# A VERY LARGE BRAZILIAN PEDIGREE WITH 11778 LEBER'S HEREDITARY OPTIC NEUROPATHY

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**ABSTRACT**

*Purpose:* We conducted extensive epidemiological, neuro-ophthalmological, psychophysical, and blood examinations on a newly discovered, very large pedigree with molecular analysis showing mtDNA mutation for Leber's hereditary optic neuropathy (LHON).

*Methods:* Four patients representing four index cases from a remote area of Brazil were sent to Sao Paulo, where complete ophthalmological examinations strongly suggested LHON. Molecular analysis of their blood demonstrated that they were LHON, homoplasmic 11778, J-haplogroup. They had an extensive family that all lived in one rural area in Brazil. To investigate this family, we drew on a number of international experts to form a team that traveled to Brazil. This field team also included several members of the Federal University of Sao Paulo, and together we evaluated 273 of the 295 family members that were still alive. We conducted epidemiological interviews emphasizing possible environmental risk factors, comprehensive neuro-ophthalmological examinations, psychophysical tests, Humphrey visual field studies, fundus photography, and blood testing for both mitochondrial genetic analysis and nuclear gene linkage analysis.

*Results:* The person representing the first-generation case immigrated from Verona, Italy, to Colatina. Subsequent generations demonstrated penetrance rates of 71%, 60%, 34%, 15%, and 9%. The percentages of males were 60%, 50%, 64%, 100%, and 100%. Age at onset varied from 10 to 64 years, and current visual acuities varied from LP to 20/400.

*Conclusions:* Almost 95% of a nearly 300-member pedigree with LHON 11778 were comprehensively studied. Analysis of environmental risk factors and a nuclear modifying factor from this group may help address the perplexing mystery of LHON: Why do only some of the genetically affected individuals manifest the disease? This fully described database may also provide an excellent opportunity for future clinical trials of any purported neuroprotective agent.

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## INTRODUCTION

Leber's hereditary optic neuropathy (LHON) is a maternally inherited form of acute or subacute loss of central vision predominantly affecting young males.[1,2] This degeneration of retinal ganglion cells and their axons is due to three prevalent pathogenic mitochondrial DNA (mtDNA) point mutations.[2] These affect nucleotide positions 11778, 3460, and 14484, respectively, in the ND4, ND1, and ND6 subunit genes of complex I. Having this mutation is necessary but not sufficient to produce blindness. If the patient has mitochondrial homoplasmy for one of these three mutations (all the mtDNA copies are mutant), there is a high predisposition for a catastrophic series of events in the optic nerve that ultimately leads to acute or subacute loss of central vision.[2,3]

These three mtDNA point mutations are pathogenic in the large majority of patients worldwide.[4-8] If the mutation is heteroplasmic (a mixture of normal/wild type and mtDNA mutation mitochondria), the percentage of the pedigree is reduced but the extent of visual impairment in those affected remains equally severe.[7]

Penetrance may be highly variable, even with the same pathogenic mutation in homoplasmic fashion within the same family.[9] Hence, environmental[1,2] and/or supplementary genetic factors, possibly in the nuclear DNA,[10] are probably needed to express the pathology as blindness. In particular, there is evidence that tobacco and alcohol consumption may act as risk factors that may trip the threshold in predisposed patients.[9,11]

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Clinically, the patient presents with unilateral or, occasionally, bilateral visual loss of acute or subacute tempo. The vision is often in the 20/400 to count fingers range with severe dyschromatopsia.[11] Fundus examination may reveal telangiectatic microangiopathy in some cases that is seen very soon after, or even before (in the fellow eye), visual loss. Indeed, these vascular features may precede the onset of bilateral asynchronous visual loss and evolve in a few weeks toward optic atrophy and permanent decrease of visual acuity.[12] In addition, an early drop out of the papillomacular bundle, an edematous appearance of the arcuate bundle nerve fiber layer, and enlarged and tortuous peripapillar vessels can be seen on fundus examination shortly after the onset of visual loss.[12] Visual field examination usually reveals cecocentral scotomas with relative preservation of the peripheral visual field. The visual loss usually stabilizes within a few months, leaving a picture of optic atrophy, more severely marked on the temporal side.[12]

Histopathological findings have been described in three cases with known mtDNA mutation.[13-16] These studies showed devastating losses of retinal ganglion cells and the corresponding nerve fiber layer in the eyes of the LHON patients. There was also a striking loss of fibers in the optic nerve with a variable and slight preservation of fibers in the far periphery. Electron microscopy revealed only very few retinal ganglion cells.[16]

Despite our extensive knowledge of the genetic and biochemical features of LHON, despite our extensive experience with its clinical presentation, and despite recent studies elucidating the morphological, morphometric, and ultrastructural features, LHON remains a great mystery. Intriguing questions include: Why does the disease selectively affect the nervous system and, more specifically, the optic nerve and, most specifically, the small fibers of the papillomacular bundle?[13] Even more mysteriously, Why does the disease have a selection bias to affect mostly men? Why are patients fine until early adulthood and then suddenly become profoundly blind in both eyes in an almost synchronous manner? Why do only some members of a genetically identical (in regard to mtDNA) pedigree manifest the disease?

To address this last question in particular, we recently undertook a field investigation to rural Brazil, where a previously undescribed and very large LHON pedigree was found. At minimum, we wished to examine the (nuclear) genetic and epigenetic factors that might trip the threshold of expression that leads to blindness.

## METHODS

We originally became aware of this extremely large pedigree when contacted (by e-mail through the International Foundation of Optic Nerve Diseases [IFOND]) by the first index case in the summer of 2001. M.O.M. was a 51-year-old woman with no visual complaints but aware of a strong family history of LHON. Her 14-year-old son had suddenly lost vision in one eye, and she went to the Internet to research her disease with the hope that recent developments afforded some treatment for her. M.O.M. displayed a great deal of knowledge about LHON as a cause of blindness, largely as a consequence or having two brothers who had become blind bilaterally many years earlier. After several e-mail exchanges, we became convinced that she was probably right about the diagnosis, and we were astonished at her estimate of a 200-member family that carried the defective mtDNA gene.

We decided to take advantage of the generosity of funding from IFOND to have M.O.M., her son, and her two brothers properly evaluated for the diagnosis of LHON. We arranged to have them all transported to the Federal University of Sao Paulo in southern Brazil. There, they were each thoroughly examined, photographed, and evaluated by psychophysical instruments. We also drew blood samples, which were sent to three independent laboratories for molecular analysis. These samples confirmed the clinical impression that all were homoplasmic for 11778 J-haplogroup and that all three of the males had the optic neuropathy.

### INDEX CASES

*Case 1*

M.O.M. was a 51-year-old woman without visual complaints. Visual acuities were 20/25 OD and 20/25 OS. FM-100 color testing did show a very mild dyschromatopsia OU without any axis. Pelli-Robson contrast sensitivity testing was borderline normal OD and normal OS. Humphrey visual field testing (24-2) was normal OU. Dilated fundus examination showed her optic discs to be flat and with evidence of slight optic atrophy OD (Figures 1A and 1B). Her left optic disc was normal. Her vessels and maculae were also normal. Clinical impression was a near-normal ophthalmological examination OD and a normal ophthalmological examination OS.

*Case 2*

P.H.M. was the 14-year-old son of M.O.M. This young man complained of bilateral visual loss, worse OS, dating back 3 weeks. He was aware that both his maternal uncles had been bilaterally blind for decades, and he knew that many other more distant members of the family had suddenly lost their vision in young adulthood. He characterized his visual loss as occurring over a period of a few days, first in the left eye and then several days later in the right. He saw a dark cloud in the center of vision and was aware

that he had problems appreciating colors, which all ran into a dark grey.

His visual acuities by ETDRS chart were 20/250 and 20/640. FM-100 color vision testing revealed very severe dyschromatopsia OU but more severe OS. Pelli-Robson contrast sensitivity testing showed moderate losses OD and very severe losses OS. Humphrey visual field testing (24-2) demonstrated large central scotomas OS worse than OD. Dilated fundus examination showed that both optic discs were abnormal (Figures 1C and 1D). There was selective loss of the papillomacular bundle and swelling of the nerve fiber layer in the arcuate bundles OD. There was also hyperemia on the nasal side. The optic disc OS was similar, but the papillomacular bundle loss was more devastating and the nerve fiber layer swelling less evident. Optic atrophy was just developing in the left eye.

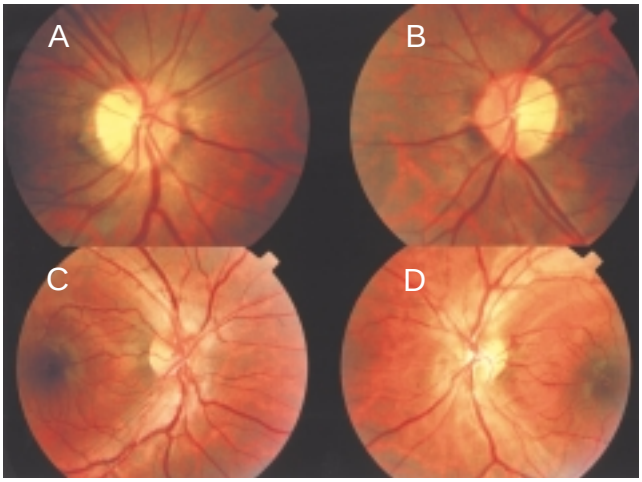


**FIGURE 1**

A and B, Index case 1. Right and left fundus of 51-year-old mother (LHON/carrier) without visual complaints. There is slight temporal pallor OD. C and D, Index case 2. Right and left fundus of 14-year-old son (LHON/affected) of index case 1. He had lost vision in both eyes 2 to 3 weeks earlier. Note swelling of arcuate nerve fiber layer and beginning of optic atrophy, especially OS.

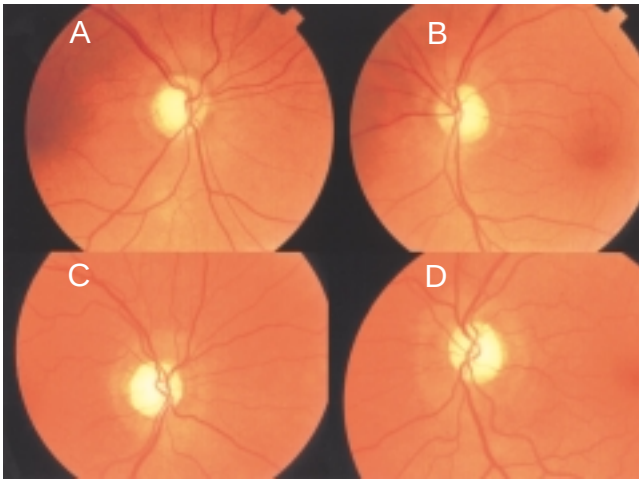


**FIGURE 2**

A and B, Index case 3. Right and left fundus of 42-year-old maternal uncle (LHON/affected) of index case 2, who had lost vision in both eyes about 7 years earlier. Note bilateral optic atrophy. C and D, Index case 4. Right and left fundus of 46-year-old maternal uncle of index case 2 (LHON/affected), who had lost vision in both eyes about 20 years before. Note severe optic atrophy OU.

Blood samples were sent to our laboratories both in Los Angeles and in Bologna. They were also sent to a commercial clinical laboratory. In all cases, the findings were that of LHON, homoplasmic mtDNA mutation for G11778A. The patient was also positive for the "secondary" mutations of T4216C and G13708A. This confirmed the clinical impression of LHON, homoplasmic 11778, a J-haplogroup.

*Case 3*

C.R.M. was the 42-year-old brother of M.O.M. He had lost his vision in February 1994, one eye a couple of weeks after the other. C.R.M. described a rapid loss of vision in each eye characterized as an inability to see straight ahead. However, he added that there may have been some slight progression of this loss over the subsequent 6 months. He described at least 26 other family members suffering visual loss presumed to be from LHON. Visual acuity by ETDRS was 10/800 OD and count fingers 1 foot OS. Color vision testing by FM-100 showed extremely severe dyschromatopsia OU. Contrast sensitivity testing showed total loss by Pelli-Robson OU. He showed complete depression on Humphrey visual field testing 24-2 OU but by confrontation demonstrated some peripheral preservation of visual field. His fundus examination OU (Figures 2A and B) revealed devastating optic atrophy with loss of the papillomacular bundle and of the superior and inferior nerve fiber layer as well. Blood test results were identical to those of his nephew P.H.M. and confirmed the clinical diagnosis of homoplasmic 11778 LHON, J-haplogroup.

*Case 4*

P.H.M. was the other brother of M.O.M. and was 46 years old. He had lost his vision in 1983 and characterized it as cloudiness that occurred almost simultaneously in both eyes, progressed slowly over a period of a year, and then stabilized. He had been a heavy drinker of alcohol until very recently. His visual acuities by ETDRS charting were 10/800 OD and 10/800 OS. Color vision by FM-100 showed very severe dyschromatopsia OU, and contrast sensitivity by Pelli-Robson demonstrated total losses OU. Testing by tangent field showed profound and large central scotomas with some preservation of the far periphery. Fundus examination showed devastating optic atrophy and a pattern of nerve fiber layer loss almost identical to that of his younger brother (Figures 2C and 2D). Blood

test results were identical to those of his brother, mother, and nephew and confirmed the clinical diagnosis of homoplasmic 11778 LHON, J-haplogroup.

Having established the diagnosis of LHON, we began the process of assembling the extent of the pedigree and planned an international field investigation. The core of this team consisted of this manuscript's authors. In particular, it included three neuro-ophthalmologists from the United States (A.A.S., P.Q.) and Italy (F.S.), a neurologist/molecular biologist (V.C.), and an ophthalmologist/epidemiologist (A.M.D.) from Italy. An ophthalmologist/epidemiologist (R.B.) and psychophysicists/epidemiologists (S.R.S. and A.B.) from the department of ophthalmology at Federal University of Sao Paulo, Brazil, were also members, and it was this group that preceded the rest of the international team by several days to make the important arrangements. Particularly critical was their identification of a very large private clinic in Colatina, Brazil, which was suitable for our needs. This private clinic was closed for 1 week and was made available to our field investigation group, as were eight technicians from that clinic who assisted with their expertise and knowledge of Portuguese. Other volunteers, including a professional phlebotomist and photographer, were all made available for us. Furthermore, we were able to get very sophisticated equipment (eg, Humphrey visual field analyzer, high-quality fundus cameras) brought to and set up in this clinic.

The international team flew to Brazil and joined the group from Federal University of Sao Paulo in Vitoria, and then we proceeded to Colatina, a small city in a rural agricultural area further inland. The selected clinic was ideal in being spacious and having 12 excellent areas for examination spread over three floors. The international team spent a day setting up these areas and discussing the nature of LHON and, in particular, this pedigree, which had already been carefully determined and characterized over seven generations and found to contain about 300 members. We also went over our expectations for data gathering.

We designed a system of two patient streams with six stations at each. The patients would register and then proceed to Station IA, where Portuguese-speaking epidemiologists would define each individual on the pedigree and ascertain that the relations were all correct. Station IB involved going over the patient's previously completed six-page questionnaire (translated into Portuguese), which covered all sorts of environmental risk factors. At Station II, 30 mL of blood was obtained from each patient. Only a small part of this blood was used for the molecular characterizations of LHON. Most of the blood was sent to the Bologna laboratory for DNA extraction for gene linkage analysis of the nuclear DNA. Station III involved extensive neuro-ophthalmological examinations by two of the three neuro-ophthalmologists. Translation was provided by one of three Portuguese-speaking MD-volunteers. Station IV involved careful psychophysical examinations, including standardized color vision, contrast sensitivity, and Amsler grid testing. Station V involved Humphrey visual field strategy 30-2 campimetry. Dilated fundus photography and occasional fluorescein angiographies were performed at Station VI. Four important members of the pedigree were too old and infirm and lived too far from any paved road to be brought to the Colatina clinic. We went to their homes and obtained confrontation visual fields and used a portable Kowa camera for color fundus photographs.

In total, we were able to find 295 living members of this pedigree (Figure 3) and to personally examine 273 of them. The extensive data acquisition was put into two large Excel spreadsheets containing either epigenetic or neuro-ophthalmologic factors. These were analyzed. Various parameters were compared, and we were able to obtain means or percentage involvements for each of three groups. In general, we compared (1) those who carried the mutant ND4 gene but had no serious visual impairment from optic neuropathy (labeled LHON/carriers in the tables), to (2) those with the LHON mutation and serious optic neuropathy (LHON/affected in the tables), and to (3) those who married into the family and had neither the mtDNA mutation nor any significant visual problems (controls). We were able to compute standard deviations for numerical data only (not percentage involvements), and we tested by chi-square test or Fisher exact test (if the number of cases was very small). Even so, these statistical treatments did not correct for several possible confounding factors that will be discussed later.

## RESULTS

### PEDIGREE

This pedigree is illustrated in Figure 3. Note that the founder was a woman born in 1861 in Verona, Italy, who immigrated to Brazil. She is depicted near the center and represents Generation I. Each subsequent generation is shown further centripetally through generation VII.

Ultimately, we found and fully examined 273 individuals of 295 living individuals identified as belonging on the pedigree. This covered seven generations. All but five of these cases clustered within 100 miles of each other between the cities of Vittoria, Colatina, and Santa Teresa. We found that the penetration of disease expression changed with these generations from over 70% in the early generations (I is the founder, so consider II), down to below 20% in the later generations V and VI (Figure 4). We also found that the percentage of cases that were male

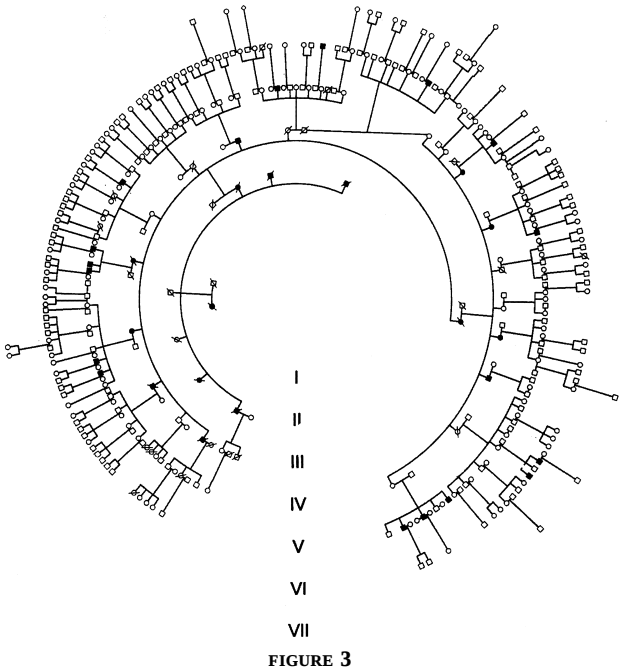


**FIGURE 3**

LHON pedigree.

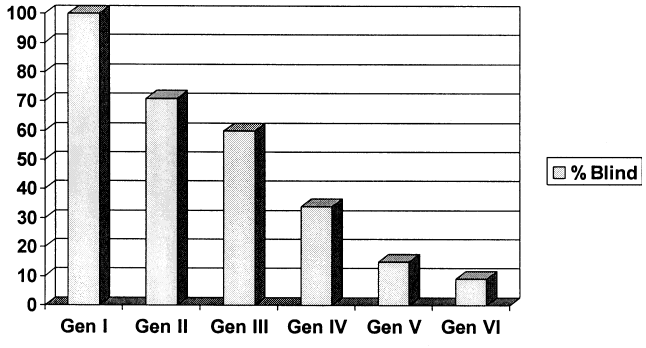


**FIGURE 4**

Penetrance rate in Leber's hereditary optic neuropathy. Chart showing percentage of those with the LHON mtDNA mutation who went blind in each generation. Penetrance rate diminished almost linearly.

changed with time. In the first generations after the immigrant founder, it ranged between 50% and 70%; however, in the last three generations it rose to nearly 100%.

**GENETICS**

Blood samples were analyzed in several laboratories. In all cases, the findings were that of LHON, homoplasmic mtDNA mutation for G11778A. The polymorphisms of T4216C and G13708A were also found to be homoplasmic. This confirmed the clinical impression of homoplasmic 11778 LHON, J-haplogroup. DNA was extracted from all of the blood samples.

**EPIGENETICS**

Various parameters of an extremely large spreadsheet could be compared both for general environmental risk factors and clinical features. Our general hypothesis was that epigenetic factors might explain why only a small subset of individuals carrying the 11778 mtDNA mutation went blind. We chose to compare several parameters that might test specific associations for each of the three groups described and noted in the tables as (1) LHON/carriers, (2) LHON/affected, and (3) controls.

Table I shows that our patients varied considerably in some of their nutritional habits. Their consumption of vegetables, beef, fish, chicken, and eggs did not, however, differ significantly between our three groups. However, in regard to the consumption of fruits, group 2, composed of LHON/affected patients who had gone blind, did consume less than the other two groups ($P<.05$ by chi-square test). In particular, group 2 averaged 1.9 fruits per day (SD, 2.4), while those in the pedigree with the same mtDNA genes who did not lose vision (group 1) averaged 3.1 (SD, 2.3) and those of the normal controls (group 3) averaged 2.9 fruits per day (SD, 2.3).

The LHON/affected group consumed more cigarettes in total (32.4 pack-years; SD, 17.2) than did group 1 (25.3 pack-years; SD, 16.4) or group 3 (14.5 pack-years; SD, 13.3). This, however, was not statistically significant.

However, as evidenced by Table II (which shows the toxic risk factors), analysis by percentage of patients smoking cigarettes did show statistical significance (by chi-square testing). We compared LHON/affected individuals (group 2) with the LHON/carriers (group 1) and found as significantly different that the blind patients were much more likely to smoke ($P<.01$). Indeed, there were fewer but yet statistically significant differences ($P<.05$) in that the LHON/carriers (group 1) were less likely to smoke than the general population (group 3).

Drinking alcohol was also much more common in LHON/affected patients who had gone blind. Sixty percent of these patients (group 2) drank heavily regularly or binged on weekends. This was a higher percentage than in either the LHON/carriers group (33.8% drank heavily) or controls (38.2% drank heavily). However, there was statistical significance only between the LHON/affected and LHON/carriers groups ($P<.01$). In general, the LHON/affected patients consumed, on average, 5 L per week of hard alcohol (usually 86 proof Cachaça).

When we compared between the groups the tendency to both smoke and drink, the differences were even more striking. Fifty percent of the LHON/affected patients did both. In contrast, only 9.5% of LHON/carriers and 11.8% of controls both smoked and drank heavily. This was statistically significant with $P<.01$ for comparisons of LHON/affected patients (group 2) to LHON/carriers (group 1) and $P<.05$ between LHON/affected patients and controls (group 3).

TABLE I: NUTRITIONAL HABITS OF STUDY GROUPS

| VARIABLE | LHON/CARRIERS MEAN ± SD (N=74) | LHON/AFFECTED MEAN ± SD (N=20) | CONTROLS MEAN ± SD (N=68) |
|---|---|---|---|
| Age | 30.6 ± 18.4 | 46.8 ± 17.2 | 44.4 ± 13.4 |
| Age at onset | | 29.2 ± 12.5 | |
| Fruits/day* | 3.1 ± 2.3 | 1.9 ± 2.4 | 2.9± 2.3 |
| Vegetables/day | 5.4 ± 2.4 | 4.6 ± 2.6 | 5.6 ±2.1 |
| Beef/day | 4.4 ± 2.5 | 5.5 ± 2.4 | 5.3 ± 2.3 |
| Fish/day | 1.0 ±1.1 | 1.3 ± 1.7 | 0.9 ± 1.2 |
| Chicken/day | 2.5 ± 1.7 | 2.8 ± 2.0 | 2.8 ± 2.0 |
| Eggs/day | 2.2 ± 2.3 | 2.4 ± 1.9 | 1.7 ± 1.6 |
| Vitamins (use) | 12.2% | 5% | 15.9% |

*$P$<.05 chi-squared for carriers versus affected versus controls.

TABLE II: TOXIC RISK FACTORS IN STUDY GROUPS

| RISK | LHON/CARRIERS (N=75) | LHON/AFFECTED (N=20) | CONTROLS (N=69) |
|---|---|---|---|
| Toxic exposure* | 9.7% | 45.0% | 16.2% |
| Cigarettes*† | 13.5% | 65.0% | 26.1% |
| ETOH* | 33.8% | 60.0% | 38.2% |
| Smoke and ETOH*† | 9.5% | 50.0% | 11.8% |

ETOH, ethanol; LHON, Leber's hereditary optic neuropathy.
*$P$<.01 carriers versus affected (chi-square test).
†$P$<.05 carriers versus controls (chi-square test).

We also questioned for other possible toxic exposure (Table II). Our questionnaire and subsequent interviews revealed that there were 12 potential toxins to which these three subject populations were exposed. For many of the toxins, only a handful of those from any of the subject population were affected, and hence statistical analysis was not possible. However, when we summed all the toxins together, it was evident that LHON/affected patients were much more likely to have been exposed. The exposure rates for group 1 (LHON/carriers), group 2 (LHON/affected), and group 3 (controls) were 9.7%, 45.0%, and 16.2%, respectively. Statistical significance was found only between the LHON/carriers and the LHON/affected groups ($P$<.05).

A variety of activities and diseases were also considered, as depicted in Table III. It was particularly notable that the presence of the mtDNA mutation of LHON or an associated gene was possibly protective in a variety of cardiovascular diseases. As the table shows, the prevalence of hypertension was 12.2%, 10%, and 24.6% for groups 1 (LHON/carriers), 2 (LHON/affected), and 3 (controls), respectively, and this was statistically significant ($P$ <.05). This was also true for diabetes mellitus, for which the rates were 2.7%, 5%, and 8.7%, respectively ($P$<.05).

Elevated cholesterol levels (>230 mg) were also lower in both groups with LHON: group 1, 8.1%; group 2, 5%; and group 3, 15.9%. Having LHON seemed protective for coronary artery disease as well, the three groups having prevalence rates of 1.4%, 5%, and 10.1%, respectively. However, these were only trends, and we could not show statistical significance. Yet, given the fact that these are all risk factors for stroke, it is not surprising that the rates of cerebral vascular disease were very low at 1.4% for LHON/carriers (group 1), 0% for the LHON/affected (group 2), and comparatively high at 8.7% for the controls (group 3). Hence there was statistical significance at $P$<.05 with regard to both LHON groups being protective against stroke in comparison to controls.

Finally, in regard to other disease states, the prevalence of reported degenerative neurologic diseases (predominantly Alzheimer's and Parkinson's diseases) was greater at 25% in LHON/affected patients (group 2) than in either the LHON/carriers at 14.9% (group 1) or controls at 10.1% (group 3).

## DISCUSSION

These results showed several remarkable associations. However, discussion of such must begin with the recognition that there were several confounding factors that preclude us from certain conclusions and even from a confident determination of what was cause and what was effect. For example, we noted that LHON patients who were not blind exercised at almost 10 times the rate of LHON patients who were blind. Did the exercise help mitigate against blindness? Or did blind patients find it more difficult to exercise (particularly playing soccer)? The fact that the controls also exercised about as much as the LHON/affected patients at least suggests that the inability to see was not the only cause and exercise the direct effect. We suspect that the very high rate of exercise in the LHON/carriers group may have been a conscious attempt to live a healthy lifestyle among a population that knew that blindness might have been around the corner. It is quite likely that several of these effects all

TABLE III: GENERAL RISK FACTORS IN STUDY GROUPS

| FACTOR | LHON/CARRIERS (%) | LHON/AFFECTED (%) | CONTROLS (%) |
|---|---|---|---|
| Exercise* | 32.0 | 5.0 | 9.1 |
| Hypertension† | 12.2 | 10.0 | 24.6 |
| Diabetes mellitus | 2.7 | 5 | 8.7 |
| High cholesterol | 8.1 | 5 | 15.9 |
| Coronary | 1.4 | 5 | 10.1 |
| CVA† | 1.4 | 0 | 8.7 |

CVA, cerebral vascular disease; LHON, Leber's hereditary optic neuropathy.
*$P$<.05 carriers versus both (chi-square test).
†$P$<.05 carriers versus controls and affected versus controls (chi-square test).

contributed to this dramatic association of low activity to LHON-associated blindness.

It should also be recognized that the three populations were heterogeneous in several important particulars. The LHON/carriers (group 1) were much younger (average age, 31) than their blinded counterparts (group 2, average age 47) or controls (average age, 44). This may have been due, in part, to the fact that the earlier generations, who were of course older, had a higher penetrance rate for blindness (see "Results" and Figure 4). Another difference was that the LHON/affected patients (group 2) were overwhelmingly male (85%) compared with LHON/carriers (35% male) and controls (46% male). This, too, is not surprising given that most published pedigrees showed strong male predominance. However, in interpretations of diet, smoking, or drinking, it would not be surprising that a group with a higher percentage of males eats less fruit and smokes and drinks alcohol more. Once again, this is a serious confounding factor that precludes us from concluding with certainty that these lifestyle differences determined who with the LHON gene went blind.

These and other confounding elements limit the conclusions that we can make in regard to the potency and exact effects of several epigenetic factors in determining which LHON patients with the homoplasmic 11778 mtDNA mutation would express the blindness. Nonetheless, the present study demonstrates that there are powerful associations between lifestyle, nutrition, toxic exposure, and risk factors that probably contribute to the crossing of a threshold that genetic predisposition sets. Furthermore, the present study gives us strong hints as to how these factors may exert an influence on this threshold.

This is true from examination of the pedigree itself. For example, we were struck by the remarkable decrease in penetrance demonstrated by each succeeding generation. This might have been due to a decrease of the risk factors, but the dramatic and smooth curve seen in Figure 4 strongly suggests the presence of a nuclear permissive gene, which may be diluted out in succeeding generations. If the permissive gene was autosomal recessive, then only the combination of both nuclear alleles and the LHON mtDNA mutation would result in blindness. The pedigree (Figure 3) demonstrates a few later branches of the genetic tree with a cluster of cases. Does this reflect happenstance, a different family lifestyle, or the doubling up of a permissive nuclear gene?

In regard to nutritional factors, the consumption of fruit did seem to confer benefit, as reflected by the fact that there was a statistically significant tendency for LHON/carriers to consume more fruit (slightly more than one extra per day) than their LHON relatives who had become blind. Fruits contain antioxidants, but so do the vegetables, which both groups seemed to consume in sufficient quantity. No other eating habit appeared to differ between the groups.

The most dramatic differences between the groups were in their habits of smoking cigarettes and drinking hard liquor. As noted in Table II, there were marked and significant differences in that those with LHON who went blind were much more likely to smoke and much more likely to drink heavily. This was most dramatic when considering those that both smoked and drank heavily. Of the LHON/affected group, 50% did both, as compared to only 9.5% of their relatives harboring the identical LHON mtDNA mutation but without the visual impairment. Smoking and drinking alcohol generate reactive oxygen species (ROS).

Other toxic exposures also seemed to make a difference. Table II also shows that 45% of the LHON/affected patients were exposed to toxins at work, while their relatives with the LHON mtDNA mutation but normal vision only had a quarter of such exposure. Further analysis of these toxins (many were pesticides and others were constituents of fertilizer) will be required.

The consequences of mitochondrial dysfunction in LHON have yet to be fully worked out. The 11778, 3460, and 14484 pathogenic mutations all affect complex I activity, and when this was replicated in a cybrid cellular model, these mtDNA mutations induced a variable impairment of mitochondrial respiratory function.[17] These effects could be mediated by a decreased release of the quinol product or by affecting proton pumping and energy conservation.[17] The common feature is that all three mutations affect the site of interaction of complex I with its natural quinone (CoQ) substrate.[18,19] As a consequence, there probably develops a chronic increase of ROS production. In fact, a number of recent publications now implicate the important role of ROS accumulation in LHON.[20-22] We think that both energy depletion and oxidative stress play roles in LHON pathogenesis. Exposure to certain toxins, such as organophosphate pesticides, may exacerbate the energy depletion problem; smoking and drinking may produce more ROS, while the lack of consumption of fruits may reduce the availability of antioxidants to deal with oxidative stress.

In regard to lifestyle issues, it is interesting that the LHON/carriers group exercised 10 times more than their LHON/affected counterparts. This difference does not simply reflect the tendency of blind individuals to not participate in sports, because the controls also failed to exercise at about the rate of the LHON/affected. It is possible that the members of the LHON/carriers group were actively attempting to preclude impending blindness by the choice of a healthy lifestyle. It is also possible that we should not overlook the benefits of exercise in LHON.

One of the benefits of exercise is on the cardiovascular system. This may account for the remarkable protection afforded the LHON/carriers against hypertension, high cholesterol, and cerebral vascular accidents (see Table III). However, even the LHON/affected patients had some measure of protection from these diseases compared with the normal controls, and yet both exercised about the same. This raises the intriguing possibility that either the mtDNA mutation of LHON itself or, more likely, an associated gene may also have a protective effect against cardiovascular disease. Indeed, Carelli and associates[23] have suggested that haplogroup J may exert a protective rather than detrimental effect in LHON and that this protection may extend to resisting some of the ravages of aging.

The increased rate of Alzheimer's disease, and especially Parkinson's, among our LHON patients was only reported and requires autopsy confirmation. However, a family with maternally inherited adult-onset Parkinsonism and multisystem degeneration has been shown to harbor the 11778 mutation.[24] Going the other direction, cerebellar ataxia has been described in two LHON families with the 11778 mutation.[25,26]

There are several optic neuropathies that produce a clinical picture very similar to that of LHON.[27] At least six classes of optic neuropathies are similar in appearance and probably also in pathophysiology[12,27] These are (1) LHON, (2) Cuban epidemic of optic neuropathy, (3) tobacco alcohol amblyopia, (4) nutritional deficiencies (especially folic acid and B12), (5) ethambutol, and (6) methanol, cyanide, and other toxins that specifically interfere with mitochondrial oxidative phosphorylation.[26] These optic neuropathies share several oddities with LHON. For example, while these diseases are metabolic and hence systemic by nature, they often have a predilection for the optic nerve and for the papillomacular bundle in particular.[12,13,28] Like LHON, these other mitochondrial optic neuropathies also share six prominent clinical features: (1) symmetrical visual losses, (2) loss of visual acuity and high spatial frequency contrast sensitivity, (3) early and profound dyschromatopsia, (4) centrocecal visual field defects, (5) temporal atrophy of the optic discs, and (6) preferential loss of the papillomacular nerve fiber layer.[27]

It is likely that for each of these mitochondrial optic neuropathies, there is, in addition to energy depletion, an accumulation of ROS. Further, through the opening of the mitochondrial permeability transition pore (mtPTP), there is a consequent release of cell death promoting factors such as cytochrome C.[27] These and similar mechanisms probably induce apoptosis in retinal ganglion cells.[29] Hence, an understanding of the role of various pro- and anti-apoptotic factors, as revealed in the study of LHON patients, may reveal principles that are generalizable to many other optic neuropathies.

The pathophysiology of mitochondrial metabolic optic neuropathies, whether congenital or acquired, suggests certain treatment strategies. Most important, of course, is removal of the offensive element.

There may also, however, be strategies for mitigating, neutralizing, or possibly even rescuing retinal ganglion cells undergoing apoptosis due to these mechanisms. In regard to LHON, several such strategies have already been attempted. For example, Idebenone not only provides an alternate pathway around the blockage of complex I, it also scavenges ROS and concentrates within the mitochondria.[30]

As mentioned, we are precluded from making any definitive statement of causality between the risk factors and disease expression, owing to the many confounds in this or any statistical analysis of the many parameters measured and analyzed. However, these data and the associations presented here should help in the generation of hypotheses that can then be tested, especially in an LHON animal model. Such an animal model may predispose to blindness but will probably need additional stressors to replicate the disease. These stressors may be developed along the lines implicated in the present paper.

The present study also does not establish the specific pathophysiological consequences of mitochondrial dysfunction. Nor does it establish the relative contributions of genetic and epigenetic factors in determining penetrance, though it does suggest that both play a role. While we have accumulated a great deal of data, covering about 90 parameters in about 300 patients, the many confounding factors discussed preclude us from making definitive statements as to which factors trigger the optic neuropathy in the 11778 mtDNA mutation (that only predisposes patients to LHON-induced blindness).

However, at minimum, we hope that this study is a good start, for it focuses attention on certain newly identified intriguing associations, between epigenetic factors and the expression of blindness in LHON. From here we can propose certain hypotheses, which can be more definitively tested by more focused follow-up examinations and in animal models. We remain optimistic that these pending studies will provide insights into the relative roles of mtDNA, nuclear DNA, and epigenetic factors in determining the cytoplasmic milieu that may lead a retinal ganglion cell down the apoptotic cascade to retinal ganglion cell death.

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## REFERENCES

1. Carelli V. Leber's hereditary optic neuropathy. In: Schapira AHV, DiMauro S, eds. *Mitochondrial Disorders in Neurology 2. Blue Books of Practical Neurology*. Burlington, Mass: Butterworth-Heinemann, 2002:115-142.
2. Chalmers RM, Schapira AHV. Clinical, biochemical and molecular genetic features of Leber's hereditary optic neuropathy. *Biochim Biophys Acta* 1999;1410:147-158.
3. Howell N. Human mitochondrial diseases: answering questions and questioning answers. *Int Rev Cytol* 1999;186:49-116.
4. Wallace DC, Singh G, Lott MT, et al. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* 1988;242:1427-1430.
5. Huoponen K, Vilkki J, Aula P, et al. A new mtDNA mutation associated with Leber hereditary optic neuropathy. *Am J Hum Genet* 1991;48:1147-1153.
6. Howell N, Bindoff LA, McCullough DA, et al. Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. *Am J Hum Genet* 1991;49:939-950.
7. Mackey D, Howell N. A variant of Leber hereditary optic neuropathy characterized by recovery of vision and by an unusual mitochondrial genetic etiology. *Am J Hum Genet* 1992;51:1218-1228.
8. Johns DR, Neufeld MJ, Park RD. An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. *Biochem Biophys Res Comm* 1992;187:1551-1557.
9. Chalmers RM, Harding AE. A case-control study of Leber's hereditary optic neuropathy. *Brain* 1996;119:1481-1486.
10. Kerrison JB, Miller NR, Hsu F, et al. A case-control study of tobacco and alcohol consumption in Leber's hereditary optic neuropathy. *Am J Ophthalmol* 2000;130:803-812.
11. Nikoskelainen EK. Clinical picture of LHON. *Clin Neurosci* 1994;2:115-120.
12. Carelli V, Ross-Cisneros FN, Sadun AA. Optic nerve degeneration and mitochondrial dysfunction: genetic and acquired optic neuropathies. *Neurochem Int* 2002;40:573-584.
13. Sadun AA, Win PH, Ross-Cisneros FN, et al. Leber's hereditary optic neuropathy differentially affects smaller axons in the optic nerve. *Trans Am Ophthalmol Soc* 2000;98:223-232.
14. Sadun AA, Kashima Y, Wurdeman AE, et al. Morphological findings in the visual system in a case of Leber's hereditary optic neuropathy. *Clin Neurosci* 1994;2:165-172.
15. Kerrison JB, Howell N, Miller NR, et al. Leber hereditary optic neuropathy. Electron microscopy and molecular genetic analysis of a case. *Ophthalmology* 1995;102:1509-1516.
16. Carelli V, Saadati HG, Madigan M, et al. Leber's hereditary optic neuropathy hystopathology suggests optic nerve axoplasmic stasis as the key pathophysiologic feature. Presented at EUROMIT 4, Queens College, Cambridge, United Kingdom, September 16-19, 1999; abstract P92.
17. Brown MD, Trounce IA, Jun AS, et al. Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778 or 14484 Leber's hereditary optic neuropathy. *J Biol Chem* 2000;275:39831-39836.
18. Carelli V, Ghelli A, Ratta M, et al. Leber's hereditary optic neuropathy: biochemical effect of the 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. *Neurology* 1997;48:1623-1632.
19. Carelli V, Ghelli A, Bucchi L, et al. Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy. *Ann Neurol* 1999;45:320-328.
20. Wong A, Cortopassi G. mtDNA mutations confer cellular sensitivity to oxidant stress that is partially rescued by the calcium depletion and cyclosporin A. *Biochem Biophys Res Commun* 1997;239:139-145.
21. Barrientos A, Moraes CT. Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J Biol Chem* 1999;274:16188-16197.
22. Klivenyi P, Karg E, Rozsa C, et al. a-Tocopherol/lipid ratio in blood is decreased in patients with Leber's hereditary optic neuropathy and asymptomatic carriers of the 11778 mtDNA mutation. *J Neurol Neurosurg Psychiatry* 2001;70:359-362.
23. Carelli V, Vergani L, Bernazzi B, et al. Respiratory function in cybrid cell lines carrying European mtDNA haplogroups: implications for Leber's hereditary optic neuropathy. *Biochim Biophys Acta* 2002.
24. Simon DK, Pulst SM, Sutton JP, et al. Familial multisystem degeneration with parkinsonism associated with the 11778 mitochondrial DNA mutation. *Neurology* 1999;53:1787-1793.
25. Funakawa I, Kato H, Terao A, et al. Cerebellar ataxia in patients with Leber's hereditary optic neuropathy. *J Neurol* 1995;242:75-77.
26. Murakami T, Mita S, Tokunaga M, et al. Hereditary cerebellar ataxia with Leber's hereditary optic neuropathy mitochondrial DNA 11778 mutation. *J Neurol Sci* 1996;142:111-113.
27. Sadun AA. Mitochondrial optic neuropathies. A model for a unifying theory. *J Neurol Neurosurg Psychiatry* 2002;72:423-425.
28. Sadun AA. Acquired mitochondrial impairment as a cause of optic nerve disease. *Trans Am Ophthalmol Soc* 1998;96:881-923.
29. Danielson SR, Wong A, Carelli V, et al. Cells bearing mutations causing Leber's hereditary optic neuropathy are sensitized to Fas-induced apoptosis. *J Biol Chem* 2002;277(8):5810-5815.
30. Carelli V, Ghelli A, Cevoli S, et al. Idebenone therapy in Leber's hereditary optic neuropathy: Report of six cases. *Neurology* 1998;50 Suppl 4:VI-02.002:A4.

## DISCUSSION

DR ALAN H. FRIEDMAN. The authors are to be congratulated on the exhaustive nature of their study and the doggedness in which they pursued it.

The purpose of this study was to investigate possible contributory factors to the development of blindness in patients with LHON. Eye examinations and blood tests were administered to determine carrier status for the LHON mutation in a large Brazilian family that had homoplasmy for LHON 11778, J-haplotype. In addition, questionnaires about dietary habits, recreational activities, concurrent medical problems, and toxic exposures were studied. The data were evaluated to focus on the differences between blind versus nonblind carriers of the mutation.

Environmental factors have been suggested as possible mechanisms in LHON expression. Wilson[1] and Cullom and colleagues[2] proposed heavy tobacco smoking and secondarily undetoxified cyanide as possible factors.

The authors wrote "at minimum, they wished to examine the (nuclear) genetic and epigenetic factors that might trip the threshold of expression that leads to blindness." No further mention is made of the nuclear factors other than "Most of the blood was sent to the Bologna laboratory for DNA extraction for gene linkage analysis of the nuclear DNA." What were the authors looking for? Was anything found?

Regarding epigenetic factors, the authors provide the results of environmental and medical history questionnaires. Consumption of fruits, exposure to toxins, cigarette smoking, alcohol consumption, and exercise tendencies differed between the LHON blind and LHON nonblind. What is not clear from the results is the timing of these differences. Were these differences present prior to the development of blindness? If so, for how long were they present? If the authors are trying to examine what might alter a phenotype, then it would be important to note if and for how long the modifying factors are present prior to the onset of the phenotypic trait. Otherwise one might argue that dietary regimens, alcohol, smoking, and exercise might change as a result of the development of blindness.

The authors also looked at penetrance rates and noted that there was significantly reduced penetrance with latter generations. It might be a good idea to include the LHON nonblind carrier/obligate carrier on the pedigree to make it easier for the reader to see the actual penetrance rates of blindness. It is also important to note the average age at which each generation became blind and the average age of each generation when the study was performed. As noted, reduced penetrance in later generations may simply have to do with the fact that the subjects are younger and have not yet developed the trait.

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## REFERENCES

1. Wilson J: Leber's hereditary optic atrophy: some clinical and aetiological considerations. *Brain* 1963; 86:346-362.
2. Cullom ME, Heher KL, Miller NR, Savino PJ, Johns DR: Leber's hereditary optic neuropathy masquerading as tobacco-alcohol amblyopia. *Arch Ophthalmol* 1993;111:1482-1485.

DR ALLAN J. FLACH. Do any of the visual fields reflect either a tobacco or a nutritional amblyopia? Did your histories pick up any suggestion of insecticide toxicity that might be contributing?

DR BRIAN R. YOUNGE. Do those of you who make a diagnosis of tobacco-alcohol amblyopia do testing to rule out Leber's disease on that individual, since they present in a similar fashion?

DR IVAN R. SCHWAB. If you assume that it's tobacco-alcohol related, this only accounts for approximately 50% of the affected individuals. What about the other 50%? You need to be very careful about making associations with ancillary points because the study wasn't designed to look at these ancillary points.

DR ALLAN J. FLACH. Dr Harrington taught us that there is no such thing as tobacco-alcohol amblyopia. Alcohol amblyopia is a form of nutritional amblyopia. Tobacco amblyopia exists, and these two diseases can be distinguished by visual field. One has a central scotoma, and the other has a scotoma that's not central. One has sloping margins, and the other does not have sloping margins to the visual field defect.

DR ALFREDO A. SADUN. I will begin with the questions of Dr Friedman. He was quite right in that the purpose of our study as presented today was to look only at the epigenetic factors. The gene-linkage analysis has not been completed; it's being done in conjunction between Bologna and Iowa, where Ed Stone has his hunches and we have ours. It will take another year to complete such an exhaustive study. Dr Friedman asked whether the risk factors were there at the time of the examinations or were they there at the time that the individual went blind. We have figured out a way of dividing the database to look at them separately. I think the more interesting aspect is

what the risk factors were at the time the patients went blind. I showed the numbers at the time that we did the examination. The historical numbers that reflect the risks at time of visual loss are less reliable owing to dependence on memory, but they indicate these same risk factors in even greater preponderance. We have now begun to analyze the data reflecting risks that occurred at the time that the patient went blind, notwithstanding the fact that their memories, of course, may not be accurate for an event that occurred 20 or 30 years ago. Dr Friedman also asked at what ages they went blind. The intuition is that if the penetrance rate keeps going down, perhaps the age at onset should also be going up, as we're shifting the threshold. We were amazed to discover that the opposite was the case. In the first few generations, people went blind in their 30s, and in the last few generations people have been going blind in their 20s, early 20s, and now as teenagers. For some reason, it's an all-or-none phenomenon which is becoming less frequent but occurring at a younger age when it does.

Dr Flach points out that there is a tremendous amount of overlap here between tobacco-alcohol amblyopia (or nutritional amblyopia) and the disease process discussed here. The visual fields are probably not the best way of making that distinction. They both present with central scotomas, although the central scotoma of tobacco-alcohol amblyopia tends to be relatively small, perhaps about 5° to 10°, whereas thats seen in this particular disease (LHON) is enormous. We are probably looking at different ways of skinning the cat by injuring mitochondria genetically and in an acquired fashion. Dr Flach also asked if we had a chance to look at the various insecticides that were used. It turns out that the pesticides probably are organophosphates, and the mechanism of action of organophosphates is, in fact, on the parasympathetic system, so that, in fact, they may aggravate the situation in the way that he has suggested.

Dr Younge reminds us it's very important to consider the overlap between the two diseases of LHON and nutritional deficiencies. I, too, have had patients who have called in after the blood tests were made available and in whom I had initially made a diagnosis of tobacco-alcohol amblyopia only to be surprised to later discover that they had Leber's. I do believe that some of these individuals may have suffered from the effects of smoking and drinking, although it's rather hard to say that this is the only cause when they're also carrying the mitochondrial deficiency. I'd rather think of it as tipping them over a threshold.

Dr Ivan Schwab suggested that one must be careful about ancillary associations when the study did not directly test this as a hypothesis. This is a very important point that I call the Feynman concern, since Richard Feynman was a Nobel Laureate who often found this mistake in the work of others. Even overwhelming statistics can't prove a point that wasn't hypothesized before the data were accrued. Hence I reemphasize and summarize from the conclusions of this present paper. The new associations from this study serve as a good start in looking at the relationship between genetic and epigenetic factors for the expression of blindness in LHON. These new hypotheses can then be definitively tested by more focused follow-up examinations in our LHON Brazilian population and in animal models.