, other allotopic gene expression of ND4 have been performed, but unlike previous approaches they used the viral vector to transport the mRNA rather then the protein into the mitochondrion. This mRNA is then translated by mitochondrial ribosomes (Bonnet et al., 2007). In order to cross the double membrane and act directly in the mitochondria, Hussain et al. (2021) use CRISPR/Cas9 technology to propose a stem-loop

element, called the RP loop, as a potential extended mitochondrial transporter for CRISPR-Cas9. By inserting a targeted guide RNA into an RP loop and producing the Cas9 enzyme with mitochondrial localization sequences, the editing procedure was carried out. In addition, a CRISPR-free method of editing mitochondrial base pairs using a bacterial toxin produced from cytidine deaminase called the DddA-derived cytosine base editor (DdCBE) allowed precise mtDNA modification in human cells without the need to get rid of copies of the gene created when specific nucleases cleave it (Cho et al., 2022; Wei et al., 2022). At least, with regard to all heteroplasmic mitochondrial diseases the clinical manifestations depend on the variation threshold resulting in a biochemical impairment. In order to address these alterations so as to bring the wild-type mitochondrial DNA copy number back above the threshold level, thus eliminating the altered mtDNA copies, several endonucleases were used. These endonucleases, which include mitochondria-targeted restriction endonucleases (mitoREs), zinc finger nucleases (mtZFNs) (Gammage et al., 2014), and mitochondrial-targeted transcription activator-like effector nucleases (mitoTALENs) (Hashimoto et al., 2015), have been shown to be able to reach mitochondria, identify pathogenic variations as targets, and selectively cause double-strand breaks in heteroplasmic mtDNA.

At the very least, preventing the transmission of the mtDNA mutation to subsequent generations could have therapeutic implications in the treatment of LHON. In this method, nuclear DNA is removed from the patient's egg or embryo and then inserted into the cytoplasm of a donor, which already contains wild-type mitochondrial DNA (Hyslop et al., 2016; Kang et al., 2016). However, this method is hampered by ongoing ethical issues and unexplored long-term effects (Shamsnajafabadi et al., 2023).

2 Scope of the thesis

The research carried out during these three years focused mainly on the development of a cellular model that can be used to better understand the patho-physiological mechanisms of LHON syndrome. Given the difficulties in manipulating mtDNA, very few animal models with mtDNA pathogenic mutations are available, preventing the possibility to study the affected tissues and organs and test therapeutic options. In 2012, the team of Doug Wallace, a pioneer in the field of mitochondrial medicine, created a mouse model with a mutation in the MT-ND6 gene. The mouse did not exhibit impaired visual responses, but acquired a disease that was very similar to LHON at the age of two years. This model was crucial in reproducing some of the distinguishing features seen in human post-mortem LHON retina. However, because the mice lack the macular region, they are ultimately unable to mimic the disease process that clinically distinguishes humans with the characteristic catastrophic development of RGC degeneration. In addition, most experiments investigating the pathomechanism of LHON to date have been performed on cybrids, lymphocytes, and fibroblasts but not in RGCs. To overcome these problems, I and other investigators exploited innovative approaches, based on the use of human-induced pluripotent stem cells as a faithful source of human neuronal cells and RGCs. The use of hiPSCs to derive terminally differentiated cells of a variety of tissues is an extraordinary approach to understanding disease mechanisms, performing drug screening, and testing gene or cell therapies. Moreover, patient-specific hiPSCs enable the study of the effects of mtDNA mutation in the context of the patient-specific nuclear background, which plays a central role in modulating disease expression, particularly in LHON. In fact, thanks to the development of these models, such as neuronal progenitors cells and neurons, it was possible to test the effect of substances such as idebenone on the cells affected by the various mutations and to study how the use of these substances alters the respiratory activity of the cells. Therefore, it will be essential to study the pathogenic mechanism of LHON disease in hiPSCs derived cell- or tissue-specific models, such as retinal ganglion cells, the target of the disease, which are not easily accessible *in vivo*.

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3 Exploiting hiPSCs in Leber's Hereditary Optic Neuropathy (LHON): Present Achievements and Future Perspectives

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More than 30 years after discovering Leber's hereditary optic neuropathy (LHON) as the first maternally inherited disease associated with homoplasmic mtDNA mutations, we still struggle to achieve effective therapies. LHON is characterized by selective degeneration of retinal ganglion cells (RGCs) and is the most frequent mitochondrial disease, which leads young people to blindness, in particular males. Despite that causative mutations are present in all tissues, only a specific cell type is affected. Our deep understanding of the pathogenic mechanisms in LHON is hampered by the lack of appropriate models since investigations have been traditionally performed in non-neuronal cells. Effective in-vitro models of LHON are now emerging, casting promise to speed our understanding of pathophysiology and test therapeutic strategies to accelerate translation into clinic. We here review the potentials of these new models and their impact on the future of LHON patients.

Keywords: Leber's hereditary optic neuropathy, human induced pluripotent stem cells, mitochondrial disorders, organoids, retinal ganglion cells (RGC)

INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is caused by maternally inherited missense point mutations of mitochondrial DNA (mtDNA) (1) and is estimated as the most-frequent mitochondrial disease (2). This blinding disorder is characterized by selective degeneration of retinal ganglion cells (RGCs), the retinal

neurons projecting their axons, which form the optic nerve to the brain. Thus, the extended loss of RGCs and their axons leads to optic nerve atrophy, with a severe defect of central vision, in most cases leaving the patient legally blind (3, 4). Almost all LHON maternal lineages present with homoplasmic mutation (100% mtDNA copies are mutant in all tissues), having one of three frequent mtDNA mutations found in over 90% of patients worldwide (m.11778G>A/MT-ND4, m.3460G>A/MT-ND1, m.14484T>C/MT-ND6), but only some individuals develop the disease. Also, despite that the homoplasmic mtDNA mutation is present in all tissues, only a cellular type, that is, RGCs, undergoes degeneration. The pathogenic mechanism leading to cell death is thus extremely tissue and cell specific (3, 4). The phenotype of these mutations characterized by defective ATP synthesis when driven by complex I substrates (5), increased oxidative stress (6, 7), and increased propensity to undergo apoptosis (8, 9) has been thoroughly investigated in cybrids, lymphocytes, and fibroblasts but not in RGCs, the disease's target, which are not easily accessible and cannot be maintained in vitro (10).

Moreover, given the difficulties in manipulating mtDNA, very few animal models with mtDNA pathogenic mutations are available (11), preventing the possibility to study the affected tissues and organs and test therapeutic options.

To overcome these issues, we and other investigators exploited innovative approaches, based on the use of human-induced pluripotent stem cells (hiPSCs) as a faithful source of human neuronal cells and RGCs.

The use of hiPSCs to obtain terminally differentiated cells of a variety of tissues is a revolutionary approach to understanding disease mechanisms, performing drug screening, and testing gene or cell therapy (12–15).

Several studies have demonstrated the possibility to generate neurons and RGCs from plated hiPSC-derived embryoid bodies (16–19). In addition, different groups developed 3D culture systems recapitulating key steps of retinal development and allowing the generation of self-organizing retinal organoids containing RGCs (15, 20–25). These models provide a bridge between traditional 2D cell culture and mouse models, representing a paraphysiologic system with pros and cons (26), but of paramount importance for modeling mtDNA- related disorders.

Modeling LHON mutations in differentiated neurons and organoids will provide not only insights into the tissue-specific disease pathogenic mechanisms, but it will offer the unique opportunity to test in-vitro pharmacological approaches in a model system much more relevant than traditional non-neuronal cell cultures, such as fibroblasts, lymphoblasts, or cybrids. Moreover, patient-specific hiPSCs allow studying the effect of the mtDNA mutation in the context of patient-specific nuclear background, which, in LHON particularly, plays a pivotal role in the modulation of disease's presentation (27).

We here discuss our experience with the generation of hiPSCs from LHON-affected patients integrated

with the data present in the literature. We particularly emphasize the translational potential for patients in exploiting LHON neuronal cells and RGCs to advance our knowledge of pathogenic mechanisms and test therapies.

REPROGRAMMING FIBROBLASTS OR PERIPHERAL BLOOD MONONUCLEAR CELLS PBMCS FROM LHON PATIENTS

Since the epochal discovery of induced pluripotent stem cells by the Yamanaka group in 2006 (28), many researchers generated hiPSC by reprogramming differentiated cells obtained from mitochondrial disease patients [MELAS syndrome (29), MERRF syndrome (30), Pearson syndrome (31); reviewed by Liang (32)]. Unexpectedly, even if LHON is the most-frequent mitochondrial disease, to date only a few groups, including ours (33), had generated LHON hiPSCs by reprogramming fibroblasts or peripheral blood mononuclear cells (PBMCs) derived from patients (34–38).

One group from Taiwan reprogrammed PBMCs from two LHON m.11778G>A patients and one LHON m.11778G>A unaffected carrier using the Sendai virus (37). The authors reported a slightly increased complex I (CI) activity, failing statistical significance, in the LHON hiPSCs, both affected and carrier, as compared to control. The authors reported a slightly increased of complex I (CI) activity in both affected and carrier LHON hiPSCs as compared to control, that failed to reach
statistical significance.

Another group reprogrammed fibroblasts from two LHON m.11778G>A patients as well as one LHON proband carrying two mutations m.4160T>C and m.14484T>C, using episomal vectors expressing six reprogramming factors OCT4, SOX2, KLF4, L-MYC, LIN28, and shRNA for p53 (34). They investigated hypothetical difficulties in reprogramming cell lines with OXPHOS defects since Yokota et al. reported reduced reprogramming efficiency in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes syndrome (MELAS) fibroblasts carrying more than 90% of the m.3243A>G mtDNA mutation (39). Hung and collaborators reprogrammed fibroblasts carrying the homoplasmic LHON mutations and found no significant differences in the number of hiPSC colonies between controls and LHON patients (21 colonies on average for the controls, and 13 colonies on average for the LHON patients). Differently, our own experience with LHON was more similar to what observed by Yokota, since we noticed that LHON fibroblasts or PMBCs are refractory to be reprogrammed to hiPSC.

Specifically, we attempted to reprogram different LHON cell lines: two m.3460G>A patients, four m.11778G>A patients, and two unaffected m.11778G>A carriers (Table 1). As shown in Table 1 and Figure 1A, the number of clones obtained was in general very low, even if numerous attempts were performed also in different laboratories. Conversely, using fibroblasts derived from healthy controls or disease's

patients affected by mitochondrial disorders, including dominant optic atrophy (OPA1 mutation), Pearson (40), and MPAN (41), we obtained on average from 10 to 20 clones of hiPSC (Table 1 and Figure 1B) per reprogramming experiment. To overcome this issue, we tested the reprogramming efficiency of LHON cells under hypoxia laboratory conditions (5% pO2, more similar to physiological oxygen tension in vivo), a condition previously used to enhance the generation of hiPSC (42), and recently demonstrated to be specifically beneficial in several OXPHOS defects, by improving disease phenotype in mice and cells (43). In fact, under traditional culturing conditions cellular models of mitochondrial respiratory-chain disease and Friedreich's ataxia showed proliferative defects, which could be reversed by lowering oxygen tension (44). In addition, hypoxia was able to prevent and even reverse the neurological phenotype in a Leigh syndrome mouse model characterized by CI deficiency due to Ndufs4 gene ablation (45). Based on this evidence and since LHON mutations were associated with reduced CI-driven ATP synthesis and increased ROS production (46), we hypothesized that hypoxic cell culture conditions during reprogramming could increase the number of hiPSC clones generated. Thus, we recently reprogrammed PBMCs derived from one LHON m.11778G>A patient and one carrier, in parallel under normoxic (11778 4a and Carrier 2a) and hypoxic (11778 4b and Carrier 2b) conditions (5% oxygen), following published procedures (42). We found that this hypoxic condition significantly increased the number of hiPSC clones generated (Table 1 and Figure 1A).

TABLE 1 | Characteristics of cell lines subjected to reprogramming.

Individuals	Cell line name	Gene mutated	Nucleotide change	Cell type reprogrammed	Reprogramming conditions	Clones obtained
LHON patients	3460 1	MT-ND1	m.3460G>A	FB	Normoxia	2
	3460 2	MT-ND1	m.3460G>A	FB	Normoxia	1
	11778 1a	MT-ND4	m.11778G>A	FB	Normoxia	0
	11778 1b	MT-ND4	m.11778G>A	FB	Normoxia	2
	11778 2a	MT-ND4	m.11778G>A	FB	Normoxia	0
	11778 2b	MT-ND4	m.11778G>A	FB	Normoxia	0
	11778 3	MT-ND4	m.11778G>A	FB	Normoxia	0
	11778 4a	MT-ND4	m.11778G>A	PBMC	Normoxia	1
	11778 4b	MT-ND4	m.11778G>A	PBMC	Hypoxia	4
	carrier 1	MT-ND4	carrier m.11778G>A	FB	Normoxia	0
	Carrier 2a	MT-ND4	carrier 90% m.11778G>A	PBMC	Normoxia	3
	Carrier 2b	MT-ND4	carrier 90% m.11778G>A	PBMC	Hypoxia	7
Disease controls	DOA	OPA1	c.1334 G>A	FB	Normoxia	13
	MPAN	C19orf12	c.172G>A	FB	Normoxia	12
	Pearson 1	mtDNA macrodeletion	m.9449_14550 del	FB	Normoxia	10
	Pearson 2	mtDNA macrodeletion	m.8469_13460 del	FB	Normoxia	16
Healthy controls	Control 1	None	none	FB	Normoxia	23
	Control 2	None	none	FB	Normoxia	20
	Control 3	None	none	PBMC	Normoxia	15

In fact, while under normoxic conditions, we obtained around nine LHON hiPSCs clones in 10 different reprogramming experiments (0.9 clones/reprogramming cycle), and this number increased, under hypoxic conditions, to 11 clones in two reprogramming experiments (5.5 clones/reprogramming cycle). Although these last results derived from only two experiments and need to be further consolidated, they indicated a statistically significant improvement of the reprogramming efficiency (Figure 1A), which remains largely below that observed for the disease control group (12.7 clones/reprogramming cycle) and for the healthy control group (19.3 clones/reprogramming cycle) (Figure 1B). This amelioration of the reprogramming efficiency is relevant not only to obtain enough biological material for further investigations but could also unravel an insight into pathogenic mechanisms, relevant for the disease, and for the development of targeted effective therapy. Remarkably, the subacute phase of LHON is hallmarked by well-known vascular changes, and ongoing discussions revolve around the issue of pseudo-hypoxic signaling that RGCs may produce as their metabolic unbalance reaches the threshold for triggering the disease, possibly underlying the microangiopathy in LHON (3,4,47, 48).

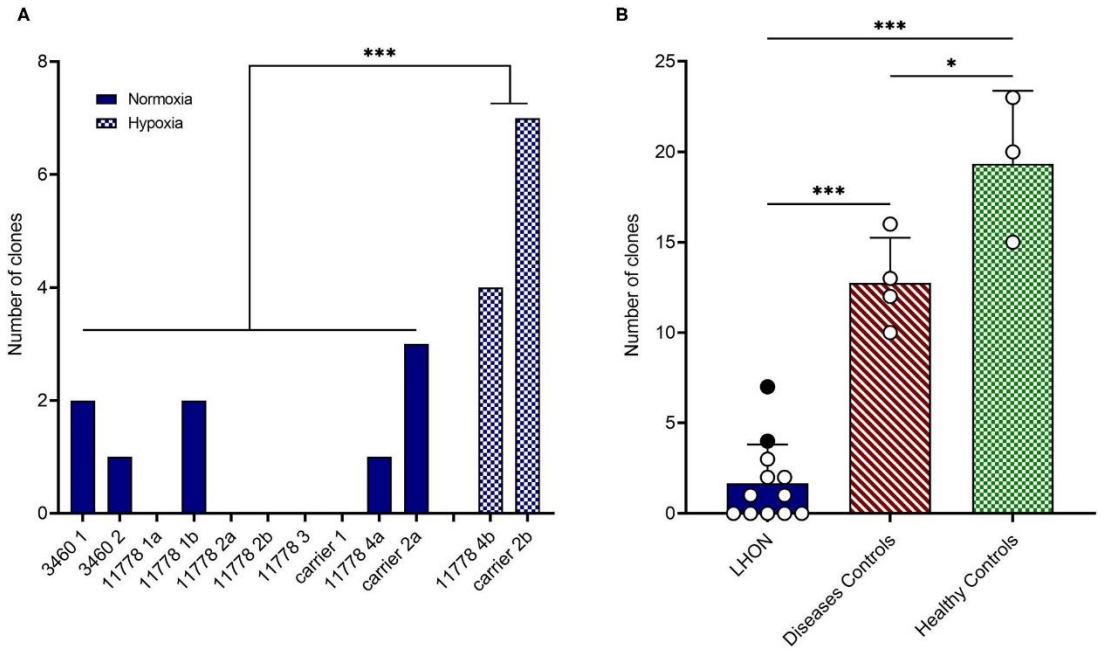

*FIGURE 1 | LHON cell lines reprogramming efficiency. (A) Number of hiPSC clones obtained by reprogramming affected or non-affected (carrier) LHON-derived fibroblasts or PBMC under normoxia (solid blue bars) or hypoxia (cross-hatched blue bars) conditions. (B) Comparison between grouped numbers of hiPSC clones obtained from LHON (solid blue bar), disease controls including non-LHON mitochondrial diseases (hatched red bar), and healthy controls (cross-hatched green bars) cell lines. The dots represent the number of clones obtained under normoxia (white dots) or hypoxia (black dots) conditions. ***p < 0.001, *p < 0.05.*

GENERATION OF RGCs FROM LHON PATIENTS

In the last years, a few protocols have been developed with the purpose to differentiate RGCs directly from patients-derived hiPSCs. However, very few of these RGCs models have been produced for LHON. The first model was reported in 2017 by the Wong group, who generated RGCs from one healthy control and one patient carrying in combination the two homoplasmic mtDNA mutations m.4160T>C and m.14484T>C. Interestingly, they used cybrid technology to also generate patients' fibroblasts homoplasmic for the wild-type mtDNA, thus creating an isogenic control hiPSCs and derived RGCs (35). They found an increased level of apoptosis in LHON RGCs not observed in the healthy and isogenic corrected RGCs, demonstrating that this phenotype was a direct consequence of the LHON mutations. Another group generated hiPSCs-derived RGCs from a m.11778G>A LHON- affected and unaffected carrier, belonging both to the same family (37). They observed enhanced mitochondrial biogenesis, decreased basal respiration, and increased oxidative stress in both affected and unaffected RGCs. However, defective neurite outgrowth was only found in the affected RGCs, while carrier cells exhibited a prominently higher expression of the gene encoding γ-synuclein. Interestingly, increased CI activity was observed in RGCs derived from the asymptomatic carrier but not from the affected patient. Differences in affected and unaffected RGCs carrying homoplasmic m.11778G>A mutation were also found by Yang et al. (49). Both lines showed increased ROS production, but only the affected cells were characterized by increased apoptosis and altered mitochondrial transport pattern along the axons, with an increase in retrograde and a decrease in stationary mitochondria. Furthermore, affected RGCs displayed a significant increase of KIF5A, a member of the kinesin-1 family KIF5, involved in the transport of mitochondria along the axons. Another study carried out on hiPSC-derived RGCs by Yang et al. (50) highlighted the possible role played by AMPA receptors and excitotoxicity in m.11778G>A LHON patients. They used a modified protocol of differentiation of hiPSCs to RGCs to obtain a highly homogeneous RGCs population. They showed how the MT-ND4-mutated LHON-RGC cells exhibited significantly reduced GluR1/R2 (subunits of AMPA receptors) and their associated scaffold proteins and the resulting different pattern of response to glutamate stimulation compared to control. Lastly, Edo et al. (51) demonstrated that hiPSC-derived RGCs can suppress the immune activity of T-cells via TGF-β, have a poor expression of HLA class I, and no expression of HLA class II (CD80 and CD86 co-stimulatory molecules), opening the possibility of using these cells in transplant without the risk of rejection.

GENERATION OF NEURONS FROM LHON PATIENTS

Almost two decades ago, the Cortopassi group generated cybrids using the neuronal precursor cell line NT2, containing mitochondria from patients with m.11778G>A and m.3460G>Amutations (52).

Differentiation of LHON-NT2 cells resulted in a decreased number of cells, reduction of mtDNA amount, and increased ROS production, compared to the parental line. To our knowledge, no hiPSCs-derived neuronal model different from RGCs has been generated to date. Although it
is clear that RGCs represent the best model to unravel LHON pathomechanisms, hiPSCs differentiation in non-RGCs neurons could be informative as well to study the selective degeneration of RGCs in patients. To maintain the transparency of the retina to light, the retinal segment of the RGCs axon is unmyelinated, increasing the energetic demand for action potential firing along this portion and making these cells particularly susceptible to energetic deficit (53). The generation of in-vitro myelinated neurons through co-cultures of Schwann cells and hiPSCs- derived neurons (54) might be informative to establish the involvement of myelin in the pathogenesis of the disease.

STATE OF THE ART ON ORGANOIDS IMPLEMENTATION

The use of 3D organoids generated in vitro from patient-derived cells may represent an important interface between in-vitro and in-vivo modeling of LHON, being more accessible and easier to obtain than mouse models and overcoming the anatomical interspecies differences between humans and rodents.

The first human brain and retinal organoids have been generated about 10 years ago from different groups (55, 56). Lancaster and colleagues successfully modeled genetic microcephaly using hiPSCs derived from patients' fibroblasts to generate brain organoids.

Only a year before, the Sasai group had generated a 3D optic structure by self-organization of cultured human embryonic stem cells (ESCs). The optic cup consisted of the retinal pigmented epithelium, and an inner neural retina correctly organized into multilayered tissue containing photoreceptors (rods and cones), interneuron precursors, and RGCs (57). Both these protocols exploited the capacity of embryoid bodies (EBs) (ESCs or hiPSCs-derived) to proceed spontaneously toward ectodermal commitment without extrinsic signaling factors, which instead are necessary for mesodermal and endodermal specifications (26, 58).

Several modifications and adjustments to the pivotal approaches of Lancaster (55, 59) and Sasai group (57, 60), have been done in the following years, essentially identifying distinct extrinsic factors to obtaining specific regions in the organoids (60, 61), or by-passing the EBs formation step (62). Moreover, improvements toward standardization are constantly evolving, such as the use of completely xeno-free culture methods (62) or the introduction of technologies allowing large-scale controlled organoids production, such as bioreactors or microfluidics chips (26). Importantly, also protocols for cryopreservation at intermediate steps of differentiation have been established, allowing the biobanking of the in-vitro-generated organoids, an additional advantage compared to animal models (26, 57, 62).

THERAPEUTIC APPROACHES

Despite the numerous clinical and pre-clinical investigations carried out to date, effective therapies for LHON are still limited. Effective means that therapy should be able to tangibly modify the disease natural history either by aborting or reverting the catastrophic wave of cell death, or at least limiting the progression so that the visual function is substantially preserved based on anatomical RGCs measurable sparing. Multiple clinical trials have been conducted in recent years, essentially targeting the main pathways involved in the pathogenic mechanism (63). Several antioxidants molecules, some of which with direct effects on mitochondrial respiration, have been tested in patients: idebenone, Coenzyme Q10 (CoQ10), EPI-743, Elamipretide, curcumin (63). To date, idebenone (Raxone®) is the only drug approved by the European Medicines Agency for LHON. It has been documented that idebenone can increase the rate of visual recovery in LHON patients after reaching a nadir of visual loss (64–66); however, its efficacy remains incomplete and variable amongst treated subjects.

The only treatment explored in LHON hiPSCs-derived RGCs was the antioxidant N-acetyl-L-cysteine, which was shown to reduce the ROS production and apoptosis, also rescuing the defective mitochondrial transport observed in the LHON cells (49).

Additional compounds targeting other pathways involved in the LHON pathogenesis (mitobiogenesis, mitophagy, mitoinflammation) have been evaluated only in patient-derived primary cells or in cybrids, such as phytoestrogens (67), rapamycin (68), papaverine, and zolpidem (69). Moreover, other potential strategies are emerging, for example, the inhibition of the miRNA181a/b, acting on both mitobiogenesis and mitophagy (70). All these pharmacological approaches should be reevaluated also in RGC to understand if they are efficacious and rapidly translatable into a therapy.

Besides pharmacological clinical trials, encouraging results are nowadays being reported by clinical trials with gene therapy for patients carrying the m.11778G>A/MT-ND4 mutation [reviewed in Amore et al. (63)], using the Adeno-Associated Virus (AAV)-mediated allotopic expression of a wild-type recoded version of the mtDNA-encoded ND4 subunit of complex I (71, 72). To better refine the efficiency of allotropic expression strategy in the context of RGCs, detailing the mitochondrial import of wild-type ND4 protein, its competition with the endogenously expressed mutant ND4, and finally the dynamics of complex I assembly of either one or the other ND4 subunits, may greatly benefit of 3D organoid modeling of LHON. This might resolve some of the criticisms previously raised by the preclinical studies (73–75). The same approach could be developed for the other LHON-related mutations, and different approaches based on gene therapy might be proposed in the future, for example, modulating the expression of modifying genes or miRNAs (70) or applying possible gene-editing strategies, as recently proposed for mtDNA (76). Similarly, the feasibility of mitochondrial import of nucleic acids, claimed by some studies

(77, 78), may benefit the use of eye/brain organoids carrying LHON mutations, reproducing those experiments and possibly paving the road for further gene therapy strategies.

DISCUSSION

In-vitro modeling of LHON through 2D cell cultures, including patient-derived hiPSCs and neurons, allowed important steps forward in the understanding of the pathogenic mechanism of this complex and fascinating disease. We here presented evidence that LHON hiPSCs are difficult to obtain as compared to other apparently more severe mitochondrial disorders, but this reduced efficiency could be improved by performing the reprogramming experiment under hypoxic conditions. This observation would deserve further investigation since obtaining a large number of hiPSCs clones is instrumental to further develop differentiated 2D cell cultures. Although 2D cell cultures show several advantages such as easy manipulation and analysis (good accessibility of nutrients and/or drugs, excellent visualization and tracking of cells at microscopy by live-cell imaging), the complex 3D architecture of in-vivo tissues is not reproduced by this method, nor are the interactions between different co- resident populations of specialized cells (79). This is particularly important for LHON, in which RGCs are the only cells affected in the retina. The application of the innovative single-cell omics on hiPSCs-derived 3D organoids can provide useful insight on the cell specificity of LHON disease. A recent study has already paved the way for this approach, performing single-cell transcriptomics on in-vitro-generated human retinal organoids and ex-vivo adult human retinas, allowing mapping of disease- associated genes to particular cell types (25). This work highlights the importance of investigating mechanisms of disease in RGCs since they could be differently regulated in the traditional cell models so far exploited. Many of the findings so far achieved in LHON should be revalidated in RGC models to assure that the right pathogenic mechanism was effectively targeted by therapies.

Modeling mitochondrial diseases caused by mtDNA mutations in animals is still challenging due to the difficulties in manipulating the mitochondrial genome (80, 81), although a new promising method has been recently described Mok et al. (76). In 2012, the group of Doug Wallace, a pioneer in the field of mitochondrial medicine, successfully generated a mouse model carrying a mutation in the MT-ND6 gene, which developed a pathology closely resembling LHON at 2 years of age, although the mouse did not show reduced visual responses (82). This model was instrumental to reproduce some of the hallmark features observed in human post-mortem LHON retina (83, 84); however, mice, because they lack the macular region, ultimately fail to reproduce the natural history that clinically characterizes humans with the characteristic catastrophic evolution of RGC neurodegeneration (3, 4).

Thus, it will be fundamental to investigate pathogenic mechanism of LHON disease in hiPSCs-derived

cell/tissue- specific models and retinal organoids might be instrumental to assess efficacy/toxicity in the pre-clinical phases. The issue of maintaining organoids in a spinning bioreactor under hypoxic conditions, with the intent of reproducing the brain endogenous developmental program, could be crucial, especially for LHON in light of our observation, but also in general for other diseases. To date, only a few brain organoids models of mitochondrial diseases have been reported, specifically for MELAS syndrome, mitochondrial neurogastrointestinal encephalomyopathy, Friedrich ataxia, and Leigh syndrome (85– 88). We think that modeling LHON with retinal organoids would provide substantial progress in the understanding of the pathogenic mechanisms and in identifying the correct targets for therapy development. To this end, testing pharmacological and gene therapy approaches with human transgenes packaged in the appropriate AAV vector constructs, currently performed in animal models with obvious problematic issues (89, 90), may benefit human-patient-derived eye/brain organoids, certainly allowing to speed translation from pre-clinical science to approval for human clinical trials of regulatory agencies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Fondazione IRCCS Istituto Neurologico Carlo Besta. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CP, AC, AI, and ID perform experiments, analyzed data, generated the table and figure, and analyzed the literature. AM, VB, and VC analyzed the literature. VT concept the manuscript architecture, supervised the analysis of the data, and of the literature. VT performed the final revision of the manuscript. All the authors draft the manuscript.

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The handling editor is currently organizing a Research Topic with one of the author VC.

4 Pathological mitophagy disrupts mitochondrial homeostasis in Leber's hereditary optic neuropathy

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SUMMARY

Leber's hereditary optic neuropathy (LHON), a disease associated with a mitochondrial DNA mutation, is characterized by blindness due to degeneration of retinal ganglion cells (RGCs) and their axons, which form the optic nerve. We show that a sustained pathological autophagy and compartment-specific mitoph- agy activity affects LHON patient-derived cells and cybrids, as well as induced pluripotent-stem-cell-derived neurons. This is variably counterbalanced by compensatory mitobiogenesis. The aberrant quality control disrupts mitochondrial homeostasis as reflected by defective bioenergetics and excessive reactive oxygen species production, a stress phenotype that ultimately challenges cell viability by increasing the rate of apoptosis. We counteract this pathological mechanism by using autophagy regulators (clozapine and chlo- roquine) and redox modulators (idebenone), as well as genetically activating mitochondrial biogenesis (PGC1-a overexpression). This study substantially advances our understanding of LHON pathophysiology, providing an integrated paradigm for pathogenesis of mitochondrial diseases and druggable targets for therapy.

INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is among the most frequent mitochondrial disorders (Bargiela et al., 2015; Yu-Wai- Man et al., 2016) and, over three decades ago, was the first to be associated with maternally inherited missense mutations affecting mitochondrial DNA (mtDNA) (Wallace et al., 1988). This blinding disease is peculiar, as it very selectively affects only retinal ganglion cells (RGCs), which are the terminal retinal neurons providing the axons to the optic nerve. RGCs undergo a catastrophic wave of neurodegeneration, leading to subacute optic nerve atrophy and severe loss of central vision (Carelli et al., 2004; Yu-Wai-Man et al., 2011). Usually, one of three common LHON mtDNA mutations, all affecting the NADH dehydrogenase (ND) subunits of complex I, are found in the homoplasmic state (100% of mtDNA is mutated) in all individuals from the maternal lineage. However, only a subset of individuals becomes affected (incomplete penetrance), most frequently young males (gender prevalence) (Carelli et al., 2004; Yu-Wai-Man et al., 2011). Com- plex I dysfunction induced by LHON mutations is reflected in decreased efficiency of oxidative phosphorylation (OXPHOS) (Baracca et al., 2005; Korsten et al., 2010) and increased reactive oxygen species (ROS) production (Beretta et al., 2004; Floreani et al., 2005; Lin et al., 2012). In vitro, if cells are forced to rely on OXPHOS for energy production by switching the culture me- dium carbon source from glucose to galactose, a loss of

viability is observed, due to increased apoptosis (Ghelli et al., 2003; Zanna et al., 2005). Furthermore, ex vivo (blood cells, muscle bi- opsies), postmortem (retinal and optic nerve specimens), and in vitro (fibroblasts) evidence pointed to increased mitobiogene- sis as a compensatory strategy to counteracting the defective phenotype induced by LHON mutations (Giordano et al., 2014). In LHON, the remarkable tissue specificity has been proposed to depend on the unique characteristic of RGC axons, which are unmyelinated in the long initial stretch of their intra-retinal tract and only then become myelinated as they cross the lamina cri- brosa at the optic nerve head (Carelli et al., 2004; Yu-Wai-Man et al., 2011). This myelination pattern implicates an asymmetric energy dependence reflected by the different mitochondrial den- sities and dynamics needed to correctly distribute the organelles along the RGC axons. Thus, the compensatory increase of mito- biogenesis must be considered in this context. In fact, small RGCs with thin axons are the most sensitive to the defective mitochondrial metabolism present in LHON (Pan et al., 2012; Sadun et al., 2000), and a larger optic disc is a protective factor (Ramos Cdo et al., 2009). The major modifying effect driving the two unexplained features of male prevalence and incomplete penetrance may depend on divergent efficiency in promoting the compensatory mitobiogenesis (Giordano et al., 2014). In particular, estrogens activate mitobiogenesis, protect- ing females (Giordano et al., 2011), and, aside from gender, the efficient activation of mitobiogenesis that some LHON mutation carriers display predicts the risk for developing the disease, allowing these carriers to be unaffected for their entire lives (Gior- dano et al., 2014). The degree of mitobiogenesis is most likely determined by specific, still unknown, genetic variants (Carelli et al., 2003) but is also highly modified by interactions with envi- ronmental factors, such as tobacco and alcohol exposure (Care- lli et al., 2016; Giordano et al., 2015). More recently, some ge- netic variants have been proposed as modifiers in LHON penetrance, and these involve different pathways occurring only in specific subgroups of families (Jiang et al., 2016; Yu et al., 2020). Overall, the scenario of LHON penetrance remains extremely complex and only partially understood. New evidence continues to link mitobiogenesis to a wider master program, regulating mitochondrial homeostasis and life cycle (Giorgi et al., 2021; Twig and Shirihai, 2011). The counter- part of mitobiogenesis is mitochondrial clearance, with the elim- ination of damaged mitochondria by autophagy, i.e., mitophagy (Patergnani and Pinton, 2015). The execution of mitophagy, its role in LHON pathogenesis, and how mitophagy coordinates with compensatory mechanisms remain largely unknown, prompting the current study, which is aimed at elucidating how mitochondrial homeostasis is disturbed by LHON-related com- plex I dysfunction, thus paving the road for new druggable ther- apeutic targets. Our results suggest that pathologically increased mitophagy prevails in LHON-affected individuals as opposed to efficient biogenesis, which characterizes the suc- cessful compensation in LHON carriers. The latter is driven by the nuclear background, whereas the pathological quality con- trol is directly dependent on the mutant mtDNA, as

supported by cybrid experiments, and this general paradigm applies across cell models, from fibroblasts to neuronal cells, and may be tar- geted by pharmacological interventions.

RESULTS

Autophagy is pathologically increased in cells from LHON-affected patients but not in those from unaffected mutation carriers.

Our starting point was to investigate how autophagy may be affected by LHON mutations in patient-derived fibroblasts, induced pluripotent stem cell (iPSC)-derived neuronal cell lines, peripheral blood mononuclear cells (PBMCs), and sera samples. To this end, we thoroughly analyzed the two most frequent homoplasmic LHON mutations, the m.3460G>A/MT-ND1 and the m.11778G>A/MT-ND4 (herein called, for the sake of brevity, 3460 and 11778 mutations). Light chain 3 (MAP1LC3, hereafter referred to as LC3) is a specific marker for monitoring autophagy. During autophagy, the cytoplasmic form of this protein (LC3-I) is cleaved and lipidated into the membrane-bound form (LC3-II), which is localized to the autophagosome (Thukral et al., 2015) and correlates with the number of autophagosomes. As an additional autophagy marker, we used the protein SQSTM1/p62, which is inversely correlated with autophagy activity (Katsuragi et al., 2015). We found that in basal conditions, compared with control cells, fibro- blasts from an LHON-affected patient (herein abbreviated as LHON-affected) with the 3460 mutation, considered the most se- vere for biochemical dysfunction (Carelli et al., 1997), presented a sustained autophagy activation (Figure 1A). To further validate these results, we used nutrient-starvation conditions, known to promote the activation of the autophagy machinery (Chen et al., 2014). Under these conditions, autophagy was signifi- cantly activated in control fibroblasts, whereas the magnitude of this response was attenuated in LHON-affected fibroblasts, suggesting that, from their high basal autophagy activity, the capacity to further increase did not exceed the top level of controls (Figure 1A). Fluorescence microscopy measurements of auto- phagy with the GFP-LC3 probe, in basal conditions and after starvation, confirmed these findings (Figure 1B). Autophagy levels in control fibroblasts (n = 4) used for this study were com- parable (Figures S1A and S1B), confirming that the differences observed with LHON-affected were dependent on the mtDNA LHON mutations. To confirm that increased autophagy is a key feature of patients affected by LHON, we also evaluated fibroblasts from two LHON-affected and the respective two brothers, unaffected mutation carriers (herein abbreviated as LHON-carrier), all harboring the most frequent but biochemically milder 11778 mutation (Carelli et al., 1997).

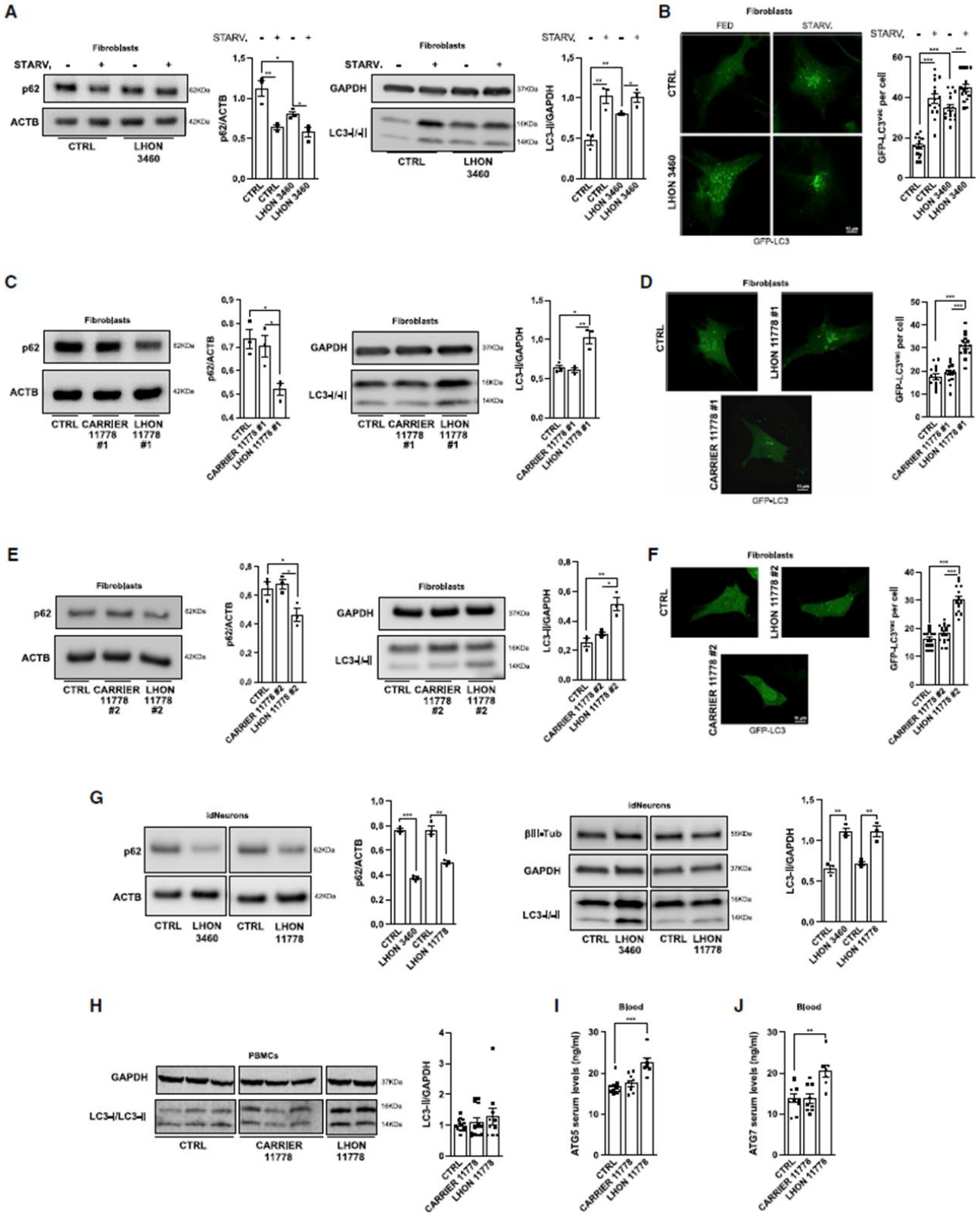


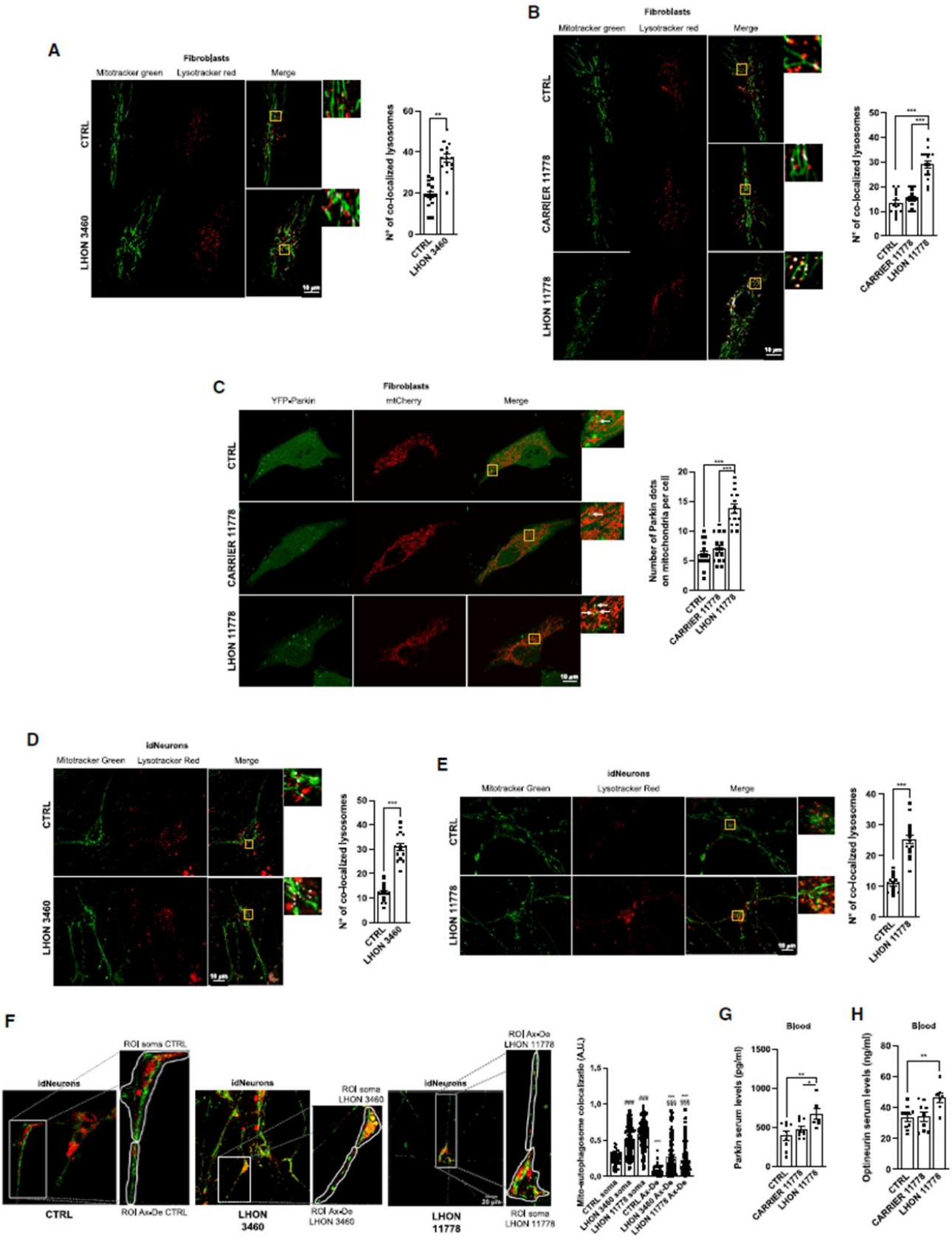
Figure 1 ***Autophagy is pathologically increased in cells from LHON-affected patients but not in unaffected mutation carriers*** *(A–F) Detection of autophagy activity through immunoblot and GFP-LC3 puncta count in LHON fibroblasts carrying 3460 (A and B) and 11778 mutations (C–F). In the latter, the autophagy levels were also analyzed in fibroblasts obtained from the two unaffected mutation-carrier brothers (identified as #1 and #2). Where not indicated, the representative western blots come from couple #2, while the*

*histograms represent the average of the data from the two pairs #1 and #2. Where indicated, cells were starved (STARV.) for 1 h.(G) Autophagy levels were also detected in idNeurons harboring LHON mutations. (H)Finally, autophagic levels were measured by immunoblot in ex vivo PBMCs obtained from healthy individuals (CTRLS) (n = 17), LHON-affected (n = 10), and LHON-carrier (n=14) (the representative image shown has been cropped to invert the order of samples loading). (I and J) ELISA was performed on serum samples from CTRLS (n=10), LHON-carrier (n = 9), and LHON-affected (n = 7) patients to detect ATG5 (I) and ATG7 (J). Data are presented as means ± SEM. n = at least 3 independent experiments for western blots or 5 visual fields per at least 3 independent samples per condition for GFP-LC3 experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.*

While significantly reduced p62 level and increased LC3-II amount were confirmed in the LHON-affected (Figures 1C–1F, respectively, for the first and second pairs of LHON-affected/carrier siblings), as seen for the 3460 mutation, the LHON-carriers had an autophagy activity similar to controls, despite the presence of the homoplasmic 11778 mutation. As LHON is primarily characterized by the subacute degeneration of RGCs, we sought to test a neuronal cell model. To this aim, we generated different transgene-free iPSC clones by using Sendai-virus-mediated expression of the four Yamanaka's factors (OCT3/4, SOX2, c-MYC, and KLF4) to reprogram the fibro- blasts of two patients carrying the 3460 and the 11778 LHON mutations, respectively, and two controls. Then, we obtained neural precursor cells (NPCs) through embryoid body formation and generated terminally differentiated neurons (Figure S1C). We assessed autophagy activity in 35-day-old iPSC-derived neurons (herein abbreviated as idNeurons) expressing specific markers such as bIII-tubulin, Map2, and NeuN (Figure S1D), and again we observed a significant reduction of p62 and a parallel increase of LC3-II in both LHON-affected carrying the 3460 and the 11778 mutations (Figure 1G). Autophagosome accumulation, and thus increased levels of LC3, may also occur due to impaired autophagosome degradation (Mizushima et al., 2010). To assess this alternative scenario, we performed autophagy flux analysis in the presence of bafilomycin A1 (Baf-A1) (100 nM for 2 h), which inhibits the late phase of autophagy (Klionsky et al., 2021). We found that Baf-A1 treatment induced abundant cleaved LC3 accumulation in all the fibroblasts analyzed (Figures S1E for 3460 mutation and S1F for 11778 mutation), indicating that the autophagy response is unaffected in our experimental conditions. To verify if pathological activation of autophagy also charaterizes LHON patients, we evaluated ex vivo LC3-II amounts in PBMCs derived from controls, LHON-affected, and LHON- carrier homoplasmic for the 11778 mutation (Figure 1H). Due to a widespread variability and a limited number of samples, we failed to observe a significant increase of LC3-II in LHON-affected compared with controls and LHON-carriers (Figure 1H). The ELISA detection of autophagy markers in blood samples represents a more sensible and reliable method to assess autophagy activity in biological samples (Patergnani et al., 2018, 2021a; Xue et al., 2020). Thus, we also evaluated serum levels of the autophagy markers ATG5 and ATG7, highlighting their significant increase only in LHON-affected (Figures 1I and 1J). Taken together, all these results demonstrated that the auto- phagy machinery is intrinsically activated in LHON-affected, whereas a compensatory mechanism must be active in LHON- carrier cells, which behaved more similarly to controls.

Selective autophagy of mitochondria (mitophagy) follows the same pattern of autophagy

Autophagy also exists in selective forms, which, in the case of mitochondria, removes damaged organelles through the targeted process of mitophagy. We assessed mitophagy by the simultaneous labeling of mitochondria with MitoTracker green and of autophagolysosomes by LysoTracker red (Kanki and Oka- moto, 2014; Patergnani and Pinton, 2015). As shown in Figures 2A and 2B, mitophagy was significantly increased in LHON-affected fibroblasts with both 3460 and 11778 mutations, whereas it was not activated in the LHON-carriers with 11778 mutation, displaying an activity similar to control cells. Upon acute mitochondrial dysfunction, the PINK1-Parkin pathway is activated by Parkin recruitment from the cytosol to the mitochondrial surface, ultimately leading to mitophagy (Narendra et al., 2010; Vives-Bauza et al., 2010). By using fluorescent microscopy techniques, we investigated Parkin recruitment to mitochondria, finding that LHON fibroblasts displayed a higher co-localization rate between Parkin and mitochondria than both control and LHON-carrier samples (Figure 2C). The assessment of mitophagy in idNeurons carrying either 3460 or 11778 mutations paralleled the fibroblasts results, displaying a significant increase in LHON-affected compared with controls (Figures 2D and 2E). Recent investigations suggested that during neurodegeneration, the excessive mitochondrial removal characterizing the neuronal cells might be region specific (Zaninello et al., 2020). We investigated this aspect by comparing the mitophagy activity measured in the body and in the axonal-dendrites regions in our massive neuronal culture. This specific growth condition prevented us from clearly identifying the axonal hillock region. Nevertheless, our analysis unveiled elevated mitophagy levels in the soma of the neurons, which also includes the axonal hillock. Although this region-specific mitophagy was a common feature shared be- tween control and LHON-affected neurons (Figure 2F), the latter showed an extremely elevated mitophagy removal of mitochondria compared with control neurons. Further support to the increased mitophagy activity is provided by the ex vivo assessment of Parkin and Optineurin in serum from circulating blood of LHON-affected patients (Figures 2G and 2H).


A
Fibroblasts
Mitotracker green
Lysotracker red
Merge
CTRL
LHON 3460
N° of co-localized lysosomes
B
Fibroblasts
Mitotracker green
Lysotracker red
Merge
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CARRIER 11778
LHON 11778
N° of co-localized lysosomes
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Figure 2 ***LHON disease is characterized by excessive mitophagy levels*** *(A, B, D, and E) Confocal microscopy assessment of mitophagy respectively in fibroblasts and 35-day-old idNeurons carrying 3460 (A and D) and 11778 (B and E) mutations were performed by loading cells with LysoTracker red and MitoTracker green to visualize lysosomes and mitochondria, respectively.(C) Mitophagy levels were analyzed by detecting the amount of fluorescent YFP-Parkin localized on the mitochondrial surface in fibroblasts from one LHON-affected patient carrying the 11778 mutation and of the non-affected (carrier) brother carrying the same 11778 mutation.(F) A similar methodological approach with LysoTracker red and MitoTracker green was used to investigate whether mito-autophagosomes were present in spe- cific regions of the idNeurons (soma and axon-dendrite (Ax-De) regions). ###p < 0.001 to CTRL soma, xxxp < 0.001 to CTRL Ax-De, ***p < 0.001 Ax-De to its own soma.(G and H) Increased levels of mitophagy marker Parkin (G) and Optineurin (H) were detected in serum samples of 11778 LHON-affected patients (n = 7) compared with CTRLS (n = 10) and 11778 LHON-carriers (n = 9).Data are presented as means ± SEM. n = at least 5 visual fields per at least 3 independent samples per condition for colocalization experiments. Data to evaluate region-specific mitophagy were obtained from 78 regions of interest (ROIs) for iPSC-derived control neurons, 297 ROIs for iPSC-derived 3460 neurons, and 225 ROIs for iPSC-derived 11778 neurons and analyzed using one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001.*

We attempted to conduct a transmission electron microscopy (TEM) assessment of RGCs in postmortem retinas from LHON- affected carrying either the 3460 or 11778 mutation; these tis- sues are not only difficult to collect, but they are processed several hours after death, thereby making it difficult to have a sufficient number for reliable quantification of the cellular events observed as well as a high resolution and quality of the samples imaged. However, acquisitions with qualitative images are available at Mendeley Data: https://doi.org/10. 17632/83vvm47z2f.1. Overall, this set of experiments documented that the increase of autophagy activity is also reflected on mitophagy, which was enhanced in LHON-affected in cultured cell models, including idNeurons. Again, the LHON-carrier did not display this phenotype, behaving much more closely to controls.

Pathological autophagy and mitophagy is transferred to cybrids with the LHON mutant mtDNA independently from the affected/carrier status

To establish whether the pathological increase in autophagy and mitophagy observed in the previous experiments is primarily driven by the mutant mtDNA, we evaluated these readouts in a cytoplasmic trans-mitochondrial hybrid (cybrid) cell model, a valuable model for investigating mtDNA-dependent phenotypes, removing the influence of the original nuclear background of the patient (King and Attardi, 1989; King et al., 1992; Vergani et al., 1995). We assessed autophagy in LHON cybrids carrying either the 3460 or 11778 mutation but also the milder m.14484T>C/MT- ND6 (herein 14484) mutation, generated from LHON-affected fibroblasts compared with mtDNA haplogroup-matched control cybrids. Furthermore, the same experiments were also per- formed in cybrids generated from a pair of discordant brothers (from #2 independent fibroblast cell lines) carrying the same 11778 homoplasmic mutation, compared with mtDNA haplogroup-matched control cybrids. In LHON cybrids, we confirmed the results previously obtained in LHON-affected fibroblasts. LHON mutations significantly increased autophagy activity (Figures 3A–3D, S2A, and S2B), and similar results were also obtained with cybrids carrying the mildest 14484 mutation (Figures S2C and S2D).

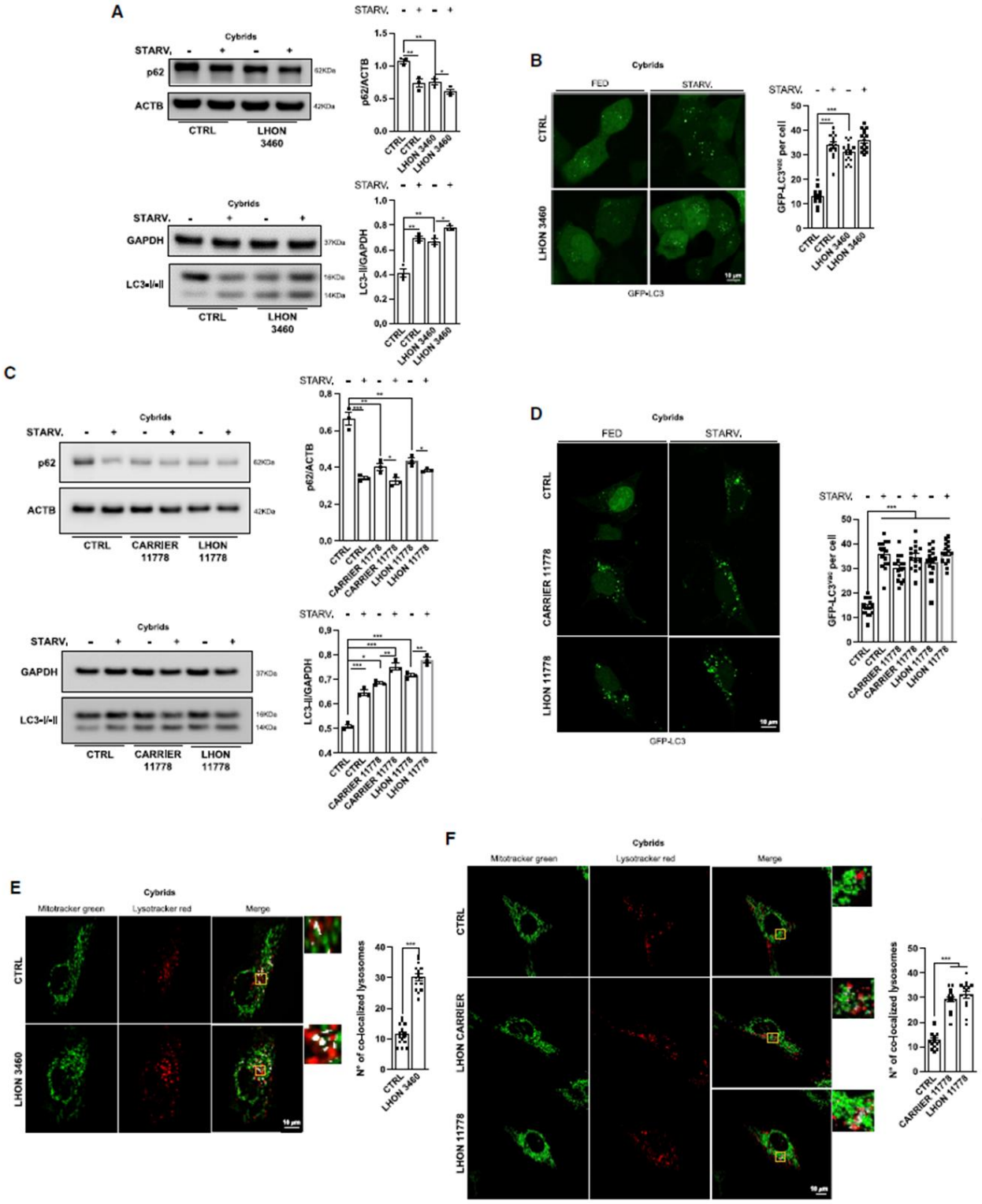

Figure 3. ***Autophagy and mitophagy results increased in cybrids*** *(A–D) Autophagy detection by immunoblot (A) and fluorescent microscopy (B) was performed in cybrids carrying 3460 and in cybrids derived from fibroblasts from one LHON-affected patient carrying the 11778 mutation and of the non-affected (carrier) brother carrying the same 11778 mutation (C and D). Where indicated, the cells were STARV. for 1 h. (E)Confocal microscopy assessment of mitophagy in control and mutant cybrids harboring 3460 LHON mutations. (F)Similar experiments were achieved in LHON-affected cybrids carrying the 11778 mutation and of the non-affected (carrier)brother carrying the same 11778 mutation. Data are presented as means ± SEM. n = at least 3 independent experiments for*

*western blots or 5 visual fields per at least 3 independent samples per condition for fluorescent microscopy experiments. *p < 0.05, **p < 0.01, and ***p < 0.001..*

In addition, the assessment of mitophagy activity in LHON cybrids carrying the 3460 or 11778 mutation also showed significantly increased mitophagy (Figures 3E, 3F, and S2E). Differently, the results obtained in LHON-carrier cybrids were discordant from those previously achieved in fibroblasts. Indeed, LHON-carrier cybrids had both autophagy (Figures 3C and 3D) and mitophagy (Figures 3F and S2E) comparable to the LHON-affected cybrids. Autophagy flux analysis, also performed by fluorescence microscopy with the tandem mCherry-GFP-LC3 construct, confirmed again that this process was unaffected and that auto- phagosomes were properly degraded (Figures S3A–S3C). To further dissect the molecular mechanisms leading to altered autophagy in LHON cells, in particular all potential factors that impinge upon global mitochondrial homeostasis and that co-regulate mitophagy, we investigated the pathways involved in the mechanistic target of rapamycin (mTOR) kinase complex and 50 adenosine monophosphate-activated protein kinase (AMPK) axis. The mTOR pathway, through phosphorylation events, strongly suppresses autophagy (Missiroli et al., 2016; Xue et al., 2020). Thus, we evaluated whether LHON mutations alter the mTOR/AMPK pathway by assessing AMPK phosphorylation levels and the regulation of mTOR-downstream targets, including the phosphorylation/inactivation of the mRNA translation repressor 4E-binding protein (4EBP1) and the autophagy regulator Unc-51 like autophagy activating kinase (ULK1), a serine/threonine (Ser/Thr) kinase that plays a specific role in clearing mitochondria (Egan et al., 2011). mTOR and AMPK- dependent ULK1 phosphorylation regulates the activity of this pathway (Kim et al., 2011) and the accumulation of dysfunctional mitochondria upon mitophagy induction (Wu et al., 2014) (Figure S3D). As shown in Figure S3E, in LHON cells, sustained autophagy involved AMPK activation and concomitant mTOR pathway inhibition, as evidenced by increased AMPK-mediated phosphorylation of ULK1 in Ser 317 and of acetyl-coenzyme A (CoA) carboxylase (ACC) and decreased phosphorylation of acetyl-eukaryotic translation initiation factor 4EBP1. In summary, the phenotype of dysfunctional autophagy and mitophagy is tightly associated with the LHON mutations, as it was faithfully transferred with mutant mtDNA in the constant nu- clear background of the cybrid cell model and separated from the influence of the original nuclear background of the patient. This phenotype was correlated with the modulation of the mTOR/AMPK pathway.

Pathological autophagy and mitophagy in LHON reflects a mitochondrial stress phenotype

Complex I dysfunction is commonly associated with increased ROS production, specifically superoxide (Fiedorczuk and Sazanov, 2018; Hirst and Roessler, 2016). We assessed ROS by using MitoSOX Red, a fluorogenic dye specifically targeted to mitochondria in live cells. All LHON-affected cells (fibroblasts and cy- brids) carrying either the 3460 (Figures 4A and 4B) or the 11778 (Figures 4C and 4D) mutation presented increased ROS levels, and a state of nutrient deprivation increased ROS (Figures 4A– 4D), corroborating that

starvation-induced autophagy is regulated by ROS (Li et al., 2013). Moreover, while ROS production in the LHON-carrier fibroblasts was equivalent to control cells (Figure 4C), excessive ROS levels characterized LHON-carrier cybrids, comparable to LHON-affected cells (Figure 4D). Notably, a harmful ROS production may be highly dependent on the electrochemical gradient that forms across the inner mitochondrial membrane and sustains mitochondrial membrane potential (Jm) (Giorgi et al., 2018b; Suski et al., 2012). As shown in Figures 4E and 4F, we found that in 3460 LHON-affected fibro- blasts and cybrids, Jm appeared significantly increased. More- over, starvation increased the Jm in controls to levels comparable to LHON cells at resting conditions, whereas it failed to affect LHON fibroblasts, which remained hyperpolarized, similar to resting conditions (Figures 4E and 4F). Comparable findings were observed in fibroblasts and cybrids carrying the 11778 mutation (Figures 4G and 4H). Consistent with the ROS results, the LHON-carrier fibroblasts had Jm equivalent to controls (Figure 4G), while LHON-carrier cybrids displayed values comparable to the LHON-affected cybrids (Figure 4H). Overall, these results demonstrate that mitochondria present a stress phenotype in LHON-affected cells characterized by increased ROS and hyperpolarization of Jm. These features were conserved in LHON-carrier cybrids but were minimal or ab- sent in LHON-carrier fibroblasts, which displayed fairly normal ROS levels and Jm similar to controls (Figures 4C and 4G), indicating again an efficient compensatory response to the LHON mutations.

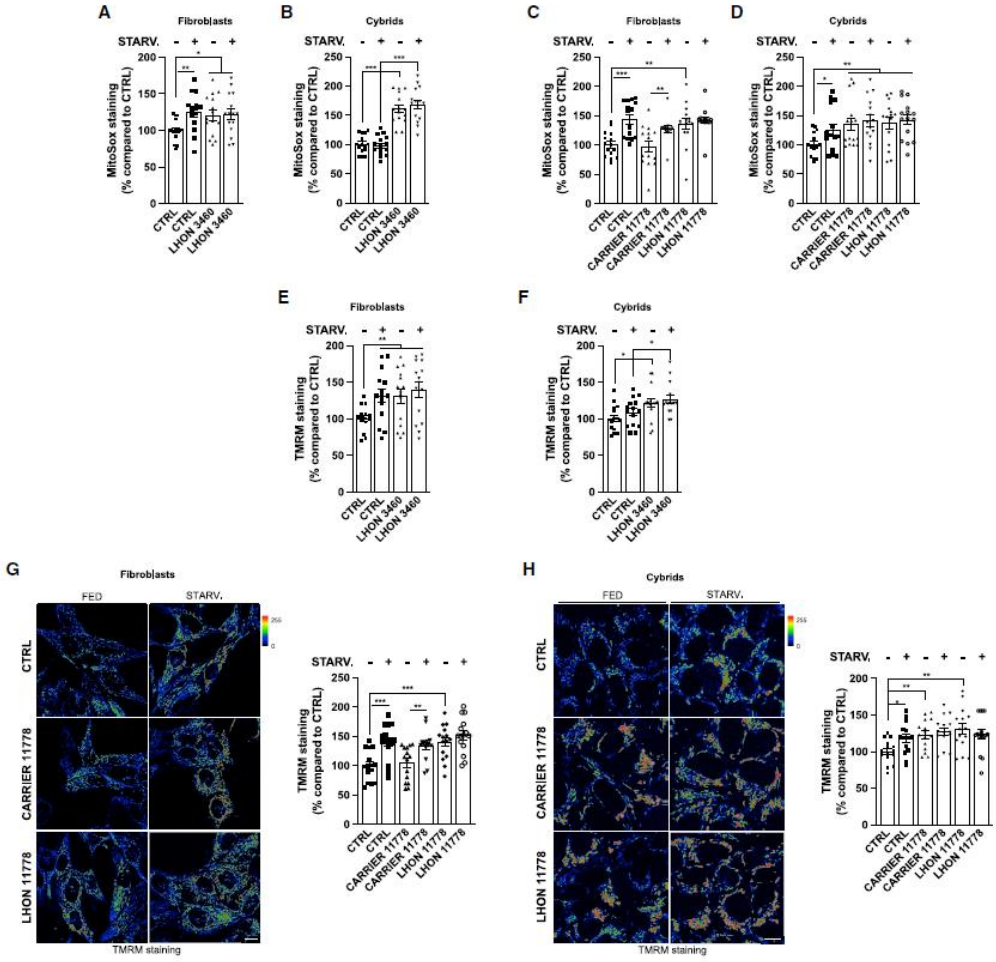

Figura 4 ***Complex I deficiency leads to altered mitochondrial function in LHON-affected individuals, which is compensated in carriers*** *(A–D) Measurements of mitochondrial ROS production in LHON-derived 3460 fibroblasts (A) and cybrids (B) and in 11778 fibroblasts (C) and cybrids (D) by using MitoSOX red probe. (E–H) The mitochondrial transmembrane potential (Jm) of 3460 and 11778 fibroblasts and cybrids was detected by using the Jm-sensitive probe TMRM. When indicated, the cells were STARV. for 30 min before TMRM loading. Data are presented as means ± SEM. n = at least 5 visual fields per at least 3 independent samples per condition. *p < 0.05, **p < 0.01, and ***p < 0.001.*

Therapeutic strategies reverse the pathologic phenotype of LHON-affected cells, balancing mitochondrial homeostasis

LHON pathogenic mutations ultimately cause impaired cell viability and increased rate of mitochondria-dependent apoptosis in cybrids, particularly when cells are pushed into a stress condition, such as in galactose medium (Ghelli et al., 2003; Zanna et al., 2005). Besides cybrids, increased propensity to apoptosis has been reported to occur also in iPSC-derived RGCs carrying an unusual combination of LHON mutations (Wong et al., 2017). We confirmed here that LHON mutations reduce the growth rate of LHON-affected fibroblasts compared with controls (Figures S4A and S4B), and this effect was transferred in cybrids (Figures S4C and S4D). This was paralleled by a significant in- crease in apoptosis of both fibroblasts and cybrids, as evidenced by the increased cleavage of poly (ADP-ribose) polymerase 1 (PARP) and caspase 3 (CASP3) (Figures S4E and S4F). The increased propensity to apoptosis also characterized the id- Neurons of LHON-affected carrying the

11778 and 3460 mutations, compared with controls (Figure S4G). Growing evidence points to an intimate relationship linking apoptosis and autophagy (Bialik et al., 2018), suggesting that the activation of autophagy and mitophagy pathways observed in LHON mutant cells may, in turn, also modulate the apoptotic machinery. Thus, we hypothesized that by modulating the autophagy and mitophagy pathways to limit their excessive activation, we could impinge therapeutically on the LHON pathogenic mechanism, correcting the apoptotic loss of cell viability. Autophagy can be finely adjusted with pharmacological interventions targeting either the early or late stages of this process (Choi et al., 2013). The most used inhibitor targeting the early stages is 3-methyladenine (3-MA); for later stages, chloroquine (CQ) and its derivatives are the most commonly used. Recently, a novel class of drugs has been added, i.e., antipsychotic and antidepressant drugs such as clozapine (CL), which acts as potent late-stage autophagy inhibitor (Park et al., 2012; Patergnani et al., 2021b). We tested the efficacy of these drugs for their capacity to inter- fere with the autophagy machinery, aware of possible re-purposing for some of these molecules. The autophagy inhibitor treatments significantly reduced the autophagy activity, assessed as LC3-II content, in both LHON-affected fibroblasts and cybrids carrying the 11778 mutation (Figures 5A and 5B). Indeed, the early-stage autophagy inhibitor 3-MA significantly decreased the LC3-II levels, whereas CQ and CL, which interfere with lysosome/autophagosome fusion, led to accumulation of LC3-II. Un- der these treatment conditions, we also found that the extent of apoptosis induction was significantly limited, as evidenced by reduced levels of both cleaved PARP and cleaved CASP3 (Figures 5C and 5D), ultimately increasing cell viability of fibro- blasts and cybrids (Figures 5E and 5F). We did not observe any cytotoxic effect of the compounds tested in either control fibroblasts or cybrids (Figure S4H).

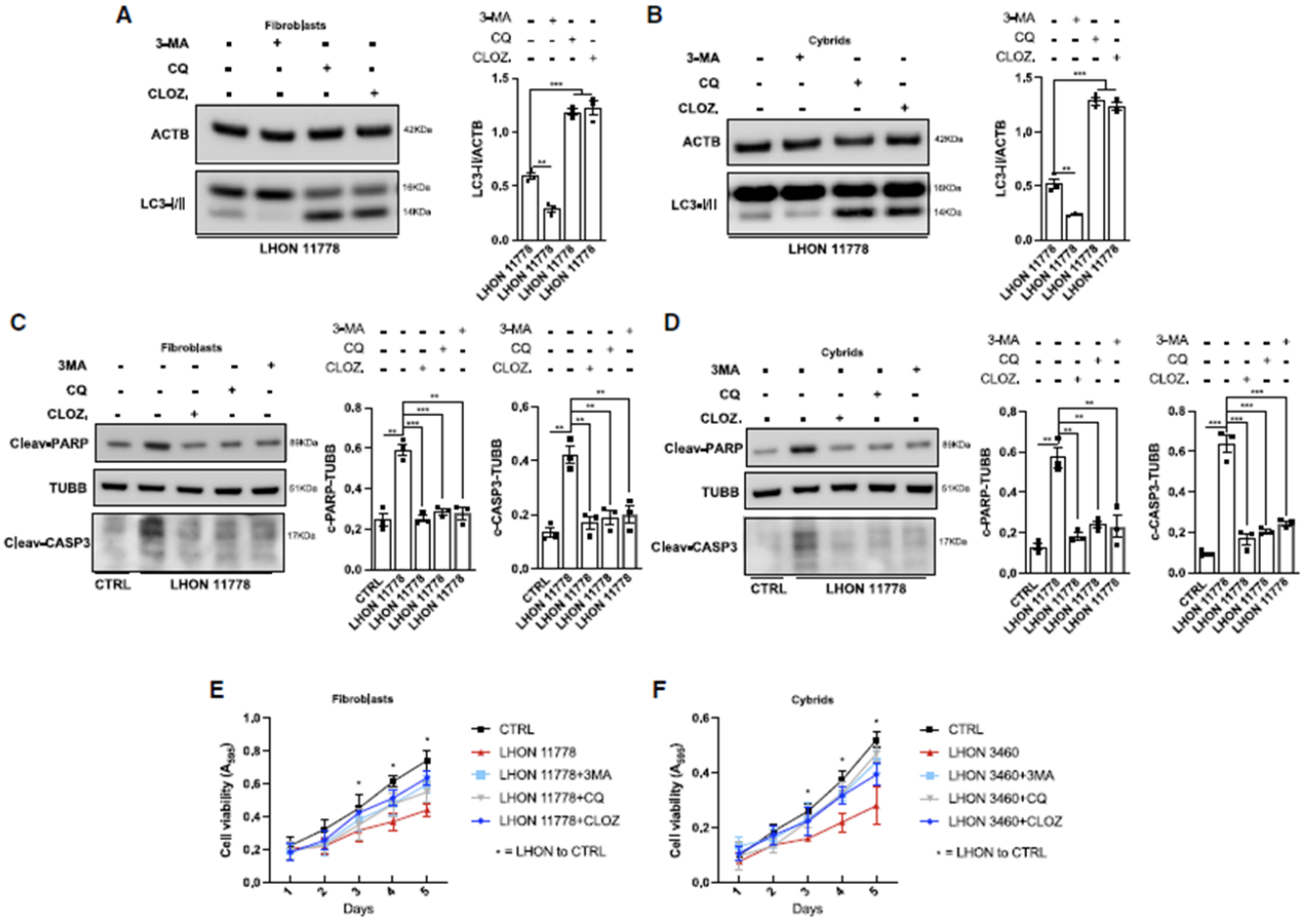

Figura 5 ***Compensatory therapeutic approaches targeting autophagy reverts LHON cells' predisposition to apoptotic death*** *(A and B) 11778 fibroblasts (A) and cybrids (B) harboring LHON mutations were treated with different autophagic inhibitors (3-MA [inhibitor of autophagy at early steps], chloroquine [CQ], and clozapine [CLOZ] [inhibitors of autophagy at late steps]). After 48 h, detection of autophagic activity through immunoblot technique was performed.(C and D) Detection of apoptotic process activity on 11778 LHON fibroblasts (C) and cybrids (D) treated with anti-autophagic agents by immunoblotting with antibodies against PARP and CAS3 apoptotic markers.(E and F) Cell viability in fibroblasts (E) and cybrids (F) pretreated with anti-autophagy compounds was performed at different time points. Data are presented as means ± SEM. n = at least 3 independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.*

Another therapeutic strategy in LHON is the administration of redox modulators, such as the short-chain benzoquinone idebenone (Gueven et al., 2021). Currently, idebenone is the only disease-specific drug approved by the European Medicine Agency (EMA) for LHON treatment (Amore et al., 2020). We tested idebenone, in its reduced form that exerts the therapeutic effect (Yu Wai-Man et al., 2017), in LHON-affected fibroblasts (Figures 6A and 6B) and idNeurons (Figures 6C and 6D), observing a marked reduction of autophagy activity and apoptotic death in the LHON-affected cells. Idebenone administration also resulted in a concomitant reduction of ROS production (Figure 6E) and Jm (Figure 6F). All these results were also confirmed in the cybrid cell model (Figures 6G–6J). Again, we did not observe any idebenone-related cytotoxic effect in control fibroblasts and cybrids (Figure S4I). Finally, increased mitochondrial biogenesis has been documented to be a key spontaneous compensatory program activated in LHON-carriers, contributing to their

lifelong unaffected state and incomplete penetrance (Giordano et al., 2014). We assessed protein expression of mitochondrial transcription factor A (TFAM) and cytochrome c oxidase (COX)-IV subunit as markers of mitochondrial mass in ex-vivo-collected PBMCs and in fibroblasts from a pair of discordant brothers carrying the 11778 mutation. Our results re-confirmed that LHON-carriers are the most efficient in compensatory mitobiogenesis (Figures S5A and S5B). Experiments aimed at detecting the amount of mtDNA confirmed the immunoblot results on mitochondrial mass (Figure S5C). Consistently, LHON-carrier fibroblasts also exhibited increased amounts of the ‘‘master regulator’’ of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma, coactivator 1 a (PGC1-a) (Scarpulla, 2011) compared with LHON-affected samples (Figure S5D). We then asked whether this compensatory biogenesis could re-establish an optimal mitochondrial turnover and, as consequence, a healthy and functional mitochondrial pool in LHON-carriers. To visualize mitochondrial turnover, we used the fluorescent protein MitoTimer, whose fluorescence shifts from green to red as the mitochondrial population ages (Hernandez et al., 2013). The fluorescence analysis demonstrated that mitochondria present in LHON-affected fibroblasts were older and less functional than mitochondria of control fibroblasts, as well as of LHON-carrier fibroblasts (Figure S5E). Differently, cybrids generated from the same pair of LHON discordant brothers had comparable amounts of TFAM, COX-IV, and PGC1-a proteins and mtDNA content (Figures S5F and S5G), indicating that the crosstalk between mtDNA and specific nuclear backgrounds drives the compensation in LHON-carrier individuals. Noticeably, the 11778 mutation in the osteosarcoma nuclear background seems to have an opposite effect on mtDNA content compared with fibroblasts, since LHON-affected cybrids showed significantly lower mtDNA levels compared with wild-type cybrids, as previously observed in Giordano et al., 2011. Moreover, LHON-carrier cybrids displayed an age of mitochondrial population (Figure S5H) similar to that of LHON-affected cybrids, being both significantly older than in controls. We thus evaluated whether an increase of mitobiogenesis might re-establish mitochondrial homeostasis in LHON cybrids. Overexpression of PGC1-a increased mitochondrial mass (Fig- ure S6A) and lowered ROS production (Figure S6B) and Jm (Figure S6C), reducing LC3 lipidation (Figure S6D) and protecting against apoptosis (Figure S6E).

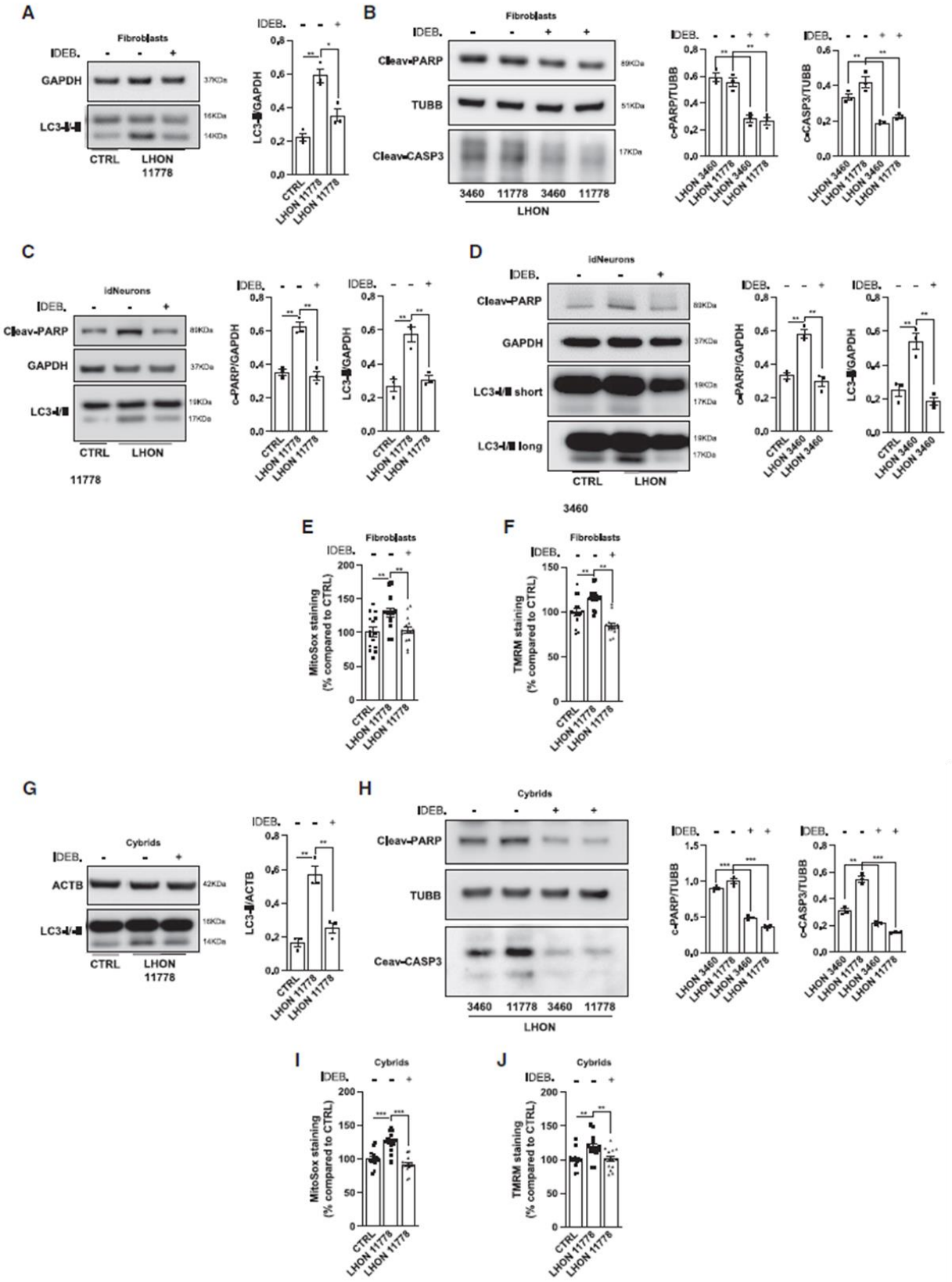

Figura 6 ***Oxidative stress modulation contributes to decrease in LHON cells' autophagic activity and apoptotic death*** *(A–D) After treatment with reduced idebenone (IDEB.) (10 mM for 3 h), fibroblasts (A and B) and iNeurons (C and D) were harvested and*

*immunoblotted for the autophagic marker LC3 and against apoptotic markers PARP and CAS3.(E and F) Measurements of mitochondrial ROS production (E) and mitochondrial transmembrane potential (F) were studied in IDEB.-treated 11778 fibroblasts. (G–J) The same idebenone treatment was repeated in cybrids, where autophagy (G), apoptosis (H), ROS production (I), and mitochondrial transmembrane potential (J) were detected. Data are presented as means ± SEM. n = at least 3 independent experiments for western blots or 5 visual fields per at least 3 independent samples per condition for fluorescent microscopy experiments. *p < 0.05, **p < 0.01, and ***p < 0.001*

DISCUSSION

The current study provides compelling evidence of a profound deregulation that targets autophagy removal of mitochondria, occurring in LHON-affected cell models, under basal conditions of cell culture, as well as in ex vivo LHON patient serum. This implicates a cellular stress phenotype documented by increased ROS production and Jm, ultimately resulting in a propensity toward apoptosis, undermining cell viability. We also show that this phenotype may be corrected by differently targeted therapeutic strategies including autophagy inhibitors acting at different stages of the autophagy mechanism (Choi et al., 2013; Park et al., 2012), redox modulators such as the EMA-approved idebenone (Amore et al., 2020; Gueven et al., 2021), and by genetically activating the mitobiogenesis program overexpressing the master regulator PGC1-a (Scarpulla, 2011). Individually, each of these therapeutic strategies was effective in our cellular models. Remarkably, all our experiments indicated that asymptomatic LHON-carriers have a naturally occurring compensation, which appears to be tightly dependent on the individual nuclear background, as this phenotype vanishes in cybrids. Ultimately, this compensatory phenotype makes LHON-carriers close to controls in all readouts analyzed despite the same LHON homoplasmic mutation as affected maternal relatives. The higher PGC1-a expression that we found only in LHON cells carrying the carrier nuclear background promotes mitochondrial biogenesis, allowing for alternative routes bypassing complex I impairment, as well as increasing the antioxidant machinery, globally compensating for the impaired OXPHOS (Giordano et al., 2014). Furthermore, the activation of the PPAR-g/PGC1-a pathway through the induction of uncoupling protein 2 (UCP2) may also impinge on mitochondrial decoupling, reducing Jm and ROS production and ultimately limiting oxidative damage and propensity to undergo apoptosis of LHON cells (Andrews et al., 2005). To reach such compensation, we envisage a better maintenance of mitochondrial homeostasis by shifting toward prevalent mitobiogenesis, which efficiently counterbalances the autophagy and mitophagy excessive activity determined by the mutant mtDNA. This highlights how these spontaneous compensatory mechanisms in RGCs, possibly prompted by yet unelucidated modifying nuclear genetic factors, are key to further refine the pathways that can be targeted therapeutically. Our understanding that autophagy pathways are highly regulated cellular mechanisms has progressed impressively (Bialik et al., 2018; Choi et al., 2013). Not surprisingly, abnormalities of both autophagy and mitophagy occur in many human diseases. Despite this, it remains unclear as to whether these mechanisms are adaptive or maladaptive in some human pathologies. In neurodegeneration, an excessive number of autophagosomes may turn toxic for neuronal cells (Choi et al.,

2013). As shown by previous studies and current results, the quality control of mitochondria is deeply integrated into a larger homeostatic program, balancing mitophagy and mitobiogenesis (Carelli et al., 2015a), strictly regulated by sensing OXPHOS efficiency and demand (Mishra et al., 2014). This in turn is tightly related to ROS production and retrograde signaling systems, key to coordinating the crosstalk of mtDNA and nuclear genome (Quiros et al., 2016). In LHON, this crosstalk may lead to very different outcomes as exemplified by the asymptomatic LHON-carriers when compared with LHON-affected brothers, which likely is deter- mined by the context of the nuclear genetic background, lost in cybrids where only the mtDNA-related phenotype is transferred. This different cellular fate, previously centered only on the efficiency of mitochondrial biogenesis (Giordano et al., 2011, 2014), is now further elucidated by our current findings taking also into account autophagy and mitophagy. Their deregulation, in particular the increased basal levels of mitophagy and reduced reservoir to further activate this quality control program, as after starvation, becomes counterproductive for the neuronal homeostasis in RGCs. Recently, modeling of optic atrophy type 1 (OPA1) deficiency in mouse, worms, and cells demonstrated the detrimental impact that excessive autophagy and mitophagy may have on neuronal architecture and suggested that an increased removal of mitochondria, particularly at the axonal hillock, depletes axons and synapses of these organelles, leading to neurodegeneration (Zaninello et al., 2020). Remarkably, our results from LHON idNeurons show a similar phenotype, with mitochondria undergoing excessive mitophagy in the soma. The increase of basal autophagy and mitophagy is a common theme in mitochondrial diseases, in particular in those with optic atrophy, as documented by others and our own studies of different mtDNA mutations affecting ND subunits of complex I (Dombi et al., 2016; Granatiero et al., 2016), and OPA1-related syndromes (Carelli et al., 2015b; Liao et al., 2017). Only one study reported results partially in contrast to this. It suggested a reduced activation of the autophagy program in LHON cybrids under stress conditions and that rapamycin was able to correct the pathologic phenotype of LHON cells (Sharma et al., 2019). The experimental design in this study differed from ours, as their data were gathered in a stress model of LHON cybrids elicited by galactose as carbon source. Intriguingly, this study reported a higher content in lysosomes of LHON cybrids in glucose culture conditions, which actually mirrors our increased autophagy and mitophagy basal levels of LHON-affected cells compared with controls, and LHON-carriers It remains challenging to clarify the mechanism leading to the observed autophagy dysregulation in LHON. All common LHON pathogenic mutations (11778, 3460, and 14484) impair complex I-driven ATP synthesis (Baracca et al., 2005). This was also assessed in vivo by phosphorus-31 magnetic resonance spectroscopy of the maximum rate of ATP production in skeletal muscle (Lodi et al., 1997). Yet, the precise biochemical consequences of LHON pathogenic mutations remain controversial (Carelli et al., 2004; Fiedorczuk and Sazanov, 2018; Yu-Wai-Man et al., 2011). There are likely multiple mechanisms to bypass the complex I impairment. The prevalent pathological mechanism in LHON is currently ascribed to

increased ROS production rather than a failure of bioenergetics (Beretta et al., 2004; Floreani et al., 2005; Lin et al., 2012). Furthermore, the paradoxical increase of Jm has been explained by the low levels of complex II-driven O2 consumption and may be prevented by coinhibition of complex II, III, or IV activity (Forkink et al., 2014). Remarkably, a deregulation in Ca2+ homeostasis was also found in LHON, as in other mitochondrial diseases (Haroon et al., 2007; Wong and Cortopassi, 1997), providing another mechanism affecting the energetic balance of cells. Either mitochondrial Ca2+ accumulation (Danese et al., 2017; Giorgi et al., 2018a) or a decrease in ATP/ ADP ratio, as well as an increased production of superoxide (O.) and hydrogen peroxide (HO), the two major ROS species, can lead to AMPK induction (Rabinovitch et al., 2017; Zmijewski et al., 2010), which ultimately orchestrates the stress response (Toyama et al., 2016). In fact, we found an increase in the active form of AMPK, strongly suggestive that the AMPK pathway is instrumental to re-establish the correct levels of ATP. Remarkably, AMPK is also strictly linked to the execution of autophagy and mitophagy (Giorgi et al., 2018a). In particular, AMPK phosphorylation of ULK1 is required to activate autophagy and targeting of mitochondria to lysosomes. Compatibly, we found in LHON-affected cells an increased concentration of the pro-autophagy phosphorylated form of ULK1, associated with excessive autophagy levels and mitochondrial removal. Our results link the AMPK sensing of cellular bioenergetic impairment and ROS overproduction in LHON with the activation of autophagy and mitophagy. The pathogenic mechanism here delineated may shed light on the massive and near synchronous death of RGCs, which occurs in LHON (Carelli et al., 2004; Yu-Wai-Man et al., 2011). There is mounting evidence that associates the autophagy pathways with cell-death programs (Galluzzi et al., 2018; Tsujimoto and Shimizu, 2005). In the RGCs, this translates into excessive levels of mitophagy that may become lethal for neuronal cells (Zaninello et al., 2020). How RGCs' sudden decompensation occurs during the conversion from asymptomatic LHON-carrier to -affected, generating a wave of rapidly propagating cell death that characterizes the subacute phase of LHON (Coussa et al., 2019; Pan et al., 2012; Sadun et al., 2000), remains under scrutiny. Fine-tuning of the balance between mitobiogenesis and mitophagy possibly distinguishes LHON-affected from -carriers and might also be the key in cases of therapeutic success. We showed that genetically boosting mitochondrial biogenesis by overexpressing PGC1-a rescued the mitochondrial stress phenotype, ultimately balancing autophagy and limiting apoptosis. Interestingly, idebenone, known for bypassing complex I, thus reducing ROS production and restoring downstream respiration, similarly mitigated autophagy and limited apoptosis. On the opposite side of the mitochondrial homeostatic balance, therapeutic agents directly hampering the autophagic activity by different mechanisms, such as 3-MA, CQ, and CL, resulted in a similar protective effect. In conclusion, we provide convincing evidence of a comprehensive mechanism for LHON implicating a stress phenotype that results from altered balance of mitochondrial biogenesis and the quality control cycle. This is mediated by AMPK sensing of the primary biochemical defect that comes from complex I dysfunction and results in

lowered bioenergetics efficiency combined with increased ROS levels, ultimately leading to increased propensity to apoptosis. This was evident in primary patient-derived cells such as fibroblasts but also transferred to cybrids, certifying the driving role of mtDNA LHON mutations, and, for the first time, was demonstrated in idNeurons, indicating the relevance of this mechanistic pathway in the cell type targeted in LHON. Remarkably, the compensatory phenotype displayed by LHON-carriers was shown to be driven by the nuclear individual background, as was lost in cybrid experiments. This compensatory phenotype derives from prevalent mitobiogenesis counteracting excessive mitophagy and globally rebalancing the stress phenotype. Finally, we provided proof of principle that manipulating, genetically or pharmacologically, this mechanistic pathway efficiently corrects the key readouts of pathology. These results cast hope for a rapid translation into clinical trials with LHON patients, as many of the drugs proposed can be repurposed to exploit these therapeutic strategies.

STAR+METHODS

Detailed methods are provided in the online version of this paper

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111124.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human samples

LHON patients, initially diagnosed by restriction fragment length polymorphism (RFLP) analysis, and healthy individuals were recruited at the IRCCS Istituto delle Scienze Neurologiche di Bologna (Bologna, Italy) for blood samples collection. The study is part of a research project approved by the institutional ethical board (Comitato Etico di Area Vasta Emilia Centro-CE-AVEC, code CE 19072), and all participants gave informed written consent. All procedures were performed according to the Declaration of Helsinki. Fibroblasts cell lines were previously generated as part of a research project (Comitato Etico dell'Azienda Ospeda- liero-Universitaria di Bologna, Policlinico Sant'Orsola Malpighi, number 123/2006/U/Sper). The generation of LHON iPSCs was approved by the Institutional ethical board (number 43-2013, date 26/6/2013) of Fondazione IRCCS Istituto Neurologico Carlo Besta.

Fibroblasts

Fibroblast lines were established from skin biopsies of four control individuals (two males of 22 and 35 years, two females of 29 and 32 years), three affected patients (two m.11778G>A males of 36 and 55 years, and one m.3460G>A female of 19 years) and two unaffected mutation-carriers (m.11778G>A males of 38 and 46 years), the respective brothers of the affected patients with the same mutation (see Table S1). Cells were grown in DMEM (EuroClone, Milano, Italy) containing 25 mM glucose supplemented with 10% fetal bovine

serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and were maintained at 37◦C in a humidified atmosphere with 5% CO2. Functional experiments were carried out on sub-confluent cell cultures with a comparable number of passages (10–20).

Induced pluripotent stem cells (iPSC)

Transgene-free induced pluripotent stem cells (iPSC) were generated, from control (male of 34 years) and two affected patient fibro- blasts (one m.3460G>A female of 19 years and one m.11778G>A male of 26 years), as previously described (Peron et al., 2020). Briefly, iPSC were generated by a CytoTune-iPS 2.0 Sendai Reprogramming Kit (Orellana et al., 2016), introducing the four transcription factors proposed by Yamanaka: OCT4, SOX2, KLF4 and c-MYC. Pluripotency of iPSC was characterized by alkaline phosphatase, PCR and immunofluorescence for the pluripotent markers Sox2, Rex1, Tra1-60, Nanog and Oct4. Integrity of nuclear and mitochondrial genomes was verified by Comparative Genomic Hybridization (CGH) array (Galizia et al., 2012) and complete mtDNA sequence by NGS, as previously described (Caporali et al., 2018).

Cybrids cell lines

Cybrid cell lines were generated as previously described in (Cock et al., 1998; Floreani et al., 2005; Ghelli et al., 2003; Zanna et al., 2005) by using enucleated fibroblasts as the mitochondria donor, and from the osteosarcoma (143B.TK—)-derived 206 cell line (female, 13 years), as the acceptor rho0 cell line (see Table S1). Cybrid cell lines were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.1 mg/mL bromodeoxyuridine. For experiments, cells were seeded at 4 x 105 cells/cm2 and incubated in DMEM containing 25 mM glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin at 37◦C in an incubator with a humidified atmosphere of 5% CO2.

Peripheral blood cells isolation and processing

Ten ml of venous blood was collected in EDTA from 17 healthy controls (8 females and 9 males, with average age 47.5 years (SD = 14.5) and 38.5 years (SD = 13.9), respectively), 10 LHON-affected (8 males with average age 38.8 years (SD = 11.6) and two females of age 49 and 59 years) and 14 LHON-carriers patients, all carrying the 11778 homoplasmic mutation (7 females and 7 males, with average age 46.4 years (SD = 10.7) and 26.1 years (SD = 5.9), respectively). PBMCs were isolated using a density gradient cell separation medium (Sigma Aldrich: Histopaque-1077), following manufacturer's instructions. Proteins were extracted from PBMCs with RIPA buffer containing proteases inhibitors cocktail (Roche: 11697498001), following standard procedures, and protein content was assessed with Bradford method.

Serum levels of ATG5, ATG7, optineurin and parkin determination

Peripheral blood from ten healthy controls (3 females and 7 males, with average age 37.6 years (SD = 17.5) and 38.1 years (SD = 15.9) respectively), seven LHON-affected (1 female of 59 years and 6 males, with average age 39 years (SD = 21.8)) and nine LHON-carriers (4 females and 5 males, with average age 35.5 years (SD = 17.3) and 37.2 years (SD = 15.5) respectively), all carrying the 11778 homoplasmic mutation, was collected in Serum Separation Tubes and centrifuged 15 min at 2900 x g. Serum samples were aliquoted and stored at -80◦C until processing. Concentrations of ATG5, ATG7, Parkin and Optineurin were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (My Biosource, San Diego, California, USA; MS7209535 for ATG5, MBS062423 for ATG7, MBS732278 for Parkin and MBS2704623 for Optineurin) following the manufacturer's instructions as previously published (Patergnani et al., 2018).

METHOD DETAILS

Immunoblotting

For immunoblotting, cells were scraped into ice-cold phosphate–buffered saline and lysed in modified 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA and protease inhibitor cocktail. After lysis on ice, homogenates were cleared via centrifugation at 12,000 g at 4◦C for 10 min. Protein extracts were quantified using the Lowry assay (Bio-Rad Laboratories). Protein extracts (20 mg for cells and 30 ug for PBMCs) were separated on 4–12% or 12% bis-Tris acrylamide gels (Life technologies: NP0323, EC6026, and NP0341) and electrotransferred to PVDF or nitrocellulose membrane according to standard procedures. Nonspecific binding sites were saturated by incubating membranes with TBS-Tween 20 (0.05%) supplemented with 5% non-fat powdered milk for 1 h. Next, membranes were incubated overnight with primary antibodies and then were assessed with appropriate HRP-labeled secondary antibodies and chemiluminescent substrate, or with fluorescent secondary antibodies.

Mitochondrial DNA content assessment and next generation sequencing

Total DNA was isolated from cell pellets using the commercial kit NucleoSpin Tissue (Macherey-Nagel, REF 740952.50). MtDNA quantification was performed by a Real Time-PCR assay based on hydrolysis probe chemistry previously used (Giordano et al., 2014). Briefly, an mtDNA fragment (MT-ND2 gene) and a nuclear DNA fragment (FASLG gene) were coamplified by multiplex PCR, and their concentration was determined by absolute quantification through a standard curve made with serial dilutions of a plasmid containing a copy of the two amplicons. Primers, probes, and conditions have been previously published (Mussini et al., 2005). The presence of LHON homoplasmic mutations, in both fibroblasts and cybrids, was assessed by complete mtDNA sequencing as previously reported (Caporali et al., 2018). The protocol consists in two overlapping long PCR amplicons (9.1 kb and 11.2 kb), amplified with PrimeSTAR Max DNA Polymerase (Takara), following

the manufacture instructions. The library was con- structed by xGen DNA Lib Prep EZ (IDT) and sequenced on MiSeq System (Illumina). Reads were aligned to the human reference mitochondrial genome (NC_012920.1) and variants were called by Mitoverse. The mtDNA haplogroup affiliations were assigned using HaploGrep2.4.0, according to PhyloTree Build 17 (www.phylotree.org). MtDNA haplogroups and private variants for each cell line are reported in Table S1.

Fluorescence microscopy and quantitative analysis of GFP-LC3 puncta

Fibroblast and cybrid cells were cultured on 24-mm glass coverslips and, at 50% confluence, were transfected with Lipofectamine reagent (Thermo Fisher Scientific: 15338100) and 1 mg of plasmid DNA (GFP-LC3). After 36 h, images were taken on a Nikon LiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging software and on a confocal laser scanning microscopy Olympus FV3000 both equipped with a 633 oil immersion objective (N.A. 1.4). For each condition, the GFP-LC3 puncta were counted in at least 25 independent visual fields.

Mitophagy assessment with LysoTracker Red and MitoTracker Green

Mitophagy experiments were performed in fibroblasts, cybrids and 35 days old idNeurons. Cells were incubated with MitoTracker Green FM (1 mM final concentration) (Thermo Fisher Scientific: M7514) for 30 min at 37◦C and then extensively washed with PBS. LysoTracker Red DND-99 (1 mM final concentration) (Thermo Fisher Scientific: L7528) was then added, and cells were immediately observed on a Nikon LiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging software and on a confocal laser scanning microscopy Olympus FV3000 both equipped with a 633 oil immersion objective (N.A. 1.4). The green and red signal colocalization rate was evaluated using the colocalization counter JACOP available in Fiji software. For each condition, the colocalization of these two signals was also determined by manual counting of fluorescent puncta. For the region-spe- cific mitophagy levels into idNeurons, Region Of interest (ROI) of soma and axon-dendrites regions, considering the entire length of the latter, were created and analyzed by the colocalization plug-in Coloc2 available in Fiji software. For each ROI the Manders' parameter was calculated.

Mitophagy assessment with YFP-Parkin and mitochondrial (mt)-Cherry

Fibroblasts and cybrid cells were cultured on 24-mm glass coverslips and, at 50% confluence, were transfected with Lipofectamine reagent (Thermo Fisher Scientific: 15338100) and 2 mg of plasmid DNA (1 mg YFP-Parkin (Addgene plasmid # 23955) (Narendra et al., 2008) and 1 ug mt-Cherry (Addgene plasmid # 55102) (Olenych et al., 2007)). After 36 h, images were taken on a confocal laser scanning microscopy Olympus FV3000 equipped with a 633 oil immersion objective (N.A. 1.4). The number of Parkin dots on mitochondria per cell and the representative images were generated by using NIH ImageJ software.

MitoTimer measurements

Cells were transfected with the pTRE-tight-MITO TIMER plasmid (Addgene plasmid # 50547) (Hernandez et al., 2013). After 36h, cells were imaged using excitation at 490 nm and 550 nm and emission of green (500–540 nm) and red (580–640 nm) fluorescence signals by using a Zeiss LSM510 confocal microscope and an Olympus scanning microscope equipped of 633 oil immersion objective (N.A. 1.4). The ratio of the fluorescence signal intensity in the red and green channels and the representative images were generated by using NIH ImageJ software as previously reported in (Morciano et al., 2021).

Autophagy induction and inhibition

The autophagy process in vitro was triggered through serum deprivation (EBSS, 30 min). The pharmacological inhibition of autophagy was performed by treating cells with 3-MA, (Merck: M9281) (2,5 mM), CL (Merck: C6305) (1 mM) or CQ (Merck: C6628) (1 mM) in DMEM supplemented with 10% FBS. After treatment, cells were fixed or lysed to detect the amount of autophagosome vesicles by fluorescence microscopy with immunoblot analysis (using an anti-LC3 antibody).

mROS measurements

Total ROS release from mitochondria was estimated fluorometrically by MitoSOX Red probe oxidation (Thermo Fisher and TaliTM Image-Based Cytometer). Fluorescence was measured using 510 ± 10 nm and 595 ± 35 nm excitation and emission wavelengths, respectively.

Measurement of Jm

The Jm was measured by labeling cells with 20 nM TMRM (Life Technologies: T-668) for 30 min at 37◦C. Images were taken on an inverted microscope (Nikon LiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped of with Elements microscope im- aging software). TMRM was excited at 560 nm, and the emission signal was collected through a 590–650-nm bandpass filter. Images were taken every 5 s with a fixed 20 ms exposure time. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 10 mM), an oxidative phosphorylation uncoupler, was added after 12 acquisitions to completely collapse the electrical gradient established by the respiratory chain.

Cell proliferation and viability assay

Cells were seeded at 15,000 cells per well in 6-well plates. Cells were seeded on 5 plates; one plate for each day. Every 24 h after seeding, cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS for 15 min. Cells were stained with 0.1% crystal violet for 20 min and then washed thrice with water. To each well, 500 mL of 10% acetic acid was added, and the cells were incubated for 20 min with shaking (extraction). Absorbance was measured at 590 nm.

Cell transfection

Where indicated, cybrid cell lines were transfected with Lipofectamine reagent (Thermo Fisher, 15338100) and 1 mg of plasmid DNA. Cells were analyzed after at least 36 h of expression.

Neural precursor cells (NPC)

NPCs were obtained by multiple steps. Embryoid body suspensions were cultured for 5 days and then plated on Matrigel-coated plates to generate neuro-ectodermal rosette structures. After 5-7 days, rosettes were picked and plated on polyornithine/laminin to generate and maintain NPC cultures [modified from (Brafman, 2015)]. NPCs were characterized after passage 3, by PCR and immunofluorescence for expression of neural precursor markers (Sox1, Pax6, Nestin) and absence of pluripotent markers (Oct4 and Nanog). To obtain iPSC-derived neurons (idNeurons), NPCs were plated on glass supports in polyornithine/laminin-coated plates. The next day, neuronal differentiation medium (DMEM/F-12, 13 N2 supplement, 13 B27 supplement, 30 ng/mL BDNF, 30 ng/mL GDNF, 1 mM DAPT, 13 penicillin/streptomycin, 13 glutamine and 13 non-essential amino acids) was added to the cells and then was changed every other day. After 35 days, the idNeurons were characterized and utilized in all the experiments here reported. Characterization was performed by immunofluorescence to verify the specific expression of typical neuronal markers such as NEUN (Millipore, MAB377), MAP2 (Cell Signaling, #4542S) and bIII-TUBULIN (GenScript, A01627). The following dilutions were used: anti-NEUN 1:1000; anti-MAP2 1:200; anti-bIII TUBULIN 1:500.

Quantitative analysis of autophagy flux

LHON fibroblasts cultured on 24-mm glass coverslips were transfected with Lipofectamine reagent (Thermo Fisher Scientific: 15338100) and 1 mg of plasmid DNA (mCherry-eGFP-LC3). After 36 h of transfection, cells were imaged at 603 magnification with a Nikon LiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging soft- ware. Obtained puncta images were merged to compare RFP and GFP signals using ImageJ software. For each condition, the co- localization of these two signals was determined by manual counting of fluorescent puncta in at least 20 independent visual fields.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed by Prism 8 (GraphPad Software Inc.). Unless otherwise specified, data are representative of at least three biologically independent experiments. The comparison between three groups by means of the one-way ANOVA test followed by Tu- key's multiple comparisons test, while the comparison between two groups using the unpaired t test of Student. p values < 0.05 were considered statistically significant and marked with asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$), as indicated in Figure legends. All data collected are represented as mean ± s.e.m. The p values of histograms that did not reach statistical significance in any of the conditions examined are reported in Table S2.

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AUTHOR CONTRIBUTIONS

P.P., V.C., C.G., V.T., A.D., S.P., and A. Maresca designed all experiments. A.D., S.P., and A. Maresca performed most of the experiments, analyzed and interpreted the data, and wrote the manuscript. A.R. and C.T. processed samples and performed electron microscopy (EM) experiments in fibroblasts. C.P., A.S., A.I., I.D.M., V.B., and V.T. generated iPSCs, NPCs, and idNeurons and performed analysis. L.C. performed mtDNA sequences. A. Maresca, C.L.M., and V.C. collected blood samples and analyzed PBMC samples from patients. V.D.D. and C.Z. generated fibroblasts cell lines. A. Maresca and V.D.D. assessed mtDNA content in fibroblasts and cybrids. A. Martinuzzi and A.C. generated cybrid cell lines. F.N.R.-C. and C.B. processed postmortem eye tissues and performed EM on RGCs. F.N.R.-C. and A.A.S. interpreted EM results of postmortem retina. M.N.M.-F., S.R.S., A.B., R.B.J., and A.A.S. collected postmortem retinal tissues. M.R.W., M.L.-A. and S.M. performed mitochondria homeostasis and autophagy and mitophagy analysis. P.P., V.C., C.G., and V.T. supervised the study and wrote the manuscript. All authors revised and approved the manuscript.

DECLARATION OF INTERESTS

V.C. and C.L.M. are both involved in clinical trials with idebenone (Santhera Pharmaceuticals) in LHON patients; V.C., C.L.M., and A.A.S. are involved in gene therapy trials with Lumevoq (GenSight Biologics) in LHON patients. V.C. and A.A.S. also serve as consultants in the advisory board of Chiesi Farmaceutici.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact: Paolo Pinton (paolo.pinton@unife.it).

Materials availability

This work did not generate any unique reagents.

Data and code availability

This paper does not report original code. All software utilized is freely or commercially available and is listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.

5 Conclusion

In vitro modelling of Leber's hereditary optic neuropathy (LHON) through 2D/3D cell cultures, which includes utilizing patient-derived human induced pluripotent stem cells (hiPSCs) and neurons, has led to significant advancements in comprehending the pathogenic mechanisms underlying this intricate and captivating disease. It is well-established that abnormalities in both autophagy and mitophagy play a role in numerous human diseases. The equilibrium, governing mitophagy and mitobiogenesis, is rigorously regulated in response to the efficiency and demand of oxidative phosphorylation (OXPHOS) processes, as elucidated by Carelli et al., (2015). Any perturbation in this balance ultimately proves detrimental to neuronal homeostasis in retinal ganglion cells (RGCs). Recent investigations involving the modelling of optic atrophy type 1 (OPA1) deficiency in mouse models, worms, and cellular systems have underscored the detrimental consequences associated with excessive autophagy and mitophagy. These studies have indicated that the excessive removal of mitochondria, particularly at the axonal hillock, results in the depletion of axons and synapses of these organelles, culminating in neurodegeneration (Zaninello et al., 2020). Intriguingly, our findings with LHON-induced neurons (idNeurons) reveal a similar phenotypic manifestation, characterized by the occurrence of excessive mitophagy within the neuronal soma (Danese et al., 2022). The increase of basal autophagy and mitophagy emerges as a recurring theme in mitochondrial diseases, particularly those characterized by optic atrophy. This is well-documented in the context of various mitochondrial DNA mutations affecting the NADH dehydrogenase subunits of complex I (Dombi et al., 2016; Granatiero et al., 2016) and OPA1-related syndromes (Carelli et al., 2015b; Liao et al., 2017). The comprehensive mechanistic framework presented here for LHON convincingly implicates a stress phenotype resulting from imbalanced mitochondrial biogenesis, as observed not only in primary patient-derived cells like fibroblasts but also in cybrids. This underscores the pivotal role of mtDNA LHON mutations and, for the first time, is demonstrated in idNeurons, thus emphasizing the relevance of this mechanistic pathway in the specific cell type affected by LHON. Furthermore, the use of patient-derived hiPSC neural progenitor cells (NPC) and neurons has facilitated the assessment of the efficacy and limitations of idebenone treatment. Taken together, these investigations illustrate how the development of a cellular model capable of replicating the onset of LHON syndrome proves highly valuable in validating pathogenic mechanisms and therapeutic outcomes previously established in cell types other than the RGCs.

6 Future Perspective

To advance our comprehension of the pathogenic mechanisms underlying the disease, two pioneering methodologies can be merged: the derivation of human induced pluripotent stem cells from individuals afflicted with Leber's Hereditary Optic Neuropathy and the employment of CRISPR/Cas9-based genome editing. These techniques collectively facilitate the generation of hiPSC lines that express a fluorescent reporter for the selective identification of retinal ganglion cells (RGCs). Subsequently, these genetically engineered hiPSCs can be directed towards differentiation into enriched RGC populations and retinal organoids. This approach provides a valuable resource for investigating the intricacies of human optic nerve biology and pathology, establishing it as an indispensable platform for the exploration of various biological pathways implicated in LHON and for drug screening. Of particular interest is the exploration of why LHON syndrome preferentially targets RGCs, leading to their specific degeneration, despite the presence of the mtDNA mutation across all mitochondrial DNA molecules. This particular facet will be further elucidated by creating a retinal-thalamic organoid, a model that closely resembles the physiological state in vitro.

To this end, hiPSC lines derived from a control individual, a line with the m.3460G>A LHON mutation, and a line with the m.11778G>A LHON mutation will be genetically modified using a CRISPR/Cas9-based strategy. This modification will introduce a fluorescent reporter into the endogenous BRN3B (POU4F2) open reading frame (ORF), allowing tracking and labelling of differentiated RGCs. BRN3B, a well-characterized transcription factor specific for RGCs, initiates its expression early in RGC differentiation and persists in adult cells, with limited expression in other body tissues. These engineered hiPSC lines will subsequently serve as the basis for developing a two-dimensional RGC cell culture. One of the major challenges encountered so far in differentiating hiPSC into RGC is the tendency of hiPSC-derived RGCs to cluster to facilitate their isolation from other cells in culture, as shown in Figure 5 (preliminary data).

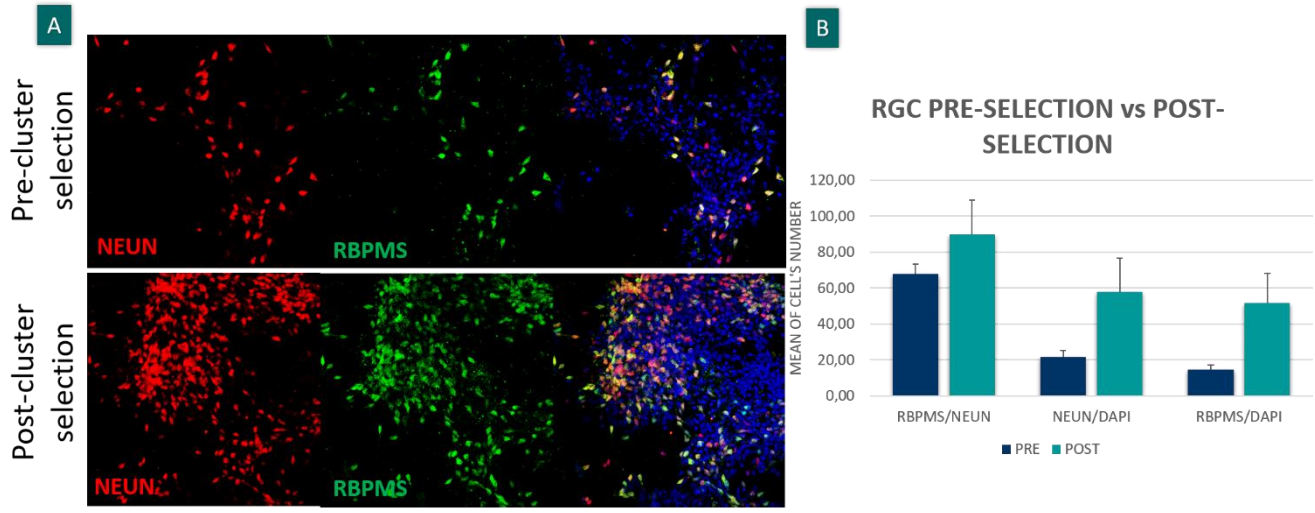

Fig. 5: (A) IF analysis to evaluate the enrichment of RGC (RBPMS) and neural cells (NeuN) after the cluster selection. (B) The amount of positive RGC cells out of the total number of neuronal cells (RBPMS/NeuN) was assessed. In addition, neuronal marker-positive cells and RGCs were related to the total number of cells (DAPI). The cells' number, pre and post selection, has been obtain with 3D Object counter ImageJ plug-in.

To construct a more sophisticated model, the engineered hiPSCs will be guided to form retinal organoids. This approach provides the opportunity to recapitulate the in vivo context. It is important to note that RGCs show rapid degeneration in both two-dimensional and three-dimensional models. A possible explanation for this phenomenon could be the lack of a specific target. Consequently, the next step is to generate a thalamic organoid that can be connected to a retinal organoid to establish RGC axon terminals. The key advantage of this model is the potential for improved RGC survival. This configuration facilitates the assessment of RGC degeneration attributed to pathology and allows for a more in-depth exploration of the pathomechanisms leading to the specific death of RGCs.

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