Analysis of genotype and phenotype in Leber Hereditary Optic Neuropathy-affected patients and asymptomatic maternal relatives

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I Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement. I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish.

I declare that this thesis is my own work. Full and informed consent was obtained from all subjects.

The Research Ethics Committee of the Royal Victoria Eye and Ear Hospital approved this study.

_____

Dr Clare Quigley

II Summary

During this study subjects who had Leber Hereditary Optic Neuropathy (LHON), or who were a first degree maternal relative of a person affected by LHON were examined and patterns of importance or clinical significance were extracted and discussed with relevance to the literature. This included analysis of mitochondrial haplogroup and optic neuropathy in asymptomatic relatives, which revealed that asymptomatic relatives with haplogroup H or HV displayed more optic neuropathy than those of other haplogroups. Optic nerve head perfusion was lower in LHON but normal overall in relatives, where evidence of optic neuropathy was found to be associated more closely with nerve fiber layer atrophy than with reduced optic nerve head perfusion. Cardiac conduction abnormalities were found in asymptomatic relatives. In conclusion, we highlight that there may be an asymptomatic cohort of patient relatives who display clinical signs of optic neuropathy and other associated signs of LHON.

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IV Dedication

To Conor,

Michael and Aoife

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VI Presentations arising from this thesis

1. Royal College of Ophthalmologists annual meeting, Glasgow, 23-26 May 2022
    - Poster presentation: Novel potential nuclear genetic modifier in Leber Hereditary Optic Neuropathy in Wolfram Syndrome-associated variant WFS-1 c.799G>A
2. Irish College of Ophthalmologists annual meeting, Killarney, 23 May 2023
    - Platform presentation: Optic nerve head perfusion and optic neuropathy in carriers of Leber Hereditary Optic Neuropathy-associated mitochondrial mutations
3. European Society of Ophthalmology annual meeting, Prague, 16 June 2023
    - Platform presentation: Optic nerve head vascular changes and optic neuropathy in carriers of LHON-causing mitochondrial mutations
4. United Kingdom Neuro-Ophthalmology Society (UKNOS) annual meeting, Newcastle, 8 February 2024, winner best poster:
    - Poster presentation: Peripapillary retinal nerve fibre layer thinning, perfusion changes and optic neuropathy in carriers of Leber Hereditary Optic Neuropathy-associated mitochondrial variants

VII Publications arising from this thesis

1. Quigley C, Stephenson KAJ, Kenna P, Cassidy L. Optic Nerve Structural and Functional Changes in LHON-Affected and Asymptomatic Maternal Relatives: Association with H and HV Mitochondrial Haplogroups. Int J Mol Sci. 2023 Jan 5;24(2):1068. doi: 10.3390/ijms24021068. PMID: 36674591

2. Quigley C, Hanrahan G, Stephenson K, Ahmed S, Mukhtar M, Cassidy L. Cardiac conduction abnormalities in Leber Hereditary Optic Neuropathy and asymptomatic maternal relatives. Eye (Lond). 2023 Oct; 37 (14): 3050-3051. doi: 10.1038/s41433-023-02466-3. PMID: 36879160

3. Quigley C, Stephenson K, Kenna P, Cassidy L. Peripapillary retinal nerve fibre layer thinning, perfusion changes and optic neuropathy in carriers of Leber Hereditary Optic Neuropathy-associated mitochondrial variants. BMJ Open Ophthalmol. 2024 Mar 11;9(1):e001295. doi: 10.1136/bmjophth-2023-001295. PMID: 38471715

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X List of Abbreviations

ATP	Adenosine triphosphate
BCVA	Best corrected visual acuity
CF	Count fingers
chLHON	Chronic Leber Hereditary Optic Neuropathy >12 months
CS	Contrast Sensitivity
ECG	Electrocardiogram
eLHON	Early subacute Leber Hereditary Optic Neuropathy <3 months
ETDRS	Early treatment of diabetic retinopathy study
F	Female
GCL	Ganglion cell layer
HM	Hand motions
IPL	Inner plexiform layer
IQR	Interquartile range
Left eye	LE
LHON	Leber Hereditary Optic Neuropathy
LogMAR	Logarithm of the minimal angle of resolution
M	Male
ms	millisecond
mtDNA	Mitochondrial DNA
NR	Nonrecordable
OCT	Optical coherence tomography
OCTA	Optical coherence tomography angiography
ON-N	Optic nerve- nasal segment
ON-NI	Optic nerve- nasal inferior segment
ON-NS	Optic nerve- nasal superior segment
ON-T	Optic nerve- temporal segment
ON-TI	Optic nerve- temporal inferior segment
ON-TS	Optic nerve- temporal superior segment
PL	Perception of light
PR-VEP	Pattern reversal- visually evoked potential
RE	Right eye
RNFL	Retinal nerve fiber layer
VA	Visual acuity
VD	Vessel density
VEP	Visual evoked potential
VF	Visual field

Chapter 1 Introduction

1.1 LHON overview

Leber Hereditary Optic Neuropathy (LHON), first described by Leber in 1871, is an inherited optic neuropathy that usually causes blindness (defined as a reduction of best corrected visual acuity (BCVA) to the equivalent of snellen 6/60 or less with both eyes open).[1] LHON is the most common primary mitochondrial disorder, with an estimated prevalence of 1:30,000-1:50,000,[2] and is caused by mitochondrial dysfunction, with one retrospective study demonstrating that over 95% of LHON pedigrees are associated with one of three mitochondrial DNA (mtDNA) pathogenic variants, including mt. 3460G>A, mt. 11778G>A, and mt. 14484T>C,[3] which affect complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial respiratory chain.[3, 4] A number of other rare LHON-causing mt DNA mutations have been described.[5-11]

Presentation of LHON typically occurs in young adulthood, with bilateral painless subacute loss of vision.[12] Usually bilateral optic atrophy becomes evident and stable blindness develops, with 70% of eyes progressing to best corrected visual acuity (BCVA) worse than 6/60 Snellen within 3 months.[13] However, there is some heterogeneity in clinical expression, whereby vision loss may uncommonly develop in childhood[14] or in late adulthood,[15, 16] and vision loss may vary from mild to severe, with some variation due to mutation type;[17, 18] vision loss in those affected by G11778 or G3460A mutations tends to be more severe than vision loss in T14484C carriers. Many carriers of pathogenic mitochondrial mutations, about 50% of males and 10% of females, never present with vision loss.[19]

1.2 LHON treatment

Treatment options for LHON are limited. Patients are advised to minimise exposure to potential optic nerve toxins including smoking and alcohol. Idebenone, an analogue of the antioxidant ubiquinone, is licensed for use in specific circumstances in LHON,[13] and shows evidence of benefit for a subgroup of patients, especially when started early within one year of onset of symptoms.[20] Idebenone's beneficial effect is mediated through a restoration in

mitochondrial function, as it bypasses the dysfunctional complex I, increasing ATP production and reducing oxidative stress.[21] Patients gain a mean of 5 letters with Idebenone treatment,[21] given standard posology is 900mg/day in three divided doses.[21]

Gene therapy has also been investigated; the RESCUE[22] and REVERSE[23] trials were randomized, double-masked, sham-controlled phase III clinical trials, assessing efficacy of intravitreal injection of rAAV2/2-*ND4* in patients with the G11778A pathogenic variant, comparing visual acuity in the injected eye to the contralateral control eye. In RESCUE the injection was given within 6 months of symptoms onset, and the primary end point was not met with no significant difference in VA between eyes. In REVERSE, treatment was given between 6 and 12 months after onset of symptoms. The primary endpoint, defined as a difference in best corrected vision between the injected and uninjected eye, was not met. Investigators noted that visual acuity improved bilaterally in the trial subjects, with 78% of subjects showing an improvement in visual acuity in both eyes at 96 weeks, of an average 15 letters in injected eyes, and 13 letters in control eyes. Animal studies have suggested that contralateral visual improvement after unilateral injection occurs due to transfer of the therapeutic viral vector DNA to the contralateral optic nerve.[24]

1.3 Variable disease expression in LHON

Important unknowns in LHON include the cause of sex bias and variable penetrance in disease expression, which may be modified by genetic or other factors. The protective mechanism for female sex is unknown. Women affected by LHON may be diagnosed later in the course of disease, presenting a potential diagnostic difficulty.[25]

Higher levels of homoplasmy of LHON-associated mtDNA carry higher risk of visual loss, with less risk of visual loss when the proportion of LHON-associated mtDNA is under 60%.[26] Each cell contains thousands of copies of mtDNA, that are usually identical (homoplasmy). A mixture of mtDNA can occur where sequences vary (heteroplasmy). In mitochondrial disease, pathogenic variants may cause disease when present at variable levels, typically at >60-90% threshold level. However LHON shows great variety in disease expression, even at 100% homoplasmic levels of pathogenic mitochondrial variant carriage.[2]

Disease penetrance and worse vision loss in G11778A and T14484C have been associated with mitochondrial halplogroup J,[27-29] and inversely associated with haplogroup H.[29, 30] Unaffected carriers have shown increased mtDNA levels raised to greater levels than controls or LHON patients, in a possible protective mechanism to compensate for reduced respiratory chain function.[31]

Observations of abnormal visual function in carriers have included reduced contrast sensitivity,[32] and reduced colour vision.[33] Abnormal optic nerve function measured by pattern reversal visually evoked potential (PR- VEP),[34] and abnormal retinal function, measured by electroretinogram has also been described.[35, 36] Measurement of optic nerve function by PR-VEP, per the standards of the International Society for Clinical Electrophysiology of Vision, includes measurement of pattern-reversal VEP in response to checkerboard stimuli with large 1 degree (°) and small 0.25° check, via scalp electrodes.[37] Electroretinogram measurement, in contrast, evokes and records retinal responses to light flashes.[38]

1.4 Optic nerve and macula optic coherence tomography (OCT) and OCT-angiography findings in LHON

A hallmark sign of LHON at the disease's earliest symptomatic stage is the appearance of peripapillary telangiectatic capillaries, with subtle nerve fiber layer swelling, visible on slit lamp biomicroscopic examination.[39, 40] The nerve fibre layer lies just deep to the internal limiting membrane of the retina, and contains the axons of retinal ganglion cells, carrying impulses to the optic nerve.[41] Over time, after onset of visual loss, nerve fiber layer swelling resolves and optic nerve atrophy and legal blindness develop.[12] Histopathological studies in humans have demonstrated mitochondrial proliferation (i.e., increased biomass) within capillaries of the optic nerve head,[42] which may occur due to a compensatory growth stimulus as mitochondrial function declines. This may underly the appearance of mild optic nerve swelling at the start of the disease process. More severe disc swelling occurs in ischaemic optic neuropathies, such as anterior ischaemic optic neuropathy.[43]

Use of optical coherence tomography (OCT) to non-invasively image the eye of a cadaver was first described in 1991.[44] In OCT a scan is generated via detection of interference of reflected light from multiple depths within a tissue.[45] The axial resolution of OCT imaging is several microns, allowing visualisation of cellular and even subcellular structures. OCT-angiography (OCT-A) adds visualisation of vasculature by detailing flow through vessels, through the analysis of variance of multiple OCT-acquired scans, see Figure 1.[46]

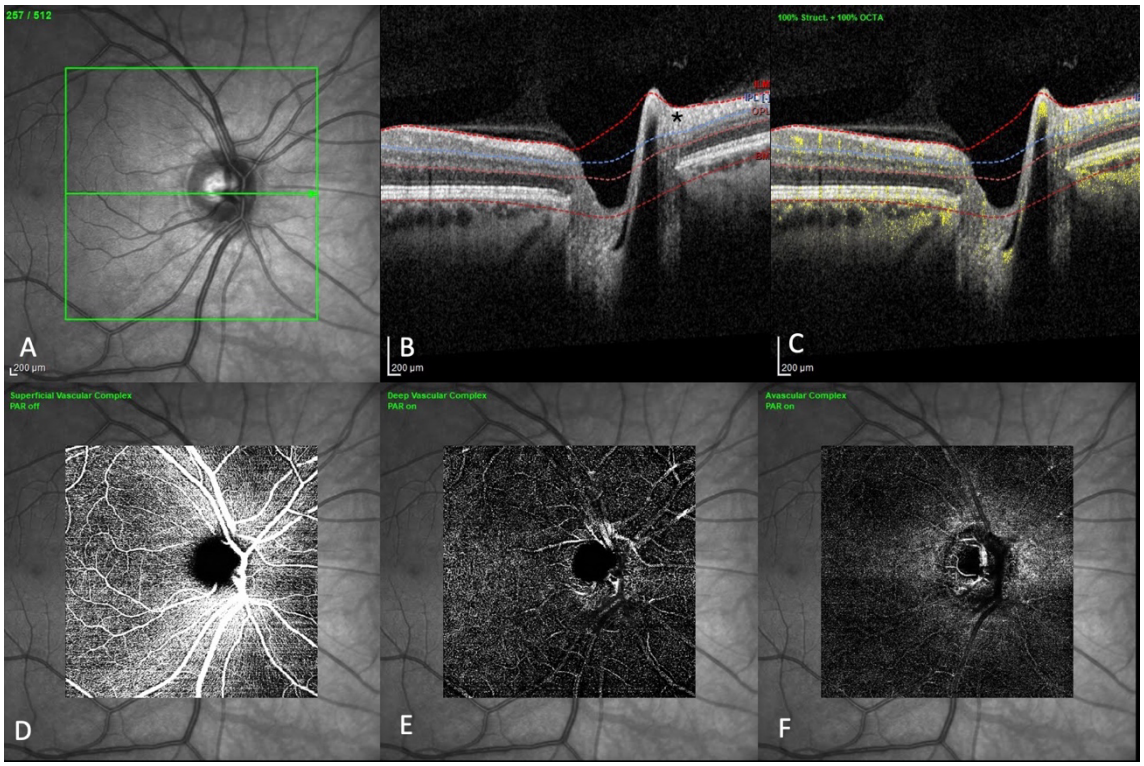


Figure 1. Optical coherence tomography (OCT) and OCT-angiography (OCT-A) images of a normal right optic nerve. En-face image (A), axial scans over optic nerve, including RNFL* (B), overlay in yellow showing OCT-A signal (C), superficial vascular complex OCT-A projected onto en-face image (D), followed by deep vascular complex (E), and avascular complex (F).

After an initial period of optic nerve swelling in LHON, atrophy develops, which is visible on OCT as degeneration of retinal ganglion cells and loss of their axons, causing thinning of the retinal nerve fiber layer (RNFL). Thinning affecting the macular choroid and retina, and the macular RNFL, and ganglion cell layer and inner plexiform layer (GCL +IPL) has been

observed in LHON-affected patients, though not in carriers.[47, 48] In unaffected carriers subtle changes at the macula may be observed, with differences in central macular morphology, including foveal pit slope, visible in unaffected carriers versus controls.[47] Increased thickness of the RNFL limited to the temporal quadrant of the optic nerve head has been observed in carriers, with more extensive RNFL thickening in males versus females.[49] This axonal thickening restricted to the papillomacular bundle may occur due to sequelae of early mitochondrial dysfunction, including reduced axonal transport and compensatory increased mitochondrial biogenesis, causing axons to increase in size. Increased variability of peripapillary RNFL thickness in carriers versus controls has also been observed,[50] representing potential transitory changes that may precede conversion to LHON and overt optic neuropathy.

In-vivo analysis of optic nerve head perfusion is possible with optical coherence tomography angiography (OCTA). Evaluation of optic nerve head perfusion at different stages of LHON has shown that lower vessel density (VD) correlates with vision loss in established LHON, and the superficial capillary layers may show reduced VD in asymptomatic individuals who carry LHON-associated mutations.[51] Further evaluation of LHON optic nerve head perfusion may be helpful in detecting patterns of variation that segregate with disease expression or severity, potentially leading to better understanding of the underlying pathophysiology. Given the large numbers of carriers of LHON-associated mutations who do not develop disease, biomarkers that could identify those at higher risk of losing vision would be helpful. Those at risk could be strongly advised to reduce exposure to environmental risk factors such as smoking and alcohol.[52, 53]

Methodology of vessel density (VD) measurement derived from OCT-A varies. In investigation of LHON, presence or absence of appearance of capillary dropout on inspection of peripapillary OCT-A images has been reported as VD;[54] in another LHON report vessel density was calculated by a method described by a group investigating primary open glaucoma, whereby VD was defined as the percentage area occupied by the large vessels and microvasculature in a particular region, calculated by AngioVue software.[55, 56]

1.5 Cardiac conduction in LHON

Cardiac arrhythmias have been described in individuals affected by LHON, and also in their maternal asymptomatic relatives, including pre-excitation syndrome on 12-lead electrocardiogram (ECG), and Wolff-Parkinson-White (WPW) syndrome,[57, 58] though the finding is described as rare.[12] ECG features of WPW include a short PR interval <120ms, see Figure 2. It has been suggested that an ECG,[12, 59] or an ECG and echocardiogram be carried out routinely as a screening measure in LHON.[60] The 12-lead ECG is a mainstay of clinical practice due to its clinical relevance, low cost, and wide availability.[61]

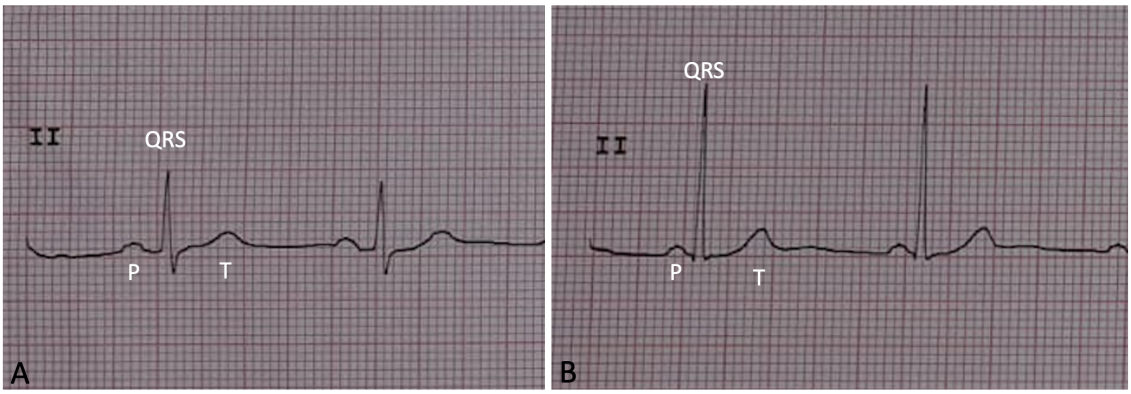


Figure 2. Normal and abnormal 12-lead electrocardiogram (ECG) intervals. The large squares correspond to 0.2 seconds, and the small squares to 0.04 seconds. The left panel shows a normal PR interval (from start of P wave to end of QRS complex) of 156 milliseconds (ms, A), and the right an abnormally short PR interval of 112ms (B).

The role of mitochondrial dysfunction causing heterogeneity of cardiac electrical activity has been implicated in cardiac arrhythmia, potentially triggering fatal arrhythmias under metabolic stress conditions.[62] Mitochondrial function is important for action potential generation in cardiac myocytes, and mitochondrial dysfunction may play a role in the heterogeneity of the stress response of cardiac muscle, mediated via ion channel dysregulation.[63]

1.6 Quality of life in LHON

LHON is known to have a severely negative impact on vision-related quality of life,[64] with a worse vision-related quality of life than those with cataract,[65, 66] strabismus,[67] nystagmus,[68] age related macular degeneration,[69] and other retinal diseases.[70] Vision loss can result in loss of independence, including inability to drive, and to read without aids.[71] However, a diagnosis of a rare disease does not guarantee a low quality of life,[72] and low mood occurs in the general population, with a background rate of a major depressive episode within the past year estimated in 19% of adults aged 18-25 in the US.[73] We aimed to assess vision-related quality of life of our cohort of 12 LHON-affected patients and 16 of their asymptomatic maternal relatives.

1.7 Study aims

In this research we aimed to 1) evaluate LHON patients who attend the neuro-ophthalmology clinic, along with maternal first degree relatives, for modifiers of disease severity, specifically mitochondrial haplogroup, 2) evaluate LHON families for 12-lead electrocardiogram abnormalities, 3) evaluate optic nerve head anatomy including retinal nerve fiber layer thickness and perfusion in LHON families.

Chapter 2 Methods

2.1 Methodology overview and study participants

The Research Ethics Committee of the Royal Victoria Eye and Ear Hospital approved this study. Attendees with LHON in the neuro-ophthalmology clinic were identified by a national Idebenone prescribing prescribing database and were invited to participate, along with their mother or another maternal first degree relative, in a genotyping-phenotyping research study. If the patient had a sibling who was interested, they were invited to also attend. For the optic nerve structural findings arm of the study, a control group was recruited. This resulted in three study groups: 1) a sample of LHON-affected participants, 2) asymptomatic maternal relatives of the LHON-affected, and 3) an unrelated control arm with normal eyes.

LHON-affected participants were divided into 2 subgroups based on symptom duration: 1) early subacute stage of disease with symptom duration <3 months (early subacute LHON, eLHON), 2) patients in the chronic stage of the disease >12 months (chronic LHON, chLHON). There were no participants included who had LHON duration between 3 and 12 months.

For each study participant, demographic data, including age and sex, and clinical data, including ophthalmic and medical history, medications, social and family history, were collected.

Controls for the optic nerve structural component of the study were recruited who had no history of diabetes, ocular or neurological disease, and who had refractive error <±3 dioptres, and normal visual acuity and intraocular pressure. For each study participant, demographic data, including age and sex, and clinical data, including ophthalmic and medical history were collected.

2.2 Vision and optic nerve function testing

Visual acuity (VA) data was recorded, including corrected distance visual acuity via Early Treatment of Diabetic Retinopathy Study (ETDRS) charts with logarithm of the minimal angle of resolution (logMAR) values, or converted from standard Snellen notation to logMAR for analysis purposes. When BCVA was reduced to counting fingers (CF), hand movements (HM) and perception of light (PL), logMAR values were substituted as 2.10, 2.40 and 2.70, respectively.[74]

For the LHON-affected and their included relatives, optic nerve function tests included colour vision testing with Ishihara plates (17), contrast acuity testing (testing at 1 meter, Thomson test chart 2016, Thomson Software Solutions), octopus visual field (Haag-Streit, UK) and standard visual-evoked potential and pattern electroretinogram, per the international society for the clinical electrophysiology of vision standard reporting.[37, 38] Visual field scotomata were classified within the study group, upon review of the variation of severity of defects present, in order to describe the spread of the data. Scotomata classification was; mild if the I2e target was seen but the field was reduced in size relative to the normal reference field; moderate if there was loss or almost loss of the I2e target, but I4e was seen; and severe if there was loss of I4e target.

Subjective change in vision was assessed for those participants who had undergone treatment with idebenone. They were directly questioned whether they had noticed that their vision was stable, better or worse, since prior to starting treatment.

2.3 Fundus examination including OCT and OCTA

For all three study groups (LHON-affected, asymptomatic relatives, and the control arm) dilated fundus examination was performed with colour optic nerve head photography (FF450plus, Carl Zeiss Meditec, Dublin, CA, USA), optical coherence tomography (OCT, Heidelberg Spectralis OCT © Heidelberg Engineering GmbH 2007, Heidelberg, Germany), and analysis using the SPECTRALIS® Glaucoma Module, that utilizes a reference normal database of eyes of 255 normal controls, of European descent.

OCTA of the peripapillary retinal nerve fiber layer (RNFL) was carried out (Heidelberg Spectralis OCT © Heidelberg Engineering GmbH 2007, Heidelberg, Germany). Peripapillary RNFL vessel density (VD) values were obtained from the OCTA scans using imageJ software.[75] The nerve fiber layer segment of a 15 x 15 degrees scan of the optic nerve head (4.4mm x 4.4mm) was extracted and this was analysed with the Vessel Analysis ImageJ plugin.[76] Briefly, the segment was binarized and VD was calculated as the proportion of white to total pixels, and then analysed using imageJ to give a percentage read-out, the RNFL perfusion (%). A normal range of RNFL perfusion (%) was calculated from the control group (n=10), who did not have ocular problems and were unrelated to the LHON group.

2.4 Genetic testing

For the LHON-affected and for their participating asymptomatic maternal relative, peripheral blood sample was drawn to carry out genetic testing at an accredited laboratory (© Blueprint Genetics, Helsinki, Finland). Genomic DNA was extracted using bead-based method, and DNA fragments were amplified by polymerase chain reaction (PCR). Bioinformatics and quality control processes were performed by Blueprint Genetics (see Appendix I). Participants had genetic sequencing performed, including sequence and copy number variation analysis of the whole mitochondrial genome (37 genes), and a panel of nuclear genes including those observed to cause neuro-ophthalmic pathology (see Appendix II). Mitochondrial haplogroup was derived from a reference phylogenetic tree,[77] utilizing the online sequence viewing software Integrated Genomics Viewer to allocate participants.[78]

2.5 Assessment of response to Idebenone

Assessment for treatment effect of Idebenone was carried out. All participants who had taken Idebenone formed a safety population (SP) from whom complication reporting could be carried out. For inclusion in a treatment-response assessment, participants who carried one of the 3 major LHON mtDNA mutations, and who had onset of symptoms within the 12 months prior to starting treatment, and who had baseline VA data formed an efficacy

population (EP). Assessment consisted of analysing whether a clinically relevant stabilisation (CRS) or a clinically relevant recovery (CRR) of vision had taken place, which is an accepted treatment effect analysis in LHON.[79] CRS was defined as baseline LogMAR VA ≤1.0 in at least one eye, maintained in this eye at the latest clinic visit. A clinically relevant recovery (CRR) of BCVA was defined as an improvement from 'off-chart' (i.e., >1.0 logMAR), to 'on-chart' by at least one full line (5 letters), or an improvement in an on-chart BCVA by at least 2 lines (10 letters, 0.2 logMAR), at latest clinical visit as compared to the nadir of visual acuity during their disease course. Adverse events after commencement of Idebenone were also recorded.

2.6 Assessment of cardiac conduction

A 12-lead ECG was performed on LHON-affected and their asymptomatic maternal relative, to assess for the presence of cardiac arrhythmia. Short PR interval was defined as duration <120ms.

2.7 Quality of life questionnaires

For the LHON-affected and asymptomatic maternal relative participants vision-related quality of life was assessed via the National Eye Institute's Visual Function Questionnaire 25 (NEI VFQ-25),[80, 81] with the 10-Item Neuro-Ophthalmic Supplement.[82] Comparative data was derived from analysis of quality of life scores of the symptomatic versus the asymptomatic LHON-variant carrier groups. Where a study participant's visual acuity precluded reading, the questions were read aloud to them by either an accompanying relative (usually the asymptomatic carrier relative) or by the study investigator (CQ).

2.8 Data analysis

Data analysis was performed using statistics software R,[83] with Kruskal Wallis ranked sum test carried out to analyse non-parametric data, Fisher exact test used to analyse categorical data, and median and interquartile range or range described. Analysis was by study subject for statistical tests unless otherwise specified. For the LHON group overall median visual

acuity for the better eye is reported. Descriptive statistics are used where statistical tests are not performed. A normal range for peripapillary VD was derived from the control eyes, utilizing mean ± 1.96 X standard deviation. Power analysis was carried out to determine sample size that would give power 0.8 in Fisher correlation test of an effect size of 0.5, of an association of mitochondrial haplogroup with presence of visual loss or optic nerve dysfunction. At a standard significance level of 0.05, two-sample comparison of proportions power calculation showed that a sample size of n=15 was required for this power level.

Chapter 3 Results

3.1 Demographic and genetic analysis

In the LHON group there were 12 subjects (3 females) from 10 pedigrees with median age 27 years, interquartile range (IQR) 19–33 years. Disease onset was at median 19, IQR 16–23.5 years. In the unaffected carrier group, comprising mothers and siblings including relatives where the LHON-affected relative was invited but unable to attend, there were 16 subjects (15 female) from 13 pedigrees, median age 56, IQR 42–61 years. In total, genetic sequencing revealed mt.11778G>A in 22 subjects, 3460G>A in 2 subjects, 14484T>C in 2 subjects, and DNAJC30 c.152A>G homozygosity in 1 subject, see Table 1.

Characteristics	**Variable**	**LHON -Affected**	**Asymptomatic Carriers**
Genetic mutation	mt. 11778G>A	9 (75%)	13 (81%)
	mt. 3460G>A	1 (8%)	1 (6%)
	mt. 14484T>C	1 (8%)	1 (6%)
	DNAJC30 c.152A>G	1 (8%)	1* (6%)
Parent country of birth: Ireland		11 (92%)	15 (94%)
Ukraine		1 (8%)	1 6%)
Sex	Female/Male	3/9	15/1
Age (years)		27 (19–33)	56 (42–61)
Age onset		19 (16–23.5)	
		6 (2–14)	
Total		12 (100%)	16

Table 1. Demographic and genetic analysis data, displayed as n (%). Age shown is median (IQR). *Mother of patient homozygous for *DNAJC30* c.152A>G.

3.2 Visual acuity data and optic nerve head structural findings

In the LHON group the median visual acuity in the better sighted eye was 1.6 logMAR, IQR 1.2, 2.1 (n = 12). In this group visual acuity was preserved in the better eye to on-chart (i.e., logMAR 1.0 or better) in 3 subjects (25%). Amblyopia was reported in one subject (8%), who was included. In the asymptomatic carriers, median visual acuity was 0.0 logMAR, IQR 0, 0.1 in the right eye, and 0.05, IQR 0.0, 0.2 in the left eye (n=16). Visual acuity worse than 0.5 logMAR was found unilaterally in 3 carriers (19%), they were among five carriers who reported a history of amblyopia (31%, who were included). The overall optic nerve head retinal nerve fiber layer (RNFL) was thinned in LHON subjects by median -54 microns, IQR -23, -63 microns in the right eye, and thinned by median -50 microns, IQR -62,-32 microns, in the left eye. In LHON the nasal RNFL was the only sector that was not thinned. Optic nerve head RNFL was normal overall in asymptomatic carriers, see Table 2.

Due to poor fixation, assessment of optic nerve head RNFL thickness was not possible on all subjects with LHON, but was measured bilaterally in 8 subjects, and unilaterally in one subject. The RNFL was reduced in all of those with chronic LHON (n = 7), whereas thickness was normal overall, with some sectors showing thickening in early LHON (n = 2), see Figure 3. Of asymptomatic relatives (n = 16), 75% had overall normal RNFL thickness bilaterally, 25% had reduction in RNFL in at least one eye, see Table 3.

Of those asymptomatic relatives with reduced optic nerve head RNFL (5 eyes of 4 asymptomatic carriers), one had normal visual acuity, and the remaining three had unilateral reduced visual acuity, on the same side as the thinning in RNFL.

Characteristics	LHON-Affected		Asymptomatic Carriers	
Female n (%)	**3 (25%)**		**15 (94%)**	
Age (years)	27 (19, 33)		56 (42, 61)	
Visual Acuity	RE	LE	RE	LE
	1.8 (1.25, 2.25)	1.8 (1.4, 2.1)	0 (0, 0.1)	0.05 (0, 0.2)
Optic nerve				
retinal nerve fiber layer (RNFL)				
Overall RNFL	−54 (−62, −23)	−50 (−62, −32)	0.5 (−9, 13)	−2 (−16, 13)
Temporal-RNFL	−48 (−38, −48)	−46 (−50, −45)	−2 (−9, 6)	−4 (−19, 4)
TS-RNFL	−83 (−97, −25)	−77 (−91, −51)	−1 (−16, 14)	−11 (−24, 23)
NS-RNFL	−44 (−54, −23)	−47 (−56, −18)	6 (−19, 21)	8 (−12, 19)
Nasal-RNFL	−25 (−40, −10)	−24 (−39, −17)	9 (−5, 18)	3 (−18, 10)
NI-RNFL	−48 (−73, −22)	−54 (−64, 2)	4 (−19, 27)	5 (−21, 17)
TI-RNFL	−96 (−107, −41)	−89 (−111, −40)	9 (−11, 20)	−15 (−45, 15)

Table 2. Demographic data and optic nerve head thickness: median deviation of optic nerve retinal nerve fiber layer (RNFL) thickness, visual acuity, sex and age of the LHON-affected and asymptomatic maternal carrier relatives in right (RE) and left (LE) eyes. Visual acuity is displayed in logMAR. The RNFL values are displayed in microns, and highlighted in green where normal and red where thinning is present. Variables including age, visual acuity, and RNFL thickness are displayed as median (1^{st} quartile, 3^{rd} quartile). Optic nerve segments include temporal, temporal-superior (TS), nasal-superior (NS), nasal-inferior (NI), and temporal-inferior (TI).

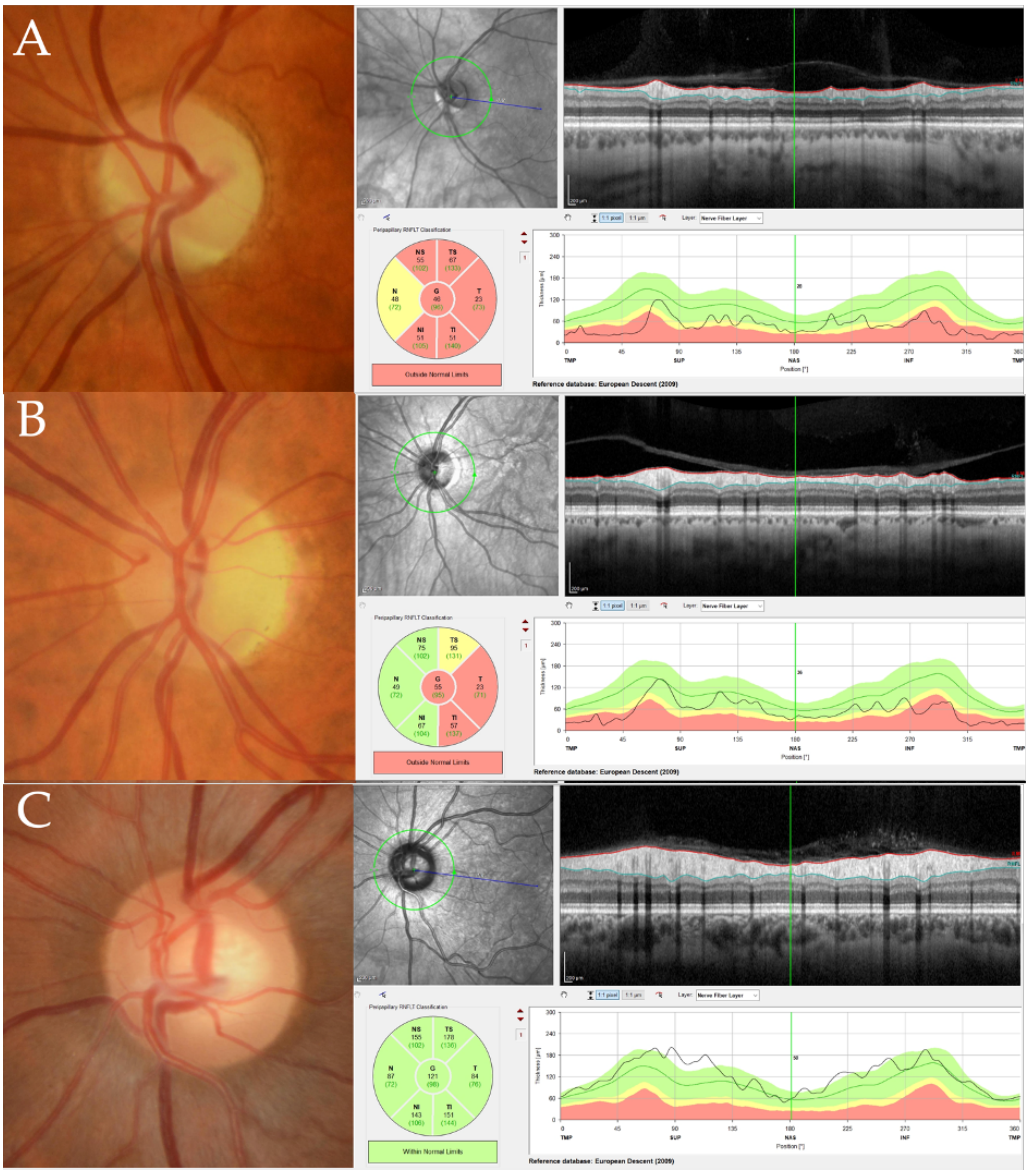


Figure 3. Optic nerve head photo of the left eye with corresponding optic nerve head retinal nerve fiber layer (RNFL) thickness. Subjects C6 chronic LHON showing established optic nerve pallor (A), U15 asymptomatic carrier with unilateral optic nerve RNFL thinning and temporal pallor (B), and U5 asymptomatic carrier with normal optic nerve findings (C).

	ID	Eye	VA	Nerve	Global	ON-T	ON-TS	ON-NS	ON-N	ON-NI	ON-TI
LHON	C1	RE	1.8	**-59**	−48	−96	−54	−30	−61	−118	
		LE	1.5	**−63**	−45	−90	−66	−40	−68	−114	
	C2	RE	2.4	**−48**	−47	−98	−35	−20	−22	−93	
		LE	2.1	**−45**	−57	−91	−35	−17	2	−88	
	C4	RE	2.7	**−74**	−56	−99	−77	−53	−70	−131	
		LE	2.4	**−71**	−57	−110	−70	−51	−56	−115	
	C6	LE	1	**−50**	−50	−66	−47	−24	−54	−89	
	C7	RE	1.8	**−64**	−48	−69	−52	−56	−94	−99	
		LE	1.8	**−58**	−46	−77	−52	−39	−64	−108	
	E1	RE	1	**6**	13	−6	−28	−2	5	50	
		LE	1	**11**	−2	15	−18	10	34	40	
	C9	RE	1.6	**−61**	−44	−97	−54	−35	−80	−103	
		LE	1.9	**−62**	−47	−95	−56	−36	−78	−111	
	C10	RE	1.4	**−32**	−49	−31	−9	−13	−34	−59	
		LE	1.3	**−32**	−46	−51	2	−24	−30	−40	
	E2	RE	1.5	**9**	−21	36	63	9	−21	12	
		LE	1.5	**8**	−33	34	53	−1	8	33	
Asymptomatic	U1	RE	0	**2**	5	−13	−21	12	3	20	
		LE	0.2	**0**	−3	−19	−32	11	12	23	
	U2	RE	0	**−39**	−47	−61	−12	−12	−38	−90	
		LE	0.2	**−42**	−47	−67	−17	−21	−35	−87	
	U3	RE	0.1	**−7**	10	1	−13	−16	−27	−2	
		LE	0.2	**−12**	0	−13	3	−19	−21	−21	
	U4	RE	0.1	**12**	−6	−8	24	16	60	−1	
		LE	0.2	**13**	−10	22	54	1	50	−8	
	U5	RE	0	**23**	13	1	38	26	42	22	
		LE	0	**23**	8	42	53	15	37	7	
	U6	RE	0.1	**−9**	−12	−23	−22	11	4	−24	
		LE	0.1	**−11**	−4	−23	−1	1	−21	−36	
	U7	RE	0.1	**4**	2	13	9	5	−20	14	
		LE	0	**7**	−1	15	12	10	3	8	
	U8	RE	0.1	**8**	−5	12	3	7	30	17	
		LE	2.4	**−26**	−52	−45	−11	5	15	−70	
	U9	RE	0	**−1**	8	−3	−29	−3	−19	32	

	LE	0	**2**	−5	−1	−14	4	4	32
U10	RE	0	**24**	11	30	10	20	62	35
	LE	0	**24**	5	34	12	22	61	34
U11	RE	0	**−9**	−12	−10	20	−10	−16	−30
	LE	0	**−10**	−9	−14	17	−17	1	−36
U12	RE	0	**17**	0	17	14	36	22	8
	LE	0.5	**14**	3	34	25	16	−30	13
U13	RE	0	**20**	−2	38	31	24	26	20
	LE	0	**−32**	11	25	44	2	23	19
U14	RE	1.8	**−24**	−57	−70	24	17	9	−78
	LE	0	**−31**	−52	−9	15	−24	−36	−70
U15	RE	0	**−5**	−2	18	−28	−13	−22	9
	LE	2.1	**−40**	−48	−36	−27	−23	−37	−80
U16	RE	0.4	**−11**	−8	−30	−27	−3	−2	−7
	LE	0	**−3**	4	−27	−3	7	11	−24

Table 3. Deviation of optic nerve head retinal nerve fiber layer (RNFL) thickness, with corresponding visual acuity, in LHON and asymptomatic carrier relatives. Visual acuity (VA) is displayed in logMAR for right eye (RE) and left eye (LE). The RNFL thickness is given in microns, with highlight colour indicating normal thickness (green), above normal (white), borderline thinning (yellow), and thinning (red). The optic nerve segments included are temporal optic nerve (ON-T), temporal superior (TS), nasal superior (NS), nasal (N), nasal inferior (NI) and temporal inferior (TI). C6 right eye is excluded from table as OCT was not available.

In LHON-affected patients, we tested for an association of sex with visual acuity preserved to on chart in at least one eye in LHON by Fisher's exact test, and found no association (analysed per participant, $p = 0.9$). We also tested for an association of sex with presence of optic neuropathy in either eye asymptomatic carriers by Fisher's exact test, and found no association (analysed per participant, $p = 0.3$)

3.3 Optic nerve function, mitochondrial variants, heteroplasmy, and haplogroup

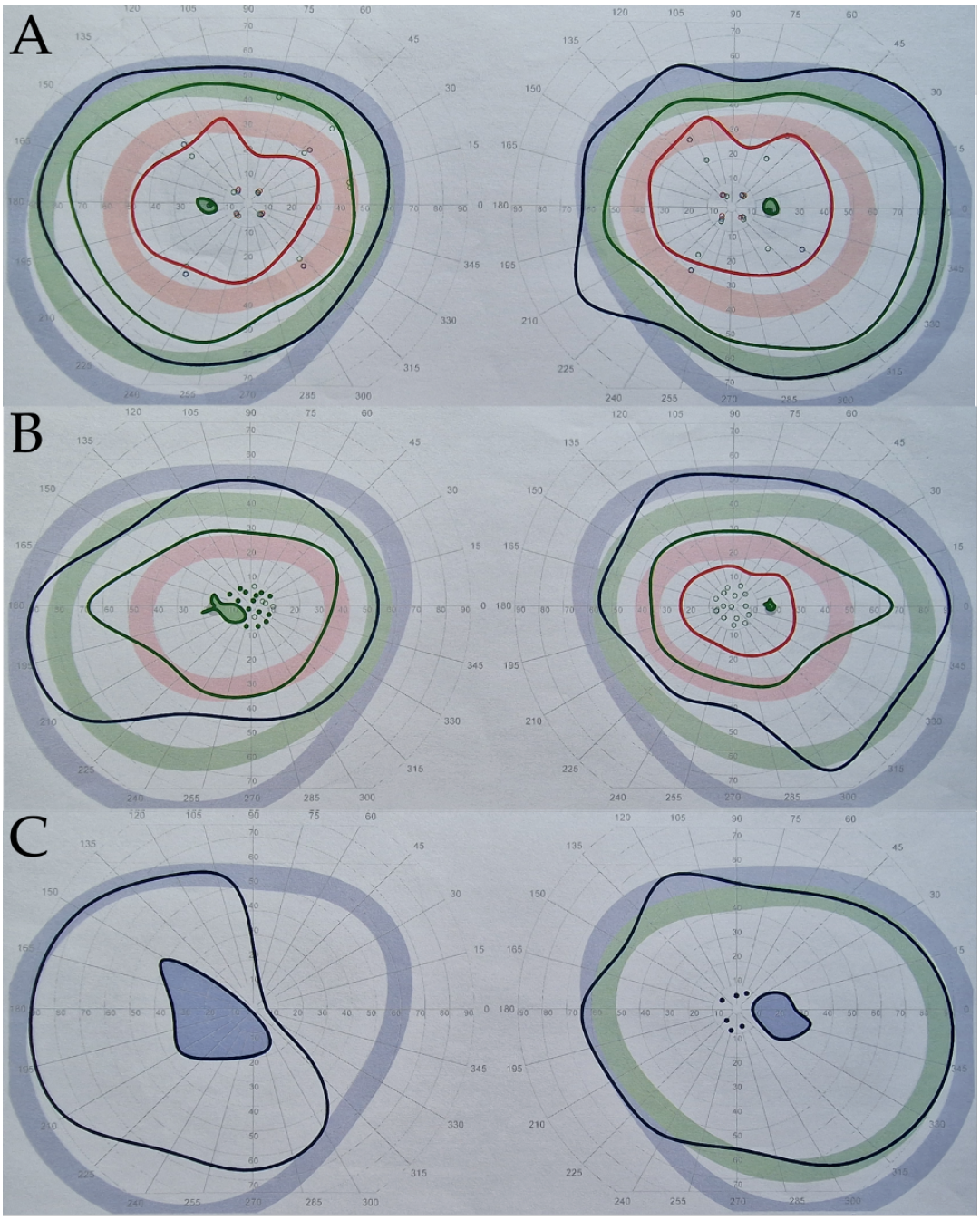
Prolonged P100 latency was present in 100% of those affected by LHON. Visual field testing varied from normal to mild scotomata (25%), through moderate (50%), to severe visual field scotomata (25%, see severity grading in methods 2.2). Carriers had normal P100 latency in 80%, with delay beyond the normal range present unilaterally in three carriers, two of whom had an abnormally high interocular difference in P100 latency greater than 10ms, and one who had prolonged P100 latency >128ms. Moderate visual field scotomata were present unilaterally in two of these carriers on the same side, and were found unilaterally in one other carrier, with remaining asymptomatic carriers having normal or mild visual field scotomata, see Figure 4. Of asymptomatic relatives who showed optic nerve RNFL thinning (n = 4), two of these eyes (50%) had abnormal P100 latency.

In the LHON group Ishihara colour vision testing (17 plates) showed a reduction in n=11 (92%) of those affected by LHON, with median Ishihara plate number in the better eye zero plates counted. There was relative preservation in one subject who had early disease (right 15/17 plates, left 16/17 plates, <3 months duration LHON). In the asymptomatic carrier group n=10 (63%) had bilateral normal colour vision, n=3 (19%) had a unilateral severe reduction (zero plates seen), and n=1 had a bilateral moderate reduction (ten plates seen). Contrast sensitivity (CS) testing in the better eye was median 0.3 in the LHON group (range 0-1.35), and median 1.5 in the beter eye of the asymptomatic carrier group (range 1.05-1.95).

Most LHON cases were homoplasmic (83%) for a known pathogenic LHON mtDNA variant, with one case heteroplasmic (C6: 11778 G>A, 80% mutant mtDNA load in peripheral blood leukocytes), and one case where the pathogenic variant was identified to be *DNAJC30* c.152A>G (C10). In asymptomatic carriers, 69% were homoplasmic, with remaining carriers showing varying heteroplasmy (10–98%), and one carrier relative where a variant was not identified (U13, mother of C10). Analysis for association of homoplasmy ≥90% with presence of optic neuropathy in asymptomatic carriers by Fisher's exact test was negative (p = 0.5). In the LHON group the most common haplogroup was HV (42%), followed by J

(33%), and among the asymptomatic carriers the most common haplogroups were HV (25%), J (25%), and H (25%), for remaining haplogroups, see Table 4.

Figure 4. Opposite page: Visual field testing results showing representative grading of visual field scotomata of a mix of asymptomatic relatives and LHON-affected subjects, with right visual field on right side and left visual field on left side of figure, I2e target in red, I4e target in green, V4e target in blue. Normal visual field bilaterally in E1 with substantially preserved I2e (A), mildly reduced vision field in right eye with central reduced sensitivity of I2e isopter, with moderate visual field scotomata in left eye in U15 with loss of I2e target (B), and bilateral severely reduced visual field with only V4e target seen in C8 (C).


A
B
C

Cohort	ID	Eye	VA	P100 Latency (Deviation)	Ishihara Colour vision	Contrast sensitivity	Visual Field Loss	Amblyopia	Mitochochondrial Mutation, Heteroplasmy			Mt Haplogroup	Pedigree
									3460 G>A	11778 G>A	14484 T>C		
LHON	C1	RE	1.8	NR	0	0	3	-		100		H	2
		LE	1.5	NR	1	0.75	3						
	C2	RE	2.4	NR	0	0	3	-		100		J	3
		LE	2.1	NR	1	0.3	3						
	C3	RE	0.9	137 (25)	1	1.05	2	-		100		J	4
		LE	0.8	149 (37)	1	1.05	2						
	C4	RE	2.7	NR	0	0	4	-		100		HV	5
		LE	2.4	NR	0	0	4						
	C5	RE	2.7	193 (81)	0	NA	4	-		100		HV	5
		LE	2.4	NR	0	NA	4						
	C6	RE	1	NR	1	0.45	3	-		80		J	6
		LE	1	NR	1	0.6	3						
	C7	RE	1.8	NR	0	0	3	-		100		HV	7
		LE	1.8	NR	0	0	3						
	E1	RE	1	NR	15	1.2	1	+		100		U	8
		LE	1	NR	16	1.35	1						
	C8	RE	2.1	NR	0	0	4	-		100		HV	7
		LE	2.1	NR	0	0	4						
	C9	RE	1.6	NR	0	0	3	-	100			I	10
		LE	1.9	NR	0	0	3						
	C10	RE	1.4	NR	0	0.15	2	-				HV	11
		LE	1.3	NR	0	0.3	2						
	E2	RE	1.5	NR	0	0.6	3	-			100	J	13
		LE	1.5	NR	7	0.6	2						
Unaffected	U1	RE	0	118 (6)	16	1.5	2	+		65		U	1
		LE	0.2	120 (8)	17	1.35	2						
	U2	RE	0	126 (14)	10	1.05	2	-		98		U	1
		LE	0.2	130 (18)	10	1.05	2						
	U3	RE	0.1	112 (0)	17	1.35	2	-		100		H	2
		LE	0.2	111 (−1)	16.5	1.35	2						
	U4	RE	0.1	107 (−5)	17	1.5	2	-		100		J	3
		LE	0.2	107 (−5)	17	1.95	1						
	U5	RE	0	NA	17	1.5	2	-		100		HV	4
		LE	0	NA	17	1.5	1						
	U6	RE	0.1	114 (2)	16	NA	1	-		100		J	5
		LE	0.1	117 (5)	16	NA	1						
Contd.	U7	RE	0.1	110 (−2)	16	1.65	1	-		88		J	6

	LE	0	108 (−4)	16	1.5	1					
U8	RE	0.1	94 (−18)	17	1.5	3	+		100	HV	7
	LE	2.4	121 (9)	0	0	1					
U9	RE	0	107 (−5)	17	1.5	1	-		100	U	8
	LE	0	114 (2)	17	1.5	1					
U10	RE	0	102 (−10)	NA	1.5	1	-		100	HV	7
	LE	0	106 (−6)	NA	1.5	1					
U11	RE	0	100 (−12)	16	1.35	1	-		100	H	9
	LE	0	106 (−6)	16	1.5	1					
U12	RE	0	98 (−14)	17	1.35	1	-	10		I	10
	LE	0.5	97 (−15)	17	1.35	1					
U13	RE	0	103 (−9)	17	1.35	1	-			HV	11
	LE	0	106 (−6)	17	1.35	11					
U14	RE	1.8	112 (0)	0	0	3	+		100	H	12
	LE	0	120 (+8)	17	1.2	2					
U15	RE	0	106 (−6)	17	1.5	2	+		100	H	12
	LE	2.1	125 (13)	0	0.45	3					
U16	RE	0.4	117 (5)	17	1.35	2	+		100	J	13
	LE	0	111 (−1)	17	1.35	2					

Table 4. Optic nerve function including logMAR visual acuity, P100 latency, colour vision (Ishihara 17 plate testing score), contrast acuity testing (measured as logCS Thomson test chart 2016) and visual field loss, and mitochondrial mutation, heteroplasmy and mitochondrial (mt) haplogroup, and pedigree, in LHON and asymptomatic maternal carrier relatives is shown. The P100 latency is given in milliseconds (ms), with deviation from mean normal value (112ms) to above 128ms, or non-recordable (NR), or interocular difference greater than 10 ms highlighted in red as delayed, or if normal is highlighted in green. Amblyopia is reported as present (+) or absent (-).

In subjects with a confirmed LHON-causing variant, and electrophysiology data, mitochondrial haplogroup H or HV versus non-H was assessed for association with vision loss; in LHON-affected patients none of these were significantly associated with vision status being 'on-chart' (i.e., logMAR ≤ 1.0) versus 'off-chart,' on Fisher's exact test (p = 0.2), however haplogroup H or HV was significantly associated with visual dysfunction in asymptomatic carriers, on Fisher's exact test (p = 0.05).

3.4 Idebenone treatment outcomes

At the time of data cut-off (January 2022), all (n = 12) LHON-affected patients had received at least one dose (900mg/day) of Idebenone and were included in the safety population (SP). Of this group, upon direct questioning to report whether vision was stable, improved, or reduced since starting idebenone treatment, 3 patients (25%) felt subjective improvement in vision while on treatment, 6 patients (50%) carried one of the 3 major LHON mtDNA mutations, had onset of symptoms within the 12 months prior to starting treatment, had baseline VA data, and had at least one follow-up visit with VA data; this was the efficacy population (EP).

In the EP of 6 patients, VA was maintained at better than logMAR 1.0 in at least one eye in 2 patients (33%) while median visual acuity in the better eye was 1.2 logMAR. Of the EP group, 1 patient (C3) had a clinically relevant recovery from nadir to latest visit (17%), who had presented with visual acuity of 1.3 logMAR in both eyes and improved to 0.9 and 0.8 logMAR in the right and left eyes, respectively, and 1 patient (C8) felt a subjective improvement in vision while on treatment. The cumulative exposure to Idebenone in the SP was 214 patient months. No adverse event was reported.

3.5 Optic nerve head perfusion in LHON

Of the cohort of 12 LHON-affected patients and 16 asymptomatic LHON relatives, OCTA image capture was not possible in eight patients and five relatives due to fixation difficulties, but was measured in four patients (four males, three with chronic LHON >12 months duration, and one with early LHON <3 months duration, median age 21, interquartile range (IQR) 17-31 years) and eleven asymptomatic relatives (ten females, median age 56, IQR 45-63 years) respectively, and was also performed in ten
controls with normal eyes (seven females, median age 48, IQR 37-56, see Figure 5). Overall, the LHON-affected were significantly younger than the asymptomatic relatives (p=0.047), and the controls were not significantly different in age from either the LHON-affected or the asymptomatic relatives (p=0.08 and p=0.2 respectively).

In the control group (20 eyes), median retinal nerve fiber layer peripapillary VD was 10.9% (IQR 10.2-12.6%), with a normal 95% confidence interval of 6.7-16.7%. Peripapillary VD was significantly lower in the LHON-affected group (n=6 eyes), median 7.9% (each successfully measured eye was included, interquartile range (IQR) 7.1-10.6%, p= 0.046). In asymptomatic LHON maternal relatives (16 eyes), all of whom carried pathogenic LHON-associated variants with varying levels of heteroplasmy, the median VD was not significantly different to that of controls, at 13.7% (IQR 10-14.7%, p=0.2), see Table 6. Relative to the normal range derived from the controls, one eye of the LHON-affected group had reduced VD at 4.1%, and of the asymptomatic LHON relatives, three eyes (19%) of three subjects had unilateral reduced VD at 6%. Raised VD was found unilaterally in two asymptomatic relatives, at 17%, one of whom also had reduced VD in the contralateral eye.

Peripapillary retinal nerve fiber layer thickness was successfully measured more than perfusion was in study subjects, see Table 5, at median 101 microns (IQR 95-106 microns) in the control group (n=20 eyes), with a normal 95% confidence interval of 86-115 microns. RNFL thickness was significantly lower in the LHON-affected group (n=16 eyes), median 51 microns (IQR 37 -77 microns, p= 0.003), compared to normal controls. In asymptomatic carrier relatives (n= 19 eyes) median RNFL thickness was also significantly reduced versus controls at 90 microns (IQR 84-98 microns, p=0.01). In the asymptomatic maternal relatives

RNFL thinning below the derived normal range was found in 7 of 19 eyes (37%) and the difference in peripapillary RNFL versus controls was statistically significant (p=0.01). There were no subjects in the asymptomatic or LHON affected group who had raised RNFL thickness above the derived normal range.

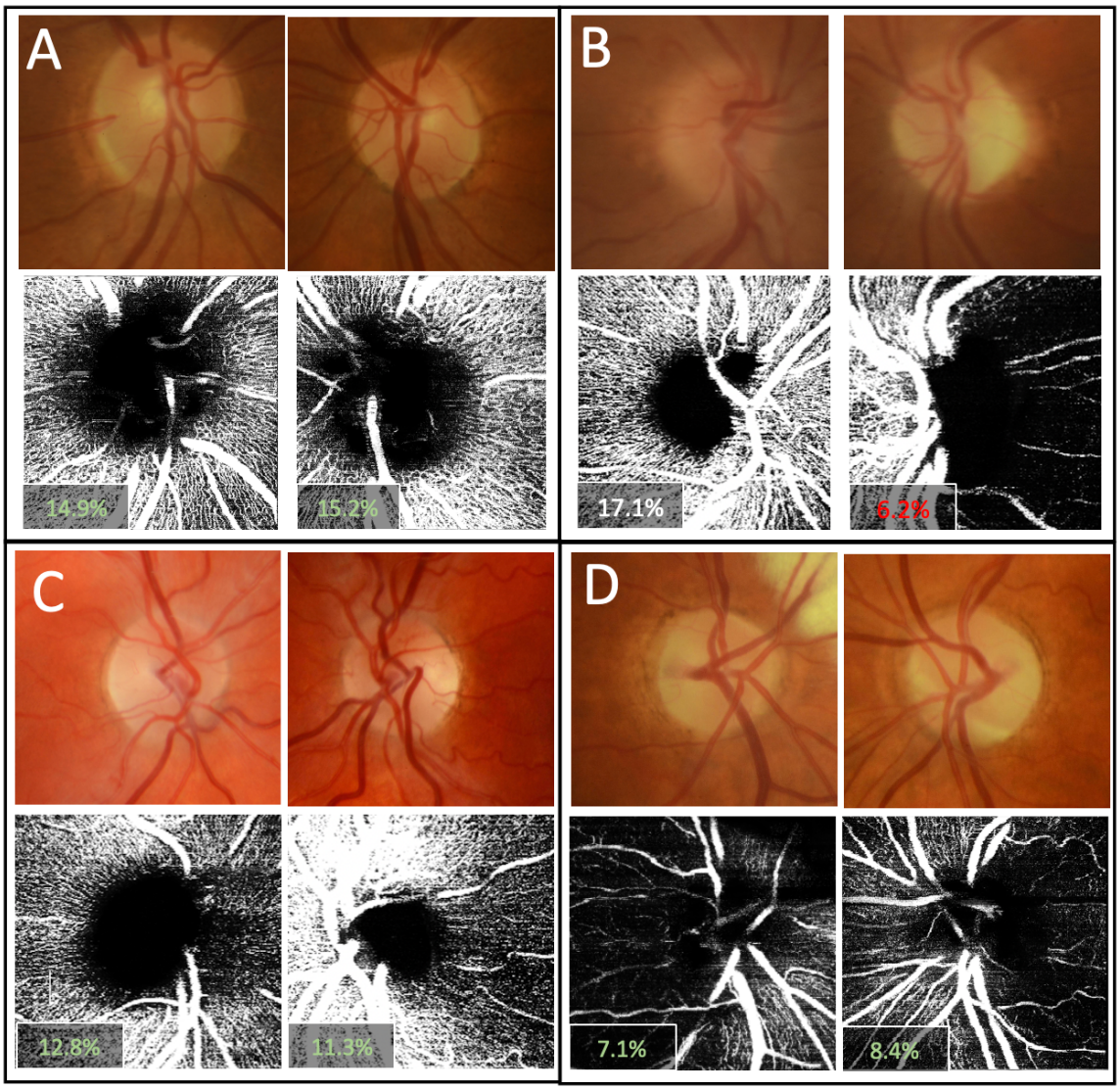


Figure 5 (i)

Figure 5 (pages 26-28) (i): Optic nerve head photo, peripapillary nerve fiber layer perfusion (page 26), **(ii), (iii)** corresponding nerve fiber layer thickness (RNFL pages 27-28) in Leber Hereditary Optic Neuropathy (LHON). The black and white perfusion images have the vessel density values displayed, green indicating normal, white indicating raised, and red indicating reduced. A, U9 asymptomatic LHON carrier relative with normal visual acuity (VA) normal perfusion on Optical Coherence Tomography Angiography (OCTA), and RNFL thickness. B, U8 asymptomatic LHON carrier relative with unilateral increased perfusion in the right eye (vessel density (VD) RNFL 17.1%) with normal VA and significantly shortened P100 latency, and reduced perfusion in left eye (6.2%), with corresponding low RNFL thickness (70 microns), with poor vision logMAR VA 2.4 and delayed P1OO latency. C, E2 LHON affected patient of duration <3 months with normal RNFL perfusion, and reduced vision in both eyes (logMAR 1.5), and thinning of RNFL left eye. D, C6 Chronic LHON affected patient with RNFL thinning, normal perfusion, and reduced logMAR VA 1.0 both eyes, and myelinated nerve fiber layer adjacent to left optic nerve head.

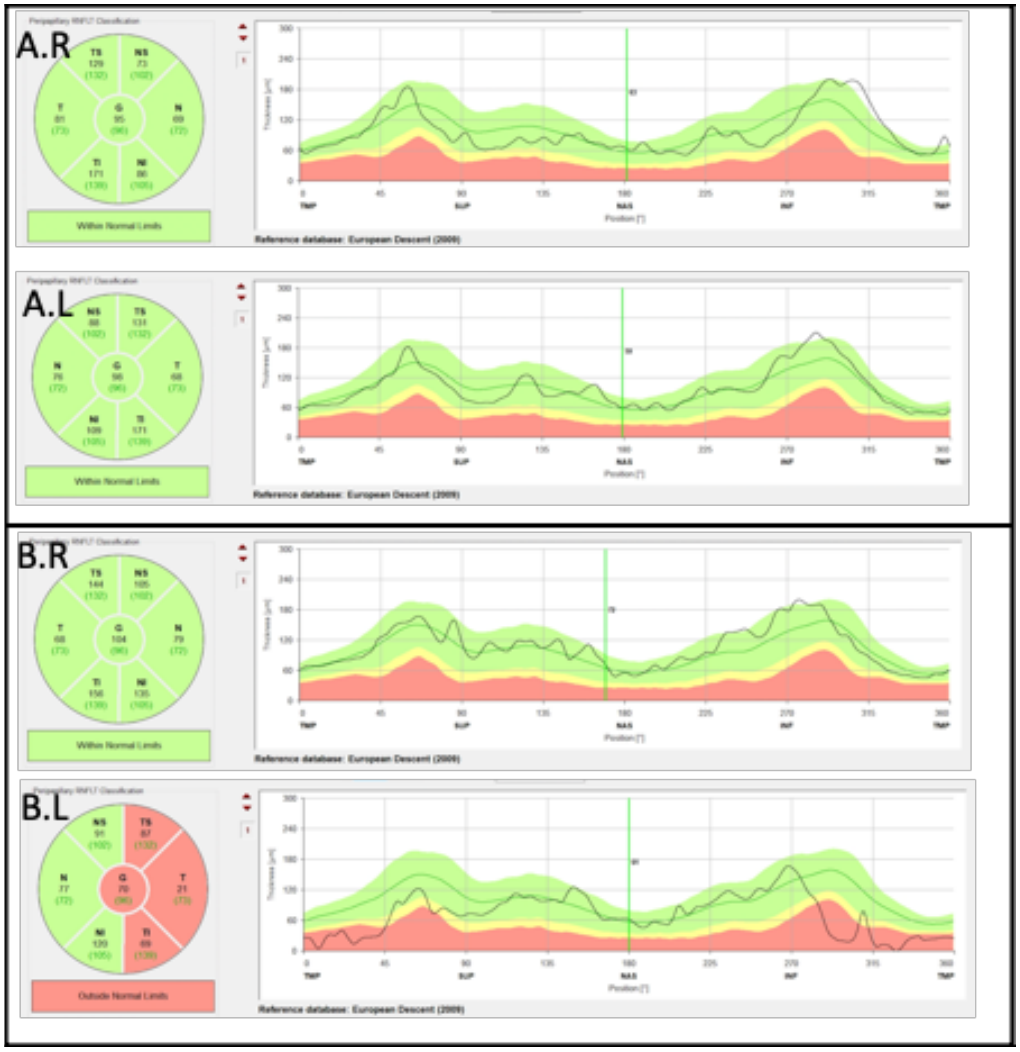

A.R
Within Normal Limits
Reference database: European Descent (2009)
A.L
Within Normal Limits
Reference database: European Descent (2009)
B.R
Within Normal Limits
Reference database: European Descent (2009)
B.L
Outside Normal Limits
Reference database: European Descent (2009)

Figure 5 (ii)

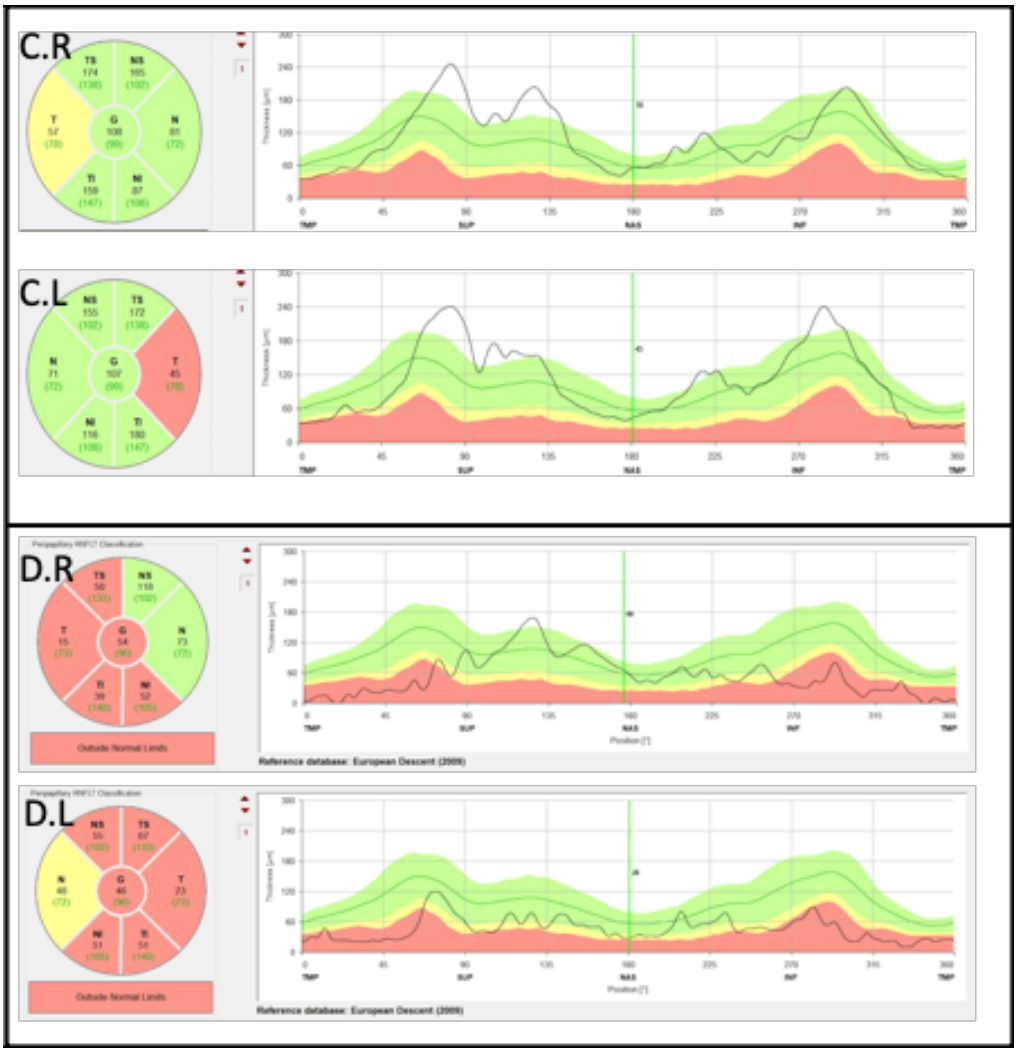

C.R
C.L
D.R
D.L
Outside Normal Limits
Reference database: European Descent (2009)

Figure 5 (iii)

	LHON-affected	Asymptomatic LHON relatives	Controls
Age	21 (17- 31) (n=4)	56 (45-63) (n=11)	48 (37-56) (n=10)
Sex	4 M/ 0 F	1 M / 10 F	3 M/ 7 F
Peripapillary vessel density	7.9% (7.1-10.6%) n=6 eyes, p= 0.046	13.7% (10.0-14.7%) n=16 eyes, p =0.166	10.9%(10.2%-12.6%) n=20 eyes
Peripapillary RNFL thickness	51 µm (37 -77 µm) p= 0.003 n=16 eyes	90 µm (84-98 µm) p=0.01 n=19 eyes	101 µm (95-106 µm) n=20 eyes

Table 5: Demographic characteristics, peripapillary vessel density (VD) and retinal nerve fiber layer (RNFL) thickness in LHON-affected (7.9%), asymptomatic LHON relatives (13.7%), and controls (10.9%). LHON Leber hereditary optic neuropathy, M male F female, IQR interquartile range, p value for comparison to control group. Age is displayed in years, peripapillary vessel density in %, and retinal nerve fiber layer thickness (RNFL) in microns, as median (interquartile range)

In those eyes with reduced peripapillary VD, visual acuity was variable, see Table 6. In the LHON-affected individual (C9) and one of the asymptomatic carriers (U8), VA was reduced to 1.6 logMAR and 2.4 logMAR respectively. The contralateral eye of U8 had increased peripapillary VD, which was associated with a short P100 latency of 94ms and normal VA. In the other asymptomatic carriers with reduced peripapillary VD, VA was normal or slightly reduced at 0.0 to 0.2 logMAR. In one asymptomatic carrier (U2) with normal VA but reduced VD, VEP was prolonged in the contralateral eye to 130ms. In one other asymptomatic relative peripapillary VD was unilaterally increased and VA and VEP were normal in both eyes.

	ID	Age	Mitochondrial pathogenic variant, % heteroplasmy	Eye	Peripapillary		Best corrected visual acuity (logMAR)	Pattern visually evoked response: P100 latency (deviation)*
					vessel density	retinal nerve fiber layer thickness (microns)		
LHON chronic	C6	50	11778 G>A, 80%	RE	7.1%	54	1	NR
	C6			LE	8.4%	46	1	NR
	C9	25	3460 G>A, 100%	RE	4.1%*	37*	1.6	NR
	C10	15	*DNAJC30*	RE	7.4%	67*	1.4	NR
LHON early	E2	17	14484 T>C, 100%	RE	12.8%	108	1.5	NR
	E2			LE	11.3%	57*	1.5	NR
Asymptomatic relatives	U1	60	11778 G>A, 65%	RE	17.1%*	98	0	118 (6)
	U1			LE	13.8%	96	0.2	120 (8)
	U2	36	11778 G>A, 98%	RE	6.0%*	58*	0	126 (14)
	U2			LE	7.4%	55*	0.2	130 (18)
	U3	72	11778 G>A, 100%	RE	13.7%	88	0.1	112 (0)
	U3			LE	10.8%	83*	0.2	111 (-1)
	U4	67	11778 G>A, 100%	RE	14.1%	107	0.1	107 (-5)
	U8	56	11778 G>A, 100%	RE	17.1%*	104	0.1	94 (-18)
	U8			LE	6.2%*	70*	2.4	121 (9)
	U9	55	11778 G>A, 100%	RE	14.9%	95	0	107 (-5)
	U9			LE	15.3%	98	0	114 (2)
	U11	37	11778 G>A, 100%	RE	14.0%	88	0	100 (-12)
	U11			LE	14.5%	87	0	106 (-6)
	U12	56	3460 G>A, 10%	RE	15.9%	113	0	98 (-14)
	U12			LE	13.2%	110	0.5	97 (-15)
	U14	36	11778 G>A, 100%	LE	6.1%*	66*	0	120 (+8)
	U15	65	11778 G>A, 100%	RE	9.3%	90	0	106 (-6)
	U16	52	14484 T>C, 100%	RE	13.0%	85*	0.4	117 (5)
	U16			LE	13.6%	93	0	111 (-1)

Table 6: Optic nerve function including logMAR visual acuity (VA) and P100 latency, peripapillary retinal nerve fiber layer (RNFL) vessel density (VD) and thickness and Leber hereditary optic neuropathy (LHON) status. LHON, C chronic >1 year duration LHON, E early <6 months duration LHON, U unaffected relative, RE right eye, LE left eye, NA non applicable, P100 latency is given in milliseconds (ms), with deviation from the mean normal reference value (112ms) shown, or result displayed as nonrecordable (NR), * denotes an abnormal value of vessel density (normal range 6.7-16.7%), or abnormal retinal nerve fiber layer value (normal range 86-115 microns).

3.6 Cardiac conduction in LHON

ECG abnormality	LHON-affected (n=12)	Asymptomatic relatives (n=16)
Short PR interval	3 (25%)	4 (25%)
ST changes	3 (25%)	3 (19%)
Q waves	1 (8%)	1 (6%)
Right axis deviation	0	1 (6%)

Table 7: Electrocardiogram findings in carriers of LHON-associated mitochondrial variants. The PR interval was defined as abnormally short if <120ms. ST changes, Q waves, and right axis deviation were detected by analysis of the ECG tracing. Values given as n (%).

We found that 58% of those affected by LHON, and 56% of their asymptomatic maternal relatives demonstrated electrocardiographic evidence of cardiac conduction abnormalities, see Table 7.

3.7 Quality of life in LHON

We assessed vision-related quality of life of LHON-affected and of carrier relatives by two questionnaires; the larger questionnaire was the general Visual Function Questionnaire-25 (appendix III), see results in Table 8. The LHON-affected were found to have significantly lower quality of life scores in general vision, near activities, distance activities, vision-specific social functioning, mental health, role difficulties, dependency, driving, colour vision, and peripheral vision. Within the second questionnaire, the neuro-supplement (Table 9), the LHON-affected were found to have lower quality of life in having difficulty in bright sunlight, parking a car, using a computer, having 'fuzzy' vision, and focusing on moving objects. There was no difference detected between the groups in general health-related quality of life, or in ocular pain, within the vision questionnaire. Within the neuro-supplement there was no difference between LHON affected and asymptomatic relatives in difficulty seeing when eyes are tired, or both eyes seeing differently, or eyelids of unusual appearance, double vision or ptosis.

	Asymptomatic relative (n=16) Median (IQR)	LHON (n=12) Median (IQR)	P value
Composite score*	91 (89-93)	51 (37-57)	<0.0001†
General Health	75 (75-100)	87.5 (75-100)	0.7
General Vision	80 (80-80)	20 (20-55)	0.0002 †
Ocular Pain	100 (100-100)	100 (75-100)	0.5
Near Activities	100 (91-100)	25 (17-42)	0.001 †
Distance Activities	100 (90-100)	29 (25-48)	0.0008 †
Vision specific:			
Social functioning	100 (100-100)	31 (25-47)	0.0003 †
Mental Health	100 (88-100)	38 (14-78)	0.003 †
Role difficulties	100 (100-100)	25 (13-63)	0.003 †
Dependency	100 (100-100)	50 (42-92)	0.005
Driving	100 (92-100)	0 (0-10)	0.002 †
Colour vision	100 (100-100)	50 (25-75)	0.0001 †
Peripheral vision	100 (100-100)	50 (31-50)	0.001 †

Table 8: Quality of life in LHON families: result of Visual Function Questionnaire 25 (VFQ-25), including analysis of LHON patients and asymptomatic first degree maternal relatives.
*composite score is average of all items except for item 1 (items 2-25)
†significantly significant ($p < 0.05$)

Neuro supplement questionnaire	Asymptomatic relative (n=16)	LHON (n=12)	P value
Difficulty when eyes tired	75 (75-100)	63 (31-75)	0.08
Difficulty in bright sunlight	100 (75-100)	25 (6-67)	0.006*
Difficulty parking car	100 (100-100)	0 (0-15)	0.002*
Difficulty using computer	100(100-100)	50 (50-88)	0.004*
Two eyes see differently	100 (0-100)	0 (0-50)	0.2
Eyelid appearance unusual	100 (100-100)	100 (50-100)	0.1
Vision fuzzy	100 (75-100)	0 (0-25)	0.005*
Trouble focusing on moving objects	100 (75-100)	25 (0-100)	0.03*
Double vision	100 (100-100)	100 (100-100)	0.4
Ptosis	100 (100-100)	100 (100-100)	0.3

Table 9: Quality of life in LHON families: result of Neuro-Ophthalmic Supplement, including analysis of LHON patients and asymptomatic first degree maternal relatives. Where responses to multiple questions feed into the score of one category of vision related quality of life the median p value for the Wilcoxon rank sum test for each response, by LHON affected or unaffected status, is given. Median score value (interquartile range) is displayed. *significantly significant ($p<0.05$)

Chapter 4 Discussion

4.1 Discussion overview

We investigated a cohort of patients affected by LHON, and also asymptomatic maternal relatives, for potential associations of expression of disease and severity of visual loss. We found that visual acuity was reduced to median 1.6 logMAR in the better-sighted eye in the LHON group. Optic neuropathy was present in a minority of asymptomatic carrier relatives, at a rate of 25% showing signs of optic neuropathy in at least one eye, with thinning of the RNFL of the optic nerve head, and in a minority of cases, reduced optic nerve head perfusion, that was associated with reduced visual function; either reduced visual acuity or delayed visually evoked response or both. This presence of optic neuropathy in asymptomatic carrier relatives was found to be associated with membership of the mitochondrial haplogroups H or HV, versus other mitochondrial haplogroups, upon evaluation of mitochondrial DNA sequence. Normal RNFL thickness correlated more closely, compared to normal RNFL perfusion, with absence of optic neuropathy in asymptomatic carrier relatives. Idebenone showed a good safety profile, with no adverse event reported over a treatment duration of 214 patient months. However, Idebenone showed limited efficacy, with 33% of those who received treatment within a year of onset of vision loss achieving clinically relevant stabilisation, with 'on chart' vision, and 17% achieving a clinical relevant recovery of vision, whereas subjective improvement of vision was reported by 25% overall with treatment. Cardiac conduction abnormalities were identified in 58% of LHON patients, and 56% of their asymptomatic relatives, though the clinical relevance of the ECG findings is difficult to interpret in the absence of echocardiogram findings.

4.2 Mitochondrial haplogroup

Mitohcondrial haplogroup has been identified as a modifier of disease expression across a range of disorders. In cardiovascular disease presence of the JT haplogroup is protective. [84] In neurological disease, the IWX haplogroup is associated with frontotemporal dementia.[85] Within ophthalmic disorders Haplogroup J has been identified as a potential risk factor for optic neuritis occurrence in multiple sclerosis,[86] and haplogroup U has been identified as

protective against primary open angle glaucoma.[87] Investigation of haplogroup membership in LHON has been previously reported in a pan-European cohort, in which membership of the H mitochondrial haplogroup was associated with relative preservation of vision.[29] Another study proposed that reduced penetrance of LHON is seen in 14484T>C in the H haplogroup, though this report included only one family.[30] Vision loss and optic nerve involvement in MS in Iran has been described in association with the H haplogroup. [88] Increased penetrance of vision loss of LHON in 11778G>A in M7b and B5 haplogroup has been suggested in Chinese studies, whereas Haplogroup F was protective.[89, 90] Penetrance of vision loss and LHON expression in 11778G>A has also been described with the J haplogroup in Italy[28] and the K haplogroup in a study in Poland.[91] In these different populations different patterns of penetrance associated with mitochondrial haplogroups are observed. The mitochondrial haplogroup association for groups H and HV that we identified is not with disease expression per se, but rather with asymptomatic signs of optic neuropathy in LHON associated mitochondrial variant carriers, that had not been assessed or identified in the other haplogroup studies. In effect, these asymptomatic individuals have relative preservation of vision compared to those with manifest LHON. At a population level, haplogroup varies. In Ireland the predominant mtDNA haplogroup in Ireland is H (40%) with significant proportions of U (12%), T (10%), J (10%) and K (10%).[92] It is likely that at a population level mitochondrial haplogroup effects may exist but that there are other important factors in LHON expression, potentially including genetic, epigenetic, and environmental exposures. Further evaluation of disease expression and severity per haplogroup in other families would be useful.

4.3 Asymptomatic relatives in LHON families

Different possibilities may explain the presence of optic neuropathy in asymptomatic carrier relatives. Most of these subjects, 75%, who were found to have optic neuropathy with reduced vision also reported ambylopia. Amblyopia can cause prolonged P-100 latency,[93] but it does not cause structural changes of the optic nerve head and does not account for these findings on OCT scanning.[94] Moreover, the prevalence of reported amblyopia in these carriers was high at 31%, compared with reported prevalence of up to 6% in international

studies,[95-98] indicating that there may be another underlying issue leading to subjective reporting of a history of amblyopia.

It is possible that the carrier's optic neuropathy is static due to impaired but not absent mitochondrial respiratory chain function causing subclinical retinal ganglion cell impairment, which has not reached the critical ATP-deprivation stage causing bilateral vision loss. However we identified three asymptomatic relatives with unilateral reduced vision worse than counting fingers. These subjects may represent an atypical LHON at a stage of asymmetric disease where one eye is involved, and has progressed beyond the early stage of subtle optic nerve head swelling to develop established unilateral RNFL thinning, but that has not yet progressed to involve the second eye. A case report has previously described a 34-year interval between losing vision first in one eye and then the other, though 97% of 2nd eye involvement in LHON occurs within 1 year.[19, 99]

Asymptomatic carrier relatives who show signs of LHON pose a diagnostic and management dilemma for clinicians. They may go on to manifest symptomatic optic neuropathy (i.e., pre-symptomatic group), or they may never progress to symptomatic 'affected' status. Distinguishing those who will progress from those who will not is not possible, thus population screening for LHON-causing mtDNA variants is neither actionable nor practical. LHON, though typically starting in early adulthood, may occur later, and onset has been described in the seventh decade of life, though 95% occur at < 50 years.[19, 100] Should relatives who show optic nerve abnormalities be treated with Idebenone, with preventative or curative intent? This is unclear. Idebenone or gene therapy are the treatments for LHON, aside from avoiding optic nerve toxins including smoking and alcohol. A clinically relevant recovery of vision with Idebenone treatment has been observed in 46–53%[20, 101] of patients treated in two different trials; however real world data often shows lower efficacy,[102] and treatment does not prevent vision loss in the second eye.[103] Our approach is to advise these asymptomatic carriers to avoid excess smoking and alcohol. Eventually if Idebenone becomes more affordable it could develop as a preventive treatment for those at risk of vision loss, who carry pathogenic mitochondrial variants. However at a present cost of €77,796 annually for treatment in Ireland, it is currently prohibitively expensive to consider such an initiative.[104]

4.4 Pathogenic mitochondrial variants

In this cohort 75% of LHON-affected individuals carried the pathogenic mitochondrial variant 11778 G>A, with other mitochondrial variants 14484T>C and 3460G>A each seen in two other individuals. We identified the causative mutation of LHON to be *DNAJC30* c.152A>G in one affected individual from Ukraine (C10). This variant has been identified in cases of autosomal recessive LHON.[5, 105, 106] The DNAJC30 protein is known to associate with respiratory chain complex I.[107] Identification of this causative variant, versus the more common mitochondrial variants, impacts on genetic counselling for the affected family. Since 24 February 2022, when the Russian invasion began, millions of refugees from the war in Ukraine are seeking safety in different countries.[108] Ophthalmologists internationally should be aware that this rare genetic mutation is described to cause autosomoal recessive LHON in this population.

4.5 Thinning RNFL in the optic nerve head in LHON families

Analysis of the peripapillary RNFL in LHON-affected and carrier relatives was carried out utilizing both the Heidelberg European Descent, in addition to comparison with an in-house control sample.

The Heidelberg European Descent OCT analysis has been described recently in analysis of optic nerve head changes in compressive versus glaucomatous optic neuropathy.[109] The patterns of optic nerve changes we observed included differences in RNFL thickness associated with LHON chronicity, with preserved or thickened nerve fiber layer present in those within the first few months of their LHON onset, versus thinned RNFL in more long-standing disease. In LHON we found that the optic nerve head nasal sector had a relatively preserved RNFL overall. This may be because the temporal optic nerve head tends to be affected first.[18, 110] Other reports have described relative preservation of nasal RNFL thickness in the optic nerve head in established LHON, with heterogeneity in the progression of temporal changes of RNFL.[111, 112] Analysis of RNFL thickness in carriers yielded more information. Thinning of the peripapillary nerve fiber layer was present either unilaterally or bilaterally in a significant proportion of asymptomatic carriers, 25% (n =

4/16). There were no subjects in the asymptomatic or LHON affected group who had raised RNFL thickness above the derived normal range.

Normal nerve fiber layer thickness compared to the control sample, with a lack of atrophy, showed good specificity for signs of normal optic nerve function (92% specificity for nerve thickness within the derived normal 95% CI). For most of these carriers, optic neuropathy in these eyes was demonstrated on visual acuity testing. However, for one of these carriers, visual acuity testing was normal bilaterally, with optic nerve dysfunction detectable only upon visually evoked potential testing, which uncovered P-100 delay. This reveals the utility of visually evoked response testing yielding an abnormal finding in an eye with normal central visual acuity testing despite structural signs of optic nerve disease on OCT. We did not find any pathological increase in thickness of the temporal optic nerve head RNFL, as has been reported in Italy, in a large sample of asymptomatic carriers.[49] Temporal nerve head thickening may be detected in early disease as mismatch between metabolic supply and demand leads to localized oedema. Not finding temporal RNFL thickening in carriers in our study may be due to our smaller sample size.

These results show that as a biomarker, monitoring of nerve fiber layer thickness may provide utility in screening those at risk of visual loss who carry LHON-associated mitochondrial variants, as it is relatively quick and easy to perform in the eye clinic. Detection of RNFL thinning in LHON may find utility in observation of asymptomatic carrier relatives who could be at risk of vision loss. Those eligible for monitoring may be as many as one in ten thousand based on population studies of LHON prevalence,[113] or as high as one in 300 based on prevalence of LHON-associated mitochondrial variants.[114] While historically there was no treatment available for LHON, monitoring and screening is of more relevance now as more treatment options become available. Idebenone is approved and has demonstrated efficacy in LHON in the RHODOS clinical trial,[21] and novel potential treatments are under development for LHON; FDA clearance of the in-vivo gene replacement therapy NFS-02 (rAAV2-*ND1*) as an investigational new drug took place in 2022.[115] Longitudinal studies are required to evaluate whether microcirculatory abnormalities may be static or progress over time.

4.6 Altered optic nerve head retinal nerve fiber layer perfusion in LHON families

In this cohort, OCTA measurement was not possible in the majority of LHON-affected patients due to fixation difficulty associated with poor vision. Of the patients who could fixate for scanning (33%), we found that the LHON-affected individuals had reduced peripapillary VD compared to controls. A greater proportion of the asymptomatic relatives were able to fixate for OCTA measurement (69%), and overall the peripapillary VD was not significantly different to that of controls. Of the asymptomatic relatives 17% were found to have reduced VD, and increased VD was observed in 10%. Abnormal VD had a variable association with visual function, and was present with a visual acuity level varying from normal visual acuity through to blindness, and also occurred in a subject with normal vision but subclinical signs of optic neuropathy including prolongation of VEP. Overall, normal nerve fiber layer thickness, with a lack of atrophy, showed greater specificity for signs of normal optic nerve function (92%), compared to a measurement of normal perfusion (66%) for normal nerve function.

A problem with utilizing OCTA measurement in LHON is obtaining measurements when visual acuity is poor and fixation is difficult, as a scan requires steady fixation for several seconds. Notwithstanding this difficulty, OCTA findings in the peripapillary circulation have been described in LHON, demonstrating variable associations including negative findings of the asymptomatic stage of LHON,[54] and reduced vessel density in established LHON.[51] Furthermore, cohorts of LHON families have been studied, demonstrating reduced vessel density in asymptomatic individuals,[54] or increased vessel density affecting the temporal optic nerve head, after correcting for retinal nerve fiber layer thickness, in asymptomatic male carrier relatives.[55]

Peripapillary RNFL vessel density was lower in LHON than in normal eyes in our study. We suspect that the reduced perfusion is secondary to atrophic changes of the optic nerve head and secondary loss of the RNFL vascular bed, resulting in ischaemia. This ischemia may be a recovery-limiting factor when considering therapies that promote mitochondrial respiratory chain function (i.e., idebenone) and reduced OCTA VD may suggest limited benefit from such treatments. Indeed each eye that showed reduced RNFL perfusion in the

LHON and asymptomatic relative groups was also found to have RNFL thinning relative to controls. In this study 75% of the LHON group could not fixate adequately for scanning and were therefore excluded. We suspect these unmeasured individuals have low peripapillary VD also based on the clinical characteristics of ONH pallor and peripapillary RNFL thinning.[116] Our control group was older than our LHON group (median age 48 versus 21 years), which may lead to the LHON group having undetected cases of lower again VD, as with advancing age there is a reduction in peripapillary VD.[117] There were sex differences in the comparison groups, with only males included in the measured LHON group. Overall VD parameters measured by OCTA do not vary by sex,[117] but LHON shows some sex-dependent penetrance with more vision loss seen in males.[118]

Asymmetric changes in VD were observed in some asymptomatic LHON relatives. One case had discordant VD changes across both optic nerve heads. This was associated with optic nerve dysfunction and poor vision of logMAR 2.4 in one eye, which had low VD, and normal vision with an abnormally short visually evoked potential of 94ms in the other eye, which had raised VD. The implications of this are not clear. Onset of LHON is usually sequential, with one eye affected within 3 months of the fellow eye, though cases of >1 decade have been reported between eyes losing vision.[99] A shortened VEP implicit time implies increased conductivity of retinal ganglion cells. As far as the authors are aware, this phenomenon is not described elsewhere and may represent compensatory increased mitochondrial biomass as mitochondrial dysfunction develops, prior to visual loss.

The measurement of peripapillary RNFL perfusion did not equate to that of RNFL thickness and each provide different data, and correlate differently with optic nerve dysfunction (either low visual acuity or delay on visually evoked potential). In this cohort lower perfusion did not give any additional insights to the aetiology of thinned RNFL. RNFL thinning may occur at an earlier stage of progressing neuropathy than vessel density changes, which may follow later when atrophy is established.

4.7 Contrast sensitivity and colour vision

Contrast sensitivity (CS) is distinct from visual acuity,[119] referring to the ability to visualize an object against a background, distinct from visual acuity, which refers to the ability to resolve small detail.[120]Despite its practical importance in mobility[121] and perceived ability in performing tasks,[122] CS is not routinely measured in eye clinics, and reference values are not well known. In LHON there is very little data on CS; a PubMed search of "contrast sensitivity" AND "LHON" gave 11 results on 18 April 2024. These published studies are heterogeneous, including reports on reduced colour CS in LHON[123] and in asymptomatic carrier relatives.[32] Methodology of CS measurement varies widely; there are reports on reduced CS in asymptomatic relatives where measurement was carried out using computer graphics,[124] and also a study that included measurement of CS in LHON using Gabor pattern stimulation.[125] A cross-sectional study of visual parameters of trial recruits in the RESCUE and REVERSE rAAV2/2-ND4 gene therapy trials reported CS using the Pelli–Robson chart, a similar method of CS measurement as that used in our study.[126]

In the LHON group in our study the median contrast sensitivity (CS) in the better eye was median 0.3 logCS, compared to median 1.5 logCS in the better eye in the asymptomatic relative group. This is comparable to the median CS measured in LHON trials RESCUE and REVERSE, which was 0.3 logCS.[126] In a small number in our LHON group, n=2 participants, the logCS was ≥1.0 This was observed with other signs of visual function parameters which were above the median in the study group. In one of the participants with relative preservation of CS who had early LHON (E1), VA logMAR was 1.0 both eyes, and colour vision (ishihara right 15/17, left 16/17), and in another participant (C3) who had preservation of VA to on chart RVA 0.9 logMAR and LVA 0.8 logMAR, though ishihara colour vision was reduced to 1/17 plates in each eye. The relevance of CS measurement remains to be seen.

In colour vision assessment, as expected, colour vision was poor in the LHON group, at median zero plates seen. In the asymptomatic carrier relatives 63% had bilateral full colour vision. In the asymptomatic group reduction in colour vision was observed with reduced visual acuity in n=3 subjects (19%). For one participant with moderately reduced colour

vision (10 plates seen each eye, U2), associated visual acuity (logMAR 0.2) was relatively preserved but VEP was prolonged to 130ms. This highlights that colour vision is a useful clinical sign of optic neuropathy, as is well known,[41] and can detect neuropathy even in cases where VA is preserved.

4.8 Demographic and social risk factors for vision loss in LHON

Male sex,[118] alcohol [127] and smoking [52] are well-established risk factors for penetrance of vision loss in LHON. Our lack of finding these associations as risk factors may be due to the small sample size and the design of our study. It is also possible that we would have detected these exposures as risk factors if we had exact details of alcohol and smoking exposure in the lead up to conversion to vision loss in LHON, but this data was not available.

4.9 Cardiac conduction abnormalities in LHON

Our findings that 56% of asymptomatic maternal relatives of LHON patients demonstrate electrocardiographic evidence of cardiac conduction abnormalities suggest that this group may be at risk of cardiac events in the absence of symptomatic optic neuropathy. The clinical relevance of these findings would be more clear if we had also conducted echocardiograms. This study was performed at rest and excluded patients with acute illness. MtDNA-associated cardiac arrhythmias may be more prevalent under physiologic stress conditions. Larger studies would be useful to assess utility and cost-effectiveness of screening (i.e., ECG ± cardiology review) for cardiac conduction abnormality in asymptomatic family members or mtDNA mutation carriers. As many as 1:300 of the normal population in the United Kingdom harbour the classic LHON-associated mtDNA variants (i.e., >225,000 people).[114]

4.10 Reduced vision-related quality of life in LHON

In vision-related quality of life, predictably, LHON patients reported lower quality of life than their asymptomatic relatives. The lowest of scores were found in relation to driving-vision. The extra ten questions of the Neuro-Supplement did not provide further useful information in this cohort, suggesting that a general vision-related questionnaire such as the NEI VFQ-

25 is sufficient in assessing quality of life in LHON. The Neuro-Supplement may be more useful in other neuro-ophthalmic disorders such as myasthenia gravis, where double vision and ptosis affect the scoring. Larger studies comparing vision-related quality of life of asymptomatic LHON carrier relatives to a normal control sample would be helpful to assess for subtle differences in quality of life, especially given the subclinical findings of optic neuropathy in the relatives.

4.11 Study limitations and strengths

Limitations of our study include that our cohort is a small heterogeneous sample of LHON patients and their older maternal relatives, including four different mutation types, the majority of whom were born in Ireland (93%), with two subjects from the same pedigree who were born in Ukraine (n = 2). A power calculation indicated that a sample size of n=15 would be required in each sample group to detect an effect size of 0.5 for correlation of mitochondrial haplogroup on presence of optic nerve dysfunction. Our sample size fell short of this with a total sample of n=28 in the LHON variant carrying group, including n=12 who have a diagnosis of LHON and n=16 asymptomatic carrier relatives, with n=10 included in the control study arm. Therefore our findings should be interpreted with caution. In our numeric analysis of structural changes of the optic nerve including thickness and perfusion of the RNFL we included each eye measured in our statistical test (Kruskal-Wallis test), which goes against an assumption of random sampling, as bilateral eyes were included for most participants. To minimised the impact of this on our results we separately derived a normal range using the normal confidence interval of our control sample, and we reported values that fell outside this normal range amongst the carriers of LHON-associated variants.

We utilised a control sample for the optic nerve head retinal nerve fiber layer thickness and perfusion arm of our study, and we also drew comparisons between LHON-affected, unaffected carriers, and the OCT optic nerve findings in the Heidelberg European Descent normative database which includes 201 healthy age-matched subjects, and for electrophysiology, an in-house normal sample normative database. We performed 12-lead ECG alone, it would have been helpful to also assess with echocardiogram. In assessing optic nerve head perfusion we evaluated the peripapillary RNFL, as the primary site of pathology,

but more information may be derived from also evaluating perfusion changes at the macula and within the papillo-macular bundle. We did not have patient or public involvement in the design of the study, nor patient reported outcome measures, which could have been very useful. Strengths of the study include the evaluation of multiple individuals who have LHON and also a group of asymptomatic maternal relatives, including 13 different pedigrees, when compared to a normative database assessed on the same imaging platform. Another strength of our study is the reporting of mitochondrial haplogroup, previously not reported from an Irish centre of treatment of LHON, and our reporting of safety data for Idebenone.

4.12 Conclusions

Carriers of LHON-associated pathogenic mitochondrial variants show signs of optic neuropathy, whether they are known to have LHON, or they are the asymptomatic maternal relatives of a confirmed case. This raises the question of what is the appropriate management for these families. The presence of these signs in asymptomatic relatives is associated with membership of the H or HV versus other mitochondrial haplogroups, and is associated with the presence of a thinned retinal nerve fiber layer at the optic nerve head. Relatives may also display cardiac conduction abnormalities. In our cohort, Idebenone treatment was well tolerated, with no adverse events, though recovery of vision was limited.

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Appendix I: Genetics testing summary

Blueprint Genetics- standard reporting: laboratory process and bioinformatics and quality control

Laboratory process: The genomic DNA was extracted from the blood sample using bead-based method. DNA quality and quantity were assessed using electrophoretic methods at Blueprint Genetics. After assessment of DNA quality, qualified genomic DNA sample was randomly fragmented using non-contact, isothermal sonochemistry
processing. Sequencing library was prepared by ligating sequencing adapters to both ends of DNA fragments. Sequencing libraries were size-selected with bead-based method to ensure optimal template size and amplified by polymerase chain
reaction (PCR). Regions of interest (exons and intronic targets) were targeted using hybridization-based target capture method. The quality of the completed sequencing library was controlled by ensuring the correct template size and quantity and to eliminate the presence of leftover primers and adapter-adapter dimers. Ready sequencing libraries that passed the quality control were sequenced using the Illumina's sequencing-by-synthesis method using paired-end sequencing (150 by
150 bases). Primary data analysis converting images into base calls and associated quality scores was carried out by the sequencing instrument using Illumina's proprietary software, generating CBCL files as the final output.These steps were
performed at Blueprint Genetics.

Bioinformatics and quality control: Base called raw sequencing data was transformed into FASTQ format using Illumina's software (bcl2fastq). Sequence reads of each sample were mapped to the human reference genome (GRCh37/hg19). Burrows-Wheeler Aligner (BWA-MEM) software was used for read alignment. Duplicate read marking, local realignment around indels, base quality score recalibration and variant calling were performed using GATK algorithms (Sentieon) for nDNA. Variant data for was annotated using a collection of tools (VcfAnno and VEP) with a variety of public variant databases including but not limited to gnomAD, ClinVar and HGMD. The median sequencing depth and coverage across the target regions for the tested sample were calculated based on MQ0 aligned reads. The sequencing run included in-process reference sample(s) for quality control, which passed our thresholds for sensitivity and specificity. The patient's sample was subjected to thorough quality control measures including assessments for contamination and sample mix-up. Copy number variations
(CNVs), defined as single exon or larger deletions or duplications (Del/Dups), were detected from the sequence analysis data using a proprietary bioinformatics pipeline. The difference between observed and expected sequencing depth at the targeted
genomic regions was calculated and regions were divided into segments with variable DNA copy number. The expected sequencing depth was obtained by using other samples processed in the same sequence analysis as a guiding reference. The
sequence data was adjusted to account for the effects of varying guanine and cytosine content. Bioinformatics and quality control processes were performed by Blueprint Genetics.

Appendix II Gene panel

The full mitochondrial genome was sequenced for each participant who was affected by LHON or was a maternal relative of an affected individual.

The panel of nuclear genes tested for all subjects included:

ACO2, AFG3L2, ANTXR1, APTX, ATAD3A, AUH, C10ORF2, C12ORF65, C19ORF12, CHN1, CISD2, DNAJC19, DNM1L, FDXR, FRMD7, GPR143, HESX1, KIF21A, LARS2, MECR, MFN2, MT-ATP6, MT-ATP8, MT-CO1, MT-CO2, MT-CO3, MT-CYB, MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6, MT- RNR1, MT-RNR2, MT-TA, MT-TC, MT-TD, MT-TE, MT-TF, MT-TG, MT-TH, MT-TI, MT-TK, MT-TL1, MT-TL2, MT-TM, MT-TN, MT-TP, MT-TQ, MT-TR, MT-TS1, MT-TS2, MT-TT, MT-TV, MT-TW, MT-TY, MTPAP, NDUFS1, NR2F1, OPA1, OPA3, OTX2, PAX6, PHOX2A, POLG, PRPS1, PUS1, ROBO3, RP1, RRM2B, RTN4IP1, SALL4, SETX, SLC25A4, SLC25A42, SLC25A46, SLC38A8, SLC52A2, SNX10, SOX2, SPG7, TIMM8A, TK2, TMEM126A, TSFM, TUBB3, TYMP, UCHL1, WFS1, YME1L1 and ZNHIT3.

For one subject, U13, an additional gene was tested:

DNAJC30

NEI-VFQ-25
Visual Functioning Questionnaire - 25

PART 1 - GENERAL HEALTH AND VISION

1. <u>In general,</u> would you say your overall <u>health</u> is:

(Circle One)

Excellent.....1
Very Good 2
Good.....3
Fair.....4
Poor 5

2. At the present time, would you say your eyesight using both eyes (with glasses or contact lenses, if you wear them) is <u>excellent</u>, <u>good</u>, <u>fair</u>, <u>poor</u>, or <u>very poor</u> or are you <u>completely blind</u>?

(Circle One)

Excellent.....1
Good.....2
Fair.....3
Poor 4
Very Poor.....5
Completely Blind.....6

3. How much of the time do you worry about your eyesight?

(Circle One)

None of the time 1

A little of the time 2

Some of the time 3

Most of the time 4

All of the time? 5

4. How much pain or discomfort have you had in and around your eyes (for example, burning, itching, or aching)? Would you say it is:

(Circle One)

None 1

Mild 2

Moderate 3

Severe, or 4

Very severe? 5

PART 2 - DIFFICULTY WITH ACTIVITIES

The next questions are about how much difficulty, if any, you have doing certain activities wearing your glasses or contact lenses if you use them for that activity.

5. How much difficulty do you have <u>reading ordinary print in newspapers</u>? Would you say you have:

(Circle One)

No difficulty at all 1

A little difficulty 2

Moderate difficulty 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

6. How much difficulty do you have doing work or hobbies that require you to <u>see well up close</u>, such as cooking, sewing, fixing things around the house, or using hand tools? Would you say:

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

7. Because of your eyesight, how much difficulty do you have <u>finding something on a crowded shelf</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

8. How much difficulty do you have <u>reading street signs or the names of</u> <u>stores</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

9. Because of your eyesight, how much difficulty do you have <u>going down steps, stairs, or curbs in dim light or at night</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight 5

Stopped doing this for other reasons or not interested in doing this 6

10. Because of your eyesight, how much difficulty do you have <u>noticing objects off to the side while you are walking along</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

11. Because of your eyesight, how much difficulty do you have <u>seeing how people react to things</u> you say?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

12. Because of your eyesight, how much difficulty do you have <u>picking</u> <u>out and matching your own clothes</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

13. Because of your eyesight, how much difficulty do you have <u>visiting</u> <u>with people in their homes, at parties, or in restaurants</u> ?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty 4

Stopped doing this because of your eyesight..... 5

Stopped doing this for other reasons or not interested in doing this 6

14. Because of your eyesight, how much difficulty do you have <u>going out</u> <u>to see movies, plays, or sports events</u>?

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty 3

Extreme difficulty 4

Stopped doing this because of your eyesight 5

Stopped doing this for other reasons or not interested in doing this 6

15. Are you currently driving, at least once in a while?

(Circle One)

Yes ..... 1 *Skip To Q 15c*

No 2

15a. IF NO: Have you never driven a car or have you given up driving?

(Circle One)

Never drove ..... 1 *Skip To Part 3, Q 17*

Gave up.....2

15b. IF YOU GAVE UP DRIVING: Was that mainly because of your eyesight, mainly for some other reason, or because of both your eyesight and other reasons?

(Circle One)

Mainly eyesight ..... 1 *Skip To Part 3, Q 17*

Mainly other reasons ..... 2 *Skip To Part 3, Q 17*

Both eyesight and other reasons ... 3 *Skip To Part 3, Q 17*

15c. IF CURRENTLY DRIVING: How much difficulty do you have driving during the daytime in familiar places? Would you say you have:

(Circle One)

No difficulty at all 1

A little difficulty 2

Moderate difficulty 3

Extreme difficulty.....4

16. How much difficulty do you have driving at night? Would you say you have:

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty..... 4

Have you stopped doing this because of your eyesight..... 5

Have you stopped doing this for other reasons or are you not interested in doing this 6

16A. How much difficulty do you have driving in difficult conditions, such as in bad weather, during rush hour, on the freeway, or in city traffic? Would you say you have:

(Circle One)

No difficulty at all 1

A little difficulty..... 2

Moderate difficulty..... 3

Extreme difficulty..... 4

Have you stopped doing this because of your eyesight..... 5

Have you stopped doing this for other reasons or are you not interested in doing this 6

PART 3: RESPONSES TO VISION PROBLEMS

The next questions are about how things you do may be affected by your vision. For each one, please circle the number to indicate whether for you the statement is true for you all, most, some, a little, or none of the time.

READ CATEGORIES:	All of the time	Most of the time	Some of the time	A little of the time	None of the time
	(Circle One On Each Line)				
17. Do you accomplish less than you would like because of your vision?	1	2	3	4	5
18. Are you limited in how long you can work or do other activities because of your vision?	1	2	3	4	5
19. How much does pain or discomfort in or around your eyes, for example, burning, itching, or aching, keep you from doing what you'd like to be doing? Would you say:	1	2	3	4	5

For each of the following statements, please circle the number to indicate whether for you the statement is definitely true, mostly true, mostly false, or definitely false for you or you are not sure.

(Circle One On Each Line)

	Definitely True	Mostly True	Not Sure	Mostly False	Definitely False
20. I stay home most of the time because of my eyesight.....	**1**	**2**	**3**	**4**	**5**
21. I feel frustrated a lot of the time because of my eyesight.....	**1**	**2**	**3**	**4**	**5**
22. I have much less control over what I do, because of my eyesight.....	**1**	**2**	**3**	**4**	**5**
23. Because of my eyesight, I have to rely too much on what other people tell me..	**1**	**2**	**3**	**4**	**5**
24. I need a lot of help from others because of my eyesight.....	**1**	**2**	**3**	**4**	**5**
25. I worry about doing things that will embarrass myself or others, because of my eyesight.....	**1**	**2**	**3**	**4**	**5**

10-ITEM NEURO-OPHTHALMIC SUPPLEMENT TO THE NEI-VFQ-25

The following are additional questions and statements about problems that involve your vision or feelings you may have about your vision condition. After each question, there will be a list of possible answers. Please choose the response that best describes your situation.

Please answer all questions as if you were wearing your glasses or contact lenses (if any). Please take as much time as you need to answer each question.

1. How much difficulty do you have performing tasks when your eyes are tired?

(Circle One)

None	1	Mild	2
Moderate	3	Severe, or	4
Very severe?	5		

2. Because of your vision, how much difficulty do you have identifying objects or performing tasks in bright sunlight?

(Circle One)

None	1	Mild	2
Moderate	3	Severe, or	4
Very severe?	5		

3. Because of your vision, how much difficulty do you have parking a car?

(Circle One)

No difficulty at all	1	A little difficulty	2
Moderate difficulty	3	Extreme difficulty	4
Stopped doing this because of your eyesight	5	Stopped doing this for other reasons or not interested in doing this	6

4. Because of your vision, how much difficulty do you have using a computer?

(Circle One)

No difficulty at all	1	A little difficulty	2
Moderate difficulty	3	Extreme difficulty	4
Stopped doing this because of your eyesight	5	Stopped doing this for other reasons or not interested in doing this	6

For each of the following statements, please indicate if it is definitely true, mostly true, mostly false, or definitely false for you or if you are not sure.

5. I have a feeling that my two eyes see differently, even with correction (glasses or contact lenses).	
	(Circle One)
Definitely true	1
Mostly true	2
Not sure	3
Mostly false	4
Definitely false	5
6. I have a feeling that my eye or eyelid appearance is unusual.	
	(Circle One)
Definitely true	1
Mostly true	2
Not sure	3
Mostly false	4
Definitely false	5

7. My vision is blurry, not clear, or "fuzzy."

For each of the following, please indicate if it is true for you all, most, some, a little, or none of the time.

(Circle One)

All of the time	1	Most of the time	2
Some of the time	3	A little of the time	4
None of the time	5		

8. I have trouble focusing on or following moving objects.

(Circle One)

All of the time	1	Most of the time	2
Some of the time	3	A little of the time	4
None of the time	5		

9. I have double vision with both eyes open that is not present when either eye is covered.

(Circle One)

All of the time	1	Most of the time	2
Some of the time	3	A little of the time	4
None of the time	5		

10. My eyelid(s) droop.

(Circle One)

All of the time	1	Most of the time	2
Some of the time	3	A little of the time	4