Published in final edited form as:

Mitochondrion. 2019 May ; 46: 187–194. doi:10.1016/j.mito.2018.06.001.

The m.11778 A>G Variant Associated with the Coexistence of Leber's Hereditary Optic Neuropathy and Multiple Sclerosis-like Illness Dysregulates the Metabolic Interplay between Mitochondrial Oxidative Phosphorylation and Glycolysis

Martine Uittenbogaard¹, Christine A Brantner², Fang ZiShui³, Lee-Jun Wong³, Andrea Gropman⁴, and Anne Chiaramello^{1,*}

¹Department of Anatomy and Regenerative Biology, George Washington University School of Medicine and Health Sciences, Washington, DC 20037, USA

²GW Nanofabrication and Imaging Center, Office of the Vice President for Research, George Washington University, Washington, DC 20052, USA

³Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

⁴Children's National Medical Center, Division of Neurogenetics and Developmental Pediatrics, Washington, DC 20010, USA

Abstract

Little is known about the molecular mechanism of the rare coexistence of Leber's Hereditary Optic Neuropathy (LHON) and multiple sclerosis (MS), also known as the Harding's syndrome. In this study, we provide novel evidence that the m.11778A>G variant causes a defective metabolic interplay between mitochondrial oxidative phosphorylation and glycolysis. We used dermal fibroblasts derived from a female proband exhibiting clinical symptoms compatible with LHON-MS due to the presence of the pathogenic m.11778A>G variant at near homoplasmic levels. Our mitochondrial morphometric analysis reveals abnormal cristae architecture. Live-cell respiratory studies show stunted metabolic potential and spare respiratory capacity, vital for cell survival upon a sudden energy demand. The m.11778 A>G variant also altered glycolytic activities with a diminished compensatory glycolysis, thereby preventing an efficient metabolic reprogramming during a mitochondrial ATP crisis. Our collective results provide evidence of limited bioenergetic flexibility in the presence of the m.11778 A>G variant. Our study sheds light on the potential pathophysiologic mechanism of the m.11778 A>G variant leading to energy crisis in this patient with the LHON-MS disease.

* Corresponding author: George Washington University School of Medicine and Health Sciences, Department of Anatomy and Regenerative Biology, 2300 I Street N.W., Washington, DC 20037, Phone number: (202) 994-2173, Fax number: (202) 994-8885, achiaram@gwu.edu.

Disclosure

The authors declare no conflicts of interest

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Mitochondria; energy reprogramming; cristae remodeling; pathogenic mitochondrial DNA variant; oxidative phosphorylation; glycolysis

1. Introduction

Leber's Hereditary Optic Neuropathy (LHON; OMIM 535000) is a maternally inherited mitochondrial respiratory disorder characterized by an acute or subacute onset of the optic neuropathy due to specific loss of retinal ganglion cells with demyelination and atrophy of the optic nerve (Bargiela and Chinnery, 2017). Most patients have bilateral visual dysfunction and remain legally blind due to a poor visual prognosis (Kirkman et al., 2009). LHON has an estimated prevalence of 1 in 30,000 with a median onset occurring between the second and third decade of life. About 90% of LHON patients harbor one of the three mitochondrial DNA (mtDNA) pathogenic variants, m.3460 A>G, m.11778 A>G, and m.14484 T>C, each affecting one of the mitochondrial-encoded subunits of the respiratory complex I, which is the first multi-subunit complex of the mitochondrial oxidative phosphorylation (OXPHOS) pathway (Wallace et al., 1988; Huoponen et al., 1991; Howell et al., 1991; Johns et al., 1992; Mackey and Howell, 1992). Among those, m.11778 A>G is the most prevalent and impairs the mitochondrial-encoded subunit ND4 of Complex I due to an arginine to histidine substitution at the position 340 (Man et al., 2002).

Most notably, LHON is distinguished by a gender bias with a male predominance of about 80 to 90% in pedigrees (Mackey et al., 1996). However, a female predominance has been observed among patients harboring one of the three LHON pathogenic variants and exhibiting LHON clinical symptoms along with clinical symptoms of multiple sclerosis (MS). This rare disease association is known as "Harding disease or Harding's syndrome" and herein referred to as LHON-MS (Harding et al., 1992; Kellar-Wood et al., 1994; Riordan-Eva et al., 1994). The m.11778 A>G variant is the most prevalent among LHON-MS female patients (Palace, 2009; Pfeffer et al., 2013). Even though our understanding of the clinical phenotype of LHON-MS is emerging, its underlying molecular pathogenic mechanism remains unknown, as metabolic studies were performed in the context of LHON using cybrid cell lines showing altered complex I activity and therefore defective ATP synthesis in keeping with *in vivo* results from ³¹P magnetic resonance spectroscopy in LHON patients (Lodi et al., 1997; Lodi et al., 2002; Carelli et al., 2004; Baracca et al., 2005).

In this study, we report the metabolic consequences in a proband presenting clinical symptoms consistent with LHON-MS. Definitive diagnosis was corroborated by mitochondrial DNA testing for the pathogenic variant pR340H of the ND4 subunit of complex I. Long-range PCR followed by massively parallel sequencing determined a near homoplasmic load of the m.11778 A>G variant. Our mitochondrial morphometric analysis of the proband's dermal fibroblasts revealed severe mitochondrial cristae defects. The proband's fibroblasts exhibited stunted mitochondrial OXPHOS activities and a decreased ability to perform glycolysis in response to a mitochondrial ATP deficit. This altered

metabolic flexibility translates into a defective switch from OXPHOS to glycolysis, thereby further aggravating the energy crisis. Taken together, our results provide insight into the pathophysiologic mechanism of the pathogenic variant m.11778 A>G associated with the rare LHON-MS mitochondrial disease.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board of the George Washington University and Children's National Medical Center and was conducted in accordance with the ethical principles of the Declaration of Helsinki of 1975 (revised 1983). Patient skin biopsy was performed only after receiving written informed consent with permission to study the derived dermal fibroblasts.

2.2. Skin biopsy and fibroblast culture

Skin biopsy was performed on the 34-year-old proband. Dermal fibroblasts were derived from 3 mm skin biopsy in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 2 mM glutamine, 2.5 mM pyruvate, 0.2 mM uridine, FGF-2 (10 ng/ml) and 20% fetal bovine serum. Uridine has previously been indicated to preserve respiratory chain deficiencies in cultured fibroblasts (Bourgeron et al., 1993). Derived dermal fibroblasts were frozen at passage 2 and never used beyond passage 10. Human primary dermal fibroblasts from a healthy adult donor (Cat# GM003377E) were obtained from the Coriell Cell Repositories (Camden, NJ).

2.3. DNA purification and determination of heteroplasmy

We used fibroblasts from the proband at passage 3 for DNA extraction using the QIAamp DNA mini kit according to the manufacturer's recommendations (Qiagen). Genetic diagnosis was determined by Sanger using total DNA isolated from blood. Heteroplasmy was determined using a Long-Range PCR (LR-PCR) Next Generation Sequencing approach (Zhang et al., 2012 and Cui et al., 2013).

2.4. Transmission electron microscopy

Skin fibroblasts from the proband were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences), 1% paraformaldehyde in 0.12 M sodium cacodylate buffer (Electron Microscopy Sciences) for 20 minutes at room temperature followed by 40 minutes on ice. Cells were then fixed for one hour in 1% osmium tetroxide (Electron Microscopy Sciences) followed by *en-bloc* staining overnight in 1% aqueous uranyl acetate. The cells were then dehydrated through a series of ethyl alcohol/deionized water solutions and propylene oxide before infiltration with Embed 812 epoxy resin. Blocks were cured for 48 hours at 60°C. Polymerized blocks were trimmed and 70 nm ultrathin sections were cut with a diamond knife on a Leica Ultramicrot EM UC7 and transferred onto 200 mesh copper grids. Sections were counterstained with 1% ethanolic uranyl acetate for 10 min and lead citrate for 2 min. Samples were imaged with a FEI Talos F200X-transmission electron microscope (FEI Company) operating at an accelerating voltage of 80 kV equipped with a CetaTM 16M camera.

2.5. Analysis of mitochondrial respiration and glycolysis

Bioenergetic status was measured using the Seahorse Extracellular Flux XFp Analyzer (Seahorse Bioscience, Agilent Technologies). Optimal cell density (5,000/well) and the uncoupler FCCP (fluoro 3-carbonyl cyanide-methoxyphenyl hydrazine; 2 μ M) were determined using the Cell Energy Phenotype Test kit. Skin fibroblasts were seeded in triplicate on poly-D lysine-coated plates and incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Prior to the assay, the supplemented DMEM medium was changed to unbuffered Base Medium supplemented with 2 mM glutamine (Invitrogen), 2 mM pyruvate (Sigma), and/or 7.1 mM glucose (Sigma) depending on the assay and adjusted to at pH 7.4 with NaOH for one hour at 37°C. Using the XFp Mito Stress Test kit, OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were measured under basal conditions and after sequential injections of oligomycin (1 μ M), FCCP (2 μ M) and a mix of rotenone and antimycin A (1 μ M) following the manufacturer's recommendations. The XFp Glycolysis Stress Test kit measures ECAR as change in pH/mmol of H⁺ to assess glycolytic functions under stress conditions (glucose starvation) after sequential injections of glucose (10 mM), oligomycin (1 μ M) and 2-DG (50 mM). The Glycolytic Rate Assay kit determines the total proton efflux rate (PER) as a result of bulk acidification and the glycolytic proton efflux correlated with lactate accumulation, an accurate measurement of basal glycolysis. Prior to the assay, the supplemented DMEM medium was changed to the XF Base medium without phenol red supplemented with 2 mM glutamine, 10 mM glucose, 1 mM pyruvate, and 5.0 mM HEPES. OCR and ECAR were measured under basal conditions and after sequential injections of rotenone/antimycin A (0.5 μ M final concentration) and 2-DG (50 mM final concentration). The data from three independent experiments, each containing three technical replicates were normalized to cell numbers and plotted as OCR (pmol/min/cell $\pm$ S.D.), ECAR (mpH/min/cell $\pm$ S.D.) and PER, as a function of time using the Seahorse Report Generator software. Statistical analyses were performed using the unpaired student t-test with p-value of less than 0.05 considered statistically significant.

3. Results

3.1. Clinical manifestations and determination of the heteroplasmic level of the pathogenic m.11778 A>G variant

The proband is a 34-year-old female who first presented with visual symptoms between June and July 2011, at the age of 27. She reported blurred vision resulting in problems with both near and distant vision. She was noted to have dilated pupils. She initially was evaluated by an optometrist who could not correct her visual acuity by refraction and recommended immediate referral to an ophthalmologist. This consultant diagnosed spasms and dry eyes and prescribed rewetting drops. She subsequently developed headaches and underwent a brain magnetic resonance imaging (MRI), which was initially normal. She was then referred to a neuro-ophthalmologist who noted a bilateral optic neuropathy, but was concerned about the diagnosis of pseudo-tumor and scheduled a lumbar puncture to check opening pressure, which was normal. The proband received solumedrol at 1 gram per day for three days.

Four years later, a contrast enhanced MRI was performed, which showed several small foci of T2 hyperintensity in the cerebral white matter (Figure 1A). Ophthalmological evaluation

revealed that the proband had a visual acuity of 20/280 OD and 20–160 OS, some color desaturation on Ishihara plates, and bilateral ceocentral scotoma on visual field testing. She was subsequently referred to a rheumatologist because of complaints of her legs feeling “heavy”, which led to the differential diagnosis of lupus, multiple sclerosis or neuromyelitis optica. She started mycophenolate mofetil and prednisone taper. In September 2011, she underwent another brain MRI, which confirmed neuromyelitis optica, and therefore started plasmapheresis. She later developed weight loss, nausea, weakness, and dizziness. In July 2015, urinary retention and bladder spasms developed leading to the need for self-catheterization. On recent examination, she has intact mental status. Her vision is poor and her optic nerves are pale. She has normal strength, tone and reflexes (2+) with no ankle clonus. Her cerebellar examination was without clonus or ataxia. Full sensory examination was intact to light touch, pinprick, proprioception, temperature and vibration.

Since her vision continued to decline, the neuro-ophthalmologist decided to have her genetically tested for mtDNA variants as a “long shot”. Using total DNA isolated from blood, Sanger sequencing revealed the presence of the pathogenic m.11778 A>G variant, which is congruent with her collective clinical manifestations. In March 2017, a skin biopsy was performed on the proband to quantify the heteroplasmic load of the m.11778 A>G variant and ascertain the pathological consequences on her bioenergetic profile. Long-range PCR followed by massively parallel sequencing showed a 94 % heteroplasmic level for the m.11778 A>G variant in the proband’s fibroblasts (Figure 1B). Since we could not acquire fibroblasts from any of the proband’s relatives, we could not determine whether this pathogenic variant occurred *de novo* or was maternally inherited. However, a three-generation pedigree of the proband indicates a family history of neurological disorders (Figure 1C). The proband is an only child. Her father being adopted, no further information is available, though he appears to be of Caucasian descent by report. The maternal side of the family is of Scottish/English and Danish ancestry. Her mother is 65 years old and healthy, who has two brothers and one sister, all of whom reportedly have high blood pressure and heart disease that developed in adulthood. The older uncle has a 50-year-old daughter with possible multiple sclerosis. This same uncle also has a 30-year-old son with “cognitive issues” that prevent him from holding a job, a 16-year-old son with the diagnosis of Asperger’s, and a third son who died in infancy as a result of choking. The sister has two daughters. The 40-year-old daughter was born with Metatarsus adductus treated with casting. The 38-year-old daughter was born with congestive heart disease, which required surgery to repair a heart valve. The maternal grandmother’s side of the family has a history of Fuch’s corneal dystrophy in the grandmother, two great-aunts, and one aunt. One great-aunt and two great-uncles have had cancer: pancreatic, throat and lung. The maternal grandfather died at the age of 56 after three heart attacks. He had four sisters and four brothers. All have since passed away; the cause of death was either stroke or heart disease.

3.2. Mitochondrial morphometric analysis of the dermal fibroblasts derived from the proband

By transmission electron microscopy, we investigated whether mitochondria from dermal fibroblasts derived from the proband exhibited ultrastructural alterations of mitochondria when compared to those of a healthy adult subject. As expected, the control subject harbored

long mitochondria with a normal morphology characterized with numerous cristae and electron-dense mitochondrial matrix (Figure 2A). Furthermore, we observed numerous mitochondrial fission-fusion events, indicative of normal mitochondrial dynamics congruent with the observed ultrastructural mitochondrial morphology (Figure 2A). In contrast, most mitochondria from the proband harboring a near homoplasmic m.11778 A>G variant were small with few and short cristae (Figure 2B). They rarely exhibited a branched mitochondrial configuration, indicative of abnormal mitochondrial connectivity and dynamics (Figure 2B). Finally, all the mitochondria from the proband's fibroblasts consistently displayed a weak electron density in their mitochondrial matrix, even in the case of the occasional long mitochondria present in the proband's fibroblasts, suggestive of impaired mitochondrial metabolic and respiratory functions (Figure 2B). Thus, our mitochondrial morphometric findings are consistent with the genetic diagnosis of near homoplasmic levels of the m.11778 A>G variant in the proband.

3.3. Functional outcome of the near homoplasmic m.11778 A>G mutation on mitochondrial respiration activities

Since the m.11778 T>G variant affects the mitochondrial-encoded subunit ND4 of the respiratory complex I, the first multisubunit complex of the OXPHOS pathway, we next investigated its functional impact on the OXPHOS metabolism in fibroblasts derived from the proband LHON-MS. As a control subject, we used dermal fibroblasts derived from a healthy adult subject since fibroblasts from any family member were not made available for the study. Using the Seahorse Extracellular Flux XFp analyzer, we measured the oxygen consumption rate (OCR), a key functional indicator of the mitochondrial ATP-coupled respiration. We initially determined the optimal cell seeding density (5,000 cells/well) and the concentration of the uncoupler FCCP (2 μ M) using the cell energy phenotype kit. Using this real-time assay, we found that the baseline metabolic phenotype of the proband's fibroblasts is lessened when compared to that of a healthy subject (Figure 3A). In addition, the proband's fibroblasts exhibited a diminished metabolic potential to respond to an energy crisis triggered by FCCP exposure (Figure 3).

We next measured several key mitochondrial bioenergetics parameters of the OXPHOS pathway (Figure 4A and B). We found a 55% decrease in the basal respiration rate of the LHON-MS fibroblasts when compared to that of a healthy subject, indicative of reduced substrate oxidation and ATP turnover (Figure 4C). Similarly, exposure to oligomycin, an inhibitor of the ATP synthase, revealed a 55% decrease in ATP-linked respiration (Figure 4C). We found that the near homoplasmic m.11778 A>G variant decreased the maximal respiration capacity and spare respiratory capacity by 57% and 60%, respectively (Figure 4C), indicative of an impaired ability to surmount a bioenergetic crisis in keeping with the results from the cell energy phenotype assay (Figure 3). Collectively, our bioenergetics results reveal that the m.11778 A>G variant significantly represses the overall OXPHOS activities.

3.4 Impaired metabolic switch from OXPHOS to glycolysis by the near homoplasmic m.11778 A>G mutation

We next investigated whether the m.11778 A>G variant could impact metabolic reprogramming from OXPHOS to glycolysis. To initiate our studies, we used the Glycolysis Stress Test assay to assess the contribution of the glycolytic pathway during an energy crisis. Glycolysis was estimated as an indirect measurement of ECAR after having starved fibroblasts from the proband and a healthy subject for glucose for one hour followed by injection of saturating amounts of glucose to fuel both glycolysis and mitochondrial OXPHOS, followed by injection of oligomycin to inhibit mitochondrial ATP synthesis, and a final injection of 2-deoxyglucose (2-DG) to inhibit glucose catabolism (Figure 5A and B). The ECAR values upon injection of glucose suggest equal glycolytic activity between the proband and healthy subject (Figure 5C). However, their glycolytic capacity in the presence of oligomycin differ with a drop by 43% in the proband's fibroblasts when compared to that of the control subject's fibroblasts (Figure 5C), indicating that the proband's fibroblasts could not sufficiently augment glycolysis to compensate for inhibition of mitochondrial ATP production. Similarly, Finally, the proband's fibroblasts exhibit a 79% reduction in glycolytic reserve upon exposure to 2-DG when compared to that of a healthy subject, demonstrating that the proband's fibroblasts did not favor glycolysis under conditions of lower ATP-linked respiration (Figure 5C).

Given that ECAR values represent bulk acidification due to protons generated from the conversion of pyruvate to lactate and from the mitochondrial CO₂ production as a by-product of the tricarboxylic acid (TCA) cycle, they overestimate the true basal glycolysis (Mookerjee et al., 2015). Thus, we complemented our analysis of the metabolic switch using the Glycolysis Rate assay to accurately quantify the total Proton Efflux Rate (PER) and the Glycolytic Proton Efflux Rate (GlycoPER) (Figure 6A). We found that the LHON-MS fibroblasts exhibited a 36% decrease in basal glycolysis when compared to that of control fibroblasts (Figure 6C). Moreover, we measured a 25% decrease in mitochondrial acidification in the LHON-MS fibroblasts (Figure 6C), congruent with the observed 40% decrease in mitochondrial OXPHOS metabolism (Figure 5). Upon complete inhibition of mitochondrial OXPHOS by blocking the activity of the respiratory complexes I and III via injection of rotenone and antimycin A (Mookerjee et al., 2016), the LHON-MS fibroblasts displayed a severe decline in compensatory glycolysis by 46% when compared to that of healthy fibroblasts (Figure 6C). This response concurs with the reduced glycolytic reserve measured by the Glycolysis Stress Test assay (Figure 5C), indicating that fibroblasts harboring the m.11778 A>G variant exhibit limited bioenergetic flexibility to switch from mitochondrial OXPHOS to glycolysis when mitochondrial ATP production is compromised.

4. Discussion

In this study, we investigated the comprehensive metabolic signature of the pathogenic variant m.11778 A>G variant from a proband affected by LHON-MS. The highly accurate technique of long-range PCR followed by massive parallel sequencing quantified this pathogenic variant at near homoplasmic level (94%) in the proband, who appears to be the only family member affected given the lack of clinical symptoms in other family members.

However, we cannot exclude that other family members harbor this pathogenic variant at low heteroplasmic levels since they were not genetically tested. More notably on a clinical point of view, four female family members have been diagnosed with Fuch's corneal dystrophy, a disease characterized by decreased expression of mitochondrial-encoded genes for the OXPHOS complexes I, III, IV, and V (ATP synthase) and nuclear genes encoding anti-oxidants, such as glutathione (Gottsch et al., 2003). More specifically, Fuch's endothelial cells exhibit declined expression levels of several mitochondrial-encoded subunits, ND2 and ND4 of complex I, cytochrome b subunit of complex III, cytochrome c oxidase subunit III of complex IV and ATPase 6 of complex V. In keeping with this trend of OXPHOS decline are missense mutations in the mitochondrial-encoded genes for the ND1 subunit of complex I and the cytochrome b subunit of complex III detected in a patient with Fuch's corneal dystrophy (Albin, 1998).

While the functional impact of the m.11778 A>G variant on the mitochondrial OXPHOS pathway has been extensively investigated in the context of LHON, very little is known about its role in the context of LHON-MS. Even though both diseases are characterized by the loss of retinal ganglion cells and optic nerve degeneration, LHON-MS is defined by a distinct clinical phenotype with neurological manifestations, such as tremor, dementia, movement disorders, peripheral neuropathy, and MS-like symptoms (Pfeffer et al., 2013). Thus, investigating the metabolic consequences of the m.11778 A>G variant using fibroblasts derived from LHON-MS patients is essential to elucidate the underlying molecular pathogenic mechanism of LHON-MS.

Our morphometric analysis of electron micrographs of mitochondria reveals that the near homoplasmic m.11778 A>G variant disrupts the architecture and abundance of mitochondrial cristae. LHON-MS mitochondria are short and contain few and small cristae. Given that cristae are modulators of the mitochondrial respiratory efficiency by housing the five respiratory complexes (Cogliati et al., 2013; Cogliati et al., 2016), such abnormal cristae ultrastructural morphology infers altered OXPHOS functions in the presence of the m.11778 A>G variant. This is further supported by the weak electron density of the mitochondrial matrix, a hallmark of impaired mitochondrial metabolic and respiratory functions as a result of decreased expression levels of mitochondrial metabolic enzymes. Our collective morphometric results are consistent with a recent mitochondrial proteomic study using LHON fibroblasts with the m.11778 A>G variant, which reveals down-regulation of proteins involved in mitochondrial metabolic pathways, cristae remodeling proteins, and OXPHOS pathway (Tun et al., 2014).

To gain knowledge on how this pathogenic variant causes metabolic disturbance, we conducted comprehensive live-cell functional analyses of mitochondrial respiratory functions using the proband's fibroblasts. We show that the near homoplasmic m.11778 A>G variant stunts the overall metabolic potential with a sharp decline in basal respiration and ATP-linked respiration resulting from decreased substrate oxidation. This is further validated with the drop in mitochondrial acidification due to diminished CO₂ production from the mitochondrial TCA cycle. Our live-cell bioenergetic results firm up the early findings from polarographic and spectrophotometric analyses of the LHON m.11778 A>G variant showing conflicting results on modulation of complex I activity spanning from subtle

to modest reduction (Larsson et al., 1991; Majander et al., 1991; Esposito et al., 1994; Carelli et al., 1997). However, this complex I dysfunction causes a decrease in malate/glutamate- or pyruvate/malate-dependent respiration by 30 to 50% (Larsson et al., 1991; Esposito et al., 1994; Carelli et al., 2004). Moreover, cybrid cell lines expressing the m.11778 A>G variant poorly grew in a medium containing galactose instead of glucose, inferring impaired OXPHOS capacity (Hofhaus et al., 1996). Polarographic and enzymatic studies using fibroblasts derived from LHON patients harboring the m.11778 A>G variant confirmed a 40% decrease in complex I activity associated with defective OXPHOS efficiency and chronic increase of oxidative stress, which is partially rescued with the analog of coenzyme Q10 idebenone (Carelli et al., 2003; Chevrollier et al., 2008; Angebault et al., 2011).

Our live-cell respiratory analysis of the mitochondrial OXPHOS pathway provides the seminal finding that the near homoplasmic m.11778 A>G variant severely impairs the spare respiratory capacity, which is vital to assume a sudden increase in energy demand for enhancing cell survival (Yadava and Nicholls, 2007). This finding is relevant to the pathogenesis of the LHON-MS disease characterized by two hallmarks derived from mitochondrial dysfunction, apoptosis of retinal ganglion cells and inflammatory demyelination causing axonal degeneration (Bargiela and Chinnery, 2017).

Until now, metabolic reprogramming by the pathogenic m.11778 A>G variant has not been investigated. We provide a novel metabolic outlook of this variant, which reveals a diminished ability to switch to glycolysis due to a curtailed compensatory glycolysis response and glycolytic reserve under conditions simulating an energy crisis. Such defective bioenergetic adaptation may compromise cellular fitness, thereby causing neuronal stress and potentially contributing to the molecular pathogenesis of LHON-MS. A recent study has uncovered that metabolic reprogramming also occurs under normal physiological conditions, as neurons take advantage of an astrocytic short-term and fast aerobic glycolysis to maximize their neural activity to prevent a shortage of ATP production during information processing (Fernández-Moncada et al., 2018). This bioenergetic plasticity is evocative of the metabolic signature of tumor cells and proliferating cells, referred to as the Warburg and Crabtree effect, characterized by enhanced glycolysis and reduced OXPHOS (Diaz-Ruiz et al., 2011).

Our metabolic analysis on this single LHON-MS patient sets the stage for future studies to confirm this metabolic disturbance in a cohort of LHON-MS patients harboring the m.11778 A>G variant at various heteroplasmic levels. Complementing these future metabolic investigations in the context of LHON will provide insight into the potential biochemical mechanistic interactions between these two disorders. Ultimately, patients with MS should be included in light of recent reports of altered respiratory chain activity in MS lesions (Campbell and Mahad, 2018).

In conclusion, the near homoplasmic m.11778 A>G pathogenic variant provokes ultrastructural defects of mitochondrial cristae, thereby impacting the mitochondrial OXPHOS pathway and curtailing the crucial spare respiratory capacity. This pathogenic variant also impairs metabolic reprogramming by altering the dynamic interplay between OXPHOS and glycolysis. Collectively, our results provide novel insight into the potential

molecular pathogenic mechanisms of the m.11778 A>G variant leading to energy crisis in this patient with the LHON-MS disease.

Acknowledgments

This work was funded by the NIH National Institute of Neurological Disorders and Stroke [NS085282 to AC], from the National Institute of Child Health and Development [1U54HD090257] and the NIH National Center for Advancing Translational Sciences [UL1TR00075].

References

- Albin RL. 1998; Fuch's corneal dystrophy in a patient with mitochondrial DNA mutations. *J Med Genet.* 35:258–259. [PubMed: 9541117]
- Angebault C, Gueguen N, Desquiret-Dumas V, Chevrollier A, Guillet V, Verny C, Cassereau J, Ferre M, Milea D, Amati-Bonneau P, Bonneau D, Procaccio V, Reynier P, Loiseau D. 2011; Idebenone increases mitochondrial complex I activity in fibroblasts from LHON patients while producing contradictory effects on respiration. *BMC Res Notes.* 4:4557.
- Baracca A, Solaini G, Sgarbi G, Lenaz G, Baruzzi A, Schapira AH, Martinuzzi A, Carelli V. 2005; Severe impairment of complex I-drive adenosine triphosphate synthesis in Leber hereditary optic neuropathy cybrids. *Arch Neurol.* 62:730–736. [PubMed: 15883259]
- Bargiela, D; Chinnery, PF. Mitochondria in neuroinflammation – Multiple sclerosis (MS), Leber hereditary optic neuropathy (LHON) and LHON-MS. *Neurosci Letters.* 2017.
- Bourgeron T, Chretien D, Rötig A, Munnich A, Rustin P. 1993; Fate and expression of the deleted mitochondrial DNA differ between human heteroplasmic skin fibroblast and Epstein-Barr virus-transformed lymphocyte cultures. *J Biol Chem.* 268:19369–19376. [PubMed: 8396136]
- Campbell G, Mahad DJ. 2018; Mitochondrial dysfunction and axon degeneration in progressive multiple sclerosis. *FEBS Letters.* 592:1113–1121. DOI: 10.1002/1873-3468.13013 [PubMed: 29453889]
- Carelli V, Ghelli A, Ratta M, Bacchilega E, Sangiorgi S, Mancini R, Leuzzi V, Cortelli P, Montagna P, Lugaresi E, Esposti MD. 1997; Leber's hereditary optic neuropathy: biochemical effect of 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. *Neurology.* 48:1623–1632. [PubMed: 9191778]
- Carelli V, Giordano C, d'Amati G. 2003; Pathogenic expression of homoplasmic mtDNA mutations needs a complex nuclear-mitochondrial interaction. *Trends Genet.* 19:257–2. [PubMed: 12711217]
- Carelli V, Rugolo M, Sgarbi G, Ghelli A, Zanna C, Baracca A, Lenaz G, Napoli E, Martinuzzi A, Solaini G. 2004; Bioenergetics shapes cellular death pathways in Leber's hereditary optic neuropathy: a model of mitochondrial neurodegeneration. *Biochem Biophys Acta.* 1658:172–179. [PubMed: 15282189]
- Chevrollier A, Guillet V, Loiseau D, Gueguen N, Pou de Crescenzo M-A, Verny C, Ferre M, Dollfus H, Odent S, Milea D, Goizet C, Amati-Bonneau P, Procaccio V, Bonneau D, Reynier P. 2008; Hereditary optic neuropathies share a common mitochondrial coupling defect. *Ann Neurol.* 63:794–798. [PubMed: 18496845]
- Cogliati S, Frezza C, Soriano ME, Varanita T, Quintana-Cabrera R, Corrado M, Cipolat S, Costa V, Casarin A, Gomes LC, Perales-Clemente E, Salviati L, Fernandez-Silva P, Enriquez JA, Scorrano L. 2013; Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell.* 155:160–171. [PubMed: 24055366]
- Cogliati S, Enriquez JA, Scorrano L. 2016; Mitochondrial cristae: where beauty meets functionality. *Trends Biochem Sci.* 41:261–273. [PubMed: 26857402]
- Diaz-Ruiz R, Rigoulet M, Devin A. 2011; The Warburg and Crabtree effects: on the origin of cancer cell energy metabolism and of yeast glucose repression. *Biochem Biophys Acta.* 1807:568–576. [PubMed: 20804724]
- Esposito MD, Carelli V, Ghelli A, Ratta M, Crimi M, Sangiorgi S, Montagna P, Lenaz G, Lugaresi E, Cortelli P. 1994; Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy. *FEBS Letters.* 352:375–379. [PubMed: 7926004]

- Fernández-Moncada, I; Ruminot, I; Robles-Maldonado, D; Alegría, K; Deitmer, JW; Barros, LF. Neuronal control of astrocytic respiration through a variant of the Crabtree effect. *Proc Natl Acad Sci USA*. 2018.
- Gottsch JD, Bowers AL, Margulies EH, Seitzman GD, Kim SW, Saha S, Jun AS, Stark WJ, Liu SH. 2003; Serial analysis of gene expression in the corneal endothelium of Fuch's dystrophy. *Invest Ophthalmol Vis Sci*. 44:594–599.
- Harding AE, Sweeney MG, Miller DH, Mumford CJ, Kellar-Wood H, Menard D, McDonald WI, Compston DAS. 1992; Occurrence of a multiple sclerosis-like illness in women who have a Leber's hereditary optic neuropathy mitochondrial DNA mutation. *Brain*. 115:979–989. [PubMed: 1393514]
- Hofhaus G, Johns DR, Hurko O, Attardi G, Chomyn A. 1996; Respiration and growth defects in transmittochondrial cell lines carrying the 11778 mutation associated with Leber's hereditary optic neuropathy. *J Biol Chem*. 271:13155–13161. [PubMed: 8662757]
- Howell N, Bindoff LA, McCullough DA, Kubacka I, Poulton J, Mackey D, Taylor L, Turnbull DM. 1991; Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. *Am J Hum Genet*. 49:939–950. [PubMed: 1928099]
- Huoponen K, Vilkki J, Aula P, Nikoskelainen EK, Savontaus ML. 1991; A new mtDNA mutation associated with Leber hereditary optic neuropathy. *Am J Hum Genet*. 48:1147–1153. [PubMed: 1674640]
- Johns DR, Neufeld MJ, Park RD. 1992; An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. *Biochem Biophys Res Commun*. 187:1551–1557. [PubMed: 1417830]
- Kellar-Wood H, Robertson N, Govan GG, Sweeney MG, Da Costa J, Harding AE. 1994; Leber's hereditary optic neuropathy mitochondrial DNA mutations in multiple sclerosis. *Ann Neurol*. 36:109–112. [PubMed: 8024249]
- Kirkman MA, Korsten A, Leonhardt M, Dimitriadis K, De Co IF, Klopstock T, Griffiths PG, Hudson G, Chinnery PF, Yu-Wai-Man P. 2009; Quality of life in patients with Leber hereditary optic neuropathy. *Invest Ophthalmol Vis Sci*. 50:3112–3115. [PubMed: 19255150]
- Larsson N-G, Andersen O, Holme E, Oldfors A, Wahlström J. 1991; Leber's hereditary optic neuropathy and complex I deficiency in muscle. *Ann Neurol*. 33:701–708.
- Lodi R, Taylor DJ, Tabrizi SJ, Kumar S, Sweeney M, Wood NW, Styles P, Radda GK, Shapira AH. 1997; In vivo skeletal muscle mitochondrial function in Leber's hereditary optic neuropathy assessed by ³¹P magnetic resonance spectroscopy. *Ann Neurol*. 42:573–579. [PubMed: 9382468]
- Lodi R, Carelli V, Cortelli P, Iotti S, Valentino ML, Barboni P, Pallotti F, Montagna P, Barbiroli B. 2002; Phosphorus MR spectroscopy shows a tissue specific in vivo distribution of biochemical expression of the G3460A mutation in Leber's hereditary optic neuropathy. *J Neurol Neurosurg Psychiatry*. 72:805–807.
- Mackey DA, Howell N. 1992; A variant of Leber hereditary optic neuropathy characterized by recovery of vision and by an unusual mitochondrial genetic etiology. *Am J Hum Genet*. 51:1218–1228. [PubMed: 1463007]
- Mackey DA, Oostra RJ, Rosenberg T, Nikoskelainen E, Bronte-Stewart J, Poulton J, Harding AE, Govan GG, Bolhuis PA, Norby S. 1996; Primary pathogenic mtDNA mutations in multigeneration pedigrees with Leber hereditary optic neuropathy. *Am J Hum Genet*. 59:481–485.
- Majander A, Huoponen K, Savontaus ML, Nikoskelainen E, Wikström M. 1991; Electron transfer properties of NADH:ubiquinone reductase in the ND1/3460 and the ND4/11778 mutations of the Leber hereditary optic neuropathy (LHON). *FEBS Lett*. 292:289–292. [PubMed: 1959619]
- Man PYW, Turnbull DM, Chinnery PF. 2002; Leber hereditary optic neuropathy. *J Med Genet*. 39:162–169. [PubMed: 11897814]
- Mookerjee SA, Goncalves RLS, Gerencser AA, Nicholls DG, Brand MD. 2015; The contributions of respiration and glycolysis to extracellular acid production. *Biochim Biophys Acta*. 1847:171–181. [PubMed: 25449966]
- Mookerjee SA, Nicholls DG, Brand MD. 2016; Determining maximum glycolytic capacity using extracellular flux measurements. *PLoS One*. 11(3):e0152016. [PubMed: 27031845]

- Palace J. 2009; Multiple sclerosis associated with Leber's hereditary optic neuropathy. *J Neurol Sciences*. 286:24–27.
- Pfeffer G, Burke A, Yu-Wai-Man P, Compston DAS, Chinnery PF. 2013; Clinical features of MS associated with Leber hereditary optic neuropathy mtDNA mutations. *Neurology*. 81:20173–2081.
- Riordan-Eva P, Sanders MD, Govan GG, Sweeney MG, Da Costa J, Harding AE. 1995; The clinical features of Leber's hereditary optic neuropathy defined by the presence of a pathogenic mitochondrial DNA mutation. *Brain*. 118:319–337. [PubMed: 7735876]
- Tun AW, Chaityarit S, Kaewsutthi S, Katanyoo W, Chuenkongkaew W, Kuwano M, Tomonaga T, Peerapittayamongkol, Thongboonkerd V, Lertrit P. 2014; Profiling the mitochondrial proteome of Leber's hereditary optic neuropathy (LHON) in Thailand: down-regulation of bioenergetics and mitochondrial protein quality control pathways in fibroblasts with the 11778G>A mutation. *PLoS ONE*. 9(9):e106779.doi: 10.1371/journal.pone.0106779 [PubMed: 25215595]
- Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJD, Nikoskelainen EK. 1988; Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*. 242:1427–1430. [PubMed: 3201231]
- Yadava N, Nicholls DG. 2007; Spare respiratory capacity rather than oxidative stress regulates glutamate excitotoxicity after partial respiratory inhibition of mitochondrial complex I with rotenone. *J Neurosci*. 27:7310–7317.

Figure 1.

Clinical and genetic analyses of the proband affected with LHON-MS. A) T2/FLAIR brain magnetic resonance imaging of the proband. Several small foci of T2 hyper intensity in the cerebral white matter were noted (yellow arrows) and without associated restricted diffusion or abnormal enhancement. B) Detection of the pathogenic m.11778 A>G variant by LR-PCR-based next generation sequencing in dermal fibroblasts of the proband. The left panel shows the gel electrophoresis of the LR-PCR products from the proband with the size of the amplicon product (16.6 kb) indicated by an arrow. The right panel shows the piled-up LR-PCR/massively parallel sequencing (MPS) result of the proband reveals that there are 92% A>G at the m.11778 position. C) Pedigree tree with the proband identified by an arrow. Family members affected by Fuch's Corneal Dystrophy is indicated by circle with a black quarter.

Figure 2.

The m.11778 A>G variant alters the morphology and abundance of cristae. A) Mitochondrial morphometric analysis by transmission electron microscopy using dermal fibroblasts of a healthy subject. The scale bar (200 nm) is indicated at the bottom right corner of each micrograph. B) Mitochondrial morphometric analysis by transmission electron microscopy using dermal fibroblasts of the proband. The scale bar (200 nm) is indicated at the bottom right corner of each micrograph.

Figure 3.

The overall metabolic signature of the pathogenic m.11778 a>G variant in the proband's fibroblasts. Basal oxygen consumption (OCR) and extracellular acidification rate (ECAR) were measured in fibroblasts from a healthy subject (blue square) and the proband (red square) and were normalized to the number of cells. Basal OCR is a measure of OXPHOS activity, while basal ECAR is a measure of glycolysis activity. The basal phenotype was determined in the absence of metabolic modulators and the stressed phenotype was determined in the presence of the uncoupler FCCP (2 μ M). The metabolic potential represents the difference between stressed OCR or ECAR over baseline OCR or ECAR.

Figure 4.

Altered mitochondrial OXPHOS pathway in the proband's fibroblasts. A) Profile of the OCR for the mitochondrial stress test. B) Real-time analysis of OCR in fibroblasts derived from a healthy subject (blue line) and the proband (red line). OCR values were normalized to the number of cells. C) Quantitative data of basal respiration, ATP-linked respiration, maximal respiration, and spare respiratory capacity. Data are represented as means $\pm$ S.D., $n=3$ of independent experiments each with three technical replicated. * indicates statistically significant differences with a p value of <0.006 between the proband and healthy subject.

Figure 5.

Reduced glycolysis activity in the proband's fibroblasts. A) Profile of the extracellular acidification rate (ECAR) from the glycolysis stress test. B) Real-time ECAR analysis in fibroblasts derived from a healthy subject (blue line) and the proband (red line). ECAR values were normalized to the number of cells. C) Quantitative data of glycolysis, glycolytic capacity, and glycolytic reserve. Data are represented as means $\pm$ S.D., $n=3$ of independent experiments each with three technical replicated. * indicates statistically significant differences with a p value of 0.002 between the proband and a healthy subject.

Figure 6.

Altered proton efflux rate in the fibroblast's proband. A) Schematic representation of proton efflux measured with the glycolytic rate assay. B) Compared total and glycolytic proton efflux rates between the proband (red) and healthy subject (blue). C) Quantitative data of basal glycolysis, compensatory glycolysis, and mitochondrial acidification. Data are represented as means $\pm$ S.D., $n=3$ of independent experiments each with three technical replicated. * and ** indicate statistically significant differences with a p value of 0.05 and 0.005, respectively between the proband and healthy subject.