Mathematically Modeling the Involvement of Axons in Leber's Hereditary Optic Neuropathy

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PURPOSE. Leber's hereditary optic neuropathy (LHON), a mitochondrial disease, has clinical manifestations that reflect the initial preferential involvement of the papillomacular bundle (PMB). The present study seeks to predict the order of axonal loss in LHON optic nerves using the Nerve Fiber Layer Stress Index (NFL-S_I), which is a novel mathematical model.

METHODS. Optic nerves were obtained postmortem from four molecularly characterized LHON patients with varying degrees of neurodegenerative changes and three age-matched controls. Tissues were cut in cross-section and stained with *p*-phenylenediamine to visualize myelin. Light microscopic images were captured in 32 regions of each optic nerve. Control and LHON tissues were evaluated by measuring axonal dimensions to generate an axonal diameter distribution map. LHON tissues were further evaluated by determining regions of total axonal depletion.

RESULTS. A size gradient was evident in the control optic nerves, with average axonal diameter increasing progressively from the temporal to nasal borders. LHON optic nerves showed an orderly loss of axons, starting inferotemporally, progressing centrally, and sparing the superonasal region until the end. Values generated from the NFL-S_I equation fit a linear regression curve ($R^2 = 0.97$; $P < 0.001$).

CONCLUSIONS. The quantitative histopathologic data from this study revealed that the PMB is most susceptible in LHON, supporting clinical findings seen early in the course of disease onset. The present study also showed that the subsequent progression of axonal loss within the optic nerve can be

predicted precisely with the NFL-S_I equation. The results presented provided further insight into the pathophysiology of LHON. (*Invest Ophthalmol Vis Sci.* 2012;53:7608-7617) DOI: 10.1167/iovs.12-10452

Leber's hereditary optic neuropathy (LHON) was the first human disease discovered to be caused by point mutations affecting mitochondrial DNA.¹ The classic clinical manifestation of LHON is the acute or subacute loss of central vision in males, generally during the second to third decades of life. The disease process initially manifests in one eye, and within weeks to months proceeds to symmetrically affect both eyes. Other clinical manifestations of LHON include visual acuities of 20/200 or worse, dyschromatopsia, diminished contrast sensitivities at high spatial frequencies, and a central scotoma on visual field testing.²⁻⁴

In the past decade significant advances were made in understanding LHON, yet many questions still remain unanswered. Central among these is why retinal ganglion cells (RGCs) are preferentially affected in a disease that targets mitochondria, energy-producing organelles that are near ubiquitous in the human body. It is not surprising that the central nervous system is involved considering that the brain, which contributes only 2% to total body weight, requires 20% of the oxygen supply.^{5,6} Furthermore, the retina, an extension of the central nervous system, has the highest relative oxygen demand of any tissue in the body.⁷ However, there may be more to the RGC that is remarkable with regard to anatomy and function that renders it exceedingly susceptible to mitochondrial dysfunction, including energy deficiency and oxidative stress.

Histologic studies have shown that there is a high density of mitochondria distributed anterior to the lamina cribrosa of the optic nerve head, with a sharp drop off in numbers posteriorly that coincides with the start of myelination.^{8,9} Furthermore, the mitochondrion has to supply the energy for its own anterograde transport from its biogenesis within the RGC body down to its location of utilization in the axon.^{10,11} Since the microtubule-associated motor proteins kinesin and dynein require considerable amounts of adenosine triphosphate (ATP) to power, the reduction of ATP can potentially trigger a cycle of dysfunctional trafficking with abnormal energy production as an initial trigger.¹²⁻¹⁴ Both the energy shortage in a highly ATP-dependent system and the disruption of a finely tuned mitochondrial network dependent on transport proteins have been shown to yield disastrous results.⁴

Interestingly, even within RGCs there is a stratification of risk, with early and preferential loss of the smallest RGCs associated with the thinnest axons in LHON patients.¹⁵ This can be clinically observed by features such as dyschromatopsia, central scotoma, and diminished contrast sensitivities at high spatial frequencies, all of which reflect the dysfunction of

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TABLE. Control and LHON Patient Characteristics

Patient Number	Sex	Age at Death	Cause of Death	Nerves Studied	Mutation Type	Disease Duration
Control #1	M	60	Lung CA	OD	—	—
Control #2	F	64	CHF	OD	—	—
Control #3	F	70	COPD	OD	—	—
LHON #1	M	59	MI	OD, OS	11778/ND4	8 y
LHON #2	M	52	MI	OD, OS	11778/ND4	23 y
LHON #3	F	68	COPD	OS	11778/ND4	30 y
LHON #4	F	75	CHF	OD, OS	3460/ND1	53 y

M, male; F, female; CA, cancer; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; MI, myocardial infarction

parvocellular RGCs in the papillomacular bundle (PMB).^{16,17} Preferential involvement of these smaller fibers has been noted in LHON as well as in other optic neuropathies such as optic neuritis.¹⁸ The present study sought to predict the order of axonal loss and test this prediction with precise quantification of disease involvement of the optic nerve in patients with LHON. To do so, a group of control optic nerves were studied to generate a baseline map of axonal diameters, and a group of LHON optic nerves were evaluated to determine the natural sequence of disease progression. The Nerve Fiber Layer Stress Index (NFL-S_I), a novel mathematical model recently described by Sadun et al, was used to quantify the severity of the disease process.¹⁹

MATERIALS AND METHODS

Postmortem Control Tissues (LHON and Controls)

Control postmortem optic nerves were obtained from the Lions Eye Bank of Oregon (Portland, OR) from three donors (Table). Only the right optic nerves from controls were analyzed for the present study. Control #1 was a 60-year-old male who died of metastatic lung cancer. Control #2 was a 64-year-old female who died of cardiogenic shock secondary to congestive heart failure. Control #3 was a 70-year-old female who died of end-stage chronic obstructive pulmonary disease with *cor pulmonale*. None of these controls had past medical history of ophthalmologic or neurodegenerative disorders.

LHON postmortem optic nerves were obtained from several sources (Table). LHON patient #1 and patient #2 were brothers in a large Brazilian maternal lineage carrying the 11778/ND4 LHON primary mutation. This extended LHON family has been characterized in a number of clinical studies.^{20–26} One proband, displaying milder neurodegenerative changes in the optic nerves, died at the age of 59 years from acute myocardial infarction. His younger brother, showing more severe neurodegenerative changes in the optic nerves died at the age of 52 years, also from an acute myocardial infarct. The other two LHON patients in this study were females, and also have been characterized previously by our group. One was a 68-year-old female harboring the 11778/ND4 mutation who died of chronic obstructive pulmonary disease complications.^{15,27,28} The last patient was a 75-year-old female carrying the 3460/ND1 mutation who died of cardiac failure.¹⁵ Bilateral optic nerves were incorporated into the present study in each LHON patient, except in the case of LHON patient #3 in whom only the left nerve was available.

Tissue Processing and Light Microscopy

Optic nerves were initially immersion-fixed in neutral buffered formalin and stored at 4°C. They were subsequently cut between 3 to 5 mm behind the globe into 1-mm thick cross-sections. A single razorblade nick was placed superiorly, and two nicks were placed temporally for orientation. The tissues were post-fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium-phosphate buffer, pH 7.4 at 4°C. Lipid fixation was performed

using 2% osmium tetroxide in 0.01 M sodium-phosphate buffer, and *en bloc* staining was performed using 1% uranyl acetate in 50 mM sodium acetate buffer. The tissues were dehydrated initially with ascending grades of ethanol, and finally with propylene oxide. An embedding medium (PELCO Eponate Kit; Ted Pella, Inc., Redding, CA) was used according to the manufacturer's instructions for final epoxy resin infiltration and embedding of the optic nerve cross-sections. Semithin sections were cut at 1 µm on a Du Pont-Sorvall MT2-B ultramicrotome, placed on glass slides with distilled water, and dried on a hot plate at low heat. Staining was performed using a 1% solution of *p*-phenylenediamine (PPD) in absolute methanol for cross-sectional myelin profiles.²⁹

Nerve tissues stained with PPD were viewed on a bright field light microscope (Axio Scope; Carl Zeiss AG, Oberkochen, Germany), and were oriented to the superior and temporal aspects using the nicks described previously. Photomicrographs were taken with a 100x objective lens (resulting in a final magnification viewed at 1000x) with oil immersion at 36 regions in each nerve based on a predetermined mathematical algorithm (Fig. 1). The algorithm used the coordinate locations of the temporal, nasal, superior, and inferior aspects of each nerve to generate a unique grid of 36 uniform rectangles to fit the specified dimensions of the nerve. This resulted in 32 non-overlapping images located within each optic nerve, and four corner images that fell outside and were then discarded. All light microscopic photos were acquired using a digital camera (RTKe SPOT 2; Diagnostic Instruments, Inc., Sterling Heights, MI) and saved to a computer for further analysis.

Image Analysis and Digital Morphometry

Photomicrographs from each control optic nerve and four LHON optic nerves (the better preserved nerve from each patient; LHON #1 right eye [OD], LHON #2 left eye [OS], LHON #3 OS, LHON #4 OS) were digitally magnified by 400x using viewing software (ImageScope; Aperio Technologies, Vista, CA). The circumference and area of each axon was quantified by hand measuring the inner border of the myelin ring via a pen display (DTU-710; Wacom Co., Saitama, Japan). Using the

FIGURE 1. (A) Schematic illustration of the optic nerve sampling methodology. The algorithm will adjust the length and width of the rectangular regions according to the unique shape of each nerve. (B) A normal human optic nerve cut in cross-section stained with *p*-paraphenylenediamine with a superimposed sampling grid (original magnification: $\times 25$). Note that the four corners (asterisks) are not included in the analysis. (Scale bar = 0.5 mm)

$$\begin{aligned}
 \text{NFL-}S_I &= [\text{Axon Energy Demand}] \div [\text{Axon Energy Supply}] \\
 &= [\text{Axon Surface Area}] \div [\text{Axon Volume}] \\
 &= [A_{SA}] \div [A_V] \\
 &= [\text{Axon Length} * \text{Circumference}] \div [\text{Axon Length} * \text{Area}] \\
 &= [L * 2\pi R] \div [L * \pi R^2] \\
 &= [2] \div [R]
 \end{aligned}$$

FIGURE 2. The NFL- S_I equation is calculated as the demand for energy divided by the available supply. Being that the primary function of RGCs is to transmit electrical potential along its axons, surface area is used as a surrogate value for its membrane transporters. Given that energy supply is generated predominantly by mitochondria, which take up cytoplasmic space, axonal volume is used as a proxy for the total energy production capability. Substituting in the geometric formulas for surface area and volume of a cylinder and simplifying common elements results in the final equation of $\text{NFL-}S_I = 2/R$. A_{SA} , axonal surface area; A_V , axonal volume; L , length; R , radius.

area generated by the software, basic mathematical calculations were performed to resolve the radius, and thus the diameter for each axon. Our previous approach of directly measuring the shortest axonal diameter was not used due to observer subjectivity and lack of axonal shape uniformity.

LHON optic nerves were further evaluated to determine regional axonal loss. Individual sectors from LHON nerves were determined to be “depleted” if fewer than 10 axons remained in the entire image since, on average, control optic nerve photomicrographs contained approximately 1000 axons per field. Regions still populated with axons and located directly adjacent to a depleted sector were characterized as “penumbral.” All remaining populated regions were then labeled as “intact.” Sectors of depletion were mapped for all seven LHON nerves and used to determine the average diameter of axons lost in each instance.

Nerve Fiber Layer Stress Index

The NFL- S_I equation described by Sadun et al condenses down to the ratio of demand versus supply, “...whereby the numerator reflects all the factors that require high energy supply by the axon and the denominator the source of that energy.”¹⁹ Because an axon’s energy expenditure is predominantly based on the propagation of action potentials^{30,31} and because depolarization and subsequent repolarization are intimately associated with plasma membrane channels and transporters, this energy demand is well represented by the prelaminar unmyelinated axonal surface area (A_{SA}). Mitochondrial population, on the other hand, is closely linked to cellular ATP production, and thus the denominator of energy supply can be represented by the number of mitochondrial units. Since mitochondrial population directly correlates to and is also constrained by axonal size, axonal volume (A_V) sets the limit for mitochondrial numbers (Fig. 2). With this model in place, simple substitution of geometric formulas for surface area and volume of a cylinder results in the equation $\text{NFL-}S_I = A_{SA}/A_V = L2\pi R/L\pi R^2$, with L being the length of the unmyelinated fiber, and R the radius of the axon. Simplifying the formula by canceling common elements reduces this formula to $\text{NFL-}S_I = 2/R$ (Fig. 2). The radius R used in the strain index for each case of LHON was calculated based on the average size of control nerve axons in the corresponding depleted zones.

RESULTS

In the first control optic nerve, 39,247 axons were measured resulting in an average axonal diameter and SD of $1.16 \pm 0.70 \mu\text{m}$ (range 0.19–6.87). In the second control optic nerve,

56,764 axons were sampled yielding an average diameter ($\pm$ SD) of $0.93 \pm 0.65 \mu\text{m}$ (range 0.15–7.80). In the third control optic nerve, 43,788 axons were measured resulting in an average diameter ($\pm$ SD) of $1.27 \pm 0.74 \mu\text{m}$ (range 0.19–8.10) (Figs. 3A–C). Combining the values of all three control optic nerves together results in an average diameter and SEM of $1.12 \pm 0.10 \mu\text{m}$. This is comparable to the average and SEM values previously found by our own group of $0.82 \pm 0.12 \mu\text{m}$,³² Mikelberg et al of $0.72 \pm 0.07 \mu\text{m}$,³³ Repka and Quigley of $0.98 \pm 0.09 \mu\text{m}$,³⁴ and Jonas et al of $1.00 \pm 0.06 \mu\text{m}$.³⁵ Extrapolated values of the total axonal population for control optic nerves #1 through #3 were 1,085,982, 899,838, and 884,468, respectively, with an average ($\pm$ SEM) of $956,762 \pm 64,762$ (Figs. 3A–C).

In LHON #1 OD, 16,076 sampled axons resulted in an average diameter ($\pm$ SD) of $1.48 \pm 1.37 \mu\text{m}$ (range 0.17–13.71). In LHON #2 OS, 7,001 axons were sampled with an average diameter ($\pm$ SD) of $1.42 \pm 1.29 \mu\text{m}$ (range 0.18–8.75). In LHON #3 OS, 3,387 sampled axons revealed an average diameter ($\pm$ SD) of $1.63 \pm 1.06 \mu\text{m}$ (range 0.22–7.39). Lastly, in LHON #4 OS, 969 measured axons had an average diameter ($\pm$ SD) of $1.78 \pm 1.15 \mu\text{m}$ (range 0.20–6.98). Extrapolated values for the total axonal population of LHON #1 OD, #2 OS, #3 OS, and #4 OS were 437,084, 80,539, 37,526, and 5,451, respectively (Figs. 3D–G). The axons measured within the penumbral region of LHON #1 OD were notably larger, with an average diameter ($\pm$ SD) of $1.90 \pm 1.60 \mu\text{m}$ (range 0.22–13.71). The extrapolated axonal population in this zone was 129,208, which was 29.6% of the total axonal population of the nerve (Fig. 3H). Similar observations of a larger penumbral average diameter compared to the aggregate average were not seen in any other morphologically assessed LHON optic nerve (data not shown).

Plotting the percentage diameter frequency of sampled axons in each control optic nerve using $0.1 \mu\text{m}$ bins produced unimodal retinal nerve fiber layer (RNFL) spectrum histograms with positively skewed tails. In controls #1 to #3, the mode for axonal diameter size was centered around $0.8 \mu\text{m}$, $0.4 \mu\text{m}$, and $0.8 \mu\text{m}$, with percentage frequencies of 9.6%, 12.2%, and 9.2%, respectively. The diameter size at which 90% of all sampled axons had been accounted for (90% cumulative frequency) was $2.1 \mu\text{m}$, $1.8 \mu\text{m}$, and $2.2 \mu\text{m}$ for controls #1 through #3, respectively (Figs. 3A–C). In LHON #1 OD, #2 OS, #3 OS, and #4 OS, the mode for axonal diameter size was $0.5 \mu\text{m}$, $0.5 \mu\text{m}$, $0.6 \mu\text{m}$, and $0.7 \mu\text{m}$, with percentage frequencies of 11.7%, 12.3%, 5.8%, and 5.9%, respectively. The 90% cumulative frequency for the same set of LHON optic nerves was reached at the axonal diameter sizes of $3.4 \mu\text{m}$, $3.3 \mu\text{m}$, $3.1 \mu\text{m}$, and $3.4 \mu\text{m}$, respectively (Figs. 3D–G). The average diameter ($\pm$ SEM) for the axonal frequency mode in control cases was $0.67 \pm 0.13 \mu\text{m}$ as compared to $0.58 \pm 0.05 \mu\text{m}$ in LHON cases ($P = 0.50$). The 90% cumulative frequency was reached at an average diameter ($\pm$ SEM) of $2.03 \pm 0.12 \mu\text{m}$ in controls, compared to $3.30 \pm 0.07 \mu\text{m}$ in LHON ($P < 0.001$). A histogram for the penumbral zone of LHON #1 OD revealed a diameter mode located at $0.5 \mu\text{m}$ with an associated frequency of 6.4%. The 90% cumulative frequency was reached at an axonal diameter size of $4.2 \mu\text{m}$. Notably, 2.8% of measured axons were larger than $6 \mu\text{m}$ (Fig. 3H).

By referencing the average axonal diameter of each sampled sector in the control optic nerves, an axonal size distribution map was generated in Figure 4A. The paired gray-scale map in Figure 4B shows the data schematically for easier appreciation of the various size gradients. The distribution of average axonal diameters by arranging the sampling grid as columns can be seen in Figure 4C. As one traverses the optic nerve from temporal-most to the nasal-most borders, the average axonal size increased gradually and can be modeled by the linear

FIGURE 3. Axonal diameter percent frequency histograms of control and LHON optic nerves. The left-side y-axis represents individual percent frequency (up to 12%), while the right-side y-axis represents cumulative percent frequency (up to 100%). The x-axis represents the axonal diameter size measured in microns, with individual bins being 0.1 μm . The x-axis scale ranges from 0 to 6 μm , with the last bin to the right representing all axons larger than 6 μm . *Black arrows* indicate the axonal diameter mode. *Open arrow heads* indicate the axonal diameter at which 90% cumulative frequency is reached. Displayed within each histogram are the average axonal diameter, SD, size range, and total axonal population. Avg, average.

regression equation $Y = 0.0639x + 0.8839$ with an $R^2 = 0.96$ ($P < 0.001$) (Fig. 4D).

A cross-sectional image of the axonal population captured in the inferotemporal region of control optic nerve #1 is depicted in Figure 5A, showing a fairly uniform distribution of small caliber axons. In contrast, Figure 5B shows a much more heterogeneous axon population located in the superonasal region from the same nerve. This shift in the distribution of axonal sizes in different regions is evident when viewed as a series of histograms. Figure 5C is a histographic mapping of

axonal size frequency within each of the 32 regions in control optic nerve #1. The sampled areas of the optic nerve that correspond to a small average axonal diameter tend to be evenly distributed around a single initial peak. In contrast, as the average axonal diameter size of a given region becomes larger, a positive skew becomes increasingly apparent.

Control optic nerve #1 and the four LHON optic nerves utilized in morphologic measurements can be seen in Figures 6A-E. The LHON nerves were ordered with respect to the degree of atrophy seen, starting with the least severe and

FIGURE 4. (A) Schematic representation of a human control optic nerve cut in cross-section. Numbers displayed are the average axonal diameters in microns calculated from three controls. (B) A gray-scale representation of the average axonal diameters. Lighter and darker shades represent a smaller and larger average axonal diameter, respectively. (C) A gray-scale representation of average axonal diameters when control optic nerve regions are organized into columns. (D) A linear regression model with the x-axis representing the columns of a control optic nerve (1 = most temporal; 6 = most nasal), and y-axis being the average axonal diameter located within that column ($P < 0.001$). Error bars represent the standard error of the mean. Col, column.

progressing to the most. This order also correlated to disease progression as determined clinically by longitudinal optical coherence tomography (OCT) RNFL studies of LHON patients.³⁶ The atrophic zone first appeared in the temporal aspect of the optic nerve, with arms that spread superiorly and inferiorly (Fig. 6B). With greater disease severity, the front of the atrophic wave continued to push nasally through the middle portions of the nerve, leaving a depleted temporal border (Fig. 6C). Eventually, the central portion of the optic nerve was completely lost, while a thin band of axons was maintained superiorly, inferiorly, and nasally at the periphery (Fig. 6D). Finally, the optic nerve rim also atrophied away, leaving a small bastion of axons in the superonasal and inferonasal corners (Fig. 6E). It is notable that the progression from one histopathologic severity to the next was very precise, and the atrophic pattern appeared to follow a set course.

Applying the same mathematical algorithm used to objectively choose sampling locations within an optic nerve, a stylized map of sectoral atrophy was created to represent each of the LHON nerves (Figs. 6G–J). These select nerves from LHON patients #1 (OD), #2 (OS), #3 (OS), and #4 (OS) lost an average of 13%, 31%, 56%, and 91% of the sampled regions, respectively. As mentioned previously, the axonal diameters were also measured in these four nerves within the sectors that remained intact, resulting in an overall average diameter ($\pm$ SD) of $1.48 \pm 1.37 \mu\text{m}$, $1.42 \pm 1.29 \mu\text{m}$, $1.63 \pm 1.06 \mu\text{m}$, and $1.78 \pm 1.15 \mu\text{m}$ in LHON #1 through #4, respectively. Considering only the penumbra regions (located adjacent to the depleted sectors), the average axonal diameter ($\pm$ SD) was $1.90 \pm 1.60 \mu\text{m}$, $1.38 \pm 1.22 \mu\text{m}$, $1.62 \pm 1.07 \mu\text{m}$, and $1.78 \pm 1.15 \mu\text{m}$ for LHON #1 through #4, respectively (Fig. 6).

FIGURE 5. (A, B) Control human optic nerve axons cut in cross-section and stained with *p*-phenylenediamine. (Scale bars = 5 μm) (A) The image reveals relative uniformity in axonal size within the inferotemporal optic nerve. (B) The superonasal optic nerve shows a much more heterogeneous pattern of axonal diameter distribution with a tendency toward larger axons. (C) Schematic representation of a control human optic nerve cut in cross-section. Nerve fiber layer spectrum histograms from control optic nerve #1 were generated for each region. The x-axis represents the axonal diameter distribution from 0 μm (left) to 6 μm (right) in 0.01 μm bins. The y-axis represents the percent frequency from 0% to 12%. All histograms have the same axes.

FIGURE 6. Histopathologic disease progression in LHON optic nerves. (A–E) Optic nerve cross-sections from control #1 and four LHON patients stained with *p*-phenylenediamine. The control optic nerve reveals a normal pattern of homogenous staining. The selection of LHON nerves are arranged in order of increasing histopathologic degeneration. The boundary between depleted and penumbral regions is outlined by solid black arrow heads. (Scale bars = 0.5 mm) (F–J) Schematic representations paired to the histopathology. A gray square represents a region that still contains axons (“intact”). An overlaid asterisk represents a “depleted” region. An overlaid black circle represents a “penumbra” region. The number of regions lost, the overall average axonal size, and the average axonal size specifically in the penumbra zone are given. Avg, average.

FIGURE 7. Linear regression equation showing the correlation between the fraction of regions lost (x-axis) in each LHON nerve and its associated stress index value (y-axis) ($P < 0.001$).

Results from applying the NFL-SI mathematical model to all seven LHON optic nerves in the present study can be seen in Figure 7. The least affected optic nerve (LHON #1 OD) lost 4 sectors out of 32 total (13%). Progressive stages of sectoral dropout was seen in LHON #1 OS (6/32; 19%), LHON #2 OS (10/32; 31%), LHON #2 OD (14/32; 44%), LHON #3 OS (18/32; 56%), LHON #4 OS (29/32; 91%), and LHON #4 OD (30/32; 94%). The average diameter size of the lost axons, from the least to the most affected nerve, was 0.98 μ m, 0.97 μ m, 1.00 μ m, 1.04 μ m, 1.05 μ m, 1.09 μ m, and 1.10 μ m. The stress index values associated with each of the nerves, from least to most affected, were 4.10, 4.12, 4.02, 3.86, 3.83, 3.65, and 3.64, respectively. Plotting these stress index values against the fraction of regions lost in each case resulted in the linear regression equation $Y = -0.5997x + 4.184$ with $R^2 = 0.97$ ($P < 0.001$).

DISCUSSION

Nerve Fiber Layer Stress Index and LHON Disease Progression

The quantitative data of the present study supported the concept that there is a preferential involvement of small axons in LHON and that this may be related to their high level of energetics paired with a relatively low energetic potential. The NFL-SI equation of demand versus supply reveals that the anatomical shortcoming of a high surface area to volume ratio, combined with its functional absence of saltatory conduction in the unmyelinated prelaminar nerve fiber layer, leaves the small RNFL axons at the highest risk for damage during the initial phases of disease onset.¹⁵ The strength of the squared correlation coefficient ($R^2 = 0.97$; $P < 0.001$) spoke to the predictive value of the stress index in assessing disease progression (Fig. 7). Although these seven nerves are from four LHON patients, we believed that given the rarity of these tissues, using fellow eyes for analysis was justified.

The first and mildest LHON optic nerve in the spectrum (LHON #1 OD) had the highest NFL-SI value, 4.10, and manifested disease predominantly in the inferotemporal

portion (Fig. 6B), which subserves the macular fiber population. Previous publications, as well as data from the current study (Fig. 4A), suggest that the inferotemporal aspect of the normal control optic nerve contains the smallest average axonal diameters.³³ Furthermore, while LHON is well known to present with a cecocentral scotoma, early Humphrey Visual Field tests have refined the location to show that the initial visual loss is actually localized slightly superotemporal to fixation.³⁷ The superotemporal region of the central 10° of vision maps to the inferonasal macular fibers, which in turn project to the inferotemporal optic nerve.³⁸

LHON #2 OS was slightly more severe and had a loss of axons with an average axonal diameter of 1.00 μ m, which corresponds to an NFL-SI value of 4.02 (Fig. 6C). Visual analysis of the histopathology revealed that the atrophy that, in the least severe case, had been confined temporally now spread centrally, involving axons both superiorly and inferiorly. However, the nasal fibers remained largely unaffected. A recent longitudinal OCT study showed that the order of RNFL involvement in LHON patients is temporal first, followed by inferior, then superior, and finally nasal.³⁶ Thus, our histopathologic observations of disease progression in the LHON optic nerve agreed with established clinical OCT findings. Furthermore, the quantitative data gathered from the current study extended these observations. Analysis of the control optic nerve as a series of columns revealed a progressive temporal to nasal increase in the average axonal diameter, modeled very well by a linear regression curve ($Y = 0.0639x + 0.8839$; $R^2 = 0.96$; $P < 0.001$) (Figs. 4C, 4D).

LHON #3 OS had moderate to severe histopathologic changes, and had a loss of axons with an average axonal diameter of 1.05 μ m, corresponding to an NFL-SI value of 3.83 (Fig. 6D). The axonal dropout shown here was as would be predicted when reviewing the progressive differences in the first two nerves. The atrophic wave front that had previously pushed centrally had, in this even more advanced case, involved the nasal fibers, completing the pattern seen in the longitudinal RNFL OCT study.³⁶ Of note for this nerve is the sparing of the fibers along the superior, inferior, and nasal edges. Our data showed that this rim of remaining fibers

correlated to regions of the control optic nerve with the largest average axonal diameters (Fig. 4A). Previous work in monkeys also has shown that the superior and inferior edges contain an increased proportion of larger diameter axons.³⁹

LHON #4 OS, the most severely affected optic nerve of the four, lost axons with an average axonal diameter of 1.09 μm , corresponding to the lowest NFL- S_I value of 3.65 (Fig. 6E). Inspection of this nerve showed a small cluster of axons preserved in the superonasal quadrant, and an even smaller group located inferonasally. Data from the current study (Fig. 4A) and previously published reports in human beings and monkeys show that these locations are associated with the highest average axonal diameters as established in control optic nerves.^{33,39,40} The fact that the smallest (inferotemporal) and largest (superonasal) axons are not aligned directly across from one another in the horizontal axis is partly due to the slightly inferior placement of the macula with respect to the optic nerve.^{33,40}

The issue of axon caliber and energy expenditure has also been addressed theoretically by Perge et al.^{41,42} They concluded that the ideal size for an optic nerve axon (saddle point in a maxima/minima relation) is approximately 0.7 μm and, indeed, that was not far from the mode of fiber size in our present data and other published nerve fiber spectra.^{32–35} However, we suggest that temporal modulation is less important than spatial resolution when dealing with the psychophysical functions most associated with the PMB, such as visual acuity, color vision, and high spatial frequency contrast sensitivity.¹⁷

Axonal Size Distribution and LHON Disease Pathophysiology

The axonal diameter frequency histograms revealed that 90% of control optic nerve axons were smaller than $2.03 \pm 0.12 \mu\text{m}$ (average $\pm$ SEM), while for LHON optic nerves the sizes approached $3.30 \pm 0.07 \mu\text{m}$ (average $\pm$ SEM) before 90% of axons were accounted for ($P < 0.001$) (Fig. 3). This finding was not surprising as we had established in this study, and in our previous work,¹⁵ that smaller axons were predominantly affected in this disease. Indeed, progression from the least severe case (LHON #1 OD) to the most severe (LHON #4 OS) revealed a gradual increase in the aggregate average axonal diameter size (Fig. 6). However, while the proportion of large fibers increased, the smallest fibers continued to be the predominant entity, as evidenced by a lack of significant difference between the control ($0.67 \pm 0.13 \mu\text{m}$; average $\pm$ SEM) versus the LHON ($0.58 \pm 0.05 \mu\text{m}$; average $\pm$ SEM) diameter frequency modes ($P = 0.50$) (Fig. 3).

Interestingly, LHON #1 OD and #2 OS (the less severe cases) appeared to have a different RNFL spectrum profile compared to LHON #3 OS and #4 OS (the more severe cases). LHON #1 OD and #2 OS had axonal diameter modes peaking at 0.5 μm (11.7%) and 0.5 μm (12.3%), respectively (Figs. 3D, 3E), which were similar to the size and frequencies of the control nerves (Figs. 3A–C). However, in LHON #3 OS and #4 OS, the axonal diameter modes of 0.6 μm (5.8%) and 0.7 μm (5.9%) were of drastically reduced frequencies (Figs. 3F, 3G), which reflected a reduction of this smaller-diameter population at late disease stages. Restricting analysis to the penumbral region of LHON #1 OD produced a RNFL spectrum histogram very similar to that of LHON #3 OS and #4 OS (Fig. 3H), further suggesting that there was an active edge to the disease process. An additional point of interest is that the axons located within the penumbra of LHON #1 OD appeared to be swollen, with a maximal axonal diameter of 13.71 μm compared to a maximum of 8.10 μm found in the controls, and a maximum of 8.75 μm in the remaining three LHON cases (Fig. 3). This

finding explained why the overall average axonal diameter size in LHON #1 OD appeared larger than that in LHON #2 OS, even though the latter had more severe histopathology (Fig. 6). However, whether this was persistent residual swelling from initial disease onset or whether it was recurrent swelling at a later time point was unclear.

It must be noted that every control optic nerve grid sector contained both large and small axons. Evidence of this could be seen in the individual histograms constructed of each sampled sector (Fig. 5C). Furthermore, studies have been performed that show the existence of both RGC types distributed within the macula as well as in the peripheral retina.^{43,44} The difference in average axonal diameter seen in each sector of the control optic nerve, thus, was due to the different proportions of each type of RGC. The question arises, then, as to why the affected sectors of LHON optic nerves were completely devoid of axons, with losses not limited to just the smallest and hence most susceptible fibers. It may be that a necessary density of small susceptible axons must be present at a particular stress index in order to initiate a locally cascading destructive event, engulfing neighbor cells regardless of size in a “common reservoir” effect. A number of different elements may mediate the aforementioned phenomenon. One possibility is in the spread of small molecules that signal apoptosis from RGC to RGC via axonal gap junctions.^{45,46} Similarly, lysis of RGCs may cause the release of neurotransmitters, such as glutamate, which can cause excitotoxicity to neighboring cells.⁴⁷ Another possibility is that as the small RGCs die, there is a reduction in trophic feedback to the associated glial cells such as astrocytes and oligodendrocytes.^{48–51} Unable to survive without a minimum threshold of RGC interactions, the death of these glial cells would also result in the loss all associated RGCs (such as those myelinated by oligodendrocytes, and those nurtured by astrocytes).

LHON also has been shown to affect blood vessels of the optic nerve head (ONH), causing endothelium to swell with mitochondria similar to what is seen in RGC axons (Ross-Cisneros FN, et al. *IOVS* 2001;3361:ARVO Abstract B503). Thus, vascular compromise can potentially lead to a loss of fibers from ischemia. Similarly, the swelling of fibers (due to an inability to effectively maintain axoplasmic transport) during the initial phases of disease onset may cause a cascade of compression akin to the pathophysiology of non-arteritic anterior ischemic optic neuropathy.^{36,52} Indeed, Ramos et al.⁵³ have shown that LHON carriers may be protected by having a larger ONH compared to affected patients. Furthermore, affected patients with a larger ONH had better visual outcomes, adding to the suggestion that a larger optic disc size may mitigate against LHON associated axonal losses.⁵³ These and other explanations of the common reservoir effect can be tested in future studies.

CONCLUSIONS

The quantitative analysis of the histopathology described in this study supported the clinical findings seen; both reveal that the papillomacular bundle is preferentially affected early in LHON. However, this detailed investigation suggested that the axonal losses in LHON spread as a wave front across the optic nerve head in an orderly manner. The nerve fiber layer stress index equation ($NFL-S_I = 2/R$) was tested and found to predict this order of fiber loss with great precision, confirming the mathematical model proposed. As in any postmortem study, we cannot ascribe with certainty any cause and effect but merely describe the association of small fibers with LHON axonal loss. We are hopeful that these results give insight into

the pathophysiologic mechanisms involved in this intriguing disease.

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